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Regulation of elastin gene transcription by proteasome dysfunction

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Kuang, Ping-Ping, and Ronald H. Goldstein. Regulation of elastin gene transcription by proteasome dysfunction. *Am J Physiol Cell Physiol* 289: C766–C773, 2005. First published April 6, 2005; doi:10.1152/ajpcell.00525.2004.—Elastin, a major extracellular matrix protein and the core component of elastic fiber, is essential to maintain lung structural integrity and normal physiological function. We previously found that the downregulation of elastin gene transcription by IL-1β is mediated via activation of NF-κB and CCAAT/enhancer binding protein (C/EBP)β, both targets of the ubiquitin-proteasome pathway. To further investigate the molecular mechanisms that underlie the control of elastin gene expression, we disrupted the ubiquitin-proteasome pathway with specific proteasome inhibitors. We found that specific proteasome inhibitors decreased the steady-state level of elastin mRNA in a dose-responsive manner. Run-on assay and promoter reporter study indicated that the proteasome inhibitor MG-132 repressed the rate of elastin transcription. MG-132 did not affect mRNA levels of NF-κB and C/EBPβ, or the nuclear presence of NF-κB, but markedly increased C/EBPβ isoforms, including liver-enriched transcriptional activating protein and liver-enriched transcriptional inhibitory protein. Addition of cycloheximide blocked these increases and the downregulation of elastin mRNA by MG-132. The MG-132-induced downregulation of elastin transcription was dependent on C/EBPβ expression as assessed with small interfering RNA. These results indicate that the ubiquitin-proteasome pathway plays an essential role in maintaining elastin gene expression in lung fibroblasts. Disruption of this pathway results in the downregulation of tropoelastin transcription via posttranscriptionally induced C/EBPβ isoforms.

interleukin-1β; lung; fibroblasts; nuclear factor-κB; CCAAT/enhancer-binding protein β

ELASTIN IS THE CORE COMPONENT of elastic fibers that is formed via extracellular assembly and cross-linking of soluble tropoelastin monomers into highly organized insoluble elastin polymers on a preformed microfibril scaffold (32, 35, 40). As an abundant, resilient extracellular matrix protein in the lung interstitium, elastin provides the proper mechanical support to conducting airway, alveolar walls, and septa and plays a pivotal role in maintaining the structural integrity and function of the lung. Tropoelastin is synthesized by lung interstitial fibroblasts in alveolar walls and by smooth muscle cells in vascular tissues (5, 44, 46). The peak expression of tropoelastin mRNA occurs in a very narrow window of postnatal development that corresponds to the period of rapid alveolarization and development of secondary septa in the neonatal lung (4). In the adult lung parenchyma, tropoelastin mRNA is minimally expressed in interstitial structures but can be reactivated under certain diseases and conditions (28, 34). Disruption of elastin gene expression may be a major contributing factor in the development of pulmonary emphysema (3, 21, 33), whereas excessive expression is often observed in fibrotic lung diseases (18).

Our previous studies (26, 27) suggested that the downregulation of elastin mRNA by IL-1β is transcriptionally mediated by NF-κB and CCAAT/enhancer binding protein (C/EBP)β pathways. NF-κB and C/EBP family protein complexes are both targeted and regulated by the ubiquitin-proteasome pathway (12, 17). The ubiquitin-proteasome pathway degrades the majority of intracellular proteins via the sequential action of two ATP-dependent functional protein complexes, the ubiquitin pathway and the proteasome (37). A variety of key regulatory proteins involved in cell proliferation, differentiation, survival, and apoptosis are substrates of the ubiquitin-proteasome pathway, implicating manipulation of proteasome dysfunction as a potential therapeutic intervention in tumorigenesis, inflammation, neurodegenerative diseases, and certain genetic diseases, including cystic fibrosis (9). Proteasome inhibitors are currently under investigation as promising anticancer drugs (1).

In this study, we investigated the biological role of the ubiquitin-proteasome pathway in the control of elastin gene expression in lung fibroblasts using well-studied proteasome inhibitors (29, 30, 36, 39). Our results indicate that the ubiquitin-proteasome pathway plays an essential role in maintaining elastin gene expression, and disruption of this pathway causes downregulation of elastin gene transcription and mRNA by posttranscriptionally upregulated C/EBPβ proteins.

EXPERIMENTAL PROCEDURES

Cell culture. Neonatal rat lung fibroblasts, also referred to as lung interstitial cells (LIC), were isolated from the lungs of 8-day-old Sprague-Dawley rat pups (Charles River Breeding Laboratory, Wilmington, MA) as previously described (26, 27). The protocol for handling the Sprague-Dawley rat pups in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine. After isolation, LIC were grown in MEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS in T-75 flasks (Falcon Plastics, Los Angeles, CA) in a humidified 5% CO_2-95% air incubator at 37°C for 3–5 days until confluence. The purity of the cultures was assessed with phase microscopy and Oil Red O staining. The first-passage cells were grown in MEM to confluence and rendered quiescent by reducing the serum content of the medium to 0.4% for 24 h before experiments. MG-132, epoxomicin, lactacystin, calpeptin, and (2S,3S)-*trans*-epoxysuccinyl-L-leucylamide-3-methylbutane ethyl ester were purchased from Calbiochem (La Jolla, CA). Recombinant human TGF-β1 and IL-1β were purchased from R&D Systems (Minneapolis, MN). Cycloheximide (CHX) and actinomycin D (ActD) were obtained from Sigma (St. Louis, MO). Human embryonic kidney 293T cells and preadipocyte

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cell lines (3T3.L1-LAP and 3T3.L1-LIP, kindly provided by Dr. Stephen R. Farmer, Boston University School of Medicine, Boston, MA) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS as described previously (27).

RNA isolation and Northern blot analysis. Cells were harvested, and total RNA was prepared with a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA samples (10 μg/lane) were electrophoresed and followed by Northern blot analysis as previously described (26, 27). Hybridization was carried out in Rapid Hybridization Buffer (Amersham, Piscataway, NJ) using ^{32}P-labeled cDNA probes for rat tropoelastin, NF-κB p56 subunit, or C/EBPβ. The ^{32}P-labeled 18S ribosome oligonucleotides (IDT, Coralville, IA) were used to assess loading equivalence and transfer efficiency.

Nuclei preparation and run-on assay. Nuclei were isolated from four P-150 dishes of cell cultures designated for each experimental group in lysis buffer [10 mM Tris-Cl (pH 7.4), 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% Nonidet P-40] with a Dounce homogenizer (Thomas, Swedesboro, NJ). After centrifugation at 500 *g* for 5 min, nuclei were resuspended in 200 μl of nuclei storage buffer [50 mM Tris-Cl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA]. Nuclear run-on reactions were carried out by incubation of 200 μl of nuclei with one volume of 2× reaction buffer [10 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl, ATP, GTP, and CTP at 2 mM, and 100 U RNasin (Promega, Madison, WI)] and 10 μl of [α-^{32}P]UTP (3,000 Ci/mmol) at 30°C for 30 min. Reactions were terminated by sequential incubation with RNase-free DNase (100 U) at 37°C for 30 min and with protease K (200 μg/ml in 1% SDS) at 42°C for 15 min. The newly synthesized ^{32}P-labeled RNA was isolated by phenol/chloroform extraction and ethanol precipitation. After washing with 70% ethanol, RNA was dissolved in TE buffer [10 mM Tris·HCl (pH 7.0), 0.1 mM EDTA]. For hybridization, labeled RNA was denatured in 0.2 N NaOH for 10 min on ice and neutralized in 0.2 M acid-free HEPES. The unincorporated [α-^{32}P]UTP was removed with NucTrap probe purification columns (Stratagene, La Jolla, CA). The plasmids (10 μg each) containing cDNA coding inserts for elastin, collagen, or GAPDH and 10 μg of pBluescript II SK vector (Stratagene) were denatured in 0.3 N NaOH at 65°C for 1 h and blotted onto the nitrocellulose membrane with a Minifold II slot blotter (Schleicher & Schuell, Keene, NH). The immobilized slot blots were prehybridized in 5× Denhardt's solution, 5× SSC, 0.5% SDS, 50 mM NaH_2PO_4 (pH 6.5), 250 μg/ml yeast tRNA, and 50% formamide at 42°C for 24 h and hybridized with equal counts (10^7 counts/min) of ^{32}P-labeled RNA at 42°C for 3 days. The slot blots were washed and exposed on X-ray films.

Reporter plasmids, transient transfection, and luciferase assay. The rat elastin promoter luciferase reporter PGL-2/118 and the TGF-β1 responsive luciferase reporter 3TP were described previously (26, 27, 38). Transient transfection and cotransfection were performed with LipofectAMINE 2000 (LF-2000; Invitrogen) according to the manufacturer's protocol. Transfection efficiency was monitored by cotransfection of pRLTK *Renilla* luciferase vector (0.25 μg/well) (Promega, Madison, WI). At 24 h after transfection, cells were conditioned with TGF-β1 (1 ng/ml), MG-132 (10 μM), or both for 20 h, and whole cell lysates were isolated, followed by luciferase assay as described previously (26, 27). Firefly and *Renilla* luciferase activity were determined with the Dual-Luciferase Reporter system (Promega) and a luminometer (TD20/20, Turner Designs, Sunnyvale, CA). Firefly luciferase values were normalized to *Renilla* luciferase values and expressed as relative firefly/*Renilla* luciferase activity. Experiments were carried out in triplicate and repeated at least three times. Statistical analyses were carried out with a two-tailed Student's *t*-test. In case of overexpression and cotransfection of liver-enriched transcriptional inhibitory protein (LIP) experiments, parallel experiments were carried out to prepare whole cell lysates for immunoprecipitation and Western blot analysis as previously described (26, 27).

Small interfering RNA design, preparation, and transfection. The design of small interfering RNA (siRNA) was according to the specific characterization of siRNA described by Elbashir et al. (14). Two 19-bp cDNA sequences were selected from the potential siRNA target sites in rat C/EBPβ transcript to generate siRNA expression constructs. They were synthesized as inverted repeats separated by a short loop sequence. The sense and antisense strands were annealed and inserted into pRNA-U6.1/Neo siRNA vector (Genescript, Scotch Plains, NJ). The sequences of these siRNA inserts (sense strand) were 5′-CTTCTACTACGAGCCCGACTTCAAGAGAGTCGGGCTCGTAGTAGAAG-3′ (siRNA-CTT) and 5′-GCTGAGCGACGAGTACAAGTTCAAGAGACTTGTACTCGTCGCTCAGC-3′ (siRNA-GCT). The corresponding siRNA constructs were named C/EBPβ siRNA-CTT and -GCT constructs, respectively, and verified by DNA sequencing. To examine the effect of these siRNA constructs on C/EBPβ expression, the siRNA construct (6 μg) was cotransfected with V5-tagged, liver-enriched, transcriptional activating protein (LAP) TOPO expression vectors (1 μg) (26) into 293T cells in P-60 dishes, using LF-2000 as described above. At 48 h after transfection, whole cell lysates were prepared and Western blot analysis was carried out with anti-V5 antibody (Invitrogen) as described previously (27). The effect of these siRNA constructs on IL-1β- or MG-132-induced downregulation of elastin transcription was tested by cotransfection of them (3 μg/well) with the elastin promoter luciferase reporter PGL-2/118 (0.5 μg/well) and the pRLTK *Renilla* luciferase vector (0.25 μg/well) into LIC in six-well plates, followed by luciferase assays and immunoprecipitation and Western blot analysis of whole cell lysates as above.

Nuclear protein extraction, immunoprecipitation, and Western blot analysis. Preparation of nuclear proteins, immunoprecipitation of V5 from transfected whole cell extracts, and Western blot analysis were performed as previously described (26, 27). Briefly, nuclear extracts (30 μg) were resolved by 4–12% gradient of SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes. Immunoprecipitation was performed with 2 μg of anti-V5 antibody (Invitrogen) as previously described (26, 27). After being blocked in 5% fat-free dry milk, membranes were subjected to incubation with anti-p65 antibody (1:500 dilution, C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-C/EBPβ antibody (1:500 dilution, C-19; Santa Cruz Biotechnology), anti-V5 antibody (1:1,000), or α-tubulin antibody (1:3,000; Sigma) at 4°C for 16 h with shaking. The immunoreactive signals were detected with ECL Plus Western blotting detection reagents (Amersham).

RESULTS

To examine the role of the ubiquitin-proteasome pathway in elastin gene expression, we used the specific proteasome inhibitor MG-132 (20, 29, 30, 39). Quiescent neonatal rat lung fibroblasts were treated with or without MG-132 (10 μM) for 20 h and subjected to RNA isolation and Northern blot analysis. We found that MG-132 markedly decreased the steady-state level of elastin mRNA (Fig. 1*A*). Densitometric analysis from six such experiments indicated that MG-132 downregulated elastin mRNA level by 85%. To verify that MG-132-induced downregulation of elastin mRNA resulted from a specific inhibition of proteasome function, we treated neonatal rat lung fibroblasts with two other structurally unrelated proteasome inhibitors that act via different mechanisms (10, 29, 35). We found that treatment with either epoxomicin or lactacystin decreased elastin mRNA in a dose-responsive manner that was consistent with their described functional activity (10, 15, 29, 35) (Fig. 1*B*). The expression of elastin mRNA was not altered by inhibitors of calcium-dependent calpain proteases (calpeptin, 10 μM) or lysosomal cysteine proteases (EST, 50

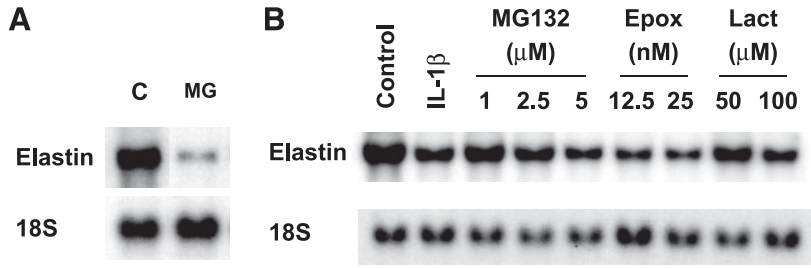

Fig. 1. *A*: inhibition of proteasome functions by MG-132 decreases the steady-state level of elastin mRNA. Quiescent lung interstitial cells (LIC) were untreated (C) or treated with 10 μM MG-132 (MG) for 20 h. Total RNA (10 μg/lane) was isolated and analyzed by Northern blot analysis using ^{32}P-labeled rat elastin cDNA. The ^{32}P-labeled 18S ribosome oligonucleotides were hybridized as a loading control. Data are representative of 6 independent experiments. *B*: effect of structurally different proteasome inhibitors on elastin mRNA levels. Quiescent LIC were untreated (C) or treated with IL-1β (250 pg/ml), MG-132 (1, 2.5, and 5 μM), epoximicin (Epox; 12.5 and 25 nM), or lactacystin (Lact; 50 and 100 μM) for 20 h. Total RNA (10 μg/lane) was prepared and subjected to sequential Northern blot analysis with ^{32}P-labeled elastin cDNA probes and oligonucleotide probe for 18S ribosome.

μM) (data not shown). These results indicated that disruption of the proteolytic function in the ubiquitin-proteasome pathway specifically downregulated elastin mRNA.

To elucidate the mechanisms whereby MG-132 repressed expression of elastin mRNA, we isolated nuclei from MG-132-treated cells and performed nuclear run-on assays. We found that MG-132 markedly decreased the rate of transcription of elastin gene (Fig. 2*A*). Densitometric analysis from three such experiments indicated that the rate of elastin gene transcription was decreased by 78%, whereas the rate of transcription of GAPDH remained unchanged. MG-132 also decreased the rate of transcription of type I collagen, but to a lesser extent. Furthermore, we analyzed elastin promoter using transient transfection, followed by MG-132 treatment and luciferase assays. MG-132 specifically decreased the luciferase activity of elastin promoter luciferase reporter PGL-2/118 by 66% (Fig. 2*B*, *left*), whereas the basal activity of the TGF-β1-responsive luciferase reporter 3TP was not affected (Fig. 2*B*, *right*). These data indicated that MG-132 inhibited elastin transcription.

To determine the effect of CHX and ActD on MG-132-induced downregulation of elastin mRNA, we pretreated neonatal rat lung fibroblasts with CHX (10 μg/ml) or ActD (15 μg/ml) for 1 h before addition of MG-132 and incubated for an additional 20 h, followed by Northern blot analysis. We found that both CHX and ActD blocked the downregulation of elastin mRNA by MG-132 (Fig. 3). CHX or ActD alone (data not shown) did not alter elastin mRNA levels. These results suggested that both new protein synthesis and global transcription were required for MG-132 to repress elastin mRNA expression.

We previously reported (26) that IL-1β activates the transcription factor NF-κB to inhibit elastin gene transcription.

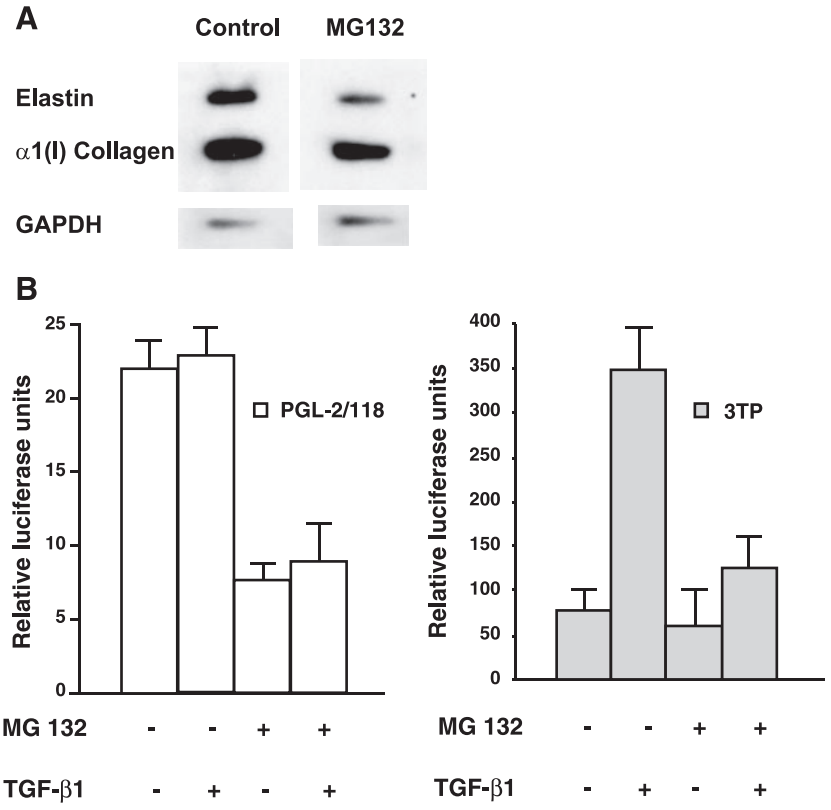

Fig. 2. Effect of MG-132 on elastin gene transcription. *A*: nuclei were isolated from LIC that were either untreated (Control) or treated with 10 μM MG-132 for 20 h, and run-on assays were performed as described in EXPERIMENTAL PROCEDURES. The labeled nuclear RNAs were hybridized to slot blots immobilized with cDNA plasmids of elastin, α1(I) collagen, GAPDH, and empty vector (pBluescript II SK; not shown). Data are representative of 3 independent experiments. *B*: effect of MG-132 on the activity of the proximal elastin promoter. LIC were cotransfected with either the elastin promoter luciferase reporter PGL-2/118 (1 μg/well; *left*) or the TGF-β-responsive luciferase reporter 3TP (1 μg/well; *right*) and pRLTK *Renilla* luciferase vector (0.25 μg/well). At 24 h after transfection, cells were treated with 10 μM MG-132, TGF-β (1 ng/ml), or both for 20 h and followed by luciferase assays with a luminometer. Firefly luciferase activities were normalized to *Renilla* luciferase activity. Data represent the average ± SE of 3 independent experiments performed in triplicate.

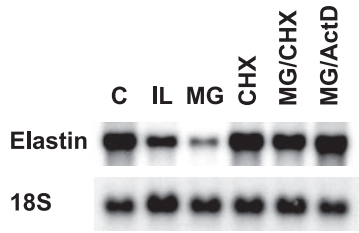

Fig. 3. Effect of cycloheximide (CHX) and actinomycin D (ActD) on the regulation of elastin mRNA by MG-132. LIC were pretreated with 10 μg/ml CHX or 15 μg/ml ActD for 1 h and then incubated with 10 μM MG-132 for an additional 20 h. Total RNA (10 μg/lane) was prepared and analyzed by Northern blot analysis using ^{32}P-labeled tropoelastin cDNA probes and 18S ribosome oligonucleotide probe.

Activation of NF-κB is mediated by degradation of its cytoplasmic inhibitory protein IκB family by the ubiquitin-proteasome pathway (41). To examine the nuclear presence of NF-κB under proteasome dysfunction, we carried out Western blot analysis using nuclear extracts from neonatal rat lung fibroblasts left untreated or treated with IL-1β or MG-132 or their combination with CHX (Fig. 4). We found that the nuclear level of NF-κB subunit p65 was not affected by MG-132 treatment (Fig. 4, *lane 7*) but was persistently increased by IL-1β, which was insensitive to CHX effect as previously described (27).

Treatment of human intestinal epithelial cells with proteasome inhibitors increased C/EBPβ nuclear protein levels and DNA binding activities (19). However, we found that MG-132 alone did not increase the level of C/EBPβ mRNA (Fig. 5, *lane 3*) and addition of MG-132 blocked increases of C/EBPβ and p65 mRNA levels by IL-1β (250 pg/ml; Fig. 5, *lanes 2* and *4*). To determine whether MG-132 increased C/EBPβ protein expression in neonatal rat lung fibroblasts, we isolated nuclear extracts after treatment with MG-132 for 20 h. Treatment with MG-132 also markedly increased two C/EBPβ LAP isoforms, full-length C/EBPβ (38 kDa) and LAP (35 kDa), and induced LIP isoform (20 kDa) (Fig. 6*A*, *lane 5*). Electrophoretic mobility shift assay was performed with a rat elastin promoter fragment (−1 to −66) and nuclear extracts from MG-132-

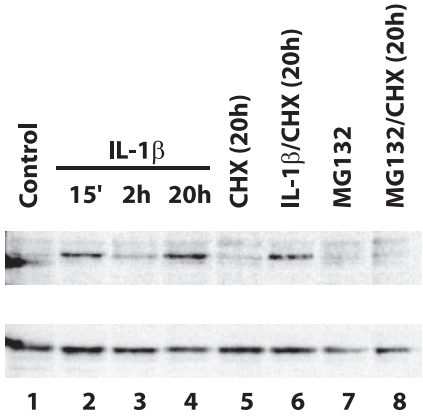

Fig. 4. Effect of MG-132 on the nuclear presence of NF-κB. LIC were untreated (Control) or treated with IL-1β (250 pg/ml), MG-132 (10 μM), CHX (10 μg/ml), or IL-1β or MG-132 in combination with CHX for indicated time periods. Nuclear extracts (30 μg/lane) were prepared and subjected to Western blot analysis with anti-p65 antibody. A cross-reactive protein (CRP) was shown, demonstrating equal loading.

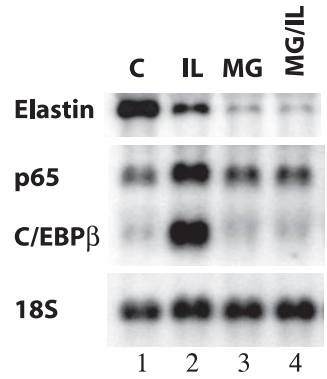

Fig. 5. Effect of MG-132 on IL-1β signaling pathway. LIC were untreated (C) or treated with IL-1β (IL; 250 pg/ml), MG-132 (10 μM), or both for 20 h. Total RNA (10 μg/lane) was isolated and sequentially analyzed by Northern blot analysis using ^{32}P-labeled cDNA probes for rat tropoelastin, p65, CCAAT/enhancer binding protein (C/EBP)β, and oligonucleotides of 18S ribosome.

treated neonatal rat lung fibroblasts, and a binding pattern of C/EBPβ proteins similar to one that we previously described (27) was found (data not shown). LIP, which lacks the NH_2-terminal activation domain but possesses the COOH-terminal DNA binding domain and the leucine zipper domain of LAP, inhibits transcription either by competing with the LAP isoforms for binding sites or by interfering with the binding of the transcriptional apparatus (11, 13). CHX and ActD (data not shown) abolished these increases of C/EBPβ proteins by MG-132 (Fig. 6*A*, *lane 6*). Notably, a unique protein complex (LIP*; Fig. 6*A*, *lane 5*) that migrates slower than LIP was only detected in the MG-132-treated but not in the IL-1β-treated nuclear extracts. These results indicated that MG-132 utilized a posttranscriptional mechanism to upregulate C/EBPβ proteins. The identity of LAP and LIP were verified by using nuclear extracts from two 3T3 L1 cell lines that constitutively express LAP or LIP (16).

To explore the possible mechanisms underlying LIP* expression, rat neonatal lung fibroblasts were transfected with a cytomegalovirus (CMV)-driven and V5-tagged LIP expression vector. The transfected cells were treated with IL-1β (250 pg/ml) or MG-132 (10 μM), and expression of LIP was determined by Western blot analysis. We detected overexpression of the fusion LIP-V5 in transfected cells but failed to detect any slow-migrating LIP*-V5 complex (Fig. 6*B*, *top*). However, MG-132 treatment induced the endogenous LIP* (Fig. 6*B*, *bottom*). These results suggest that expression of the slow-migrating LIP (LIP*) required the components from the 5′ untranslated region (UTR) of the C/EBPβ gene that is critical for differential expression of LAP and LIP (2, 7).

We examined the interaction of overexpression of LIP and treatment with IL-1β or MG-132 on the activity of the elastin promoter luciferase reporter construct (PGL-2/118). We found that overexpression of LIP markedly decreased the luciferase activity of PGL-2/118 as we reported previously (27), and this decrease was further potentiated by addition of either IL-1β or MG-132 (Fig. 6*C*, *top*). Expression of LIP-V5 fusion protein and endogenous C/EBPβ isoforms was demonstrated by immunoprecipitation using V5 antibody (Fig. 6*C*, *middle*) and Western blot analysis using C/EBPβ antibody (Fig. 6*C*, *bottom*), respectively.

We previously demonstrated (27) that the truncated C/EBPβ protein, LIP, decreases the rate of elastin transcription. To further define the relationship between C/EBPβ expression and proteasome dysfunction on the elastin transcription, we used siRNA techniques. The siRNA sequences were selected according to Elbashir et al. (14), and the double-stranded siRNA was inserted into pRNA-U6.1/Neo siRNA vector. The silencing effect of these siRNA constructs on C/EBPβ expression

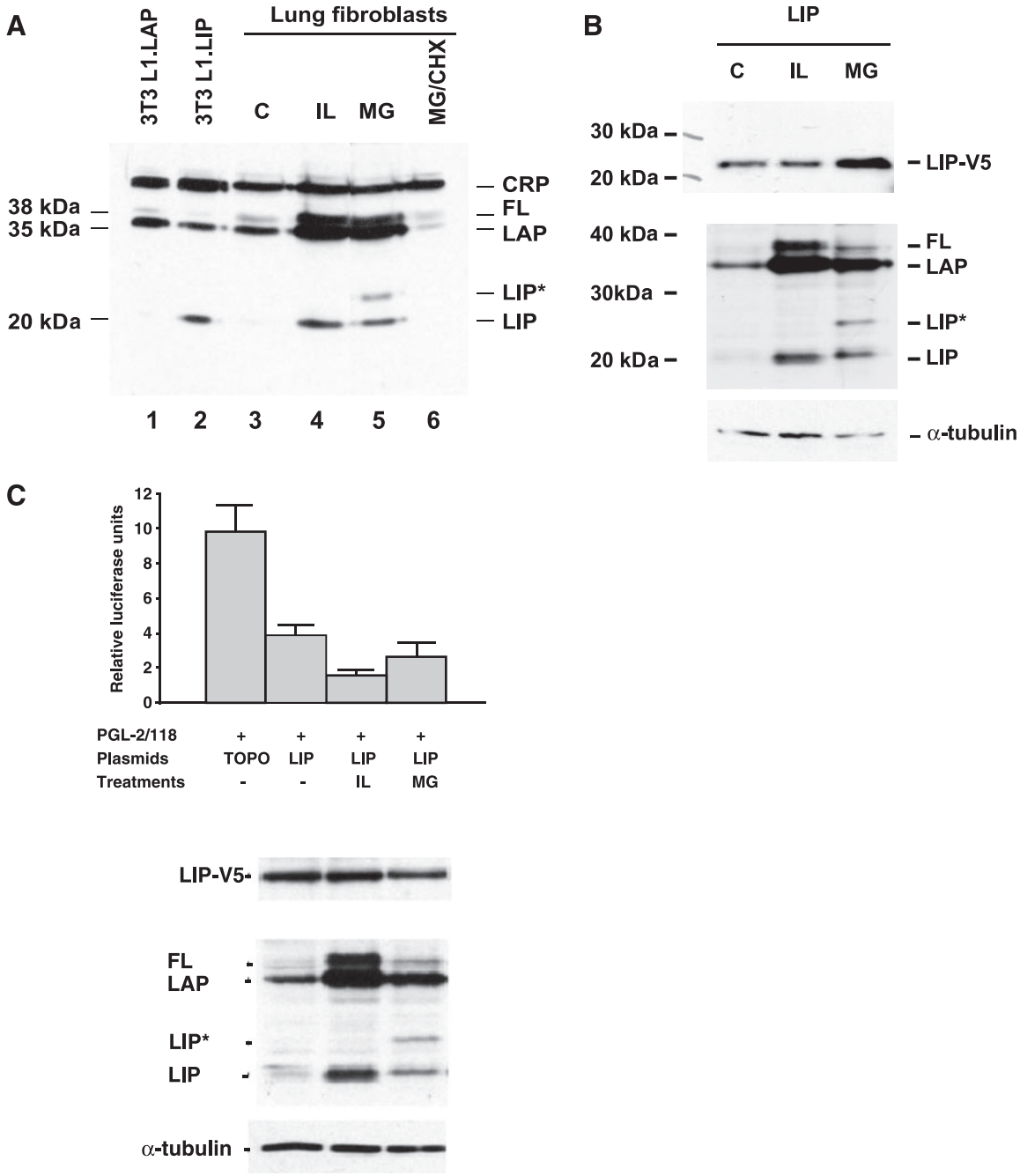

Fig. 6. MG-132-stimulated expression of C/EBPβ isoforms. *A*: LIC were untreated (C) or treated with IL-1β (250 pg/ml), MG-132 (10 μM), or MG-132 + 10 μg/ml CHX for 20 h. Nuclear extracts (30 μg/lane) were prepared and subjected to Western blot analysis with anti-C/EBPβ antibody. Nuclear extracts from 3T3 preadipocyte cell lines that stably express liver-enriched transcriptional activating protein (LAP) (3T3 L1.LAP) or liver-enriched transcriptional inhibitory protein LIP (3T3 L1.LAP) were used to confirm the identity of LAP and LIP. Also indicated were the full-length C/EBPβ (FL) and a CRP, demonstrating equal loading. *B*: LIC were transfected with pcDNATOPO-LIP-V5 expression vector and treated with IL-1β (250 pg/ml) or MG-132 (10 μM). Immunoprecipitation of whole cell lysates using anti-V5 antibody (*top*) and Western blot analysis using anti-C/EBPβ antibody (*middle*) or α-tubulin antibody (*bottom*) were performed. *C*: elastin promoter luciferase reporter PGL-2/118 and pRLTK *Renilla* luciferase vector (0.25 μg/well) were cotransfected with either parent vector pcDNATOP (TOPO) or pcDNATOP-LIP-V5 (LIP) expression vector. The cotransfected cells were treated with IL-1β or MG-132, and Western blot analysis was performed as in *B*. The firefly luciferase activity was measured and determined by normalization to the *Renilla* luciferase value as relative luciferase units (±SE of 3 independent experiments performed in triplicate) (*top*). The expression of LIP-V5 fusion protein and the C/EBPβ isoforms induced by IL-1β and MG-132 are indicated.

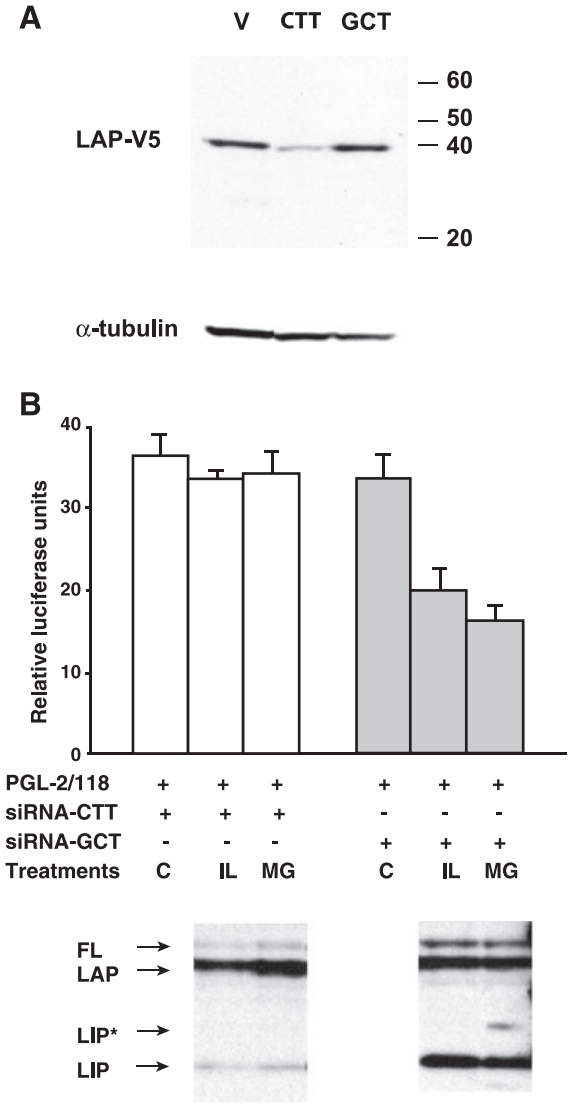

Fig. 7. Effect of C/EBPβ siRNA on ectopic C/EBPβ protein expression and elastin promoter activity. *A*: small interference RNA (siRNA)-CTT or -GCT construct (6 μg) was cotransfected with V5-tagged LAP-expressing plasmids (1 μg) into 293T cells in P-60 dishes. At 48 h after transfection, whole cell lysates (80 μg/lane) were prepared and subjected to Western blot analysis using anti-V5 antibody. *B*: proximal elastin promoter luciferase reporter PGL-2/118 (0.5 μg/well), siRNA-CTT or -GCT construct (3 μg/well), and pRLTK *Renilla* luciferase vector (0.25 μg/well) were cotransfected. At 24 h after transfection, cells were untreated (C) or treated with IL-1β (250 pg/ml) or MG-132 (10 μM) for 20 h. Firefly luciferase activities were measured and determined by normalization to *Renilla* luciferase activity as relative luciferase units (±SE of 4 independent experiments performed in triplicate) (*top*). The effect of siRNA on endogenous C/EBPβ protein expression was determined by Western blot analysis of whole cell lysates isolated from parallel experiments using anti-C/EBPβ antibody (*bottom*).

was tested by cotransfection with LAP-V5 TOPO expression vector into 293T cells and analyzed by Western blot analysis. C/EBPβ siRNA-CTT construct suppressed the expression of LAP-V5, whereas C/EBPβ siRNA-GCT construct had no effect on LAP-V5 expression (Fig. 7*A*). The effect of siRNA-CTT construct on MG-132- or IL-1β-induced elastin promoter inhibition was examined by cotransfection of elastin promoter luciferase reporter PGL-2/118 and the siRNA construct into neonatal rat lung fibroblasts. Overexpression of C/EBPβ siRNA-CTT construct completely blocked the decrease of luciferase activities by MG-132 or IL-1β, whereas C/EBPβ siRNA-GCT construct had only minimal effect (Fig. 7*B*, *top*). The effect of these siRNA constructs on endogenous C/EBPβ proteins was demonstrated by Western blot analysis of whole cell lysates with C/EBPβ antibody (Fig. 7*B*, *bottom*).

DISCUSSION

We demonstrate herein that proteasome inhibitors specifically decrease the steady-state level of elastin mRNA in neonatal rat lung fibroblasts. Treatment with MG-132 decreased the rate of elastin transcription as determined by nuclear run-on assay and promoter analysis. Inhibition of protein synthesis by CHX and inhibition of transcription by ActD blocked the downregulation of elastin mRNA by MG-132. The level of C/EBPβ proteins, but not mRNA, was strongly increased by MG-132, which was abolished by CHX. Overexpression of an effective C/EBPβ siRNA abrogated the inhibition of elastin promoter activity by MG-132. These results indicate that disruption of the proteolytic function in the ubiquitin-proteasome pathway inhibits tropoelastin gene transcription via upregulation of C/EBPβ proteins and suggest that the ubiquitin-proteasome pathway plays an essential role during maintenance of elastin homeostasis.

In vivo, C/EBPβ proteins dimerize among themselves or with other C/EBP family proteins and exist as homo- or heterodimers. Self-dimerization or dimerization with other C/EBP family members increases the stability of C/EBPβ proteins and protects them from ubiquitin-proteasome degradation (17). Treatment with MG-132 did not increase the level of ectopic expressed C/EBPβ or generate any higher-molecular-weight form of C/EBPβ (ubiquitin linked), whereas the levels of ectopic expressed Ig/EBP and C/EBP homologous protein (CHOP), which lack self-dimerization and exist as monomers, were markedly increased and ubiquitin-marked by MG-132 treatment (17). These findings indicate that the proteasome dysfunction does not upregulate C/EBPβ proteins in lung fibroblasts via decreases in degradation. The molecular mechanisms underlying turnover of the functional dimeric C/EBPβ complexes and regulation of their availability are not understood. During transcription initiation, DNA-bound C/EBPβ complexes may be specifically subjected to various modifications, such as phosphorylation, acetylation, or methylation by coregulators such as p300/CBP or other components in general transcription machinery. Such modifications may lead to a conformational change of C/EBPβ that causes loss of their DNA binding activity or dimerization ability. Inhibition of DNA binding activity may temporarily disable dimeric C/EBPβ complexes to function as transcriptional regulators, whereas permanent loss of dimerization property could be an essential step for irreversible termination by the ubiquitin-proteasome pathway.

Our results showed that the expression pattern of C/EBPβ protein induced by MG-132 was similar to that induced by IL-1β, except for the appearance, in MG-132-treated nuclear extracts, of a unique anti-C/EBPβ reactive complex (LIP*) with a slightly higher molecular weight than LIP. There is no additional potential in-frame translation start codon ATG located between ATG of LAP and ATG of LIP. We did not detect any ubiquitin-marked C/EBPβ proteins (higher molec-

ular weight), suggesting that the turnover of C/EBPβ proteins is not mediated by the ubiquitin-proteasome pathway and indicating that certain posttranslational modification may specifically occur to LIP during proteasome dysfunction. The 5′ UTR of C/EBPβ gene is critical for the differential expression of C/EBPβ proteins (2, 7). The expression of slow-migrating LIP* may be controlled by the components of 5′ UTR in the C/EBPβ gene, because MG-132 failed to induce such a slow-migrating LIP*-V5 complex from rat neonatal lung fibroblasts that were transfected with a CMV-driven and V5-tagged LIP expression vector. The biological properties of this modification are not clear and are currently under investigation in this laboratory. It has been reported that the addition of the small ubiquitin-like modifier (SUMO) to both C/EBPα and C/EBPβ LAPs can specifically alter their functional roles during regulation of gene transcription (24, 43). It is possible that LIP can also be subjected to sumoylation to modulate its gene transcriptional regulation.

Proteasome inhibitors increased levels of C/EBPβ isoforms and their DNA binding activities in human intestinal epithelial cells (19). It was reported that proteasome dysfunction caused endoplasmic reticulum (ER) stress (6, 23), a cellular response to accumulation of unfolded and denatured proteins that are normally degraded by the ubiquitin-proteasome pathway. Interestingly, C/EBPβ gene transcription was induced via an unfolded protein response element harbored in its 3′ UTR (8). However, our data showed that MG-132 did not alter C/EBPβ mRNA but markedly increased the levels of LAPs and induced LIP expression that were CHX sensitive. These results suggest that, in these rat lung fibroblasts, ER stress induced by MG-132 may increase C/EBPβ mRNA translation or its stability. The LIP isoform lacks most of the NH_2-terminal transactivation domain, enabling it to act as a dominant-negative isoform for LAP isoform and other bZIP transcription factors. LIP is generated by alternative translational initiation at the third in-frame AUG during a variety of important cellular processes in response to physiological and pathological stimulations (2, 7, 13, 42, 45). The specific RNA binding protein CUG-BP1, which binds to CUG repeats within the 5′ UTR of C/EBPβ mRNA, may function as a key regulator of LIP expression (2). In addition, two short open reading frames located in this 5′ UTR may also be essential for induction of truncated C/EBP isoforms (7). It is possible that these molecular mechanisms may be utilized to initiate LIP translation under the inhibition of proteolytic function of proteasome.

We previously demonstrated (26, 27) that IL-1β-induced upregulation of C/EBPβ proteins LAPs and LIP and downregulation of tropoelastin transcription are dependent on NF-κB activation. Proinflammatory stimuli failed to induce C/EBPβ mRNA in $p65^{-/-}$ fibroblasts (25). Interestingly, upregulation of C/EBPβ proteins by MG-132 appears to be independent of the NF-κB pathway, because MG-132 increased neither the steady-state level of p65 mRNA nor the nuclear NF-κB level (Fig. 4). Moreover, MG-132 blocked the activation of NF-κB by IL-1β, as indicated by abrogation of increases of C/EBPβ and p65 mRNA (Fig. 5, *lane 4*). Activation of NF-κB pathway by cytokines, such as IL-1β and TNF-α, occurs via ubiquitin-proteasome pathway-dependent degradation of its cytoplasmic inhibitory protein IκB family (22).

We have also shown (27) that IL-1β inhibits elastin transcription by increasing C/EBPβ isoforms and binding of the inhibitory complexes LIP-LAP and LIP-LIP to a GCAAT element located within the proximal elastin promoter at −56 to −62 bp. In the present study, we used an RNA interference technique and further demonstrated that downregulation of elastin gene transcription by MG-132 or IL-1β is dependent on the expression of C/EBPβ proteins. The molecular basis of this C/EBPβ-dependent inhibition of elastin transcription is not known. Both transactivation and leucine zipper domains of C/EBPβ are required for cooperative activation of rat CYP2D5 gene by Sp1 and C/EBPβ LAP isoforms (29). Therefore, LIP-containing inhibitory complexes may recruit other mediator complexes to change DNA structure in the elastin promoter to disrupt the association of Sp1 enhancesome or indirectly block its transactivation of transcriptional apparatus. In summary, for the first time, we demonstrate here that the proteolytic function of the ubiquitin-proteasome pathway is essential for normal elastin gene expression. Disruption of this pathway may cause an ER stress response to upregulate C/EBPβ proteins LAPs and LIP and downregulate elastin biosynthesis.

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