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Kuang, Ping-Ping, John L. Berk, David C. Rishikof, Judith A. Foster, Donald E. Humphries, Dennis A. Ricupero, and Ronald H. Goldstein. NF-κB induced by IL-1β inhibits elastin transcription and myofibroblast phenotype. *Am J Physiol Cell Physiol* 283: C58–C65, 2002; 10.1152/ajpcell.00314.2001.—Interleukin (IL)-1β released after lung injury regulates the production of extracellular matrix components. We found that IL-1β treatment reduced the rate of elastin gene transcription by 74% in neonatal rat lung fibroblasts. Deletion analysis of the rat elastin promoter detected a *cis*-acting element located at −118 to −102 bp that strongly bound Sp1 and Sp3 but not nuclear factor (NF)-κB. This element mediated IL-1β-induced inhibition of the elastin promoter. IL-1β treatment did not affect the level of Sp1 but did induce translocation of the p65 subunit of NF-κB. Overexpression of p65 decreased elastin promoter activity and markedly reduced elastin mRNA. Immunoprecipitation studies indicated an interaction between the p65 subunit and Sp1 protein. Microarray analysis of mRNA isolated after overexpression of p65 or treatment with IL-1β revealed downregulation of α-smooth muscle actin and calponin mRNAs. Expression of these genes is associated with the myofibroblast phenotype. These results indicate that IL-1β activates the nuclear localization of NF-κB that subsequently interacts with Sp1 to downregulate elastin transcription and expression of the myofibroblast phenotype.

nuclear factor-κB; interleukin-1β; Sp1

ELASTIN IS a major structural protein in the lung. It is found most notably in alveolar walls and blood vessels. Tropoelastin, a soluble precursor, is synthesized in alveolar structures by interstitial fibroblasts and in vascular tissue by smooth muscle cells (4, 34, 35). Elastin synthesis in the pulmonary parenchyma of the rodent lung is highest during alveolarization. This process usually begins in the postnatal period and decreases with maturity (4, 25). Disruption of elastin deposition during development results in failure of alveolar formation (19). In the adult lung parenchyma, elastin mRNA is minimally expressed in interstitial structures but can be reactivated during the development of pulmonary emphysema or fibrosis (18, 20).

We previously reported (20) that elastin and collagen mRNA levels are upregulated after bleomycin treatment of rodent lungs. This expression was confined primarily to myofibroblasts. The myofibroblast phenotype is characterized by α-smooth muscle actin expression (28). Myofibroblasts appear to be responsible for matrix deposition during wound healing (31). In the adult lung, elastin mRNA levels can be modulated by effector substances released from macrophages or resident interstitial cells or from the extracellular matrix after proteolytic injury. Elastin mRNA can be upregulated by insulin-like growth factor, transforming growth factor (TGF)-β, and retinoic acid (13, 16, 22) and downregulated by basic fibroblast growth factor (7) and interleukin (IL)-1β (3).

IL-1β affects gene transcription via several different families of *trans*-acting factors including AP-1 proteins, nuclear factor (NF)/IL-6-related factors [CCAAT box/enhancer binding protein (C/EBP)-α and C/EBP-β], and NF-κB protein complexes. NF-κB binds to DNA as a homo- or heterodimer in a number of cell types (33). The p50/p65 dimeric complex is the most transcriptionally active and abundant NF-κB isoform found in cells. The p65 component contains two or three independent transactivation domains. In quiescent cells, NF-κB is maintained in the inactive state in the cytosol bound to inhibitor IκBα/IκBβ. In the present studies, we found that IL-1β treatment decreased elastin transcription and induced large increases in the nuclear localization of the p65 subunit of NF-κB levels. Overexpression of the NF-κB component p65 resulted in the downregulation of elastin mRNA levels and the myofibroblast phenotype.

METHODS

Cell culture. Lung fibroblasts were isolated from the lungs of 8-day-old Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) as previously described (3). The fibroblasts were maintained in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 0.37 g sodium pyruvate/100 ml, 100 U penicillin/ml, and 100 μg streptomycin/ml in a humidified 5% CO_2-95% air

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incubator at 37°C. Confluent cultures were rendered quiescent by reducing the serum content of the medium to 0.4% for 24 h. The purity of the cultures was assessed with phase microscopy and Oil Red O staining.

Transfection procedures. Luciferase reporter constructs driven by the elastin promoter were prepared from a 1,028-bp rat elastin promoter (kindly provided by Dr. Charles D. Boyd, University of Hawaii, Honolulu, HI). Elastin promoter fragments −990 to −1, −535 to −1, −216 to −1, −133 to −1, −118 to −1, −115 to −1, −114 to −1, −102 to −1, and −66 to −1 (relative to the elastin translational start site) were inserted into PGL-2 basic luciferase reporter plasmid (Promega, Madison, WI), referred to as 990-Lux, 535-Lux, 216-Lux, 133-Lux, 118-Lux, 115-Lux, 114-Lux, 102-Lux, and 66-Lux, respectively. The 114A mutant involved changing the flanking sequences for the wild-type GC box from CTCCCACCCGCCCTCTC to CTCCATTCCGCCCACTC as previously described (14). The 114C mutant involved changing the wild-type sequence to CTCCGCCCCGCCCCCTC. All constructs were verified by sequencing. Transient transfection and luciferase assays were performed in triplicate as previously described (17).

RNA isolation and analysis. Total cellular RNA prepared with the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol was used for Northern blotting or microarray analysis. We used a rat microarray chip (Affymetrix rat genome RG-U34A containing 7,000 genes). The analysis was performed by the Partners Gene Array Technology Center (Brigham and Woman's Hospital, Boston, MA) with the standard Affymetrix protocol.

Western blot analysis. Confluent cultures of fibroblasts were treated with IL-1β (250 pg/ml). Cells were homogenized in *buffer A* [20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.1% NP-40, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mg leupeptin, 1 mg pepstatin, and 2 mM Na_3VO_4]. The homogenate was centrifuged (500 *g*, 5 min), and the pellets were suspended in *buffer C* (*buffer A* with 25% glycerol). Nuclear proteins were extracted in 300 mM NaCl on ice for 30 min followed by centrifugation (17,000 *g*, 20 min, 4°C). Cytoplasmic and nuclear extracts were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with anti-IκBα (New England Biolabs, Beverly, MA) or anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Nuclear run-on analysis. Isolated nuclei (~5×10^6 nuclei/sample) were resuspended in 200 μl of glycerol buffer containing 50 mM Tris-Cl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol and stored in liquid nitrogen. The nuclear run-on transcription assay was performed according to the methods outlined by Greenberg and Ziff (9) and Groudine et al. (10) with modifications as previously described (17). cDNA inserts coding for elastin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were bound to a nitrocellulose filter with a slot blot apparatus.

Electrophoretic mobility shift assay. Nuclear extracts from confluent cultures of fibroblasts that were untreated or treated with IL-1β (R&D Systems, Minneapolis, MN) were allowed to bind to radiolabeled double-stranded DNA containing consensus binding sequences of the targeted transcription factor. Each binding reaction (20 μl) included labeled DNA (1×10^5 cpm/0.5–1 ng) and 10 μg of nuclear extracts in solution with 1 μg of poly(dI-dC), 10 mM HEPES, pH 7.9, 20% glycerol, 0.1% NP-40, 70 mM NaCl, 1 mM EDTA, and 1 mM DTT. After incubation at room temperature for 30 min, DNA-protein complexes were resolved on a preelectrophoresed 4% nondenaturing polyacrylamide gel. For supershift experiments, antibodies (Santa Cruz Biotechnology) and nuclear extracts were incubated for 30 min at 4°C before binding reactions were initiated.

Immunoprecipitation. Nuclear extracts (500 μg) were incubated with 10 μg of anti-p65 antibodies (Santa Cruz Biotechnology) in lysis buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 0.25 mM PMSF, 1 mg/ml aprotinin, leupeptin, and pepstatin, and 2 mM Na_3VO_4) at 4°C for 16 h with end-to-end rotation. The precipitates were collected by centrifugation and washed with lysis buffer at 4°C, boiled in 50 μl of electrophoresis sample buffer, separated in 8% SDS-PAGE, and transferred to nitrocellulose membrane. Western blot analysis was performed with anti-Sp1 and anti-p65 antibodies.

Oligonucleotide agarose conjugate pull-down assay. Sp1 consensus or Sp1 mutated oligonucleotide-agarose conjugate (100 μg) was incubated with 500 μg of nuclear extracts (IL-1β treated or untreated) in binding buffer [10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 50 μg/ml poly(dI-dC), 1 mg/ml aprotinin, leupeptin, and pepstatin, and 2 mM Na_3VO_4] at room temperature for 2 h with gentle rotation. Samples were centrifuged (17,000 *g*) for 3 min and washed three times with binding buffer at 4°C, proteins were eluted with 300 μl of elution buffer (binding buffer supplemented with 150 mM NaCl), and the supernatants were concentrated with Centricon YM-3 (Millipore, Bedford, MA).

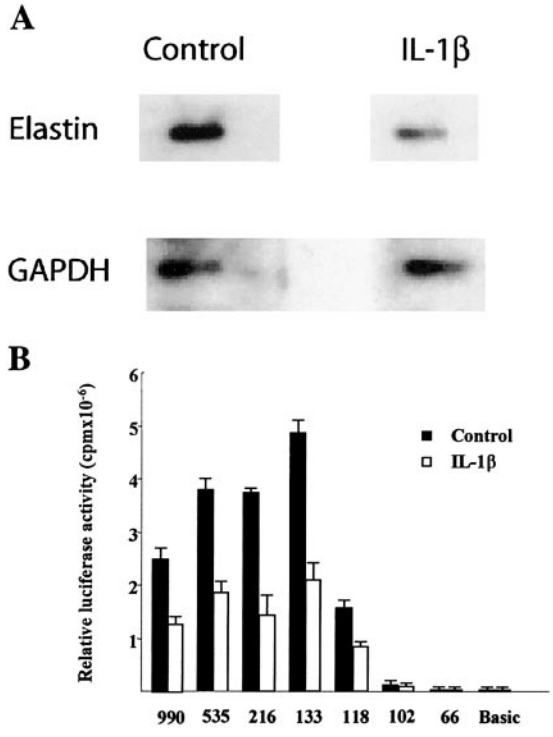

Fig. 1. Effect of interleukin (IL)-1β on elastin transcription. *A*: rate of transcription was assessed by nuclear run-on assay. Confluent cultures of lung myofibroblasts were incubated without (control) or with IL-1β (250 pg/ml). After 24 h, the nuclei were harvested and the level of transcription was assessed for elastin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are representative of 4 experiments. *B*: effect of IL-1β on the transcriptional activity of the rat elastin promoter. Neonatal rat lung fibroblasts were transfected with indicated elastin promoter fragments linked to a luciferase reporter (and with β-galactosidase). The cultures were untreated (control) or treated for 24 h with IL-1β (250 pg/ml). Luciferase activity was adjusted for β-galactosidase activity. Data are means + SE; $n = 3$.

Proteins were resolved by SDS-PAGE and subsequently identified by Western blot analysis.

RESULTS

We previously reported (3) that IL-1β treatment decreased elastin mRNA and protein levels in rat neonatal lung myofibroblasts. To assess the effect of IL-1β on the rate of transcription of the elastin gene, we performed nuclear run-on assays. We found that IL-1β treatment caused a marked decrease in the rate of elastin gene transcription (Fig. 1*A*). Densitometry analysis from four such experiments revealed that the rate of transcription of the elastin gene was reduced by 74%, whereas the rate of transcription of GAPDH did not change. No hybridization was detected to plasmids without inserts. These results parallel the effect of IL-1β on steady-state elastin mRNA levels (3).

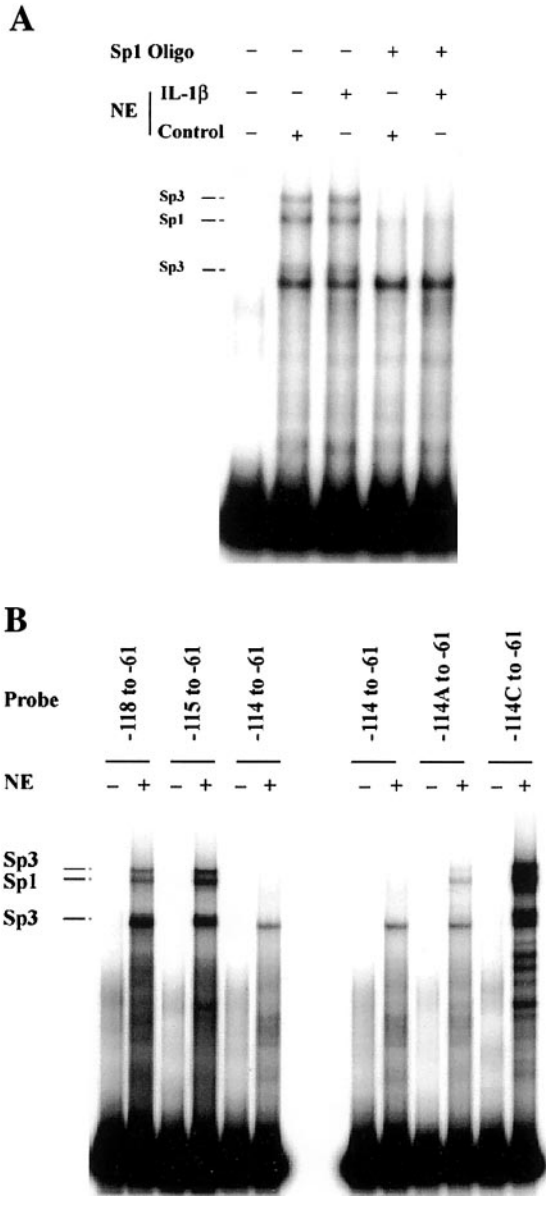

Fig. 2. Effect of IL-1β on nuclear protein binding to elastin promoter fragments. Nuclear extracts (NE) isolated from untreated (control) and IL-1β-treated cultures were bound to ^{32}P-labeled elastin promoter fragments. The bound complexes were resolved on a 4% native polyacrylamide gel. *A*: competition was performed with the 118-bp elastin promoter fragment and unlabeled oligonucleotides (100-fold excess) encoding consensus Sp1 sequence as shown. *B*: binding of Sp1 and Sp3 to mutated elastin promoter fragments. Wild-type fragments are shown on *left*, and mutated fragments (−114A and −114C) are shown on *right*. Results are representative of 3 experiments.

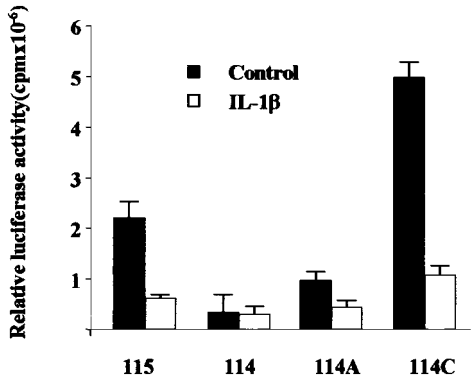

Fig. 3. Transcriptional activity of wild-type and mutated rat elastin promoter-luciferase constructs. Subconfluent cultures of fibroblasts were transfected with 2 μg of luciferase reporter constructs as indicated. Transfection efficiency was assessed by cotransfection with β-galactosidase. The cultures were untreated (control) or treated with IL-1β (250 pg/ml). After 24 h, the luciferase activity generated by the elastin promoter fragments was assessed. Data are means + SE; $n = 4$.

To identify the IL-1β-responsive elements in the elastin promoter, fragments of the elastin promoter were synthesized by PCR and cloned into the luciferase reporter construct (Lux). We assessed the effect of IL-1β (250 pg/ml) on the transcriptional activity of these various promoter constructs after transfection (Fig. 1*B*). High levels of luciferase activity were detected in quiescent fibroblasts that were transfected with constructs containing the elastin promoter sequence from −1 (relative to the translational start site) to −118 bp or larger. Treatment with IL-1β resulted in a 50–60% decrease of luciferase activity generated by these constructs. In contrast, the basal luciferase activities were 15- and 20-fold lower in the fibroblasts transfected with the 102-Lux reporter or the 66-Lux reporter, respectively, and decreased <10% after treatment with IL-1β. These data suggest that a *cis*-element located between −118 and −102 bp plays a major role in the inhibition of elastin gene expression by IL-1β in neonatal rat lung fibroblasts. IL-1β did not affect the transcriptional activity of a control luciferase vector utilizing an SV-40 promoter (data not shown).

To determine the mechanism by which IL-1β attenuated the basal activity of the elastin promoter, the binding of nuclear proteins from untreated and IL-1β-treated fibroblasts to the promoter was examined by electrophoretic mobility shift assay (EMSA) with the elastin promoter fragment −118 to −61 bp (Fig. 2*A*). Nuclear proteins from IL-1β-treated fibroblasts formed complexes comparable to the complexes observed with nuclear extracts from untreated fibroblasts. The spec-

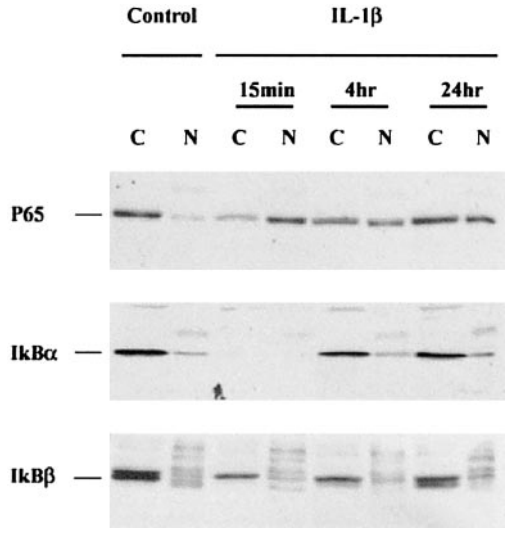

Fig. 4. Expression of p65, IκBα, and IκBβ after IL-1β treatment. Cytosolic (C) and nuclear (N) proteins were isolated from confluent cultures of fibroblasts at various times after IL-1β exposure and analyzed by Western blotting. Membranes were probed with an antibody directed against the p65 subunit of NF-κB, an antibody against IκBα, or an antibody directed against IκBβ. Data are representative of 3 experiments.

ificity of Sp1 binding was demonstrated by successful competition with an oligonucleotide containing the Sp1 binding consensus sequence (Fig. 2*A*) and by the addition of Sp1 and Sp3 antibodies (data not shown).

To further explore this unique region, we performed EMSA with additional elastin promoter fragments. We found that the −118 to −61 bp and the −115 to −61 fragments bound Sp1 and Sp3. In contrast, the binding of Sp1 and Sp3 to the −114 to −61 bp fragment was markedly attenuated (Fig. 2*B*). The elastin promoter region −118 to −102 bp contains three overlapping elements that bind Sp1: CTCCC, CACCC, and CGCCC. Although the affinity of Sp1 varies, each of these elements can serve as partial Sp1 binding sites to control the expression of a wide variety of genes (24). However, the −114 to −61 fragment did not bind Sp1 or Sp3. To further examine the elastin promoter sequence −114 to −102 bp, the flanking nucleotides were mutated so that the GC box was flanked by either an AT-rich sequence (designated 114A) or a GC-rich sequence (designated 114C). The flanking regions were shown to contribute to Sp1 binding (14). Sp1 binding to the 114A fragment was slightly increased, whereas Sp1 binding to the 114C fragment was dramatically increased.

The contribution of Sp1 binding to promoter activity was assessed with luciferase assays. The luciferase reporter driven by wild-type (115-Lux and 114-Lux) or mutated (114A-Lux and 114C-Lux) promoter fragments was transfected into fibroblasts. The 115-Lux reporter generated high basal luciferase activity, whereas the 114-Lux reporter showed a five- to sixfold reduction in basal luciferase activity (Fig. 3). Notably, the mutated 114C-Lux reporter displayed a large increase in basal luciferase activity and the 114A-Lux reporter showed a small increase in luciferase activity. The luciferase activities of the 114C-Lux, 114A-Lux, and 115-Lux reporters were significantly decreased by IL-1β treatment, whereas IL-1β treatment minimally decreased luciferase activity of the 114-Lux reporter.

We examined the activation of NF-κB by IL-1β by following the nuclear accumulation of the p65 subunit

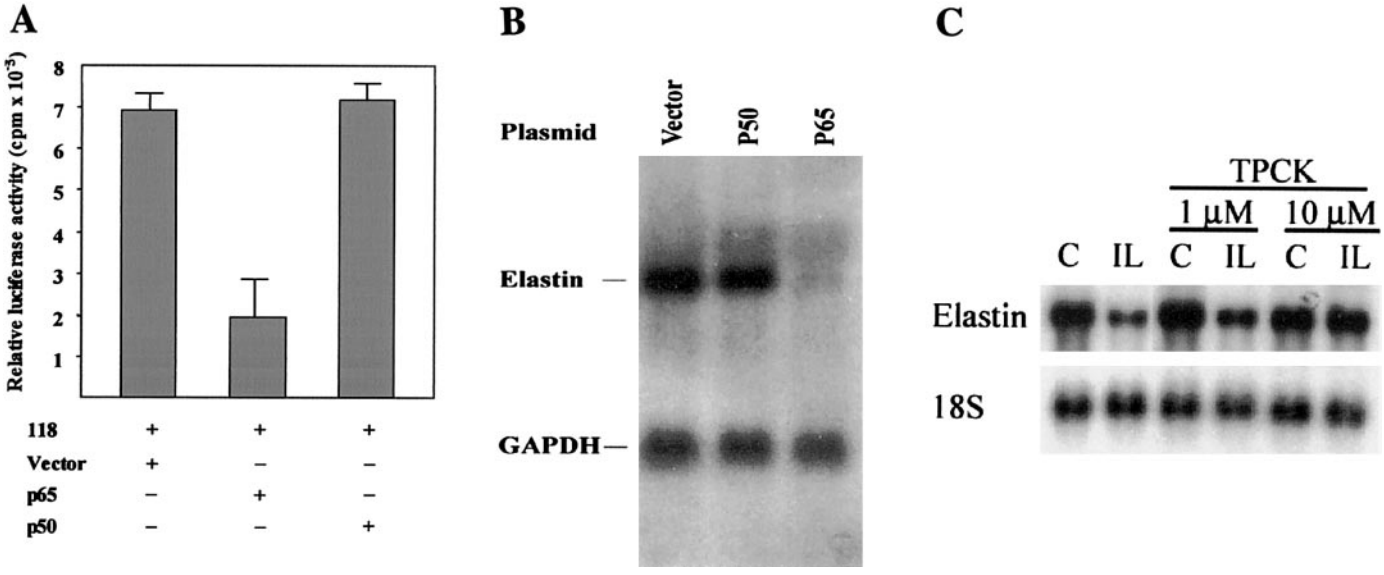

Fig. 5. Effect of overexpression of the p65 subunit on the activity of the elastin promoter. *A*: myofibroblasts were cotransfected with the 118-Lux elastin promoter construct and either the p65 or p50 expression construct or empty vector. After 24 h, luciferase activity was determined. Data are means + SE; $n = 4$. *B*: elastin mRNA levels after transfection of p50 or p65 expression vectors. After 24 h, the RNA was isolated and probed by Northern blotting for elastin and GAPDH. Data are representative of 3 experiments. *C*: elastin mRNA levels after treatment with IL-1β and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) at the indicated concentrations. After 24 h, the RNA was isolated and probed by Northern blotting for elastin and 18S. C, control (untreated); cpm, counts/min.

and the levels of IκBα and IκBβ in the cytosol. Western blot analysis indicated that 15 min after stimulation with IL-1β, p65 appeared in the nucleus with a decrease of p65 in the cytosol (Fig. 4). Concomitant with the movement of p65, the IκBα subunit disappeared from the cytosol. After 4 h, the level of p65 found in the nucleus remained unchanged but the level of p65 in the cytosol increased. After 24 h, the distribution of p65 and IκBα remained comparable to that observed at 4 h. The distribution and level of expression of IκBβ was not affected by treatment with IL-1β.

EMSA did not detect binding of p65 or p50 NF-κB subunits to the elastin promoter (data not shown). However, Sp1 interacts with certain transcription factors including the p65 subunit of NF-κB (2, 5, 26, 27). To determine the effect of p65 on elastin gene expression, fibroblasts were transfected with both a p65 expression vector and the elastin promoter driving the luciferase reporter 118-Lux (Fig. 5*A*). Cotransfection of p65 but not p50 markedly decreased the luciferase activity of the 118-Lux reporter (73%; $P < 0.05$). Thus the overexpression of the p65 subunit attenuated luciferase activity of the elastin promoter. In addition, the steady-state level of elastin mRNA was strongly reduced in fibroblasts transfected with the p65 expression vector but only slightly decreased in fibroblasts transfected with the p50 subunit (Fig. 5*B*). Addition of *N*-tosyl-L-phenylalanine chloromethyl ketone, an inhibitor of IκB degradation (36), blocked the IL-1β-induced decreases in elastin mRNA levels (Fig. 5*C*).

To demonstrate an interaction between Sp1 and the p65 subunit of NF-κB, nuclear extracts were immunoprecipitated with an anti-p65 antibody. The immunoprecipitated proteins were Western blotted with anti-p65 antibodies and anti-Sp1 antibodies. Sp1 was observed in the anti-p65 immunoprecipitate of the nuclear extracts from untreated and IL-1β-treated fibroblasts. We found that p65 was increased in nuclear extracts derived from IL-1β-treated cells (Fig. 6*A*). We also examined the interaction of Sp1 with the p65 subunit by using agarose beads conjugated to a consensus Sp1 binding oligonucleotide or an oligonucleotide that does not bind Sp1. Nuclear extracts from untreated and IL-1β-treated fibroblasts were incubated with the conjugated beads, and Western blotting identified the bound proteins. Sp1 bound to the agarose beads incubated with the nuclear extracts from both the untreated and IL-1β-treated fibroblasts. In contrast, the p65 subunit was not detected in the nuclear extracts from untreated fibroblasts but was detected in the complex formed by the oligonucleotide-agarose and the nuclear extracts of IL-1β-treated fibroblasts (Fig. 6*B*). Sp1 and p65 could not be detected in the complexes formed by the non-Sp1-binding oligonucleotide-agarose incubated with nuclear extracts from either untreated or IL-1β-treated fibroblasts.

Treatment of fibroblasts with IL-1β is reported to inhibit expression of the myofibroblast phenotype as assessed by levels of α-smooth muscle actin mRNA (37). To determine the effect of p65 overexpression and IL-1β treatment on the myofibroblast phenotype, total

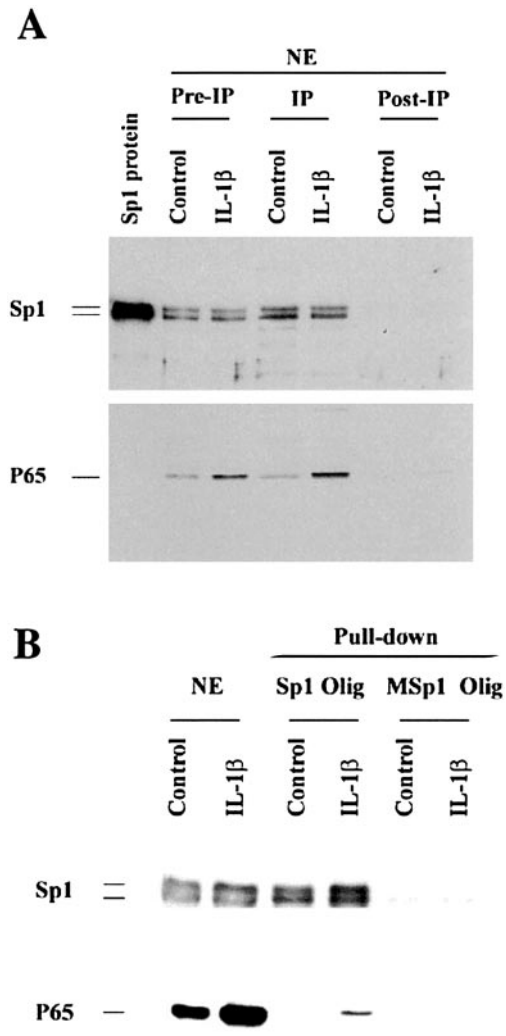

Fig. 6. Interaction between p65 and Sp1. Nuclear proteins (NE) were isolated from confluent cultures of myofibroblasts that were untreated (control) or treated with IL-1β (250 pg/ml) for 24 h. *A*: NE were immunoprecipitated (IP) with an anti-p65 antibody. Western blotting was performed with Sp1 and p65 antibodies as shown. *B*: NE were incubated with wild-type or mutated Sp1 (MSp1)-binding oligonucleotides conjugated to agarose beads (pull-down assay) and isolated by centrifugation. The complexes were Western blotted with anti-p65 or anti-Sp1 antibodies.

RNA was isolated and analyzed by microchip gene array. Overexpression of p65 and treatment with IL-1β increased levels of several genes known to be transactivated by NF-κB including cyclooxygenase-2 and inducible nitric oxide synthase (Table 1). In addition, we found that overexpression of p65 and treatment with IL-1β caused dramatic decreases in mRNA associated with the myofibroblast phenotype including α-smooth muscle actin and calponin (23, 31). Northern blot analysis confirmed that IL-1β treatment decreased α-smooth muscle actin and calponin mRNA levels (Fig. 7). Results from three separate experiments indicated that α-smooth muscle actin mRNA levels were decreased by 85 ± 7% and calponin mRNA by 63 ± 9% (mean ± SE; $n = 3$).

Table 1. *Microchip array analyses*

Gene Description (accession number)	Overexpression of p65, fold change from empty vector control	IL-1β, fold change from untreated control
Ornithine decarboxylase (J04792)	+14	+29
Macrophage inflammatory protein-2 (U45965)	+11	+192
Chemokine CX3C (AF030358)	+9	+28
Inducible nitric oxide synthase (U03699)	+8	+9
Prostacyclin receptor (D28966)	+6	+3
Cyclooxygenase-2 (L25925)	+6	+46
CC chemokine ST38 precursor (AF053312)	+5	+134
Decorin (X59859)	+5	+10
α-Smooth muscle actin (X06801)	−17	−40
α1(I) collagen (Z78279)	−16	−3
Tropoelastin (J04035)	−12	−4
α1(III) collagen (X70369)	−12	−4
Connexin 40 (AF022136)	−11	+9
G protein-coupled thrombin receptor (M81642)	−8	−10
Tenascin (U09401)	−5	+11
Lysyl oxidase (S66184)	−5	−1
Calponin (D14437)	−5	−13
Myosin regulatory light chain isoform C (S77900)	−5	−38
Vascular cell adhesion molecule-1 (X63722)	−4	−2
α-Tropomyosin 2 (M60666)	−4	−6
Tropmyosin (TM-4) (M15474)	−3	−5

Lung fibroblasts were transfected with either an empty vector or p65 expression construct, additional cultures were untreated or treated with interleukin (IL)-1β (250 pg/ml). After 24 h, the RNA was isolated and probed with a microchip array as described in text. Fold change is shown as compared with transfected empty vector or the untreated cultures, respectively.

DISCUSSION

Myofibroblasts synthesize extracellular matrix components during alveolar development and fibrogenic reactions (31). Neonatal rat lung fibroblasts express the myofibroblast phenotype in culture (33). We previously reported (3) that IL-1β reduced the steady-state levels for elastin mRNA. In the present study, we found that this effect was mediated primarily by decreases in the rate of elastin gene transcription. IL-1β treatment induced a 74% decrease in transcriptional activity as assessed by nuclear run-on assay. Similar to the human elastin gene, the proximal region of the rat elastin promoter is GC rich and contains an atypical TATA box sequence (ATAAA) (21). This sequence serves as a functional TATA box motif in other genes such as the β-globin gene, as it may in the elastin gene.

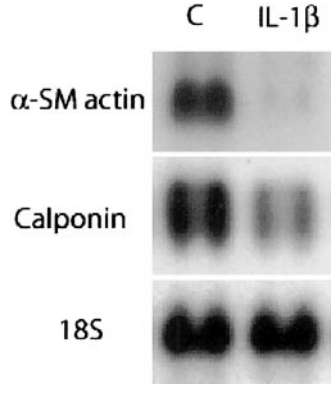

Fig. 7. Levels of α-smooth muscle actin (α-SM actin) and calponin mRNA after treatment with IL-1β. Confluent cultures of lung fibroblasts were incubated without (C) or with IL-1β (250 pg/ml). After 24 h, the RNA was isolated and probed by Northern blotting for α-smooth muscle actin, calponin, and 18S.

Multiple transcription initiation sites are located within the first 50 bp upstream of the translational start site. The promoter also contains a CAAT sequence (located at −56 bp from the translational start site) and several Sp1 binding sites (8, 15).

We found that Sp1 binding to the region between −118 bp and −102 bp relative to the transcriptional start site largely determined the activity of the proximal rat elastin promoter. This region of the promoter also mediated inhibition by IL-1β. Promoter activity was decreased and IL-1β sensitivity was abolished by deletions that eliminated Sp1 binding to this region of the promoter. Notably, increases in promoter activity and IL-1β sensitivity were generated by mutations that increased the binding of Sp1 to this previously unresponsive promoter region. IL-1β treatment did not affect the expression of Sp1 and Sp3 or binding of Sp1 to the elastin promoter but upregulated nuclear localization of the p65 subunit of NF-κB. In addition, overexpression of the p65 subunit of NF-κB decreased elastin promoter activity and steady-state levels of elastin mRNA.

Interactions between Sp1 and p65 have been described previously (2, 5, 26, 27). NF-κB interacts with Sp1 to regulate the activity of specific promoters via several distinct mechanisms. These mechanisms include binding to adjacent or overlapping regulatory sites (2, 27) and physical interactions between the transcription factors (5, 11, 26). In vivo, the protein-protein interactions may disrupt DNA binding when the affinity of transcription factors for their binding site decreases because of associated chromatin structures (1). Alternatively, the assembled complex may interfere with interactions with other transcription factors or the transcriptional apparatus itself. Our studies indicate that p65 and Sp1 do not compete for binding because p65 did not disrupt Sp1 binding

to the proximal elastin promoter. However, immunoprecipitation studies with nuclear extracts from IL-1β-treated cultures demonstrate an interaction between Sp1 and p65.

IL-1β activates NF-κB by inducing phosphorylation of IκB with subsequent release and nuclear translocation of p65. We find that IL-1β treatment induces a large and sustained increase in intranuclear p65 in lung fibroblasts. A similar finding was reported by others (12). High levels of p65 persisted in the nucleus despite increases in IκB in the cytoplasm in the hours after IL-1β stimulation. Recent studies suggest that intranuclear cytokine-activated acetylation of NF-κB subunits prevents binding of IκB and subsequent inactivation (6).

Gene array analysis indicated that overexpression of p65 and treatment with IL-1β predictably increased several species of mRNA including inducible nitric oxide synthase and cyclooxygenase-2 (30, 32). Importantly, inspection of the array data revealed downregulation of many genes associated with the myofibroblast phenotype. Expression of α-smooth muscle actin mRNA, a classic marker for this phenotype (28, 31), was markedly decreased in both arrays. These results are consistent with the previous observation that IL-1β downregulates expression of α-smooth muscle actin in neonatal rat fibroblasts (37). It is possible that NF-κB functions through a common mechanism involving Sp1 to coordinately regulate these genes. It is noteworthy in this regard that NF-κB, particularly the p65 component, can bind to Sp1 proteins and interfere with the transcriptional rate of α1(I) collagen (29). Activation of other transcription factors by IL-1β likely accounts for differences in gene expression between the two arrays. Together, our results suggest that elastin mRNA expression is a feature of the myofibroblast phenotype and that IL-1β-induced nuclear localization of NF-κB dramatically decreases elastin transcription and expression of the myofibroblast phenotype.

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REFERENCES

1. **Adams CC and Workman JL.** Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol Cell Biol* 15: 1405–1421, 1995.
2. **Algarte M, Kwon H, Genin P, and Hiscott J.** Identification by in vivo genomic footprinting of a transcriptional switch containing NF-kappaB and Sp1 that regulates the IkappaBalpha promoter. *Mol Cell Biol* 19: 6140–6153, 1999.
3. **Berk JL, Franzblau C, and Goldstein RH.** Recombinant interleukin-lβ inhibits elastin formation by a rat lung fibroblast subtype. *J Biol Chem* 266: 3192–3197, 1991.
4. **Bruce MC and Honaker CE.** Transcriptional regulation of tropoelastin expression in rat lung fibroblasts: changes with age and hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 274: L940–L950, 1998.
5. **Chapman NR and Perkins ND.** Inhibition of the RelA(p65) NF-kappaB subunit by Egr-1. *J Biol Chem* 275: 4719–4725, 2000.
6. **Chen L-f, Fischle W, Verdin E, and Greene WC.** Duration of nuclear NF-κB action regulated by reversible acetylation. *Science* 293: 1653–1557, 2001.
7. **Davidson JM, Zoia O, and Liu JM.** Modulation of transforming growth factor-beta 1 stimulated elastin and collagen production and proliferation in porcine vascular smooth muscle cells and skin fibroblasts by basic fibroblast growth factor, transforming growth factor-alpha, and insulin-like growth factor-I. *J Cell Physiol* 155: 149–156, 1993.
8. **Fazio MJ, Kahari VM, Bashir MM, Saitta B, Rosenbloom J, and Uitto J.** Regulation of elastin gene expression: evidence for functional promoter activity in the 5′-flanking region of the human gene. *J Invest Dermatol* 94: 191–196, 1990.
9. **Greenberg ME and Ziff EB.** Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature* 311: 433–438, 1984.
10. **Groudine M, Peretz M, and Weintraub H.** Transcriptional regulation of hemoglobin switching in chicken embryos. *Mol Cell Biol* 1: 281–288, 1981.
11. **Hirano F, Tanaka H, Hirano Y, Hiramoto M, Handa H, Makino I, and Scheidereit C.** Functional interference of Sp1 and NF-kappaB through the same DNA binding site. *Mol Cell Biol* 18: 1266–1274, 1998.
12. **Hohmann HP, Remy R, Scheidereit C, and van Loon AP.** Maintenance of NF-kappa B activity is dependent on protein synthesis and the continuous presence of external stimuli. *Mol Cell Biol* 11: 259–266, 1991.
13. **Jensen DE, Rich CB, Terpstra AJ, Farmer SR, and Foster JA.** Transcriptional regulation of the elastin gene by insulin-like growth factor-I involves disruption of Sp1 binding. Evidence for the role of Rb in mediating Sp1 binding in aortic smooth muscle cells. *J Biol Chem* 270: 6555–6563, 1995.
14. **Kadonaga JT, Jones KA, and Tjian R.** Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem Sci* 111: 20–23, 1986.
15. **Kähäri VM, Fazio MJ, Chen YQ, Bashir MM, Rosenbloom J, and Uitto J.** Deletion analyses of 5′-flanking region of the human elastin gene. Delineation of functional promoter and regulatory *cis*-elements. *J Biol Chem* 265: 9485–9490, 1990.
16. **Kähäri VM, Olsen DR, Rhudy RW, Carillo P, Chen YQ, and Uitto J.** Transforming growth factor-β upregulates elastin gene expression in human skin fibroblasts. Evidence for a post transcriptional modulation. *Lab Invest* 66: 580–588, 1992.
17. **Krupsky M, Fine A, Kuang PP, Berk JL, and Goldstein RH.** Regulation of type I collagen production by insulin and transforming growth factor-β in human lung fibroblasts. *Connect Tissue Res* 203: 1020–1027, 1996.
18. **Kuhn C, Yu SY, Chraplyvy M, Linder HE, and Senior RM.** Induction of emphysema with elastase. II. Changes in connective tissue. *Lab Invest* 34: 372–380, 1976.
19. **Lindahl P, Karlsson L, Hellstrom M, Gebre-Medhin S, Willetts K, Heath JK, and Betsholz C.** Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. *Development* 124: 3943–3953, 1997.
20. **Lucey EC, Ngo HQ, Agarwal A, Smith BD, Snider BD, Snider GL, and Goldstein RH.** Differential expression of elastin and α1(I) collagen mRNA in mice with bleomycin induced fibrosis. *Lab Invest* 74: 12–20, 1996.
21. **Manohar A and Anwar RA.** Evidence for the presence of a functional TATA box (ATAAA) sequence in the gene for the bovine elastin. *Biochim Biophys Acta* 1219: 233–236, 1994.
22. **McGowen SE and McNamer R.** Transforming growth factor-β increases elastin production in neonatal rat lung fibroblasts. *Am J Respir Cell Mol Biol* 3: 369–376, 1990.
23. **Miano JM, Carlson MJ, Spencer JA, and Misra RP.** Serum response factor-dependent regulation of the smooth muscle calponin gene. *J Biol Chem* 275: 9814–9822, 2000.
24. **Michelotti EF, Tomonaga T, Krutzsch H, and Levens D.** Cellular nucleic acid binding protein regulates the CT element of the human c-myc protooncogene. *J Biol Chem* 270: 9494–9499, 1995.

25. **Myers B, Dubick M, Last JA, and Rucker RB.** Elastin synthesis during postnatal development in the rat. *Biochim Biophys Acta* 761: 17–22, 1983.
26. **Perkins ND, Agranoff AB, Pascal E, and Nabel GJ.** An interaction between the DNA binding domains of RelA(p65) and Sp1 mediates human immunodeficiency virus gene activation. *Mol Cell Biol* 14: 6570–6583, 1994.
27. **Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, and Nabel GJ.** A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. *EMBO J* 12: 3551–3558, 1993.
28. **Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, and West AB.** Myofibroblasts I. Paracrine cells important in health and disease. *Am J Physiol Cell Physiol* 277: C1–C9, 1999.
29. **Rippe RA, Schrum LW, Stefanovic B, Solis-Herruzo JA, and Brenner DA.** NF-kappaB inhibits expression of the alpha1(I) collagen gene. *DNA Cell Biol* 18: 751–761, 1999.
30. **Schmedtje JF, Ji YS, Liu WL, DuBois RN, and Runge MS.** Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 272: 601–608, 1997.
31. **Serini G and Gabbiani G.** Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 250: 273–283, 1999.
32. **Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM, Billiar TR, and Geller DA.** Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem* 273: 15148–15156, 1998.
33. **Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, and Miyamoto S.** Rel/NF-kappa B/Ikappa B family: intimate tales of association and dissociation. *Genes Dev* 9: 2723–2735, 1995.
34. **Vrhovski B and Weiss AS.** Biochemistry of tropoelastin. *Eur J Biochem* 258: 1–18, 1998.
35. **Wolfe BL, Rich CB, Goud HD, Terpstra AJ, Bashir M, Rosenbloom J, Sonnenshein GE, and Foster JA.** Insulin-like growth factor-I regulates transcription of the elastin gene. *J Biol Chem* 268: 12418–12426, 1993.
36. **Wu M, Lee H, Bellas RE, Schauer SL, Arsura M, Katz D, FitzGerald MJ, Rothstein DH, Sherr DH, and Sonenshein GE.** Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. *EMBO J*: 15 4682–4690, 1996.
37. **Zhang HY, Gharaee-Kermani M, and Phan SH.** Regulation of lung fibroblast alpha-smooth muscle actin expression, contractile phenotype, and apoptosis by IL-1beta. *J Immunol* 158: 1392–1399, 1997.