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Potential Role of Nuclear Factor κB and Reactive Oxygen Species in cAMP and Cytokine Regulation of *Surfactant Protein-A* Gene Expression in Lung Type II Cells

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The human surfactant protein-A2 (*hSP-A2*) gene is developmentally regulated, expressed in type II pneumonocytes, and induced by cAMP. cAMP induction of *hSP-A2* expression is O_2 dependent and mediated by increased phosphorylation, DNA binding, and transcriptional activation of thyroid transcription factor-1 (TTF-1). The TTF-1-binding element (TBE) at −175 bp contains a reverse-oriented nuclear factor-κB (NF-κB) binding site. IL-1 increased SP-A expression in lung type II cells and had additive stimulatory effects with cAMP. Nuclear extracts from cAMP- or IL-1-treated type II cells manifested increased binding to NF-κB consensus and TBE probes; cAMP and IL-1 had additive effects. Competitive and antibody supershift EMSA revealed that NF-κB and TTF-1 interact with TBE. IL-1 treatment of type II cells caused rapid (1 h) increases in nuclear levels of NF-κB (p50 and p65) and in binding to NF-κB and TBE probes; nuclear levels of TTF-1 were unaffected. Bt_2cAMP increased binding to NF-κB and TBE probes more slowly; no changes in nuclear levels of p50, p65, or TTF-1 were evident, suggesting that IL-1 and cAMP act by different mechanisms. A role for endogenous NF-κB in cAMP and IL-1 regulation of SP-A was suggested by findings that dominant-negative forms of inhibitor of κB reduced binding of type II cell nuclear proteins to TBE and inhibited SP-A expression. In cotransfection assays, NF-κB and TTF-1 cooperatively interacted at TBE to stimulate *SP-A* promoter activity; this was further enhanced by IL-1. In coimmunoprecipitation assays using type II cell nuclear extracts, TTF-1 was found to interact with p65 *in vivo*. Finally, antioxidant inhibitors of NF-κB reduced type II cell nuclear protein binding to TBE and blocked stimulatory effects of cAMP on *SP-A* expression. This provides intriguing evidence that permissive effects of O_2/reactive oxygen species on cAMP regulation of *SP-A* expression may be mediated by cooperative interactions of TTF-1 and NF-κB at the TBE. (*Molecular Endocrinology* 16: 1428–1440, 2002)

PULMONARY SURFACTANT, a developmentally regulated lipoprotein synthesized exclusively in lung alveolar type II cells, acts to reduce alveolar surface tension and is essential for normal breathing. Inadequate surfactant production by lungs of premature newborns can result in respiratory distress syndrome, a leading cause of neonatal morbidity and mortality. To define mechanisms involved in type II cell-specific, developmental, and hormonal regulation of surfactant synthesis, we have focused on the major surfactant protein, SP-A, which is lung specific, expressed in type II cells, and developmentally regulated in fetal lung in concert with surfactant phospholipid synthesis (1). SP-A, a C-type lectin, plays an important role in immune defense within the alveolus by binding to a variety of bacterial, fungal, and viral pathogens and facilitating their uptake by alveolar macrophages, as well as by enhancing macrophage migration and cytokine production (see Ref. 2 for review). The importance of SP-A in host-defense within the alveolus is supported by the finding that mice homozygous for a targeted deletion of the *SP-A* gene manifested an increased susceptibility to infection by Group B *streptococcus* and *Pneumocystis carinii*, as well as defective clearance of *S. aureus*, *P. aeruginosa*, and *K. pneumoniae* (see Ref. 3 for review).

Type II cell differentiation and SP-A expression in human fetal lung are induced by hormones and factors that increase cAMP; inductive effects of cAMP are dependent upon a critical O_2 tension. When midgestation human fetal lung explants are cultured in a 20% O_2 environment, epithelial cells of prealveolar ducts differentiate spontaneously into type II cells that produce SP-A; the rate of type II cell differentiation and *SP-A* gene expression are markedly induced by cAMP treatment (4). By contrast, when cultured in 1–2% O_2, type II cells fail to differentiate and no stimulatory

Abbreviations: Bt_2cAMP, Dibutyryl cAMP; CBP, CREB-binding protein; CMV, cytomegalovirus; DN, dominant-negative; βgal, β-galactosidase; GST, gluatathione-*S*-transferase; hGH, human GH; IκB, inhibitor of κB; m.o.i., multiplicity of infection; NAC, *N*-acetyl cysteine; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; PKA-cat, PKA catalytic subunit; ROS, reactive oxygen species; RSV, Rous sarcoma virus; SP, surfactant protein; SRC-1, steroid receptor coactivator-1; TBE, TTF-1-binding element; TTF-1, thyroid transcription factor-1.

effects of cAMP on SP-A expression are apparent (5). Bt_2cAMP stimulation of SP-A expression is evident only at O_2 concentrations ≥10%. These findings led us to postulate that increased vascularization of fetal lung during the third trimester of gestation results in enhanced O_2 availability to type II cell precursors, facilitating cAMP induction of type II cell differentiation and of surfactant protein gene expression (5).

In studies using transgenic mice (6) and transfected type II cells (7–11), we found that as little as approximately 400 bp of 5′-flanking region from the rabbit, baboon, and human *SP-A* genes mediates lung type II cell-specific, developmental and cAMP-regulated expression. This region contains four response elements that are highly conserved in *SP-A* genes of various species. These include an element that likely binds a member of the nuclear receptor family (7–9), an E-box (12) that binds upstream stimulatory factor-1 (13) and -2 (Gao, E., and C. Mendelson, unpublished observations), a GT-box, which binds Sp1 and other related transcription factors (10), and a thyroid transcription factor-1 (TTF-1) binding element (TBE) for the homeodomain factor, TTF-1 (11). Mutagenesis of any one of these elements markedly reduces basal and cAMP induction of *SP-A* promoter activity in transfected type II cells, suggesting the cooperative interaction of these transcription factors.

TTF-1, which is expressed selectively in developing thyroid, diencephalon, and lung epithelium from the earliest stages of organogenesis (14), is crucial for morphogenesis of thyroid, anterior pituitary, and lung parenchyma (15). In studies using deoxyribonuclease I footprinting and EMSA, we identified three elements (TBE1, 2, and 3) within a 255-bp region upstream of the baboon *SP-A2* gene that specifically bound TTF-1 (11). We observed that TBE1, which lies approximately 175-bp upstream of the *SP-A* gene, is the most highly conserved TBE among the *SP-A* genes of various species, exhibited the strongest footprint (11) and was most critical for basal and cAMP induction of *SP-A* promoter activity in transfected type II cells (16). cAMP, acting through PKA, was found to enhance TTF-1 phosphorylation and transcriptional activity, as well as binding of type II cell nuclear proteins to TBE1 (16). Although TTF-1 is expressed in lung epithelium from the earliest phases of development, *SP-A* expression is initiated only after approximately 75% of gestation is completed (17). We have postulated that developmental changes in TTF-1 phosphorylation by PKA may enhance its binding to this TBE and its interaction with other critical transcription factors and coactivators, resulting in increased *SP-A* gene transcription.

In characterizing TBE1, we noted that it contained a reverse-oriented binding site for NF-κB, a transcription factor known to be activated by inflammatory cytokines (see Ref. 18 for review), PKA (19, 20), and reactive oxygen species (ROS) (21). ROS are required for cytokine-mediated phosphorylation and degradation of inhibitor of κB (IκB), the inhibitory partner of NF-κB. In women with intraamniotic infection associated with chorioamnionitis, amniotic fluid levels of IL-1α and IL-1β are increased (22). The finding that the incidence of respiratory distress syndrome is decreased in infants born prematurely to such women supports a possible role of bacterially produced cytokines in fetal lung maturation. In studies by Bry *et al.* (23), it was found that IL-1α administered intraamniotically to fetal rabbits increased expression of SP-A and SP-B by the fetal lung, and enhanced dynamic lung compliance; IL-1α treatment of fetal rabbit lung explants increased SP-A expression (24). Interferon-γ, a proinflammatory cytokine also known to activate NF-κB (see Ref. 18 for review), increased SP-A expression in midgestation human fetal lung explants (25).

In light of the role of NF-κB as a mediator of cytokine action, the reported effects of IL-1 on surfactant protein expression, and the presence of a putative NF-κB response element within TBE1, we sought to determine whether NF-κB is a component of the complex of type II cell nuclear proteins that bind to this TBE to activate *SP-A* promoter activity. We observed that NF-κB proteins p65 and p50, as well as TTF-1, were present in the complex of nuclear proteins bound to TBE1 and that cAMP and IL-1 had additive stimulatory effects on TBE-binding activity. In cell transfection studies, we found that p50, p65, and TTF-1 cooperatively increased *SP-A* promoter activity; this was further enhanced by IL-1 treatment. By coimmunoprecipitation, TTF-1 was found to interact with p65 in type II cell nuclear extracts. Furthermore, dominant-negative (DN) forms of IκB-α and IκB-β inhibited cAMP and IL-1 induction of *SP-A* expression and the binding of type II cell nuclear proteins to the TBE. Finally, antioxidant inhibitors of NF-κB markedly reduced both TBE-binding activity and SP-A expression. The results of our studies suggest that permissive effects of O_2 on cAMP induction of *SP-A* gene expression may be mediated by cooperative actions of TTF-1 and NF-κB at the TBE.

RESULTS

IL-1α and IL-1β Increase the Levels of Immunoreactive SP-A in Human Fetal Type II Cells and Have an Additive Stimulatory Effect with Bt_2cAMP

As mentioned earlier, in studies using cultured fetal rabbit lung explants, it was found that the cytokine IL-1α stimulated SP-A expression (24). To analyze the effects of IL-1 on SP-A expression in human fetal lung, as well as effects of IL-1 in combination with cAMP, human fetal lung type II cells were cultured for 3 d in control medium, in medium containing Bt_2cAMP (1 mM), IL-1α or β (10 ng/ml), or Bt_2cAMP and IL-1 in combination. SP-A levels in cytoplasmic fractions isolated from these cells were analyzed by immunoblotting. A representative immunoblot is shown in Fig. 1A, and the results of densitometric scans of immunoblots

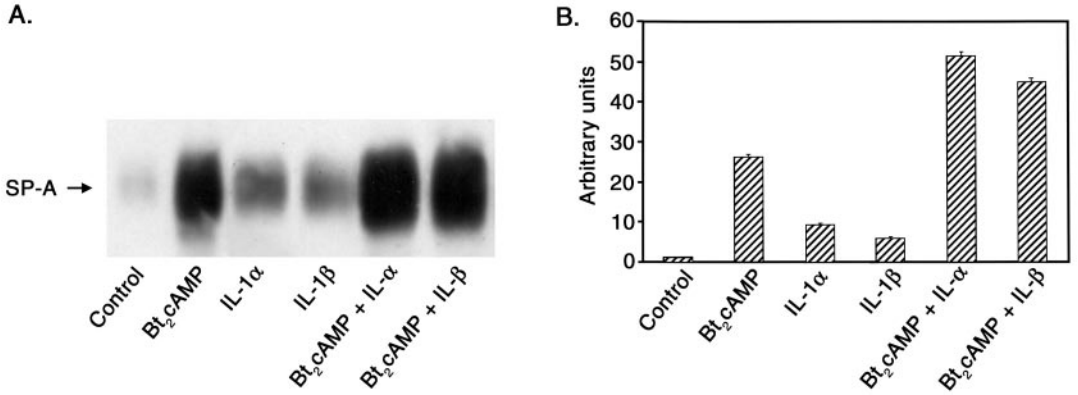

Fig. 1. Effects of IL-1α and IL-1β in the Absence or Presence of Bt_2cAMP on the Levels of Immunoreactive SP-A in Human Fetal Type II Cells in Primary Culture

Human fetal lung type II cells were cultured for 3 d in control medium, in medium containing Bt_2cAMP (1 mM), IL-1α, or IL-1β (10 ng/ml), or Bt_2cAMP and IL-1 in combination. SP-A levels in cytoplasmic fractions isolated from these cells were analyzed by immunoblotting using antihuman SP-A IgG (4). A, Autoradiogram of a representative immunoblot. B, Summarized results of densitometric scans of immunoblots from three independent experiments. Scanned values for the treated samples in each experiment are presented relative to those of the control sample, which is given a value of 1. Data are the mean ± SEM of scanned values from the three experiments.

from three independent experiments are shown in Fig. 1B. As we observed previously (26), immunoreactive SP-A was present at relatively low levels in the fetal type II cells cultured in control medium and was markedly stimulated by Bt_2cAMP treatment. Incubation of the cells in medium containing IL-1α or β alone also caused a pronounced induction of SP-A expression as compared with control cells. When cells were incubated with Bt_2cAMP and IL-1α or β in combination, the levels of immunoreactive SP-A were greater than those of cells treated with either factor alone (Fig. 1). IL-1α and IL-1β have 62% identity in amino acid sequence and elicit highly comparable biological responses. In light of the similarity in effects of IL-1α and IL-1β on SP-A expression, subsequent experiments were carried out using IL-1α.

TBE1 Contains a Reverse-Oriented NF-κB Binding Site

In previous studies, we found that the TBE1 (heretofore referred to as TBE) specifically binds TTF-1 (11) and is critical for basal and cAMP induction of *SP-A* promoter activity (16). cAMP acting through PKA enhances TTF-1 phosphorylation and transcriptional activity, as well as binding of type II cell nuclear proteins to the TBE (16). In characterizing the TBE and surrounding nucleotides, we noted the presence of an overlapping reverse-oriented binding site for NF-κB (Fig. 2). A reverse-oriented NF-κB response element was previously characterized within the 5′-flanking region of the murine *P-selectin* gene (27). This site, which was found to mediate TNF-α and lipopolysaccharide enhancement of *P-selectin* expression, was proposed to bind p65 homodimers preferentially (27). The TBE core sequence (CTCAAG) and flanking nucleotides shown in Fig. 2 are perfectly conserved in the

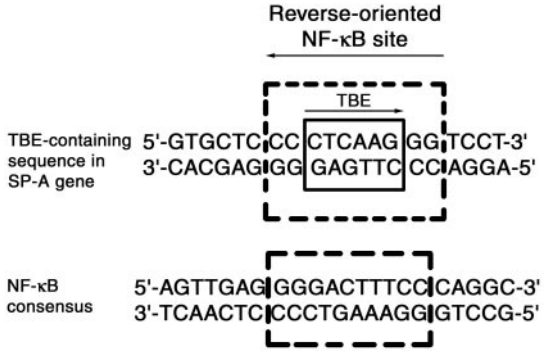

Fig. 2. Comparison of the Nucleotide Sequences of the Major TTF-1 Response Element (TBE) in the *bSP-A2* Gene and a Consensus Binding Site for NF-κB

Upper panel, *SP-A* 5′-flanking region between −185 and −165 bp. The TBE core sequence is enclosed by a *solid line*. The sequence of a reverse-oriented NF-κB consensus element is enclosed by a *dashed line*. *Lower panel*, Sequence of an oligonucleotide containing a NF-κB consensus binding site, which is enclosed by a *dashed line*.

bSP-A1, *bSP-A2*, *hSP-A1*, and *hSP-A2* genes (11). In previous studies, we found that mutation of the TBE to 5′. . . GTGCTCCCCTgtgcGGTCCT. . . 3′ (mutated sequence is *lowercase* and *underlined*) prevented nuclear protein binding and markedly reduced transcriptional activity (11, 16). This mutation also falls within the reverse-oriented NF-κB consensus binding sequence.

Bt_2cAMP and IL-1α and β Increase Binding of Human Type II Nuclear Proteins to a NF-κB Consensus Element and to the TBE

In light of the inductive effects of IL-1 on SP-A expression and the presence of a reverse-oriented NF-κB

response element overlapping the TBE, it was of interest to analyze effects of IL-1 ± Bt_2cAMP on TBE-binding activity and to determine whether NF-κB proteins p50 and p65 interact with the TBE. We first compared binding of nuclear proteins from human fetal type II cells incubated for 3 d in control medium, or in medium containing Bt_2cAMP, IL-1α, or IL-1α + Bt_2cAMP to the TBE and to an NF-κB binding consensus oligonucleotide using EMSA. As can be seen, nuclear extracts from type II cells bound to the NF-κB oligonucleotide as two complexes (Fig. 3A). Treatment of cells with either Bt_2cAMP or with IL-1α alone caused an increase in binding activity for the NF-κB consensus probe. Binding activity was further increased in nuclear extracts of type II cells incubated with Bt_2cAMP and IL-1α, in combination. Pyrrolidine dithiocarbamate (PDTC), an antioxidant inhibitor of NF-κB that scavenges ROS, reduced binding activity for the NF-κB consensus probe of nuclear extracts from type II cells incubated in the presence of Bt_2cAMP, IL-1α, or Bt_2cAMP + IL-1α. Essentially identical effects were observed when type II cells were incubated with IL-1β in place of IL-1α (data not shown).

Binding activity of the type II cell nuclear extracts from this experiment also was analyzed by EMSA using radiolabeled TBE as probe (Fig. 3B). In contrast to the binding pattern observed using the NF-κB consensus sequence, as probe, type II cell nuclear extracts bound to the TBE as a single broad band. TBE binding activity also was increased in nuclear extracts from Bt_2cAMP- and IL-1α-treated cells; binding was further increased in nuclear extracts of type II cells incubated with Bt_2cAMP + IL-1α. Of great interest, was the finding that the NF-κB inhibitor, PTDC, also decreased TBE-binding activity of nuclear extracts from Bt_2cAMP-, IL-1- and IL-1 + Bt_2cAMP-treated type II cells, suggesting that NF-κB may bind to the TBE. PDTC had no discernible effect on the level of TBE-binding activity in type II cells cultured in control medium (data not shown). Cells treated with PDTC were refractile and appeared viable. Furthermore, the amount of nuclear protein isolated from PDTC-treated cells was similar to that obtained from cells not treated with PDTC. In other experiments, we found that treatment of type II cells with the antioxidant inhibitor of NF-κB, *N*-acetylcysteine (NAC), also markedly decreased binding of nuclear proteins to the TBE (data not shown).

The NF-κB Inhibitor PDTC Reduces SP-A Expression in Human Fetal Lung Type II Cells and Antagonizes the Stimulatory Effect of Bt_2cAMP

As shown in Fig. 3, the antioxidant PDTC inhibited the binding of type II cell nuclear proteins to the NF-κB consensus oligonucleotide and to the TBE and prevented the stimulatory effects of IL-1 and Bt_2cAMP. In consideration of these findings, it was of interest to evaluate the effect of PDTC on SP-A expression in human fetal type II cells incubated with Bt_2cAMP and IL-1. When type II cells were incubated with PDTC in the absence of Bt_2cAMP or IL-1α for 72 h, the levels of immunoreactive SP-A were similar to those of control type II cells (Fig. 4). On the other hand, PDTC blocked the stimulatory effects of Bt_2cAMP and IL-1α on SP-A expression. It should be noted that treatment of human fetal type II cells with NAC also caused a marked inhibition of cAMP and IL-1 induction of SP-A expression (data not shown). These findings support our

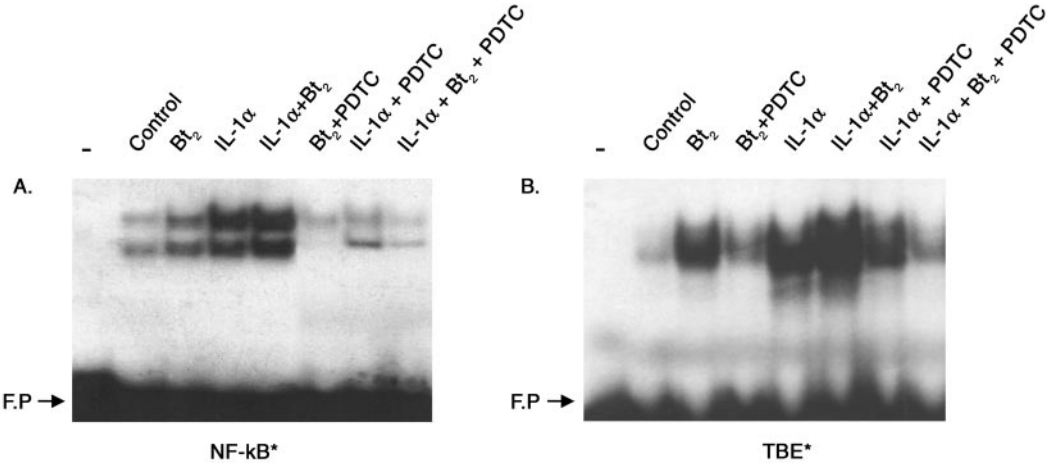

Fig. 3. Effects of Bt_2cAMP and IL-1α, Added Alone and in Combination, in the Absence or Presence of PDTC, on the Binding of Human Fetal Lung Type II Cell Nuclear Proteins to Radiolabeled Oligonucleotides Containing a NF-κB Consensus Binding Site (A) and TBE (B) from the *bSP-A2* Gene

Human fetal lung type II cells were incubated for 3 d in control medium or with Bt_2cAMP (Bt_2, 1 mM), IL-1α (10 ng/ml) or IL-1α + Bt_2cAMP, in the absence or presence of the antioxidant PDTC (25 μM). Binding activities of nuclear extracts isolated from these cells for ^{32}P-labeled oligonucleotides containing a NF-κB consensus sequence (A) and TBE from the *bSP-A2* gene (B) were analyzed by EMSA. The sequences of the oligonucleotides used in the EMSA are presented in Fig. 2. Shown is an autoradiogram of a representative EMSA from an experiment that was repeated five times with similar results.

previous observations that O_2 plays a permissive role in cAMP induction of *SP-A* gene expression (5) and also suggest a role of ROS in mediating the stimulatory effects of IL-1. The observation that PDTC reduced cAMP and IL-1 induction of TBE-binding activity (Fig. 3) also suggests that permissive effects of ROS on induction of SP-A expression are mediated, in part, by their effects on the binding of nuclear proteins to the TBE.

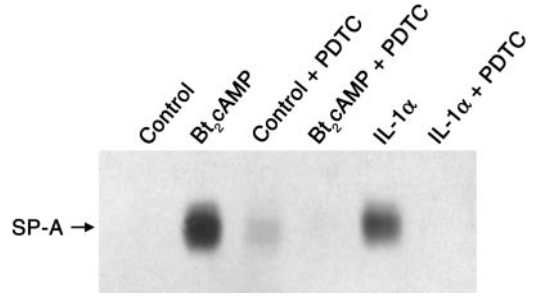

Fig. 4. Effects of the Antioxidant PDTC on SP-A Expression in Human Fetal Type II Cells

Human fetal type II cells were cultured for 3 d in the absence or presence of Bt_2cAMP or IL-1α, in the absence or presence of PDTC (25 μM). Cytoplasmic fractions from these cells were analyzed for immunoreactive SP-A by immunoblotting using specific antibodies. Shown is an autoradiogram of a representative immunoblot from an experiment that was repeated three times with similar results.

NF-κB is a Component of the Complex of Type II Cell Nuclear Proteins that Binds to the TBE

In light of the presence of a reverse-oriented NF-κB response element overlapping the TBE, and the effects of cytokines and PDTC on binding of type II cell nuclear proteins to the TBE, it was of interest to determine whether NF-κB proteins are components of the complex that binds to this site. To achieve this, we used competitive EMSA and antibody-mediated supershift EMSA using TBE as radiolabeled probe and nuclear proteins from human fetal type II cells incubated for 3 d with Bt_2cAMP. As can be seen, both nonradiolabeled NF-κB consensus oligonucleotide and TBE competed with ^{32}P-labeled TBE for binding to type II cell nuclear proteins in a concentration-dependent manner (Fig. 5A). A 1000-fold excess of an unrelated oligonucleotide (Nonsp. cold) failed to compete with the radiolabeled TBE for nuclear protein binding (Fig. 5B). Although this suggests that NF-κB proteins may have the capacity to bind to the TBE, it should be noted that the NF-κB consensus oligonucleotide contains the sequence CTCAAC on the bottom strand in a 5′ to 3′ orientation. Because this is

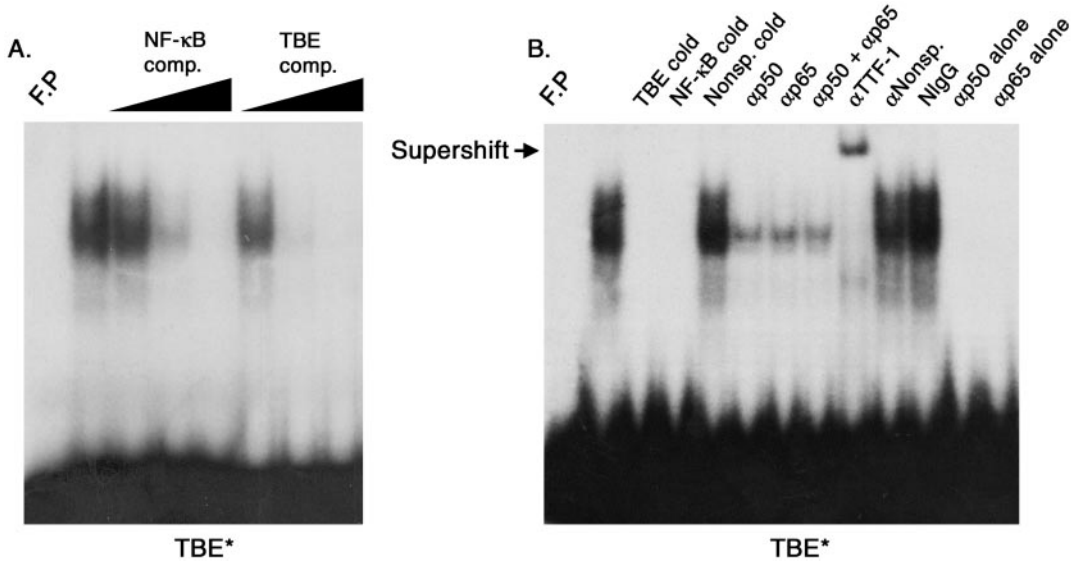

Fig. 5. Competitive and Antibody-Mediated Supershift EMSA of Type II Cell Nuclear Protein Binding to Radiolabeled TBE

Human fetal lung type II cells were incubated in medium containing Bt_2cAMP for 3 d. A, Type II cell nuclear proteins were incubated with ^{32}P-labeled TBE in the absence or presence of nonradiolabeled NF-κB consensus or TBE oligonucleotides at 10-, 100-, and 1000-fold molar excess (indicated by the wedge). B, The nuclear extracts and radiolabeled TBE probe were incubated with a 1000-fold excess of the following nonradiolabeled oligonucleotides: TBE (TBE cold); NF-κB consensus (NF-κB cold), oligonucleotide containing the sequence 5′. . . TGCAGGGCCCAAGGACCTGGGCCATC. . . 3′ (Nonsp. cold). Alternatively, the nuclear extracts and radiolabeled TBE probe were incubated with antisera (α) to p50, p65, p50+p65, TTF-1, CCAAT/enhancer binding protein-β (C/EBP-β) (nonspecific antibody; αNonsp.), or with nonimmune IgG. To ensure that the antibodies to p50 and p65 did not interact directly with the radiolabeled TBE probe, parallel incubations were carried out using radiolabeled TBE and antisera to p50 and p65 in the absence of nuclear protein. The reaction mixtures were then analyzed by EMSA. Shown is an autoradiogram of a representative experiment; the majority of the conditions were analyzed in four independent experiments with similar results.

similar to the TTF-1 binding consensus (CTNNAG), TTF-1 may have the capacity to bind to the NF-κB consensus sequence. Antibodies to NF-κB proteins, p65 and p50, added alone or in combination considerably reduced the intensity of the TBE-nuclear protein complex (Fig. 5B), whereas antibodies to TTF-1 caused an apparent supershift of the binding complex. On the other hand, antibodies to the transcription factor C/EBP-β (αNonsp.) and nonimmune IgG had no effect (Fig. 5B) to alter the intensity or mobility of the binding complex. Antibodies to p50 and p65 failed to interact with the TBE probe in the absence of type II cell nuclear proteins (αp50 alone, αp65 alone). These findings suggest that NF-κB proteins p50 and p65 are components, together with TTF-1, of the complex of type II cell nuclear proteins binding to the TBE.

In previous studies, we observed specific binding of a bacterially expressed TTF-1 homeodomain-containing peptide to the TBE (11). In the present study, we assessed the binding to the TBE and to the NF-κB consensus oligonucleotide of *in vitro* transcribed/translated p50 and p65 proteins, alone and in combination. However, the expressed NF-κB proteins failed to bind to either oligonucleotide probe (data not shown). This suggests that extensive posttranslational modification of NF-κB is required for DNA binding activity. Therefore, it is uncertain as to whether p50 and p65 bind directly to the TBE, or whether they bind indirectly through their interactions with other proteins.

IL-1 Treatment of Human Fetal Type II Cells Causes a Rapid Induction of Nuclear Levels of Immunoreactive p50 and p65

In previous studies, we observed that cAMP had only a modest or even negligible effect to increase the rate of synthesis and nuclear levels of immunoreactive TTF-1. Rather, the marked stimulatory effect of Bt_2cAMP on TTF-1-binding activity was due to an induction of TTF-1 phosphorylation (16). In the case of NF-κB, however, it has been reported that cytokine-mediated activation is associated with increased nuclear translocation, as well as by increased phosphorylation of p65 by PKA (19, 20). In the present study, we analyzed the levels of p50, p65, and TTF-1 in type II cell nuclear extracts after 1, 3, 6, 24, and 72 h of incubation in the absence or presence of Bt_2cAMP or IL-1α. In this experiment, all type II cells were incubated for 72 h in serum-free culture medium; Bt_2cAMP and IL-1 were added to the medium at the various time points before harvesting the cells. As can be seen, Bt_2cAMP had little or no effect to alter nuclear levels of p50, p65 or TTF-1 at any of the time points studied (Fig. 6A). On the other hand, IL-1α had a rapid effect (within 1 h) to increase nuclear levels of p50 and p65. In the case of p50, the stimulatory effect of IL-1α persisted throughout the 72-h incubation period. By contrast, the stimulatory effect of IL-1α on p65 levels was evident after 1–6 h of incubation but was greatly diminished after 24 h. Nuclear levels of immunoreactive TTF-1 were relatively unaffected by IL-1α at time points up to and including 6 h of incubation. However, after 24 h of incubation, the levels of immunoreactive TTF-1 in nuclear extracts of IL-1α-treated type II cells were markedly reduced as compared with untreated cells or cells incubated with Bt_2cAMP alone (Fig. 6A).

Nuclear extracts from type II cells in the same time-course experiment were analyzed for DNA binding activity using radiolabeled NF-κB consensus (Fig. 6B) and TBE (Fig. 6C) oligonucleotides as probes. Interestingly, nuclear protein binding activity for both NF-κB and TBE probes was maximally induced by IL-1α after 1 h of incubation. This level of binding activity was maintained throughout the 72 h incubation period (Fig. 6, B and C). In nuclear extracts from Bt_2cAMP-treated cells, a modest stimulatory effect on binding activity was evident within 3 h of incubation; maximal induction of binding activity by Bt_2cAMP was observed after 24 h of treatment and persisted throughout the 72-h incubation period (Fig. 6, B and C). Therefore, it is evident that binding activity remained elevated in nuclear extracts from IL-1-treated cells after 24 h of treatment, despite the decline in nuclear levels of p65 and TTF-1 proteins.

The NF-κB Proteins p50 and p65 Act Synergistically with TTF-1 to Increase *SP-A* Promoter Activity in Transfected A549 Cells

To determine whether NF-κB p50 and p65 functionally interact with TTF-1 at the TBE to regulate *SP-A* promoter activity, A549 lung adenocarcinoma cells were transfected with a fusion gene comprised of three copies of the TBE fused to the first 50 bp of 5′-flanking DNA and +40 bp of the first exon of the *bSP-A2* gene, linked upstream of the *human GH* (*hGH*) structural gene, as reporter (*TBE_3:hGH*). The *bSP*-A2 gene contains three TBEs (11). Although these differ in sequence, all have been found to bind expressed TTF-1 (11). As mentioned above, only TBE1 is highly conserved in sequence among the *SP-A* genes of various species and contains the reverse NF-κB site. The *TBE_3:hGH* reporter gene construct (which contains three tandem TBE1 sequences) was used to amplify the signal and to mimic the number of TBE sequences in the baboon and human *SP-A* genes. A549 cells were used for transfection because they do not express detectable levels of TTF-1 (16). The cells were cotransfected with expression vectors containing p50, p65, or TTF-1, alone or in various combinations, in the absence or presence of IL-1β; cytomegalovirus (*CMV): β-galactosidase (β-gal*) was cotransfected as a control for transfection efficiency. Transcription of the reporter gene was evaluated by measuring the hGH that accumulated in the culture medium over a 24-h period, 39 h after transfection. As can be seen, transfection of p50 and p65 alone and in combination caused a modest induction of *SP-A* promoter activity, whereas TTF-1 alone increased *SP-A* promoter activity approximately 3-fold (Fig. 7A). In cells cotransfected with TTF-1 and either p50 or p65, there was an additive effect on *SP-A*

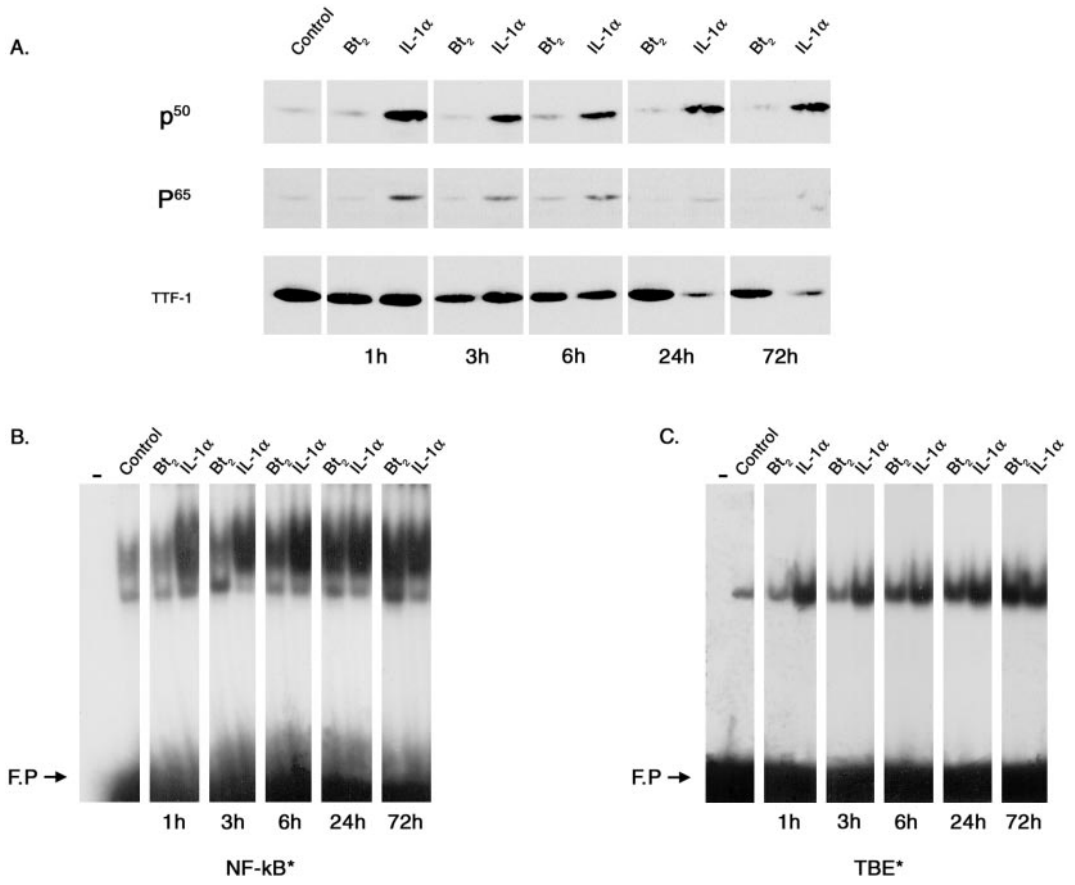

Fig. 6. Time Course of the Effects of Bt_2cAMP and IL-1α on the Levels of Immunoreactive p50, p65, and TTF-1 in Nuclear Extracts of Type II Cells and on Nuclear Protein Binding Activity for NF-κB and TBE Probes

Human fetal type II cells were cultured for 1, 3, 6, 24, and 72 h in control medium, in medium containing Bt_2cAMP (Bt_2, 1 mM), IL-1α (10 ng/ml), or Bt_2cAMP plus IL-1α. The levels of immunoreactive p50, p65 and TTF-1 in nuclear extracts were analyzed by immunoblotting using specific antibodies (A). Binding activities of type II cell nuclear proteins for radiolabeled NF-κB consensus (B) and TBE (C) oligonucleotides were analyzed by EMSA. Shown are autoradiograms of immunoblots and EMSAs from a typical experiment that was repeated five times with similar results.

promoter activity; an even greater inductive effect was evident when p50, p65, and TTF-1 were cotransfected in combination (Fig. 7A). In independent transfection experiments, A549 cells cotransfected with *TBE_3:hGH* with or without expression vectors for TTF-1, p50+p65 (NF-κB), alone and in combination were cultured in the absence or presence of IL-1β (10 ng/ml). As can be seen, IL-1β markedly enhanced the inductive effects on *bSP-A2* promoter activity of TTF-1 and NF-κB, transfected alone and in combination (Fig. 7B). These findings indicate that NF-κB proteins and TTF-1 cooperatively interact at the TBE to stimulate *SP-A* promoter activity, and that the inductive effects of these transcription factors are enhanced by IL-1.

DN Forms of IκB-α and IκB-β Inhibit Type II Cell Nuclear Protein Binding to the NF-κB Consensus and TBE Oligonucleotides and Inhibit SP-A Expression

The data presented thus far, suggest that NF-κB proteins act together with TTF-1 at the TBE to mediate induction of *SP-A* gene expression. To further define the role of endogenous NF-κB in cAMP and IL-1 induction of type II cell nuclear protein binding activity and SP-A expression, human fetal type II cells in primary culture were infected with recombinant adenoviruses expressing DN forms of IκB-α or IκB-β, or with a recombinant adenovirus containing *CMV:βgal* as a control. The DN isoforms of IκB contain mutations in the IκB kinase phosphorylation sites and are resistant to degradation by the ubiquitin/proteasome pathway (28). This enables the overexpressed IκB proteins to sequester p65/p50 complexes within the cell, blocking activation of NF-κB. Overexpression of DN IκB-α and -β was confirmed by immunoblotting (data not shown). As can be seen in the immunoblot in Fig. 8A, both DN forms of IκB inhibited Bt_2cAMP and Bt_2cAMP + IL-1α induction of SP-A expression as compared with type II cells infected with the recombinant adenovirus containing *CMV:βgal*. It should be noted that an inductive effect of IL-1α alone on SP-A expression was evident upon longer exposure of the immunoblot,

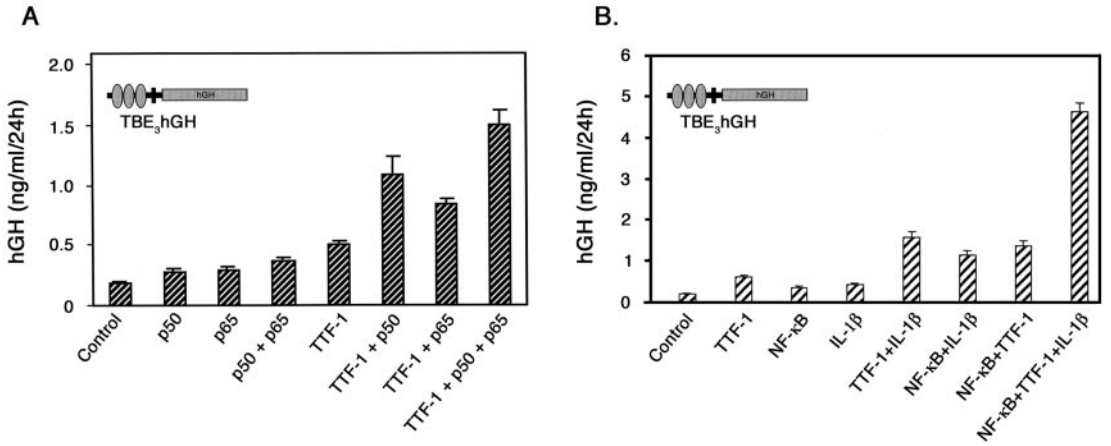

Fig. 7. Effects of p50, p65, and TTF-1, Overexpressed Alone and in Various Combinations in the Absence or Presence of IL-1β, on Expression of a Cotransfected *TBE$_3$:hGH* Reporter Gene in A549 Lung Adenocarcinoma Cells

A549 lung adenocarcinoma cells were transfected with a fusion gene comprised of three TTF-1 binding elements fused upstream of 50 bp of 5′-flanking DNA from the b*SP-A2* gene + 40 bp of the first exon, linked upstream of the *hGH* structural gene, as reporter (*TBE$_3$hGH*). A, The A549 cells were cotransfected either with empty expression vector (Control) or with expression vectors containing p50, p65, or TTF-1, alone or in various combinations. B, In an independent experiment, the A549 cells were cotransfected with empty expression vector (Control) or with expression vectors for TTF-1, p50+p65 (NF-κB), or TTF-1 + NF-κB in the absence or presence of IL-1β (10 ng/ml). *CMV:β-gal* was cotransfected into all dishes as a control for transfection efficiency. Transcription of the reporter gene was evaluated by measuring the hGH that accumulated in the culture medium over a 24-h period, 39 h after transfection. Values were corrected for transfection efficiency. Data shown are the mean ± SEM of corrected values from three independent experiments, each conducted in triplicate.

and this increase was antagonized by infection with the DN-IκB-expressing adenoviruses. Comparable inhibitory effects of the DN-IκB isoforms were observed on nuclear protein binding to both NF-κB (Fig. 8B) and TBE (Fig. 8C) probes. An inhibitory effect of DN-IκB on TBE-binding activity of control nuclear extracts was also evident, suggesting that endogenous NF-κB is important for basal levels of DNA binding activity. Together, these findings further suggest the importance of endogenous NF-κB proteins in cAMP and cytokine induction of *SP-A* gene expression.

TTF-1 and p65 Interact *in Vivo*

From the findings presented thus far, it is evident that TTF-1 and NF-κB proteins cooperatively interact at the TBE to stimulate *SP-A* promoter activity. In antibody-mediated supershift EMSA (Fig. 5), we have obtained evidence that p50 and p65 interact with the TBE; however, we have been unable to demonstrate that expressed p65 and p50 bind to the TBE directly. Because the expressed proteins also failed to bind to the NF-κB consensus, we believe that this may be due to their inadequate posttranslational modification. To determine whether TTF-1 has the ability to interact with p50 and p65 *in vivo*, we carried out coimmunoprecipitation assays using type II cells incubated with Bt_2cAMP + IL-1α for 24 h. Nuclear extracts from the treated cells were immunoprecipitated using either preimmune rabbit IgG or rabbit antibodies to TTF-1, normal rabbit IgG or rabbit polyclonal antibodies to p50 and p65. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed for p65 by immunoblotting. As can be seen in the immunoblot in Fig. 9, p65 was found to strongly interact with itself and with its heterodimeric partner, p50. Interestingly, p65 was also found to interact with TTF-1. Essentially no p65 was observed in the control IgG lanes. When the blot was reprobed using antibody to p50, no interaction with TTF-1 was evident, although there was strong interaction with itself and with p65 (data not shown). These findings indicate that TTF-1 may interact with NF-κB through direct binding to p65.

DISCUSSION

SP-A is a lung-specific member of the collectin family that is believed to serve a role in innate immune defense within the alveolus by activating macrophages (3). In the present study, we found that the inflammatory cytokines IL-1α and IL-1β increased SP-A expression in human fetal lung type II cells and had an additive stimulatory effect with Bt_2cAMP. These effects on SP-A expression were associated with comparable inductive effects on binding of type II cell nuclear proteins to the TBE, a response element known to bind the homeodomain transcription factor TTF-1 (11), which is critical for cAMP induction of *SP-A* promoter activity (16). We found these stimulatory effects of IL-1 to be of interest in light of the presence within the TBE of an overlapping reverse-oriented binding sequence for NF-κB, a transcription factor complex that medi-

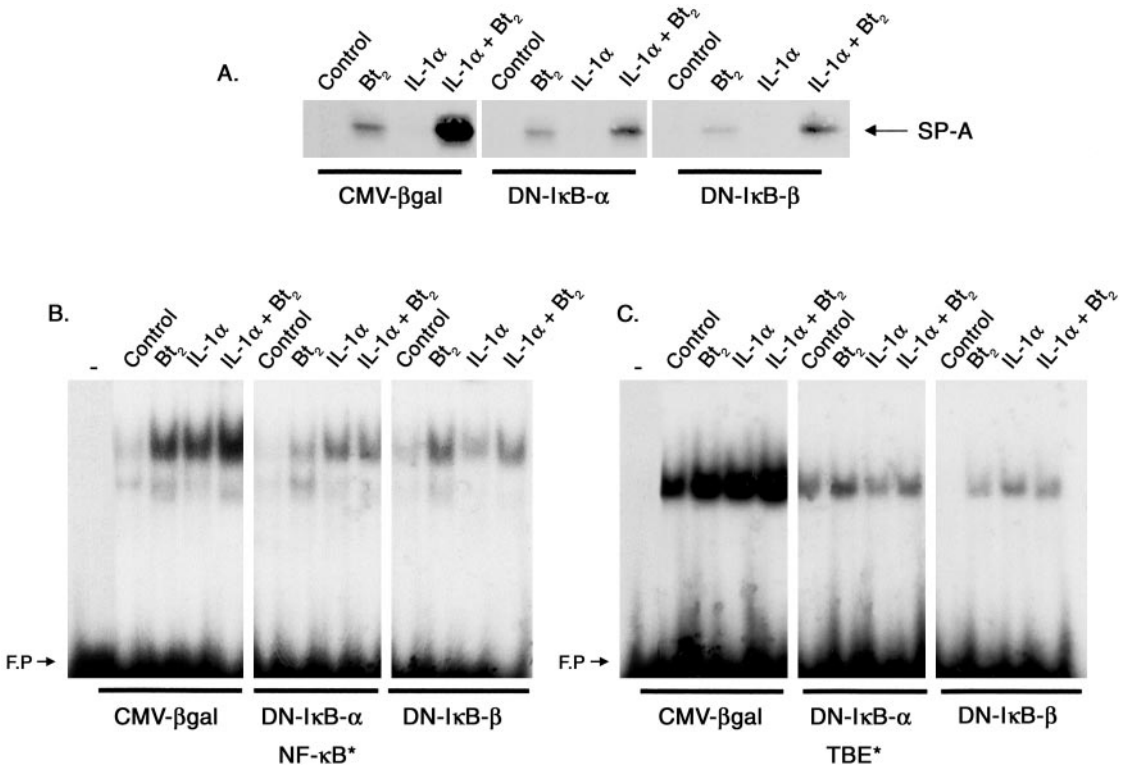

Fig. 8. Effects of Overexpression of DN Forms of IκB-α and IκB-β on SP-A Expression and on Type II Cell Nuclear Protein Binding Activity for NF-κB and TBE Probes

Human fetal type II cells in control medium were infected with recombinant adenoviruses containing *CMV* expression vectors for DN forms of IκB-α or IκB-β (m.o.i. = 10), or with a control virus expressing *CMV:βgal* (m.o.i. = 10) for 1 h. The cells were washed and placed in serum-free culture medium containing no additions (Control), or in media containing Bt_2cAMP (Bt_2, 1 mM), IL-1α (10 ng/ml) or IL-1α plus Bt_2cAMP. After 72 h of incubation, type II cell cytoplasmic fractions were analyzed for SP-A by immunoblotting (A); nuclear extracts were analyzed for binding to radiolabeled NF-κB (B) and TBE (C) probes by EMSA. Shown are representative autoradiograms from the same experiment. This experiment was repeated three times with similar results.

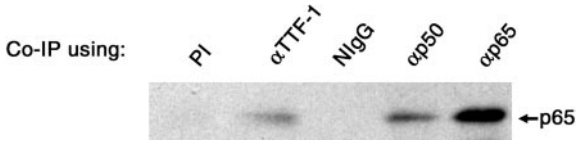

Fig. 9. Analysis of p65 Interaction with TTF-1, p50 and p65 in Human Fetal Type II Cell Nuclear Extracts by Coimmunoprecipitation

Nuclear extracts isolated from human fetal lung type II cells cultured in medium containing Bt_2cAMP + IL-1α for 24 h were subjected to an immunoprecipitation protocol using rabbit antibodies to TTF-1, p50 or p65. Parallel samples were run using either rabbit preimmune IgG or nonimmune IgG, as controls. The immunoprecipitated samples were then analyzed for the presence of p65 by immunoblotting. Shown is an autoradiogram of an immunoblot from an experiment that was repeated twice with the same results.

ates the actions of IL-1 and other cytokines on expression of genes involved in immune regulation and inflammation (18). IL-1 and Bt_2cAMP treatment also increased binding of type II cell nuclear proteins to a NF-κB consensus sequence; again, an additive stimulatory effect on binding activity was observed in cells treated with these factors in combination. The finding that antibodies to NF-κB proteins p50 and p65 and TTF-1 reduced the binding of type II cell nuclear proteins to radiolabeled TBE suggests that NF-κB exists together with TTF-1 in the complex that binds to this element. The potential role of endogenous NF-κB in the regulation of *SP-A* gene expression was further supported by the significant finding that overexpression of DN forms of IκB-α or IκB-β inhibited cAMP and IL-1 induction of nuclear protein binding to the TBE and reduced SP-A expression in cultured type II cells.

A functional reverse-oriented NF-κB site was first described in the 5′-flanking region of the murine *P-selectin* gene (27). This novel sequence, which was found to be required for optimal induction by TNF-α and lipopolysaccharide of *P-selectin* promoter activity, was suggested to preferentially bind p65 homodimers (27). It was proposed that this unusual element is functional because it binds to symmetrical homodimers, which do not require an orientation-specific NF-κB binding site for transcriptional activation (27). At present, it is not known as to whether TTF-1 and NF-κB proteins have the capacity to bind

simultaneously to the TBE site. In previous studies, we found that expressed TTF-1 homeodomain peptide binds specifically to the TBE (11). In the present study, we were unable to ascertain whether *in vitro* transcribed/translated p50 or p65 have the capacity to bind to the TBE directly. However, the expressed proteins also failed to bind to the NF-κB consensus sequence, suggesting that additional posttranslational modification of the NF-κB proteins is required for binding activity. On the other hand, our observations in cotransfection assays that p50/p65 and TTF-1 act synergistically at the TBE to increase *SP-A* promoter activity suggest that all three factors cooperatively interact at the TBE. In coimmunoprecipitation assays using nuclear extracts from human type II cells treated with Bt_2cAMP + IL-1α, we observed that TTF-1 and p65 interact *in vivo*, whereas interaction of TTF-1 with p50 was not detected. These findings suggest that p65/p50 heterodimer may interact with TTF-1 at the TBE through TTF-1·p65 binding.

In recent studies, we observed that TTF-1 binds to the coactivators CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) *in vitro* (29). CBP and SRC-1 were found to synergistically interact with TTF-1 at the TBE to increase *SP-A* promoter activity (29). It also has been reported that p65 interacts with CBP and its structural homolog, p300 (19, 30); phosphorylation of p65 by PKA was found to increase transcriptional activity by enhancing its association with CBP (19). On the other hand, p50 was observed to interact with SRC-1, and this interaction was found to enhance NF-κB-mediated transcription (31). In light of these findings, we suggest that TTF-1 and NF-κB proteins may form a transcriptional activation complex at the TBE that is stabilized by bivalent or multivalent interactions with coactivators, which in turn mediate interaction and stabilization of the basal transcription complex and cause an opening of chromatin structure through their intrinsic histone acetylase activities.

To begin to compare the mechanisms whereby Bt_2cAMP and IL-1 induce SP-A expression, their effects on nuclear levels of p65, p50, and TTF-1 and on nuclear protein binding activity were analyzed as a function of incubation time. Type II cell nuclear protein binding to both NF-κB and TBE probes was found to be maximally induced by IL-1 within 1 h of incubation and remained elevated throughout the 72-h incubation period. This rapid induction of binding activity by IL-1 was associated with an equally rapid increase in nuclear levels of p50 and p65. Whereas the stimulatory effect of IL-1 on p50 levels persisted throughout the 72-h incubation period, nuclear levels of p65 were reduced to those of control type II cells after 24 and 72 h of incubation. This decline in nuclear levels of p65 in IL-1-treated type II cells may be caused by NF-κB-induced activation of IκB-α gene expression (see Ref. 18 for review) and reaccumulation of NF-κB/IκB complexes in the cytoplasm. Interestingly, the decrease in p65 levels was associated with a marked decrease in nuclear levels of TTF-1.

In contrast to the extremely rapid inductive effects of IL-1 on NF-κB consensus and TBE binding, a stimulatory effect of Bt_2cAMP was evident after 3 h of incubation; maximal levels were reached only after 24 h and were maintained throughout the 72-h incubation period. cAMP treatment of type II cells had little or no effect to alter nuclear levels of TTF-1, p50, or p65. Previously, we found that the stimulatory effect of Bt_2cAMP on TBE binding activity in cultured type II cells was associated with increased phosphorylation of TTF-1; phosphatase treatment of nuclear extracts abolished DNA binding activity (16). In A549 cell transfection studies, PKA catalytic subunit (PKA-cat) increased TTF-1 transcriptional activity (16). It has also been reported that cytokines (32) and PKA (20) increase p65 phosphorylation and DNA binding activity. Our finding that binding of nuclear proteins to both NF-κB and TBE probes remains elevated in IL-1-treated cells after 24 and 72 h of treatment, whereas nuclear levels of p65 and TTF-1 decline suggests that posttranslational modification of these proteins is a primary factor in their DNA binding activity. It also should be noted that TTF-1 is surprisingly abundant for a transcription factor. It, therefore, is possible that only a small pool of the total nuclear TTF-1 is involved in DNA binding and that this pool is unaffected by IL-1. The present findings, therefore, suggest that IL-1 and cAMP act through NF-κB and TTF-1 to increase SP-A expression by different mechanisms, and that posttranslational modification of TTF-1 and p65 may have a greater influence on DNA binding and transcriptional activity than their absolute levels within the nucleus.

Activation of the NF-κB pathway by a variety of factors, including cytokines, lipopolysaccharide, and ROS, results from phosphorylation, ubiquitination, and degradation of the inhibitory IκB subunit and the release of active NF-κB p50/p65 heterodimer to the nucleus (see Ref. 18 for review). The p65 component of the released and active NF-κB heterodimer is phosphorylated at a specific PKA site, which enhances its DNA binding and transcriptional activity (20). Recently, it was reported that PKA-cat is associated in an inactive complex with IκB-α and -β in association with the NF-κB complex. Stimulatory factors that promote degradation of IκB cause the release and activation of the PKA-cat with phosphorylation of NF-κB p65 (20). This provides a mechanism for a cAMP-independent, cytokine-mediated activation of PKA and of NF-κB. These findings, together with those of the present study, suggest that cAMP and IL-1 may exert additive effects on *SP-A* gene expression by facilitating increased activation of PKA with associated phosphorylation, DNA binding and transcriptional activation of both TTF-1 and NF-κB. Our findings further suggest that activated TTF-1 and NF-κB proteins exert their stimulatory effects on *SP-A* gene expression through binding to the TBE.

As mentioned above, we previously observed that O_2 has a dose-dependent, permissive effect on cAMP induction of *SP-A* gene expression in cultured human

fetal lung (5). In the present study, we found that treatment of cultured type II cells with the antioxidant inhibitors of NF-κB, PDTC, and NAC, reduced nuclear protein binding to the NF-κB consensus oligonucleotide and to the TBE, and blocked cAMP induction of *SP-A* gene expression. Our findings provide intriguing evidence that permissive effects of O_2 on cAMP induction of *SP-A* gene expression may be mediated by cooperative interaction of TTF-1 and NF-κB at the TBE. NF-κB is considered to be a redox-sensitive transcription factor; ROS appear to serve a permissive role in the activation of NF-κB in response to a variety of inflammatory mediators in a number of different cell types (18, 21, 33). Antioxidants, such as PDTC and NAC, block the activation of NF-κB by various cytokines, lipopolysaccharide, and other inducers, suggesting the involvement of a common oxygen-sensitive intermediate; however, the precise step(s) sensitive to oxidative stress has not been determined. On the other hand, it has been proposed that one or more steps involving phosphorylation of IκB by IκB kinases and/or its degradation via the proteasome pathway may be regulated by oxidative stress (21). In recent studies using fetal rat type II cells, it was found that NAC, a glutathione precursor and scavenger of ROS, decreased nuclear binding activity for an NF-κB consensus sequence, while increasing DNA binding to a consensus sequence for hypoxia-inducible transcription factor-1α (34). It was observed in those studies that binding of type II cell nuclear proteins to an NF-κB consensus sequence was activated by subjecting the cells to acute increases in O_2 tension in association with an increase in the ratio of reduced to oxidized glutathione.

In summary, we have observed that cAMP and IL-1 have additive stimulatory effects on TBE-binding activity and on SP-A expression in human fetal lung type II cells. NF-κB and TTF-1 proteins appear to cooperatively interact at the TBE to increase *SP-A* promoter activity. We also found that cAMP and IL-1 induction of TBE-binding activity and SP-A expression is strongly influenced by the redox state of the type II cell. ROS are likely to increase in epithelial cells of developing lung in association with increased vasculogenesis and O_2 delivery, and with associated decreases in cellular glutathione (35) and glutathione-*S*-transferases (GSTs) (36). Our findings suggest that developmental increases in O_2 availability and ROS may serve a permissive role in cAMP and cytokine-mediated induction of *SP-A* gene expression through enhanced binding, activation and cooperative interaction of TTF-1 and NF-κB at the TBE.

MATERIALS AND METHODS

Plasmids and Antisera

An expression vector (p*CMV5*/*TTF-1*) containing a full-length cDNA encoding baboon TTF-1 (11) was constructed as described previously (16). Expression vectors for NF-κB proteins p50 and p65 [*Rous sarcoma virus* (*RSV*)/*p50* and *RSV/p65*] were kindly provided by Dr. Richard Gaynor (UT Southwestern). A *human GH* (*hGH*) reporter plasmid (*TBE_3SP-A:hGH*) containing a concatamer of three DNA repeats of *bSP-A2* gene 5′-flanking sequences between −185 and −165 bp, containing TBE1 (*underlined*) (henceforth referred to as TBE, 5′-GTGCTCCCCTCAAGGGTCCTA-3′) (11), was constructed as described previously (16). Recombinant adenovirus DN forms of IκB-α and IκB-β containing mutations in the IκB kinase serine phosphorylation sites (28) were generously provided by Dr. Richard Gaynor (UT Southwestern). A recombinant adenovirus containing *CMV:βgal* was used as a control.

TTF-1 antiserum was raised in rabbits against bacterially expressed GST-TTF-1_{1-50} peptide containing the N-terminal 50 amino acids of bTTF-1 (11). The antiserum was adsorbed with GST peptide to remove GST-specific antibodies. Specific rabbit polyclonal antibodies were raised against human SP-A protein isolated from alveolar lavage of a patient with alveolar proteinosis (4). Rabbit antisera against NF-κB p50 and p65 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Preparation and Culture of Human Fetal Lung Explants, Type II Cells, and Human Adenocarcinoma Cell Lines

Type II cells were isolated from cultured midgestation human fetal lung explants, as described previously (26). Briefly, lung tissues from midgestation human abortuses were maintained in organ culture in serum-free Waymouth's MB752/1 medium (Life Technologies, Inc., Grand Island, NY) in the presence of Bt_2cAMP to promote type II cell differentiation (4). After 3 d of culture, tissues were digested with collagenase; isolated cells were treated with diethylaminoethyl-dextran, and plated at a density of 5–9 × 10^6 cells/60-mm dish. The cells were maintained overnight in Waymouth's medium containing 10% FBS, and then incubated for up to 3 d in serum-free Waymouth's medium in the absence or presence of Bt_2cAMP (1 mM), IL-1α (10 ng/ml), IL-1β (10 ng/ml), or Bt_2cAMP and IL-1 in combination. In some experiments, type II cells treated with cytokines and cAMP also were incubated with or without PDTC (25 μM; Sigma, St. Louis, MO). The human lung adenocarcinoma cell line A549 (ATCC CCL 185) was maintained in Waymouth's medium containing 10% FBS.

EMSAs

Nuclear extracts were prepared from lung type II cells as described previously (37). Protein concentrations were determined by a modified Bradford assay (Bio-Rad Laboratories, Inc., Richmond, CA). Double-stranded oligonucleotides corresponding to the TBE (TBE, *underlined*) and flanking sequences (5′-GTGCTCCCCTCAAGGGTCCT-3′) and an oligonucleotide corresponding to an NF-κB consensus binding site (*underlined*) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Integrated DNA Technologies, Inc., Coralville, IA) were end-labeled using [γ^{32}P]ATP (ICN Biomedicals, Costa Mesa, CA) and used as probes. Nuclear proteins (7 μg) were incubated with ^{32}P-labeled TBE oligonucleotide for 30 min at room temperature in reaction buffer (20 mM HEPES, pH 7.6; 75 mM KCl; 0.2 mM EDTA; 20% glycerol) and 1 μg of poly(deoxyinosine-deoxycytidine)-poly(deoxyinosine-deoxycytidine) (Amersham Pharmacia Biotech, Piscataway, NJ) as nonspecific competitor. Protein-DNA complexes were separated from free probe on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography. For DNA competition EMSA, nonradiolabeled double-stranded oligonucleotides in 10- to 1000-fold molar excess of the radiolabeled probe were added simultaneously with radiolabeled probe. Supershift EMSA was performed by a 30-min incubation at room temperature of nuclear extracts with 1 μl of antisera to TTF-1, p50, or p65 before incubation with the radiolabeled probe.

Immunoblot Analysis

Human fetal type II cells were cultured for up to 3 d in serum-free medium, in medium containing Bt_2cAMP (1 mM), or with IL-1α or β (10 ng/ml) alone or in combination with Bt_2cAMP. The cells were scraped from the dishes and homogenized in ice-cold PBS containing protease inhibitor cocktail (1 tablet/10 ml) (Roche Molecular Biochemicals, Indianapolis, IN). Proteins (20 μg) from nuclear and cytoplasmic (supernatant) fractions obtained during isolation of nuclei for EMSA (37) were separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes as described previously (4). The membranes were then analyzed for SP-A, TTF-1, p50, and p65 using specific antisera and an Enhanced Chemiluminescence System according to manufacturer's recommendations (Amersham Pharmacia Biotech).

Coimmunoprecipitation Assay

Nuclear extracts were prepared from human fetal lung type II cells maintained in medium containing Bt_2cAMP + IL-1α (10 ng/ml) for 24 h. Nuclear proteins (200 μg) were diluted in 500 μl of RIPA buffer (50 mM Tris, pH 7.4; 1 mM EDTA; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; protease inhibitor cocktail) and precleared with 50 μl of protein A/G plus agarose (Santa Cruz Biotechnology, Inc.) for 1 h at 4 C. Precleared samples were then incubated either with TTF-1 antiserum (5 μl), or with antisera to p50, p65, or the corresponding preimmune serum or normal IgG for 2 h at 4 C. The immune complexes were recovered upon incubation with 50 μl of protein A/G plus agarose overnight at 4 C. Pellets containing the immunoprecipitated proteins were collected by centrifugation at 2500 rpm for 5 min at 4 C and washed three times with cold PBS containing protease inhibitor cocktail. After the final wash, the pellet was resuspended in sample buffer and subjected to SDS-PAGE followed by immunoblot analysis using p65 or p50 as the primary antibody.

Transient Transfections

Before transfection, A549 cells were grown to logarithmic phase (70% confluence) in Waymouth's MB752/1 medium containing 10% FBS in 35-mm culture dishes. The cells were transfected with 2 μg of the *(TBE)$_3$SP-A:hGH* fusion gene, together with either 1 μg of *pCMV5/TTF-1* or the empty expression vector (*pCMV5*), or 1 μg of *RSV/p50*, *RSV/p65* alone or in combination and compensatory amounts of *RSV* empty vector. All cells were cotransfected with 0.25 μg *RSV/β-gal* as an internal control for transfection efficiency. The plasmids were combined with 8 μl of Fugene (Roche Molecular Biochemicals) in Waymouth's MB752/1 medium and incubated at room temperature for 30 min. The cells were washed once with medium and incubated with reporter and expression plasmid/Fugene mixtures in 1 ml of serum-free Waymouth's MB752/1 medium for 15 h at 37 C. The medium was then removed, fresh Waymouth's medium (1 ml) was added to each well with or without IL-1β (10 ng/ml), and the cells were incubated at 37 C for 24 h. Media were then collected and assayed for hGH content by RIA (Nichols Institute Diagnostics, San Juan Capistrano, CA). Variations in transfection efficiency were corrected by normalizing hGH to β-gal activity, which was assayed using a Galato-light kit (Tropix, Inc., Bedford, MA).

Infection of Type II Cells with Recombinant Adenoviruses

Human fetal type II cells were infected with recombinant adenoviruses as described previously (7). Briefly, the cells were incubated for 1 h with recombinant adenoviruses expressing either DN-IκB-α (*CMV:DN-IκB-α*), IκB-β (*CMV:DN-IκB-β*) (28) (multiplicity of infection, m.o.i. = 10) or with *CMV:βgal* (m.o.i. = 10), as a control, followed by incubation in the absence or presence of Bt_2cAMP, IL-1α, or IL-1α + Bt_2cAMP for 3 d.

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