Glucocorticoid inhibition of SP-A gene expression in lung type II cells is mediated via the TTF-1-binding element

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Alcorn, Joseph L., Kazi N. Islam, Pampee P. Young, and Carole R. Mendelson. Glucocorticoid inhibition of SP-A gene expression in lung type II cells is mediated via the TTF-1-binding element. Am J Physiol Lung Cell Mol Physiol 286: L767–L776, 2004. —Induction of surfactant protein-A (SP-A) gene expression in fetal lung type II cells by cAMP and IL-1 is mediated by increased binding of thyroid transcription factor-1 (TTF-1) and NF-κB proteins p50 and p65 to the TTF-1-binding element (TBE) at ∼183 bp. In type II cell transfections, dexamethasone (Dex) markedly inhibits cAMP-induced expression of rabbit SP-A/human growth hormone (hGH) fusion genes containing as little as ∼300 bp of the SP-A 5′-flanking sequence. Dex inhibition is blocked by RU-486, suggesting a role of the glucocorticoid receptor (GR). The present study was undertaken to define the mechanisms for GR inhibition of SP-A expression. Cotransfection of primary cultures of type II cells with a GR expression vector abrogated cAMP induction of SP-A promoter activity while, at the same time, causing a 60-fold induction of cotransfected mouse mammary tumor virus (MMTV) promoter. In lung cells transfected with a fusion gene containing three TBEs fused to the basal SP-A promoter, Dex prevented the stimulatory effect of IL-1 on TTF-1 induction of SP-A promoter activity, suggesting that the GR inhibits SP-A promoter activity through the TBE. In gel shift assays using nuclear extracts from human fetal type II cells cultured in the absence or presence of cAMP, Dex markedly reduced binding of nuclear proteins to the TBE and blocked the stimulatory effect of cAMP on TBE-binding activity. Our finding that Dex increased expression of the NF-κB inhibitory partner IκB-α suggests that the decrease in TBE-binding activity may be caused, in part, by GR inhibition of NF-κB interaction with this site.

surfactant protein A; fetal lung; glucocorticoid receptor; cAMP; thyroid transcription factor-1

PULMONARY SURFACANT, a developmentally regulated phospholipid-rich lipoprotein produced by type II pneumocytes, reduces surface tension at the alveolar air-liquid interface and prevents alveolar collapse. In fetal lung, type II cells containing stored surfactant are first evident only after ∼70% of gestation is completed. The fundamental discoveries that administration of synthetic glucocorticoids to fetal lambs resulted in accelerated lung maturation (37) and that glucocorticoid treatment of fetal rabbits enhanced surfactant production and accelerated appearance of type II cells (32, 59) led to numerous studies that supported the concept that glucocorticoids enhance surfactant glycero phospholipid synthesis (6, 44). On the basis of such findings, the antenatal administration of glucocorticoids has been used clinically to enhance lung maturation and prevent respiratory distress syndrome in infants of mothers likely to deliver preterm (38). A number of factors, including gestational age, gender, and treatment regimen, influence the effectiveness of antenatal glucocorticoid regulation of the lipid and protein components of surfactant (7). Surfactant glycerophospholipid synthesis is, in fact, under multifactorial control; in addition to glucocorticoids, a number of other steroid and polypeptide hormones, as well as cAMP, are important in its regulation (44).

Surfactant is primarily composed of phosphatidylcholine, with lesser amounts of other glycerophospholipids and cholesterol. Surfactant also contains four associated proteins, surfactant protein (SP) A, SP-B, SP-C, and SP-D, which are expressed in a lung-specific manner and developmentally regulated in fetal lung tissue (22, 44). Synthesis of the major surfactant protein, SP-A, a C-type lectin that plays an important role in immune defense within the lung alveolus, is initiated in fetal lung during the third trimester of gestation in concert with augmented surfactant glycerophospholipid synthesis (45, 57). cAMP increases expression of the SP-A gene in rabbit (9, 45), baboon (52), and human (49) fetal lung tissue in culture. The stimulatory effects of cAMP appear primarily to be mediated at the level of SP-A gene transcription (9, 11).

In studies using transgenic mice and transfected type II cells, we found that a ∼400-bp region flanking the 5′-end of the SP-A gene mediates lung type II cell-specific and appropriate developmental regulation of expression (2). Within this region, five response elements, each of which is critical for basal and cAMP induction of SP-A promoter activity, have been identified (63). One of these, termed the TBE [thyroid transcription factor (TTF)-1-binding element], binds the homeodomain transcription factor TTF-1/Nkx2.1 (34, 35). cAMP acting through protein kinase A causes increased TTF-1 phosphorylation, TBE binding, and transcriptional activity (34, 62). We recently found that treatment of human fetal type II cells with the cytokine IL-1, a known activator of NF-κB, increases SP-A expression and has additive stimulatory effects with cAMP. Interestingly, TBE at approximately ∼180 bp within the human and baboon SP-A2 (hSP-A2 and bSP-A2) 5′-flanking regions contains a “reverse-oriented” NF-κB-binding site; IL-1 and cAMP treatment of human fetal type II cells enhances binding of NF-κB proteins p50 and p65, together with TTF-1, to the TBE (27). Although we were unable to ascertain whether p65 and p50 bind to the TBE directly, in coimmunoprecipitation assays it was found that p65 interacts with TTF-1 in type...
II cells (27). Furthermore, p65 and p50 act cooperatively with TTF-1 to stimulate SP-A promoter activity (27).

Glucocorticoids have complex effects on SP-A expression that may be species specific and dependent on the stage of development at which treatment is initiated. In studies using midgestation human fetal lung explants, we observed that dexamethasone (Dex) caused a dose-dependent induction of hSP-A gene transcription and acted synergistically with dibutyryl cAMP (DBcAMP) (11, 12); however, in the same tissues, Dex caused a dose-dependent decrease in the levels of SP-A mRNA and protein and antagonized the stimulatory effect of DBcAMP on SP-A mRNA and protein levels (48). This apparent inhibitory effect of glucocorticoids is due to a dominant action to decrease SP-A mRNA stability (11, 12, 24). The inhibitory effects of Dex on SP-A mRNA stability were found to be dose dependent, completely reversible, and blocked by the glucocorticoid receptor (GR) antagonist RU-486 (12). In contrast to the rabbit, which has a single-copy SP-A gene (10, 14), the genomes of humans (30, 39) and baboons (18) contain two highly similar SP-A genes, SP-A1 and SP-A2. We previously observed that the hSP-A2 gene is more responsive to the inductive effects of cAMP and the inhibitory effects of glucocorticoids than is hSP-A1 (40).

In studies using lung explants from 21-day fetal rabbits (full term = 31 days), 10^{-7} M Dex caused an acute (6–24 h) inhibition of SP-A gene transcription and reduced the magnitude of the stimulatory effect of DBcAMP (9). However, after 48–72 h of incubation, a stimulatory effect of glucocorticoid was observed, and an additive effect with DBcAMP on SP-A gene transcription was found (9). In type II cell transfection studies, we previously observed that Dex markedly inhibited cAMP-induced expression of SP-A (rSP-A); human growth hormone (hGH) gene containing as little as 380 bp of the SP-A 5' flanking DNA (1). The finding that this effect of Dex was half-maximal at 10^{-10} M and blocked by the GR antagonist RU-486 suggests a role of the GR in Dex inhibition of SP-A promoter activity. Although Dex alone had little effect on basal expression of the SP-A: hGH fusion gene constructs, it markedly antagonized the stimulatory action of cAMP (1).

It was our objective in this study to define the mechanisms for the effects of glucocorticoids acting through the GR on SP-A promoter activity. We have focused on TBE in light of its importance in cAMP and IL-1 induction of SP-A gene expression and because TBE interacts with TTF-1 and NF-κB proteins p65 and p50 (27). Glucocorticoids acting through the GR have been reported to antagonize NF-κB transcriptional activity via several mechanisms that appear to be cell type specific (17). Our findings suggest that, in the absence of a functional glucocorticoid-response element (GRE) within ~1,000 bp upstream of the SP-A transcription initiation site, the inhibitory effects of Dex acting through the GR are mediated via GR inhibition of the binding of type II cell nuclear proteins to the TBE. Furthermore, the inhibitory actions of glucocorticoids may be mediated, in part, through GR-induced expression of IκB-α, an inhibitory partner of NF-κB, thereby reducing the levels of p65 and p50 available to bind to the TBE.

**MATERIALS AND METHODS**

Fusion genes and recombinant adenoviruses. Fusion genes and expression vectors incorporated into the genomes of recombinant adenoviruses and used in the present study are shown in Fig. 1. DNA containing the promoterless hGH gene, as reporter (53), was fused downstream of rSP-A genes comprising 20 bp of exon I and 990 bp of exon I 5'-flanking DNA (rSP-A_{990}:hGH), as described previously (1), and to the mouse mammary tumor virus (MMTV) long terminal repeat (LTR; MMTV_{770}:hGH), a sequence known to contain functional GREs (19). rSP-A_{990}:TK:hGH for analysis of effects of the GR on SP-A 5'-flanking sequences acting on a heterologous promoter. Several fusion genes were also constructed that contained various amounts of 5'-flanking DNA from the hSP-A2 gene and +20 bp of the rabbit surfactant protein A (rSP-A) gene plus 20 bp of the human growth hormone (hGH) structural gene. These genes were utilized to analyze effects of dexamethasone (Dex) on the human SP-A2 (hSP-A2) gene promoter: hSP-A2_{1500}:hGH, hSP-A2_{900}:hGH, and hSP-A2_{47}:hGH, which contained 1500, 990, and 47 bp of 5'-flanking sequence and 20 bp of the 1st exon from the hSP-A2 gene fused to the hGH structural gene. These fusion genes were incorporated into the genome of a recombinant human adenovirus (Ad5), as previously described (1). TBE_{3}:hGH: fusion gene comprising 3 repeats of the baboon SP-A2 (bSP-A2) gene 5'-flanking sequence between +185 and +165 bp containing thyroid transcription factor-1 (TTF-1)-binding element (TBE)-1 (underlined: 5'-GTGGCTCACCTCAAGGTCCCTCA-3') subcloned upstream of 50 bp of hSP-A2 5'-flanking sequence and +40 bp of hSP-A2 exon I fused to hGH.

Fig. 1. Schematic diagram of fusion genes incorporated into recombinant adenoviruses. CMV:hGR: a full-length cDNA encoding the human glucocorticoid receptor (hGR) was placed under control of 600 bp of the ubiquitously expressed cytomegalovirus (CMV) promoter. MMTV_{770}:hGH: human growth hormone (hGH) structural gene (used as a reporter of promoter activity) was placed under control of 770 bp of the mouse mammary tumor virus (MMTV) long terminal repeat. rSP-A_{990}:hGH: hGH structural gene was placed under control of 990 bp of DNA flanking the 5'-end of the rabbit surfactant protein A (rSP-A) gene plus 20 bp of SP-A exon I, TK:hGH: 250-bp herpes simplex virus thymidine kinase (TK) promoter, which is unaffected by glucocorticoids or cAMP, was subcloned upstream of the hGH structural gene, rSP-A_{900}:TK:hGH: ~990 to ~47 bp of the rSP-A 5'-flanking sequence was fused upstream of the TK promoter. Several fusion genes were utilized to analyze effects of dexamethasone (Dex) on the human SP-A2 (hSP-A2) gene promoter: hSP-A2_{1500}:hGH, hSP-A2_{900}:hGH, and hSP-A2_{47}:hGH, which contained 1500, 990, and 47 bp of 5'-flanking sequence and 20 bp of the 1st exon from the hSP-A2 gene fused to the hGH structural gene. These fusion genes were incorporated into the genome of a recombinant human adenovirus (Ad5), as previously described (1). TBE_{3}:hGH: fusion gene comprising 3 repeats of the baboon SP-A2 (bSP-A2) gene 5'-flanking sequence between +185 and +165 bp containing thyroid transcription factor-1 (TTF-1)-binding element (TBE)-1 (underlined: 5'-GTGGCTCACCTCAAGGTCCCTCA-3') subcloned upstream of 50 bp of hSP-A2 5'-flanking sequence and +40 bp of hSP-A2 exon I fused to hGH.
bp of the first exon fused upstream of the promoterless hGH gene (64) to evaluate the effect of Dex on hSP-A2 promoter activity. These fusion genes include hSP-A2–1500:hGH, hSP-A2–990:hGH, and hSP-A2–378:hGH. The cDNA encoding the human GR (hGR) (23) was placed under the control of the strong, ubiquitously expressed cytomegalovirus (CMV) E1 promoter (CMV:hGR) (60). These fusion genes were incorporated independently into the genomes of recombinant replication-defective adenoviruses using methods described by Graham and Prevec (21). An SP-A promoter construct was also generated that consisted of a concatamer of three repeats of the hSP-A2 gene 5'-flanking sequence between −185 and −165 bp containing TBE1 (underlined: 5'-GTGCTCCCCCTCAAAGGTCTCTA-3') subcloned upstream of 50 bp of the hSP-A2 5'-flanking sequence and +40 bp of hSP-A2 exon I, fused to hGH (TBE5:hGH) (34). This construct was introduced into A549 cells using standard transfection methodology (see below).

**Organ culture of fetal lung tissues, type II pneumocyte isolation, and primary culture.** The procedure used to isolate and maintain human, rat, and rabbit fetal type II pneumocytes in primary culture has been described previously (4). Midgestation human fetal lung tissues were obtained from Advanced Bioscience Resources (Alameda, CA) in accordance with the Donors Anatomical Gift Act of the State of California. Rat tissues were obtained from the Animal Welfare Committee of the University of Texas Southwestern Medical Center at Dallas. Lung tissues from 18-day-gestation-age Sprague-Dawley fetal rats were obtained in accordance with the guidelines established by the Animal Welfare Information Center and approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas. The tissues were minced and cultured in serum-free Waymouth's MB752/1 medium (GIBCO BRL, Gaithersburg, MD) in the presence of 1 nM DbcAMP (Boehringer Mannheim, Indianapolis, IN). After 3–5 days of organ culture with daily medium changes, lung explants were dissociated by digestion with collagenase type I (0.5 mg/ml; Sigma Chemical, St. Louis, MO) and collagenase type IA (0.5 mg/ml; Sigma Chemical). After collagenase digestion, the cell suspension was depleted of fibroblasts (4) and plated onto 60-mm tissue culture dishes or on Transwell coverslips (Nunc, Naperville, IL) that were coated with extracellular matrix prepared from Madin-Darby canine kidney cells (CR L 6253, American Type Culture Collection; 2–5 × 10⁶ cells/60-mm dish) (4). Plated epithelial cell-enriched cultures were incubated overnight in Wayne's medium with 10% (vol/vol) FBS (GIBCO BRL). Dishes were washed twice with medium to eliminate dead and nonadherent cells and then incubated in Wayne's MB752/1 medium without FBS. The plating density of the cells after overnight incubation was ∼50–60%.

**Infection of human type II pneumocytes with recombinant adenoviruses.** Type II pneumocytes were infected with recombinant replication-defective human adenoviruses containing the appropriate fusion genes, as described previously (1, 4). Briefly, type II pneumocytes that were plated onto 60-mm extracellular matrix-coated dishes and maintained overnight in Wayne's MB752/1 medium containing 10% FBS were then washed several times with medium to remove nonadherent cells and debris and incubated for 1 h with recombinant adenovirus. The adenovirus-containing medium was then removed and replaced with fresh medium without serum.

**Cell culture and transfection.** The lung adenocarcinoma cell line A549 (36) was maintained in Wayne's MB752/1 medium containing 10% FBS. The recombinant DNA was introduced into the cell line by infection using recombinant adenoviruses as described for type II cells in primary culture or by standard transfection techniques. In transfection studies to analyze the effects of Dex and the GR on TTF-1 and IL-1 induction of SP-A promoter activity with the (TBE)pSP-A:hGH fusion gene reporter. A549 cells were grown to logarithmic phase (70% confluence) in Wayne's MB752/1 medium containing 10% FBS in 35-mm culture dishes. The cells were transfected with 2 μg of the (TBE)pSP-A:hGH fusion gene, together with 1 μg of pCMV5/TTF-1 or the empty expression vector (pCMV5) or with 1 μg of respiratory syncytial virus (RSV)p50 or RSVp65, alone or in combination, and compensatory amounts of RSV empty vector. All cells were cotransfected with 0.25 μg of RSVβ GALDS (β-gal) as an internal control for transfection efficiency. The plasmids were combined with 8 μl of FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) in Wayne'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 mixture in 1 ml of serum-free Wayne's MB752/1 medium for 15 h at 37°C. The medium was then removed, fresh Wayne'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 radioimmunoassay (Nichols Institute, 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, Bedford, MA).

**Immunoblot analysis.** Cellular proteins were isolated as described previously (3). Briefly, cells were scraped from the plates with a rubber policeman and homogenized in ice-cold homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) with 10 strokes in a Teflon-glass homogenizer. The homogenized samples were centrifuged at 600 g for 5 min to sediment nuclei and debris, and the resulting supernatant was assayed for protein content. Cellular proteins (20 μg) were subjected to one-dimensional SDS-PAGE, and the separated proteins were transferred to nitrocellulose by electrotransfer. The proteins on the resulting blots were analyzed for SP-A and β-actin content as described previously (45) using antibodies to hSP-A (49) and to β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The resulting immune complexes were visualized using the ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were subjected to autoradiography using Kodak X-OMAT radiographic film for visualization of the resulting protein-antibody complexes.

**Quantitative analysis of hGH production in transfected human type II pneumocytes and cell lines.** Media from transfected cells or from cells infected with the recombinant adenoviruses containing promoter fusion constructs with hGH reporter genes were collected at 24-h intervals. The concentration of hGH in the medium was quantitated by radioimmunoassay using an Allegro hGH kit (Nichols Institute Diagnostics).

**Electrophoretic mobility shift assays.** Nuclear extracts were prepared from lung type II cells as described previously (33). Protein concentrations were determined by a modified Bradford assay (Bio-Rad, Richmond, CA). Double-stranded oligonucleotides corresponding to TBE, underlined, and flanking sequences (5'-GTGGCTCCCTCAAAGGTCTCTA-3') and to an NF-kB consensus binding site (underlined: 5'-GTGGAGGCGACCTCCCCAGGCGC-3'; Integrated DNA Technologies, Coralville, IA) were end labeled using [γ-32P]ATP (ICN, Costa Mesa, CA) and used as probes. Nuclear proteins (7 μg) were incubated with 32P-labeled TBE and NF-kB oligonucleotides for 30 min at room temperature in reaction buffer (20 mM HEPES, pH 7.6, 75 mM KCl, 0.2 mM EDTA, and 20% glycerol) and 1 μg of poly(dl-dc)-poly(dl-dc) (Pharmacia) as nonspecific competitor. Protein-DNA complexes were separated from free probe on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

**RESULTS**

Dex acting through the GR inhibits SP-A promoter activity and stimulates MMTV promoter activity in type II pneumocytes in primary culture. Previously, we observed that Dex antagonized the stimulatory effect of DBcAMP on expression of rSP-A:hGH fusion genes containing 1,766, 990, and 378 bp
of SP-A 5'-flanking DNA; the effect of Dex was half-maximal at $10^{-9}$ M and blocked by the GR antagonist RU-486 (1). To determine whether Dex had the capacity to stimulate gene expression in lung type II cells when acting through classical GREs, we compared effects of Dex with or without GR overexpression on SP-A promoter activity with effects on activation of the MMTV promoter, which is known to be positively regulated by glucocorticoids. MMTV promoter activity is regulated by direct binding of the GR to cognate GREs within the LTR (19). Consequently, recombinant adenoviruses containing the hGR coding sequence under control of the ubiquitously expressed CMV E1 promoter CMV:hGR and a fusion gene consisting of 770 bp of the glucocorticoid-responsive MMTV LTR linked to the hGH structural gene as reporter (MMTV/770:hGH) were created (Fig. 1). Rat type II cells in primary culture were infected with recombinant adenoviruses containing rSP-A/990:hGH (containing 990 bp of 5'-flanking DNA from the rSP-A gene linked to hGH) or MMTV/770:hGH reporter constructs with or without cotransfected CMV:hGR. The transfected cells were incubated in the absence or presence of DBcAMP, Dex, or both for up to 5 days in culture, and the amount of hGH secreted into the medium during each 24-h period was determined by radioimmunoassay. In type II cells transfected with SP-A/990:hGH, Dex had little effect on fusion gene expression compared with cells incubated in control medium (Fig. 2A). Similar findings were obtained in type II cells cotransfected with SP-A/990:hGH and CMV:hGR (Fig. 2B). As we observed previously, DBcAMP increased fusion gene expression >25-fold (Fig. 2, A and B), whereas Dex antagonized the stimulatory effect of DBcAMP by ~70% (Fig. 2A). In the absence of Dex treatment, coexpression of CMV:hGR had little effect on SP-A/990:hGH expression in cells treated with DBcAMP; however, hGR overexpression enhanced the suppressive effect of Dex on cAMP stimulation of SP-A promoter activity, reducing hGH production by 99% compared with DBcAMP alone (Fig. 2B). These findings suggest that the antagonistic effect of Dex is mediated through the GR.

In type II cells transfected with MMTV/770:hGH, Dex caused a modest increase in hGH production compared with untreated cells (Fig. 2C). Interestingly, DBcAMP increased MMTV/770:hGH expression in a pattern similar to that observed in cells transfected with SP-A/990:hGH. In cells cotransfected with CMV:hGR, Dex caused a rapid and marked increase in MMTV/770:hGH expression; no further stimulation was observed in cells coincubated with DBcAMP + Dex (Fig. 2D).
2D). These findings suggest that cultured rat type II cells contain the cofactors required to mediate GR stimulation of MMTV expression; however, it is evident that these cells contain low levels of endogenous GR. On the other hand, it appears that the low endogenous levels of GR in rat type II cells are sufficient to mediate pronounced glucocorticoid inhibition of cAMP induction of SP-A promoter activity.

To ascertain whether the inhibitory effect of Dex was mediated by interaction of the GR with SP-A 5′-flanking sequences upstream of the TATA box or with sequences within the hGH structural gene, fetal rat type II cells were infected with recombinant adenoviruses containing an hGH fusion gene comprising SP-A 5′-flanking sequences from −990 to −47 bp (excluding the TATA box and +20 bp of exon 1) fused upstream of the 250-bp herpes simplex virus TK promoter (TK:SP-A−990:hGH) or with the 250-bp TK promoter fused to hGH (TK:hGH) (Fig. 1) (42). Whereas no effect of Dex or cAMP was observed on expression of TK:hGH, Dex caused a 50% inhibition of cAMP-induced SP-A−990:TK:hGH expression (data not shown), suggesting that Dex inhibition is mediated through SP-A 5′-flanking sequences between −47 and −990 bp. These findings differ from those of Hoover and colleagues (25), who reported that a region between 770 and 990 bp of exon I fused upstream of the 250-bp herpes simplex virus TK promoter (TK:SP-A−990:hGH) or with the 250-bp TK promoter fused to hGH (TK:hGH) (Fig. 1) (42). Whereas no effect of Dex or cAMP was observed on expression of TK:hGH, Dex caused a 50% inhibition of cAMP-induced SP-A−990:TK:hGH expression (data not shown), suggesting that Dex inhibition is mediated through SP-A 5′-flanking sequences between −47 and −990 bp. These findings differ from those of Hoover and colleagues (25), who reported that a region between −32 and +63 bp of the hSP-A1 gene mediates Dex repression of transcription.

Effects of Dex on SP-A and MMTV promoter activity in rat type II cells and A549 lung adenocarcinoma cells are likely dependent on endogenous levels of GR. In light of the apparently low levels of endogenous GR in primary cultures of lung type II cells, we performed similar experiments in the human lung adenocarcinoma cell line A549, which is presumed to be of type II cell origin. The A549 cells were cultured and infected in a manner identical to that used for the rat type II cells. Cells were infected with recombinant adenoviruses containing SP-A−990:hGH or MMTV−770:hGH fusion genes with or without cotransfection with CMV:hGR. The cells were cultured for 5 days in the absence or presence of DBcAMP, Dex, or both, and the amounts of hGH secreted into the medium over a 24-h period between days 4 and 5 of culture were determined by radioimmunounassay. The data shown in Fig. 3A for rat type II cells correspond to the results obtained after 5 days of culture that are shown in Fig. 2.

In contrast to findings in primary cultures of rat type II cells and as reported previously (1), DBcAMP only modestly increased SP-A promoter activity in A549 cells (Fig. 3). On the other hand, the effect of Dex to stimulate MMTV−770:hGH expression and to inhibit SP-A−990:hGH expression in the absence of cotransfected GR was much more pronounced in the A549 cells than in the primary cultures of rat type II cells, suggesting elevated levels of endogenous functional GR in the former (Fig. 3B). Accordingly, in the A549 cells, cotransfection of CMV:GR had a relatively modest effect to further increase Dex inhibition of SP-A promoter activity and Dex stimulation of MMTV promoter activity (Fig. 3B).

Glucocorticoid inhibition of hSP-A2 promoter activity is mediated by sequences within 296 bp upstream of the transcription initiation site and is associated with decreased binding of type II cell nuclear proteins to the TBE. Because the inhibitory effect of Dex on SP-A promoter activity in rat type II cells is manifest only in the presence of DBcAMP, it is likely that the effect of Dex is mediated by GR inhibition of DNA binding and/or transcriptional activity of a cAMP-activated transcription factor. In previous studies, we observed that Dex antagonized cAMP stimulation of SP-A:hGH fusion genes containing as little as 378 bp of rSP-A 5′-flanking sequence (1), suggesting that the transcription factor(s) with which the GR interacts lies within the proximal 5′-flanking region. In recent studies, we found that the TBEs of the hSP-A2 and hSP-A2 genes, which contain a reverse-oriented NF-κB-binding site and bind NF-κB proteins p65 and p50 with TTF-1 (27), play an important role in cAMP and IL-1 stimulation of SP-A promoter activity (27). Because the GR has been reported to interact with p65 (61) and to block NF-κB transcriptional activity (17), it was of interest to determine whether the inhibitory effect of Dex is mediated via the TBE. Although the TBE is highly conserved among the SP-A genes of a variety of species (43), we elected to focus our studies on the TBE of the hSP-A2 gene because of our prior studies on its characterization.
It was first important to evaluate the effect of Dex on hSP-A promoter activity and to delineate the genomic region that mediates glucocorticoid inhibition. As we observed using the rSP-A promoter constructs (Figs. 2 and 3), Dex antagonized cAMP induction of hSP-A2:hGH expression in transfected human fetal type II cells (Fig. 4). The inhibitory effect of Dex on cAMP-induced hSP-A2 promoter activity appears to be mediated by sequences between −47 and −296 bp of hSP-A2 5′-flanking DNA. In contrast to findings using rat type II cells (Figs. 2 and 3), Dex inhibited basal levels of hSP-A2:hGH expression in the human fetal type II cells (Fig. 4). This may be due, in part, to the high basal levels of fusion gene expression in the human compared with the rat type II cells. As reported previously, the hSP-A2 5′-flanking region between −296 and −1500 bp appears to contain “silencer” element(s) (64). Despite the presence of these putative silencers, an inhibitory effect of Dex on basal and cAMP-induced expression was evident (Fig. 4). A silencer region also has been reported upstream of the rat SP-A gene (56).

Because the −296-bp genomic region contains the TBE, it was of interest to analyze the effect of Dex on binding of type II cell nuclear proteins to this element using electrophoretic mobility shift assays; nuclear protein binding to a consensus NF-κB response element was also studied for comparison.

Cytoplasmic fractions of these cells were analyzed for SP-A content by immunoblotting. As reported previously, cAMP treatment of human fetal type II cells caused a pronounced increase in SP-A protein levels compared with untreated cells; Dex, which had little effect on SP-A levels in the absence of cAMP treatment, markedly inhibited cAMP induction of SP-A expression (Fig. 5A) (48, 49). However, the mechanisms for this inhibitory effect of Dex on SP-A protein levels are complex and mediated, at least in part, by its action to decrease SP-A mRNA stability (11). As observed previously, cAMP increased nuclear protein binding to the TBE (Fig. 5B) (27, 34), as well as to the NF-κB consensus sequence (Fig. 5C) (27). Interestingly, Dex inhibited the binding of type II cell nuclear proteins to the TBE and NF-κB response elements and markedly antagonized the stimulatory effect of DBcAMP on nuclear protein binding to these sites (Fig. 5, B and C). These findings suggest that Dex inhibition of SP-A promoter activity is mediated, in part, by reduced binding of TTF-1 and/or NF-κB to the TBE.

Dex inhibits TTF-1 and IL-1 stimulation of SP-A promoter activity acting through the TBE. To directly assess whether GR and Dex inhibit SP-A promoter activity through the TBE, A549 cells, which do not express endogenous TTF-1 (34) but contain NF-κB (unpublished observations), were transfected with a
fused gene comprising three TBEs fused upstream of −50/+40 bp of promoter/first exon DNA from the hSP-A2 gene, linked upstream of the hGH structural gene, as reporter (TBE: hGH). A549 cells were cotransfected with empty CMV expression vector and/or CMV expression vector containing TTF-1 or hGR; β-gal was cotransfected as a control for transfection efficiency. After 15 h, cells were incubated for 24 h with IL-1β (10 ng/ml) or Dex (10−7 M) or both. IL-1β has previously been reported to increase NF-κB binding and transcriptional activity in A549 cells (29). On the other hand, effects of cAMP were not analyzed in the present study, because cAMP has little or no effect on SP-A expression these cells (Fig. 3), likely because of a deficiency in protein kinase A activity (34). Reporter gene expression was evaluated by measuring the hGH that accumulated in the culture medium during this period. As we observed previously (27), TTF-1 caused a twofold induction of SP-A promoter activity; a similar fold stimulation was observed in cells incubated with IL-1β (Fig. 6). In cells transfected with CMV:TTF-1 and treated with IL-1β, SP-A promoter activity was increased more than fourfold. The stimulatory effects of TTF-1 and/or IL-1β were markedly inhibited by overexpression of hGR in the presence of Dex (Fig. 6). The GR had no effect in the absence of Dex (data not shown). These findings suggest that the inhibitory effect of Dex/GR on SP-A promoter activity is mediated through the TBE. Furthermore, the finding that Dex/GR inhibited IL-1β-induced fusion gene expression in the absence of TTF-1 cotransfection suggests that glucocorticoid inhibition of hSP-A2 promoter activity is likely mediated by GR inhibition of endogenous NF-κB DNA-binding activity.

**DISCUSSION**

The regulation of SP-A gene expression by glucocorticoids is complex and multifaceted, involving transcriptional and posttranscriptional effects (11, 12, 24, 26), and may be mediated by direct and indirect actions of the steroid on type II cells. In type II cell transfection studies using rabbit (1) or human SP-A:hGH fusion gene constructs, we consistently observed an inhibitory effect of Dex on cAMP stimulation of SP-A promoter activity. The inhibitory effect of Dex appears to be mediated by sequences within −380 (1) and −300 bp upstream of the rSP-A and hSP-A gene transcription initiation sites, respectively. This genomic region contains a number of highly conserved response elements, each of which is essential for cAMP induction of SP-A promoter activity (46).

In the present study, we have begun to investigate the mechanisms for the inhibitory effects of glucocorticoids on SP-A gene transcription in lung type II cells. In type II cell transfection studies, we compared the effect of Dex in the absence or presence of overexpressed hGR on SP-A promoter activity and on activation of the MMTV promoter, which is known to be glucocorticoid responsive (19). Whereas Dex in the presence of overexpressed GR markedly inhibited cAMP induction of SP-A promoter activity, a marked stimulatory

**Dex stimulates IκB-α expression in human fetal lung type II cells.** Glucocorticoids have been reported to increase IκB-α expression in certain systems (5, 51); however, this effect appears to be cell type and promoter specific (17). Glucocorticoid induction of IκB is suggested to cause increased retention of NF-κB p50 and p65 in inactive complexes within the cytoplasm. To further define the mechanism(s) by which Dex inhibits SP-A gene expression, human fetal lung type II cells were cultured for 3 days in control medium or in medium containing DBcAMP, Dex, or IL-1α, alone and in various combinations. Cytoplasmatic fractions from these cells were isolated and analyzed for IκB-α by immunoblotting. Levels of IκB-α protein were relatively unaffected by IL-1α or DBcAMP, added alone or in combination (Fig. 7). By contrast, Dex markedly stimulated IκB-α levels in the absence or presence of DBcAMP (Fig. 7). Interestingly, Dex failed to stimulate IκB-α levels in type II cells treated with IL-1α or IL-1α + DBcAMP. These findings suggest that Dex antagonism of basal and cAMP-induced SP-A expression may be mediated, in part, by increased IκB expression, resulting in inhibition of NF-κB transcriptional activity. On the other hand, Dex antagonism of IL-1- or IL-1 + DBcAMP-induced SP-A expression cannot be explained by this mechanism.
The lung cell transfection studies reported here also revealed that functional levels of endogenous GR in primary cultures of fetal rat type II cells are quite low. Although a pronounced inhibitory effect of Dex on cAMP-induced SP-A promoter activity was observed in rat type II cells in the absence of cotransfected CMV:hGR, a robust induction by Dex of MMTV promoter activity required hGR overexpression. These findings suggest that the inhibitory action of glucocorticoids on cAMP induction of SP-A expression requires lower concentrations of GR than does Dex stimulation of MMTV promoter activity. On the other hand, the finding that Dex markedly inhibited SP-A promoter activity and stimulated MMTV promoter activity in A549 cells in the absence of cotransfected GR indicates that the adenocarcinoma cell line contains relatively high levels of functional GR.

The results of a number of morphological studies indicate that the GR is localized to mesenchyme and epithelium in fetal lung (16). An autoradiographic study of [3H]Dex binding in sections of fetal mouse lung at various stages of development and in human fetal lung at 8 wk of gestation revealed that mesenchyme directly adjacent to the more proximal portions of the bronchiolar network was heavily labeled, whereas the epithelium destined to later differentiate into bronchi and bronchioles was relatively unlabeled. Nuclear localization of Dex binding to distal portions of the growing epithelium destined to become alveolar ducts and alveoli was also evident (8). By in situ hybridization, GR mRNA levels were found to increase in embryonic mouse lung from embryonic day 13 to embryonic day 15 or 17; expression was mainly localized to the mesenchyme, whereas ductular epithelium was less intensely labeled (31). In another study of fetal mouse lung during the later stages of gestation, the GR was localized to mesenchyme adjacent to the bronchial and air sac epithelia (28). These findings suggest that glucocorticoid actions on fetal lung development may be mediated by indirect effects on lung mesenchyme to reduce expression of inhibitory factors and/or enhancement of stimulatory factors. It has been suggested that glucocorticoids act on the fetal lung fibroblast to promote synthesis of a “differentiation factor” termed fibroblast pneumocyte factor, which acts on lung epithelium to enhance alveolar type II cell differentiation and surfactant synthesis (55). Fibroblast pneumocyte factor has not been isolated and characterized.

The finding that mice homozygous for targeted deletion of the GR gene (GR−/−) die within several hours of birth as a result of respiratory failure caused by atelectatic underdeveloped lungs (15) emphasizes the importance of GR in lung development. It was suggested that the lack of GR impaired development of the terminal bronchioles and alveoli beyond embryonic day 15.5. Of importance, however, was the finding of comparable numbers of alveolar type II cells and apparently normal levels of mRNA encoding SP-A, SP-B, and SP-C in lung tissues of newborn GR−/− mice compared with heterozygous or wild-type animals (15). On the other hand, mice homozygous for a point mutation in the GR that prevents its dimerization and DNA binding are viable, despite lack of inducibility of a number of GR-regulated genes. This suggests that the actions of glucocorticoids to enhance lung development occur through DNA-binding-independent mechanisms (50).

In the present study, we found that Dex/GR inhibition of SP-A promoter activity was most pronounced in lung cells that manifested elevated basal and/or cAMP induction of SP-A promoter activity. GR repression of activator protein-1- and NF-κB-induced gene expression has been reported to be mediated by the interaction of GR with these stimulatory transcription factors (50). In this regard, it is of interest that Dex treatment of human fetal lung type II cells inhibited nuclear protein binding to the TBE and prevented the stimulatory effect of cAMP on TBE-binding activity. In addition to the homeodomain transcription factor TTF-1, the TBE also interacts with NF-κB p50 and p65 (27). Although we have yet to obtain evidence that NF-κB binds directly to the TBE, by antibody-mediated supershift electrophoretic mobility shift assay, we have found that p50 and p65 interact with TTF-1 at this site. Furthermore, in coimmunoprecipitation studies using type II cell nuclear extracts, we observed that TTF-1 and p65 interact in vivo (27). TTF-1 and NF-κB also were found to cooperatively interact through the TBE to increase SP-A promoter activity; this was further enhanced by IL-1 (27). Our finding, in the present study, that Dex/GR acted through the TBE to inhibit the stimulatory effects of TTF-1 and IL-1 on SP-A promoter activity in transfected lung A549 adenocarcinoma cells suggests that glucocorticoids acting through GR may inhibit SP-A expression by blocking binding and transcriptional activity of TTF-1 and NF-κB at the TBE. As mentioned earlier, A549 cells do not express endogenous TTF-1 (34), although they do contain NF-κB. Because the inhibitory effect of Dex/GR on SP-A promoter activity was manifest in IL-1-treated cells in the absence of TTF-1 cotransfection, our findings suggest that glucocorticoids exert their inhibitory actions in A549 cells through interaction with NF-κB.

We have not determined whether the GR interacts directly with TTF-1 in type II cells to block its DNA binding and transcriptional activity. However, the GR has been found to inhibit prolactin gene expression in nonpituitary cells through a response element that binds the POU-homeodomain transcription factor Oct-1 and the homeodomain transcription factor Pbx-1 (58). Interestingly, addition of in vitro translated GR DNA-binding domain to nuclear extracts prevented the binding of Oct-1 and Pbx-1 to this response element (58). A similar mechanism, involving interaction of the GR with Oct-1 bound to its response element, has been proposed for glucocorticoid inhibition of the mouse gonadotropin-releasing hormone promoter (13).

As mentioned above, there is abundant evidence to suggest that glucocorticoids exert their anti-inflammatory and immunosuppressive actions on cells by GR antagonism of NF-κB-mediated transactivation and that NF-κB antagonizes the actions of glucocorticoids by inhibition of GR function (17). Several mechanisms, however, that appear to be cell type and promoter specific have been proposed to explain these phenomena (17): 1) direct (61) or indirect [through cAMP response element-binding protein (CBP)] (41) interaction between the NH2 terminus of p65 and the DNA-binding domain of the GR, which prevents functional association with the preinitiation complex and blocks transactivation, 2) competi-
tion between GR and NF-κB for limiting amounts of coactivators, CBP/p300 and steroid receptor coactivator (SRC)-1 (54), and/or J) glucocorticoid/GR induction of IκB-α gene expression (5, 51).

In the present study, we have observed that Dex increased IκB-α expression in human fetal type II cells cultured in the absence or presence of DBcAMP. It is suggested that the resulting increase in IκB-α protein causes trapping of p65 and p50 in inactive complexes within the cytoplasm, blocking their transcriptional activity. Accordingly, we recently found that overexpression of dominant-negative, phosphorylation-defective mutant forms of IκB in lung type II cells reduced binding of nuclear proteins to the TBE and inhibited cAMP and IL-1 induction of SP-A expression (27). On the other hand, an effect of Dex to increase IκB expression was not manifest in type II cells treated with cAMP alone or in combination with IL-1. This is most likely due to a dominant effect of IL-1 to increase degradation of IκB. Because Dex inhibition of nuclear protein binding to the TBE and of SP-A promoter activity was also manifest in cells treated with IL-1, it is improbable that Dex antagonism of IL-1, alone or in combination with DBcAMP stimulation of SP-A expression, can be explained by IκB induction.

We recently reported that the coactivators CBP/p300 and SRC-1, which contain intrinsic histone acetyltransferase activity, interact directly with TTF-1 and act synergetically to increase SP-A promoter activity (63). cAMP treatment of cultured type II cells was found to increase TTF-1 acetylation. This suggests that cAMP-mediated TTF-1 phosphorylation facilitates interaction with CBP and SRC-1, resulting in TTF-1 hyperacetylation, which further enhances its DNA-binding and transcriptional activity (63). In light of the importance of these coactivators in TTF-1, as well as NF-κB (20, 47) transcriptional activity, it is possible that GR antagonism of cAMP and IL-1 induction of SP-A gene expression may be mediated, in part, by competition for essential coactivators and formation of a repressed chromatin structure.

In summary, our present findings suggest that glucocorticoid inhibition of SP-A gene transcription is complex and occurs at several levels. The inhibitory actions of glucocorticoids do not appear to be mediated by direct binding of GR to DNA sequences surrounding the SP-A promoter. Rather, negative effects of glucocorticoids are likely caused by GR inhibition of binding and transcriptional activity of NF-κB and/or TTF-1 at the TBE, a response element that is crucial for cAMP and IL-1 induction of SP-A expression. Glucocorticoid-induced expression of the NF-κB inhibitor IκB-α may also play a role in Dex/GR inhibition of cAMP-stimulated SP-A promoter activity. In consideration of previous findings that the coactivators CBP/p300 and SRC-1 interact with NF-κB (20, 47) and TTF-1 and play an important role in the induction of SP-A promoter activity (63), it also is likely that negative effects of Dex/GR are mediated, in part, by competition for limiting amounts of these coactivators.

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REFERENCES


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