Phorbolester Down-regulation of Lung Surfactant Protein B Gene Expression by Cytoplasmic Trapping of Thyroid Transcription Factor-1 and Hepatocyte Nuclear Factor 3

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The lung-specific surfactant protein B (SP-B) is essential for surfactant function and normal respiration. We investigated the role of thyroid transcription factor-1 (TTF-1) and hepatocyte nuclear factor 3 (HNF3) in the down-regulation of SP-B gene expression by phorbol ester in pulmonary adenocarcinoma H441 cells. Responsiveness to 12-O-tetradecanoylphorbol-13-acetate (TPA) localized to the SP-B proximal promoter (−140/−65 bp) and specifically to binding sites for TTF-1 and HNF3, which act as cell-specific enhancers of SP-B expression. Treatment of cells with TPA (10 nM) caused a time-dependent decrease in both TTF-1 and HNF3 in nuclear extracts and accumulation of both factors in the cytoplasm as assessed by electromobility shift, Western, Southwestern, and immunofluorescence assays. Treatment did not alter the mRNA content or DNA binding activity for either transcription factor. We conclude that down-regulation of SP-B gene expression by phorbol ester involves cytoplasmic trapping and loss of TTF-1 and HNF3 from the nucleus. This mechanism of action is independent of AP-1 and other transcription factors known to be influenced by phorbol ester.

Surfactant protein B (SP-B), an 8-kDa hydrophobic protein secreted by epithelial cells of the lung, enhances the formation and stability of the surface active phospholipid film which prevents collapse of lung alveoli during respiration. Expression of SP-B is essential for the surface tension lowering properties of pulmonary surfactant and for normal respiratory function. Absence or inactivation of SP-B in animal models results in respiratory distress and/or respiratory failure, and inherited deficiency of SP-B is a lethal disorder of infants (1).

Expression of the SP-B gene is limited to the lung and is developmentally regulated at both transcriptional and post-transcriptional levels (2, 3). Recent studies of the SP-B gene promoter have identified important roles for two transcription factors in the cell selective expression of the gene (4–6). Thyroid transcription factor-1 (TTF-1), a member of the NKx2 family of homeodomain transcription factors, binds to sites in both the proximal and more distal SP-B promoter and transactivates the promoter in vitro. TTF-1 also transactivates expression of two other surfactant protein genes, SP-A and SP-C (7, 8), a clara cell-specific protein (CCSP) (9), as well as the thyroid-specific thyroglobulin, thyroperoxidase, and thyrotrpin receptor genes (10–12). Consistent with its proposed roles in lung organogenesis and epithelial cell differentiation, expression of TTF-1 in developing lung has a temporal-spatial distribution pattern similar to that of SP-B and in adult lung is expressed only in type II cells and subsets of nonciliated bronchiolar (clara) cells (13).

The other known transcription factor affecting expression of the SP-B gene is hepatocyte nuclear factor 3 (HNF3), which is a member of the forkhead winged helix family of transcription factors. HNF3 binds to the proximal region of the human SP-B promoter adjacent to the TTF-1 binding sites and enhances promoter activity (5). The HNF3 family, consisting of isoforms, α, β, and γ, also act as enhancers for the lung-specific CCSP gene (14, 15) as well as numerous liver-specific genes (16).

In liver and thyroid, respectively, HNF3 and TTF-1 have been implicated in the response of tissue-specific genes to hormones and other agents. Down-regulation of hepatic cholesterol 7α-hydroxylase by insulin and phorbol ester localizes to the promoter region, which contains a HNF3 binding sequence (17), and an intact HNF3 binding site is necessary for glucocorticoid regulation of IGFBP-1 (18). In thyroid cells, down-regulation of thyroid-specific genes by thyrotropin, cAMP, v-Ras activation, interferon-γ, or phorbol ester is associated with a decrease in either TTF-1 content (19, 20) or binding activity (10, 21, 22). At present there is no information regarding the possible role of TTF-1 and/or HNF3 in hormonal modulation of lung-specific proteins.

Development of the fetal lung, including synthesis of surfactant components, is regulated by a variety of hormones and other factors (23). Expression of the SP-B gene is increased by glucocorticoids and activators of cAMP and inhibited by transforming growth factor-β, tumor necrosis factor-α, insulin, and phorbol ester (2, 24–27). The mechanism of action of 12-O-tetradecanoylphorbol-13-acetate (TPA) on SP-B gene expression was initially examined in pulmonary adenocarcinoma NCI
H441 cells, and evidence was obtained for post-transcriptional regulation mediated by cis-acting elements in the 3’translated region (24, 28). Subsequent studies found that TPA decreased the transcription rate of the SP-B gene in both H441 cells and fetal lung (29), and a deletional study of the SP-B gene promoter localized TPA responsiveness to the proximal promoter region (30). This region (~140 to 7 bp) of the promoter does not contain AP-1 sites which are known to mediate many effects of TPA through binding of the nuclear protooncogenes fos and jun. Since the TPA-responsive region of the promoter includes the binding sites for both TTF-1 and HNF3, which are known to activate SP-B gene transcription, we proposed that the inhibitory effects of phorbol ester were mediated through one or both of these transcription factors.

In the present report we have further localized TPA responsiveness to the TTF-1 and HNF3 cis-acting elements of the SP-B promoter and demonstrate that TPA treatment causes a loss of both transcription factors from the nucleus and their accumulation in the cell cytoplasm. Preliminary results from this study have been reported in abstracts (31, 32).

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media, antibiotics, and fetal calf serum were obtained from the Cell Center Facility, University of Pennsylvania. Restriction and other DNA enzymes were purchased from Promega Corp. (Madison, WI) or from Pharmacia Biotech Inc. TPA, hormones, and other biochemicals were obtained from Sigma. Reinforced nicoce-lulose membrane (Duralose) and pliopetides were purchased from Stratagene (La Jolla, CA). NCI H441, HeLa, and A549 cells were obtained from American Type Culture Collection (Rockville, MD). Antibodies to human HNF3, Polyclonal anti-TTF-1 antibody was a gift from P. Minoo (University of California, Los Angeles, CA). Antibodies to human HNF3α, HNF3β, and HNF3γ were obtained from J. E. Darnell (Rockefeller University, New York).

**cDNA Probes**—A 600-bp 5’ end human SP-B cDNA probe was obtained by digesting full-length cDNA with BamHI (34). A 500-bp 5’ end TTF-1 probe was excised by EcoRI digestion from a full-length mouse TTF-1 cDNA obtained from R. Di Lauro (Naples, Italy). A 620-bp rat HNF3 β cDNA, which shares ~90% homology with the human counterpart and contains the homologous region of HNF3α, was excised by EcoRI and BamHI (gift of W. S. Chen, Rockefeller University, New York). Finally, a TTF-1 cDNA probe was obtained using [32P]dCTP and Ready To Go DNA labeling kit from Pharmacia.

**Plasmids**—Plasmid RSV-CAT contains the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter expressing the bacterial chloramphenical acetyltransferase (CAT) gene (35). The plasmidless pOOCO harbors herpes simplex thymidine kinase (TK) promoter fragment (~109 to +55 nucleotides) inserted into the BglII site of pOOCO (37). The plasmid 6RL expressing lacZ with plasmids containing CAT (38). We have previously described preparation of the plasmid ~100 to 44CO2, containing SP-B genomic DNA linked to CAT (30).

A series of SP-B 5’ flanking sequences (~404 to 35, ~404 to 255, ~217 to 141, ~140 to 65 and ~217 to 53) were prepared by polymerase chain reaction amplification and confirmed by sequencing (Table 1). These DNA fragments were linked to TTF-1 and HNF3 promoters, and AP-2 oligonucleotides were synthesized commercially (Integrated DNA Technologies, Inc. Coralville, IA) and were purified by Bio-Gel columns. Double-stranded (ds) TTF-1 oligonucleotides were designed with 5’ HindIII and 3’ Sall compatible ends, while HNF3 ds oligonucleotides were made with 5’ SalI and 3’ Xbal compatible ends (Table 1). After annealing, the ds oligonucleotides were cloned into compatible ends in pOOCO. For the TTF-1/HNF3 construct, the two oligonucleotides were linked followed by ligation into the HindIII/XbaI sites of pOOCO.

**Cell Culture**—The NCI H441 and A549 cells were grown in Dulbecco’s modified Eagle’s medium and HeLa cells were grown in modified Eagle’s medium. Both media were supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (2 mg/ml). H441 cells were plated 1–2 days prior to treatment with combinations of dexamethasone (50 μM), TPA (0.1–30 μM), and 8-bromo-cAMP (0.1 mM) plus isobutylmethylxanthine (0.1 mM). For experiments involving dexamethasone plus TPA treatment, cells were incubated with dexamethasone (50 μM) for 30 min, TPA for 2 h, and washout experiments were carried with TPA for different time periods (1, 2, 4, 8, and 24 h) prior to harvest.

**Transient Transfections**—Test plasmid DNA (2.5 μg) was mixed with 1 μg of internal control plasmid 6RL and coprecipitated by the CaCl2 procedure (37). Total DNA in each treatment, including vector alone, was kept constant at 5 μg with the addition of BlueScript plasmid. Coprecipitates were added directly to cells and incubated for 18–20 h at 37°C. Subsequently cells were washed twice with phosphate-buffered saline, RPMI medium with 10% fetal bovine serum added, and incubation continued for 48–54 h. To test the effect of TPA on expression of different test plasmids, cells were grown in 10 μM TPA following transfection. Total protein, β-galactosidase, and CAT assays were determined in cells as described (38). CAT activity was determined by β-galactosidase activity for comparison between different plasmid constituents in untreated cultures. Since TPA treatment increases the expression of β-galactosidase, comparisons of CAT activity in the presence and absence of TPA used non-normalized data.

**DNA Preparation and Northern Blot Analysis**—Total RNA was extracted from H441 cells grown in TPA + dexamethasone by the phenol-guanidinium isothiocyanate method (39) and poly(A)+ RNA was isolated using the PolyATtract® mRNA isolation system (Promega Corp.). Either 20 μg of total RNA or 2 μg of poly(A)+ RNA was used for Northern blot analysis. Contents of SP-B, TTF-1, HNF3β, and β-actin mRNAs were determined by hybridization at 42°C with labeled cDNA probes for. For dot blot hybridization analysis, serial dilutions of RNA were immobilized on membranes using a 96-well dot blot apparatus and hybridized as above. Blots were exposed to DuPont reflection film with intensifying screens for 1–4 days. The autoradiograms were scanned using a densitometer (Hoeffer GS300, Hoeffer Scientific Instruments, San Francisco, CA) and relative densities calculated from the linear portions of the dose-response curve for each RNA sample.

**Isolation of Nuclear and Cytoplasmic Proteins**—Nuclear extracts were prepared from cells growing in the medium described above with minor modifications. Briefly, H441 cells (~107) were washed twice in cold phosphate-buffered saline and resuspended in 1 ml of cold buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40). After 10 min on ice the lysate was centrifuged at 500 × g for 3 min. The supernatant was designated as the cytoplasmic fraction and was saved. The pellet was resuspended in buffer B (250 mM KCl, 250 mM sucrose, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM each of leupeptin and pepstatin) with frequent vortexing for 15 min at 4°C. After centrifugation at 20,000 × g for 5 min to obtain a purified nuclear pellet. Nuclear extract was isolated by resuspending the pellet in 25 μl of buffer B (25 mM HEPES, pH 7.6, 1 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mM each of leupeptin and pepstatin) with 10% glycerol, stored at –80°C, thawed, and 10 μl of total nuclear extract was added to 1 μl of antibody (TTF-1, HNF3α, HNF3β, or HNF3γ, diluted 1:10 or 1:100) or preimmune antiserum. For competitor analysis cold probes (1 μg, 10 μg, and 20 μg) were added along with labeled probe, nuclear extract, and binding buffer and incubated for 15 min on ice. Products were analyzed on low ionic strength gels (4.5 mM Tris borate, 1 mM EDTA, pH 8.0), 5% Long Ranger (FMI Bioproducts, Rockland, ME) with 0.25% glycerol and

**Electrophoretic Mobility Shift Assay (EMSA)**—A modified method of Hennighausen and Lubon (41) was adopted to identify DNA-protein interactions. The end-labeled probe (10,000 cpm) was incubated with 2 μg of nuclear protein in a reaction mix with binding buffer (0.5 μg of poly(dI-dC), 0.5 μg of sonicated salmon sperm DNA, 10 mM HEPES, pH 7.6, 80 mM KCl, 1 mM EDTA, 1 mM EGTA, and 6% glycerol) and incubated on ice for 20 min. For cytoplasmic EMSA experiments the same amount of probe was incubated with 20 μg of cytoplasmic protein with binding buffer. For supershift analysis, the probe and nuclear extract were incubated on ice in a total volume of 25 μl for 10 min at 4°C for additional 15 min at 22°C after addition of 1 μg of antibody (TTF-1, HNF3α, HNF3β, or HNF3γ, diluted 1:10 or 1:100) or preimmune antiserum. For competitor analysis cold probes (1 ×, 10 ×, and 100 ×) were added along with labeled probe, nuclear extract, and binding buffer and incubated for 15 min on ice. Products were analyzed on low

**Supplementary Information**

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autoradiography performed. To quantitate the amount of probe in a retarded complex, the area on the gel with a retarded band was excised and counts/min determined in a scintillation counter.

**DNA and Oligonucleotide Probes for EMSA**— The 76- and 77-bp fragments of the SP-B gene 5′-flanking sequence corresponding to −1039 to −65 and −217 to −141 bp upstream of the transcription start site and the commercially synthesized double-stranded oligonucleotides designated TTF-1, HNF3, and AP-2 (Table I) were used as probes for EMSA. Mixtures of complementary oligonucleotides were heated initially to 95 °C for 5 min and annealed at incubating serially at 65 °C, 37 °C, and on ice for 10 min each, 1–2 pmol of DNA or oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega Corp.) and purified using Sephadex G-50 columns.

**SouthWestern blots**—SouthWestern blot analysis was done following the modified method of Singh et al. (42). Nuclear extracts were dialyzed at 4 °C in excess volume of dialysis buffer without glycerol for 6 h with two to three buffer changes. Twenty-five µg of protein of nuclear extracts were separated on 12% SDS (TTF-1 and AP-2) or 4.5–14% gradient native (HNF3) PAGE, and proteins were transferred electrophoretically to Duralose membrane as described (33). Membranes were blocked in 3% milk powder in Tris-buffered saline and denatured with serial dilutions (1.5, 0.75, 0.375, 0.18, 0.09 µl) of guanidium chloride in HEPEs, pH 7.0, buffer. Denatured membranes were blocked again for 30 min and incubated overnight with end-labeled TTF-1, HNF3, or AP2 ds oligonucleotides in binding buffer (0.2 M Tris, pH 7.2, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml of poly(dI-dC), and 1 µg of sonicated salmon sperm DNA). Blots were washed and exposed to x-ray film with intensifying screen for 4 to 5 days.

**Immunolocalization**—H441 and HeLa cells were plated on 10-cm culture dishes at 30–35% confluence; after 1 day the cells were treated with 10 nM TPA for 24 h. Cells were fixed in methanol for 20 min at 20 °C, washed twice with phosphate-buffered saline, and immunostained with rabbit polyclonal anti-SP-B, -TTF-1, -HNF3 antibodies (1:500 dilution) or non-immune serum (1:250) overnight at 4 °C, followed by Cy3-conjugated goat anti-rabbit IgG secondary antibody treatment for 2 h at 22 °C. Stained cells were observed on an inverted microscope with a fluorescent light source and photographed.

**Western Blot Assay for HNF3 Proteins**—A cytoplasmic fraction was recovered from H441 cells, and 25 µg of protein were separated by SDS-PAGE, and proteins were transferred electrophoretically to Duralose membrane. Immuno blotting was performed with antibodies to HNF3α, HNF3β (1:1000) and HNF3γ (1:500), and goat anti-rabbit IgG antisera conjugated with horseradish peroxidase (1:10,000). Immunoreactive products were detected on a x-ray film using enhanced chemiluminescence according to the manufacturer’s directions (NEN Life Science Products). Bands were quantitated by scanning densitometer.

**Statistics**—In all experiments we included two to three replicates, and the experimental results were confirmed in two or more experiments. Data analysis was performed with Statview 512+ (Abacus Concepts, Calabasas, CA) and analyzed by Student’s t test or analysis of variance, compared by Fisher Exact Test. Data are expressed as mean ± S.E. except where otherwise noted.

**RESULTS**

**TPA Responsiveness Localizes to Proximal 5′-Flanking Elements of the SP-B Gene**—We have shown previously that treatment of transfected H441 cells with TPA inhibited the expression of CAT reporter gene driven by the SP-B gene promoter fragment –1039/+431 (30); similar responsiveness was found with lacZ as a reporter gene. Deletional studies using SP-B-CAT constructs localized TPA responsiveness to the proximal promoter (30), however, studies of TPA inhibition with further deletions of the 5′-flanking region were not possible because of low basal SP-B promoter activity. We therefore used the heterologous TK minimal promoter to test TPA responsiveness of linked SP-B DNA elements that did not contain the SP-B gene TATA box (−33/−30).

To establish TPA responsiveness of SP-B DNA fragments linked to a heterologous promoter, we initially performed TPA dose-response studies. H441 cells were grown with different concentrations of TPA for 48 h after transfecting with SP-B3 TK-CAT or SP-B1039/−431)CAT and treated with TPA at the concentrations shown. CAT activity was determined after 48 h. Data are expressed as percent of control (diluent) and are mean values from a representative experiment with replicate samples.

**Fig. 1.** TPA responsiveness of constructs containing SP-B DNA linked to heterologous promoter. A, TPA dose response. H441 cells were transfected with SP-B1039/−431)CAT or SP-B1039/−431)TK-CAT and treated with TPA at the concentrations shown. CAT activity was determined after 48 h. Data are expressed as percent of control (diluent) and are mean values ± S.E. for two to six experiments with replicate determinations. *p < 0.01 versus TK-CAT. B, responsiveness in different cell lines. Cells were transfected with TK-CAT alone or with SP-B1039/−431)TK-CAT and treated with TPA (10 nM) or diluent (control). Data are expressed as TPA effect (percent of control) and are mean values ± S.E. values from 3–19 experiments. C, deletional analysis of proximal SP-B promoter. H441 cells were transfected with plasmids containing different 5′-flanking fragments of the SP-B gene linked to TK promoter and CAT reporter gene. Cells were cultured in the presence of 10 nM TPA and extracts assayed for CAT activity. TPA responsiveness localizes to −140/−65 bp above the transcription start site of the SP-B gene. Data are expressed as percent of control and are mean ± S.E. values from 3–19 experiments.

B1039/−431)CAT or with SP-B1039/−431)TK-CAT. Inhibition was comparable with the two constructs; half-maximal inhibition occurred at 0.5 nM TPA, and CAT activity was 10% of control at 10 nM TPA (Fig. 1A).

Cell specificity of the TPA response was examined in H441 and A549 cells, both derived from lung adenocarcinomas, and HeLa cells. After transfection with TK-CAT, TPA treatment (10 nM, 24 h) decreased CAT activity in H441 cells, increased activity in A549 cells, and had no effect in HeLa cells (Fig. 1B). Compared with these control results, transfection with plasmids containing the SP-B DNA fragment resulted in TPA inhibition of CAT activity in H441 and A549 cells but did not

2 B. C. Planer and P. L. Ballard, unpublished observations.
To localize more precisely the region for TPA responsiveness, we transfected H441 cells with plasmids containing different fragments of SP-B proximal promoter, generated by polymerase chain reaction, linked to TK-CAT (Fig. 1C). In these experiments TPA reduced the strength of TK promoter alone (TK-CAT) to ~50% of control. In cells transfected with plasmids containing TK promoter plus SP-B fragments ~404/~35, ~140/~65, and ~217/~53, TPA reduced CAT activity to <12% of control, similar in magnitude to the inhibition observed with the SP-B promoter (~1039/~431-CAT). TPA did not decrease CAT expression for constructs containing the SP-B sequences ~404/~255 and ~217/~141. These results indicate that the TPA responsive element(s) in the SP-B gene reside within bp ~140/~65 and that TPA responsiveness occurs independent of promoter function consistent with the expected properties of a regulated enhancer element.

**TPA Responsiveness Localizes to TTF-1 and HNF3 Enhancer Elements**—The proximal 5'-flanking region of SP-B gene contains 2 TTF-1 binding sites and an HNF3 binding site that function as cell line-specific enhancer elements (5). To determine whether TPA acts at these sites, we transfected H441 cells with plasmids containing the two TTF-1 binding sites (~112/~89) and/or the HNF3 binding site (~89/~72) linked TK-CAT (see Table I). Constructs containing both the HNF3 and TTF-1 binding sites increased CAT activity ~10-fold compared with TK-CAT alone, comparable with the enhancer response observed with SP-B fragment ~140/~65 (Fig. 2A). Promoter activity of TK-CAT was enhanced 4~5-fold with plasmids containing either the TTF-1 or HNF3 binding sites. These findings suggest that the TTF-1 and HNF3 elements influence SP-B gene transcription independently and additively, consistent with data using site-directed mutagenesis of the promoter (5).

Responsiveness of the TTF-1 and HNF3 sequences to TPA is shown in Fig. 2B. TPA treatment reduced CAT activity by ~90% in cells transfected with plasmids containing enhancer sequences compared with ~25% decrease in cells transfected with TK-CAT alone. We observed a similar reduction in CAT activity (~89%) in cells transfected with the SP-B (~1039/~431)-CAT construct. Thus, TTF-1 and HNF3 sites appear to be similarly sensitive to TPA.

**TPA Reduces Binding Activity of Nuclear Factors for SP-B Proximal Promoter**—We used EMSA to investigate the effect of TPA on binding activity of nuclear factors for the SP-B promoter. Transcriptional down-regulation of SP-B could result from binding of a TPA-induced inhibitory protein and/or from reduced binding of existing transcription factors. Nuclear extracts were isolated from treated and untreated H441 cells, and EMSA was carried out using as a probe the SP-B (~404/~65) DNA fragment, which is TPA-responsive by transfection analysis. We observed three retarded complexes using nuclear extracts from cultured cells (Fig. 3A). These bands were not affected by
treatment of cells with dexamethasone and/or cyclic AMP, hormones which increase SP-B gene expression, but were consistently reduced after TPA treatment (Fig. 3A). Prior treatment of nuclear extracts with proteinase K or 10% SDS, or preincubation at 65 °C for 10 min, completely eliminated the retarded bands (data not shown), suggesting that the binding factors are heat-labile proteins. EMSA was also performed with labeled upstream (−217/−141 Fig. 3B) and downstream (−57/−38, see Fig. 5A) SP-B DNA fragments; single bands were observed with both probes, and these did not change with TPA treatment. Thus, the EMSA data indicate that the TPA effect is associated with loss of proteins binding to the −140/−65 region of the SP-B gene.

Retarded Bands on EMSA Are TTF-1 and HNF3—We determined the identity of the TPA-responsive proteins in the EMSA analysis using competition (Fig. 4A) and supershift (Fig. 4B) approaches. For competitor analysis, end-labeled SP-B (−140/−65) probe was incubated with 1 ×, 10 ×, and 100 × excess of unlabeled ds oligonucleotides corresponding to TTF-1 or HNF3 binding sites. Excess TTF-1 oligonucleotide eliminated the lowest of the three retarded bands, while the upper two bands were either reduced or eliminated with the HNF3 oligonucleotide (Fig. 4A). These results tentatively identified the bottom band as a TTF-1–DNA complex and the top two bands as HNF3–DNA complexes.

To immunologically identify the proteins in retarded bands, labeled SP-B (−140/−65 bp) probe was incubated with nuclear extract followed by an additional incubation with non-immune or specific antiserum (Fig. 4B). No supershifted band was observed on EMSA with the addition of preimmune serum (Fig. 4B, non-immune serum) or with HNF3–β antibody (not shown). The smallest of the retarded complexes was partially supershifted with TTF-1 polyclonal antibody at both 1:10 and 1:100 dilutions (Fig. 4B, TTF-1). With HNF3α antibody, intensity of the middle band was reduced and two supershifted bands were observed; a partial shift occurred at 1:100 antibody dilution, and a complete shift was seen at 1:10 dilution (Fig. 4B, HNF3α). The higher supershifted band that was observed with the greater concentration of HNF3α antibody may be an heterodimeric complex of HNF3α and γ proteins. Complete shift of the top retarded band was observed with HNF3γ antibody at both concentrations (Fig. 4B, HNF3γ). Thus, competitor and supershift analyses identify the TPA-responsive bands on EMSA as TTF-1 and HNF3.

TPA Decreases TTF-1 and HNF3 in Nuclear Extract—To further characterize the effect of TPA we used the TTF-1 and HNF3 oligonucleotides (Table I) as probes in EMSA experiments. We found two retarded bands with TTF-1 oligonucleotide (Fig. 5A, TTF-1) as observed previously (5); intensity of both bands was reduced significantly by 8 h of TPA treatment and was almost eliminated by 24 h. HNF3 oligonucleotide probe also produced two retarded bands, perhaps representing dimers of HNF3 isoforms (Fig. 5A, HNF3). HNF3 binding ac-
activity in both bands decreased significantly in nuclear extracts after 8 h of TPA treatment and was usually not detected after 24 h.

For comparison we also determined the binding activity of the same nuclear extracts for the AP-2 oligonucleotide probe, representing bp −57/−38 of SP-B promoter downstream of the TFF-1 and HNF3 binding sites. EMSA with the AP-2 probe produced one strong retarded band (Fig. 5A, AP-2) and two additional retarded bands with longer exposure (not shown). None of the retarded bands with AP-2 probe were affected by TPA treatment of cells.

To quantitate the reduction in TFF-1 and HNF3 binding activities, we excised the retarded bands from gels and counted the radioactivity. TPA treatment caused a similar, time-dependent decrease in both binding activities (Fig. 5B). The reduction was statistically significant after 8 h of TPA treatment and was <10% of control at the 24 h time point. These findings suggest that transcriptional down-regulation of SP-B by TPA results from reduced binding of TFF-1 and HNF3 transcription factors to the SP-B proximal promoter.

We confirmed the changes in DNA binding activity by South-Western analysis. Nuclear extracts were isolated from H441 cells grown in TPA for different time periods. Proteins were separated on a 12% SDS-PAGE, transferred to membrane, and probed with radiolabeled TFF-1 oligonucleotide, which bound to a single band of expected size (~39 kDa). The autoradiographic signal decreased significantly after 8 h of TPA treatment and was very weak after 24 h (Fig. 6, TFF-1). For analysis of HNF3, proteins were separated on a non-denaturing gel. HNF3 oligonucleotide probe bound to three distinct bands with untreated nuclear extract (Fig. 6, HNF3), and reduced binding of probe to all three HNF3 bands was observed with ≥6 h of TPA treatment. Apparent differences between control and early TPA time points in this gel were not reproducible and probably resulted from the variability in protein loading noted by Coomassie staining. As a control, blots were also hybridized with AP-2 oligonucleotide probe. A 52-kDa band was observed, which was not significantly altered by TPA treatment (Fig. 6, AP-2).

To examine content of the transcription factors, we carried out Western blot analyses of nuclear extract. A satisfactory signal was not achieved with TFF-1 antibody. Gels probed with antibodies to HNF3α and HNF3γ revealed bands of ~51 and ~36 kDa, respectively, which decreased in intensity in nuclear extracts from cells treated with TPA for 8 and 24 h compared with control (data not shown). Thus, decreased HNF3 DNA binding activity after TPA treatment is due to reduced content of HNF3 proteins in nuclear extracts. Loss of transcription factor from nuclei could result from either decreased expression of the factor or a change in intracellular localization. Experiments were carried out to address these two possibilities.

Content of TFF-1 and HNF3 mRNAs Is Not Affected by TPA—To determine the effect of TPA on expression of TFF-1 and HNF3 genes, we isolated total and poly(A)⁺ RNA from H441 cells grown in 10 nM TPA for different time periods. On Northern analysis, probing with TFF-1 cDNA produced a single band of 2.6 kilobases, which did not change in intensity.
mRNA levels remained unchanged over 24 h (Fig. 7B) levels by 8 h of TPA treatment, whereas TTF-1 and HNF3 activities were consistent during prolonged TPA treatment. The intensity of both bands increases after 2 or more h exposure to TPA. Right, HNF3 probe. A weak band is observed in control cytoplasmic fraction and the signal increases with TPA treatment.

Fig. 7. Effect of TPA on TTF-1 and HNF3 mRNA. A, Northern blot. Total and poly (A)+ RNA was isolated from H441 cells exposed to 10 nM TPA for different time periods and 2 µg of poly(A)+ RNA were used for Northern blot analysis and hybridized with 32P-labeled HNF3 or TTF-1 cDNA. The signals for both TTF-1 and HNF3 were similar for all samples, except for the TTF 2-h band, which had less RNA loaded by probing for actin (not shown). B, quantitative analysis of mRNA content. The abundance of different mRNAs was quantitated by dot blot analysis using total RNA for SP-B and poly(A)+ RNA for TTF-1 and HNF3. Results were quantitated densitometrically, and data are represented as percent of change over control. SP-B mRNA content was reduced significantly by 8 h of TPA treatment, while TTF-1 and HNF3 mRNAs were not affected by TPA. Mean ± S.E.; n = 3 experiments.

with TPA treatment for 24 h (Fig. 7A, TTF-1). Likewise a single 2.8-kilobase, TPA-insensitive band was identified on probing with HNF3 cDNA (Fig. 7A, HNF3). Using dot blot hybridization analysis we found the expected reduction in SP-B mRNA levels by 8 h of TPA treatment, whereas TTF-1 and HNF3 mRNA levels remained unchanged over 24 h (Fig. 7B). Thus, decreased nuclear content of TTF-1 and HNF3 is not due to reduced expression of the genes.

TTF-1 and HNF3 Accumulate in the Cytoplasm of TPA-treated Cells—EMSA was performed with cytoplasmic fraction to examine the effect of TPA treatment on TTF-1 and HNF3 DNA binding activities in the cytoplasm. As with the nuclear extracts, two retarded bands were observed with TTF-1 probe (Fig. 8, TTF-1). A single retarded band was found with HNF3 probe on short exposure of gels to film (Fig. 8, HNF3), and a second band of lower mobility was seen after longer exposure (not shown). Low levels of TTF-1 and HNF3 binding activities were present in the cytoplasmic fractions from control cells, and activities of both transcription factors were consistently increased with ≈2 h of TPA treatment (Fig. 8). These results could reflect a change in either DNA binding activity or content of the proteins.

To examine localization of immunoreactive HNF3, TTF-1, and SP-B in intact H441 cells, we performed immunostaining using fluorescently labeled secondary antibodies (Fig. 9). Control, untreated H441 cells stained with HNF3α (Fig. 9B) or TTF-1 (Fig. 9F) antibodies showed strong fluorescence in the nucleus and minimal cytoplasmic signal. After 24 h of TPA treatment there was little nuclear fluorescence and the appearance of cytoplasmic fluorescence, which was distributed primarily in a perinuclear fashion for both HNF3α (Fig. 9D) and TTF-1 (Fig. 9H); similar results were found for HNF3γ (not shown). In general, fluorescence was stronger with HNF3 stained cells compared with TTF-1 stained cells. Using SP-B antibody a bright orange fluorescence was observed in the cytoplasm of most control cells (Fig. 9J), which was nearly eliminated in cells treated with TPA for 48 h (Fig. 9L). Staining of H441 cells with non-immune serum did not produce any fluorescence (Fig. 9N). Similarly, no fluorescence with either TTF-1 or HNF3 antibodies was noticed in HeLa cells (not shown), confirming other reports that these cells lack both transcription factors (5). No effect of TPA treatment on cell structure was observed by phase microscopy (left panels of Fig. 9).

Finally, we carried out Western analysis of cytoplasmic proteins using TTF-1 and HNF3 antibodies. Similar to findings with nuclear extracts, staining of cytoplasmic Western blots with TTF-1 antibody did not result in satisfactory results. Blots probed with HNF3α and HNF3γ antibodies identified an expected 51-kDa band and a weak band at 36 kDa, respectively; intensity of both of these bands increased with time of TPA treatment (Fig. 10). Probing of Western blots with HNF3β identified a single protein band of 47 kDa, which did not change during prolonged TPA treatment. The identity of the higher molecular weight bands observed with HNF3α and HNF3β antibodies is not known. These findings by protein blotting are in agreement with the EMSA and Southwestern data, and together the results suggest that TPA reduces SP-B gene transcription by causing cytoplasmic trapping of TTF-1 and HNF3.

DISCUSSION

Previous studies of SP-B gene expression in H441 cells established the strong inhibitory effect of phorbol esters (24, 27), and we recently found that this effect involved transcriptional down-regulation mediated by regulatory elements in the proximal promoter region (29, 30). In the current study we provide evidence that TPA causes a loss of nuclear TTF-1 and HNF3, which are trans-activators of SP-B gene expression, secondary to cytoplasmic trapping of the proteins without alteration of their mRNA contents or DNA binding activities. Regulation of
gene expression by coordinate effects on the intracellular distribution of two transcription factors represents a previously undescribed mechanism for the action of phorbol esters. By deletional analysis and use of heterologous promoter constructs the TPA responsive region of the SP-B gene was localized to bp 2140/265 upstream of transcription start site. AP1 sites, which mediate many transcriptional responses of PKC activation, lie outside this region of the SP-B gene, suggesting that c-Fos and c-Jun are not involved in this TPA effect. This region of the promoter does contain binding sites for TTF-1 and HNF3, which act as lung cell specific enhancers of SP-B gene expression (4, 5). We initially explored two possibilities of TPA action: 1) induction of a repressor protein binding to this region and 2) decreased binding of existing transcription factors. Down-regulation of gonadotropin-releasing hormone by TPA, for example, involves induction of a DNA protein complex with kinetics consistent with repressor action (43). Using EMSA we were unable to detect new protein binding to the 2140–265-bp region after TPA treatment. The three retarded bands observed with this probe were identified as TTF-1 and HNF3 by supershift and oligonucleotide competition approaches. The second

FIG. 9. Cellular localization of HNF3, TTF-1, and SP-B before and after TPA treatment. H441 cells were cultured with 50 nM dexamethasone and then exposed to 10 nM TPA or diluent (Control) for 24 h and immunostained. The left-hand column shows phase contrast photographs corresponding to the immunofluorescent micrographs in the right-hand column. B and D, HNF3 immunofluorescence. Fluorescence with HNF3α polyclonal antibody was localized to nuclei in control cells and was predominantly cytoplasmic after TPA treatment. F and H, TTF-1 immunofluorescence. When TTF-1 polyclonal antiserum was used, fluorescence was localized to nuclei in control cells, while primarily perinuclear cytoplasmic staining was observed after TPA treatment. J and L, SP-B immunofluorescence. Intense cytoplasmic staining was observed with SP-B polyclonal antibody in H441 before but not after TPA treatment (48 h). N, non-immune serum. No staining was observed when non-immune serum replaced the primary antibody. The final magnification for all micrographs is 800 × except for K and L, which were photographed at a higher magnification (final × 1200) to better observe the low level of cytoplasmic fluorescence.

FIG. 10. Western blot analysis of cytoplasmic HNF3 proteins. Cytoplasmic fractions were isolated from untreated cells (control) or cells treated with 10 nM TPA for 1–48 h. 25 μg of protein were separated by SDS-PAGE, transferred onto Duralose membrane, and immunostained with HNF3α, β, and γ polyclonal antibodies. Detection of the HNF3γ band required longer exposure to film than for the other isoforms. A, representative Western blot. HNF3α (51 kDa) and γ (36 kDa) proteins increased with time of TPA treatment, whereas TPA had no apparent effect on HNF3β (47 kDa) proteins. B, quantitation of Western analysis. The HNF3α-, HNF3β-, and HNF3γ-specific bands were quantitated by scanning densitometry and represented as percent increase over control. Both HNF3α and HNF3γ bands increased ~10-fold with TPA treatment, while there was no effect on HNF3β protein. Mean ± S.E., n = 3–4 experiments.
posibility was supported by the observation that all three shifted bands decreased in intensity after TPA treatment.

Several lines of evidence from our study, examining both DNA binding activity and immunoreactive protein, indicated that TPA caused a loss of TTF-1 and HNF3 from the nucleus and accumulation in the cytoplasm. The increase in cytoplasmic TTF-1 and HNF3 (by 2 h) preceded loss of the factors from the nucleus, consistent with failure of nuclear translocation as the primary event. Transport of proteins from the cytoplasm to the nucleus is an important regulated event for many nuclear proteins including selected transcription factors (44), and for many proteins a sequence of basic amino acids serves as a nuclear localization signal (45). Nuclear import can be modulated by the phosphorylation of sites adjacent to the localization signal, either inhibiting nuclear transport (e.g. lamin B2, protein kinase A catalytic subunit, and viral Jun) or increasing translocation as described for lamin A (22, 46–48). Activity of a localization signal can also be regulated by protein-protein interaction, either blocking import as in the case of 1α,β binding to NF-αB or conferring nuclear localization as occurs with chaperones (49–51). Regulated control of nuclear localization is important during the cell cycle, in developmental processes such as occurs with MyoD during mesodermal induction and with inflammatory responses mediated through NF-αB (49, 52). Nuclear localization of HNF3β has been mapped to the winged helix DNA binding domain (53), but a localization signal has not been reported for TTF-1.

Although not directly addressed in the current study, it is likely that changes in the phosphorylation state of TTF-1 and/or HNF3 are involved in the response of these transcription factors to TPA treatment. Zannini et al. (54) mapped seven serine phosphorylation sites in TTF-1 and observed phosphorylation with PKC in vitro; however, effects of PKC on DNA binding and/or transactivation activity were not examined. Other studies found that TTF-1 DNA binding activity was phosphorylation-dependent and that inactivation by phosphorylation action was reversed by treatment with protein kinase A and cAMP (10, 11). The modification of TTF-1 activity by v-Ras also appears to involve phosphorylation as indicated by the finding that v-Ras activation excludes the protein kinase A catalytic subunit from the nucleus (22). The HNF3 α and β isoforms are phosphorylated in HepG2 cells, but a role in nuclear localization or transcripational activity has not been determined (53). With regard to TPA effects in lung cells, further studies are required to determine whether the phosphorylation state of TTF-1 and HNF3 is altered and whether this represents a direct or indirect effect of PKC.

A consistent finding in the current study was the parallel effect of TPA on TTF-1 and HNF3. The nature of the linkage between these two transcription factors is not known. It is possible that both proteins undergo the same alteration in phosphorylation state mediated by PKC, sharing a common mechanism for nuclear translocation. Alternatively, only one of the transcription factors could be the target of PKC-mediated phosphorylation with nuclear transport of the other factor dependent on TTF-1/HNF3 heterodimerization. Finally, PKC could mediate phosphorylation of a separate protein, which would serve to either inhibit or promote nuclear transport. It is important to note, however, that the parallel responses for the two factors does not reflect a general change in transcription factor binding to the proximal promoter, since we found no change with TPA treatment in protein binding to DNA regions just upstream and downstream of the TTF-1/HNF3 binding sites.

Our finding that TPA-induced cytoplasmic trapping of TTF-1 is supported by data from studies in thyroid cells (22). Exposure of a differentiated thyroid cell line to 16 nM TPA for 3 days reduced TTF-1 binding activity in nuclear extracts as well as the activity of transfected thyroglobulin promoter. This response was reversible and recovery of TTF-1 binding activity was not inhibited by the presence of cycloheximide. By contrast, treatment of cells with a high concentration of TPA (350 nM), which down-regulates PKC, caused relatively little loss of TTF-1 binding activity, consistent with the proposal that activation of PKC is responsible for the loss of nuclear TTF-1 binding activity. Although intracellular distribution of TTF-1 was not examined in this study, the response to TPA is consistent with cytoplasmic trapping as we have observed. It is likely that cytoplasmic trapping of TTF-1 and/or HNF3 in response to TPA also operates in regulation of other genes that are activated by these transcription factors. In the lung, TTF-1 is an important transactivating factor for expression of SP-A and SP-C in type II cells and CCSP in bronchiolar Clara cells (7–9). Furthermore, TPA inhibits both SP-A (24, 27, 29) and SP-C gene expression acting at the level of transcription. In hepatic cells, expression of T4 binding globulin, apolipoprotein A-1, and CYP7A genes are inhibited by TPA and responsiveness mapped to promoter regions containing HNF binding sites (17, 55, 56). The possible role of altered intracellular distribution of transcription factors in the regulation of these and potentially other genes awaits further investigation.

We examined the three isoforms of HNF3 with regard to TPA responsiveness. Using specific antibodies, we identified the α and γ isoforms of HNF3 in nuclear extracts by both supershift and Western analysis; content of both isoforms increased in the cytoplasmic fraction after TPA treatment, but there was no change in levels of HNF3β. Previous studies also identified HNF3α as the isoform binding to human and rabbit SP-B promoter (5, 15, 57); however, Bohinski et al. (5) reported that only the α isoform was expressed in H441 cells. The molecular weights of immunoreactive HNF proteins in the current study were consistent with the stated specificity of the antibodies that we used. Thus, additional analysis of HNF3 isoforms is warranted.

The SP-B gene also contains three additional TTF-1 binding sequences further upstream in the 5′-flanking region (−439/−331), which recently were shown to both bind TTF-1 and to enhance heterologous promoter activity (6). Since inhibition of SP-B promoter activity by TPA was equivalent for plasmid constructs with or without this upstream region (30), the proximal TTF-1 sites appear to be primary. It is possible, however, that TTF-1 binds to the upstream sites with a higher affinity than for the proximal sites and that the residual promoter activity found in the presence of TPA represents the enhancer effect of TTF-1 at the distal sites.

The physiologic relevance of TPA down-regulation of surfactant protein gene expression may relate to lung growth and differentiation as well as the effects of inflammatory processes in the lung. Like TPA, transforming growth factor-β and platelet-derived growth factor both activate PKC and, in the developing lung, regulate cell proliferation, epithelial cell differentiation, and synthesis of lung matrix proteins. In particular, transforming growth factor-β modulates pulmonary type II cell differentiation and decreases synthesis of both surfactant phospholipids and associated proteins (26, 58–61). Various inflammatory mediators, including endotoxins, cytokines, and free oxygen radicals, modulate gene expression by activating PKC, and tumor necrosis factor-α decreases SP-A and SP-B gene expression in studies with both H441 cells and cultured fetal lung (27).
Our findings lead us to propose the following model for TPA-mediated effects on expression of SP-B. Exposure of lung cells to low concentrations of TPA activates PKC, resulting in phosphorylation of newly synthesized and resident TTF-1 and/or HNF3 in the cytoplasm. Phosphorylation could occur directly via PKC or indirectly through a phosphorylation cascade and results in inactivation of the nuclear translocation signal on one or both factors. With translocation to the nucleus blocked, cytoplasmic levels of the factors increase and nuclear content decreases secondary to degradation and/or nuclear export. Transactivation of SP-B gene expression diminishes to a low level reflecting the strong enhancer role of the two factors. This model predicts that reversibility of TPA inhibition does not require new protein synthesis, and this has been observed in preliminary experiments. An alternative explanation for cytoplasmic trapping of the transcription factors could involve phosphorylation of proteins serving as chaperones or transport functions rather than direct phosphorylation of TTF-1/HNF3. The mechanism for cytoplasmic trapping is currently under investigation.

In summary, we have found that TPA acts post-translationally to down-regulate SP-B gene expression by cytoplasmic trapping of both TTF-1 and HNF3 without a loss of DNA binding activity. This mechanism of phorbol ester regulation of gene expression is distinct from previously described processes involving AP-1, AP-2, NF-κB, and octamer-binding protein. Cytoplasmic trapping of TTF-1 and HNF3 may also be involved in PKC-mediated down-regulation of thyroid-specific and liver-specific genes, respectively.

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