Retinoblastoma Protein Complexes With C/EBP Proteins and Activates C/EBP-Mediated Transcription

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Abstract   The retinoblastoma protein (RB) recruits histone deacetylase (HDAC) to repress E2F-mediated transactivation that plays a critical role in cell cycle regulation. RB is also involved in activation of expression of a number of tissue specific- and differentiation-related genes. In this study, we examined the mechanism by which RB stimulated the expression of a differentiation-related gene, the surfactant protein D (SP-D), which plays important roles in innate host defense and the regulation of surfactant homeostasis. We demonstrated that RB specifically stimulated the activity of human SP-D gene promoter. The RB family member, p107 but not p130, also increased SP-D promoter activity. Activation by RB was mediated through a NF-IL6 (C/EBPβ) binding motif in the human SP-D promoter, and this sequence specifically bound to C/EBPα, C/EBPβ, and C/EBPδ. RB formed stable complexes with all three C/EBP family members. RB small pocket (amino acid residues 379–792), but not the C-pocket (amino acid residues 792–928), was necessary and sufficient for its interaction with C/EBP proteins. Furthermore, we demonstrated that the complexes containing RB and C/EBP proteins directly interacted with C-EBP binding site on DNA. These findings indicate that RB plays a positive, selective, and direct role in the C/EBP-dependent transcriptional regulation of human SP-D expression. J. Cell. Biochem. 83: 414–425, 2001.   © 2001 Wiley-Liss, Inc.

Key words: retinoblastoma protein; C/EBP; transcription; differentiation; surfactant protein D

Inactivation of retinoblastoma protein (RB) has been documented in many human tumors and cancers [Weinberg, 1995; Dyson, 1998]. RB plays a critical role in cell cycle regulation by physical association with the members of the E2F transcription factors to form active repressor complexes [Weintraub et al., 1995; Zhang et al., 1999]. Loss of RB-mediated transactivation repression is thought to be critical in tumorigenesis [for review see Harbour and Dean, 2000].

RB not only negatively regulates cell proliferation but also is important in cell differentiation and development. It is not clear, however, whether RB-mediated transrepression of E2F activity accounts for the RB function in cell differentiation and development. RB has been implicated in activating transcription of a number of genes associated with cell differentiation. For instance, RB interacts with the human bromoprotein (hBrm) to potentiate glucocorticoid receptor–mediated transactivation [Singh et al., 1995]. In addition, RB positively regulates adipocyte differentiation by binding to C/EBPα and activates transcription of the UCP-1 promoter in preadipocytes [Chen et al., 1996a]. Differentiation of monocytes is associated with RB binding to NF-IL6 which is also called C/EBPβ [Chen et al., 1996b]. Furthermore, RB associates directly with the transcription factor MyoD in promoting muscle cell differentiation [Halevy et al., 1995; Skapek et al., 1995].

The RB family consists of RB and the related proteins p107 and p130. It has been shown that

the expression of RB and the closely related proteins p107 and p130 is developmentally, but not necessarily coordinately regulated [Cobrinik et al., 1996; Jiang et al., 1997] and that these proteins are expressed in a tissue and cell-specific fashion [Dyson, 1998].

RB has been implicated in lung cell proliferation and differentiation. It has been shown that RB expression is induced during fetal lung maturation or terminal growth arrest of a conditionally immortalized fetal rat lung epithelial cell line [Levine et al., 1998]. It is well documented that loss of RB expression is closely associated with the development of small cell lung carcinoma [Yokota et al., 1988; Ookawa et al., 1993; Dean et al., 1994].

The surfactant-associated proteins include SP-A, SP-B, SP-C, and SP-D that are expressed in epithelial cells of the peripheral lung in a developmentally regulated fashion [Xu et al., 1995]. The surfactant-associated proteins are associated with lung epithelial type II cell differentiation and are important in lung alveolar function and immune response [for reviews see Crouch et al., 2000; Lawson and Reid, 2000]. SP-A and SP-D are highly homologous collagenous lectins. Both proteins are believed to participate in the pulmonary response to lung injury, and the production of both proteins can be increased in response to lung injury and infection. Although the SP-A and SP-D genes are expressed late in gestation in the same distal lung epithelial cells, they appear to be differentially regulated [Mendelson et al., 1991; McCormick and Mendelson, 1994; Smith et al., 1995; Rust et al., 1996]. Importantly, surfactant protein expression is closely correlated with the expression of the members of the CAAT-enhancing binding proteins (C/EBP) [Li et al., 1995].

C/EBP (CCAAT/enhancer-binding protein) is a family of bZIP (basic region-leucine zipper) proteins consisting of three subfamilies, α, β (also called NF-IL6), and δ. Because of their high similarity in the basic region, all three members, C/EBPα, C/EBPβ, and C/EBPδ have been shown to interact with virtually identical DNA sequences [Cao et al., 1991; Williams et al., 1991; Osada et al., 1996]. Furthermore, the C/EBP members can readily form heterodimers with one another that are capable of binding to C/EBP-biding motif. C/EBP factors play important roles in the cell-specific gene expression and in cell differentiation, most notably in liver,

fat, and monocytes [Wu et al., 1995; Darlington et al., 1998; Diehl, 1998; Lekstrom-Himes and Xanthopoulos, 1998; Poli, 1998].

A body of evidence suggests that C/EBP proteins are involved in lung cell differentiation. C/EBPα is expressed in lung alveolar type II cells and, to a less extent, in bronchiolar Clara cells [Li et al., 1995]. In the embryonic rat lung, C/EBPα expression closely correlates with the expression of SP-A in the alveolar epithelial cells and the expression of CCSP in the bronchiolar epithelium [Li et al., 1995]. Interestingly, both SP-A and SP-D gene promoter sequences contain C/EBP (NF-IL6) binding sites [Crouch et al., 2000]. Furthermore, C/EBPα knockout mice exhibit immature lung [Wang et al., 1995] as well as alveolar abnormalities with hyperproliferation of epithelial cells [Flodby et al., 1996]. In addition, other members of C/EBP proteins may play important roles in the lung development, as well. For example, C/EBPδ appears to be most abundant in the mouse lung [Cao et al., 1991; Sugahara et al., 1999]. C/EBPδ is localized primarily to alveolar epithelial cells and is rapidly induced during differentiation of human fetal lung in culture [Breed et al., 1997].

In this study, we demonstrated that RB functions as a co-activator in upregulation of tissue specific SP-D gene expression. RB and p107, but not p130, specifically stimulated human SP-D gene reporter activity. In addition, we demonstrated that RB directly interacts with C/EBPα, C/EBPβ as well as C/EBPδ and that RB-C/EBP complexes can directly bind to DNA containing C/EBP binding site.

MATERIALS AND METHODS

Cell Culture and Transfection

MLE-15 cells are embryonic lung epithelial cells immortalized by SV40 T with expression of endogenous surfactant proteins A, B, and C [Wikenheiser et al., 1993]. HeLa and MLE-15 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and L-glutamine and 10% heat-inactivated fetal bovine serum (FetalClone I, Hyclone, Logan, Utah), penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator. Cells grown to 40–60% confluence were used in transient transfection by a calcium phosphate protocol. Briefly, cells were cotransfected with a DNA

mixture containing 2 μg of the pCMV-β-galactosidase plasmid, 7.5 μg of either a rat SP-A/CAT or a human SP-D/CAT reporter plasmid in the absence or presence of an indicated amount of pCMV-pRB. Thirty-six hours after transfection, cells were collected and lysed in Promega lysis buffer (Promega, Madison, WI). Aliquots of cell lysates were assayed for CAT and β-galactosidase activities, respectively.

Cell Extracts and Western Blotting Analysis

Nuclear extract of HeLa and MLE-15 cells were essentially performed according to the protocol of Rana and Farmer [Rana et al., 1995]. Briefly, cells were washed with ice-cold PBS and lysed with NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40) on ice for 15 min. Cell pellets were collected by centrifugation at 1,000g for 5 min at 4°C, washed with the NP-40 lysis buffer, and resuspended in a nuclear extraction buffer (20 mM HEPES pH.7.9, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 350 mM NaCl, 25% glycerol, 0.5 mM DTT, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μg/ml PMSF). After incubation on ice for 20 min and centrifugation at 14000 rpm for 15 min at 4°C, the supernatants were collected and stored at −80°C.

For Western blot analyses, proteins were separated by electrophoresis on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with 4% non-fat dry milk in TBST (10 mM Tris-HCl pH.8.0, 165 mM NaCl, 0.25% Tween 20). Antibodies used in this study, C/EBPα (14AA), C/EBPβ (C-19), or C/EBPδ (M-17), were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Proteins were visualized with an enhanced chemoluminescence system (ECL, Amersham, Piscataway, NJ).

Electrophoresis Mobility Shift Assay (EMSA)

A reaction mixture contained 10 μg of nuclear extract proteins, 1 μl of radiolabeled double stranded DNA oligo, 3 μg of poly(dI/dC), 5 μg of bovine serum albumin, and 4 μl of binding buffer (20 mM Tris-HCl pH7.5, 5 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 125 μM $MnCl_2$, and 10% glycerol) in a total volume of 20 μl. The reaction mixture was incubated at room temperature for 30 min prior to electrophoresis on non-denaturing 6% polyacrylamide gels in Tris-borate-EDTA buffer (90 mM Tris-borate, 2 mM

EDTA HEPES [pH 8.0]). In supershift studies, nuclear extracts were pre-incubated for 10 min at room temperature with a specific GST-RB fusion protein (2 μg) prior to the addition of the ^{32}P-radiolabeled probe. Unlabeled wild type or mutant oligo DNA was used at 50-fold molar excess for binding competition of the ^{32}P-labeled oligo. A wild-type 40 base-pair oligonucleotide, SP-D(WT), contains the DNA sequences from −355 to −316 in the human SP-D promoter (5′GCAGTTGTGAGTTCC**TTTTGCAAT**CGC-TGTAGGTCATTGT3′) [Rust et al., 1996]. This oligonucleotide contains the consensus NF-IL6 binding site (**TT/GNNGNAAT/G**). A mutant oligonucleotide, SP-D(mt) with the NF-IL6 binding site scrambled (5′GCAGTTGTGA-GTTCC**GTATATTCT**CGCTGTAGGTCATTG-T3′), was used in parallel.

The double stranded DNA oligo was radiolabeled with [γ-^{32}P]dATP (New England BioLab) using T4 polynucleotide kinase.

RESULTS

RB Activates SP-D Gene

Since the human SP-D gene contains a number of putative RB-responsive elements including a NF-IL6 site (−340/−323) and two half GRE sites (Fig. 1) [Rust et al., 1996], we wondered whether RB can function as a transcription co-activator for human SP-D gene. Using a CAT-reporter construct in which the CAT expression is under the control of the SP-D 5′ promoter segment (SP-D698), we examined the effect of RB on the SP-D698 reporter activity in the lung epithelial MLE-15 cells [Wikenheiser et al., 1993]. As shown in Figure 2A, when co-transfected to MLE-15 cells, RB stimulated SP-D698/CAT reporter activity more than three fold, whereas RB had only marginal effect on SP-A236/CAT reporter activity despite the presence of a consensus NF-IL6 binding site (TTCTGCAAT) n the SP-A promoter region (−184/−176) [Lacaze-Masmonteil et al., 1992]. The positive effect of RB on the SP-D698/CAT reporter was also observed in HeLa cells (data not shown).

Because p107 and p130 are members of the RB family, we examined whether p107 or p130 have similar effects. As shown in Figure 2B, co-transfection of p107 led to a reproducible simulation on the SP-D698/CAT reporter activity. However, there was no stimulation by p130 despite the expression of p130 in the cells was
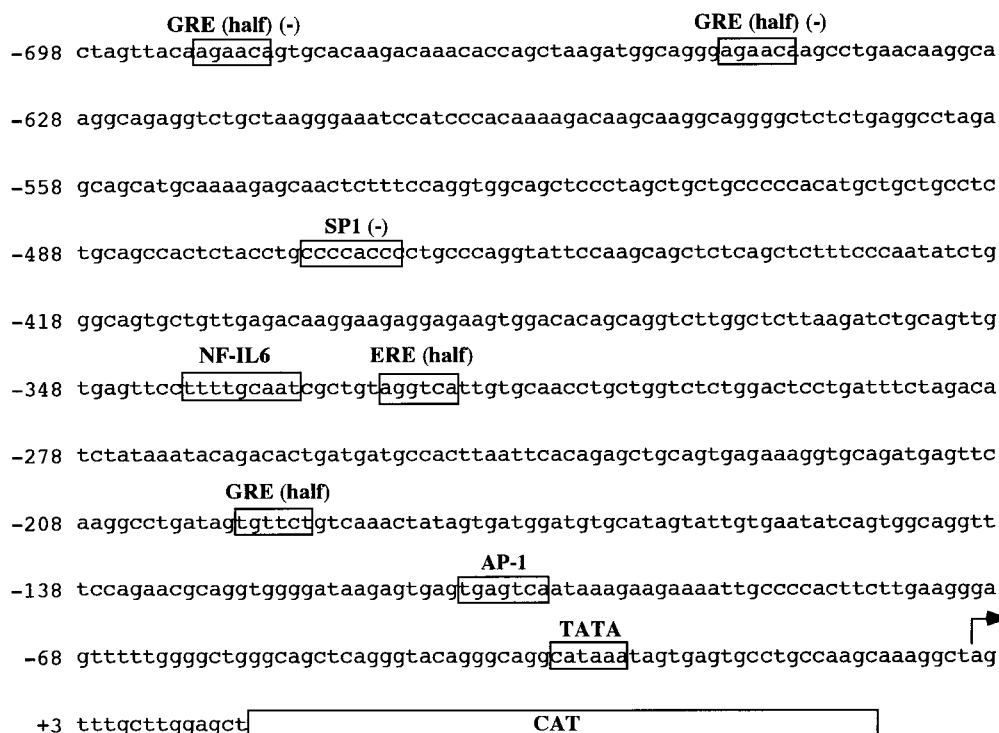
GRE (half) (-)   GRE (half) (-)
-698 ctagttac|agaaca|gtgcacaagacaaacaccagctaagatggcagg|agaac|agcctgaacaaggca

-628 aggcagaggtctgctaagggaaatccatcccacaaaagacaagcaaggcaggggctctctgaggcctaga

-558 gcagcatgcaaaagagcaactctttccaggtggcagctccctagctgctgcccccacatgctgctgcctc

                          SP1 (-)
-488 tgcagccactctacctg|ccccaccc|ctgcccaggtattccaagcagctctcagctctttcccaatatctg

-418 ggcagtgctgttgagacaaggaagaggagaagtggacacagcaggtcttggctcttaagatctgcagttg

              NF-IL6   ERE (half)
-348 tgagttcc|ttttgcaat|cgctgt|aggtca|ttgtgcaacctgctggtctctggactcctgatttctagaca

-278 tctataaatacagacactgatgatgccacttaattcacagagctgcagtgagaaaggtgcagatgagttc

            GRE (half)
-208 aaggcctgatag|tgttct|gtcaaactatagtgatggatgtgcatagtattgtgaatatcagtggcaggtt

                            AP-1
-138 tccagaacgcaggtggggataagagtgag|tgagtca|ataaagaagaaaattgccccacttcttgaaggga

                      TATA
-68 gtttttggggctgggcagctcagggtacagggcagg|cataaa|tagtgagtgcctgccaagcaaaggctag

+3 tttgcttggagct| CAT |

Fig. 1. DNA sequence and several putative regulatory elements in 5′ untranslated region of the human SP-D gene. A putative TATA sequence is indicated. The transcriptional start site is indicated by an arrow [Rust et al., 1996].

comparable with that of RB and p107 (Fig. 2B, inserted).

Dose-Dependent RB Activation of SP-D Requires the Presence of a C/EBP Binding Motif

We next examined whether the RB stimulatory effect on human SP-D promoter is dose-dependent. MLE-15 cells were co-transfected with a fixed amount of the SP-D698/CAT reporter and an increasing amount of pCMV-RB (0–10 µg). RB-mediated stimulation was evidently dose-dependent (Fig. 3A). In contrast, despite of an increasing amount of input RB plasmid, RB had no significant effect on the SP-D161/CAT reporter construct (Fig. 3A). Since SP-D161/CAT is a deletion construct that contains only 161 base pairs upstream of the transcriptional start site and lacks the putative NF-IL6 (C/EBPβ)-binding site [Rust et al., 1996], the data suggested that the NF-IL6 site is an important RB-responsive element. To further examine this possibility, we constructed a targeted deletion mutant (SP-D698/CATΔ) which lacks the 9 bp (TTTTGCAAT) C/EBP-binding site in the SP-D698 promoter region. When tested for RB effect, SP-D698Δ/

CAT apparently lost the RB-responsiveness (Fig. 3B).

C/EBP Proteins Specifically Bind to the C/EBP-Binding Site in the Human SP-D Promoter

Since it is likely that RB functions as a transcription co-activator for SP-D gene, we sought to identify specific proteins that may mediate the RB stimulatory effect. We analyzed the expression of C/EBP proteins in both MLE-15 and HeLa cells. As indicated in Figure 4A, both MLE-15 (M) and HeLa (H) cells expressed C/EBPβ including two major protein species (38 and 18 kDa) and one minor (42 kDa) protein species. Expression of C/EBPδ was also detected in both cell lines. Two C/EBPα protein species (42 and 30 kDa) were readily detected in MLE-15 cells but no immunoreactive C/EBPα was found in HeLa cells indicating that HeLa cells lack the detectable expression of C/EBPα.

Next, we performed EMSA to examine whether C/EBP proteins bind to the C/EBP-binding motif in the human SP-D promoter. Using a ^{32}P-labled 40 bp double-stranded DNA oligo (−355/−316 in the human SP-D promoter) as probe, we detected at least three major
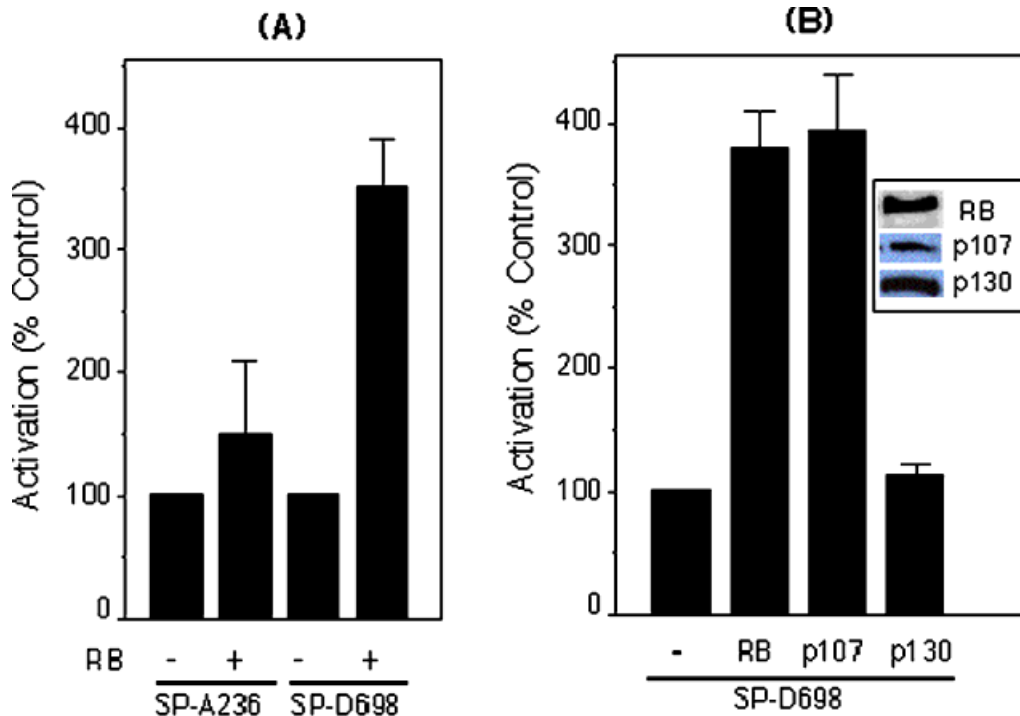
(A)

(B)

Fig. 2. RB specifically upregulates SP-D/CAT reporter activity. A CAT reporter containing either a 698 bps of the human SP-D (SP-D698) promoter (see Fig. 1) or a 236 bps of the rat SP-A promoter (SP-A236) was used in cotransfection experiments. **A:** Exponentially growing lung epithelial MLE-15 cells were transfected with a DNA mixture containing 2 µg of pCMV-β-galactosidase plasmid, 7.5 µg of either the SP-A236/CAT or the SP-D698/CAT reporter plasmid DNA in the absence or presence of 5 µg of pCMV-RB plasmid per 100 mm plate. In the mocked transfections, 5 µg of pCMV plasmid was used instead. **B:** MLE-15 cells were co-transfected with DNA mixtures containing 2 µg

of pCMV-β-galactosidase, 7.5 µg of SP-D698/CAT reporter in the presence of either 5 µg of pCMV-RB, pCMV-p107 or pCMV-130. Aliquots of cell lysates were assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase activities. The corrected CAT activities are presented as fold activation. Data shown is the mean from at least three independent experiments in duplicate with standard derivations. Protein expression in the transient transfection experiments were confirmed (inserted in panel B) by Western blot analysis using antibodies specific for RB, p107 and p130, respectively (purchased from Santa Cruz).

specific protein-DNA complexes from MLE-15 nuclear extracts (Fig. 4B, arrowheads). In contrast, only two major protein-DNA complexes were observed in HeLa nuclear extracts (Fig. 5A), consistent with the observation that HeLa cells lack C/EBPα expression. To demonstrate the specificity of the protein-DNA complexes, we performed competition analysis. The wild-type oligo, SP-D(wt), completely eliminated all three major proteins-DNA complexes (Fig. 4B, lane 3) whereas the mutant oligo, SP-D(mt) that contains a scrammed sequence in the 9 bp C/EBP-binding site, was unable to do so (Fig. 4B, lane 4). Furthermore, addition of a 34 bp DNA oligo derived from the G-CSF promoter sequence (−183 to −150), which contains a functional NF-IL6 (C/EBP) site [Natsuka et al., 1992; Tanaka et al., 1995], also exhibited effective competition (Fig. 4B, lane 2). Similar competition was observed using HeLa

cell lysate (Fig. 5A, lanes 2–4). Thus, we conclude that the human SP-D promoter segment (−355/−316) contains a functional NF-IL6 (C/EBP) binding site.

Next, we performed antibody supershift assays to further examine the specificity of the protein-DNA complexes. Addition of a control rabbit IgG antibody in the reaction mixture had no effect on the protein-DNA complexes (Fig. 4C, lane 2). However, a C/EBPα-specific rabbit polyclonal antibody caused the complete disappearance of the second protein-DNA complex, a decrease in the fastest migrated DNA-protein complex, and the appearance of a slower migrating band (Fig. 4C, lane 3). A C/EBPβ-specific antibody supershifted the fastest migrating protein-DNA complex (Fig. 4C, lane 4), and an antibody specific for C/EBPδ caused the disappearance of the upper band (Fig. 4C, lane 5). These data indicate that the C/EBP
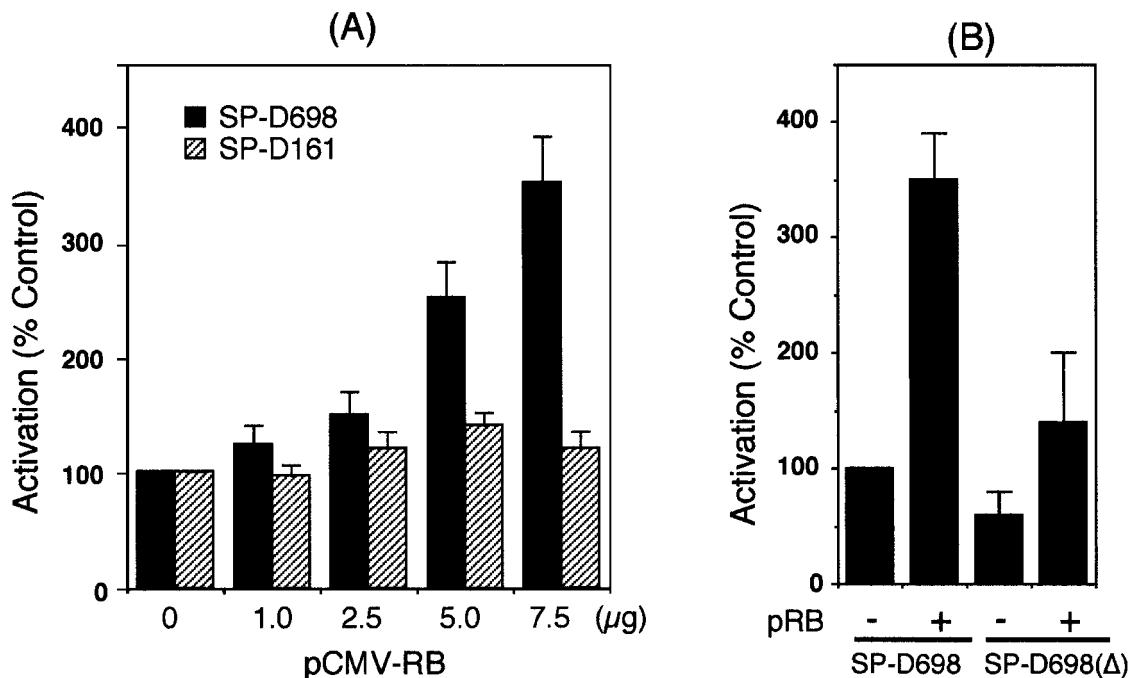
(A)

(B)

Fig. 3. RB-mediated upregulation of SP-D is dose-dependent and requires presence of the NF-IL6 site (−340/−332). **A:** MLE-15 cells were transfected with a DNA-mixture containing 2 μg of pCMV-β-galactosidase, 7.5 μg of either SP-D698/CAT reporter or SP-D161/CAT and an increasing amount of pCMV-RB, as indicated. **B:** MLE-15 cells were transfected with 2 μg of pCMV-

β-galactosidase, 7.5 μg SP-D698 or a deletion mutant (SP-D698Δ) which lacks the NF-IL6 site (TTTTGCAAT), in the absence or presence of 5 μg of pCMV-RB. The corrected CAT activities are presented as fold activation. Data shown is the mean with standard derivations from at least three independent experiments in duplicate.

proteins can specifically bind the human SP-D promoter.

Similar experiments were performed using HeLa cell extracts. Because HeLa cells express C/EBPβ and C/EBPδ, but not C/EBPα (Fig. 4A), we could only detect two protein-DNA protein complexes that were supershifted with antibodies to C/EBPβ and C/EBPδ, respectively (data not shown).

C/EBP Proteins Specifically Interact With the RB Small Pocket and the C/EBP-RB Protein Complexes Directly Bind to DNA

Because RB stimulatory effect on SP-D promoter requires the presence of the NF-IL6 site, we examined the interactions between RB and the C/EBP proteins by EMSAs. As expected, two specific protein-DNA complexes were identified from HeLa cell lysate (Fig. 5A, lane 1). Addition of the GST-RB large pocket [GST-RB(379–928)] or GST-RB small pocket [GST-RB(379–792)], led to a supershift of these two protein-DNA complexes, respectively (Fig. 5A, lanes 5 and 6). In contrast, addition of a comparable amount of RB C-pocket [GST-

RB(792–928)], or GST alone, had no effect on these two protein-DNA complexes (Fig. 5A, lanes 7 and 8). Similarly, GST-RB(379–928) led to supershifts of both C/EBPα-DNA and C/EBPβ-DNA complexes and the loss of the C/EBPδ-DNA complex in MLE-15 cells (Fig. 5B, lane 4), whereas GST alone (Fig. 5B, lane 2) or GST-RBC(792-928) (Fig. 5B, lane 3) had no effect on the protein-DNA complexes. Addition of an antibody specific for C/EBPα (Fig. 5B, compares lanes 5 and 6) or C/EBPβ (Fig. 5B, compare lanes 7 and 8) further supershifted C/EBP-RB-DNA complexes (indicated by open arrows). These data indicate that RB interacts with C/EBP proteins and the RB-C/EBP protein complexes can directly bind to DNA.

Because RB interacts with C/EBP-DNA complexes in nuclear extracts, we next examined whether RB can form stable complexes with C/EBP proteins within intact epithelial cells. Since RB expression in MLE-15 cells is low (data not shown), we could not detect reproducible RB-C/EBP-DNA complexes using antibody supershift assay. We, therefore, transiently transfected MLE-15 cells with pCMV-RB,
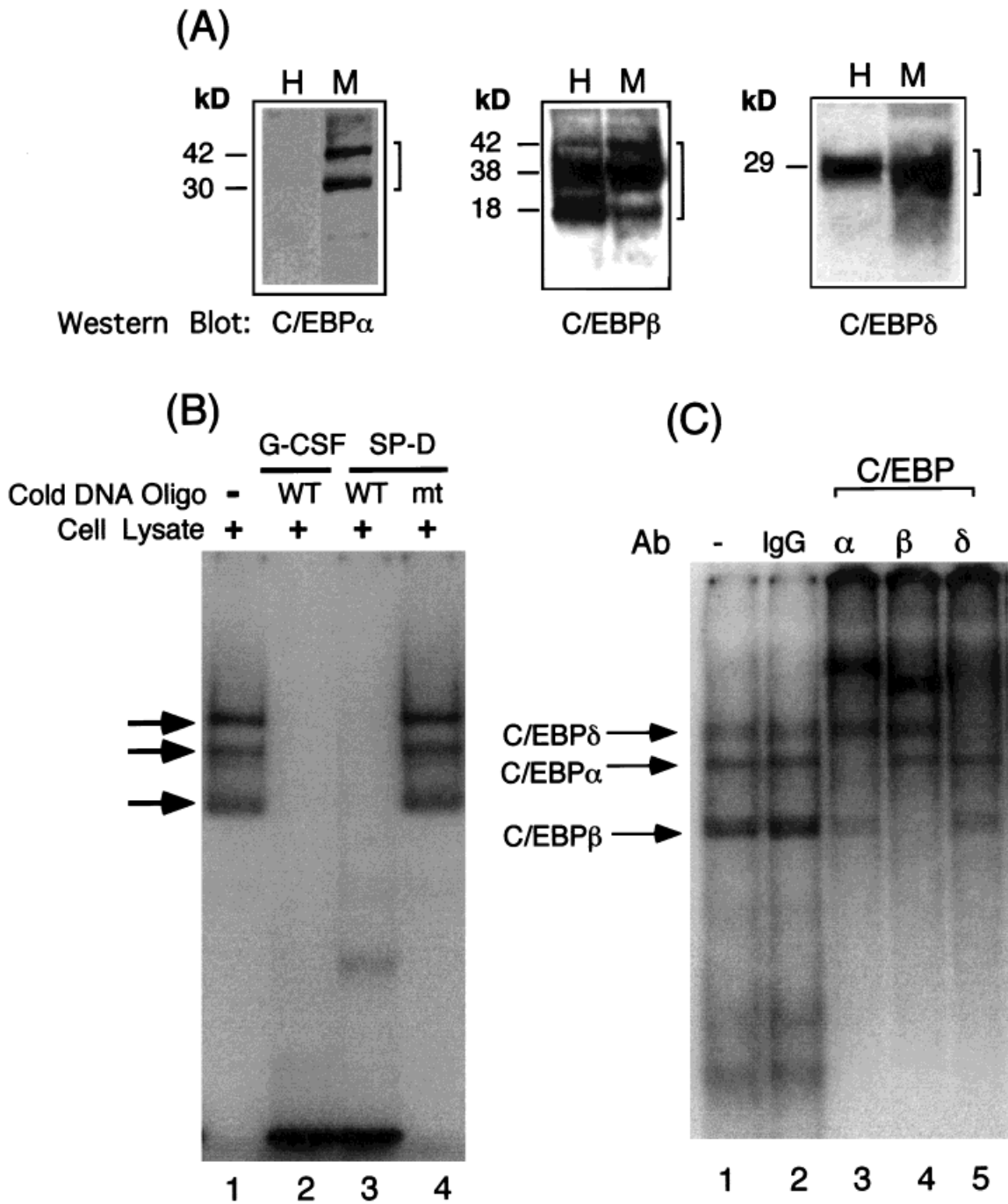
Fig. 4. C/EBP proteins interact with the NF-IL6 binding site in the human SP-D promoter. **A:** Expression of C/EBP proteins in HeLa and MLE-15 cells. Equal amounts (50 μg) of whole cell lysates from HeLa cells (H) or MLE-15 cells (M) cells were separated on a 10% SDS–PAGE and Western blot analyses were performed using antibodies specific for C/EBPα (14AA), C/EBPβ (C-19), or C/EBPδ (M-17), respectively. All antibodies were purchased from Santa Cruz Biotech, Inc. **B:** At least three cellular proteins interact with the NF-IL6 site in the SP-D promoter. Aliquots of nuclear extract (6 μg) derived from MLE-15 cells were used for EMSA, using ^{32}P-labeled, double-stranded DNA oligo (SP-D) that contains the NF-IL6 site (−355/−315) as probe. Fifty fold molar access of cold DNA oligos were used in competition assays. The mutant SP-D DNA oligos (SP-Dmt) contain a scrambled sequence at the NF-IL6 site (−340/−332). The G-CSF oligo contains a NF-IL6 binding site, which has been documented previously [Natsuka et al., 1992]. **C:** C/EBP proteins interact with the NF-IL site. Aliquots of MLE-15 nuclear extracts were incubated with 1 μl of either rabbit IgG, or polyclonal antibody specific for C/EBPα, C/EBPβ, or C/EBPδ prior to the addition of the probe. The reaction mixtures were separated on a 4% non-denaturing acrylamide gel and visualized by autoradiography. Specific DNA-protein-antibody complexes are indicated by arrows.
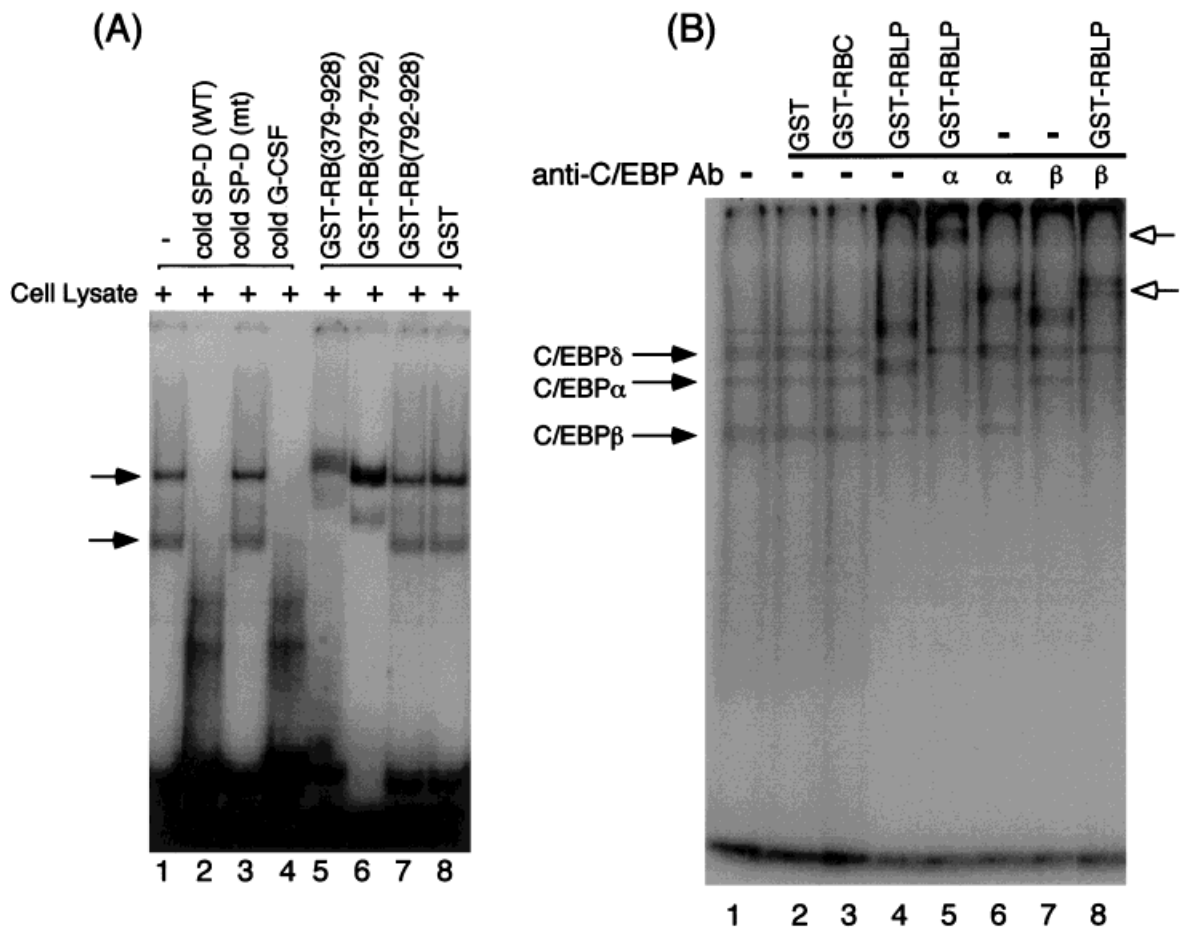
Fig. 5. RB-C/EBP protein complexes directly interact with DNA. Equal amounts (6 µg) of whole cell extracts from HeLa cells (**panel A**) or nuclear extracts from MLE-15 cells (**panel B**) were used in EMSA using ^{32}P-labeled human SP-D (WT) (−355/ −315) as probe. Fifty fold molar access of the cold SP-D (WT), SP-D(mt) or the G-CSF oligo was used in the competition assay (panel A, lanes 2–4). For assessing RB-C/EBP protein interaction, 1µg of the purified GST-RBLP (panel A, lane 5), GST-RBSP (panel A, lane 6), GST-RBC (panel A, lane 7), or GST protein (panel A, lane 8) was added in the reaction mixture prior to the addition of the ^{32}P-labeled probe. In antibody-supershifting experiments, 1 µl of polyclonal antibody specific for C/EBPα, C/EBPβ, or C/EBPδ was added to the reaction mixture and incubated for 2 h prior to addition of the ^{32}P-labeled probe. The reaction mixtures were separated on a 4% non-denaturing acrylamide gel and visualized by autoradiography. Specific C/EBP protein-DNA complexes are indicated by arrows with solid heads. The RB-C/EBP-DNA complexes are indicated by arrows with hollow heads.

which encodes the wild-type human RB. RB protein expression in the transfected cells was confirmed by Western blot analysis (data not shown). Nuclear extracts from pCMV-RB transfected MLE-15 cells were used in EMSA. To identify specific RB-C/EBP protein complexes, the purified GST-RB(379–928) fusion protein was used in EMSA (duplicated in Fig. 6, lanes 2 and 3), which led to the formation of two specific RB-C/EBP protein complexes (Fig. 6, compare lane 1 with lanes 2 and 3). Expression of human RB proteins in MLE-15 cells led to two additional DNA-protein complexes (Fig. 6, open arrows) in addition to the three C/EBP-DNA protein complexes (solid arrows). These data

indicate that RB-C/EBP protein complexes can directly bind to DNA.

DISCUSSION

RB has been documented to function as a tumor suppressor protein by negatively regulating cell cycle G1 exit [Weinberg, 1995]. RB associates E2Fs and recruits histone deacetylase (HDAC), resulting in transcriptional repression [Weintraub et al., 1995; Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998]. Thus, the primary function of RB in cell cycle regulation is to act as a transcriptional corepressor. On the other hand, RB can function
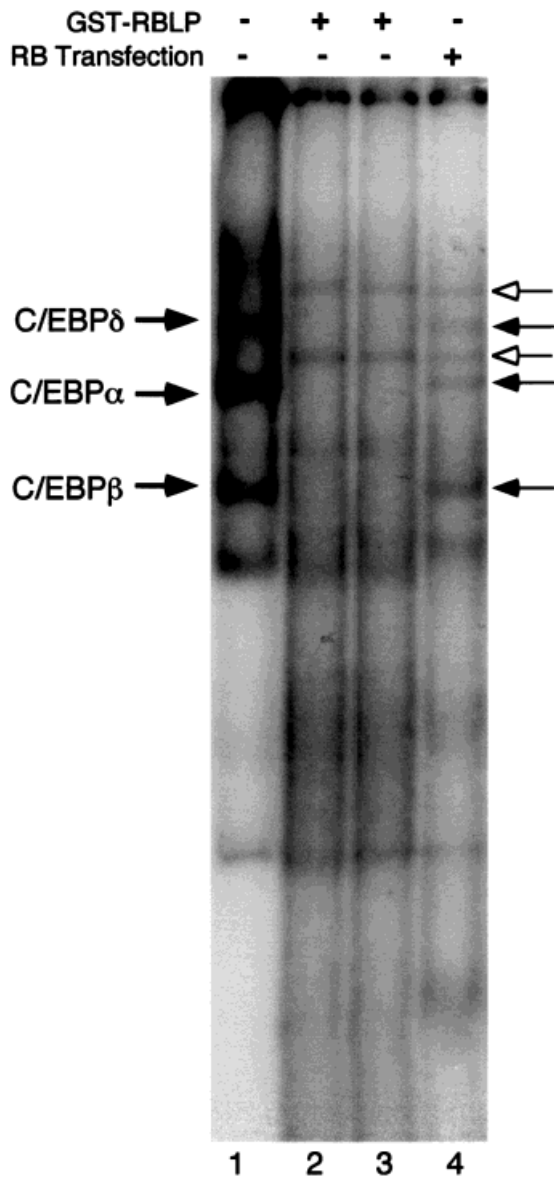
GST-RBLP   -   +   +   -
RB Transfection   -   -   -   +

C/EBPδ →

C/EBPα →

C/EBPβ →

1   2   3   4

Fig. 6. RB forms complexes with C/EBP proteins in vivo. Pre-confluent (40–60%) MLE-15 cells were transfected with 5 µg of pCMV-RB plasmid using calcium phosphate procedure. Thirty-six hours after transfection, nuclear extracts were prepared. Six micrograms of proteins from mock transfected (**lanes 1–3**) or RB-transfected MLE-15 cell lysate (lane 4) was used in a reaction mixture, using ^{32}P-labeled human SP-D (WT) (−355/−315) as probe. As controls for RB-C/EBP-DNA complexes, 1 µg of purified GST-RB protein (duplicated in lanes 2 and 3) was added in the reaction mixture prior to the addition of the ^{32}P-labeled probe. The reaction mixtures were separated on a 4% non-denaturing acrylamide gel and visualized by autoradiography. Specific C/EBP-DNA complexes are indicated by arrows with solid heads. The newly formed RB-C/EBP-DNA complexes in RB-transfected cell lysate are indicated by arrows with hollow heads

as transcription co-activator through its physical interaction with selective transcriptional factors, such as hBrm and C/EBP [Singh et al., 1995; Chen et al., 1996a; Chen et al., 1996b]. Recently, it has been shown that RB interacts with Jun to activate Jun-mediated transcription through an AP-1 consensus sequence [Nishitani et al., 1999]. RB can also interact with AP2 to activate transcription [Batsche et al., 1998]. In this study, we demonstrated that RB performs a positive regulatory function in regulating differentiation-specific gene expression through direct interaction with C/EBP-DNA complexes, and that p107 but not p130, likely functions in a similar fashion.

RB-Mediated Transcriptional Activation is not Cell Type-Specific

RB-mediated stimulation of human SP-D gene expression is observed in both non-lung cells (HeLa) and lung epithelial cells (MLE-15). This is consistent with recent studies indicating that SP-D is not a lung specific protein [Crouch et al., 2000]. Overexpression of RB can stimulate gene transcription in both cell lines, suggesting that the components involved in RB-mediated transcriptional activation are common in both cell types and, therefore, unlikely to be tissue specific.

Like RB, p107 but not p130 stimulates SP-D promoter activity. It is known that RB, p107, and p130 have overlapping but different functions at different cell cycle stages by targeting different E2F family members [Hurford et al., 1997; Dyson, 1998]. Importantly, the RB family genes are differentially regulated during development. It has been noted that the E2F-5 knockout mice display a specific developmental abnormality in the choroid plexus that is not present in either RB null mice nor in p107 null mice [Lindeman et al., 1998]. This suggests that p130, a primary partner of E2F-5, could have functions in development and cell differentiation that differ from RB and p107.

RB Selectively Activates Promoter Activity Through the C/EBP Element at −340

RB activation of SP-D gene expression requires the NF-IL6 binding motif (TTTTGCAAT) located in the 5′ promoter region of SP-D. Deletion of the sequence totally eliminates the RB effect on reporter activity (Fig. 4). Interestingly, the rat SP-A promoter, which contains a perfect NF-IL6 consensus sequence

(TTCTGCAAT) at position -184 to -176 in the promoter region [Smith et al., 1995], is not activated by RB. One possibility for this selectivity of RB-mediated transactivation is that RB functionally interacts not only the C/EBP site but also the adjacent DNA elements. Alternatively, RB may also target on other regulatory proteins that functionally interact with certain corresponding elements adjacent to the C/EBP site.

RB-C/EBP Complexes Bind Directly to DNA

It has been reported that that RB directly interacts with NF-IL6 (i.e., C/EBPβ) [Chen et al., 1996a; Chen et al., 1996b]. This interaction between RB and NF-IL6 occurs when U937 cells differentiate along the monocyte/macrophage lineage. Consequently, RB enhances C/EBP proteins to bind to its cognate DNA sequences and thus to increase C/EBP-mediated transcription [Chen et al., 1996b]. Here, we have demonstrated that RB specifically interacts with three major C/EBP isoforms and that RB-C/EBP protein complexes can directly interact with DNA. This particular finding implies, although does not prove, that RB might be directly involved in the transcriptional machinery that activates transcription.

It is interesting to note that RB activates C/EBP-mediated increases in SP-D promoter activity in the absence of C/EBPα in HeLa cells, suggesting that C/EBPβ and C/EBPδ, through their interaction with RB, can activate transcription. This possibility is supported by the C/EBP-specific antibody supershifts of nuclear extracts that bind to the NF-IL6 (C/EBPβ) in RB-transfected cells. Interestingly, it appears that C/EBPα and C/EBPδ are reciprocally expressed in lung alveolar type II cell proliferation and in acute lung injury [Sugahara et al., 1999]. Thus, RB may function in interaction with different C/EBP factors in regulating cell proliferation and differentiation.

Physiological Relevance of RB-Mediated Activation on SP-D Gene

The surfactant proteins A and D (SP-A and SP-D) are primarily found in the surfactant on the pulmonary epithelial cells. They can also be found in cells lining the gastrointestinal tract. There is accumulating evidence that SP-D, like SP-A, is an important component of the innate immune response to microbial pathogens. SP-D interacts directly with glycoconjugates and/or lipid moieties on the surface of microorganisms, which in turn initiates a variety of biological reactions. Additionally, SP-A and SP-D are also implicated in inflammation as immunomodulators [Crouch et al., 2000; Lawson and Reid, 2000]. It has been shown that a recombinant SP-D protein inhibits human T lymphocyte proliferation and IL-2 production, suggesting that SP-D may contribute to the suppression of stimulated T cell proliferation [Borron et al., 1998; Wang et al., 1998]. Mice lacking SP-D exhibit chronic inflammation, emphysem, and fibrosis on the lung, suggesting that SP-D may, directly or indirectly, influence the state of activation of alveolar macrophages [Wert et al., 2000]. The observation that RB can activate SP-D gene expression raises an interesting possibility that RB might be involved in non-acquired host responses to infection. Co-activation by RB and C/EBPs on the expression of SP-D, but not SP-A, suggests a mechanism for differential regulation on surfactant genes.

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