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Article **Pulmonology**

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Keratinocyte growth factor and the transcription factors C/EBPα, C/EBPδ, and SREBP-1c regulate fatty acid synthesis in alveolar type II cells

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Strategies to stimulate endogenous surfactant production require a detailed understanding of the regulation of lipogenesis in alveolar type II cells. We developed culture conditions in which keratinocyte growth factor (KGF) stimulates fatty acid and phospholipid synthesis. KGF stimulated acetate incorporation into phosphatidylcholine, disaturated phosphatidylcholin, and phosphatidylglycerol more than 5% rat serum alone. To determine the mRNA levels of lipogenic enzymes and transport proteins, we analyzed gene expression by oligonucleotide microarrays. KGF increased the mRNA levels for fatty acid synthase, stearoyl-CoA desaturase-1 (SCD-1), and epidermal fatty acid–binding protein more than rat serum alone. In addition, KGF increased the mRNA levels of the transcription factors CCAAT/enhancer-binding protein α (C/EBPα) and C/EBPδ as well as SREBP-1c (ADD-1), but not PPARγ. These changes in C/EBPα and C/EBPδ were confirmed by in situ hybridization. SCD-1 was also found to be highly expressed in alveolar type II cells in vivo. Furthermore, KGF increased protein levels of fatty acid synthase, C/EBPα, C/EBPδ, SREBP-1, epidermal fatty acid–binding protein, and SCD. Finally, the liver X receptor agonist T0901317 increased acetate incorporation and SREBP-1 but not SREBP-2 protein levels. In summary, KGF stimulates lipogenesis in type II cells by a coordinated expression of lipogenic enzymes and transport proteins regulated by C/EBP isoforms and SREBP-1c.

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Introduction

Pulmonary surfactant lowers the surface tension at the air/liquid interface in the lung and prevents alveolar instability, small airway closure, and alveolar flooding. Surfactant is composed predominantly of phospholipids, especially phosphatidylcholine and phosphatidylglycerol, and the surfactant proteins (SP-A, SP-B, SP-C, and SP-D). The phospholipid that is most responsible for the low surface tension is dipalmitoylphosphatidylcholine. Although the pathways for fatty acid and phospholipid synthesis in type II cells have been defined previously through metabolic labeling experiments, relatively little is known about the regulation of these pathways (1–5).

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Nonstandard abbreviations used: surfactant protein (SP); rat serum (RS); keratinocyte growth factor (KGF); disaturated phosphatidylcholine (DSPC); CCAAT/enhancer-binding protein (C/EBP); liver X receptor (LXR); epidermal fatty acid–binding protein (E-FABP); stearoyl-CoA desaturase (SCD); charcoal-stripped FBS (CS-FBS); dexamethasone (Dex); ribonuclease protection assay (RPA); 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2).

Understanding the regulation of lipid synthesis is important for the development of new therapeutic strategies to increase endogenous surfactant production. Surfactant deficiency or dysfunction is thought to be important in respiratory deficiency of the newborn, acute lung injury/acute respiratory distress syndrome, diffuse pulmonary fibrosis, and diseases of small airways such as asthma and bronchiolitis. Although treatment with exogenous surfactant is highly effective in respiratory distress syndrome of the newborn and partially effective in acute lung injury/acute respiratory distress syndrome, this form of therapy is expensive, requires intubation, and is not practical in milder forms of disease. If it were possible to stimulate the production of endogenous surfactant, this therapy would likely be beneficial in a variety of diffuse parenchymal diseases or diseases of small airways. A means of stimulating the production of surfactant phospholipids in the mature lung is not known.

A major problem for studies on the regulation of lipogenesis in adult type II cells has been the requirement of a culture system that maintains or induces type II cell differentiation. We have recently developed a system that permits differentiation in vitro (6). Rat alveolar cells are plated on a matrix of type I collagen and Matrigel, and the cells are cultured with rat serum and keratinocyte growth factor (KGF, FGF-7). In this system, type II cells maintain relatively high levels of

expression of the SPs (SP-A, SP-B, SP-C, and SP-D), form a polarized epithelium, and secrete phospholipids in response to agonists. In addition, we have also shown that KGF stimulates acetate incorporation into surfactant phospholipids in type II cells cultured on Matrigel, another system for maintaining differentiation (7), and Chelly et al. have reported that KGF stimulates lipogenesis in fetal type II cells (8).

In the current study we sought to define conditions that would permit the synthesis of the surfactant phospholipids, especially disaturated phosphatidylcholine (DSPC) and phosphatidylglycerol, and to determine the expression of key transcription factors, lipogenic enzymes, and transport proteins responsible for lipogenesis in type II cells. For this purpose, we performed gene expression profiling using high-density oligonucleotide arrays, which provides a broad analysis of many lipogenic enzymes and of potential transcription factors responsible for their regulation, and verified the results by real-time PCR, in situ hybridization, and immunoblotting.

Methods

Materials. The source of most of the reagents is stated in the description of the individual methods. Human recombinant KGF was purchased from Promega Corp. (Madison, Wisconsin, USA) or R&D Systems Inc. (Minneapolis, Minnesota, USA). Preparation of the rabbit polyclonal antibodies against rat SP-A and rat SP-D, provided by Dennis Voelker and Mandy Evans (National Jewish Medical and Research Center), has been previously described (9). Antibodies to CCAAT/enhancer-binding protein α (C/EBPα), C/EBPβ, and C/EBPδ were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA), polyclonal antibodies to rat SREBP-1 and SREBP-2 were generous gifts of Joel Goldstein (University of Texas Southwestern Medical Center, Dallas, Texas, USA), and additional mAb's to SREBP-1 and SREBP-2 were used as culture supernatants from cell lines obtained from the American Type Culture Collection (Manassas, Virginia, USA) and from Doug Thewke (East Tennessee State University, Johnson City, Tennessee, USA). Antibody to fatty acid synthase was a gift of Stuart Smith (Oakland Children's Medical Center, Oakland, California, USA), and antibody to epidermal fatty acid–binding protein (E-FABP) was a gift of Marino DeLeon (Loma Linda University, Loma Linda, California, USA). The PPARγ agonist 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 and the liver X receptor (LXR) agonist T0901317 were purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). We thank Masaki Takiguchi (Chiba University, Chiba, Japan) for murine C/EBPα, C/EBPβ, and C/EBPδ cDNAs, and James Ntambi (University of Wisconsin, Madison, Wisconsin, USA) for murine stearoyl-CoA desaturase (SCD) cDNAs and antibody to SCD.

Type II cell isolation. Alveolar type II cells were isolated from specific pathogen–free adult male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, Indiana, USA) by dissociation with porcine pancreatic elastase (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) and partial purification on discontinuous metrizamide gradients as previously described (10).

Culture in the apical-access system. Type II cells were plated on a filter insert (Millicell-CM, 30-mm diameter; Millipore Corp., Bedford, Massachusetts, USA) that had been coated with 0.4 ml of a 4:1 (vol/vol) mixture of rat tail collagen and Engelbreth-Holm-Swarm (EHS) tumor matrix (Matrigel; Collaborative Biomedical Products, Bedford, Massachusetts, USA) (6). The mixture was prepared at 4°C and allowed to gel at 37°C and contained approximately 0.8 mg rat tail collagen and 2 mg EHS protein per ml. Two and one-half million viable cells were plated in 1 ml of DMEM containing 5% rat serum (RS; Pel-Freez Biologicals, Rogers, Arkansas, USA), 2 mM glutamine, 2.5 μg/ml amphotericin B, 100 μg/ml streptomycin, 100 μg/ml penicillin G, and 10 μg/ml gentamicin (all from GIBCO BRL, Life Technologies Inc., Rockville, Maryland, USA; or Sigma-Aldrich, St. Louis, Missouri, USA), and 2.0 ml of the same medium was added outside the insert of each well. After attachment for 20–24 hours, the monolayers were rinsed, and then 0.4 ml of the specified medium was added to the apical surface and 2.0 ml was added outside the insert. In different experiments the medium contained combinations of 1% charcoal-stripped FBS (CS-FBS), 5% RS, 10 ng/ml KGF, or 10^{-8} M dexamethasone (Dex). The six-well plates were then placed on a rocking platform inside a humidified incubator gassed with 10% CO_2. The plates were rocked about five times per minute, and the medium was changed every 48 hours.

Culture on Matrigel. In the apical-access system described above, the cells attach, flatten, dedifferentiate, and then proliferate and differentiate to form a more cuboidal monolayer. To prevent the initial flattening, spreading, and dedifferentiation, cells were plated directly on Matrigel, where they form spherules with the apical surface facing inward and the basolateral surface facing outward (6). Type II cells were resuspended in DMEM containing 5% RS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10 μg/ml gentamicin sulfate. Cells were plated into the wells of six-well cluster dishes (Becton Dickinson Labware, Franklin Lakes, New Jersey, USA) coated with 1 ml of Matrigel that had been diluted with DMEM (1:2 vol/vol, DMEM/Matrigel). Type II cells were seeded into each well at a density of 5×10^5 viable cells/cm^2; the day of isolation was considered day 0 of culture.

Labeling and analysis of lipids. Lipid synthesis by type II cells cultured under the various conditions was performed as described previously (7). Briefly, the day before harvest, the medium was changed, and the cells were incubated with 10 μCi/ml [1-^{14}C]acetate (specific activity 25–60 μCi/mmol; ICN Biomedicals Inc., Aurora, Ohio, USA) for the final 24 hours. The acetate-labeling protocol was designed to approximate equilibrium labeling conditions of newly synthesized lipid

so that the percentage of individual newly synthesized phospholipids could be ascertained with small samples. For the choline-incorporation studies, the cells were incubated with 5 μCi/ml [^{3}H] choline for 5 hours in DMEM (27 μM choline). Lipids were extracted with methanol and chloroform and separated by two-dimensional thin-layer chromatography on silica gel 60 plates (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). The first solvent was 130 ml chloroform, 50 ml methanol, and 20 ml acetic acid, and the second solvent was 130 ml chloroform, 50 ml methanol, and 20 ml formic acid. Individual phospholipid spots were scraped directly into scintillation vials, their incorporated radioactivity was determined, and then the incorporation into each phospholipid species as a percentage of total phospholipids was calculated (7). The percentage of DSPC was determined as described previously (5). In other experiments, cells were extracted with methanol and chloroform, and lipid phosphorus was measured.

Measurement of DNA. To harvest type II cells for the DNA assay, the collagen-Matrigel gel was teased off the insert and placed in a polypropylene tube. The matrix was digested by incubation with 1 ml of a 1:4 (vol/vol) mixture of 5 mg/ml type I collagenase (Worthington Biochemical Corp., Lakewood, New Jersey, USA) in DMEM with 10% FBS and 50 U/ml dispase (Becton Dickinson Labware). The cells were collected in saline, sedimented, resuspended, and washed once before resuspension in phosphate buffer containing EDTA for the DNA analysis. The suspension was frozen and stored at –20°C. After thawing, the cells were sonicated and the DNA content was determined fluorometrically (11).

Oligonucleotide microarray analysis. Gene expression profiling was used to identify genes that were regulated by KGF. Cells were cultured for 6 days in the apical-access system with 1% CS-FBS alone, or with the addition of 10 ng/ml KGF, 5% RS, KGF plus 5% RS, or KGF plus 5% RS plus 10^{-8} M Dex. The Dex was present for the last 4 days of culture (6). The cells were directly lysed into 4 M guanidinium isothiocyanate, 0.5% *N*-laurylsarcosine, and 0.1 M β-mercaptoethanol in 25 mM sodium citrate buffer. Total cellular RNA was isolated by centrifugation through a 5.7-M CsCl cushion at 150,000 *g* for 18 hours. The RNA was treated with RNase-free DNase I (Ambion Inc., Austin, Texas, USA) to insure that only RNA was analyzed. Isolated RNA was used to generate cRNA (complementary RNA) according to the standard protocol supplied by Affymetrix (Santa Clara, California, USA). The quality of the cRNA was evaluated by control hybridization to Affymetrix Test Chips containing probes built to match the 5′, middle, and 3′ sequences of housekeeping genes. Fluorescently labeled cRNA samples from cell preparations were hybridized to the Affymetrix rat U34 oligonucleotide arrays, containing about 8,000 full-length genes and expressed-sequence tags, using standard Affymetrix protocols. All chips were scanned using Affymetrix software and normalized, and the relative expression was quantified as relative differences and ratios. Analysis was also performed using GeneSpring 3.2.8 and 4.1 software (Silicon Genetics, Redwood City, California, USA). Each chip was normalized to itself. Expression level of each gene on the chip was quantified and ranked based on the absolute difference in normalized expression between KGF-treated and untreated cells.

Real-time PCR measurement. Type II cells in the apical-access culture system were directly lysed into 4 M guanidinium isothiocyanate, 0.5% *N*-laurylsarcosine, and 0.1 M β-mercaptoethanol in 25 mM sodium citrate buffer. Total cellular RNA was isolated as described for the microarray analyses and treated with 4 units of RNase-free DNase I (Ambion Inc.) for 0.5 hours at 37°C. Total RNA (2 μg) in 100 μl of reverse transcription reaction mix was used to synthesize cDNA, using TaqMan reverse transcription reagents kit according to the manufacturer's directions (Applied Biosystems, Branchburg, New Jersey, USA). Random hexamers were used as primers. The reactions were incubated at 25°C for 10 minutes, at 48°C for 30 minutes, and at 95°C for 5 minutes, and then stored at –20°C until use. Real-time PCR primer and probe sets were selected for each cDNA using Primer Express software (version 1.5; Applied Biosystems) (Table 1). For primer design, the sequences for cDNAs were obtained from GenBank. Real-time PCR reactions were performed according to the manufacturer's directions with TaqMan PCR Core Reagents kit (Applied Biosystems) using two-step RT-PCR reactions on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The rat GAPDH primer/probe set (Applied Biosystems) was used to normalize all assays. Samples were run in triplicate. The reactions were quantitated by selection of the amplification cycle during which the PCR product of interest was detected to be accumulating logarithmically (the threshold cycle [C_T]). Before use of this method, validation experiments were performed to demonstrate that the efficiency of target amplification and the efficiency of reference amplification were approximately equal. Serial dilution of total RNA was performed from 16 ng to 0.25 ng, and ΔC_T values for target and reference were obtained. For the real-time PCR assay, 2–4 ng of input RNA was used.

Ribonuclease protection assay for SREBP. Because SREBP-1a and SREBP-1c are so similar, their mRNA relative concentrations were determined by a ribonuclease protection assay (RPA) as described previously (12). Plasmid constructs containing cDNA for rat SREBP-1 and SREBP-2 were obtained from Iichiro Shimomura and Joel Goldstein (University of Texas Southwestern Medical Center). Radiolabeled antisense riboprobes were prepared, and the RPA was performed as previously described (7). The protected fragments for the single SREBP-1 riboprobe were resolved into two bands. The protected fragments for SREBP-2, SREBP-1a, and SREBP-1c corresponded to bands of 520 bp, 257 bp, and 160 bp, respectively.

Table 1
Probes and primers for RT-PCR

		Forward primer	Reverse primer	Probe
Transcription factors				
C/EBPα	(NM_012524)	GGCGGGAACGCAACAA	TCCACGTTGCGCTGTTTG	CTTTATCTCGGCTCTTGCGCACCG
C/EBPβ	(NM_02415)	AAGCTGAGCGACGAGTACAAGA	GTCAGCTCCAGCACCTTGTG	CAACAACATCGCGGTGCGCA
C/EBPδ	(NM_013154)	CGACCCCTGCCATGTATGAC	GGCAGCCATGGAATCAATGT	AGAGCGCCATCGACTTCAGCGC
SREBP-1	(ADD-1; L16995)	TGCCCTAAGGGTCAAAACCA	TGGCGGGCACTACTTAGGAA	CCCAGAGCCTTGCACTTCTTGACACG
SREBP-2	(XM_216989)	GCTGCAGATCCCGCAGTAC	GGTGGATGAGGGAGAGAAGGT	CGGTCATTCAGCCAGGTCCCATTG
PPARγ	(AB011365)	CTTGGCCATATTTATAGCTGTCATTATT	TGTCCTCGATGGGCTTCAC	TGGAGACCGCCCAGGCTTGC
Fatty acid synthesis and transport				
ACC	(AB004329)	GAATTTGTCACCCGCTTTGG	TGGAGCGCATCCACTTGA	CGGTGCTCATCGCCAATAATGGTATCG
FAS	(M76767)	GGACATGGTCACAGACGATGAC	GTCGAACTTGGACAGATCCTTCA	CTTAGGCAACCCATAGAGCCCAGCCT
SCD-1	(J02585)	CCTTAACCCTGAGATCCCGTAGA	AGCCCATAAAAGATTTCTGCAAA	CTGATGATCCTCCAGCCAGCCTCTTG
SCD-2	(AB032243)	CAGCGTGCCTCCTCTCCTAAT	TGTGTTGCCATATTTTAGTGTCACA	AGTTCCACGGCTGTCACCCAATCAG
E-FABP	(S69874)	GCAACAACCTCACCGTCAAA	TCTCTCCCAAGGTGCAAGAAA	ACGGTCGTCTTCACCGTGCTCTCA
Glycerolipid synthesis				
GPAT	(AF021348)	ATCCAACACCATCCCTGACATC	AGTGACCTTCGATTATTCGATCA	TGGTCATCCCTGTGGGCATCTCG
DGAT	(AF296131)	TCTTCCTACCGGGATGTCAATC	TCCCTGCAGACACAGCTTTG	TTGACCCTTCGCTGGCGGCA
CCT	(U03490)	GCGGAGCACCGGATTG	TGTCTGATGTGGAGATGCCTTCT	TCGTCGCTCCCTGCCGAAGAGT
CDS	(AB009999)	CAGAGGAGATTCCGATGTTCCT	CCAGACAGGGCTTTCTTGAGA	TCCCACCATCCTCAGACAGGACCC
PIS	(D82928)	GGCCATGCTCGACATGCT	AGGGCCAGGTTGACCAAGA	ACGGACCGTTGCGCCACCA
PGPS	(BG663105)	AGAGGTGAACGGCTTCTTTGG	CACTGTAGAACTGTCGCTCAATGTG	CCGGCGCCATCCCAGCTG

ACC, Acetyl-CoA Carboxylase; FAS, fatty acid synthase; SCD-1, Stearoyl-CoA desaturase-1; SCD-2, Stearoyl-CoA desaturase-2; E-FABP, epidermal fatty acid–binding protein; DGAT, diglyceride acyltransferase; GPAT, glycerol-3P acyltransferase; CCT, CTP:phosphocholine cytidylyltransferase; CDS, CDP-diacylglycerol synthase; PIS, phosphatidylinositol synthase; PGPS, phosphatidylglycerol phosphate synthase.

In situ hybridization. In situ hybridization was performed as described previously (13, 14). Briefly, the Millicell inserts were fixed in freshly prepared 4% paraformaldehyde and transferred to 70% ethanol for embedding in paraffin. Radiolabeled sense and antisense riboprobes were transcribed from PCR amplified cDNAs that were previously cloned into plasmid pGEM-4Z (Promega Corp.). Gene-specific in situ hybridization probes for rat SCD-1 and SCD-2 were prepared by PCR. Full-length cDNA was prepared from isolated rat type II cells. Primers were based on the reported sequence for SCD-1 (accession no. J02585) and for SCD-2 (accession no. AB032243). The forward primers included a *Bam*HI restriction site added to the 5′ end, and the backward primers included an *Eco*RI restriction site added to the 5′ end to facilitate directional cloning into pGEM-4Z. The primers for SCD-1 were 5′-CGGATCCTGGCAGGGCAGGAAATAGTG-3′ coding sense and 5′-GGAATTCGATTGGGCTACAGGGACAGTAAAG-3′ coding antisense. The SCD-1 probe corresponded to the 1,003 bases from nucleotides 2,277–3,280 in the 3′-untranslated region of the gene. The primers for SCD-2 were 5′-CGGATCCCCCCTTCTATCTTCTTTTCCCG-3′ coding sense and 5′-GGAATTCTGGCTTCAAACTCAGAGACCCACC-3′ coding antisense. The SCD-2 probe corresponded to the 684 bases from nucleotides

Table 2
Fold increase in acetate incorporation into lipids

Lipid	Basal medium	KGF	RS	KGF + RS
Total lipids	1.0	16.9 ± 1.9[A]	0.9 ± 0.1	11.4 ± 1.8[A]
Neutral lipids	1.0	2.4 ± 0.2[A]	0.9 ± 0.1	1.8 ± 0.2[A]
Phospholipids	1.0	34.3 ± 5.3[A]	0.9 ± 0.2	23.2 ± 4.6[A]
Phosphatidylcholine	1.0	37.5 ± 7.4[A]	0.8 ± 0.2	28.8 ± 7.4[A]
Disaturated phosphatidylcholine	1.0	37.0 ± 8.2[A]	0.7 ± 0.2	38.5 ± 9.1[A]
Phosphatidylglycerol	1.0	46.4 ± 11.8[B]	1.1 ± 0.2	85.6 ± 14.0[A]
Phosphatidylinositol	1.0	33.2 ± 1.2[A]	1.3 ± 0.4	10.7 ± 1.6[A]
Phosphatidylserine	1.0	15.4 ± 0.8[A]	1.1 ± 0.2	4.3 ± 0.2[A]
Phosphatidylethanolamine	1.0	57.9 ± 16.5[A]	1.4 ± 0.2	15.5 ± 3.6
Sphingomyelin	1.0	18.8 ± 3.5[A]	2.2 ± 0.6	13.7 ± 3.2[A]

Alveolar type II cells were cultured for 7 days, the final 6 days with or without the additions of KGF or RS and the final 24 hours with 10 μCi/ml ^{14}C-acetate. The data are expressed as cpm per microgram DNA to adjust for the increased DNA in cultures with KGF. The results are presented as the fold increase compared with 1% CS-FBS (basal medium). Data are expressed as the mean ± SE for four independent experiments. [A]$P < 0.01$ and [B]$P < 0.05$ increase relative to basal medium.

Table 3
Percent distribution of acetate incorporation

	Basal medium[A]	KGF	RS	KGF + RS
% Total lipids				
Neutral lipids	53.4 ± 2.4	7.8 ± 0.2[B]	54.0 ± 4.8	8.7 ± 0.3[B]
Phospholipids	46.6 ± 2.4	92.2 ± 0.2[B]	46.0 ± 4.8	91.3 ± 0.3[B]
% of Phospholipids				
Phosphatidylcholine	73.7 ± 4.2	77.3 ± 0.6	61.0 ± 3.0[C]	85.3 ± 1.2
Phosphatidylglycerol	0.8 ± 0.1	1.1 ± 0.3	1.0 ± 0.1	2.9 ± 0.3[B]
Phosphatidylinositol	4.6 ± 1.0	4.2 ± 0.1	5.5 ± 0.1	2.1 ± 0.2[C]
Phosphatidylserine	4.9 ± 0.8	2.2 ± 0.1[C]	5.8 ± 0.6	1.0 ± 0.1[B]
Phosphatidylethanolamine	7.9 ± 0.8	11.9 ± 0.3[C]	12.9 ± 0.8[B]	5.3 ± 0.7
Sphingomyelin	8.1 ± 2.7	3.3 ± 0.2	13.8 ± 1.4	3.5 ± 1.0
% of Phosphatidylcholine				
Disaturated phosphatidylcholine	55.4 ± 2.5	53.4 ± 2.0	53.1 ± 1.9	75.2 ± 1.1[B]

Alveolar type II cells were cultured for 7 days, the final 6 days in basal medium with or without KGF or RS and the final 24 hours with 10 μCi/ml ^{14}C-acetate. The results are expressed as percentages of total lipids, phospholipids, or phosphatidylcholine. The data for ^{14}C-acetate incorporation are expressed as the mean ± SE for four independent experiments. [A]Basal medium is 1% CS-FBS in DMEM. [B]$P < 0.01$ and [C]$P < 0.05$ vs. basal medium.

1,609–2,293 in the 3′-untranslated region of the gene. The vectors were linearized with *Bam*HI and transcribed using SP6 polymerase for antisense riboprobes. The vectors were linearized with *Eco*RI and transcribed using T7 polymerase for sense control riboprobes. Riboprobes were transcribed with [^{33}P] UTP as described previously (15). Hybridization with radiolabeled sense riboprobes was done as a control. Sections were hybridized and processed as described previously (13, 14).

Western immunoblotting. Cells were lysed using ice-cold buffer composed of 10 mM Tris-HCl (pH 8); 50 mM NaCl; 0.5% Na deoxycholate; 0.2% SDS (all from Sigma-Aldrich); 1% Nonidet P-40 (United States Biochemical Corp., Cleveland, Ohio, USA); 1× protease inhibitor cocktail (catalog no. 214262; Pharmingen, San Diego, California, USA) containing benzamidine-HCl, phenanthrolene, aprotinin, leupeptin, pepstatin A, and PMSF; 1× phosphatase inhibitor cocktail 2 (catalog no. P5726; Sigma-Aldrich) containing Na orthovanadate, Na molybdate, Na tartrate, and imidazole; and 25 μg/ml ALLN (*N*-acetyl-Leu-Leu-Nle-CHO; Calbiochem-Novabiochem Corp., San Diego, California, USA). Dishes containing cultures in the apical-access system were placed on ice, the medium was removed, and the cell/matrix layer was carefully rinsed with ice-cold PBS. Ice-cold lysis buffer (0.2–0.4 ml) was applied to the cell layer for 15 minutes on ice. The cell layer was extracted a second time with lysis buffer, and the extracts were pooled. DNA was sheared using a syringe and 25-gauge needle, and the insoluble material was removed by centrifugation at 14,000 *g* for 10 minutes. Dishes containing cultures in the Matrigel system were placed on ice, the medium was removed, and the cells were recovered after dissolving of the matrix with MatriSperse (Becton Dickinson Labware) at 4°C according to manufacturer's instructions. The final washed cell pellets were suspended in 0.5 ml of lysis buffer on ice for 30 minutes with vortexing. The DNA was sheared, and insoluble material was removed as above. One part 4× SDS-PAGE reducing Laemmli sample buffer was added to three parts lysate. The mixtures were boiled for 5 minutes and stored at –20°C until used. Aliquots of the lysates in reducing sample buffer were layered onto precast 8–16% Tris-glycine polyacrylamide slab gels, and the proteins were separated by electrophoresis in a Novex XCell MiniCell (Invitrogen Corp., Carlsbad, California, USA). The volumes of lysates were adjusted to load equal amounts of actin protein in each lane. Nonspecific binding sites on the nitrocellulose membranes were blocked by incubation of the blots in 5% nonfat dry milk in TTBS (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween-20, pH 7.5) at 4°C overnight. Primary antibodies to specific proteins (see *Materials* above) were diluted in 5% BSA in TTBS and incubated for 1 hour at room temperature with rocking. HRP-conjugated

Table 4
Percent distribution of acetate incorporation

	KGF + RS	KGF + RS + Dex
% of Total phospholipids		
Phosphatidylcholine	81.3 ± 1.2	78.40 ± 0.9[B]
Phosphatidylglycerol	2.6 ± 0.2	5.89 ± 0.4[A]
Phosphatidylinositol	3.2 ± 0.3	2.09 ± 0.2[B]
Phosphatidylethanolamine	6.8 ± 0.5	8.00 ± 0.5
Phosphatidylserine	1.4 ± 0.1	1.33 ± 0.1
Sphingomyelin	4.8 ± 0.5	4.29 ± 0.3
% of Phosphatidylcholine		
Disaturated phosphatidylcholine	70.7 ± 1.4	72.3 ± 0.9

Alveolar type II cells were cultured for 7 days, the final 6 days with or without 10^{-8} M Dex and the final 24 hours with 10 μCi/ml ^{14}C-acetate. The data are expressed as percentage of phospholipids or phosphatidylcholine. Values for the addition of Dex that differ from those with RS + KGF alone are indicated by [A]$P < 0.01$ and [B]$P < 0.05$. The data are the mean ± SE for five independent experiments.

Table 5
Phospholipid content and choline incorporation

Condition	Phospholipid content			Choline incorporation				
	μg DNA/well	nmol PLP/well	nmol PLP/μg DNA	10^4 cpm PC/well	10^4 cpm PC/μg DNA	10^4 cpm DSPC/well	10^4 cpm DSPC/μg DNA	DSPC as % PC
RS	4.7 ± 0.3	37.1 ± 1.4	7.95 ± 0.40	21.0 ± 6.0	4.23 ± 0.32	5.9 ± 1.4	1.21± 0.11	28.7± 1.6
RS + KGF	22.4 ± 1.7[A]	171.4 ± 7.0[A]	7.72 ± 0.45	126.7 ± 14.3[A]	5.83 ± 0.36	55.6 ± 9.3[A]	2.57 ± 0.23[A]	43.7± 1.7[A]
RS + KGF + Dex	15.6 ± 1.3[A]	115.0 ± 2.5[A]	7.41 ± 0.35	112.1 ± 13.1[A]	7.44 ± 0.41	53.6 ± 8.0[A]	3.57 ± 0.30[A]	47.8 ± 2.0[A]

Alveolar type II cells were cultured for 7 days, the final 6 days with or without KGF and the final 4 days with or without Dex. The data are the mean ± SE for seven independent experiments for measurement of PLP and five experiments for choline incorporation. [A]$P < 0.01$ vs. RS. PLP, phospholipid phosphorus; PC, phosphatidylcholine.

secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) were applied for 30 minutes at room temperature. Antigens were detected by enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and exposure to Hyperfilm (Amersham Pharmacia Biotech).

LXR agonist experiments. Type II cells were cultured on Matrigel as previously described except that 24-well culture plates (Becton Dickinson Labware) with 0.3 ml Matrigel per well were used and the cultures were maintained for a total of 4 days. After 24 hours of plating on day 1 in DMEM plus 5% RS, the cells were rinsed three times with DMEM without serum, and 1 ml of culture medium containing 1% CS-FBS with or without 20 ng/ml KGF was added. The medium was changed again on day 2, and various doses of the LXR agonist T0901317 were added along with appropriate DMSO controls. For analysis of lipid synthesis, 10 μCi of ^{14}C-acetate was added on day 3, 24 hours prior to harvest. Cells were collected and processed as described previously.

Data analyses. A repeated-measures ANOVA was used to determine whether the means of outcome variables were different among treatments. The primary objective was to determine whether there was an effect of KGF, and for these analyses Dunnett's multiple-comparison method was used. Values are presented as the means ± SE, and a two-tailed P value less than 0.05 is considered statistically significant for all comparisons.

Results

Acetate incorporation. In order to study the differentiated functions of alveolar type II cells, we developed a culture system that maintains the expression of the SPs. In this culture system, type II cells secrete phosphatidylcholine into the apical fluid and have phospholipid inclusions that can be visualized by phosphine 3R or electron microscopy (6). To assess lipogenesis, type II cells were incubated with ^{14}C-acetate for the final 24 hours, which is from day 6 to day 7 of culture. KGF greatly stimulated acetate incorporation into phospholipids, whether in the presence of 1% CS-FBS or 5% RS (Table 2). In addition, as shown in Table 3, KGF markedly changed the lipids synthesized from acetate. There was a large increase in the incorporation into phospholipids and a corresponding proportional decrease in incorporation into neutral lipids. In 5% RS, KGF also increased the percent incorporation into phosphatidylglycerol and DSPC, two phospholipids found in relatively high concentrations in surfactant (Table 3). However, since the

Table 6
Microarray analysis

Gene	Accession number	Fold increase	
		KGF Basal medium	KGF + RS Rat serum
Differentiation markers			
SP-A	M33201	400.6 ± 66.8	18.0 ± 1.7
SP-B	AI170350	127.1 ± 16.1	11.8 ± 0.7
SP-C	X14221	41.6 ± 1.3	6.3 ± 0.5
SP-D	M81231	5.4 ± 0.5	2.5 ± 0.2
Alkaline phosphatase	X16038	24.7 ± 6.9	5.9 ± 0.3
Lipogenic enzymes			
Acetyl-CoA carboxylase	AB004329	ND	ND
Fatty acid synthase	M76767	5.5 ± 0.8	5.0 ± 1.6
Malic enzyme	M26594	1.5 ± 0.2	0.7 ± 0.1
ATP citrate lyase	J05210	2.5 ± 0.4	2.4 ± 0.4
SCD-1	J02585	101.9 ± 24.5	42.5 ± 16.5
SCD-2	AF036761	66.6 ± 24.8	26.0 ± 13.0
E-FABP	S69874	6.5 ± 1.2	7.7 ± 0.7
Acyl-CoA synthase	D85189	4.5 ± 0.7	3.6 ± 0.1
HMG-CoA reductase	X55286	2.1 ± 0.1	3.2 ± 0.6
Glycerol-3P acyltransferase	U36772	I	0.8 ± 0.8
Choline kinase	D37884	I	I
Phosphatidylinositol synthase	D82928	0.8 ± 0.1	0.5 ± 0.1
CDP-diacylglycerol synthase	AB009999	1.6 ± 0.1	1.9 ± 0.2
Transcription factors			
C/EBPα	X12752	I	I
C/EBPδ	AI045030	I	21.6 ± 9.2
PPARγ	AB011365	0.9 ± 0.1	0.2 ± 0.2
SREBP-1c (ADD-1)	L16995	I	I
TTF-1 (Nkx2-1)	X53858	1.4 ± 0.2	1.4 ± 0.2

The data are expressed as fold change (mean ± SEM, $n = 3$). For values whose basal level is low, the fold increase cannot be calculated reliably and is designated I for indeterminate. Some values were below the detection limits and are designated ND for not detected.

Table 7
Real-time PCR

Gene	Fold increase KGF Basal medium	KGF + RS Rat serum
Transcription factors		
C/EBPα	12.50 ± 1.93[A]	13.38 ± 3.54[B]
C/EBPβ	1.05 ± 0.24	1.18 ± 034
C/EBPδ	5.20 ± 0.60[A]	4.59 ± 0.74[A]
SREBP-1 (ADD-1)	6.54 ± 3.92	2.98 ± 1.00
SREBP-2	1.25 ± 0.22	1.67 ± 0.25[B]
Fatty acid synthesis and transport		
Acetyl-CoA carboxylase	4.35 ± 1.15[B]	1.98 ± 0.39
Fatty acid synthase	7.38 ± 2.66	6.62 ± 1.48[B]
SCD-1	147 ± 39[B]	162 ± 52[B]
SCD-2	9.38 ± 1.78[A]	6.22 ± 1.06[A]
E-FABP	15.04 ± 2.85[A]	44.97 ± 28.95
Glycerolipid synthesis		
Glycerol-3P acyltransferase	3.60 ± 0.37[A]	3.48 ± 0.54[A]
Diacylglycerol acyltransferase	0.74 ± 0.14	0.60 ± 0.10[B]
CTP:phosphocholine cytidylyltransferase	0.66 ± 0.10[B]	0.94 ± 0.36
CDP-diacylglycerol synthase	1.78 ± 0.28[B]	2.07 ± 0.58
Phosphatidylinositol synthase	0.78 ± 0.08	0.62 ± 0.10[B]
Phosphatidylglycerol phosphate synthase	2.04 ± 0.18[A]	2.91 ± 0.49[B]

Type II cells were cultured in the apical-access system, and mRNA was quantified by real-time PCR with the primers and probes listed in Table 1. For these studies, all expression is normalized to GAPDH as stated in Methods. The significant ratios are designated $^{A}P < 0.01$ and $^{B}P < 0.05$. The values are the mean ± SE for six independent experiments.

percentage incorporation into phosphatidylglycerol was modest under these conditions and significantly less than found in surfactant, additional studies were done to define conditions to increase the relative incorporation into phosphatidylglycerol. Addition of Dex further increased the percentage incorporation into phosphatidylglycerol (Table 4). Dex is also required for stimulated secretion of phospholipids and a more normal appearance of lamellar bodies, as visualized with electron microscopy (6). Hence, KGF greatly stimulated acetate incorporation into phospholipids under all conditions, but the maximal percentage increase of incorporation into DSPC and phosphatidylglycerol required the combination of KGF, Dex, and RS.

Studies of acetate incorporation primarily reflect de novo fatty acid synthesis and the subsequent conversion of newly synthesized fatty acids, especially palmitate, into phospholipids. To address phosphatidylcholine synthesis more directly, we performed additional studies with radiolabeled choline. Compared with RS alone, KGF plus RS, and KGF plus Dex plus RS significantly increased the incorporation of choline into phosphatidylcholine per well but not per microgram DNA (Table 5). There was, however, increased incorporation into DSPC per well and per microgram DNA in the presence of KGF plus Dex. KGF and KGF plus Dex also greatly increased the total mass of phospholipid per well but not the amount of phospholipid per microgram DNA (Table 5).

Microarray gene profiling. To determine the alterations in mRNA levels of lipogenic enzymes and transcription factors, gene expression profiling was done with Affymetrix oligonucleotide microarrays. Type II cells were plated in the apical-access system on the mixture of Matrigel and rat tail collagen (6). On day 1 of culture, the cells were washed and the medium was changed with the addition of 1% CS-FBS, 1% CS-FBS plus KGF, 1% CS-FBS plus 5% RS, 1% CS-FBS plus KGF plus RS, or 1% CS-FBS plus KGF plus RS plus 10^{-8} M Dex (last 4 days). The medium was changed every 2 days, and the RNA was isolated on day 7 of culture. In these cultures, KGF greatly stimulated the mRNA levels for selected differentiation markers and lipogenic enzymes in the presence of 1% CS-FBS or 5% RS, as shown in Table 6. There was a marked elevation of the mRNA levels for the SPs and alkaline phosphatase. A variety of lipogenic enzymes and transport proteins were also identified. In both 1% CS-FBS and 5% RS, KGF increased mRNA levels of fatty acid synthase, SCD-1 and SCD-2, E-FABP, and acyl-CoA synthase. However, for genes such as transcription factors that are expressed at low abundance, it was difficult to calculate a fold increase, because the value was very low in basal media. Hence, relative fold increases for some genes could not be calculated with the GeneSpring software and are listed as indeterminate (Table 5). However, in the data analyzed with the Affymetrix software, it was possible to observe reproducible increases in the mRNA levels of C/EBPα and SREBP-1c (ADD-1, or adipocyte determination differentiation–dependent factor-1) (data not shown), whereas there were no apparent increases for TTF-1 (Nkx2-1), HNF-3, or PPARγ. The addition of Dex for the last 4 days of culture produced no marked changes in mRNA levels compared with KGF plus RS.

Real-time PCR verification. Our next goal was to confirm these observations by an independent method and to evaluate the mRNA levels of transcription factors and other lipogenic enzymes that were expressed at low levels or not represented on the microarrays. We therefore designed primers and probes (Table 1) for a variety of relevant genes (Table 7). These primers and probes were designed from known rat genes or, in the case of phosphatidylglycerol phosphate synthase, from a rat expressed-sequence tag that was nearly identical to the hamster gene for phosphatidylglycerol phosphate synthase (accession no. AB016930). Real-time PCR verified the results observed with the oligonucleotide microarrays, as shown in Table 7. In addition, the mRNA for the C/EBP isoforms could be measured. There was an increase in C/EBPα and C/EBPδ, but not C/EBPβ. In terms of fatty acid synthesis and transport, there was a modest increase of mRNA for acetyl-CoA carboxylase in the absence of RS, an increase in fatty acid synthase, a marked increase in SCD-1, a smaller increase in SCD-2, and a significant increase in E-FABP in the absence of RS.

By this approach, more of the enzymes involved in glycerolipid synthesis could also be evaluated. There was an increase in glycerol-3P acyltransferase and a modest increase in phosphatidylglycerol phosphate synthase. However, there was no increase in CTP:phosphocholine cytidyltransferase. KGF decreased the mRNA values for diacylglycerol acyltransferase and phosphatidylinositol synthase in the presence of 5% RS.

RPA for SREBP-1a, -1c, and -2. From the real-time PCR observations, the increase in mRNA for SREBP-1 was not statistically significant. In addition, the primers and probes that we used do not differentiate SREBP-1a from SREBP-1c. To determine whether SREBP-1a or SREBP-1c was altered by KGF, we used an RPA. SREBP-1a and SREBP-1c are nearly identical but can be distinguished by this RPA (16, 17). As shown in Figure 1, KGF increases the expression of SREBP-1c mRNA but not SREBP-2 or SREBP-1a mRNA. The increase was statistically significant only in the condition of KGF plus Dex. However, both SREBP-2 and SREBP-1a are expressed in type II cells.

Confirmation and localization of mRNA expression by in situ hybridization. To confirm these observations and to extend them to the intact lung, we evaluated the expression of C/EBPα, C/EBPβ, C/EBPδ, SREBP-1, SREBP-2, SCD-1, SCD-2, and fatty acid synthase by in situ hybridization in our cultures, in normal rat lungs, and in lungs from rats that had been instilled with an adenovirus that expressed KGF (Ad-KGF) to produce marked type II cell hyperplasia (9). These studies were designed to confirm the increase in C/EBP isoforms, SREBP-1 and -2, and SCD-1 and -2 in the cultured cells and to localize their expression in the intact lung. Only a portion of these data is shown in Figure 2. There was an obvious increase in C/EBPα in type II cells cultured with KGF (Figure 2, a–d), which confirms the real-time PCR data. C/EBPα was also expressed in hyperplastic type II cells produced by Ad-KGF (data not shown). There were smaller increases for C/EBPδ and C/EBPβ in our cultures. C/EBPβ was expressed at high levels in macrophages in vivo, and C/EBPδ showed variable expression in the lung, as described previously (data not shown) (18). SREBP-1 was not detected by in situ in our cultures or in the intact lung, presumably because of the low level of expression. There was a modest increase in expression of SREBP-2 with KGF in culture (data not shown). SREBP-2 was expressed in many lung cells at low levels and was not restricted to type II cells. There were increases in expression of both SCD-1 and SCD-2 in the cultured type II cells with KGF (Figure 2, e–l). SCD-2 appeared to be more highly expressed than SCD-1. In vivo SCD-1 was expressed in alveolar cells and had the same pattern of distribution as SP-C in both rat and murine lungs, and hence the SCD-1–expressing cells were considered to be type II cells (Figure 2, m and n). However, SCD-2 was expressed more widely and did not appear to be restricted to type II cells (data not shown). The results from in situ hybridization confirmed the importance in C/EBPα in the response to KGF and demonstrated the expression of both SCD-1 and SCD-2 in type II cells.

Confirmation of protein expression by immunoblotting. To demonstrate the effect of KGF on protein expression, we used both the apical-access and the Matrigel culture systems. Most of the regulation of SREBP-1 and SREBP-2 is by proteolytic processing and should be more sensitive than alterations in mRNA levels (19). As shown in Figure 3, KGF increased C/EBPα, C/EBPδ, SREBP-1, fatty acid synthase, E-FABP, and SP-D in the apical-access cultures. KGF also increased the mature 68-kDa form of SREBP-1 as well as the precursor form.

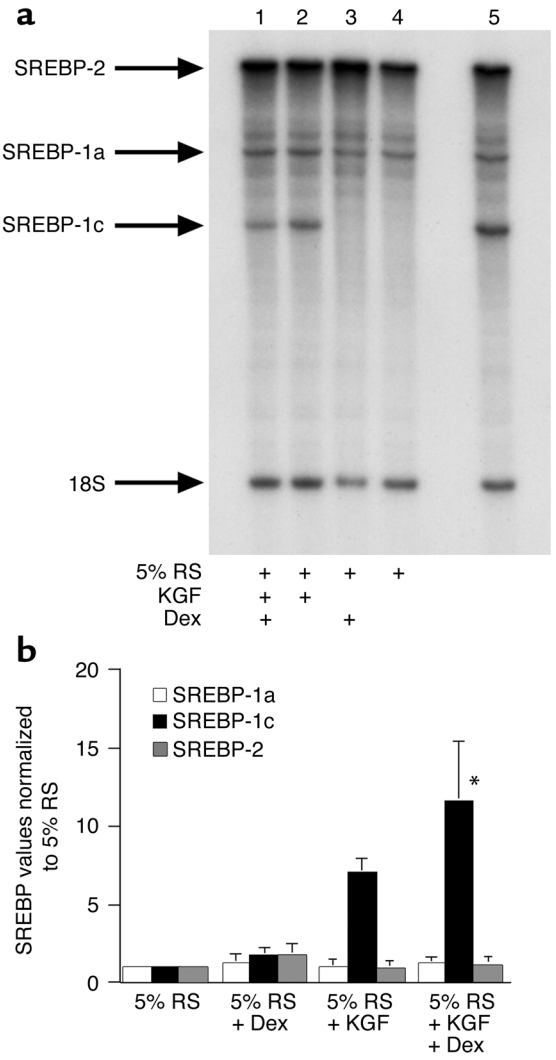

Figure 1
SREBP-1c mRNA level is increased in the differentiated cultures. Type II cells were cultured in 5% RS with and without 10 ng/ml KGF and 10^{-8} M Dex in the apical-access system. The mRNA levels were determined by an RPA as described in Methods. The values were normalized to 18S RNA and then compared with the value for 5% RS, which was given a level of 1. (**a**) A representative RPA. Lanes 1–4 show samples from type II cells cultured in the apical-access system and harvested on day 7. Lane 5 shows a sample from freshly isolated type II cells. (**b**) The summary of three independent experiments normalized to 5% RS. *$P < 0.05$ after adjustment for repeated-measures ANOVA.

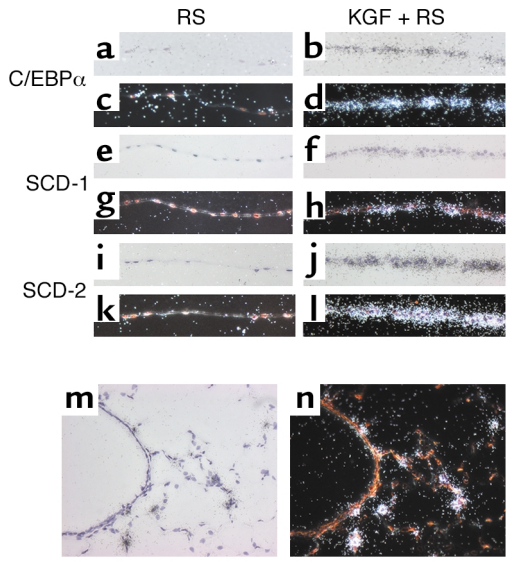

Figure 2
C/EBPα, SCD-1, and SCD-2 mRNA are increased in type II cells in response to KGF. (**a**–**l**) Type II cells were cultured in 5% RS with or without KGF for 6 days in the apical-access system. The monolayers were fixed with 4% paraformaldehyde, processed for in situ hybridization with sense and antisense probes, and stained with hematoxylin. In **a** (bright field) and **c** (dark field), the results for RS alone are shown. The expression is very low but higher than with the sense probe (data not shown). In **b** (bright field) and **d** (dark field), there is increased expression of C/EBPα in the presence of RS plus KGF. Similarly, KGF stimulates the expression of SCD-1 (**e**–**h**) and SCD-2 (**i**–**l**). (**m** and **n**) The expression of SCD-1 is shown in the normal lung in bright field (**m**) and dark field (**n**). Specific cells in the alveolar region are heavily labeled, and the distribution of these cells is the same as that of SP-C, a marker restricted to type II cells. The micrographs were taken at ×400.

This confirms the mRNA expression and indicates that SREBP-1 is more important than SREBP-2 in the response to KGF. One problem with the apical-access system is that it is difficult to get the cells off the matrix, and our extracts contained some rat serum proteins that crossreacted with our antibodies, especially SCD. If we used collagenase to dissolve the gels, we degraded some of the SP-A and SP-D. To circumvent these problems, we used the Matrigel system and dissolved the gel with MatriSperse. With cells grown on Matrigel, we were also able to demonstrate that KGF increased protein levels of C/EBPα, C/EBPδ, SREBP-1, fatty acid synthase, and E-FABP, but not SREBP-2. The data are not shown, because the results are identical to those in Figure 3. In addition, we were able to demonstrate an increase in SCD with KGF by immunoblotting in the Matrigel system (data not shown).

PPARγ. In the liver and adipocytes, PPARγ is an important transcription factor for many lipogenic enzymes (20, 21). However, in the gene profiling studies on microarrays, there was no increase in the mRNA for PPARγ. In addition, in six independent experiments, there was no increase in PPARγ by real-time PCR with KGF (data not shown). We also sought to determine the amount of PPARγ protein present and whether there was any increase in protein expression in our differentiated cultures by immunoblotting. PPARγ was present in macrophages and in liver, but relatively little was detected in lung or in type II cells. We then sought to determine whether lipogenesis, as measured by acetate incorporation, was increased by the PPARγ agonist 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2). In three independent experiments, there was no increase in acetate incorporation with 10 and 40 nM 15d-PGJ_2 in type II cells treated for 48 or 96 hours before harvest on day 7 of culture, when the cells were cultured in RS. The PPARγ agonist could not substitute for KGF in our system.

Stimulation of fatty acid synthesis by the LXR agonist T0901317. To determine whether direct stimulation of SREBP could reproduce the effects of KGF, the LXR agonist T0901317 was evaluated in the presence and absence of KGF. For these studies, the type II cells were plated on Matrigel in the presence of 5% RS, washed, and then cultured for 3 days with 1% CS-FBS with and without KGF (Figure 4). T0901317 was added for the last 48 hours of culture. At several doses, the LXR agonist increased acetate incorporation in the absence of KGF, but it increased acetate incorporation in the presence of KGF only at the highest dose tested. Concomitantly, there was an increase in SREBP-1 protein but not C/EBPα or SREBP-2. There was also an increase in fatty acid synthase by immunoblotting. Hence, direct stimulation of SREBP by the LXR agonist T0901317 stimulated fatty acid synthesis in these cultures.

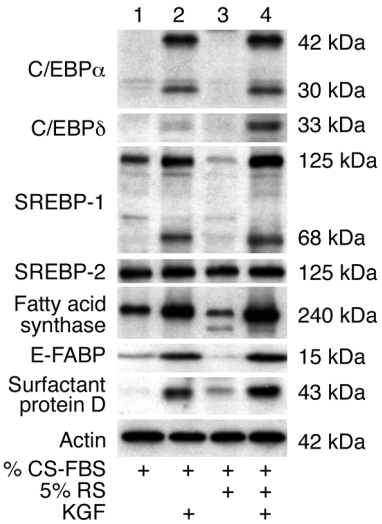

Figure 3
C/EBPα, C/EBPδ, and SREBP-1 protein levels are increased in the differentiated cultures. Type II cells were cultured in 1% CS-FBS alone (lane 1), plus KGF (lane 2), plus 5% RS (lane 3), or plus 5% RS and KGF (lane 4) for 6 days in the apical-access system under air/liquid conditions. The cells were extracted and the protein expression measured by Western analysis as described in Methods. Because matrix proteins within the gel precluded a reliable measurement of cellular protein, loading of individual lanes was based on the amount of actin. A representative example of at least three determinations is shown.

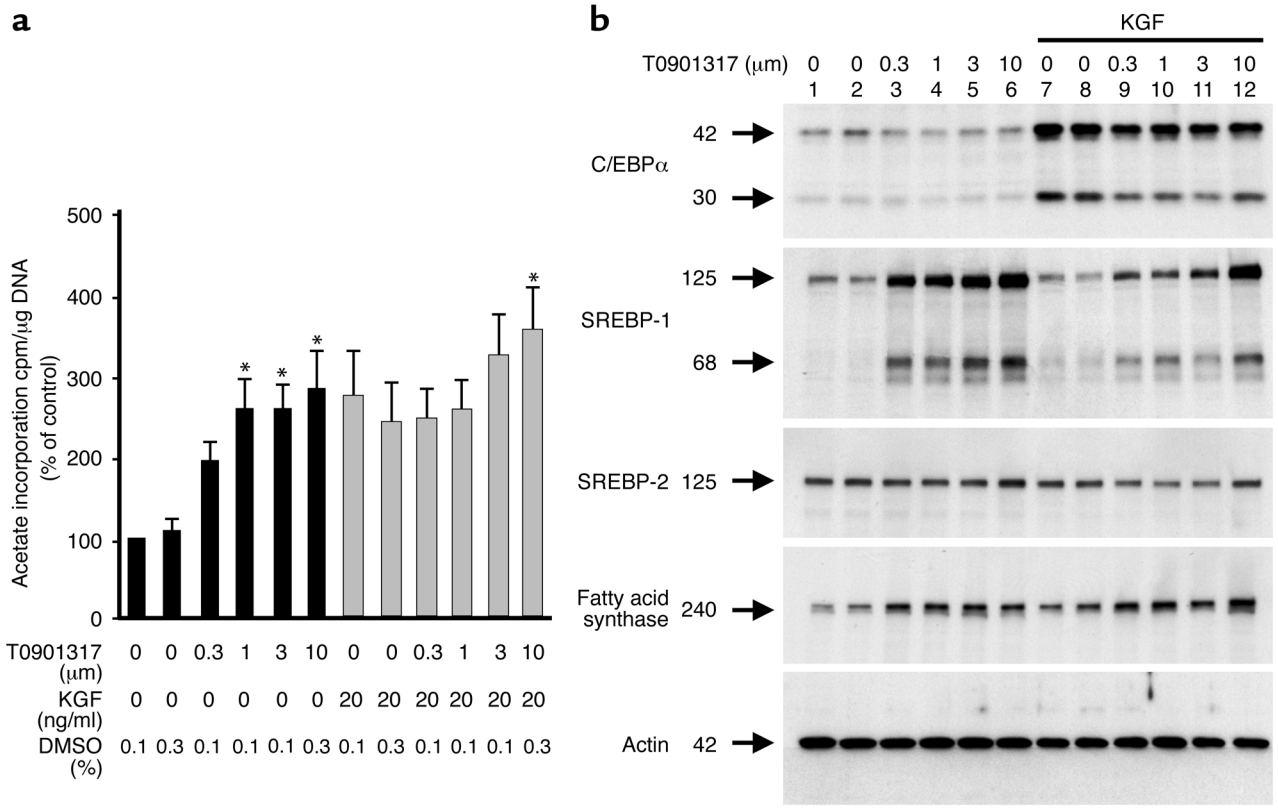

Figure 4
Fatty acid synthesis is stimulated by the LXR agonist T0901317. Type II cells were plated on Matrigel in 5% RS and then cultured in 1% CS-FBS with or without KGF and with or without varying doses of T0901317 and appropriate vehicle controls. (**a**) T0901317 increased acetate incorporation in a dose-dependent manner in 1% CS-FBS and at the highest dose in the presence of KGF. The results are normalized to the value of 1% CS-FBS and are means ± SEM for five independent experiments. *$P < 0.05$ vs. the appropriate DMSO control without the LXR agonist. (**b**) Protein expression is shown by immunoblotting. The conditions are the same as in **a**. Lanes 1, 2, 7, and 8 contain DMSO without T0901317 as shown in **a**. Protein loading is normalized to the amount of actin. A representative sample of five independent experiments is shown.

Discussion

Although the pathways for synthesis of the phospholipids of surfactant have been defined by metabolic labeling experiments, relatively little is known about the regulation of these pathways. By analysis of type II cells under different states of differentiation, it is possible to define the relative contribution of specific enzymes and their regulation. In our culture systems, KGF greatly stimulates fatty acid synthesis and differentiation of rat type II cells.

Our studies indicate the importance of C/EBPα, C/EBPδ, and SREBP-1c as transcription factors and of fatty acid synthase, SCD, E-FABP, and glycerol-3P acyltransferase as components of the lipogenic response. In addition, the LXR agonist T0901317 increased the expression of SREBP-1 and, thereby, fatty acid synthase and acetate incorporation. To our knowledge, these are the first studies to indicate that SREBP-1c, SCD-1, and SCD-2 are part of the lipogenic response in type II cells. However, it should be recognized that we have identified only a few of the genes involved in the lipogenic response. Recent studies on lipogenesis differentiation in adipocytes identified over 1,200 genes activated during differentiation (22); in our studies, about 250 genes were increased more than twofold by KGF in the presence of 1% CS-FBS or 5% RS, and about an equal number were decreased. The ultimate regulation of lipogenesis for the production of pulmonary surfactant is likely to be very complex. Nevertheless, the results with KGF and the LXR agonist suggest that pharmacologic regulation of the phospholipid component of the surfactant system is possible.

Regulation of lipogenesis in type II cells is similar to that of lipogenesis in adipocytes, but the transcription factors involved appear to be different. Preadipocytes can be stimulated to differentiate with Dex, cAMP, and IBMX (a phosphodiesterase inhibitor) over 6 days (23). In adipocytes, at least three families of transcription factors are important for fatty acid synthesis: C/EBP isoforms, SREBP-1c, and PPARγ. In our system, 6 days are also required to stimulate lipogenesis. One major difference is that type II cells convert fatty acids into phospholipids for secretion and the adipocytes convert fatty acids into triglyceride for intracellular storage. Our studies indicate that C/EBPα, C/EBPδ, and SREBP-1c are important, whereas PPARγ and C/EBPβ appear not to be involved.

C/EBPα appears to be especially important in the lipogenic response in type II cells. The mRNA expression

and protein expression of C/EBPα are increased in the differentiated type II cell cultures. C/EBPα is also important for the expression of SP-A (24) and SP-D (25). Gene-targeted mice with a deletion of C/EBPα die shortly after birth and have diffuse type II cell hyperplasia (26, 27). The neonatal death of these mice has been attributed to hypoglycemia, and the type II cell hyperplasia to the growth-inhibiting properties of C/EBPα. However, a more profound pulmonary insufficiency may be present. C/EBPα is also known to be critical for the stimulation of lipogenesis in adipocytes and is the one transcription factor that is both necessary and sufficient (20, 23). In contrast, there was relatively little change in C/EBPβ at the mRNA or the protein level in type II cells, whereas C/EBPβ increases early in the lipogenic response in adipocytes (21). In the adult lung, C/EBPβ is not restricted to the epithelium but is highly expressed in macrophages. C/EBPδ is also likely to be important in lipogenesis and increases by real-time PCR, by in situ hybridization, and by immunoblotting. However, it is expressed at a lower level than C/EBPα.

SREBP-1c also appears to be important in type II cells. SREBP-1a, SREBP-1c, and SREBP-2 are all expressed in type II cells, and these transcription factors are known to regulate fatty acid and cholesterol metabolism in fat cells and liver (12, 28, 29). In general, SREBP-1c regulates fatty acid synthesis and SREBP-2 regulates cholesterol synthesis (19). KGF increased SREBP-1c mRNA levels, whereas there was little change in SREBP-1a and SREBP-2. The LXR agonist T0901317 increased SREBP-1 protein expression and acetate incorporation to levels similar to those observed with KGF. Nevertheless, it is unlikely that SREBP-1c is absolutely required for lipogenesis in the lung, since gene targeting of SREBP-1 results in marked alterations in lipid metabolism in the liver but no pulmonary phenotype has been reported (30). However, detailed studies of surfactant metabolism in these gene-targeted mice have not been reported. In addition, SREBP-2 appears to be expressed in type II cells at a higher level than SREBP-1 and hence could compensate for a deletion of SREBP-1a, SREBP-1c, or both.

In adipocytes, PPARγ is an important regulator of lipogenesis, and its activity can be increased by the PPAR agonist 15d-PGJ_2 (31, 32). However, in whole lung we detected relatively little PPARγ by immunoblotting, although it was readily detected in alveolar macrophages and liver. No change in PPARγ mRNA was detected in the gene profiling studies or with real-time PCR, and little protein was detected in isolated type II cells. In addition, there was no significant stimulation of acetate incorporation in type II cells in response to the PPARγ agonist 15d-PGJ_2. Previously, Michael et al. reported the expression of PPARγ-1 in human and rabbit type II cells, but there was no increase in the protein level with differentiation, whereas there was an increase in the mRNA level (33).

Based on our culture studies, C/EBPα, C/EBPδ, and SREBP-1c appear to be important in the regulation of fatty acid synthesis in type II cells, whereas C/EBPβ and PPARγ do not appear to be physiologically important. In addition, the regulation of fatty acid synthesis in type II cells is different from the regulation of fatty acid synthesis in adipocytes. Future studies will be required to define the relative importance of SREBP-1c and C/EBPα in the regulation of fatty acid synthesis in type II cells.

One surprising observation in the microarray studies was the marked increase in expression of SCD in cells treated with KGF. SCD is a lipogenic enzyme that heretofore was not thought to be important in surfactant synthesis. SCD converts stearate into oleate and palmitate into palmitoleate (34, 35). Although it has been known for several decades that the phosphatidylcholine in pulmonary surfactant has a relatively high concentration of palmitoleate compared with phosphatidylcholine from other sources, this observation has not received much consideration (36). There are two SCDs in rodents, SCD-1 and SCD-2. Both enzymes are expressed in the lungs (37, 38). By in situ hybridization in murine lung, SCD-1 appears to be specifically and highly expressed in type II cells, whereas SCD-2 is expressed in many cell types. SCD is likely to be important in lipogenesis in type II cells, based on the increase in mRNA during differentiation, the importance of the deacylation-reacylation pathway for phosphatidylcholine synthesis, and the relative abundance of palmitoleate in pulmonary surfactant. SCD is also a key enzyme that is highly regulated during lipogenesis in adipocytes (35).

KGF did not markedly alter the mRNA levels of enzymes related to phospholipid synthesis, compared with changes in enzymes involved in fatty acid synthesis. By real-time PCR, there was an increase in glycerol-3P acyltransferase, which is the gatekeeper of glycerolipid synthesis. However, there was no increase in phosphocholine cytidylyltransferase, which is thought to be the rate-limiting enzyme in phosphatidylcholine synthesis (1). But this is not unexpected, since it is regulated primarily posttranslationally (39, 40). In terms of phosphatidylglycerol synthesis, there was a modest increase in CDP-diacylglycerol synthase, an increase in phosphatidylglycerol phosphate synthase, and a slight decrease in phosphatidylinositol synthase. These changes would favor the synthesis of phosphatidylglycerol. There are also several important enzymes in phospholipid synthesis, such as lysophosphatidylcholine acyltransferase whose genes have not been isolated and cannot be evaluated at the present time. We also recognize that changes in enzymatic activity were not measured, and that post-transcriptional activation of certain enzymes is important and would be missed by these analyses.

Our studies used two different culture systems, one with Matrigel on which the type II cells form spherules and the other on Millicells where cells form a monolayer (6, 7). The effect of KGF was documented in both systems. In the Matrigel system, the cells do not have to proliferate to become differentiated. The apical-access system is more complex, because differentiation requires proliferation and takes several days longer. In the current experiments we used both systems for acetate incorporation and immunoblotting to verify the basic observations.

An important issue that will require additional studies is the linkage between KGF signaling and lipogenesis. It is highly likely that C/EBPα, C/EBPδ, and SREBP-1c are involved in fatty acid synthesis in type II cells. However, the signaling pathways between the KGF receptor and activation of these transcription factors are not known. The signaling pathways stimulated by KGF are poorly understood, in general and in type II cells in particular. In rat type II cells, KGF stimulates extracellular signal–regulated kinase (ERK; p42, p44 MAPK) and the PI3K and Akt/protein kinase B pathways (41). However, the precise linkage between these pathways and the regulation of lipogenesis is not known.

In summary, KGF stimulates lipogenesis in alveolar type II cells, and this involves increased expression of several enzymes involved in fatty acid biosynthesis. The signaling pathways whereby KGF stimulates lipogenesis likely involve C/EBPα, C/EBPδ, and SREBP-1c. Three of the main proteins that are increased in the lipogenic response in type II cells are fatty acid synthase, SCD, and E-FABP.

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1. Batenburg, J.J. 1992. Surfactant phospholipids: synthesis and storage. *Am. J. Physiol.* **262**:L367–L385.
2. Rooney, S.A., Young, S.L., and Mendelson, C.R. 1994. Molecular and cellular processing of lung surfactant. *FASEB J.* **8**:957–967.
3. van Golde, L.M.G., Batenburg, J.J., and Robertson, B. 1988. The pulmonary surfactant system: biochemical aspects and functional significance. *Physiol. Rev.* **68**:374–455.
4. Mason, R.J., and Dobbs, L.G. 1980. Synthesis of phosphatidylcholine and phosphatidylglycerol by alveolar type II cells in primary culture. *J. Biol. Chem.* **255**:5101–5107.
5. Mason, R.J., and Nellenbogen, J. 1984. Synthesis of saturated phosphatidylcholine and phosphatidylglycerol by freshly isolated rat alveolar type II cells. *Biochim. Biophys. Acta.* **794**:392–402.
6. Mason, R.J., et al. 2002. Maintenance of surfactant protein A and D secretion by rat alveolar type II cells in vitro. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**:L249–L258.
7. Shannon, J.M., Pan, T., Nielsen, L.D., Edeen, K.E., and Mason, R.J. 2001. Lung fibroblasts improve differentiation of rat type II cells in primary culture. *Am. J. Respir. Cell Mol. Biol.* **24**:235–244.
8. Chelly, N., et al. 1999. Keratinocyte growth factor enhances maturation of fetal rat lung type II cells. *Am. J. Respir. Cell Mol. Biol.* **20**:423–432.
9. Morikawa, O., et al. 2000. Effect of adenovector-mediated gene transfer of keratinocyte growth factor on the proliferation of alveolar type II cells in vitro and in vivo. *Am. J. Respir. Cell Mol. Biol.* **23**:626–635.
10. Dobbs, L.G., and Mason, R.J. 1979. Pulmonary alveolar type II cells isolated from rats. Release of phosphatidylcholine in response to beta-adrenergic stimulation. *J. Clin. Invest.* **63**:378–387.
11. Labarca, C., and Paigen, K. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**:344–352.
12. Shimomura, I., Shimano, H., Horton, J.D., Goldstein, J.L., and Brown, M.S. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J. Clin. Invest.* **99**:838–845.
13. Pan, T., et al. 2002. Serum SP-D is a marker of lung injury in rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**:L824–L832.
14. Deterding, R.R., and Shannon, J.M. 1995. Proliferation and differentiation of fetal rat pulmonary epithelium in the absence of mesenchyme. *J. Clin. Invest.* **95**:2963–2972.
15. Fehrenbach, H., Fehrenbach, A., Pan, T., Kasper, M., and Mason, R.J. 2002. Keratinocyte growth factor-induced proliferation of rat airway epithelium is restricted to Clara cells in vivo. *Eur. Respir. J.* **20**:1185–1197.
16. DeBose-Boyd, R.A., Ou, J., Goldstein, J.L., and Brown, M.S. 2001. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proc. Natl. Acad. Sci. U. S. A.* **98**:1477–1482.
17. Shimomura, I., et al. 1999. Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc. Natl. Acad. Sci. U. S. A.* **96**:13656–13661.
18. Sugahara, K., Sadohara, T., Sugita, M., Iyama, K., and Takiguchi, M. 1999. Differential expression of CCAAT enhancer binding protein family in rat alveolar epithelial cell proliferation and in acute lung injury. *Cell Tissue Res.* **297**:261–270.
19. Horton, J.D., Goldstein, J.L., and Brown, M.S. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109**:1125–1131. doi:10.1172/JCI200215593.
20. Gregoire, F.M., Smas, C.M., and Sul, H.S. 1998. Understanding adipocyte differentiation. *Physiol. Rev.* **78**:783–809.
21. Morrison, R.F., and Farmer, S.R. 1999. Insights into the transcriptional control of adipocyte differentiation. *J. Cell. Biochem.* **75**(Suppl. 32–33):59–67.
22. Soukas, A., Socci, N.D., Saatkamp, B.D., Novelli, S., and Friedman, J.M. 2001. Distinct transcriptional profiles of adipogenesis in vivo and in vitro. *J. Biol. Chem.* **276**:34167–34174.
23. Mandrup, S., and Lane, M.D. 1997. Regulating adipogenesis. *J. Biol. Chem.* **272**:5367–5370.
24. Li, F., et al. 1995. Correlation of expression of transcription factor C/EBPα and surfactant protein genes in lung cells. *Am. J. Physiol.* **269**:L241–L247.
25. He, Y., and Crouch, E. 2002. Surfactant protein D gene regulation. Interactions among the conserved CCAAT/enhancer-binding protein elements. *J. Biol. Chem.* **277**:19530–19537.
26. Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L., and Xanthopoulos, K.G. 1996. Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. *J. Biol. Chem.* **271**:24753–24760.
27. Sugahara, K., et al. 2001. Mice lacking CCAAt/enhancer-binding protein-alpha show hyperproliferation of alveolar type II cells and increased surfactant protein mRNAs. *Cell Tissue Res.* **306**:57–63.
28. Kersten, S. 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep.* **2**:282–286.
29. Edwards, P.A., Tabor, D., Kast, H.R., and Venkateswaran, A. 2000. Regulation of gene expression by SREBP and SCAP. *Biochim. Biophys. Acta.* **1529**:103–113.
30. Shimano, H., et al. 1997. Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. *J. Clin. Invest.* **100**:2115–2124.
31. Michalik, L., and Wahli, W. 1999. Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions. *Curr. Opin. Biotechnol.* **10**:564–570.
32. Latruffe, N., and Vamecq, J. 1997. Peroxisome proliferators and peroxisome proliferator activated receptors (PPARs) as regulators of lipid metabolism. *Biochimie.* **79**:81–94.
33. Michael, L.F., Lazar, M.A., and Mendelson, C.R. 1997. Peroxisome proliferator-activated receptor gamma1 expression is induced during cyclic adenosine monophosphate-stimulated differentiation of alveolar type II pneumonocytes. *Endocrinology.* **138**:3695–3703.
34. Ntambi, J.M. 1999. Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J. Lipid Res.* **40**:1549–1558.
35. Kim, Y.C., and Ntambi, J.M. 1999. Regulation of stearoyl-CoA desaturase genes: role in cellular metabolism and preadipocyte differentiation. *Biochem. Biophys. Res. Commun.* **266**:1–4.
36. Clements, J.A., Nellenbogen, J., and Traham, H.J. 1970. Pulmonary surfactant and evolution of the lungs. *Science.* **169**:603–604.
37. Kaestner, K.H., Ntambi, J.M., Kelly, T.J., Jr., and Lane, M.D. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes. A second differentially expressed gene encoding stearoyl-CoA desaturase. *J. Biol. Chem.* **264**:14755–14761.
38. Baba, H., Fuss, B., Watson, J.B., Zane, L.T., and Macklin, W.B. 1994. Identification of novel mRNAs expressed in oligodendrocytes. *Neurochem. Res.* **19**:1091–1099.
39. Lykidis, A., and Jackowski, S. 2001. Regulation of mammalian cell membrane biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **65**:361–393.
40. Kent, C. 1997. CTP:phosphocholine cytidylyltransferase. *Biochim. Biophys. Acta.* **1348**:79–90.
41. Portnoy, J., and Mason, R.J. 2002. KGF utilizes both AKT/PKB and ERK signaling pathways in alveolar type II epithelial cells in vitro. *Am. J. Respir. Crit. Care Med.* **165**:A429. (Abstr.)