KGF induces lipogenic genes through a PI3K and JNK/SREBP-1 pathway in H292 cells

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Abstract Lipid synthesis is required for cell growth and is subject to pharmacologic regulation. Keratinocyte growth factor (KGF) stimulates proliferation and lipogenesis in H292 cells, a pulmonary epithelial cancer cell line, but the signaling pathways are not known. KGF stimulated the expression of the transcription factors sterol-regulatory element binding protein-1 (SREBP-1), CCAAT/enhancer binding protein α (C/EBPα), and C/EBPδ and two key enzymes involved in lipogenesis, FAS and stearoyl coenzyme A desaturase-1 (SCD-1). We found that KGF induced rapid activation of Akt, p70 S6K, JNK, and extracellular signal-regulated (ERK). Induction of SREBP-1, SCD-1, and FAS by KGF was inhibited by the JNK inhibitor SP600125 and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 but not by the ERK inhibitor PD98059. Using FAS and SCD-1-luciferase promoter constructs, we observed that KGF stimulated the transcription of these promoters and that exogenous cholesterol inhibited the induction. Mutation of the SREBP-1 binding site in the SCD-1 promoter abolished the effect of KGF on SCD-1 transcription. In addition, overexpression of active SREBP-1 directly stimulated SCD-1 and FAS. Conversely, adenovirus-mediated overexpression of a dominant negative form of SREBP-1 inhibited the KGF effect on FAS and SCD-1 expression. In summary, we conclude that KGF requires both PI3K and JNK signaling pathways to induce SREBP-1, which in turn induces SCD-1 and FAS expression in H292 cells.—Chang, Y., J. Wang, X. Lu, D. P. Thewke, and R. J. Mason. **KGF induces lipogenic genes through a PI3K and JNK/SREBP-1 pathway in H292 cells. *J. Lipid Res.* 2005.** 46: **2624–2635.**

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Keratinocyte growth factor (KGF; FGF-7) is a potent and relatively specific mitogen for epithelial cells, including some adenocarcinomas (1–3). Recently, recombinant KGF (palifermin) has been shown to treat oral ulcers produced by cytotoxic drugs used in cancer therapy (4). In the lung, KGF stimulates proliferation, lipogenesis, and surfactant production in type II alveolar cells (5–7). In addition, administration of KGF also protects the lung against a variety of injuries, including bleomycin, hyperoxia, radiation, and acid instillation (8–11). KGF interacts with its specific receptor, KGFR, a tyrosine kinase receptor, to initiate a cascade of signaling pathways. In this cascade, rapid changes in the phosphorylation state of some signaling proteins and transcription factors mediate the subsequent effects of KGF, which include proliferation, cell differentiation, and lipogenesis (12). Because KGF can stimulate lipogenesis required for both membrane biogenesis and surfactant production, we sought to determine whether the pathways involved and the lipogenic enzymes stimulated were similar in the two processes.

Lipogenesis is necessary for cell proliferation and growth to form new organelles and membranes. Certain cancers, including breast, colon, prostate, liver, lung, bladder, and stomach cancer, have been reported to have high levels of lipid synthesis and expression of fatty acid synthase and stearoyl CoA desaturase (SCD) (13–17). More importantly, inhibition of FAS and SCD delays disease progression (18–21). However, the regulation of lipogenesis is a complex process. In adipocytes, there are three classes of transcription factors that influence lipogenesis: adipocyte determination differentiation factor/sterol response element binding protein family members [sterol-regulatory element binding proteins (SREBPs)]; the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ); and CCAAT/enhancer binding protein (C/EBP) family mem-

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Abbreviations: Akt, Akt/protein kinase B; C/EBP, CCAAT/enhancer binding protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; KGF, keratinocyte growth factor; KGFR, keratinocyte growth factor receptor; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PFU, plaque forming units; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPARγ, peroxisome proliferator-activated receptor γ; SCAP, sterol-regulatory element binding protein cleavage-activating protein; SCD-1, stearoyl coenzyme A desaturase-1; SREBP, sterol-regulatory element binding protein.

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bers (22, 23). SREBPs, consisting of SREBP-1a, SREBP-1c, and SREBP-2, have been identified as important transcription factors regulating lipogenesis (24). SREBP-1 has two isoforms derived from a single gene through the use of alternative transcription start sites and splicing. SREBP-1 is synthesized as a 128 kDa precursor protein, which is bound to the endoplasmic reticulum and the nuclear envelope by SREBP cleavage-activating protein (SCAP) in the presence of sterol. Upon activation, the SREBP/SCAP complex migrates to the Golgi. The active 68 kDa mature SREBP is released by a sequential two-step cleavage process and translocates into the nucleus and promotes the transcription of many lipogenic genes (25). Genes that have been shown to be regulated at the transcriptional level by SREBPs include those involved in cholesterol homeostasis (LDL receptor, HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase) and fatty acid synthesis (fatty acid synthase, acetyl-CoA carboxylase, SCD, and ATP citrate-lyase). In general, SREBP-1 regulates fatty acid synthesis and SREBP-2 is responsible for cholesterol synthesis (26–31).

SCD, a microsomal enzyme, catalyzes the desaturation of fatty acyl-CoAs. Stearoyl-CoA and palmitoyl-CoA are converted to oleoyl-CoA and palmitoleoyl-CoA, respectively (32). In the mouse, four SCD genes have been identified (32–35). Human SCD is a single gene that yields two transcripts resulting from the use of alternative polyadenylation sites present in the 3′ untranslated region (36). SCD is expressed at a higher level in certain tumors compared with normal tissues, and inhibition of SCD delays tumor development in mice (17).

FAS is a key multifunctional enzyme that catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA. Insulin stimulates the transcription of FAS in liver and adipose tissues, and glucose and glucagon also regulate its expression (37–40). Increased fatty acid synthase expression and fatty acid synthesis is also common in some human cancers. A variety of human cancers, including cancers of prostate, breast, ovary, and colon, express increased levels of FAS (41–43). Most studies of FAS and SCD expression have focused on liver and adipose tissue. Regulation of FAS and SCD in alveolar type II cells is modulated through SREBP-1c (5).

The goals of this study were to define the KGF signaling pathways involved in lipogenesis in a human pulmonary tumor cell line that does not produce surfactant. Recently, we showed that KGF increases SREBP-1c, C/EBPα, C/EBPδ, and lipogenic enzymes as it stimulates surfactant production in primary cultures of rat type II cells (5, 44). In the current study, we sought to determine whether KGF induces the same family of transcription factors and lipogenic enzymes for lipogenesis required for cell growth as it does for lipogenesis for surfactant production. We chose H292 cells, a cell line derived from a metastatic pulmonary mucoepidermoid carcinoma that proliferates in response to KGF (45). A better understanding of the molecular basis of lipogenesis might also help in the development of therapeutic interventions for cancer. In addition, signaling pathways in H292 cells may provide insight into KGF signaling and lipogenesis in alveolar type II cells. We demonstrate here that KGF stimulates FAS and SCD, the two rate-limiting enzymes in lipogenesis, through an SREBP-1 pathway in H292 cells. Our data also indicate that phosphatidylinositol 3-kinase (PI3K) and c-Jun N-terminal kinase (JNK) signaling pathways are involved in SREBP-1 activation, as judged from the effects of the inhibitors LY294002 and SP600125. These findings define pathways for the development of inhibitors to modulate lipid metabolism and potentially inhibit the growth of some cancers. Regulation of lipogenesis by KGF appears to be similar in H292 cells, a cancer cell line, and primary cultures of alveolar type II cells.

MATERIALS AND METHODS

Materials

Human recombinant KGF was purchased from R&D Systems, Inc. (Minneapolis, MN). Antibody to C/EBPα was purchased from Active Motif (San Diego, CA). Antibodies to actin, C/EBPβ, and C/EBPδ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to SREBP-1 was purified from culture supernatants from a cell line obtained from the American Type Culture Collection (Manassas, VA). Antibody to human SCD was purchased from Alpha Diagnostic International, Inc. (San Antonio, TX). Antibody to fatty acid synthase was a gift from Stuart Smith (Oakland Children's Medical Center, Oakland, CA). Antibodies to extracellular signal-regulated kinase (ERK), JNK, S6K, Akt, p-ERK, p-Akt, p-JNK, and p-S6K were purchased from Cell Signaling Technology (Beverly, MA). The FAS luciferase reporter construct, the pGL-3 plasmid, and the H292 cell line were gifts of Hong-Bing Shu (National Jewish Medical and Research Center, Denver, CO), and the β-galactosidase reporter plasmid, pCMV-β-galactosidase, was obtained from BD Biosciences Clontech (Palo Alto, CA). pGL-2-SCD was generously provided by Peter Edwards (University of California, Los Angeles). The adenoviruses containing LacZ and active and negative SREBP-1 were obtained from Jerry Schaack and Jed Friedman, respectively (University of Colorado Health Science Center, Denver, CO). All primers and probes were synthesized at the National Jewish Medical and Research Center. The sources of most of the reagents are stated in the descriptions of the individual methods. DMEM and FBS were purchased from GIBCO-BRL and Irvine Scientific (Santa Ana, CA), respectively. DMSO, penicillin, streptomycin, cholesterol, and 25-hydroxycholesterol were purchased from Sigma (St. Louis, MO).

Cell proliferation assay

H292 cells were plated at a density of 0.5 million cells/well on a six-well plate in DMEM with 10% FBS. One day later, the cells were washed twice with PBS, and then 2 ml of DMEM with 1% FBS in the presence or absence of KGF was added to each well. Media and fresh KGF were added every other day. Cells were washed with PBS and trypsinized every 2 days. The cells were pelleted by centrifugation (1,000 rpm for 10 min), resuspended in an assay buffer (containing 10 mM NaH_2PO_4, 40 mM Na_2HPO_4, 2 M NaCl, and 2 mM EDTA), and sonicated, and then the DNA was measured by fluorimetry.

Lipid synthesis measurements

H292 cells were seeded on six-well plates in DMEM with 10% FBS at a density of 0.8 million cells/well. Twenty-four hours later, the cells were rinsed three times with PBS and cultured in the

medium containing 0.2% FBS for 16 h, then treated with or without KGF (20 ng/ml) for 48 h. [1-^{14}C]acetate (10 μCi, 5 μCi/ml; MP Biomedicals, Irvine, CA) was added to each well during the last 4 h of culture. Cells were rinsed three times with PBS, scraped off in 1.8 ml of PBS, and extracted by the Bligh and Dyer method (46). Phospholipids and neutral lipids were separated by thin-layer chromatography using silica gel H250 plates (Analtech, Inc., Newark, DE) developed with chloroform-methanol-acetic acid-water (100:50:16:5, v/v) for phospholipid and G250 plates (Analtech, Inc.) developed with hexane-diethylether-acetic acid (120:50:1.5, v/v) for neutral lipids. Each sample was run in the presence of phospholipid and neutral lipid standards. The plates were exposed to iodine vapor, and individual lipids spots were scraped into vials for scintillation counting.

Western blot analysis

Cells were lysed using ice-cold RIPA 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, OH); 1× protease inhibitor cocktail (catalog No. 214262; Pharmingen, San Diego, CA) containing benzamidine-HCl, phenanthrolene, aprotinin, leupeptin, pepstatin A, and PMSF; 1× phosphatase inhibitor cocktail 2 (catalog No. P5725; 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, CA). Culture dishes were placed on ice, the medium was removed, and the cells were rinsed twice with PBS. Ice-cold lysis buffer (200 μl) was applied to cells. Cells were removed with a cell scraper. DNA was sheared using a syringe and a 25 gauge needle. The insoluble material was removed by centrifugation at 14,000 *g* for 20 min. The protein concentration was measured with BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). One part 4× SDS-PAGE reducing Laemmli sample buffer was added to three parts lysate. The mixture was boiled for 10 min 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, CA). Nonspecific binding of proteins to the nitrocellulose membranes was 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 were diluted in 5% BSA or 5% nonfat dry milk in TTBS and incubated overnight at 4°C with rocking. HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were applied for 1 h at room temperature. Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Hyperfilm (Amersham Pharmacia Biotech).

Quantitative real-time PCR and RT-PCR

To determine whether KGF could affect mRNA levels of SREBP-1a, SREBP-1c, SCD-1, and FAS, H292 cells were plated at a density of 1 million cells/well on six-well plates in 2 ml of DMEM containing 10% FBS. One day later, the medium was replaced with DMEM containing 0.2% FBS. After the overnight incubation in 0.2% FBS, KGF was added to each well and incubated for 6, 12, and 24 h. To inhibit transcription and protein synthesis, cells were pretreated for 30 min with different concentrations of cycloheximide and actinomycin D before adding KGF. After a 12 h incubation, total RNA was extracted from cultured cells with an RNeasy Mini Kit (Qiagen, Valencia, CA). Isolated RNA samples were treated with 4 units of RNase-free DNase I (Promega, Madison, WI) for 30 min at 37°C to remove any genomic DNA contamination. Total RNA (2 μg) was used to synthesize cDNA with the TaqMan reverse transcription reagents kit (Applied Biosystems, Branchburg, NJ) in a final volume of 100 μl according to the manufacturer's instructions. Random hexamers were used as primers in the reverse transcription reaction. The reactions were incubated at 25°C for 10 min, at 48°C for 30 min, and at 95°C for 5 min, then stored at −20°C until use. Primers and probes for real-time PCR for human SREBP-1, SCD-1, and FAS were designed using Primers Express software (version 1.5a; Applied Biosystems). For human SREBP-1a, 5′-CAG CGA GGC GGC TTT GGA-3′ and 5′-TCT TCG ATG TCG GTC AGC A-3′ were selected for the forward and reverse primers, respectively. The probe sequence was 5′-CAG ATC GCA CGG CTC GCC CAG C-3′. For human SREBP-1c, 5′-CGG AGC CAT GGA TTG CAC T-3′ and 5′-TAG GCC AGG GAA GTC ACT G-3′ were selected for the forward and reverse primers, respectively. The probe sequence was 5′-AAG ACA TGC TTC AGC TTA TCA ACA ACC AA-3′. For human SCD-1, 5′-CAC CAC ATT CTT CAT TGA TTG CA-3′ and 5′-ATG GCG GCC TTG GAG ACT-3′ were used as forward and reverse primers, respectively. The probe sequence was 5′-CCG CCC TCG GTC TGG CCT ATG-3′. For human FAS, 5′-GAA CTC CTT GGC GGA AGA GA-3′ and 5′-GGA CCC CGT GGA ATG TCA-3′ were used as forward and reverse primers, respectively. The probe sequence was 5′-CAC CCG CTC GGC ATG GCT ATC TT-3′. FAM fluorescent dye was used as the reporter for SREBP-1, SCD-1, and FAS, whereas VIC was used as the reporter for the GAPDH reference (Applied Biosystems). Samples were run in triplicate. The reactions were quantitated by selection of the amplification cycle during which the PCR product of interest was accumulating logarithmically. Data were analyzed with the comparative threshold cycle method to achieve the results of relative quantitation.

To determine whether the rat dominant negative SREBP-1 was expressed in human H292 cells, total RNA was isolated from H292 cells with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Four micrograms of RNA was reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Single-strand cDNA was then amplified by PCR with specific forward and reverse primers for the adenovirus-mediated dominant negative rat SREBP-1: 5′-GGA GGA CCC AAG GTG ACA-3′ and 5′-GGA GCC AGG GTG TTG ATG-3′, respectively. The PCR product was then separated on a 1% agarose gel run in 1× Tris-buffered EDTA in the presence of ethidium bromide.

Signaling pathway activation by KGF

To study early signaling events, we seeded H292 cells on six-well plates in DMEM with 10% FBS. The cells were allowed to attach for 24 h and then gently rinsed with PBS to remove nonadherent cells. The 10% FBS medium was replaced with DMEM containing 0.2% FBS to minimize the basal activity of signaling pathways. After an overnight incubation in 0.2% FBS, KGF was added to each well and incubated for various time periods. At the end of the incubation, we washed the cells twice with PBS followed by lysis with 200 μl of RIPA buffer as described above. Protein quantitation (BCA assay) was performed on an aliquot of the supernatant. Equal protein loading was ascertained by normalizing quantities to nonphosphorylated ERK, JNK, Akt, and p70 S6K, respectively. Phosphospecific and total antibodies to ERK, JNK, Akt, and p70 S6K were used for Western blotting as described above.

Transient transfections and reporter gene assays

Cells were seeded on six-well plates in DMEM with 10% FBS at a density of 0.8 million cells/well. The next day, 10 μl of SuperFect Transfection Reagent (Qiagen) was added to 3 μg of DNA in 100 μl of DMEM without serum to allow the formation of the transfection complexes. For each complex, 600 μl of DMEM con-

taining 1% FBS was added to the reaction tube and then added to one of the wells on a six-well plate. The cells were incubated with the transfection complexes for 10 h, and then the medium was removed and fresh medium with 1% FBS with either KGF (20 ng/ml) or sterols (10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol; repressing medium) was added. Each transfection was performed in triplicate. To normalize for transfection efficiency, 0.5 μg of CMV-β-galactosidase luciferase reporter plasmid was added to each transfection. Approximately 60 h after transfection, luciferase reporter assays were performed using a luciferase assay kit (BD Biosciences) by following the manufacturer's protocol. β-Galactosidase activity was measured using the Galacto-Light chemiluminescent kit (TROPIX, Bedford, MA). Luciferase activity were normalized on the basis of β-galactosidase luciferase expression levels.

SCD-1 promoter-reporter gene constructs

The functional SRE in pGL-2-SCD-1 was mutated using the QuikChange kit from Stratagene (La Jolla, CA) and oligonucleotides exactly as described by Tabor et al. (29). The 614 bp SCD-1 promoter and SRE mutant fragments were excised by digestion with *Xho*I/*Hind*III and subcloned into the *Xho*I/*Hind*III sites of pGL-3 basic to produce pSCD-1 and pSCD-1-mut, respectively. The constructs were sequenced before use.

Treatment with recombinant adenoviruses

The recombinant adenovirus expressing the transcriptionally active N-terminal fragment (amino acids 1–403) of SREBP-1c was kindly provided by Jed Friedman and was originally constructed by Foretz and colleagues (47). The recombinant adenovirus expressing the dominant negative form of rat SREBP-1c was constructed as described previously (48). On day 0, cells were seeded on six-well plates at a density of 1 million cells/well in DMEM supplemented with 10% FBS. On day 1, the medium was removed, and 1 ml of fresh medium supplemented with 2% heat-inactivated FBS was added. The recombinant adenoviruses expressing LacZ and dominant active or dominant negative SREBP-1 were added at titers ranging from 20 to 100 plaque-forming units (PFU)/cell. Cultures were incubated for 4 h on a rocker. Then, 1 ml of serum-free DMEM was added to each well. On day 2, medium was removed and cells were incubated with 2 ml of fresh DMEM supplemented with 0.5% FBS in the absence or presence of KGF. On days 3 and 4, RNA and protein, respectively, were harvested as described above.

RESULTS

KGF stimulates proliferation and lipogenesis in H292 cells

To study how KGF stimulates lipid synthesis in human lung epithelial cells, we evaluated several different cell lines. KGF did not alter cell growth or lipogenic genes in H441 and 16HBE cells, although both cell lines expressed KGFR (data not shown). However, KGF did stimulate NCI-H292 cells, which is a human lung cancer cell line derived from a pulmonary mucoepidermoid carcinoma (45). To determine whether KGFR was expressed in H292 cells, we first checked its transcripts by RT-PCR using specific primers and sequenced the PCR product. H292 cells express KGFR (data not shown). Immunoblot analysis of cell lysates also identified KGFR protein in H292 cells (data not shown). Because KGF stimulates the proliferation of primary rat alveolar type II cells (12), we determined whether KGF could stimulate the proliferation of H292 cells. Our results indicate that KGF significantly stimulated the proliferation of H292 cells in 1% FBS (**Fig. 1A**). KGF also stimulated proliferation in 5% FBS (data not shown). This proliferative effect is similar to that seen in alveolar type II cells (6).

We next studied whether KGF stimulated lipogenesis. Cells were cultured with and without KGF and then incubated with [1-^{14}C]acetate for 4 h. The lipids were extracted and analyzed by thin-layer chromatography. KGF increased acetate incorporation into both phospholipids and neutral lipids as expected for membrane biogenesis (Fig. 1B, C).

KGF activates several signaling pathways in H292 cells

Several growth factor signaling pathways are involved in cell proliferation and differentiation. An important pathway is the mitogen-activated protein kinase (MAPK) cascade, which includes the Ras/MEKK/ERK, JNK, and p38 pathways. Another critical signaling pathway induced by growth factors is the PI3K/Akt and p70 S6K pathways. In H292 cells, KGF stimulated ERK kinase, as demonstrated by protein phosphorylation. Peak activation occurred after 10 min of exposure (**Fig. 2A**). Generally, growth factors stimulate the phosphorylation of two members of the p42/p44 family, the extracellular regulated kinases [ERK-1 (p44) and ERK-2 (p42)] (12). However, in H292 cells, it appears that KGF activates p42 but not p44. Immunoblotting with anti-total p42/p44 antibody indicates that the total amount of p42/p44 was unaltered. We next investigated whether KGF activated Akt and p70 S6K, which are downstream targets of PI3K. Western blot analysis revealed the activation of both p70 S6K and Akt. Phosphorylation of both occurred between 5 and 15 min of exposure (Fig. 2B, C). Immunoblotting with anti-total Akt and p70 S6K antibodies showed that the total amount of Akt and p70 S6K remained similar in all samples. We also found that KGF activated the phosphorylation of JNK1 (Fig. 2D).

SREBP-1 and C/EBP transcription factors are involved in lipogenesis stimulated by KGF in H292 cells

Next, we studied the expression of three classes of transcription factors involved in lipogenesis. SREBP-1 is important for lipogenesis and regulates the transcription of genes involved in cholesterol and fatty acid metabolism. Immunoblotting demonstrated that SREBP-1 was induced by KGF (**Fig. 3A**). KGF increased the expression of mature SREBP-1 (68 kDa) by 2.1-, 1.6-, and 1.2-fold after 2, 4, and 6 days of treatment, respectively. The SREBP-1 precursor protein (125 kDa) was induced by 1.3-, 1.2-, and 1.2-fold after 2, 4, and 6 days of treatment with KGF. During adipocyte differentiation, C/EBPβ and C/EBPδ are induced at an early stage of adipogenesis and are required for maximal lipogenesis, and C/EBPα is induced relatively late in differentiation (49). In H292 cells, C/EBPβ was induced by 1.5-fold by KGF only on day 2 (Fig. 3A). C/EBPα was induced by 3.3-, 2.8-, and 1.9-fold after 2, 4, and 6 days of treatment, respectively, and C/EBPδ was induced by 1.8-, 2.1-, and 2.2-fold after 2, 4, and 6 days (Fig. 3A). However, PPARγ protein was not altered by KGF (data not shown). These results thus dif-

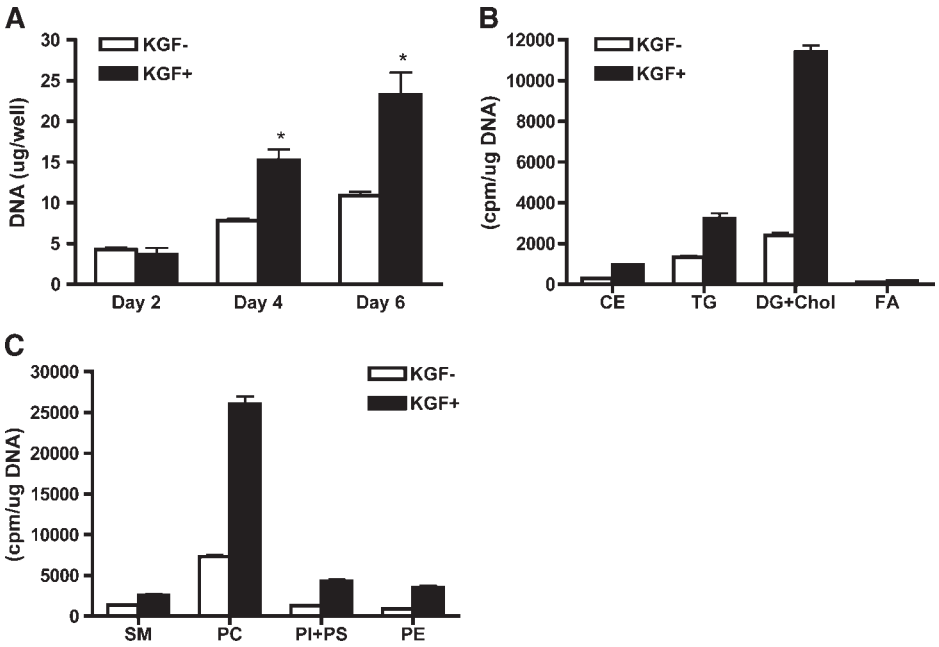

Fig. 1. Keratinocyte growth factor (KGF) stimulates proliferation and lipogenesis in H292 cells. A: KGF stimulates H292 cell proliferation. H292 cells were plated at a density of 1 million/well in DMEM supplemented with 10% FBS. Cells were cultured for the indicated time intervals in the presence or absence of KGF (20 ng/ml) in DMEM with 1% FBS. KGF stimulated proliferation as measured by DNA per cell on days 4 and 6. These data are from one representative experiment of a set of three independent experiments. Asterisks indicate significantly different results from control ($P < 0.05$). B, C: KGF stimulates H292 cell lipogenesis. H292 cells were cultured in medium containing 0.2% BSA for 16 h and then were incubated with or without KGF for 48 h. Incorporation of [1-^{14}C]acetate was measured during the last 4 h of culture. Each condition was tested in triplicate. B shows the results for neutral lipids, and C shows the results for phospholipids. Results from one of two similar experiments are shown. CE, cholesteryl ester; Chol, cholesterol; DG, diglyceride; FA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TG, triglyceride. Error bars indicate the standard error of the mean.

fer from those observed with adipocyte differentiation (49, 50). In conclusion, KGF induces some key transcription factors involved in lipogenesis, including C/EBPα, C/EBPβ, C/EBPδ, and mature SREBP-1 but not PPARγ.

Because SREBP-1 is a transcription factor capable of inducing the expression of two key lipogenic enzymes, FAS and SCD-1, and because SREBP-1 can be induced by KGF, we determined whether FAS and SCD-1 were also induced

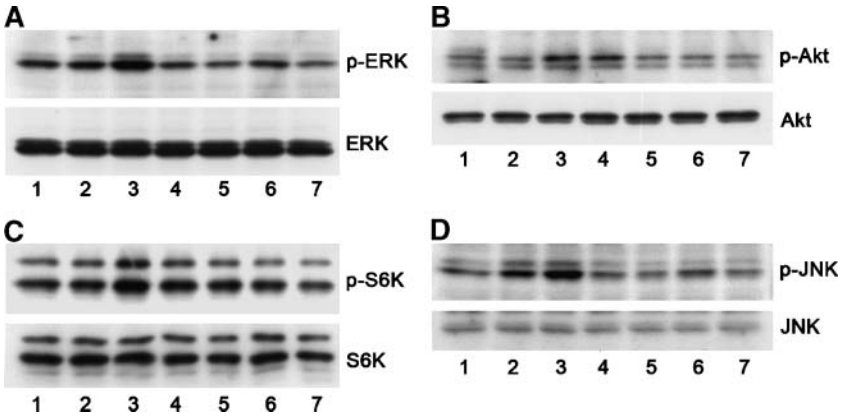

Fig. 2. KGF activates ERK, Akt, S6K, and JNK. H292 cells were cultured in DMEM as described in Materials and Methods. KGF (20 ng/ml) was added for different time intervals. Signaling pathways were identified by the use of phosphospecific antibody in Western analyses. A: Phosphorylated ERK and total ERK. B: Phosphorylated Akt and total Akt. C: Phosphorylated S6K and total S6K. D: Phosphorylated JNK1 and total JNK1. Lane 1 represents unstimulated cells used as a control. Lanes 2–7 represent a time course of H292 cells exposed to KGF. Cells were harvested at 5, 10, 15, 30, 60, and 120 min after the addition of KGF. All pathways appeared to be maximally stimulated at 10–15 min after the addition of KGF. Western blots are from one of three independent experiments.

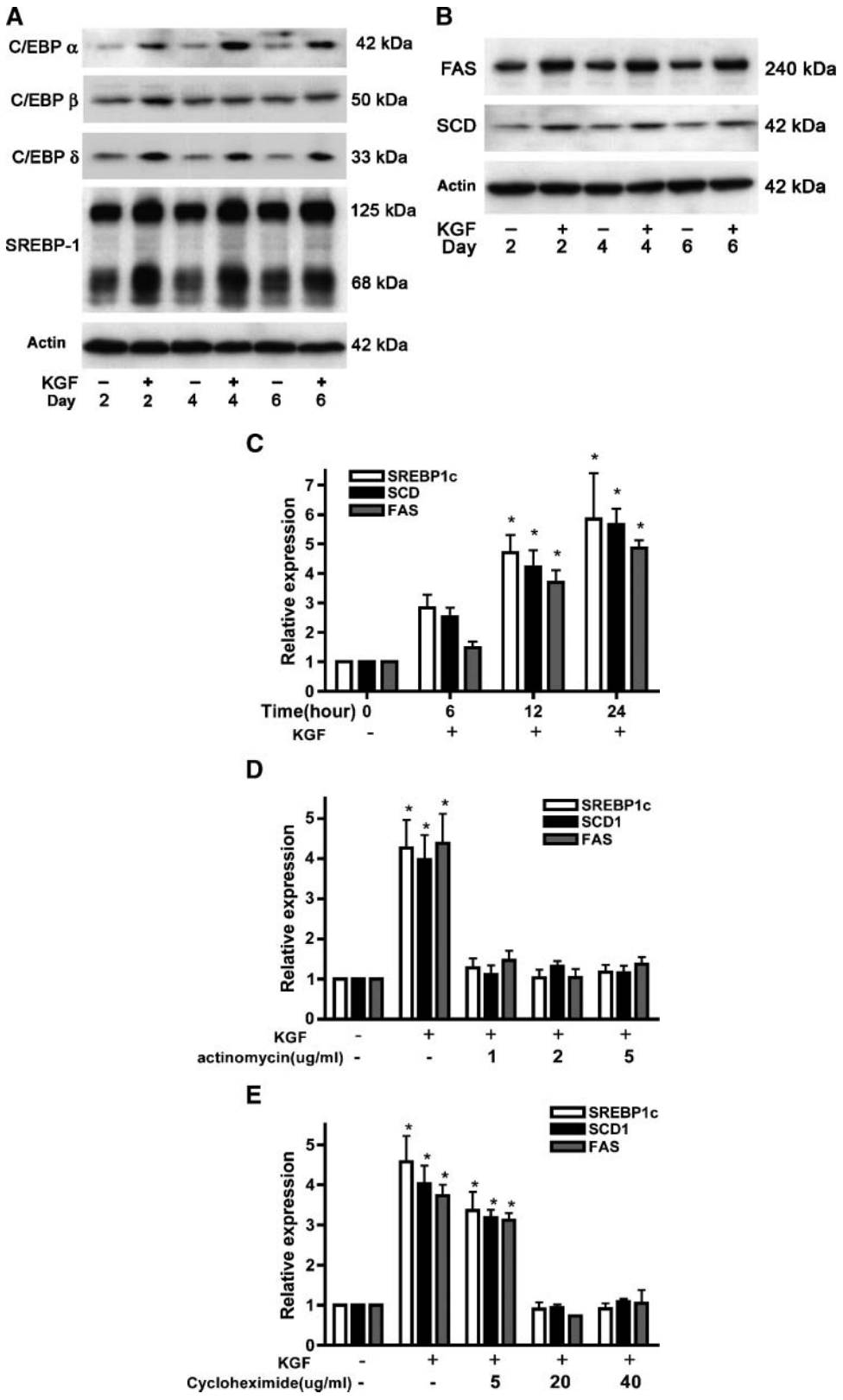

Fig. 3. The transcription factors CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ, C/EBPδ, and sterol-regulatory element binding protein-1 (SREBP-1) and the lipogenic enzymes FAS and stearoyl coenzyme A desaturase-1 (SCD-1) are induced by KGF. H292 cells were cultured in DMEM with 0.2% FBS in the presence or absence of 20 ng/ml KGF and inhibitors for the indicated times as described in Materials and Methods. The relative intensities of bands on the Western blot were determined using NIH Image 1.62 software and normalized using actin band intensity. The results are described in the text. A: Total protein was separated by SDS-PAGE and analyzed for C/EBPα, C/EBPβ, C/EBPδ, and SREBP-1. Actin was used to show the similar amount of protein loaded in different lanes. C/EBPβ appeared to increase only on day 2, whereas the other transcription factors were increased at all time points. B: KGF increased protein level of FAS and SCD-1 at all time points. C: Time course of mRNA expression of SREBP-1c, SCD-1, and FAS induced by KGF and measured by quantitative real-time PCR. D, E: Effect of actinomycin D and cycloheximide on the KGF-mediated upregulation of SREBP-1c, SCD-1, and FAS. GAPDH was used as an internal control. Asterisks indicate statistically significant differences compared with controls ($P < 0.05$). Results were obtained from three independent experiments performed in triplicate. Error bars indicate the standard error of the mean.

by KGF. High levels of FAS and SCD-1 expression are found in some tumor cells (17, 41–43). The protein level of FAS was induced by 1.5-, 1.5-, and 1.4-fold after treatment with KGF for 2, 4, and 6 days, respectively. SCD-1 was induced by 1.4-, 1.3-, and 2-fold on days 2, 4, and 6 of treatment (Fig. 3B). We also determined whether these genes could be upregulated by KGF in a shorter time. Our Western blot results indicated that after 12 h, KGF could increase protein levels of SREBP-1, FAS, and SCD-1 (data not shown). By quantitative real-time PCR, KGF also increased the mRNA levels of SREBP-1c, SCD-1, and FAS (Fig. 3C). However, SREBP-1a and SREBP-2 mRNA levels were not affected by KGF (data not shown). To identify the mechanism by which KGF upregulates the expression of these genes, H292 cells were pretreated with actinomycin D, a transcriptional inhibitor, or cycloheximide, an inhibitor of protein synthesis, for 30 min before exposure to KGF. As shown in Fig. 3D, E, actinomycin D treatment completely blocked the KGF-induced increase in SREBP-1c, SCD-1, and FAS mRNA levels. High concentrations of cycloheximide also blocked the KGF-mediated upregulation of these genes. These data indicate that KGF increases the expression of these genes at the transcriptional level and that the activation requires new protein synthesis.

PI3K and JNK mediate the effect of KGF on SREBP-1

As mentioned above, four major signaling mediators can be activated by KGF. To assess whether these kinases are involved in the regulation of SREBP-1 by KGF, we treated the cells with specific small inhibitory molecules, LY294002, SP600125, and PD98059, that target PI3K, JNK, and MEK1, respectively. LY294002 and SP600125, which blocked Akt phosphorylation and JNK phosphorylation in response to KGF (data not shown), blocked the stimulation of mature SREBP-1 (**Fig. 4A**, **B**). Although PD98059 completely blocked the phosphorylation of ERK induced by KGF (data not shown), the downstream target of MEK1, PD98059, had no significant effect on the stimula-

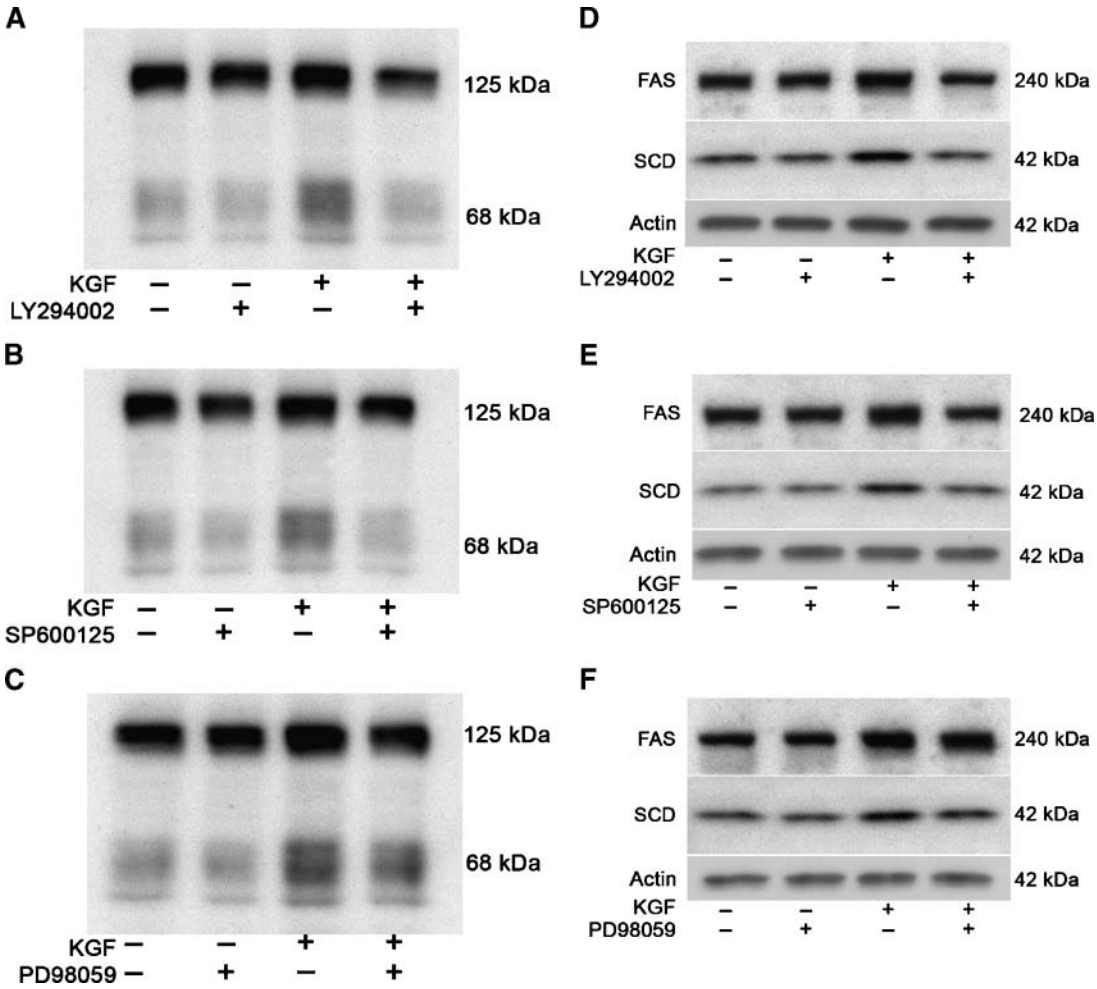

Fig. 4. Phosphatidylinositol 3-kinase (PI3K) and JNK signaling pathway inhibitors block KGF induction of SREBP-1, SCD-1, and FAS. H292 cells were cultured in DMEM with 0.2% FBS in the presence or absence of KGF (20 ng/ml) and/or different inhibitors (40 μM PD98059, 40 μM LY294002, or 10 μM SP600125) for 24 h. The cells were harvested and protein immunoblots were probed with anti-SREBP-1 (A–C), anti-SCD-1, and anti-FAS (D–F). DMSO was held constant at 0.1% in all conditions. The PI3K and JNK inhibitors blocked the stimulation of SREBP, SCD-1, and FAS, whereas the ERK inhibitor did not. Actin was used to show the similar amount of protein loaded in different lanes. The relative intensities of bands on the Western blot were determined using NIH Image 1.62 software and normalized using actin band intensity. The results are described in the text and are representative of three separate experiments.

tion of mature SREBP-1 expression by KGF (Fig. 4C). Because mature SREBP-1 could also be increased within 12 h after KGF treatment (data not shown), we also found that these inhibitors could block the KGF effect on SREBP-1 at this time. Our results indicate that the effect of KGF on mature SREBP-1 was blocked after cells were exposed to LY294002 and SP600125 for 12 h, but PD98059 did not abolish the effect of KGF (data not shown). These results indicate that JNK and PI3K act upstream of SREBP-1 and that both of these signaling pathways are important in the activation of lipogenic enzymes by KGF. We also studied whether the upregulation of C/EBPα and C/EBPδ could be blocked by LY294002 and SP600125. Both of these inhibitors abolished the KGF stimulation of C/EBPα and C/EBPδ (data not shown). These results also indicate that the effects of KGF on lipogenesis are complex and involve the activation of cross-talk between multiple signal transduction pathways.

Blocking the induction of SREBP-1 through PI3K and JNK1 by KGF abolishes the effect of KGF on SCD-1 and FAS

Because blocking PI3K and JNK by specific inhibitors blocks the induction of mature SREBP-1 by KGF, we also studied whether the inhibitors can block the induction of FAS and SCD-1 by KGF. SREBP-1c is a key transcription factor in the regulation of FAS and SCD-1. We treated cells as described above. KGF induced FAS and SCD-1 by 1.4- and 1.5-fold, respectively (Fig. 4D–F). The PI3K and JNK inhibitors completely blocked the KGF effect on FAS and SCD-1 (Fig. 4D, E). However, blocking of the MAPK signaling pathway by PD98059 did not significantly alter the effect of KGF on SCD-1 and FAS (Fig. 4F). We also determined whether treatment of H292 cells with various inhibitors at a shorter time (12 h) could block the effect of KGF on SCD-1 and FAS. At this shorter time point, LY294002 and SP600125 also blocked the KGF effect, whereas PD98059 did not (data not shown). Because the KGF effect on the expression of SREBP-1, SCD-1, and FAS could be blocked by LY294002 and SP600125, we wondered whether these inhibitors could affect the KGF-dependent proliferation in H292 cells. Our results indicate that both of these inhibitors abolished the proliferative effect of KGF (data not shown). These results suggest that KGF regulates the expression of FAS and SCD-1 through KGFR/PI3K and JNK/SREBP-1 pathways.

KGF stimulates the transcription of FAS and SCD-1 promoter-reporter constructs

To investigate the effect of KGF on SREBP transcriptional activity, we performed luciferase reporter experiments with specific gene promoters. The FAS and SCD-1 promoter fragments present in the constructs harbor a SREBP binding site (SRE). The SRE was altered in the SCD-1 mutant promoter, so that SREBP-1 cannot bind to the SCD-1 mutant promoter-reporter construct (29). H292 cells were transiently transfected with these promoter-reporter constructs and then incubated in the presence or absence of KGF. KGF increased FAS and SCD-1 reporter activity by ~2-fold (**Fig. 5A**, **B**). Furthermore, mutation of the SRE in the SCD-1 promoter abolished the effect of KGF on SCD-1 transcription. These findings indicate that stimulation of FAS and SCD-1 expression takes place at least in part at the transcriptional level and is mediated by *cis*-acting elements present in the proximal FAS and SCD-1 promoters.

Exogenous cholesterol inhibits the induction of FAS and SCD-1 expression by KGF

To demonstrate that KGF activated the FAS gene promoter in a SREBP-dependent manner, we used cholesterol and 25-hydroxycholesterol, which inhibit the cleavage of the SREBP-1 precursor, to form mature SREBP-1.

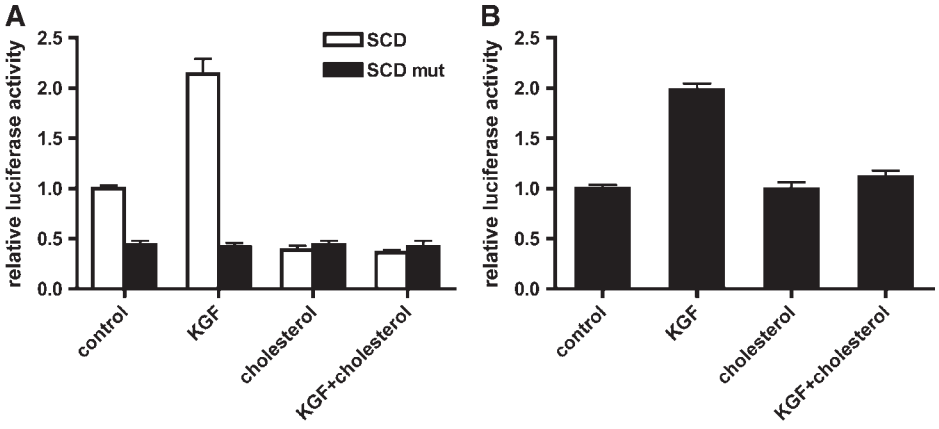

Fig. 5. Cholesterol abrogated the stimulation of transcription of FAS and SCD-1 by KGF. H292 cells were transfected with promoter-reporter and β-galactosidase plasmids. Cells were then cultured in the presence or absence of KGF and/or cholesterol for 60 h. Luciferase reporter gene assays were performed as described in Materials and Methods. A: Averages of relative luciferase activities (normalized by β-galactosidase activities) from one representative experiment in which each transfection was performed in triplicate. KGF induced SCD-1 transcription, and the effect was blocked with exogenous cholesterol. KGF was not able to stimulate the SCD-1 promoter without the SRE site (SCD mutant). B: KGF induced FAS transcription, and the effect was blocked by exogenous cholesterol. The results are representative of three separate experiments. Error bars indicate the standard error of the mean.

When cellular sterol levels are low, two distinct proteolytic events result in the release of the mature 68 kDa N-terminal fragment of SREBP-1. The mature protein translocates to the nucleus, binds to the promoter of target genes, and activates the transcription. Conversely, when levels of cellular sterol are high, proteolytic processing of SREBP is diminished, nuclear levels of the mature proteins decline, and transcription of target genes is low (25). Promoter-reporter constructs of FAS, SCD-1, and mutant SCD-1 were transiently transfected into H292 cells together with a plasmid encoding β-galactosidase under the control of the CMV promoter. The cells were then incubated in medium containing 1% FBS and sterol (10 μg/ml cholesterol and 1.0 μg/ml 25-hydroxycholesterol), lysed, and assayed for luciferase activity. The β-galactosidase activity was used to normalize luciferase activity for minor differences in transfection efficiencies. The exogenous sterols abrogated the stimulation of FAS and SCD-1 by KGF (Fig. 5A, B). Hence, our data indicate that SREBP processing is required for the transcriptional effects of KGF on FAS and SCD-1.

An adenovirus expressing active SREBP-1 increases the expression of SCD-1 and FAS

To further substantiate the concept that stimulation of SREBP-1 expression could reproduce the effect of KGF on SCD-1 and FAS, an adenovirus expressing the active N-terminal fragment of SREBP-1 (44 kDa) (47) was used. This protein translocates to the nucleus and binds to sterol-regulatory elements of target genes to activate expression, independent of cellular sterol levels. Infection of H292 cells with an adenovirus expressing the active form of SREBP-1 increased the expression of SCD-1 and FAS (**Fig. 6**). To ascertain whether this induction of FAS and SCD-1 expression was attributable to active SREBP-1 expression and not to nonspecific effects related to the viral infection, we used an adenovirus expressing LacZ as a control virus. Infection with this control virus had little influence on the expression of SCD-1 and FAS. The endogenous SREBP-1 expression remained unchanged by the adenoviruses.

Overexpression of a dominant negative form of SREBP-1 blocks the induction of SCD-1 and FAS by KGF

To further test the hypothesis that SREBP-1 is involved in KGF activation of SCD-1 and FAS, we introduced a dominant negative form of SREBP-1 into H292 cells. The dominant negative form of SREBP-1, consisting of the N-terminal fragment of SREBP-1 (amino acids 1–403), contains an alanine mutation at amino acid 320. The ability of this dominant negative construct to counteract the transcriptional activity of wild-type SREBP-1 has been established previously (48). We used an adenoviral system to deliver efficiently the gene (Ad-dn-SREBP-1) into H292 cells. By RT-PCR, we first used primers specific to rat SREBP-1 to check whether the dominant negative form of SREBP-1 mediated by adenovirus could be expressed in human H292 cells. As shown in **Fig. 7**, Ad-dn-SREBP-1 could express the rat exogenous gene in H292 cells. The induction of SCD-1 and FAS by KGF was completely abolished by Ad-dn-SREBP-1. To ensure that the reduction of SCD-1 and FAS expression was the result of dominant negative SREBP-1 expression and not of nonspecific effects related to the viral infection, we used an adenovirus ex-

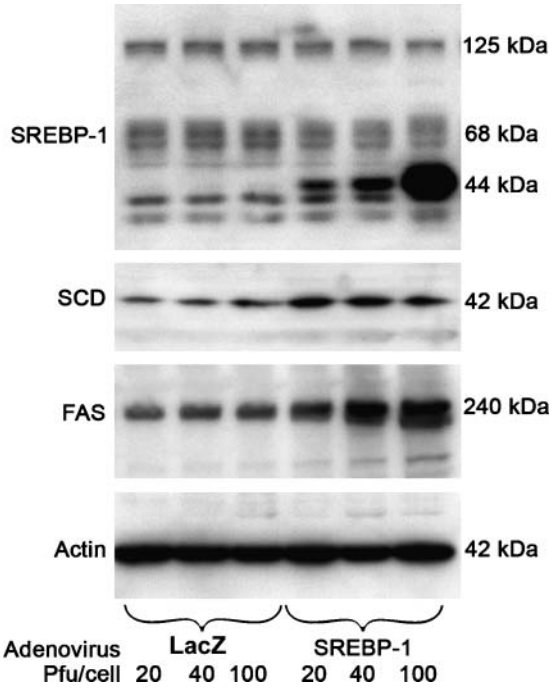

Fig. 6. Ad-SREBP-1 increases FAS and SCD-1. H292 cells were infected with an adenovirus expressing an active form of SREBP-1 [20, 40, and 100 plaque-forming units (PFU)/cell] or with an adenovirus expressing LacZ as a control (20, 40, and 100 PFU/cell) in DMEM with 2% FBS for 4 h. The infected cells were incubated for 12 h in DMEM with 1% FBS, then the medium was removed and fresh medium containing 0.5% FBS was added for 48 h. Cells were harvested and examined for the expression of SCD-1 and FAS, endogenous SREBP-1, (125 kDa and 68 kDa) and active SREBP-1 (44 kDa). Representative Western blots of three independent experiments are shown.

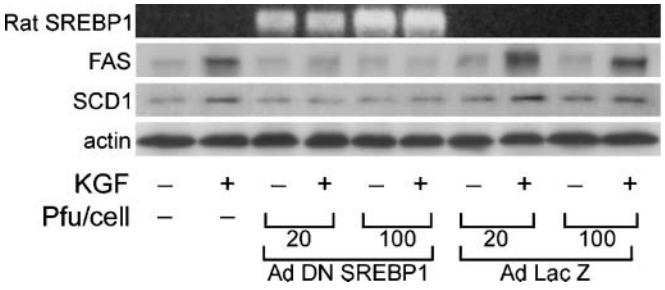

Fig. 7. The dominant negative form of SREBP-1c abolished the increased SCD-1 and FAS expression by KGF. H292 cells were infected with an adenovirus expressing a dominant negative rat SREBP-1c [20 and 100 plaque-forming units (PFU)/cell] or with an adenoviruses expressing LacZ as a control (20 and 100 PFU/cell) in DMEM with 2% FBS for 4 h. The infected cells were incubated for 12 h in DMEM with 1% FBS, then the medium was removed and fresh medium containing 0.5% FBS in the absence or presence of KGF was added for 24 and 48 h. RNA and protein were harvested and examined for the expression of exogenous dominant negative SREBP-1c (44 kDa), SCD-1, and FAS. Representative RT-PCR results and Western blots of three independent experiments are shown.

pressing LacZ as a control. As shown in Fig. 7, infection with this control virus had no effect on the stimulation of FAS and SCD-1 expression by KGF.

DISCUSSION

This is the first study to link KGF signaling pathways to lipogenesis. Previously, we found that KGF could induce the proliferation and lipogenesis of alveolar type II cells in primary culture. KGF stimulated fatty acid, phospholipid, and surfactant protein synthesis (5). In alveolar type II cells, KGF stimulates ERK and Akt pathways with subsequent increases in C/EBPα, C/EBPδ, and SREBP-1c, ultimately resulting in the increased expression of FAS, SCD-1, SCD-2, and epidermal fatty acid binding protein. However, the signaling pathways between the KGF receptor, activation of transcription factors, and enzymes involved in fatty acid synthesis were not defined. Because of some limitations of primary culture systems, we used H292 cells to further define the signaling pathways. We found that KGF stimulates proliferation and fatty acid synthesis in H292 cells. The signaling pathways, the transcription factors involved, and the lipogenic enzymes stimulated appear to be similar regardless of whether the resultant lipids are to be used for cell growth and the formation of new cellular membranes or for the synthesis of surfactant, a secreted product.

In H292 cells, KGF activated ERK, JNK, Akt, and S6K signaling pathways. Induction of SREBP-1 and subsequent upregulation of FAS and SCD-1 by KGF was blocked by inhibitors of PI3K and JNK signaling pathways. These data indicate that KGF induced FAS and SCD-1 through a PI3K and JNK/SREBP-1 pathway in H292 cells. However, there is a time delay between the activation of PI3K, JNK, and SREBP-1 and the final increases in FAS and SCD-1. Undoubtedly, there are numerous intervening steps that have not been defined. In addition, there are likely additional proteins regulated as part of lipogenesis that have not been identified. In lipogenesis in adipocytes, >1,259 genes change by >3-fold in expression, and there may be >100 transcription factors and signaling molecules involved in the process (51). Hence, lipogenesis is very complex, and concordance in some findings does not mean that there will not be discordance in other findings.

The current model of SREBP-1 regulation holds that the 125 kDa SREBP-1 precursor is anchored to intracellular membranes as a complex with SCAP. SCAP acts as a sterol sensor. When cells are deprived of sterols, SCAP is activated and escorts SREBP to the Golgi complex. In the Golgi, SREBPs are activated by sequential proteolytic cleavage by two proteases, site 1 protease and site 2 protease. When cells are overloaded with sterol, the SCAP-SREBP complex fails to move to the Golgi and SREBPs are not processed (25). FAS and SCD have been shown to be regulated by SREBP-1. In the liver, the effects of insulin on SREBP-1 expression involved mainly the PI3K pathway (37). However, glucose-induced maturation of SREBP-1 was not prevented by the inhibition of PI3K or MAPK; this suggests that these pathway are not involved (40). Our results indicate that the JNK and PI3K signaling pathways are involved in the KGF induction of SREBP-1. Addition of the specific JNK inhibitor SP600125 and the specific PI3K inhibitor LY294002 abolished the induction of SREBP, whereas the ERK inhibitor PD98059 did not. However, the mechanism of how PI3K and JNK signaling leads to SREBP activation is not known. In general, SREBP stimulation is thought to require some endogenous lipid ligand. If KGF induces the production of a lipid ligand, it has not been identified to our knowledge. Nevertheless, this process of growth factor stimulation of proliferation and lipogenesis through the PI3K and SREBP pathways is likely a general phenomenon and has also been reported for the stimulation of normal human fibroblasts by platelet-derived growth factor (PDGF) (52). The PDGF effect on SCD was also blocked by exogenous sterols and a PI3K inhibitor and was unaffected by an ERK inhibitor. Because exogenous sterols blocked the formation of mature SREBP stimulated by KGF or PDGF, the proteolytic processing of SREBP is activated, not just the phosphorylation of the mature SREBP. In addition, it is possible that other signaling pathways (e.g., protein kinase C) also affect the induction of SREBP-1 by KGF, but we did not evaluate this possibility.

Because SREBP-1 is a critical transcription factor for SCD-1 and FAS, we wondered whether the inhibition of SREBP-1 would block the induction of the two enzymes. Inhibition of SREBP-1 blocked the induction of SCD-1 and FAS by KGF, which suggests that induction of lipogenesis by KGF requires SREBP-1. In addition, KGF-induced activation of the SCD-1 promoter required a SREBP binding site, and the addition of cholesterol to inhibit SREBP activation blocked the effect of KGF on FAS and SCD-1. Our data indicate that KGF induces SCD-1 and FAS through a SREBP pathway and that this pathway is necessary for activation.

In adipocytes, three classes of transcription factors, including C/EBPs, PPARγ, and SREBPs, are critical in lipogenesis (22, 23). Regulation of lipogenesis in type II cells is similar to that of lipogenesis in adipocytes. Our previous results in type II cells indicate that KGF induces SREBP-1c, C/EBPα, and C/EBPδ but not C/EBPβ or PPARγ (5). C/EBPα and C/EBPδ were upregulated by KGF from day 2 to day 6 in H292 cells. In adipocytes, PPARγ is an important regulator of lipogenesis. Its activity can be upregulated by PPARγ agonists (53). The mRNA or protein levels of PPARγ were not changed in type II cells by KGF (5). In H292 cells, KGF did not stimulate the expression of PPARγ (data not shown). It appears that PPARγ is not critical for lipogenesis in type II cells or in H292 cells. In H292, KGF increases fatty acid synthesis, and this process also involves SREBP-1, C/EBPα, and C/EBPδ but apparently not PPARγ. However, direct studies with PPARγ agonists were not done.

MAPK and PI3K pathways are important pathways involved in the proliferation and differentiation of some cells. The inhibitors of these two signaling pathways can block the proliferation of type II cells induced by KGF

(12). Our data indicate that KGF activated all of these signaling proteins, including ERK, JNK, Akt, and S6K, rapidly. Phosphorylation of these proteins peaked 10 min after stimulation. Previous results showed that KGF stimulated the phosphorylation of two members of the MAPK family, ERK-1 (p44) and ERK-2 (p42) (12). However, our data show that KGF activated only ERK-2, and not ERK-1, in the H292 cell line. In bronchial epithelial cells (16HBE cells), this antibody showed activation of both ERK-1 and ERK-2 by KGF (data not shown); hence, the observation in H292 cells was not attributable to the antibody used in these experiments.

The JNK signaling pathway belongs to the MAPK family. Activated JNK may directly phosphorylate transcription factors. JNK is also involved in cell proliferation and apoptosis (54–56). The JNK family comprises JNK1, JNK2, and JNK3. JNK1 and JNK2 show a broad tissue distribution, whereas JNK3 is expressed predominantly in neurons but also in cardiac muscle and testes. Our results suggest that KGF activates JNK1 (46 kDa). Because the antibody against phospho-JNK that we used does not cross-react with JNK2 (55 kDa), we do not know whether JNK2 is activated by KGF. Akt, the PI3K distal effector, has been implicated in lipogenesis and in protection against lung injury (57). S6K, the other PI3K distal effector, is suggested to be involved in wound healing of corneal epithelial cells (58). Activation of S6K is also important for cell proliferation. S6K has been shown to be involved in cell cycle progression, gene transcription, and protein translation (59). Our data indicate that both isoforms of S6K, p70 and p85, are activated by KGF.

In summary, KGF activates the ERK, JNK, and PI3K signaling pathways in H292 cells. KGF induces the important transcription factors involved in lipogenesis, including C/EBPα, C/EBPβ, C/EBPδ, and SREBP-1, and two key lipogenic enzymes, SCD-1 and FAS. KGF signaling pathways for lipogenesis involve both JNK and PI3K and the subsequent activation of SREBP-1. Cholesterol blocks the effect of KGF on FAS through the SREBP-1 pathway. Finally, overexpression of dominant negative SREBP-1 inhibits the KGF effect on FAS and SCD-1, and overexpression of active SREBP-1 stimulates the expression of SCD-1 and FAS. Hence, KGF induces FAS and SCD-1 through the KGF/KGFR/JNK and PI3K/SREBP-1 pathways. The transcription factors activated and the enzymes of fatty acid synthesis induced are similar regardless of whether the fatty acids are used for membrane biogenesis in H292 cells or pulmonary surfactant production in alveolar type II cells.

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