Platelet-derived growth factor stimulates membrane lipid synthesis through activation of phosphatidylinositol 3-kinase and sterol regulatory element-binding proteins

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We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with platelet-derived growth factor (PDGF) using cDNA microarrays. 103 significantly regulated transcripts that had not been previously linked to PDGF signaling were identified. Among them, a cluster of genes involved in fatty acid and cholesterol biosynthesis, including steroyl-CoA desaturase (SCD), fatty acid synthase, and hydroxymethylglutaryl-CoA synthase (HMGCS), was up-regulated by PDGF after 24 h of treatment, and their expression correlated with increased membrane lipid production. These genes are known to be controlled by sterol regulatory element-binding proteins (SREBP). PDGF increased the amount of mature SREBP-1 and regulated the promoters of SCD and HMGCS in an SREBP-dependent manner. In line with these results, blocking SREBP processing by addition of 25-hydroxycholesterol blunted the effects of PDGF on lipogenic enzymes. SREBP activation was dependent on the phosphatidylinositol 3-kinase (PI3K) pathway, as judged from the effects of the inhibitor LY294002 and mutation of the PDGF receptor tyrosines that bind the PI3K adaptor subunit p85. Fibroblast growth factors (FGF-2 and FGF-4) and other growth factors mimicked the effects of PDGF on NIH3T3 and human fibroblasts. In conclusion, our results suggest that growth factors induce membrane lipid synthesis via the activation SREBP and PI3K.

Growth factors stimulate cell division by promoting the transition from G₀ to S in the cell cycle. One such factor, platelet-derived growth factor (PDGF), was initially characterized as a potent mitogen for fibroblasts and other cells of mesenchymal origin (1). PDGF consists of a family of dimeric ligands that are produced from four different genes, namely PDGF-A, -B, -C, and -D. They exert their cellular effects by binding to and dimerizing PDGF α- and/or β-receptors, which belong to the receptor-tyrosine kinase family (2, 3). The PDGF isoforms play important roles during embryonic development and in wound healing (1, 4). In addition, an improper activation of PDGF receptors contributes to the development of fibrosis, atherosclerosis, and certain forms of cancer (1).

Fibroblast growth receptors, including the PDGF receptors, use a common set of signal transduction cascades (5). One of the best characterized pathways involves the sequential activation of Ras, RAF, MEK, and the ERK mitogen-activated protein kinase, which phosphorylates multiple substrates, many of which are transcription factors. A second important signaling event depends on the re-localization of phosphatidylinositol 3-kinase (PI3K) to the plasma membrane, which leads to the production of phosphatidylinositol 3,4,5-trisphosphate and the activation of downstream effectors such as protein kinase B (PKB, also called Akt), and p70 S6 kinase (6). Both ERK and PI3K play essential roles in the mitogenic response to growth factors (1, 7). Recently, Jones and Kazlauskas (8) proposed that the G₀ to S transition requires two waves of signaling; one that follows immediately after growth factor stimulation and is mediated by ERK and c-Myc, and a second wave that occurs about 8-h later and overlaps temporally with the cell cycle program (8). PI3K activation appeared to be essential during the second phase.

Most studies have focused on the mechanism by which growth factors stimulate DNA synthesis and mitosis. Besides these essential aspects of the cell cycle, cell proliferation also requires the replication of all other cell constituents. It was recently suggested that this process is in part mediated by PI3K, which was shown to stimulate protein synthesis and to contribute to cell size increase (9). The mechanisms of production of other cell constituents, such as membrane lipids, during proliferation are not well understood.

Lipogenic enzymes are controlled at multiple levels, such as gene expression, protein stability, and enzymatic activity. One of the most important groups of transcription factors that govern the expression of these enzymes is constituted by sterol regulatory element-binding proteins, i.e. SREBP-1α, -1c, and -2 (10, 11). SREBP-1c differs from SREBP-1α only in the N-terminal hydroxymethylglutaryl-CoA synthase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; RPLP0, ribosomal protein, large, P0; SCD, stearoyl-CoA desaturase; SLC20A1, solute carrier family 20, member 1; SREBP, sterol regulatory element-binding proteins; ERK, extracellular signal-regulated kinase.

This paper is available online at http://www.jbc.org
minus, which is encoded by an alternative exon 1 of the SREBP-1 gene. These factors are produced as precursor proteins embedded in the endoplasmic reticulum membrane as a complex with the cholesterol sensor SCAP (10, 12). SCAP is required to escort SREBP in the Golgi apparatus, where two specific proteases, S1P/SKI-1 and S2P, cleave SREBP, releasing a soluble active transcription factor that up-regulates the expression of genes involved in cholesterol and fatty acid biosynthesis, as well as NADPH production (13, 14). In a negative feedback mechanism, cholesterol promotes the binding of SCAP to INSIG-1 and -2, which prevents SREBP movement to the Golgi and its processing (12, 15). Using mutant mammalian cell lines, Goldstein et al. (16) have demonstrated that several components of the SREBP pathway are required for long term proliferation, unless the cells are provided with key lipid metabolites, such as oleic acid, cholesterol, and mevalonate (16). Whether SREBP is regulated during cell growth is, however, not known.

In this report, we analyzed changes in gene expression in fibroblasts stimulated by PDGF, using cDNA microarrays. After 1–4 h of stimulation, we observed altered expression mainly of previously characterized immediate early genes (17, 18). Among the genes induced after longer periods of PDGF treatment were genes involved in lipid biosynthesis, which required the activation of SREBP transcription factors, and correlated with increased membrane lipid synthesis. This cascade of events was also triggered by other growth factors and was found to depend on the activation of the PI3K pathway.

MATERIALS AND METHODS

Cell Culture and Reagents—AG01518 foreskin fibroblasts (Coriell Institute for Medical Research, Camden, NJ) from passages 11–19 were cultured in modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum and glucose. For starvation and treatments, cells were washed and incubated in medium containing glucose and either used for total lipid determinations (see below), or processed as described (20). Whether SREBP is regulated during cell growth is, however, not known.

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SREBP Activation by Growth Factors

We evaluated whether activation of the SREBP pathway is triggered by growth factors and whether SREBP-dependent gene expression is regulated by growth factors. We used AG01518 foreskin fibroblasts (Coriell Institute for Medical Research, Camden, NJ) from passages 11–19. Subconfluent AG01518 fibroblasts were transfected using LipofectAMINE PLUS (Invitrogen), and HepG2 with calcium phosphate precipitates, as described (20). The indicated promoters cloned in the pGL2-Basic luciferase reporter (Invitrogen) were cotransfected with pBE-chEF1-RUS’ (β-galactosidase reporter, Invitrogen, 125 ng) and pTKCIII as carrier DNA. Cells were washed, incubated in serum-free medium for 36 h, in the presence or absence of PDGF-BB (50 ng/ml), lysed, and processed as described (20). Data are presented as the average ratio between the luciferase activity and the β-galactosidase content, with standard deviations from duplicate transfections. All experiments were repeated at least three times.

Lipid Synthesis Measurements—Subconfluent AG01518 fibroblasts were starved for 16 h, and treated with or without PDGF-BB (50 ng/ml) for 20 h. [1-14C]acetate (5Ci/ml) was then added, and incubation was prolonged for 4 h. Cells were washed, trypsinized, centrifuged, and resuspended in PBS, and lipids were extracted with chloroform/methanol 2:1. Four volumes of 0.9% NaCl were added to separate the two phases. The chloroform phase was dried under a nitrogen flux, and either used for total lipid determinations (see below), or hydrolyzed for 1 h at 80 °C in 1 ml of methanol containing 1 m KOH. Cholesterol and other neutral lipids were extracted with hexane. The lower phase was then neutralized and mixed with chloroform to extract fatty acids. Fractions were dried and analyzed by thin layer chromatography using silica gel 60 plates (Merck), which were developed with hexane/diethyl ether/acetic acid (87:20:1, vol/vol), in the presence of standards (oleic acid, stearic acid, cholesterol, lanosterol, and squalene, all from Sigma). Unsalted fatty acids were separated from saturated fatty acids by plates impregnated with AgNO3, as described (21). Radioactivity was quantified using a Fuji phosphorimager, and standards were visualized in iodine vapors or by charring. The radioactivity of each fraction was also measured in a β counter. The analysis of non-hydrolyzed lipids was performed as described (22). The initial chloroform extract was loaded on an anion exchange column (Supleco). Neutral lipids were eluted with chloroform/isopropanol alcohol (2:1) and phospholipids with methanol. Each fraction was dried and analyzed by thin layer chromatography. The plates were developed in chloroform/methanol (2:1) only to 2 cm from bottom edge, followed by development with chloroform/ethanol/triethylamine/water (30:34:30:8) for 4 cm, thoroughly dried, and then developed in hexane/diethyl ether (50:50). Spots were visualized as above, and identified by comparison with migration standards: phospholipid mix (Supleco), triolein, cholesterol oleate, cholesterol, lanosterol (all from Sigma). Total protein contents of the initial samples were measured using the BCA kit (Pierce) and used to normalize the results.

Immunoprecipitations and Western Blots—Subconfluent AG01518 cells were starved as described above and stimulated with PDGF-BB for the indicated period of time. N-Acetyl-leucyl-leucyl-norleucinal (25 μg/ml, Calbiochem) was added to the medium 2 h before harvest (23). Cells were washed, scraped in 2 ml of PBS, centrifuged, and lysed (1% Triton, 0.2% sodium deoxycholate, 140 mM NaCl, 10% glycerol, 50 mM Tris, pH 8, 1 mM Pefabloc, 1 mM Trasylol, 1 mM NaF, 25 μM N-Acetyl-leucyl-leucyl-norleucinal). Lysates were immunoprecipitated by using polyclonal antibody raised against SREBP-2 and cleared by centrifugation. SREBP-2 was immunoprecipitated from 1 mg of total proteins with 10 μg of ID2 antibody and protein G-Sepharose (Amersham Biosciences). Immunoprecipitates or equal amounts of total protein extracts were analyzed by SDS-PAGE followed by Western blotting.
## SREBP Activation by Growth Factors

### Gene regulation by PDGF in AG01518 fibroblasts

AG01518 fibroblasts were stimulated with PDGF-BB for 1, 4, 10, or 24 h and analyzed using microarrays as described under “Materials and Methods.” The value that corresponds to the largest change (peak) is shown with S.E. A more detailed table including all uncharacterized ESTs is available at www.icp.ucl.ac.be/mexp/pdgf.

| Name | Synonyms/description | Ensembl reference | Peak | Published |
|------|----------------------|-------------------|------|-----------|
|      |                      |                   | Time | Value ± S.E. | PDGF | SREBP |
| **Transcription factors** | | | | | |
| NB4A1 | Nuclear receptor | ENSG00000123358 | 1 | 8.20 ± 0.39<sup>a</sup> | +<sup>b</sup> |
| EGR3 | Pilot, early growth response 3 | ENSG00000179388 | 1 | 4.44 ± 0.54 | + |
| JUNB | Immediate early response 3 interleukin-3 promoter | ENSG00000171223 | 1 | 4.30 ± 0.49 | + |
| IER3 | Transcriptional activator | ENSG00000171243 | 1 | 2.83 ± 0.69 | + |
| NFIL3 | C1S Complement component | ENSG00000180599 | 1 | 1.87 ± 0.67 | + |
| TIEG | TGF-β inducible early protein | ENSG00000155090 | 1 | 2.46 ± 0.28 | + |
| IRF1 | Interferon regulatory factor 1 | ENSG0000125347 | 1 | 2.30 ± 0.32<sup>a</sup> | + |
| JUN | Sterol regulatory element-binding factor 2 | ENSG00000100152 | 24 | 2.11 ± 0.47<sup>a</sup> | + |
| MYC | Inhibitor of DNA binding 2 | ENSG000000115738 | 24 | 0.52 ± 0.03 | + |
| SREBP2 | Sterol regulatory element-binding factor 2 | ENSG00000138764 | 4 | 0.52 ± 0.03 | + |
| ID2 | Nuclear receptor inhibitor 2 | ENSG00000137285 | 24 | 2.99 ± 0.35 | + |
|  | Tubulin α 8 | ENSG00000070490 | 24 | 5.19 ± 0.44 | + |
| TUBA1 | Tubulin α 1 | ENSG00000167552 | 24 | 4.94 ± 0.61 | + |
| TUBB | Tubulin β | ENSG00000137285 | 24 | 2.99 ± 0.35 | + |
| TUBB5 | Tubulin β 5 | ENSG00000158395 | 24 | 2.85 ± 0.30 | + |
| KRT3A | Keratin, hair acidic 3A | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| Cytoskeleton | | | | | |
| TUBA8 | Cytokeratin 15 | ENSG00000137285 | 24 | 2.99 ± 0.35 | + |
| TUBB | Moesin | ENSG00000158395 | 24 | 2.85 ± 0.30 | + |
| TUBB5 | Keratin, hair, acidic, 1 | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| TUBA3 | Keratin, hair, acidic 1 | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| TUBB | Actin-related protein 3 | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| KRT3A | Paxillin | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| TUBA8 | Leuvin | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| TUBB | Connexin 43 | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
| TUBB | Connexin 43 | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
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| TUBB | Connexin 43 | ENSG00000106333 | 24 | 2.03 ± 0.31 | + |
Fatty acid synthesis
SCD Stearoyl-CoA desaturase 2A4
FASN Fatty acid synthase 2A4
ACAT2 Acetyl-Coenzyme A acetyltransferase 2
ACLY ATP citrate lyase
MGLL Lipophospholipase-like
BACH Long chain acyl-CoA hydrolase
PKM2 Pyruvate kinase M1
PKLR Pyruvate kinase type L
PPAP2B Phosphatic acid phosphatase type 2B

Other metabolic pathways
SFRS7 9G8 splicing factor
SFRS3 pre-mRNA splicing factor
ANPEP Aminopeptidase N
ENO1 α-Enolase
NP Purine nucleoside phosphorylase
TGM2 Transglutaminase
UBE2N Ubiquitin-conjugating enzyme E2N
CSC Cathepsin C
MT1 Metallothionein 1L
MT1K Metallothionein-1K
HYOU1 Oxygen-regulated protein ORP150
NP Purine nucleoside phosphorylase
SFRS7 9G8 splicing factor
PKM2 Pyruvate kinase M1
PKLR Pyruvate kinase type L
ALDH1A1 Aldehyde dehydrogenase 1

Stress & redox
DUSP1 MKP1 phosphatase
ARHB Rho B
DUSP4 Dual specificity protein tyrosine phosphatase
DUSP5 Dual specificity protein tyrosine phosphatase
STK15 Serine/threonine kinase 15
DUSP6 PST1 phosphatase
SH3BP5 SH3-domain binding protein 5
SGK Serum/glucocorticoid regulated kinase

GADD45A Growth arrest and DNA-damage-inducible α
MT1 Metallothionein 1L
MT1B Metallothionein 1B
MT3 Metallothionein-III
MT1K Metallothionein-1K
GLRX Glutaredoxin
HYOU1 Oxygen-regulated protein ORP150
DDB2 DNA damage-binding protein 2
GADD45B Growth arrest and DNA damage inducible
TXNIP Thioredoxin interacting protein

Miscellaneous
NOL5A Nucleolar protein 5A
DDX17 Probable RNA helicase
HISTH2A Histone H2A.L
CTSK Cathepsin K
CTSF Cathepsin F
ADM Preproadrenomedullin
DAAM2 Dishevelled-3, activator of morphogenesis
PGS11 Pregnancy-specific glycoprotein 13
PMP22 Peripheral myelin protein 22, gas3
RDC1 Orphan G protein-coupled receptor

* Data were confirmed by Northern blotting or quantitative PCR.
* +, genes that were previously shown to be regulated by PDGF or SREBP.

RESULTS

Regulation of Gene Expression by PDGF in Fibroblasts—In order to gain knowledge about gene regulation by PDGF, we performed microarray experiments with Hver1.2.1 chips, which were provided by the Sanger Institute Microarray facility, and contain 9932 single-stranded cDNAs. Human foreskin fibroblasts were starved in serum-free medium for 16 h, and then stimulated by PDGF-BB (10 ng/ml) for 1, 4, 10, or 24 h. Cells that were left untreated for the same period of time were used as control for each time point. RNA was extracted, and used as a template to synthesize cDNA in the presence of dCTP-Cy3 or dCTP-Cy5. These probes were used for competitive hybridization of microarrays, as described under "Materials and Methods." We selected genes that were significantly regulated by PDGF-BB by at least 1.75-fold, at one or more time points. We found 127 genes that fulfilled these criteria, usually at only one time point. They included 24 that had previously been linked to PDGF signaling in various models. Notably, PDGF-AB elicited an almost identical transcriptional program (data not shown). To further validate our data, the expression of 18 genes was checked by Northern blotting and/or quantitative PCR. Overall, we found a close concordance between cDNA microarray data and PCR or Northern blot data.

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with the microarray analysis underestimating the amplitude of each specific gene response.

Of the 127 genes regulated by PDGF-BB, 18 genes have no known cellular function, while the others could be classified into 12 clusters (Table I). Transcription factors constituted the most prominent class of genes regulated after 1 h of stimulation by PDGF and included well characterized proto-oncogenes such as c-Jun, c-JunB, and c-Myc. Several molecules involved in signaling were also up-regulated early, including the dual specificity phosphatases DUSP-1, -4, -5, and -6, which have been shown to inhibit ERK activation, in a negative feedback loop (24). Stimulation of fibroblasts for 24 h affected the expression of genes connected with the extracellular matrix and cytoskeleton, which might contribute to the effects of PDGF on connective tissue contraction. PDGF treatment also increased the expression of two phosphate transporters, SLC20A1 and SLC20A2, which may account for the old observation that growth factors stimulate phosphate uptake by fibroblasts (25).

The largest group comprised 28 genes connected to metabolism, including 17 genes implicated in the biosynthesis of fatty acids or cholesterol. Stearoyl-coenzyme A desaturase (SCD), which catalyzes the transformation of saturated fatty acids into monounsaturated fatty acids, was the gene induced to the highest extent by PDGF after 24 h (8.0 ± 2.2-fold). It was also regu-
lated by PDGF in NIH3T3 and AG01523 fibroblasts (data not shown). This cluster also included fatty acid synthase (FAS), hydroxymethylglutaryl-coenzyme A synthase (HMGCS), and reductase (HMGCR). Altogether, our microarray data point to a major effect of PDGF on lipid biosynthesis pathways.

**Stimulation of Membrane Lipid Production by PDGF**—We next investigated whether the effects of PDGF on the expression of lipogenic and cholesterogenic enzymes correlated with a change in lipid biosynthesis in AG01518 fibroblasts. To analyze newly synthesized lipids, cells were labeled with [14C]acetate for 4 h before harvest, after which lipids were extracted, hydrolyzed, and analyzed by thin layer chromatography. In line with our microarray results, PDGF had a striking effect on unsaturated fatty acid synthesis, and a moderate effect on the production of cholesterol and saturated fatty acids (Fig. 1A). To determine if fatty acids were incorporated into phospholipids or triglycerides, we also analyzed non-hydrolyzed lipid extracts. We observed that PDGF stimulated the synthesis of phospholipids and free sterols, i.e. cholesterol and lanosterol, a cholesterol precursor (Fig. 1B). Triglyceride levels were not affected.

**Fig. 3. Stimulation of SREBP-driven promoters by PDGF.** A, NIH3T3 cells were transfected with constructs consisting of the indicated gene promoters cloned in front of a luciferase reporter. Cells were then cultured in serum-free medium in the presence or absence of PDGF-BB for 36 h. Luciferase content was measured from sample lysates. B, cells were co-transfected with a dominant negative SREBP-1 transgene (SREBP Δ90, 100 ng) or a control plasmid (mock), and a reporter construct containing the HMGCS promoter, the SCD2 promoter, or a mutated version of the SCD2 promoter in which the sterol-responsive element has been disrupted (SCD2-mutA) (32). C, HepG2 cells were co-transfected with the PDGF β-receptor (20 ng) and either a luciferase reporter driven by the HMGCS promoter or by multiple STAT3-responsive elements (APRE) and treated as above.
by PDGF, and cholesterol esters were not detectable, indicating that PDGF did not promote fat storage, but rather membrane lipid synthesis. Importantly, the proportions of the different types of membrane lipids were not modified by PDGF. Altogether, these observations likely reflect the duplication of cellular membranes, which should accompany the mitogenic effect of PDGF on fibroblasts. Our data also suggest that PDGF affects the saturation index of fatty acids in these cells.

**Activation of SREBP upon PDGF Treatment**—To gain insights into the mechanism by which PDGF regulated the expression of lipid biosynthesis enzymes, we searched for common transcription factors that could control the corresponding genes. Based on published work (10, 11, 14), thirteen of them, including SCD, HMGCR, and HMGCS, are known targets of a group of closely related transcription factors, the sterol regulatory element-binding protein (SREBP)-1 and -2 (Table I). In addition, 24-dehydrocholesterol reductase (DHCR24) encodes an enzyme that lies in the cholesterol biosynthesis pathway and is therefore a likely target for SREBP, which has been suggested to regulate all genes involved in that process (26).

Fibroblast stimulation with PDGF-BB also enhanced the expression of two other SREBP target genes, i.e. the cytochrome c (Table I), and glucose-6-phosphate dehydrogenase genes (1.71 ± 0.32). Based on quantitative PCR experiments, the low density lipoprotein receptor gene was also up-regulated about 5-fold (data not shown) as previously published (27). Altogether, PDGF had a significant effect on all genes that are known to be controlled by SREBP-1 and -2 and that are represented on the Hver1.2.1 arrays.

Our microarray data indicated that PDGF regulated the SREBP-2 gene, itself. To analyze the effect of PDGF on the expression of SREBP genes, we set up a quantitative PCR experiment with probes specific for each SREBP family member. The results presented in Fig. 2A demonstrated that the abundance of the SREBP-1c and SREBP-2 transcripts were increased upon PDGF stimulation, at the same time or slightly after those encoding SCD and HMGCR. The alternative transcript encoding SREBP-1a was expressed at a low level in AG01518. We next checked whether PDGF could influence the expression of other components of the SREBP activation pathway. In Western blot experiments, we observed that PDGF did not modify the levels of the SCAP regulatory protein and the proteases S1P/SKI-1 and S2P (data not shown). INSIG-1, which acts as an inhibitor of the pathway in a negative feedback loop, was induced 4.5-fold by PDGF stimulation for 24 h, as one would expect if SREBP is activated (12, 15). INSIG-2 was not significantly affected (not shown). Altogether, our data suggested that PDGF regulated INSIG-1, SREBP-1c, and -2 genes. Since these genes are controlled by SREBP itself in a feedback manner, it is likely that their regulation in AG01518 cells is a consequence of the post-translational activation of SREBP by PDGF (15, 28, 29).

To test if PDGF treatment resulted in an increase in the amount of mature SREBP, we performed Western blots with specific monoclonal antibodies. The level of mature SREBP-1 was significantly increased as early as 3 h after PDGF treatment and reached a peak after 18 h (Fig. 2B). The SREBP-1 precursor protein was increased after 12 h at the same time as

![Fig. 4. Sterols block SREBP processing and the regulation of lipid metabolism genes by PDGF. AG01518 fibroblasts were starved in medium containing 0.1% bovine serum albumin, and stimulated with PDGF-BB in the presence of a mixture of sterols (cholesterol 10 µg/ml and 25-hydroxycholesterol 1 µg/ml) or carrier alone (0.2% ethanol), as indicated, for 24 h. A, SREBP-1 and immunoprecipitated SREBP-2 were analyzed by Western blotting with specific antibodies. B, RNA was extracted and analyzed by Northern blotting (10 µg/lane) as described (43), with the radioactive probes corresponding to SCD, acetyl-coenzyme A acetyltransferase 2 (ACAT2), SLC20A1 phosphate transporter, or β-actin. C, HepG2 cells were transfected with the PDGFβ receptor and the indicated luciferase reporter constructs and processed as described in Fig. 3. As a control, cells were transfected with a construct encoding the mature nuclear form of SREBP-1 (nSREBP1).](image-url)
SCD, in line with our observations at the RNA level. Two SREBP-2 species were immunoprecipitated from fibroblasts lysates: a 130-kDa protein that corresponds the precursor form, and a 95-kDa protein, which may represent a modified form of mature SREBP-2 (Fig. 2C). The latter was increased by PDGF stimulation for 12 h. The SREBP-2 precursor protein was also slightly increased. In conclusion, PDGF induces a rapid increase in mature SREBP-1 protein, which is followed by an increase in SREBP-2 and SCD. In addition, the amount of SREBP precursor proteins also increased upon prolonged PDGF stimulation.

**Regulation of SCD and HMGCS Promoters by PDGF in a SREBP-dependent Manner**—To investigate the effect of PDGF on SREBP transcriptional activity, we performed luciferase reporter experiments with the gene promoters of HMGCS, FAS, and SCD, which contain SREBP binding sites (12, 30). A construct containing the promoter of the low density lipoprotein receptor gene was also used because it had been shown to be regulated by PDGF (31). In NIH3T3 mouse fibroblasts, PDGF-BB enhanced the activity of all four promoters (Fig. 3A). In mice, two ubiquitous SCD isoforms have been described, SCD1 and SCD2, both of which were regulated by PDGF at the promoter level (not shown). We next used a dominant negative variant, SREBPΔ90, which correlates to the mature form of SREBP-1 devoid of its N-terminal transactivation domain, and which neutralizes both SREBP-1 and -2 (32). When co-expressed, this construct inhibited the stimulation of SCD2 and HMGCS promoters by PDGF (Fig. 3B). In addition, a SCD2 promoter in which the SREBP binding site has been mutated did not respond to PDGF. Similar results were obtained in HepG2 cells transfected with the PDGF β-receptor (Fig. 3C and data not shown). As a control, SREBPΔ90 did not affect the regulation by PDGF of a STAT3-responsive promoter containing multiple APRE sites.

To further demonstrate that PDGF activated lipogenic gene promoters in a SREBP-dependent manner, we used 25-hydroxycholesterol, which prevented the cleavage of SREBP-1 and -2 precursors in human fibroblasts (Fig. 4A), as shown previously in many cell lines (16). This compound abrogated the regulation of SCD and ACAT2, another SREBP target gene, by PDGF (Fig. 4B). By contrast, it did not interfere with the induction of the phosphate transporter SLC20A1. Addition of 25-hydroxycholesterol also blocked the PDGF-induced stimulation of the HMGCS promoter, but not that of the APRE promoter (Fig. 4C). As a further control, we showed that sterols had little effect on the activation of the HMGCS promoter by co-transfection of a construct encoding an active form of SREBP-1, which does not require endogenous processing (Fig. 4C). In conclusion, our data suggested that SREBP processing was required for the transcriptional effects of PDGF on lipid metabolism enzymes.

**PI3K Mediates the Effects of PDGF on SREBP and Lipid Biosynthesis Genes**—As mentioned above, two major signaling mediators have been linked to growth stimulation by PDGF, i.e. PI3K and ERK. To assess if these kinases were involved in the regulation of SREBP target genes by PDGF, we treated cells with specific small inhibitory molecules, LY294002 and U0126 that target PI3K and MEK, respectively. Although it completely blocked the phosphorylation of ERK (data not shown), U0126 had no effect on the stimulation of SCD and HMGCS expression by PDGF, and only partially inhibited HMGCR expression (Fig. 5). By contrast, LY294002 was as effective as sterols in blocking the effect of PDGF. In addition, inhibition of mTOR, which has also been shown to control cell size, by rapamycin partially inhibited the up-regulation of SCD, HMGCR, and HMGCS by PDGF (Fig. 5). LY294002 also blocked the PDGF-induced increase in the amount of mature SREBP-1 protein, indicating that PI3K acted upstream of SREBP-1 (Fig. 6A). As expected, the same inhibitor decreased the biosynthesis of unsaturated fatty acids and cholesterol (Fig. 6B). The production of saturated fatty acids was also affected to a lesser extent.

The activation of PI3K by PDGF requires the binding of the regulatory subunit p85 to phosphorylated tyrosines 740 and 751 of the human PDGF β-receptor. In HepG2 cells transfected with the Y740/751F PDGF β-receptor, PDGF elicited a reduced activation of the HMGCS promoter, compared with cells transfected with the wild-type receptor (Fig. 6C). Taken together, these findings support the notion that PI3K is an important mediator of the activation of lipid biosynthesis by PDGF.

**Stimulation of SREBP Target Gene Expression by other Growth Factors**—If our observations reflect membrane lipid synthesis associated with cell growth, stimulation of fibroblasts with other growth factors would be expected to recapitulate the effects of PDGF. We first measured the activity of the SCD2 and HMGCS promoters in a luciferase assay using a NIH3T3 clone stably transfected with the epidermal growth factor (EGF) receptor. Under these experimental conditions, EGF, fibroblast growth factor (FGF)-4 and high doses of insulin were at least as potent as PDGF-BB (Fig. 7A). In line with these results, treatment of human fibroblasts with FGF-4, insulin, as

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2 J. B. Demoulin, unpublished data.
well as FGF-2 and PDGF-AB, induced the expression of SCD, and increased the amount of mature SREBP-1, as demonstrated by Western blotting (Fig. 7B). PDGF-BB was, however, more potent than other growth factors in these cells. These results were mirrored at the RNA level, as illustrated by the regulation of SCD, HMGCR, and HMGCS by FGF-4 (Fig. 7C).

**DISCUSSION**

In the present report, we identified a sequence of events that lead to the synthesis of cellular membranes when fibroblasts enter the cell cycle. This pathway involves the PI3K-dependent activation of SREBP by growth factors, such as PDGF-BB. The role of SREBP was demonstrated by the following observations: (i) the transcriptional program elicited by PDGF presented an SREBP signature, with virtually all known SREBP target genes up-regulated by PDGF; (ii) PDGF stimulated the activity of SREBP-driven promoters, which could be inhibited by a dominant negative SREBP construct; (iii) PDGF treatment quickly increased the amount of mature SREBP-1; and (iv) blocking SREBP processing by sterols prevented PDGF action.

A role for SREBP in membrane lipid production was recently demonstrated in *Drosophila*, where SREBP processing is directly controlled by phospholipids (33). In mammalian cells, the importance of the SREBP pathway in long term proliferation was suggested by analysis of mutant CHO cell lines which lack SCAP, SIP, or S2P and are auxotrophic for cholesterol, mevalonate, and unsaturated fatty acids (16, 23). Here, we show that SREBP-1 is activated early after growth stimulation, before mitosis. Importantly, our study does not exclude the existence of additional mechanisms of lipid metabolism regulation by growth factors, independently of SREBP.

Since SCD and FAS are regulated to a higher extent by PDGF, and the corresponding promoters are more sensitive to SREBP-1 compared with SREBP-2 (30), SREBP-1 might play a predominant role, which is compatible with its kinetics of activation after PDGF stimulation. However, the phenotype of SREBP-1-deficient mice, which are viable, argues against a unique role for SREBP-1 in cell growth in vivo (13). By contrast, inactivation of the gene encoding SREBP-2 results in early embryonic lethality (13). Whether PDGF also activates SREBP-2 should be further clarified. We observed the appearance of an intermediate molecular weight SREBP-2 protein, which was recognized by an antibody raised against mature SREBP-2, and disappeared upon addition of sterols, as one would expect from the *bona fide* mature form of SREBP-2.

Recently, it was demonstrated that SREBP proteins are substrates for multiple post-translational modifications, including phosphorylation, acetylation, ubiquitination, and sumoylation, some of which significantly affect SREBP migration when analyzed by SDS-PAGE (20, 34, 35).

Our data point to the PI3K pathway as a link between PDGF receptors and activation of SREBP, based on the effect of LY294002 and the consequences of specific receptor mutations. In line with these results, SREBP regulation by insulin in hepatocytes depends on the PI3K pathway (36, 37). Several studies have also stressed the role of ERK in SREBP activation in cell lines and *in vitro* (31, 35, 38). However, the MEK inhibitor U0126 had no effect on SCD and HMGCS regulation by PDGF in fibroblasts. Finally, the effect of rapamycin suggested an involvement of mTOR, which cooperates with PI3K-dependent effectors to phosphorylate p70 S6 kinase and 4EBP1, and
thereby controls protein synthesis and cell size (39). Although cell size increase obviously requires membrane synthesis, to our knowledge, that aspect had not been studied before. Our observations show that the same signaling effectors may control protein synthesis and membrane lipid production in a coordinate manner during cell growth. Further studies will have to determine how growth factor-activated PI3K affects SREBP.

The inhibitory effect of sterols indicated that SREBP activation by PDGF required SREBP precursor processing in a cholesterol-sensitive manner. It is possible that PDGF treatment increases SREBP cleavage, for instance by increasing the transport of cholesterol from the endoplasmic reticulum to the plasma membrane. Alternatively, since active SREBP proteins are quickly degraded, stabilization of mature SREBP by PDGF would produce the same effect. Given the importance of membrane synthesis for cell growth, it is possible that the regulation of the SREBP pathway occurs at multiple levels.

SCD was the gene that showed the largest change in expression after 24 h of PDGF treatment. As a result, PDGF induced more pronounced increase in the production of unsaturated fatty acids compared with saturated fatty acids. The insertion of more rigid fatty acids in the membranes is expected to increase membrane fluidity, and might affect cell responsiveness to various stimuli (40). In addition, it was suggested that SCD is expressed at a higher level in certain tumors compared with normal tissues (41), and that tumors have a higher oleic to stearic acid ratio. Our results imply that SCD induction by activation of growth factor-regulated pathways in tumors might be responsible for these observations. Interestingly, inhibition of SCD delayed tumor development in an in vivo murine model (42). Independently of SCD expression, SREBP activation has also been studied in various tumor cell lines. In that respect, Swinnen et al. (38) reported that overactivation of SREBP-1 by EGF in prostate cancer cells resulted in FAS overexpression and triglyceride accumulation. Based on the present data, we speculate that activation of SREBP by growth factors may play a broader role in tumor cell proliferation, by controlling membrane synthesis. Future work should assess if an increased lipid biosynthesis could contribute to the role of PDGF in the development of other diseases, such as atherosclerosis (1).

In conclusion, our data show that PDGF and other growth factors activate SREBP, which enhances the expression of lipogenic enzymes, leading to the synthesis of new membranes. This sequence of events appears to depend on PI3K activation, providing a link between lipid, protein, and DNA synthesis during cell growth.

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