Oncogenic Ha-Ras Transformation Modulates the Transcription of the CTP:Phosphocholine Cytidyltransferase α Gene via p42/44MAPK and Transcription Factor Sp3*

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Marica Bakovic‡‡, Kristin Waite§§, and Dennis E. Vance¶

From the Department of Biochemistry and Canadian Institutes of Health Research Group on Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

We have shown previously that expression of the murine CTP:phosphocholine cytidyltransferase (CT) α gene is regulated during cell proliferation (Golfman, L. S., Bakovic, M., and Vance, D. E. (2001) J. Biol. Chem. 276, 43688–43692). We have now characterized the role of Ha-Ras in the transcriptional regulation of the CTα gene. The expression of CTα and CTβ2 proteins and mRNAs was stimulated in C3H10T1/2 murine fibroblasts expressing oncogenic Ha-Ras. Incubation of cells with the specific inhibitor (PD98059) of p42/44MAPK decreased expression of both CT isoforms. Transfection of fibroblasts with CTα promoter-luciferase constructs resulted in an 2-fold enhanced luciferase expression in Ha-Ras-transformed, compared with nontransformed, fibroblasts. Electromobility shift assays indicated enhanced binding of the Sp3 transcription factor to the CTα promoter in Ha-Ras-transformed cells. Expression of several forms of Sp3 was increased in nuclear extracts of Ha-Ras-transformed fibroblasts compared with nontransformed cells. Tyrosine phosphorylation of one Sp3 form was decreased, whereas phosphorylation of two other forms of Sp3 was increased in nuclear extracts of Ha-Ras-transformed cells. When control fibroblasts were transfected with a Sp3-expressing plasmid, an enhanced expression of CTα and CTβ was observed. However, the expression of CTα or CTβ was not increased in Ha-Ras-transformed cells transfected with a Sp3 plasmid presumably because expression was already maximally enhanced. The results suggest that Sp3 is a downstream effector of a Ras/p42/44MAPK signaling pathway which increases CTα gene transcription.

Phosphatidylcholine (PC) is the most abundant phospholipid in mammalian cellular membranes. Besides having a structural role in membranes and lipoproteins (1–3), PC plays an important role in signal transduction as a source of lipid second messengers (1–3). In all nucleated cells, PC is made primarily through the CDP-choline pathway in which the key enzyme is CTP:phosphocholine cytidyltransferase (CT) (4–6).

Two genes that encode CT activity have been identified and characterized. CTA is expressed in many cells and tissues (7–13) and has a predicted structure that contains catalytic, phosphorylation, and lipid binding domains as well as a nuclear localization sequence (8, 14–21). Recently, CTβ has been identified in human tissues and appears to exist as two splice variants, CTβ1 and CTβ2, differing at their C termini (22, 23). Like CTα, CTβ1/2 contains catalytic and lipid binding domains. However, CTβ1 lacks the phosphorylation domain, and both CTβ1 and CTβ2 lack the nuclear localization sequence (23).

In the last several years, studies have shown that CT can be regulated at both the transcriptional level and post-transcriptionally. CT mRNA is increased after partial hepatectomy in rats (13), after stimulation with colony-stimulating factor 1 in macrophages (24), and during development and growth (25–28). It remains to be determined whether these increases in the message levels are the result of CTα and/or CTβ1/2 mRNA stability, an increase in gene transcription, or a combination of both.

The murine CTα gene (Ctpct) was cloned and characterized by Tang and co-workers (29). The Ctpct promoter contains several putative elements for binding transcription factors, including Ap1, an overlapping site for nuclear factor-xB, E2F, and Elk1, one sterol response element, as well as three elements for Sp-related factors (30). We have shown that Sp1, Sp2, and Sp3 bind competitively to three GC-rich elements and that relative promoter activity depends upon the abundance of these factors (31). We further established that transcription enhancer factor-4 can bind to an upstream regulatory element (~103–82) and enhance the CTα gene expression through its interactions with the basal transcriptional machinery (32). In agreement with the finding that lipoprotein deficiency induces the expression of CTα mRNA and protein in alveolar type II epithelial cells (33), and our observation that the CTα promoter contains a putative sterol response element (30), studies by Kast et al. (34) indicate a role for cholesterol/sterol response element-binding protein and the functionality of the sterol response element in the regulation of CTα gene expression in Chinese hamster ovary cells and THP-1 cells. On the other hand, Lagace et al. (35) have shown that cholesterol/sterol response element-binding protein can stimulate PC biosynthesis.
Regulation of the CTα Gene by Ha-Ras

The importance of PC metabolism in cell proliferation has been under intense investigation, and it has become clear that the GTP exchange protein, Ras, might play an important role in linking these two processes (38–44). Both PC synthesis and degradation are stimulated in Ras-transformed C3H10T1/2 murine embryonic fibroblasts (45), NIH-3T3 fibroblasts (46), and in human keratinocyte cell line, HaCaT (47). The oncogenic transformation in mouse cells leads to increased activity of choline kinase and decreased activity of CT (45, 46), whereas in human keratinocytes CT activity and choline uptake were increased, but choline kinase activity did not change (47). Based upon those data and our finding that CTα promoter activity and CTα mRNA increased after growth stimulation by serum (36), we investigated the role of Ha-Ras in the regulation of the CTα gene. We demonstrate that the Ras/p42/44MAPK signaling pathway plays a role in the regulation of expression of both CTα and CTβ which is at least partially mediated by the transcription factor Sp3.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—All reagents were molecular biology grade. The inhibitors of p38MAPK (SB202190) and MEK1 (PD98059) were from Calbiochem. The transfection reagent DOTAP was from Avanti Polar Lipids (Birmingham, AL). Phospho-MAPK antibody sampler containing phospho-p42/44MAPK, phospho-p38MAPK, and phospho-SAPK/JNK-specific rabbit polyclonal antibodies was from New England Biolabs, Mississauga, Canada. A murine monoclonal phospho-tyrosine-specific antibody, 4G10, was from BD Biosciences, Canada. Cell culture media and reagents were from Invitrogen.

Plasmid Constructs—5-Deletion luciferase reporter constructs encoding the murine CTα promoter, LUC.C5 (-2068/+38), LUC.C7 (-1268/+38), LUC.C8 (-201/+38) and LUC.D1 (-90/+38), LUC.D2 (-130/+38), and LUC.D3 (-52/+38), and the vector enabling expression of β-galactosidase, pβKΔGal, have been described previously (30). The Sp1 expression plasmid pSpCsp1 and the pPacO-control vector were gifts from Dr. R. Tjian (48). The Sp1 expression plasmid pSpCsp1 and the pPacO-control vector were from BD Biosciences, Canada. Cell culture media and reagents were from Invitrogen.

Cell Culture and MAP Kinase Inhibition—Murine embryonic fibroblasts (C3H10T1/2) and the Ha-Ras-transformed clone ras11A were kindly provided by Dr. C. Kent (University of Michigan) (45). They were grown at 37 °C in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum, 100 units/ml streptomycin, and 100 units/ml penicillin under a humidified 5% CO2 atmosphere at 37 °C. The ras11A cell line is neomycin-resistant and was grown in the presence of 400 μg/ml G418. During experiments, G418 was not added to the medium, as recommended (45). The MAP kinase inhibitors PD98059 and SB202190 were dissolved in dimethyl sulfoxide. An aliquot of each inhibitor solution was added to the medium, and the final concentration of the vehicle in the medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle.

Transient Transfections—Fibroblasts were transfected using a DOTAP liposomal method (30). Control and Ha-Ras-transformed fibroblasts were plated at a density of 2 × 106 cells/60-mm dish and transfected the next day with 2.5 μg of specific C3a-luciferase reporter plasmid or with or without the indicated amounts of Sp1 and Sp3 expression vector, pPD200. Transfected cells were grown overnight in normal medium, then growth was arrested in a low serum medium (0.5% fetal calf serum) for 2 additional days. The arrested cells were stimulated to grow by the addition of 10% serum and 24 h later collected for further analysis. The pBkΔGal vector, encoding β-galactosidase, was cotransfected as an internal control to measure the efficiency of transfection efficiency. Luciferase and β-galactosidase activities were measured using a luciferase and β-galactosidase assay system (Promega). The amount of cellular protein was measured by the Bio-Rad method.

CT Enzymatic Activity—CT activity in total cell homogenates, cytosol, and microsomes was assayed in the presence of PCl-α-leaf vesicles by monitoring the conversion of phosphol[3H]choline to CDP-[3H]choline as described previously (36). Briefly, cells were collected in a homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 100 μM NAD, and 100 μg/ml each leupeptin and aprotinin), sonicated for 25 s at 4 °C, and the lysate was either stored at −70 °C or added immediately (25 μg of protein) to a CT-activity assay buffer (57.8 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1.78 mM EDTA, 8.9 mM magnesium acetate) containing 1.5 mM [3H]labeled phosphocholine, 3 mM CTP, and 0.2 mM PCl-α-leaf (1:1) vesicles in a final volume of 100 μl. The reaction was incubated 15 min at 37 °C and stopped by boiling for 2 min. The supernatant was collected by centrifugation at 500 × g for 5 min and an aliquot was spotted on a Silica gel G0 thin-layer plate. The plate was developed in a solvent mixture of methanol, 0.6% NaCl, and saturated ammonium (10:10:0.9, v/v/v) and CDP-[3H]choline quantified by liquid scintillation counting.

Immunoblotting Analysis of CTα and CTβ—Cell lysates from control and Ha-Ras-transformed cells (20–50 μg of protein) were prepared as described previously (36) and separated on 10% denaturing polyacrylamide gels. The proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) at −150 mA and 4 °C for 1 h. After checking for protein loading with Ponceau S dye, the membranes were incubated overnight with 5% skim milk in TTBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20). The membranes were washed in TTBS and incubated at 25 °C for 2 h with an antibody, followed by washing at 35 °C for 2 h with polyvalent antibodies raised against CT (anti-M), CTα, CTβ1/2, and/or CTβ2. The CT (anti-M) antibody was a rabbit polyclonal antibody directed against a peptide corresponding to amino acids 256–288 (M domain) of rat liver CTα from Dr. R. Cornell (51). The anti-CTβ rabbit polyclonal antibody, corresponding to the first 17 amino acids of human CTα, the rabbit anti-human CTβ1/2 (B2 epitope) antibody, corresponding to amino acids 5–22 of CTβ1/2, and the rabbit anti-human CTβ2 antibody (B3 epitope), corresponding to amino acids 347–365 of CTβ2, were all gifts from Dr. S. Jackowski (22, 23). Immunoblotting was performed by incubation of the membranes with either anti-M (1:2,000), anti-CTα (1:1,000), anti-CTβ1/2 (1:1,000) or CTβ2 (1:5,000) as the primary antibody. The membranes were washed five or six times for 10 min each six times for 1–2 min. The membranes were blocked with goat anti-rabbit antisera (1:5,000 dilution; horseradish peroxidase-conjugated (Roche Molecular Biochemicals) at room temperature for 1 h. The membranes were washed five or six times with TTBS, developed with enhanced chemiluminescence reagent (Pierce), and exposed to XAR-5 film (Kodak). To reprobe the blots, the membranes were stripped in 100 mM mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, at 50 °C for 30 min and subjected to the above immunoblotting procedure.

Activation States of p42/44MAPK and p38MAPK—Nuclear extracts were prepared at described (30, 52). Equal aliquots of nuclear proteins or cell lysates (50 μg) from control and transformed cells were separated by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes. To investigate the activation state of different MAP kinases in response to Ha-Ras transformation, we employed specific antibodies directed against the phosphorylated forms of p42/44MAPK and p38MAPK. The immunoblotting procedure was as described above for CT.

Immunoprecipitation and Immunoblot Analysis of Sp3-related Proteins—Nuclear proteins were prepared as described (30, 52). Cells grown in 100-mm dishes were scraped into 1 ml of an immunoprecipitation buffer (phosphate-buffered saline (PBS) containing 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1 mM diethiothreitol). The cell debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C. Total proteins (50 μg) or nuclear proteins (100 μg) from control and Ha-Ras-transformed cells were incubated with 2 μg of Sp3-specific polyclonal antibody (H-225, 200 μg/ml; sc-13018, Santa Cruz Biotechnology) for 1 h at 4 °C. Subsequently, 20 μg of protein A-agarose was added, and the samples were mixed gently overnight at 4 °C. The immunoprecipitates were collected by centrifugation at 10,000 × g for 5 min at 4 °C. Immunoprecipitates were washed four times with PBS. Finally, the pellet was resuspended in 40 μl of electrophoresis sample buffer and boiled for 2–3 min. Immunoprecipitated proteins were resolved on a 12% SDS-polyacrylamide gel. For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane that was blocked with PBS containing 6% powdered milk, 0.5% polyvinylpyrrolidone, and 0.1% Triton X-100 for 1 h at room temperature. After three washes with PBS containing 0.5% Tween 20, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:2,000) for 1 h, washed with PBS-Tween, and proteins were visualized using enhanced chemilumi-
Phosphorylated Sp3 was detected using an anti-phosphoryl-
ated antibody (PY-20 at 1:1,000 dilution) by reprobing the same blot
using a protocol similar to that described for CT. In some experiments,
Sp3-related proteins and tyrosine phosphorylation were measured di-
rectly by immunoblotting (31).

Electromobility Shift Assays—Nuclear extracts from Ha-Ras and
control fibroblasts were prepared 24 h after serum stimulation as de-
scribed (30, 52). The double stranded CTs promoter probes D1 (−95/
+38) and D2 (−130/+38) containing Sp binding sites were prepared
from LUC.D1 and LUC.D2 reporter vectors by restriction digestion and
purification (30). The promoter fragments were 3′-end labeled with[
32P]dCTP and Klenow polymerase and the binding of Sp1 and Sp3
analyzed after separation of DNA-protein complexes on 5% nondena-
turing polyacrylamide gels followed by autoradiography (30, 31).

RNA Preparation and Reverse Transcriptase-mediated PCR of CTs—
Total RNA from control and Ha-Ras-transformed cells grown in the
presence or absence of MAP kinase inhibitors was extracted using TRIAZOL reagent (Invitrogen) (36). The RNA was reverse transcribed
with a first strand cDNA synthesis kit (Superscript II, Invitrogen)
according to the manufacturer’s protocol. The reverse-transcribed
mRNA (0.5–3 μg) was amplified using PCR primed with forward (5′-
ATGGACAGTGTTCAGGCAA-3′) and reverse (5′-GGGGTTACTA-
AAAGTTCAACTTCA-3′) primers corresponding to the CTs gene. The
signal from the CTs mRNA transcript was normalized to the signal obtained from glyceraldehyde-3-phosphate dehydrogenase as a control using
the primer pair 5′-TCCACACCTGTCTTGCTGTA-3′ (forward) and 5′-
ACCACTGCTAGCGACTAC-3′ (reverse) or with the signal from cyclo-
philin using the primers 5′-TCTTTCTCTGCTGTCTTGCCATTCC-3′
(forward) and 5′-TCCAAAGGACAGGAAAAACTT-3′ (reverse). 30 cy-
cles of PCR amplification at 95 °C for 1 min, 45° for 1 min, and 72 °C for
2 min produced ~200-bp fragments for CTs and ~250-bp fragments for
glyceraldehyde-3-phosphate dehydrogenase; 33 cycles at 94 °C for 1
min, 60 °C for 2 min, 72 °C for 2 min produced ~300-bp fragments for
cyclophilin.

Statistical Analysis of Data—Calculations of the average values,
standard deviations, and the comparison of means by Student’s t test
were performed by Scion Image acquisition and analysis software (Scion
Inc.).

RESULTS
Expression of Ha-Ras Increases Active p44/p42MAPK—It is well
documented that deregulated cell proliferation is a conse-
quency of activated Ras signaling through the MAP kinase
cascade, which is a critical component of the proliferative re-
sponse (39–41). Activated Ras signals directly through Raf
kinase with the subsequent activation of MEK1/2 kinases and
results in the phosphorylation and activation of p44/p42MAPK.
Typically, the Raf/MEK1/p42/p44MAPK pathway is strongly
stimulated by growth factors and mitogenic stimuli, whereas in
contrast, two other signaling pathways, mediated by p38MAPK
and p46/p54JNK, are activated primarily by cellular stresses that
include heat, UV radiation, and hypoxia (42–44, 53) and usu-
ally are antiproliferative and apoptotic (54–56). We, there-
fore, determined whether p44/p42MAPK was activated to a greater
extent in fibroblasts constitutively expressing oncogenic
Ha-Ras. The phosphorylation of p44/p42MAPK was analyzed by
antibodies that specifically detected phosphorylated, and therefore
active, forms of p44/42MAPK and p38MAPK. Using the anti-phospho-p42/44MAPK antibody we observed bands corre-
sponding to p42/p44MAPK in the nuclear proteins from both
control and Ha-Ras-transformed cells (Fig. 1). The nuclear
proteins from transformed cells contained significantly more
phosphorylated p42/44MAPK, whereas p38MAPK was only
weakly phosphorylated in Ha-Ras-transformed cells relative to
control cells (Fig. 1).

Ha-Ras Transformation Decreases Total CT Enzymatic Ac-
tivity but Increases CTa and CTb2 Protein Mass in a p42/
p44MAPK-dependent Manner—It was of interest to know whether or not CT activity was affected by the Ras-signaling pathway.
Table 1 shows total CT enzymatic activities in control and
Ha-Ras-transformed cells stimulated by serum in the presence

| CT activitya |
|----------------|
| Control cells | Ha-Ras cells |
| (nmol/mg) |
| Total | 5.31 ± 1.53 (n = 6) | 3.43 ± 0.5 (n = 6) |
| MEK1/2 inhibitorb | | |
| PD98059 (μM) | (n = 4) | |
| 0 | 4.95 ± 0.39 | |
| 5 | 4.90 ± 0.82 | |
| 50 | 5.35 ± 0.49 | |
| 150 | 5.33 ± 0.48 | 3.77 ± 0.45 (n = 2) |
| p38MAPK inhibitorb | | |
| SB202190 (μM) | (n = 4) | (n = 2) |
| 5 | 5.42 ± 0.75 | 3.8 ± 0.72 |
| 10 | 5.23 ± 0.42 | 4.4 ± 0.0 |
| 20 | 5.37 ± 0.41 | 4.3 ± 0.33 |

*a Determined 24 h after 10% serum stimulation of arrested cells.
*b After cell arrest for 2 days in low serum (0.5%), both inhibitors were
added in the presence of 10% serum, and activity was determined 24 h
later. The amounts of MEK and p38 inhibitors used are indicated.

and absence of specific inhibitors of p42/p44MAPK and p38MAPK
The flavone compound PD98059 is a specific inhibitor of
MEK1/2 kinase and has been used extensively for investigating
the physiological function of p42/p44MAPK (57). The pyridylimi-
dazole compound SB202190 is a p38MAPK inhibitor (58, 59).
Neither compound inhibits other known related kinases (57–
59). The results shown in Table I reveal that, similar to published
data (45), in Ha-Ras-transformed cells CT activity was 65% (p < 0.05) less than in the control cells. An equivalent
decrease was also observed in the cytosol and microsomes of
Ha-Ras-transformed cells compared with control cells (data not
shown). Paradoxically, in neither control cells nor Ha-Ras-
transformed cells was the CT activity modified by kinase in-
hibitors, suggesting a complex regulation of CT activity by Ras
signaling (Table I).

We next performed immunoblotting analyses of the different
CT isoforms (Fig. 2A). Densitometric analysis of the immuno-
reactive bands showed that CTa protein levels increased 1.8 ±
0.2-fold in Ha-Ras-expressing cells relative to control cells.
With the CTb2-specific antibody (Fig. 2B) Ha-Ras-transformed
cells contained significantly higher amounts (1.5 ± 0.1-fold) of
immunoreactive CTb2 protein than did the control cells (CTb1
was not detectable by the CTb1/2 antibody).

Because cells constitutively expressing Ha-Ras contained
more active nuclear p42/44MAPK activity compared with the
control cells (Fig. 1), we investigated whether or not the

![FIG. 1. Nuclear p42/p44MAPK, but not p38MAPK, is activated in
Ha-Ras-transformed CHI10T1/2 fibroblasts. Control and Ras-
transformed fibroblasts were grown in 10% serum-containing medium.
Cell lysates were harvested 24 h after serum treatment and nuclear
extracts prepared. The phosphorylation of p42/44MAPK and p38MAPK
was determined by immunoblotting. Each lane contains 50 μg of
nuclear protein. Immunoblots were probed with anti-phospho-p42/p44-
MAPK antibody and anti-phospho-p38MAPK antibody. The immuno-
blots were also stained with Ponceau S to verify equal loading of protein
in each lane (not shown). Results were similar in two independent
experiments.](http://www.jbc.org/doi/figure-pdf/10.1074/jbc.C117.147551)

Table I

| CT activitya |
|----------------|
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| (nmol/mg) |
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| 0 | 4.95 ± 0.39 | |
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| 150 | 5.33 ± 0.48 | 3.77 ± 0.45 (n = 2) |
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| 5 | 5.42 ± 0.75 | 3.8 ± 0.72 |
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| 20 | 5.37 ± 0.41 | 4.3 ± 0.33 |

*a Determined 24 h after 10% serum stimulation of arrested cells.
*b After cell arrest for 2 days in low serum (0.5%), both inhibitors were
added in the presence of 10% serum, and activity was determined 24 h
later. The amounts of MEK and p38 inhibitors used are indicated.
MEK1/2 inhibitor PD98059 altered the amount of CTα and CTβ2 proteins (Fig. 2). Because MEK1/2 phosphorylates and activates p42/p44MAPK, the inhibitor would be expected to inhibit the phosphorylation of p42/p44MAPK and their downstream targets. The addition of the 20–150 μM MEK1/2 inhibitor to the cells reduced the amount of CTα (Fig. 2A) and CTβ2 (Fig. 2B) in a dose-dependent manner. The magnitude of inhibition by 150 μM PD98059 in control cells was 37 and 50% for CTα and CTβ2, respectively, and in Ha-Ras-transformed cells was 61 and 87% for CTα and CTβ2, respectively.

CTα mRNA Is Increased in Ha-Ras-transformed Cells in a p42/44MAPK-dependent Manner—Based upon the above results and our previous findings that serum growth factors stimulate Ha-Ras transformation (A) and our previous findings that serum growth factors stimulate Ha-Ras transformation (A), we investigated whether CTα mRNA was increased by overexpression of Ha-Ras. Fig. 3A shows that CTα mRNA is increased in Ha-Ras-transformed cells in the absence of serum (2.5–2.8-fold) as well as after serum stimulation (4.0–6.5-fold).

Fig. 3B demonstrates that 150 μM p42/44MAPK inhibitor (PD98059) reduces CTα mRNA expression by 70% in both control and Ha-Ras cells. The p38MAPK inhibitor SB202190 did not decrease CTα mRNA expression in control and Ha-Ras cells. Together, these results suggest that the increase in CTα protein mass shown in Fig. 2 is caused by an increased transcription of the CTα gene in Ha-Ras-transformed cells. This increase is p42/44MAPK-dependent and is increased further by serum.

CTα Promoter Activity Is Increased in Cells Overexpressing Ha-Ras—To examine further the transcriptional regulation of the CTα gene, we investigated CTα promoter activity in transiently transfected cells using a series of truncated promoter-luciferase reporters (Fig. 4A). We have shown previously that C3H10T1/2 fibroblasts support the activation of CTα promoter-luciferase constructs (30, 31). Fig. 4A shows that CTα promoter activity in the Ha-Ras-transformed cells both have the ability to direct luciferase gene expression, as expected (31). Interestingly, the promoter activity of each of the promoter-reporter constructs tested, including the basal promoter construct −52/+38 (LUC.D3), is persistently 1.5–3-fold higher in the Ha-Ras cells than in control cells, suggesting that the basal promoter region is sufficient to drive the increased luciferase expression in the cells constitutively expressing Ha-Ras.

Sp3 Binding to the CTα Promoter Is Increased in Ha-Ras-transformed Cells—The basal promoter region of the CTα gene contains complex, overlapping binding sites for nuclear factor-κB, Elk1, E2F, and Sp1. Previously, we investigated the role of the Sp site located at position −39/−9 (30, 31). The role of Sp1, Sp2, and Sp3 in binding to this site was established by mutation and overexpression analysis in insect and mammalian cells, including C3H10T1/2 murine fibroblasts (30, 31). We have demonstrated previously that Sp1 and Sp3 specifically and competitively bind to this promoter region as well as to two other regions of the CTα promoter at −88/−50 and −148/−128. We therefore performed electromobility shift assays with nuclear proteins from control and Ha-Ras-transformed cells to determine whether the increase in CTα promoter activity in the Ha-Ras cells was caused by changes in protein binding to the promoter. Fig. 4B shows that the protein profile from control and Ha-Ras cells is the same, suggesting that stimulation by Ha-Ras transformation of CTα promoter activity is not the result of the binding of a new transcription factor. We demonstrated previously by supershift analysis in C3H10T1/2 cells using antibodies specific for the Sp1 and Sp3 proteins that these proteins bind the promoter probe −90/+38 (30, 31). From Fig. 4B, it can be seen that there is a stronger binding of the Sp3-related nuclear proteins in the transfected, compared with the control, cells. A similar binding profile was observed when a different promoter probe, −130/+38, which contained Sp binding sites, was used (data not shown). From these results and from our previously published data (30, 31), we conclude that the increase in CTα promoter activity in the Ha-Ras-transformed cells shown in Fig. 4A is, at least in part, a consequence of an increased binding of the transcription factor Sp3.

Ha-Ras Transformation Increases Sp3 Protein Expression and Modifies Sp3 Proteins Post-translationally—We next investigated whether the increase in Sp3 binding to the promoter (Fig. 4B) was caused by an increase in the levels of Sp3 protein and/or a consequence of differences in post-translational modifications of Sp3. The existence of multiple Sp3 proteins has been shown to be caused by the presence of an internal translation initiation start site, which results in a truncated Sp3 that has opposing activity to the full-length Sp3 protein (50, 60). We therefore immunoprecipitated proteins from cell lysates and nuclear extracts from transfected and control cells using a Sp3-specific antibody. Fig. 5 (lanes 3 and 4) clearly demonstrates that in nuclear extracts from Ha-Ras-transformed cells the amounts of both the full-length Sp3 (a1, 116 kDa, a2, 97 kDa) and truncated Sp3 (b1, 70 kDa and b2, 66 kDa) are increased compared with control cells. We also investigated the role of Sp1, Sp2, and Sp3 in binding to this site was established by mutation and overexpression analysis in insect and mammalian cells, including C3H10T1/2 murine fibroblasts (30, 31). We have demonstrated previously that Sp1 and Sp3 specifically and competitively bind to this promoter region as well as to two other regions of the CTα promoter at −88/−50 and −148/−128. We therefore performed electromobility shift assays with nuclear proteins from control and Ha-Ras-transformed cells to determine whether the increase in CTα promoter activity in the Ha-Ras cells was caused by changes in protein binding to the promoter. Fig. 4B shows that the protein profile from control and Ha-Ras cells is the same, suggesting that stimulation by Ha-Ras transformation of CTα promoter activity is not the result of the binding of a new transcription factor. We demonstrated previously by supershift analysis in C3H10T1/2 cells using antibodies specific for the Sp1 and Sp3 proteins that these proteins bind the promoter probe −90/+38 (30, 31). From Fig. 4B, it can be seen that there is a stronger binding of the Sp3-related nuclear proteins in the transfected, compared with the control, cells. A similar binding profile was observed when a different promoter probe, −130/+38, which contained Sp binding sites, was used (data not shown). From these results and from our previously published data (30, 31), we conclude that the increase in CTα promoter activity in the Ha-Ras-transformed cells shown in Fig. 4A is, at least in part, a consequence of an increased binding of the transcription factor Sp3.
kDa) are increased compared with control cells. The doublets typically observed for each isoform, a1/a2 and b1/b2, likely result from differences in mobility of post-translationally modified Sp3 proteins, primarily from differences in phosphorylation. In the nucleus of Ha-Ras-transformed cells 2.1-fold more of the slowest migrating form of Sp3, a1, is present than in control nuclei. Moreover, the a2 form is 1.4–1.9-fold more abundant in Ha-Ras-transformed cells than in control cells. However, the a3 form of Sp3 (−90 kDa), which represents yet another modified full-length Sp3 protein, was more dominant in control cells than in Ha-Ras-transformed cells (2.2-fold more in lysates and 2.1-fold more in the nuclear extract). No differences in the amounts of the truncated Sp3 b1 and b2 forms between control and Ha-Ras-transformed cells were observed in the whole cell lysates. However, in nuclear extracts the amount of the b1 form was increased by 50%, and the b2 form was increased by 30% in Ha-Ras cells relative to control cells.

Next, we inspected the murine Sp3 (GenBank XP_130306) for putative phosphorylation sites by using the NetPhos 2.0 algorithm (61). The computer analysis did not reveal any con-
Regulation of the CTα Gene by Ha-Ras

We have found that in C3H10T1/2 fibroblasts Ha-Ras activates the expression of Sp3 via the p42/44 MAPK kinase pathway, thereby increasing the amounts of CTα and CTβ2 mRNA and protein. Our data indicate that when Sp3 binds to one or more sites on the CTα promoter, transcription of the CTα gene is stimulated. Because the CTβ promoter has not yet been characterized, it is not possible to determine whether Sp3 directly or indirectly governs the increased amount of CTβ2 in the Ha-Ras-transformed cells.

Mechanistic Studies on Activation of the CTα Gene—The focus of the current study was to elucidate the mechanism by which the expression of CTα mRNA and protein is increased in Ha-Ras-transformed fibroblasts. Previously, we established that growth stimulation by serum increased the expression of CTα mRNA during the cell cycle (36). The results presented here demonstrate that the p42/44 MAPK signaling pathway is responsible for the serum-induced increase in CTα mRNA and protein that is further magnified by constitutive activation of p42/44 MAPK in transformed cells. In accordance with those results, when signaling via p42/44 MAPK was inhibited, or when serum was eliminated from the growth medium, the expression of CTα mRNA and protein was decreased. To identify the regulatory cis-acting elements responsible for the Ras/p42/44 MAPK regulation of the CTα gene, we used luciferase-reporter mutants spanning the promoter region from −201 bp to +38 bp. We demonstrated that the promoter activity for all constructs was persistently higher in transformed cells.

Previously we established that the CTα basal promoter is completely inactive if transcription is not supported by Sp1 or Sp3 nuclear factors, as in insect cells naturally lacking those factors (31). Transient expression of Sp1 or Sp3 initiates CTα basal transcription in insect cells, suggesting that the interactions of Sp1 and Sp3 with the basal promoter and general transcription factors are critical for the CTα transcriptional activation (31). The basal CTα promoter Sp element is located in the vicinity of the transcription initiation site, at position −22/−15 bp, and could bind Sp1 and Sp3 together with other nuclear proteins, possibly including the basal transcription factors (30, 31). We have now established that Sp3 from transformed cells binds stronger to the CTα promoter than Sp3 from untransformed cells and that Sp1 binds equally in both cells (Fig. 4B), suggesting that Sp3, not Sp1, could be solely responsible for the up-regulation of CTα gene in transformed cells.

The stronger binding of Sp3 to the CTα promoter in transformed cells is probably a combination of increased mass and modified phosphorylation of several Sp3 protein species. Sp3 exists in two main isoforms (50) of which we showed that the full-length Sp3 a (stimulatory form) is primarily overexpressed and post-translationally modified by Ha-Ras/p42/44 MAPK. The

DISCUSSION

served tyrosine phosphorylation sites in the Sp3 protein. There are, however, two nonconsensus tyrosine kinase sites at positions 87 (LQGNYIQSP) and 347 (CGKVYGTKS) which prompted our further analysis, shown in Fig. 5 (lanes 1 and 2). The immunoprecipitated Sp3 proteins were reprobed with an anti-phosphotyrosine antibody and compared with the Sp3 immunoblots. Differences in phosphorylation of Sp3 proteins between control and Ha-Ras-transformed cells are evident in both whole cell lysates and nuclear extracts. In addition, an unknown protein of molecular mass >150 kDa was immunoprecipitated which was more highly phosphorylated in the Ha-Ras-transformed cells. Furthermore, Fig. 5 (lanes 1 and 2) shows less phosphorylation of Sp3 a1 and more phosphorylation of Sp3 a3 in nuclear extracts from the transformed cells than from the control cells. Phosphorylation of Sp3 a3 and Sp3 b1/b2 was not observed. The results in Fig. 5 indicate that the full-length Sp3 a3 and an unknown protein that coimmunoprecipitates with Sp3, are more phosphorylated in the Ha-Ras-transformed cells, but other Sp3 forms are less phosphorylated compared with control cells. Furthermore, the amounts of full-length Sp3 (a1 and a2) and truncated Sp3 (b1 and b2) are augmented by Ha-Ras transformation resulting in stronger binding of these Sp3 species to the CTα promoter (Fig. 4B).

Based upon our previous results (30, 31), which demonstrated that Sp1 also plays an important role in the regulation of expression of Ctpct, we next determined whether the amount of Sp1 was increased by increased expression of Ha-Ras. We found, however, that the amount of Sp1 protein was unchanged (data not shown), consistent with the data in Fig. 4B that show no difference in binding of Sp1 to the promoter probe between control and Ha-Ras-transformed cells.

Transient Expression of Sp3 Increases the Amount of Both CTα and CTβ2 Protein—Mutation and transfection analyses (31) revealed that Sp3 is functionally equivalent to Sp1 and can act as an independent transcriptional activator of the CTα promoter. We have also demonstrated that overexpression of Sp3 in mammalian cells (including C3H10T1/2 murine embryo fibroblasts) stimulates CTα promoter-luciferase reporter activity (31). Thus, to determine whether Sp3 increases the amount of CT proteins we analyzed the expression of CT proteins after transfection of control and Ha-Ras fibroblasts with various amounts (0–12 μg) of the Sp3-expression plasmid, pPacSp3. The levels of both CTα (1.2-fold and 1.4-fold) and CTβ2 (1.15-fold and 1.5-fold) proteins increased in experiments with 3 and 6 μg of the pPacSp3 plasmid, respectively, for control cells only (Fig. 6). No increase in CTα and CTβ2 proteins was apparent in Ha-Ras cells after transfection with 3 and 6 μg of pPacSp3 because CT expression was already increased. At higher plasmid concentration, 12 μg of pPacSp3, the CTα protein decreased to the “basal” levels (transfections with empty plasmid pPacO), and in case of CTβ2 the detectable protein declined below the basal level (0.7-fold and 0.5-fold in the Ha Ras and control cells, respectively). These results show for the first time that expression of Sp3 increases the level of CTα and CTβ2 proteins (Fig. 6).

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truncated form Sp3\(^b\) (repressive form) was increased modestly by the Ha-Ras transformation and was not affected by tyrosine phosphorylation (Fig. 5). At lower Sp3\(^\text{cDNA}\) concentrations the protein expression of both CT\(\alpha\) and CT\(\beta\) increased in control cells, whereas in transformed cells, overexpression of Sp3 did not alter the CT\(\alpha\) and CT\(\beta\) levels because they were already elevated. However, at higher Sp3\(^\text{cDNA}\) concentrations, the levels of CT\(\alpha\) and CT\(\beta\) were decreased below or returned to the basal level even in transformed cells, suggesting that Sp3 also acted as a transcriptional repressor. A dual property of Sp3 has been manifested in previous studies for the regulation of other genes (62, 63) including our own work with the Sp3\(^\text{gene}\) regulation of CT\(\alpha\) promoter in insect cells (31). That Sp3 is a multiple regulator is also supported by the findings that at higher concentrations, Sp3 is normally inhibitory by a DNA binding-independent mechanism that involves competition for components of the basal transcriptional machinery (62) or titration of the promoter-specific transcription factors (64).

Given the overwhelming evidence for the role of Sp1 in growth regulation and tumor development (62, 63), it is surprising that Sp3 is solely implicated in the regulation of CT\(\alpha\) during oncogenic transformation and that neither the Sp1 binding nor abundance appears to be important for CT\(\alpha\) gene expression. Sp1 is a well known target for Ras/p42/44MAPK activation by phosphorylation, which typically does not involve changes in the Sp1 protein mass (65–68). Even as a target for p42/44MAPK, we report that Sp1 is not a significant regulator of CT\(\alpha\) gene expression after oncogenic transformation and that Sp3 is predominately regulating CT\(\alpha\) by means of the Ras/p42/44MAPK signaling pathway. To our knowledge this is the first time that Sp3, independently from Sp1, appears to regulate gene expression by means of the Ras/p42/44MAPK signaling pathway. Interestingly, ERK2 (p42MAPK) but not ERK1 (p44MAPK) could also be preferentially stimulated by Sp3, not Sp1 (69), which taken together with our findings suggests the existence of a positive feedback mechanism for the observed selective up-regulation of Sp3 protein in transformed cells. The most recent evidence from gene disruption of Sp3 (70) suggests that Sp3 has a distinct regulatory function from Sp1 and other members of the broader Sp1/Krüppel-like family of transcription factors (62). Sp3 is essential for postnatal survival, and Sp3-deficient embryos are growth-retarded and die at birth of respiratory failure (70). The observed breathing defect remains obscure, and surfactant protein expression was not found to be different from that in the Sp3\(^w\) wild type embryos (70). However, given that CT\(\alpha\) plays a central regulatory role in the production of the lung phospholipid surfactant, PC, it may be that the deletion of the Sp3 gene would diminish CT\(\alpha\) expression and, thus, PC production causing lung failure. It is highly likely that Sp3 deletion will also abolish the expression of CT\(\beta\); however, the precise mechanisms for how Sp3 regulates the expression of CT\(\beta\) will remain unknown until the promoter of the murine CT\(\beta\) gene is isolated and fully characterized.

**Ha-Ras Activation of CT\(\alpha\) Expression**—Expression of constitutively active Ras induces cellular proliferation and usually is transforming. The mechanism by which Ras mediates these effects is believed to be through a direct interaction with downstream effectors resulting in a persistent activation of MAP kinase signaling pathways (39–41). It is well established that one characteristic of transformed cells is perturbation of lipid synthetic and degradative pathways (71–73), suggesting an active role of lipids in oncogenic transformation. Expression of oncogenic Ras correlates with increased levels of phosphocholeine and phosphoethanolamine (45, 73–75), diacylglycerols (76), inositol phosphates (77), and arachidonic acid (78). The stimulated degradation of phospholipids is accompanied by an increased biosynthesis (45, 73–75), demonstrating an accelerated turnover of lipids in transformed cells. Increased amounts of phosphocholeline and phosphoethanolamine have been implicated in tumor development (78), during which choline/ethanolamine kinases are typically up-regulated, whereas enzymes involved in the subsequent metabolism of phosphocholeline and phosphoethanolamine (i.e., CTP:phosphocholeline- and phosphoethanolamine cytidylyltransferases) are either up-regulated (47) or down-regulated (45, 46) depending on the cell type.

Our data show that in Ha-Ras-transformed fibroblasts, the levels of CT\(\alpha\) and CT\(\beta\) proteins and mRNAs are increased relative to control cells. However, we were surprised that the increased amounts of CT\(\alpha\) and CT\(\beta\) proteins in the Ha-Ras-transformed cells did not correlate with an increase in CT activity. On the contrary, CT activity was significantly lower in transformed cells than in control cells even though more CT protein was present. Other studies have also reported that CT activity is lower in Ha-Ras-transformed cells than in control cells (45). Moreover, CT activity was not affected by the specific inhibitor of p42/44MAPK (PD98059) in transformed cells (that had constitutively active p42/44MAPK) or in control cells in which p42/44MAPK was activated by serum. The inhibitor PD98059, however, decreased the protein mass of both CT\(\alpha\) and CT\(\beta\) as would be expected if their expression were regulated by p42/44MAPK. Thus, Ha-Ras/p42/44MAPK signaling simultaneously increases the amount of CT protein yet decreases CT activity.

CT phosphorylation has been studied extensively. It has been clearly established that soluble, inactive forms of CT are more highly phosphorylated than are membrane-bound, active forms (79, 80). CT activity also varies inversely with CT phosphorylation during the cell-cycle (81), and the translocation of CT between membranes and cytosol is influenced by phosphorylation/dephosphorylation (4, 79, 80). CT dephosphorylation is secondary to its association with membranes, suggesting that CT interactions with lipids could be a more significant factor for enzymatic activity than phosphorylation (82, 83). Insulin and epidermal growth factor stimulate the phosphorylation of

![Fig. 6. The amount of CT\(\alpha\) protein is increased by transfection of cDNA encoding Sp3. Sp3 proteins were expressed in control and Ha-Ras-transformed cells by transient transfection of 3, 6, and 12 \(\mu\)g of pPacSp3 or 12 \(\mu\)g of pPacO control vector. The total amount of DNA was kept constant at 12 \(\mu\)g by the addition of the appropriate amounts of empty control vector. After 48 h, cells were harvested and 50 \(\mu\)g of proteins from cell lysates was loaded onto 7% SDS-polyacrylamide gels. The expression of CT\(\alpha\) and CT\(\beta\) were assessed by immunoblotting. Two independent experiments were performed with similar results.](http://www.jbc.org/doi/abs/10.1074/jbc.M107120200)

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- Insulin and epidermal growth factor stimuli...
Ample evidence suggests that the cellular lipid content is to the modification of CT activity in Ha-Ras-transformed cells. CTβ has been discovered only recently (22, 23), and its post-translational regulation by phosphorylation has not been reported. However, CTβ2 phosphorylation domains are highly homologous to the phosphorylation domains of CTα and possibly could be targeted by similar mechanisms. Which of the numerous Ser/Thr phosphorylation sites of CTα and CTβ are selective targets for Ras/p42/44MAPK signaling is presently unknown.

Alteration in lipid composition can also post-translationally regulate CTα (and possibly CTβ) activity and might contribute to the modification of CT activity in Ha-Ras-transformed cells. Ample evidence suggests that the cellular lipid content is changed after oncogenic transformation (45–47, 71–73, 76). For example, recent data show that increased cell proliferation and activation of the p42/44MAPK signaling cascade alter the expression and/or activity of several genes of phospholipid metabolism, including phospholipase C (76), phospholipase A2 (71), the level and/or phosphorylation of lipid transport proteins, apo-A1 (85), apoC-III (86), low density lipoprotein receptor (87), and the activity of lipid-related transcription factors sterol response element-binding protein (88) and peroxisomal proliferator activated receptor-γ (84) which regulate many genes involved in lipid metabolism, perhaps including the CTα gene (34).

Whether the decreased CT activity in Ha-Ras-transformed cells is linked to direct post-translational modifications of both CT isoforms, differences in the type, content and/or location of regulatory lipids, or other mechanisms, would be difficult to resolve.

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Oncogenic Ha-Ras Transformation Modulates the Transcription of the CTP:Phosphocholine Cytidyltransferase α Gene via p42/44 MAPK and Transcription Factor Sp3

Marica Bakovic, Kristin Waite and Dennis E. Vance

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