Evidence for Phosphorylation of CTP:Phosphocholine Cytidyltransferase by Multiple Proline-directed Protein Kinases*

(Received for publication, September 27, 1995, and in revised form, January 19, 1996)

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Reversible phosphorylation of CTP:phosphocholine cytidyltransferase, the rate-limiting enzyme of phosphatidylcholine biosynthesis, is thought to play a role in regulating its activity. In the present study, the hypothesis that proline-directed kinases play a major role in phosphorylating cytidyltransferase is substantiated using a c-Ha-ras-transfected clone of the human keratinocyte cell line HaCaT. Cellular extracts from epidermal growth factor-stimulated HaCaT cells and from ras-transfected HaCaT cells phosphorylated cytidyltransferase much stronger as compared with extracts from quiescent HaCaT cells. The tryptic phosphopeptide pattern of cytidyltransferase phosphorylated by cell-free extracts from ras-transfected HaCaT cells was similar compared with the patterns of cytidyltransferase phosphorylated by p44\textsuperscript{mapk} mitogen-activated protein kinase and p34\textsuperscript{cdc2} kinase in vitro, whereas in the case of casein kinase II the pattern was different. Furthermore, in c-Ha-ras-transfected HaCaT cells the in vivo phosphorylation state of cytidyltransferase was 2-fold higher as compared with untransfected HaCaT cells. This higher phosphorylation of cytidyltransferase in the ras-transfected clone was reduced to a level below the phosphorylation of cytidyltransferase in untransfected cells, using olomoucine, a specific inhibitor of proline-directed kinases. The reduced phosphorylation of cytidyltransferase in olomoucine-treated cells correlated with an enhanced stimulation of enzyme activity by oleic acid.

In mammalian cells, the main pathway for the biosynthesis of phosphatidylcholine (PC) is via CDP-choline and CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) (CT) is the rate-limiting enzyme of this pathway (I). In addition to the structural function as a component of cellular membranes, PC has been identified to be involved into signal transduction via the PC cycle (2). In a recent study, the coordination of PC metabolism with the cell cycle was investigated, and evidence was provided that the net biosynthesis of PC is restricted to the S-phase of the cell cycle (3).

CT exists as a soluble, inactive form that can be activated by translocation to membranes. Many mechanisms that modulate this translocation process have been firmly established (4–7), and reversible phosphorylation of CT has been shown to influence translocation of CT between cytosol and membranes (7–10). In many systems cytosolic CT is highly phosphorylated, whereas the membrane-bound, active form of CT is dephosphorylated. However, in a recent study it was shown that dephosphorylation of CT is not required for membrane binding (11).

The physiological role of phosphorylation and the kinases involved in this process are still discussed. Whereas CT is a substrate for CAMP-dependent kinase in vitro (12), neither CAMP-dependent protein kinase (13–15) nor protein kinase C (16) seem to phosphorylate CT in vivo. In rat hepatocytes and HeLa cells only serine residues of CT are phosphorylated in vivo (14, 17), and the phosphorylation sites of CT from rat liver were identified (18). This study revealed that phosphorylation of CT is confined to the carboxyl-terminal region of CT and that many serine residues reside in potential sites for proline-directed kinases. We have shown recently that growth factors can stimulate phosphorylation of CT in HeLa cells and that CT is a substrate for p44\textsuperscript{mapk} MAP kinase in vitro (19), suggesting that the ras/Raf/MAP kinase-signaling pathway is involved in this process.

In the present study, we investigated the phosphorylation of CT in vivo and in vitro. In ras-transfected HaCaT cells, the phosphorylation of CT was increased by 2-fold as compared with the phosphorylation of CT in untransfected cells. The enhanced phosphorylation of CT in ras-transfected cells was reduced in the presence of olomoucine, a specific inhibitor of proline-directed kinases, such as p34\textsuperscript{cdc2} and p44\textsuperscript{mapk} MAP kinase (20). Using this experimental approach we could show that phosphorylation of CT interferes with the activation of the enzyme by oleic acid and protects the enzyme against proteolytic digestion. Furthermore, cell-free extracts from quiescent and EGF-stimulated HaCaT cells as well as ras-transfected HaCaT cells were phosphorylated in vitro. The tryptic phosphopeptide patterns of CT phosphorylated with cell-free extracts from HaCaT and ras-transfected HaCaT cells were compared with the patterns of CT phosphorylated with purified MAP kinase, cyclin-dependent kinase, and casein kinase II in vitro. The results presented here substantiate the hypothesis that p44\textsuperscript{mapk} MAP kinase is involved in the phosphorylation of CT, but other proline-directed kinases, such as p34\textsuperscript{cdc2} kinase, represent probable candidates as well.

**EXPERIMENTAL PROCEDURES**

Materials—The ECL immunoblotting detection reagent and [methyl-\textsuperscript{14}C]phosphocholine (2.04 GBq/mmol) were from Amersham (Braun-
mixed with wild type linear AcNPV DNA (3:1 ratio) and cotransfected into SF21 cells using cationic liposomes, according to the manufacturer's instructions. The culture supernatant was collected, titered, and used to infect a lawn of SF21 cells that were overlaid with agarose. Blue plaques were picked and subjected to three cycles of plaque purification until cells with inclusion bodies were not detected. For expression of C5, 9 × 10^6 SF21 cells in a 15-cm-diameter dish were infected with recombinant baculovirus in TC-100 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Expression of CT was assayed by Western blot analysis and measurement of CT activity. 64 h after infection, SF21 cells were collected in medium, pelleted, and stored at

-80°C.

In vitro Phosphorylation of Cytidylyltransferase—Rat liver CT expressed in SF21 insect cells was used for in vitro phosphorylations. 20 nmol/min of CT activity was immunoprecipitated using the antibody SA 2 (25) bound to protein A-Sepharose. To immunoprecipitate CT, 50 µl of antiserum SA 2 were incubated with 5 mg of protein A-Sepharose in 2 ml of buffer 1 (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) for 3 h at 4°C after which the antibody coupled protein A-Sepharose was sedimented by centrifugation and incubated overnight at 4°C with homogenates of infected SF21 cells. The protein A-Sepharose was centrifuged at 13,000 × g for 2 min, and the supernatant was discarded. The immune complex was washed three times with buffer 1 and one time with phosphate-buffered saline. CT was phosphorylated with

CT in 50 mM Tris-HCl (pH 7.7) for 15 or 30 min at 30°C. The immune complex was washed again three times with buffer 1 and one time with phosphorylation buffer containing 15 mM MOPS (pH 7.2), 2 mM diithiothreitol, 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 2 mM EGTA, 5 mM NaF, and 1 mM sodium vanadate. Then phosphorylation was performed in the presence of different kinases or cell-free extracts and 100 µM [γ-32P]ATP (20 µCi/assay) at 37°C in a total volume of 20 µl. After 30 min the reaction was stopped by adding SDS sample buffer, and the mixtures were loaded on SDS-PAGE (10% acrylamide) and electrophoresed. Proteins were transferred to nitrocellulose membrane, and the membrane was exposed to Kodak XAR 5 film to detect cytidylyltransferase. Bands corresponding to CT were excised, and incorporated radioactivity was determined by scintillation counting.

Two-dimensional Peptide Mapping of Phosphorylated Cytidylyltransferase—Digestion of CT and peptide separation was performed as described by Boyle et al. (27). Briefly, bands corresponding to CT were excised from the nitrocellulose membrane, and the strips were incubated with 0.5% polyvinylpyrrolidone (PVP-360) in 100 mM acetic acid to prevent adsorption of trypsin to the nitrocellulose membrane. The membrane pieces were washed three times with water and subsequently incubated in 200 µl of 50 mM ammonium bicarbonate (pH 8.2) containing 10 µg of L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin. Additional fresh trypsin (10 µg) was added after 2 and 4 h of incubation. The total incubation time was 6 h. The membrane pieces were washed, and the combined supernatants were dried in a SpeedVac and washed by three cycles of resuspended with purified water and dried using 200 µl of distilled water for each cycle. Samples were resuspended in 5 µl of electrophoresis buffer (water/acetic acid/formic acid, 897:78:25, v/v/v) and spotted on cellulose thin layer chromatography sheets (0.1-mm thickness). Separation of the phosphopeptides was accomplished by electrophoresis at 1000 V for 45 min followed by ascending chromatography in n-butanol/water/pyridine/acetic acid (15: 12:10:3, v/v/v/v) for 7 h.

Other Procedures—CT activity was measured by a modified method of Sohal and Corneli (28) using liposomes containing 400 µM PC and different concentrations of oleic acid as described previously (15). Protein was determined by the BCA assay (29) with BSA as standard. Statistical comparisons were made in these studies with Student's t-test.

RESULTS

c-Ha-ras Transfection Alters MAP Kinase Activation in HaCaT Cells—Activation of the MAP kinase cascade was measured by incubating the cells with EGF, and MAP kinase activity was assessed by measuring the rate of phosphorylation of MBP, a known substrate of MAP kinases. Fig. 1A shows the phosphorylation of MBP after 2 and 10 min of EGF stimulation in untransfected HaCaT cells. After 10 min of EGF treatment, phosphorylation of MBP was 220% as compared with the control. In contrast, MBP was highly phosphorylated without EGF stimulation in ras-transfected HaCaT cells, and the addition of
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EGF had no further stimulating effect on phosphorylation of MBP. After 10 min of EGF treatment, incorporation of phosphate into MBP was 95% of basal activity (determined by scintillation counting of excised bands).

MAP kinases are activated by phosphorylation (30). It is known that the phosphorylation of both isoforms, p42mpk and p44mpk, leads to a mobility shift in the SDS gel. As shown in the Western blot of Fig. 1B, p42mpk and p44mpk are expressed in HaCaT cells and become phosphorylated time-dependently after EGF stimulation. In ras-transfected HaCaT cells, both isoforms of MAP kinase appear as phosphorylated, high molecular weight forms that were not influenced by EGF stimulation.

Phosphorylation of Cytidylyltransferase in Vivo—To investigate the phosphorylation state of CT in cell culture experiments, HaCaT cells and the ras-transfected clone were metabolically labeled with \(^{33}\)Porthophosphoric acid. After 2 h of incubation time, phosphorylation of CT was elevated by about 2-fold in ras-transfected cells as compared with the phosphorylation of CT in untransfected HaCaT cells (Fig. 2). In addition, experiments of the activity of CT was assayed in HaCaT cells and ras-transfected HaCaT cells. In the absence of exogenous liposomes CT activity was 0.31 ± 0.05 nmol/min/mg of protein in HaCaT cells and 0.37 ± 0.03 nmol/min/mg of protein in ras-transfected cells (n = 3), indicating that the increased phosphorylation of CT in ras-transfected cells did not have a significant effect on its basal activity.
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Degradation products of CT with an apparent molecular mass below 30 kDa were observed after 20 min in extracts from olomoucine-treated cells. On the other hand, no degradation products of this size were observed in extracts from control cells.

Phosphorylation of Cytidylyltransferase with Cell-free Extracts of HaCaT Cells in Vitro—To substantiate the hypothesis that proline-directed kinases are involved in the phosphorylation of CT, immunoprecipitated CT was incubated with cell-free extracts from HaCaT cells and ras-transfected HaCaT cells (corresponding to the conditions shown in Fig. 1). Phosphorylation of CT was very low when extracts from quiescent HaCaT cells were used in the assay, and the phosphorylation was significantly stimulated by pretreatment of quiescent HaCaT cells with 100 ng/ml EGF. On the other hand, extracts from quiescent ras-transfected HaCaT cells strongly phosphorylated CT, and pretreatment of ras-transfected cells with 100 ng/ml EGF did not cause higher phosphorylation of CT (Fig. 5). The weak phosphorylation of immunoprecipitated CT in the absence of cytosol was probably due to the activity of endogenous kinases (see also Fig. 6) and did not disturb the in vitro assay.

Additionally, the effect of olomoucine on the phosphorylation of CT by cellular extracts from ras-transfected cells in vitro was investigated. The strong phosphorylation of CT catalyzed by cellular extracts from ras-transfected HaCaT cells was reduced in the presence of olomoucine far more potently than in the presence of the unspecific kinase inhibitor iso-olomoucine. To verify that olomoucine inhibits p44mpk MAP kinase and p34cdc2 kinase and not casein kinase II in our system, we investigated the effect of olomoucine on the phosphorylation of CT by the purified kinases in vitro. Although the phosphorylation of CT is reduced in the case of p44mpk MAP kinase and p34cdc2 kinase, olomoucine has no inhibitory effect on casein kinase II activity (see Table I).

Phosphorylation of Cytidylyltransferase by Different Kinases In Vitro—In order to investigate the phosphorylation of CT in more detail, we used three different kinases in an in vitro phosphorylation assay. Fig. 6 illustrates that immunoprecipitated CT is phosphorylated by MAP kinase (p44mpk), cyclin-dependent protein kinase (p34cdc2), and casein kinase II. In all assays equal kinase activities were used (2 × 10⁻¹¹ μmol/min). As also mentioned in the previous section, phosphorylation of CT in the absence of exogenous kinases was very low. The radioactivity incorporated into CT was determined by scintillation counting of excised bands, and the background phosphorylation in the control was defined as 100%. In relation to the control, the incorporation of phosphate into CT catalyzed by p44mpk and p34cdc2 was 333 and 420%, respectively, whereas incorporation of phosphate into CT was only 165% in the case of casein kinase II.

In another set of experiments, immunoprecipitated CT was phosphorylated by MAP kinase, cyclin-dependent kinase, casein kinase II, and cell-free extracts from quiescent HaCaT cells and ras-transfected HaCaT cells, followed by digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and two-dimensional separation of phosphorylated peptides. In order to obtain a sufficiently high ³²P label in the phosphorylated peptides, the purified kinases were used in this assay at the highest specific activity available. The peptide maps obtained in the presence of purified kinases were compared with the patterns of digested CT phosphorylated by cell-free extracts from quiescent HaCaT cells and ras-transfected HaCaT cells (Fig. 7). Confirming the results in the previous section, phosphorylation of the different peptides by extracts from ras-transfected cells was higher as compared with phosphorylation by extracts from normal HaCaT cells. However, with the exception of spots 10 and 12, the patterns of HaCaT cells and ras-transfected HaCaT cells were identical. Furthermore, the patterns obtained by phosphorylation with MAP kinase and cdc2 kinase (especially the spots numbered 2, 3, 4, 5, 6, 7, 10, and 12) resembled the pattern of ras-transfected

**Fig. 3.** Effect of reduced CT phosphorylation on oleic acid-mediated enzyme activation. 6 × 10⁶ ras-transfected HaCaT cells were incubated with 100 μM olomoucine or 0.25% Me₃SO as controls for 4 h. Subsequently, the cells were homogenized in 1 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.06% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride by use of a tight-fitting Dounce homogenizer, and CT activity was measured as described. The values are given in percentages of basal activity determined in the presence of liposomes containing no oleic acid. In extracts of cells treated with Me₃SO, basal activity was 0.85 ± 0.22 nmol CDP-choline formed per ml, and in extracts of cells treated with 100 μM olomoucine basal activity was 0.72 ± 0.2 nmol CDP-choline formed per ml ± S.D. (n = 3). * significantly different from controls at p < 0.02. ** significantly different from controls at p < 0.01.

**Fig. 4.** Effect of reduced CT phosphorylation on enzyme stability. 6 × 10⁶ ras-transfected HaCaT cells were incubated with 100 μM olomoucine or 0.25% Me₃SO as controls for 4 h. Subsequently, the cells were homogenized on ice in 1 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 100 μM olomoucine, 100 μM o-acetid acid, and 10 mM NaF by use of a tight-fitting Dounce homogenizer. Control extraction buffer contained 0.25% Me₃SO instead of 100 μM olomoucine. Cellular extracts were then incubated at 37°C for the indicated time periods, and CT and degradation products were analyzed by Western blot using CT antibody SA 2 (1:3000).
Phosphorylation of cytidylyltransferase by different kinases in vitro. Rat liver CT expressed in Sf21 insect cells was immunoprecipitated using protein A-Sepharose and antibody SA 2. The washed immune complex was dephosphorylated with alkaline phosphatase and incubated with 2 x 10^{-11} \mu mol/min p44 MAP kinase, p34^{\text{cdc2}} kinase, and casein kinase II for 30 min at 30 °C. Phosphorylated CT was separated by SDS-PAGE. A representative autoradiogram from two independent experiments is shown.

HaCaT cells. In contrast, when casein kinase II was used in the assay two predominant phosphopeptides were obtained (spots 4 and 7), resulting in a different phosphopeptide pattern as compared with the other patterns.

**DISCUSSION**

Reversible phosphorylation is a universal mechanism regulating enzymatic processes in eukaryotic cells. It has been known for some time that CT is regulated by reversible phosphorylation (1, 7, 9, 31). However, the kinases involved in this process remain to be identified. Many approaches have used different protein kinase activators (13, 14, 16) and kinase inhibitors (15) as well as phosphatase inhibitors (10) to investigate the phosphorylation of CT. In the present paper, a different approach is presented using the human keratinocyte cell line HaCaT and a c-Ha-ras-transfected clone of this cell line. In untransfected HaCaT cells, MAP kinases became activated rapidly after EGF stimulation of the cells. On the other hand, MAP kinases were already fully activated in ras-transfected HaCaT cells without EGF stimulation, confirming the well-known effect that EGF treatment and ras transfection stimulate MAP kinase activity (30). Cell culture experiments revealed that ras-transfected HaCaT cells contained a highly phosphorylated form of endogenous CT, suggesting that the activation of MAP kinases might also be important for the phosphorylation of CT in vivo. The increased phosphorylation of CT in ras-transfected cells did not influence CT activity when determined in the absence of stimulating liposomes. However, we could clearly demonstrate that activation of CT by oleic acid was enhanced when phosphorylation of the enzyme was reduced by olomoucine treatment. This is in accordance with previous findings by Yang and Jacobowski (32), who showed that a mutant of CT lacking the COOH-terminal phosphorylation domain is much more sensitive to stimulation by lipids when compared with the wild type enzyme. Furthermore, we tested the hypothesis that phosphorylation of CT influences the stability of the enzyme. Using extracts from olomoucine-treated cells that mainly contained dephosphorylated CT, we could demonstrate that CT was more susceptible to digestion by endogenous proteases in vitro. In this context it has been shown that down-regulation of CT by cholecystokinin treatment of pancreatic acinar cells correlates with a decrease in CT phosphatase levels, indicating that phosphorylation of CT protects the enzyme from degradation (33).

Phosphorylation of CT by extracts from quiescent HaCaT cells was very low, and EGF stimulation as well as ras transfection of HaCaT cells obviously activated kinases or inactivated phosphatases. As a consequence, the phosphorylation of CT by extracts from EGF-stimulated or ras-transfected HaCaT cells was much stronger. Additionally, olomoucine, a purine analogue that was shown to specifically inhibit cyclin-dependent kinases and p44^{\text{mpk}} MAP kinase (20) reduced phosphorylation of CT in vivo and in vitro, suggesting an involvement of proline-directed kinases in this process.

Phosphorylation of CT by extracts from ras-transfected HaCaT cells has finally been ruled out. Because the cDNA of rat liver CT contains many consensus phosphorylation sequences for proline-directed kinases (like p34^{\text{cdc2}} kinase and MAP kinase) and one consensus sequence for casein kinase II (34), we tested these kinases in their ability to phosphorylate rat liver CT in vitro. All three kinases were able to phosphorylate CT, and p34^{\text{cdc2}} kinase catalyzed the strongest incorporation of \textsuperscript{32}P into CT. The advantage of the approach presented here, using rat liver CT as a substrate for phosphorylation by cellular extracts and purified kinases, is that the tryptic phosphopeptide patterns of CT can be compared with each other. Using this system, difficulties arising from differences in the amino acid sequence of CT from different species are ruled out. The number of different spots obtained was similar to patterns presented by other groups, using rat liver CT as well (18). Interestingly, the pattern obtained after phosphorylation of CT by cellular extracts from ras-transfected HaCaT cells was similar...
to the patterns obtained after phosphorylation by purified p44MAP kinase and p34^cdc2 kinase. On the other hand, phosphorylation of CT by casein kinase II revealed a different tryptic phosphopeptide pattern, suggesting that this kinase plays a minor role in phosphorylating CT. In this context, it is interesting to note that a mutant of CT with an alanine residue in the potential phosphorylation site for casein kinase II showed the same properties as compared with the wild type enzyme (35).

The findings presented here, together with results showing that growth factors stimulate phosphorylation of CT in HeLa cells (19), substantiate the hypothesis that proline-directed kinases, such as MAP kinases or cyclin-dependent kinases, are involved in the phosphorylation of CT.

Acknowledgments—We thank Dr. R. Cornell (Simon Fraser University, Burnaby, BC, Canada) for the rat CT cDNA clone, Dr. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) for providing HaCaT cells, and M. Draeger (Freie Universität Berlin) for expert assistance with the thin layer electrophoresis.

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J. Biol. Chem. 1996, 271:9955-9961.
doi: 10.1074/jbc.271.17.9955

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