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Growth factors stimulate phosphorylation of CTP:phosphocholine cytidylyltransferase in HeLa cells

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Abstract The effect of insulin and epidermal growth factor on the phosphorylation of CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) was investigated in HeLa cells. For the first time, cytidylyltransferase phosphorylation was shown to be influenced by growth factors in cell culture experiments. The rephosphorylation of cytidylyltransferase after an oleate-mediated dephosphorylation and translocation to membranes was increased after 2 min in the presence of insulin or epidermal growth factor by 99% and 76%, respectively, compared with controls. However, the increased phosphorylation of cytidylyltransferase did not have an effect on its subcellular distribution. Furthermore, purified cytidylyltransferase preincubated with alkaline phosphatase is a substrate for $p44^{mapk}$, a member of the mitogen-activated protein (MAP) kinase family downstream of the growth factor receptors, in vitro. In accordance with the in vivo data, in vitro phosphorylation of cytidylyltransferase by $p44^{mapk}$ occurred after 2 min.

Key words: CTP:phosphocholine cytidylyltransferase; Growth factor; MAP kinase

1. Introduction

Phosphatidylcholine (PC) is the most abundant phospholipid in mammalian cell membranes, playing an important role in both membrane structure and signal transduction [1]. In these cells PC is synthesized mainly by the CDP-choline pathway, for which CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) (CT) is a rate-limiting enzyme [2–4]. An important regulatory mechanism for the control of CT activity is the translocation of the enzyme between cytosol and membranes, especially in HeLa cells [5,6]. In most cases the cytosolic form of CT appears to be an inactive reservoir which can be translocated reversibly to membranes, where it becomes activated by interaction with phospholipids in the membrane [7]. The translocation process can be modulated by various signals [for review see 8] and the involvement of reversible phosphorylation has been discussed [9–11].

Concerning the kinases that phosphorylate CT there exists some controversy. Whereas CT is a substrate for cAMP-dependent protein kinase (PKA) in vitro [12], neither PKA nor protein kinase C (PKC) seem to phosphorylate CT in vivo [13–17]. In rat hepatocytes and HeLa cells only serine-residues of CT are phosphorylated in vivo [15,17]. The cDNA of rat liver CT shows repeated serine/proline-rich domains [18], resembling potential phosphorylation sites for proline-directed protein kinases like mitogen-activated protein kinases (MAP kinases) [19,20] or cyclin-dependent protein kinases [21]. Recently, most of these serines were identified to be phosphorylated in vivo [22]. MAP kinases, also described as extracellular signal-regulated kinases (ERK), comprise a family of serine/threonine protein kinases conserved through evolution in all eukaryotic cells, that participate in signal transduction pathways initiated by many extracellular stimuli [23]. In HeLa cells, two highly related mammalian MAP kinase isoforms, $p44^{mapk}$ and $p42^{mapk}$, are activated within minutes in response to growth factors [24].

In the present study, we demonstrate that growth factors stimulate CT phosphorylation in HeLA cells. Since MAP kinases are known to be activated in response to growth factors in HeLa cells, we tested if a typical MAP kinase ($p44^{mapk}$) phosphorylates CT in vitro. After dephosphorylation of purified CT, we observe an incorporation of phosphate into CT catalyzed by $p44^{mapk}$ in a time- and dose-dependent manner.

2. Materials and methods

2.1. Materials

[*methyl*-^{14}C]Phosphocholine (2.04 GBq/nmol), [γ^{32}P]ATP and the ECL immunoblotting detection reagent were from Amersham (Braunschweig, Germany). Carrier-free $^{32}PO_4$ (10 mCi/ml) was purchased from DuPont (Bad Homburg, Germany). Digitonin, fatty acid-free bovine serum albumin (BSA), agarose-conjugated alkaline phosphatase, anti-phosphotyrosine monoclonal mouse antibody, insulin from bovine pancreas, epidermal growth factor (EGF) from mouse submaxillary glands, protein A-Sepharose, and oleic acid were from Sigma (München, Germany). Silicagel 60 high-performance thin-layer chromatography plates and reagents were purchased from Merck (Darmstadt, Germany). For quantification of radioactivity a radioscanner (LB2821 HR; Berthold, Wildbad, Germany) was used. Peroxidase-conjugated swine anti-rabbit antibody was from Dakopatts (Hamburg, Germany) and the nitrocellulose-membrane (0.45 μm) was from Schleicher and Schüll (Dassel, Germany). MAP kinase ($p44^{mapk}$, purified enzyme from sea star) was purchased from UBI (Lake Placid, New York, USA). Polyclonal antibodies were raised against two peptide fragments of CT (CT peptide 1–17 and CT peptide 247–257) according to the cDNA published by Kalmar et al. [18], as described and characterized [25].

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Abbreviations: BCA, bicinchoninic acid; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; CT, cytidylyltransferase; MAP kinase, mitogen-activated protein kinase; MOPS, 4-morpholinopropanesulfonic acid; PC, phosphatidylcholine; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EDTA, ethylenediamine tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; $p42^{mapk}$/$p44^{mapk}$, p42/p44 isoforms of mitogen-activated protein kinase.

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2.2. Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.58 g/l glutamine, 110 000 units/l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc, Denmark). Media and culture reagents were from Gibco (Karlsruhe, Germany) and antibiotics from Boehringer (Mannheim, Germany). Confluent cells were further cultured in DMEM containing 0.5% FCS for 30 h prior to experiments with growth factors.

2.3. Solutions

PBS buffer contained 150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1 mM KH_2PO_4, pH 7.2. A 100 mM oleate stock solution was prepared in 0.12 M KOH containing 95% ethanol as described [6]. For oleate treatment, the ethanol was evaporated under N_2 gas and the fatty acid was resuspended in 0.33% fatty acid free BSA in DMEM or phosphate-free DMEM (for the in vivo ^{32}P-labeling experiments), sonicated in a bath sonicator for 10 min and used immediately.

2.4. ^{32}P-labeling of cells

HeLa cells were grown to confluence in 60 mm-diameter dishes. The serum-starved cells were washed twice with 5 ml of phosphate-free DMEM and then incubated for 2 h in 2 ml of the same medium containing 1 mCi of carrier-free $^{32}PO_4$ and 0.33% BSA. At that time 0.5 ml oleate-containing medium was added to a final concentration of 0.5 mM. After 1 h preincubation, the medium was removed and the cells were washed twice with 5 ml PBS. The test medium, containing the indicated growth factors and 0.33% fatty acid free BSA, was added for 2 min. The dishes were washed twice with ice-cold PBS, and the cells were fractionated by treatment with digitonin release buffer as described in the next section.

2.5. Digitonin permeabilization of cells

Cells were incubated with digitonin release buffer containing 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.8 mg/ml digitonin, 50 mM NaF, 50 μM sodium vanadate, 3.3 mM EDTA, 3.3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin and 2 μg/ml pepstatin for 2 min at 4°C. The soluble and particulate fractions of cells were separated as described previously [16].

2.6. Immunoblotting

For phosphotyrosine immunoblotting stimulated cells were lysed by sonication in 200 μl of 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 50 μM sodium vanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin and 2 μg/ml pepstatin. Insoluble material was removed by centrifugation at 13,000 × *g* for 5 min at 4°C. The protein samples were mixed with SDS sample buffer [26] and separated by 10% SDS-PAGE. After transfer to nitrocellulose membrane the blot was incubated with 5% non-fat dry milk in PBS for 1 h at room temperature to block non-specific binding. The membrane was then incubated with a monoclonal anti-phosphotyrosine antibody (1:2500, Sigma) in PBS containing 0.1% Tween 20 overnight at 4°C. After washing three times for 5 min with PBS/Tween 20 the antibody reactions were detected using horseradish peroxidase-conjugated antibodies and the ECL chemiluminescent detection reagents according to the manufacturer's instructions.

The same procedure was used to detect purified CT with an antiserum against CT peptide 247–257.

2.7. Immunoprecipitation of ^{32}P-labeled cytidylyltransferase

To immunoprecipitate CT, the soluble and particulate fractions (500 μl each) obtained by digitonin treatment were preincubated 2 h at 4°C with 100 mg Sepharose CL-4B (Pharmacia) prewashed with 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) after which the sepharose was sedimented by centrifugation. 50 μl of antiserum against CT peptide 247–257 was incubated with 2 ml protein A-Sepharose in buffer 1 for 2 h at 4°C after which the antibody coupled protein A-Sepharose was sedimented by centrifugation and incubated overnight at 4°C with the precleared supernatant of the first step. The protein A-Sepharose was centrifuged at 13,000 × *g* for 2 min, and the supernatant was discarded. Finally the Sepharose containing immunoprecipitant was washed three times with buffer 1, three times with buffer containing 50 mM Tris, pH 7.4, 1 M NaCl and 0.1% Triton X-100 and one time with PBS. The washed Sepharose was boiled in 2-mercaptoethanol containing SDS gel sample buffer, to dissociate immunoprecipitant. The immunoprecipitated protein samples were separated on 10% SDS-PAGE and the dried gels were exposed to Kodak XAR-5 film for 72 h at −70°C. Protein bands were quantified by use of a videodensitometer (Fischer Biotech, Reiskirchen, Germany).

2.8. CTP:phosphocholine cytidylyltransferase assay

CT activity was measured by a modified method of Sohal and Cornell [27] as described previously [16]. One unit of enzyme activity is defined as 1 nmol of CDP-choline formed per min.

2.9. In vitro phosphorylation of cytidylyltransferase

CT was purified from rat liver as described [28]. 1 μg of purified CT was used in each reaction. Dephosphorylation of purified CT was achieved by treatment with 5 U of agarose-bound alkaline phosphatase in the presence of 50 mM Tris-HCl, pH 7.7 and 2 mM dithiothreitol (DTT) for 30 min at 37°C. Alkaline phosphatase was removed by centrifugation at 13,000 × *g* for 5 min. The phosphorylation reaction was performed in the presence of 15 mM MOPS, pH 7.2, 2 mM DTT, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 2 mM EGTA, 50 μM ATP containing [γ-^{32}P]ATP (specific radioactivity 2.5 μCi/nmol), 5 mM NaF, 1 mM sodium vanadate and different amounts of purified MAP kinase ($p44^{mapk}$). Mixtures were incubated for up to 2 h at 30°C. The reactions were stopped by adding SDS sample buffer and the mixtures were loaded on SDS-PAGE (10% acrylamide) and electrophoresed. In some experiments, CT was immunoprecipitated prior to electrophoresis using antiserum against CT peptide 1–17. The dried gels were exposed to Kodak XAR-5 film for 24 h at −70°C.

3. Results and discussion

3.1. The effect of insulin and EGF on the activation of MAP kinases

It has been shown in HeLa cells that both MAP kinase isoforms, $p42^{mapk}$ and $p44^{mapk}$ become activated in response to growth factors within minutes [24]. To determine the time-dependent stimulation of protein tyrosine phosphorylation in our system we incubated serum-starved HeLa cells with insulin or EGF for different time-periods. A rapid stimulation of tyrosine phosphorylation of proteins in the 41–44 kDa range (indicated by the arrow) takes place after 2 min of growth factor treatment (Fig. 1). This stimulation is sustained over a time period of 15 min.

3.2. Rephosphorylation of cytidylyltransferase in the presence of insulin and EGF

To investigate the possible role of PKA or PKC in phosphorylating CT different approaches have used cAMP-analogues

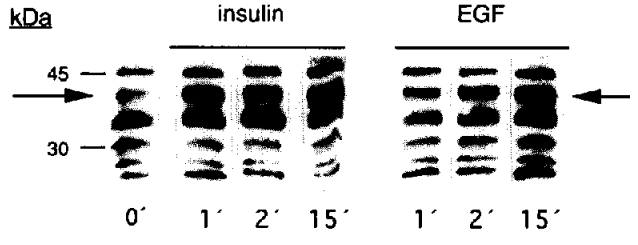

Fig. 1. Time-dependent stimulation of p42 tyrosine phosphorylation in HeLa cells. Serum-starved HeLa cells were stimulated with insulin (100 nM) or EGF (100 ng/ml) for 1, 2 and 15 min. After lysing the cells by sonication 40 μg of cell lysate was analyzed by SDS-PAGE, and phosphotyrosine-containing proteins were identified by immunoblotting as described in section 2. Positions of molecular weight standards and p42 are indicated. The results shown are representative of three independent experiments.

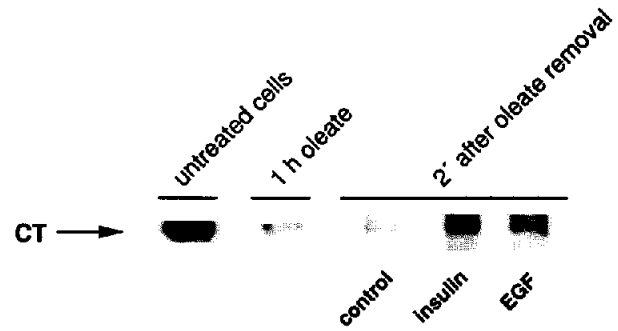

Fig. 2. Effect of insulin and EGF on the phosphorylation of cytidylyltransferase. Serum-starved HeLa cells were incubated in phosphate-free DMEM containing 1 mCi/60 mm dish $^{32}P_i$ (carrier-free) and 0.33% fatty acid free BSA for 2 h. Oleate-containing medium was added to a final concentration of 0.5 mM and the incubation was continued for 1 h. The medium was removed and the cells were washed twice with 5 ml PBS. At this time the test medium containing 0.33% fatty acid free BSA and the indicated growth factors (100 nM insulin or 100 ng/ml EGF, respectively) was added for 2 min. The cells were incubated with digitonin release buffer for 5 min and CT was immunoprecipitated using CT-peptide antiserum. The immunoprecipitated protein samples were separated by 10% SDS-PAGE and the gels were exposed to Kodak XAR-5 film for 72 h. This experiment was repeated twice with similar results. Phosphorylation of CT was quantified by densitometric video-scanning.

[14], cholera toxin [15], the selective inhibitor of cAMP-dependent protein kinase (PKA) H-89 [16] or phorbol esters [13,17]. However, differences in the overall phosphorylation of CT could not be observed in any of these cases. Since cytosolic CT is already extensively phosphorylated in untreated HeLa cells, we used a different starting-point where CT is almost completely dephosphorylated by incubation of cells with oleate for 1 h. Then, the medium was removed and replaced by a medium containing 0.33% fatty acid free BSA and insulin or EGF, respectively. The rephosphorylation of cytosolic CT was increased after 2 min in the presence of insulin or EGF by 99 ± 20% and 76 ± 15%, respectively, as compared with controls containing only BSA (Fig. 2). Membrane-associated CT is not phosphorylated at all, neither in treated nor in untreated cells (data not shown).

3.3. *The effect of insulin-/EGF-treatment on the subcellular distribution of cytidylyltransferase*

Since a significant difference in the phosphorylation of cytosolic CT was observed 2 min after oleate removal, the distribution of CT under these conditions was determined. The cytosolic and particulate fractions were separated by treatment with digitonin-release buffer and the activity of fully activated enzyme was measured in both fractions. While 83.3 ± 3% of CT is located in the cytosol in untreated cells almost the whole enzyme pool (95 ± 2%) translocates to membranes following 1 h incubation with 0.5 mM oleate. Two minutes after oleate removal 68.2 ± 5% retranslocates back to the cytosol in controls and only slightly less does so in insulin- or EGF-treated cells (61.5 ± 1% and 62.3 ± 1.3%, respectively). Therefore, the increased phosphorylation of CT observed in insulin/EGF-treated cells does not significantly influence the distribution of the enzyme between cytosol and membranes. In this context, experimental evidence was provided by another group that the phosphorylated form of CT can bind to membranes [29].

3.4. *Phosphorylation of cytidylyltransferase by MAP kinase in vitro*

It was tested if CT is a substrate for MAP kinases in vitro for two reasons: first, the cDNA of rat liver CT contains potential phosphorylation sites for proline-directed protein kinases and, second, growth factors are known to activate $p42^{mapk}$ and $p44^{mapk}$ within minutes, especially in HeLa cells [24]. CT was purified from rat liver cytosol and incubated with $p44^{mapk}$. The CT preparation did not contain any intrinsic protein kinase activity (Fig. 3, lane 1). After a preceding dephosphorylation of CT with alkaline phosphatase an incorporation of phosphate into CT catalyzed by $p44^{mapk}$ in vitro was observed (Fig. 3, lane 4) suggesting that most phosphorylation sites are already occupied in cytosolic CT from rat liver. The $p44^{mapk}$ showed extensive autophosphorylation in the absence of CT as a substrate protein (Fig. 3, lane 2). Interestingly, autophosphorylation of $p44^{mapk}$ was markedly reduced in the presence of CT. This might be due to competition of the phosphorylation sites of CT and $p44^{mapk}$.

In another set of experiments, we used different amounts of $p44^{mapk}$ to determine the optimum kinase concentration. 1 ng of pure $p44^{mapk}$ per μl assay volume was found to be the most effective concentration (data not shown).

3.5. *Time course of incorporation of ^{32}P into cytidylyltransferase*

To further investigate the CT specificity of $p44^{mapk}$, a time course of ^{32}P incorporation into CT was performed using the optimum kinase concentration of 1 ng/μl. After 2 min of incubation CT was significantly phosphorylated, supporting our in vivo data that rephosphorylation of CT is very fast. The incorporation of ^{32}P into CT reached a plateau after 120 min (Fig. 4) with a stoichiometry of 0.15 mol phosphate per mol CT subunit. This substoichiometric in vitro phosphorylation is in accordance with results of other groups using PKA [12]. Furthermore, an additional slower migrating band appeared after 10 min of incubation which might represent another form of CT containing a higher level of phosphorylation as reported previously [30,31]. However, the possibility that the slower

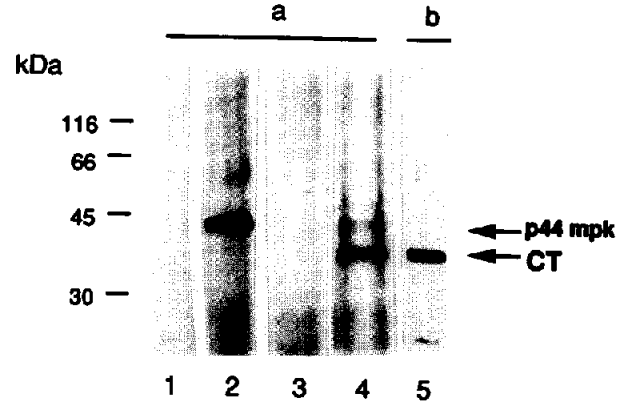

Fig. 3. Phosphorylation of cytidylyltransferase by MAP kinase in vitro. 1 μg CT was incubated with [γ-^{32}P]ATP in the absence (lane 1) or presence (lane 3) of $p44^{mapk}$ for 120 min at 30°C. Lane 2 represents the incubation of $p44^{mapk}$ with [γ-^{32}P]ATP in the absence of CT and lane 4 shows the incubation of [γ-^{32}P]ATP and $p44^{mapk}$ with 1 μg CT that was initially dephosphorylated with alkaline phosphatase. The proteins were then analyzed on SDS-PAGE and the gel was exposed to autoradiography (a) as described in section 2. (b) Western blot showing CT probed with antiserum against CT peptide 247–257 (1:3000).

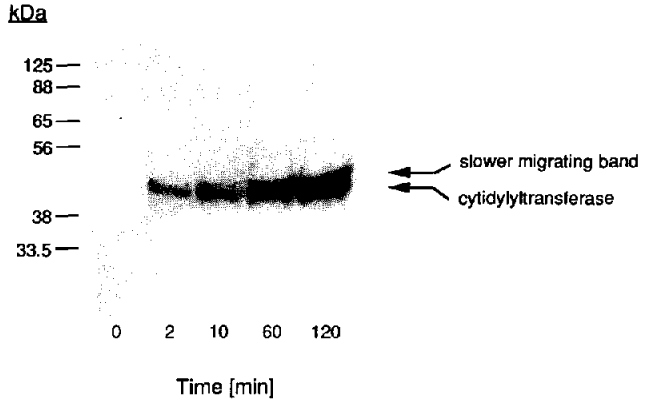

Fig. 4. Time-dependent phosphorylation of cytidylyltransferase by MAP kinase in vitro. Dephosphorylated CT was incubated with [γ-^{32}P]ATP in the presence of p44^{mapk} (1 ng/μl assay volume) for different time intervals. CT was immunoprecipitated and subsequently analyzed on SDS-PAGE as described in section 2 and a representative autoradiogram is shown. For stoichiometric calculations, bands corresponding to CT were excised from the gel and counted.

migrating band represents a coprecipitated protein can not be excluded.

In conclusion, we provide evidence that the phosphorylation of CT is connected with stimulation of cells by growth factors, supposedly through activation of MAP kinases.

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