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Effects of Altered Phosphorylation Sites on the Properties of CTP:Phosphocholine Cytidylyltransferase*

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To investigate the role of phosphorylation and dephosphorylation in modulating the activity and location of CTP:phosphocholine cytidylyltransferase, we used site-directed mutagenesis to construct four mutant forms of cytidylyltransferase. These forms were 5SP → AP, in which five of the seven Ser-Pro sequences were converted to Ala-Pro; 7SP → AP, in which all of the seven Ser-Pro sequences converted to Ala-Pro; 16S → A, in which all sixteen Ser residues that can be phosphorylated in wild type cytidylyltransferase were converted to Ala; and 16S → E, in which all sixteen Ser residues were converted to Glu. The mutant enzymes were expressed in the strain 58 Chinese hamster ovary cell line, which is temperature-sensitive for growth and cytidylyltransferase activity. All mutant enzyme forms were enzymatically as active as the wild type when assayed under optimal conditions. In untreated cells, more of the Ser → Ala mutants were membrane-associated than in cells expressing wild type enzyme, consistent with the phosphorylation state of the enzyme affecting its affinity for membranes. About half of the 16S → A mutant remained soluble, however, indicating that dephosphorylation alone does not trigger membrane association. Although the amount of membrane-associated enzyme in the 16S → A mutant was about 10-fold greater than that of wild type, phosphatidylcholine synthesis was increased by only about 75%, suggesting that membrane association does not necessarily cause full activation. All mutant forms, including the 16S → E mutant, translocated to the particulate fraction upon oleate treatment, indicating that a high negative charge in the phosphorylation region does not preclude association of cytidylyltransferase with membranes. All mutant enzymes were able to support growth of strain 58 at 40 °C, and the rate of phosphatidylcholine synthesis was not greatly altered in the cell lines expressing mutant cytidylyltransferase forms. These results are consistent with a role for phosphorylation in the equilibrium distribution of cytidylyltransferase but suggest that changes in enzyme activity and location are not triggered exclusively by changes in the phosphorylation state.

CTP:phosphocholine cytidylyltransferase catalyzes the conversion of phosphocholine to CDP-choline, an important regulatory step for phosphatidylcholine biosynthesis in mammalian cells (1). Cytidylyltransferase is mainly regulated at the post-transcriptional level, although alterations in mRNA levels for cytidylyltransferase have been reported (2, 3). Cytidylyltransferase is a nuclear protein that exists in both soluble and nuclear envelope-associated forms (4–6). In normal cells most of the enzyme is in the soluble, relatively inactive form. Activation of cytidylyltransferase is accompanied by conversion to the particulate form (1) or, in some cells, to a soluble lipoprotein complex (7–9). Activation and translocation of cytidylyltransferase to the membrane can be achieved by treating cells with fatty acids (10, 11) or phosphatidylcholine-specific phospholipase C (12, 13), choline deficiency (14), or supplementation with choline analogs (15).

Soluble cytidylyltransferase is highly phosphorylated (16); 15–16 Ser residues near the carboxyl terminus have been determined to be the phosphorylation sites in baculovirus-expressed cytidylyltransferase (17). Activation and translocation to the membrane are accompanied by extensive dephosphorylation (18, 19). Although it appears that phosphorylation and dephosphorylation are key components in the inactivation and activation of cytidylyltransferase, the precise contribution of the phosphorylation state of cytidylyltransferase to its activity and location are presently unknown. Treatment of CHO[1] cells with okadaic acid, an inhibitor of protein phosphatase 1 and 2A, prevents dephosphorylation and translocation of cytidylyltransferase in response to phospholipase C, indicating that protein dephosphorylation is a necessary component of activation (18). It is not clear, however, whether the protein that must be dephosphorylated is cytidylyltransferase or another protein involved in the transduction of the signal from exogenous phospholipase C to cytidylyltransferase. Okadaic acid does not block translocation and dephosphorylation of cytidylyltransferase in HeLa cells stimulated with oleate (19), suggesting either that the target of okadaic acid in the CHO cell system is a protein distinct from cytidylyltransferase or that different protein phosphatases activate cytidylyltransferase in the different cell systems.

To approach the role of protein phosphorylation in the function and location of cytidylyltransferase, we have chosen to use site-directed mutagenesis of the phosphorylation sites followed by expression of the mutant enzymes in a heterologous cell. We previously found that a suitable expression system is the strain 58 CHO cell line, in which endogenous cytidylyltransferase is temperature-sensitive and is present at very low levels even at the permissive temperature (5, 20, 21). We first mutated a putative casein kinase II site, Ser^{362}, to Ala and found that this change did not affect the activity of cytidylyltransferase, its ability to translocate upon stimulation of cells expressing the mutant, or its ability to support growth of the transfected cells

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[1] The abbreviations used are: CHO, Chinese hamster ovary; PCR, polymerase chain reaction.

at 40 °C (22). Subsequent analysis of additional mutants in which only a single site was mutated also revealed little, if any, change in cytidylyltransferase function or location.[2] In the present study, we characterized mutants in which 5–16 phosphorylation sites were altered. We report that, although these modifications dramatically altered the phosphorylation state of cytidylyltransferase and in some cases modified its location, the activation state of the enzyme was not appreciably altered.

EXPERIMENTAL PROCEDURES

Materials—Sodium oleate, Ham's F-12 medium, and phosphate-free Dulbecco's modified Eagle's medium were from Sigma. $^{32}P_i$ (400–800 mCi/ml) was from ICN. [*methyl*-^{3}H]Choline chloride and [*methyl*-^{14}C]choline chloride were from Amersham Corp. Protein A agarose, Lipofectin reagent, and G418 were from Life Technologies, Inc. QIAGEN supplied a plasmid purification kit. The pCMV5 vector was a gift from Dr. David Russell, University of Texas. All other reagents were from previously described sources (17, 19).

Construction of Phosphorylation Site Mutants—To make the 5SP → AP and 7SP → AP mutants, the *Hin*dIII-*Xba*I fragment of cytidylyltransferase from pCMV4RCCT (22) was transferred into the pAlter vector of the Promega Altered Sites mutagenesis system. Site-directed mutagenesis was carried out to construct a 4SP → AP mutant; the oligonucleotides were 5′-ATGAGTAGGGGCGCTGCTGGGAGCCTGCTTGGA for S319A,S323A and 5′-TGCTGGGGCGGAAGATGGGGCAGTCTTGC for S343A,S347A. The *Hin*dIII-*Xba*I fragment was then transferred to M13mp19, and the Amersham *in vitro* mutagenesis kit was used to convert Ser^{315} to Ala with the oligonucleotide 5′-GCTTGGGAGCGATGGCCT to give 5SP → AP. The 7SP → AP mutation was then constructed with 5SP → AP as template and 5′-GGAGGGGGCGGGGGCGCGCTCAT as the mutagenic oligonucleotide to introduce S329A and S331A. The resulting mutant constructs were sequenced entirely to ensure that only the desired mutants were obtained. The *Hin*dIII-*Xba*I fragments were then cloned into the *Hin*dIII-*Xba*I site of pCMV5.

The strategy for making the 16S → A and 16S → E mutants was to use multiple-round PCR to make a mutated fragment that became longer at the COOH terminus with each round; the COOH-terminal primer for the second through the fifth rounds overlapped with the COOH-terminal primer from the previous round. The first round introduced mutations at Ser residues 315, 319, 321, 322, and 323; the second round introduced mutations at Ser residues 329, 331, and 333; the third round introduced mutations at Ser residues 339, 343, 345, 346, and 347; the fourth round introduced mutations at Ser residues 350 and 352; and the fifth round introduced the mutation at Ser residue 362. The template for the PCR was pCMV5CT, which was constructed by subcloning the *Hin*dIII-*Xba*I fragment of cytidylyltransferase from pCMV4RCCT into the pCMV5 *Hin*dIII-*Xba*I site. The PCR program was 92 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min for 20 cycles for each round. The NH_2-terminal primer for all rounds, corresponding to the first 16 base pairs of the coding region for cytidylyltransferase, was 5′-CGCGGATCCAGATCTATGGGATGCACAGAGTTCA. For the first round, wild type rat liver cytidylyltransferase cDNA in plasmid pCMV5CT was used as template; the COOH-terminal primers were 5′-GAGTAGGGGCGGCGGCGGGAGCCTGCTTGGGAGCGATGGCCTGCAGCAT for 16S → A and 5′-ATGAGTAGGTTCCTCTTCGGGTTCCTGCTTGGGTTCGATGGCCTGCAGCAT for 16S → E. The PCR product from the first round was separated from wild type template by agarose gel electrophoresis and used as the second round PCR template. For the second round the COOH-terminal primers were 5′-GGCCACCGAAAGGCGGGGGCGGGGGCGCGCTCATGAGTAGGGGCGGCGG for 16S → A and 5′-GAAGGGCCACCGAAATTCAGGCTCGGGTTCGCGCTCATGAGTAGGTTCCTC for 16S → E. The template for the third, fourth, and fifth rounds were the PCR products from the previous rounds. The COOH-terminal primers for the third round were 5′-TGGGGCGGCAGCTGGGGCAGTCTTGCCAGCGAAGGGCCACCGAAAGGCG for 16S → A and 5′-TTCTGCTGGTTCCTCTTCTGGTTCAGTCTTGCCTTCGAAGGGCCACCGAAA for 16S → E. The COOH-terminal primers for the fourth round were 5′-CACAGGTCACAGCCTTGCACCTAGCGAGAGCTGCTGGGGCGGCAGCTGG for 16S → A and 5′-GATGTCACAGGTCACAGCCTTGCACCTTTCGAGTTCTTGCTGGTTCCTC for 15S → E. The COOH-terminal primers for the fifth round were 5′-TGCTCTAGATTAGTCCTCTTCATCCTCGGCGATGTCACAGGTCACAGCCT for 16S → A and 5′-TGCTCTAGATTAGTCCTCTTCATCCTCTTCGATGTCACAGGTCAC for 16S → E.

The final PCR products were purified and subcloned into the *Bam*HI and *Xba*I sites of M13mp19. The 0.6-kilobase pair *Eco*RV-*Xba*I fragment of the COOH-terminal portion of the cDNA (23) was sequenced to ensure that no undesired mutation occurred during the multiple-round reactions. The *Eco*RV-*Xba*I portions were then subcloned into pCMV5CT, from which the *Eco*RV-*Xba*I portion had been removed; the resulting plasmids were pCMV5CT16S → A and pCMV5CT16S → E. All of the constructed plasmids were purified with the QIAGEN Maxi-kit.

Cell Culture—CHO strain 58 cells were cultured with Ham's F-12 medium plus 10% fetal bovine serum at 34 °C with 5% CO_2. Stably transfected cell lines were cultured with the same medium for experiments and with the addition of 20 mM HEPES and 0.8 mg/ml G418 for regular maintenance. Cells were plated at 1×10^6 cells/60-mm dish for 1 day at 34 °C before experiments unless otherwise specified.

Stable Transfection—Wild type and mutant constructs of cytidylyltransferase were transfected into CHO 58 cells with Lipofectin as recommended by the manufacturer. Cells were plated at 2×10^5 cells/60-mm dish, cultured for 1 day, and then incubated with Lipofectin and the DNA overnight. The transfection was stopped by the addition of medium containing 20% fetal bovine serum, after which the cells were incubated in normal medium for 2 days. The cells were then trypsinized and subcultured in four 150-mm dishes with 0.8 mg/ml G418. Single colonies were picked, replated, and screened by indirect immunofluorescence (19) for expression of cytidylyltransferase.

Cell Labeling—For measuring the rate of choline incorporation into phosphatidylcholine, cells were plated at 1×10^6 cells/60-mm dish at 34 °C for 1 day. The cells were then washed twice with calcium- and magnesium-free phosphate-buffered saline and incubated in 1.5 ml of culture medium containing 2 μCi/ml [^{3}H]choline for 0.5–2 h at either 34 or 40 °C. The cells were then washed twice with calcium- and magnesium-free phosphate-buffered saline and harvested by scraping into 1.0 ml of H_2O at 0 °C. Lipids were extracted from 0.8 ml of the cell extract by the Bligh-Dyer method (24). The chloroform phase containing the lipids was dried and counted.

For measuring pools of phosphatidylcholine and aqueous choline metabolites, cell were plated at 2×10^5 cells/60-mm dish and incubated at 34 °C for 1 day. The cells were then washed twice with calcium- and magnesium-free phosphate-buffered saline, fed with culture medium containing 0.5 μCi/ml [^{14}C]choline, and incubated at 34 °C for 1 more day. Maintaining the cells in the labeled medium, two dishes of each cell line were then kept at 34 °C for an additional day, while two other dishes were incubated at 40 °C for the additional day. The cells were then washed twice with calcium- and magnesium-free phosphate-buffered saline and harvested by scraping into 1 ml of H_2O. Lipids and aqueous metabolites were separated by Bligh-Dyer extraction, and the lipid phase was dried and counted. Under these conditions 90% of the lipid-associated label was in phosphatidylcholine. The aqueous phase was dried, dissolved in 60 μl of methanol:H_2O (1:1), and chromatographed on a silica gel G thin layer plate in methanol:5% NaCl:ammonia (50:50:1). The plates were dried and exposed to a PhosphorImager screen, and metabolites were quantitated in a Molecular Dynamics PhosphorImager.

Labeling and Immunoprecipitation of Cytidylyltransferase—Labeling, immunoprecipitation, and detection of cytidylyltransferase by Western blotting and autoradiography were performed as described (19). Two-dimensional peptide mapping was performed as described (17); the electrophoresis buffer was 1% ammonium bicarbonate.

Immunoblots—Western blots were performed as described (19) except that a 1:6000 dilution of amino-terminal antiserum was used as the first antibody.

Growth Curve—Cells were plated at 2.3×10^4 cells/60-mm dish and incubated for 1 day at 34 °C. The cells were then incubated at 40 °C for up to 5 days. Viable cell counts were determined by counting cells excluding trypan blue. Two dishes of each cell line were counted each day.

Enzymatic Assay—Cells were fractionated into soluble and particulate fractions by use of digitonin (19). Cytidylyltransferase activity was determined with previously described conditions for enzyme incubation (25) and binding of the product to charcoal (6).

Oleate Treatment—Cells were treated with 0.25 mM sodium oleate in F12 medium without serum for 20 min at 34 °C. Under these conditions the cells were viable as determined by the exclusion of trypan blue, and the translocation of cytidylyltransferase was reversible by transfer to fresh medium without oleate.

Protein Assay—Protein was determined by the Bradford method (26).

[2] Y. Wang, F. Shi, and C. Kent, unpublished observations.

TABLE I
Sites of alteration of cytidylyltransferase by site-directed mutagenesis

Name	Mutation
Wild type	^{315}SPKQSPSSSPTHERSPSPSFRWPFSGKTSPSSSPASLSRCKAVTCDISEDEED
5SP → AP	^{315}APKQAPSSAPTHERSPSPSFRWPFSGKTAPSSAPASLSRCKAVTCDISEDEED
7SP → AP	^{315}APKQAPSSAPTHERAPAPSFRWPFSGKTAPSSAPASLSRCKAVTCDISEDEED
16S → A	^{315}APKQAPAAAPTHERAPAPAFRWPFAGKTAPAAAPAALARCKAVTCDIAEDEED
16S → E	^{315}EPKQEPEEEPTHEREPEPEFRWPFEGKTEPEEEPAELERCKAVTCDIEEDEED

RESULTS

Construction of Phosphorylation Site Mutants—In order to test the effect of phosphorylation on the properties of cytidylyltransferase, we introduced mutations in phosphorylation sites by site-directed mutagenesis as described under "Experimental Procedures." The sequences encoded by the wild type and mutant constructs are listed in Table I. In the 5SP → AP mutant, the Ser residues followed by Pro that are found in the repeated SPSSSP sequence plus an additional Ser followed by Pro at position 315 were changed to Ala. In the 7SP → AP mutant, the remaining two Ser residues followed by Pro were similarly modified. All 16 Ser residues from position 315 to the COOH terminus were mutated to Ala in 16S → A, so that the resulting mutant cytidylyltransferase could not be phosphorylated. The same residues were changed to Glu in 16S → E in an attempt to mimic the negatively charged phosphorylated enzyme. Stable cell lines of strain 58 CHO cells transfected with each mutant were isolated and screened with indirect immunofluorescence to monitor purity of the clones. Cytidylyltransferase was nuclear in all clones expressing either wild type cytidylyltransferase or the phosphorylation site mutants.

^{32}P-Labeling of Phosphorylation Site Mutants—To determine the level of phosphorylation of the cytidylyltransferase mutants, the cells expressing the mutant enzymes were labeled with $^{32}P_i$, and the mutant enzymes were immunoprecipitated and analyzed by Western blotting and autoradiography (Fig. 1). As expected, there was no phosphorylation detected in the mutants in which all 16 phosphorylation sites were mutated either to Ala or Glu. The extents of phosphorylation of 5SP → AP and 7 SP → AP were considerably decreased. The low degree of phosphorylation in the latter two mutants suggested that one or more Pro-directed protein kinases may be important in determining whether or not other sites are modified.

The labeled phosphopeptides in the partially phosphorylated cytidylyltransferase mutants were analyzed by two-dimensional peptide mapping (Fig. 2). Even though 11 phosphorylation sites remain in the 5SP → AP mutant, only two major phosphopeptides were observed for 5SP → AP. When the 5SP → AP peptides were mixed with those of the wild type, the two major peptides from 5SP → AP did not coincide with the major peptides of wild type cytidylyltransferase. Only one major phosphorylated peptide was observed with 7SP → AP. From the position of this peptide, it appears to correspond to the COOH-terminal tryptic pepide, which contains a putative casein kinase II site (22). The minor phosphopeptide does not appear to coincide with peptides from the wild type. Because of the low amount of label associated with 7SP → AP, there was insufficient material to mix with wild type peptides.

Activities and Subcellular Distributions of Phosphorylation Site Mutants—To determine if the changes in phosphorylation sites dramatically affected the catalytic ability of cytidylyltransferase, soluble and particulate fractions were assayed under optimal conditions in the presence of lipids, and the activity was normalized to the amount of cytidylyltransferase protein in the fraction as determined by immunoblotting (Table II). Under these conditions, the activities of the cytidylyltransferase mutants were essentially the same as the activity of wild type cytidylyltransferase. The mutant enzymes required lipids for maximal activity in the assay *in vitro*, as does the wild type enzyme; the extent of activation of the mutant enzymes by lipids was the same as that of the wild type enzyme (data not shown).

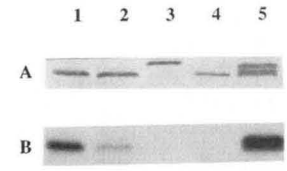

FIG. 1. **Phosphorylation levels of wild type and mutant forms of cytidylyltransferase.** Cells were incubated with $^{32}P_i$, and cytidylyltransferase was immunoprecipitated as described under "Experimental Procedures." The samples were then separated by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting (*A*), and the blot was subjected to autoradiography (*B*). *Lane 1*, 5SP → AP; *lane 2*, 7SP → AP; *lane 3*, 16S → E; *lane 4*, 16S → A; *lane 5*, wild type.

To study the effects of the phosphorylation site mutations on the partitioning of cytidylyltransferase between soluble and membrane fractions, the cell lines expressing wild type and mutant cytidylyltransferase constructs were harvested by digitonin extraction. Cytidylyltransferase activity was determined in the presence of lipids (Table III), and the mass of cytidylyltransferase was determined by Western blotting (Fig. 3). As judged by the activity measurements, mutation of Ser to Ala affected the distribution of cytidylyltransferase in a cumulative manner (Table III), with the amount of membrane-associated enzyme increasing from 5% with the wild type to about 20, 30, and 40% with 5SP → AP, 7SP → AP, and 16S → A, respectively. It is notable that removal of all the phosphorylation sites, however, did not cause complete translocation to the membrane of 16S → A; about 60% of the 16S → A mutant enzyme remained soluble. As expected, conversion of all the sites to Glu resulted in a normal, soluble distribution in unstimulated cells. The Western blots agreed with the activity measurements regarding the subcellular distributions and expression levels of the mutants (Fig. 3). The 16S → E mutants had a considerably slower mobility than the 16S → A mutant. This decreased mobility is similar to the effect of phosphorylation on protein mobility and is consistent with the higher charge of this mutant cytidylyltransferase.

Translocation of Phosphorylation Mutants in Response to Fatty Acid Treatment—Fatty acid treatment causes translocation and activation of cytidylyltransferase in HeLa and other cultured cells (11, 27, 28). CHO cells are much more sensitive to oleate treatment than HeLa cells, but we determined that cells remained viable when treated with 0.25 mM oleate for 20 min, which is sufficient for translocation of cytidylyltransferase. To ask if the phosphorylation mutants could translocate in response to cell stimulation, the cell lines were treated with oleate, and the distributions were determined by activity measurements and Western blots. Oleate stimulated translocation

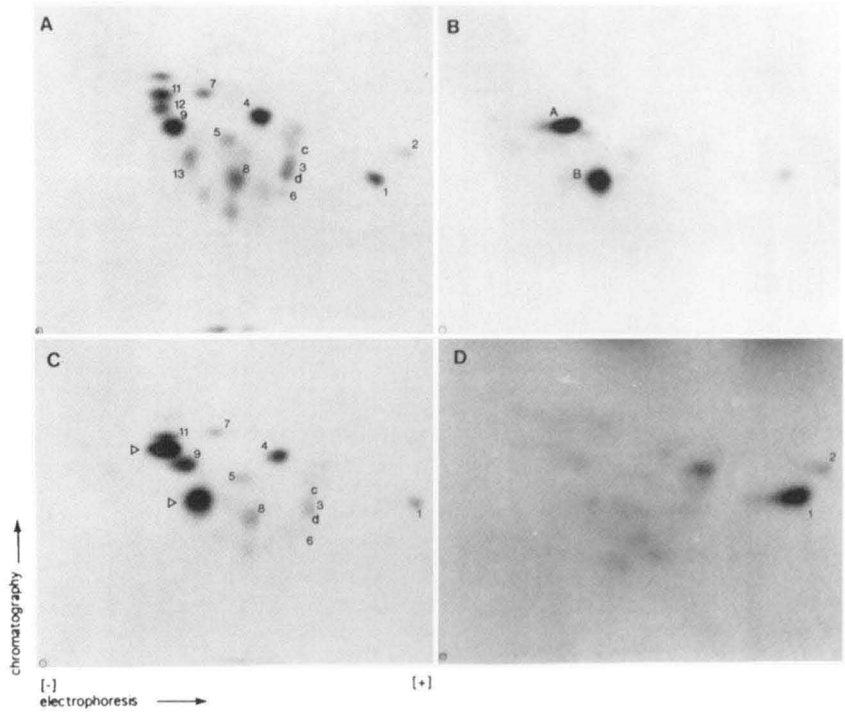

FIG. 2. **Phosphopeptide maps of ^{32}P-labeled cytidylyltransferase.** Labeled samples were immunoprecipitated and blotted as described for Fig. 1. After the labeled cytidylyltransferase bands were detected by autoradiography, the bands on the blots were excised, digested with trypsin, and subjected to two-dimensional mapping as described under "Experimental Procedures." *A*, wild type. *B*, 5SP → AP. *C*, a mixture of 5SP → AP and wild type. *D*, 7SP-AP.

TABLE II
Activity of wild type and mutants normalized to cytidylyltransferase protein

Cells were harvested in digitonin buffer as soluble and particulate fractions. Samples in which the enzyme was most abundant (*e.g.* soluble in control cells and particulate in oleate-treated cells) were assayed in the presence of lipids as described under "Experimental Procedures." The amount of cytidylyltransferase protein in each sample was determined by immunoblotting, detecting by chemiluminescence, and then quantitating with a Bio-Rad Molecular Imager. Specific activity was expressed as nmol of CDP-choline produced per min/relative protein density. Data are the averages ± half the range from duplicate determinations. The experiment was performed twice with similar results.

	Wild type	16S → A	16S → E	7SP → AP
Soluble −oleate	32.6 ± 1.1	35.3 ± 4.0	34.4 ± 4.6	34.2 ± 5.5
Particulate +oleate	19.5 ± 2.7	16.7 ± 1.4	21.7 ± 1.0	16.2 ± 0.7

TABLE III
Specific activities and distributions of wild type and mutant cytidylyltransferases following treatment with oleate

Cells were incubated with or without 0.25 mM sodium oleate for 20 min at 40 °C and harvested into soluble and particulate fractions by digitonin extraction. Enzyme activity was assayed in the presence of lipid and divided by the total protein in the indicated fraction. Specific activity values are the averages ± half the ranges of duplicate determinations. Numbers in parentheses are the percentages of total cytidylyltransferase in each fraction. This experiment was performed twice with similar distributions, except that 5SP → AP was included in only one experiment.

	−Oleate		+Oleate	
	Soluble	Particulate	Soluble	Particulate
	nmol × min^{-1} × mg protein^{-1}			
Wild type	6.9 ± 0.6 (95)	0.3 ± 0.1 (05)	1.6 ± 0.5 (18)	6.4 ± 1.1 (82)
5SP → AP	7.8 ± 0.2 (81)	4.3 ± 0.0 (19)	2.2 ± 0.6 (23)	13.7 ± 1.5 (77)
7SP → AP	7.9 ± 0.8 (72)	7.2 ± 0.6 (28)	1.6 ± 0.0 (18)	14.3 ± 1.8 (82)
16S → A	2.6 ± 0.4 (61)	5.2 ± 2.2 (39)	1.3 ± 0.7 (24)	7.8 ± 0.8 (76)
16S → E	30.1 ± 3.8 (94)	1.6 ± 0.4 (6)	26.9 ± 1.3 (49)	18.7 ± 0.9 (51)

of 77% of wild type cytidylyltransferase to the particulate fraction (Table III), and this correlated with a shift in mobility from multiple bands in the soluble fraction to a faster migrating band in the particulate fraction (Fig. 3). All of the Ser to Ala mutants translocated to a similar extent as the wild type enzyme. A considerable amount of the 16S → E mutant also translocated in response to oleate. Although the percentage of total 16S → E enzyme that translocated appeared less than that in wild type, the actual amount of membrane-associated enzyme in the 16S → E mutant was about three times higher than in wild type due to the higher level of expression of this mutant. Thus, the high negative charge in the phosphorylation region of the 16S → E mutant did not preclude its association with the membrane.

Abilities of Phosphorylation Mutants to Complement the Defects in Strain 58—Because of its temperature-sensitive cytidylyltransferase, the strain 58 cell line cannot grow or synthesize phosphatidylcholine at 40 °C (20, 21). Expression of rat liver cytidylyltransferase in strain 58 cells, however, corrects the defect and allows both phosphatidylcholine synthesis and growth at 40 °C (22). All phosphorylation site mutants were capable of supporting growth of strain 58 CHO cells at 40 °C (Fig. 4), consistent with the fact that these mutant enzymes retain catalytic activity (Table II). At either 34 °C or 40 °C, all cell lines expressing wild type or mutant cytidylyltransferases incorporated radiolabeled choline into phosphatidylcholine far better than did untransfected strain 58 control (Fig. 5). The rates of choline incorporation relative to wild type were somewhat variable for most mutants; cells expressing 16S → A, however, always had a somewhat higher rate of choline incorporation than the wild type and other mutants. The increased choline incorporation in 16S → A, however, was far less than expected, considering there was about 10-fold more membrane-associated enzyme in these cells (Table III). In a total of six labeling experiments, including one pulse-chase protocol, the rate of phosphatidylcholine synthesis was 74 ± 46% higher than in the wild type. This suggests that cytidylyltransferase can be membrane-associated and still be relatively inactive.

The levels of aqueous choline metabolites were also determined at both 34 and 40 °C. As expected, the phosphocholine

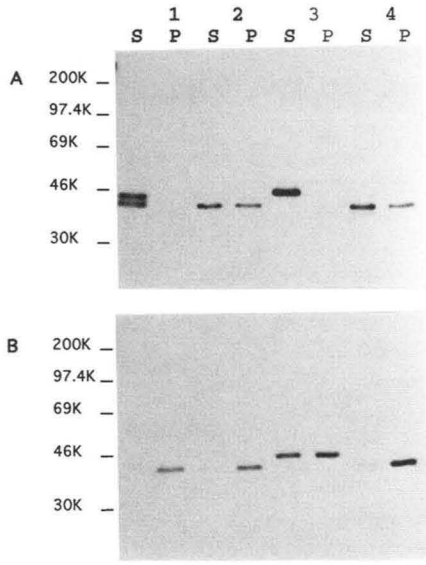

FIG. 3. **Translocation of wild type and mutants in response to oleate treatment.** Cells were plated for 1 day and then treated with 0.25 mM sodium oleate in Ham's F-12 medium for 20 min. The cells were then washed and separated by digitonin extraction into soluble (*S*) and particulate (*P*) fractions of equal volumes. Samples were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting; equal volumes of all fractions were loaded, except for the use of 0.25 volume for 16S → E *S* minus oleate and 16S → E *S* and *P* plus oleate and 0.5 volume for 16S → E *P* minus oleate. *A*, control cells. *B*, oleate-treated cells. *Lanes 1*, wild type; *lanes 2*, 16S → A; *lanes 3*, 16S → E; *lanes 4*; 7SP → AP.

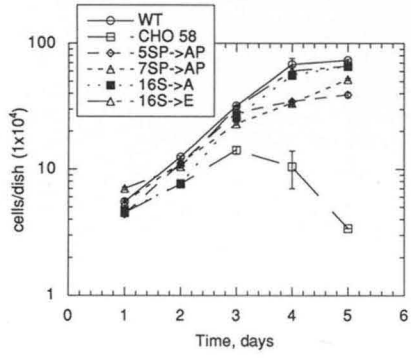

FIG. 4. **Cell growth at 40 °C.** Cells were left at 34 °C for 1 day after plating and then shifted to 40 °C for up to 5 days. Viable cells were counted as described under "Experimental Procedures." *WT*, wild type; *CHO 58*, untransfected strain 58 cells.

level was high and the CDP-choline level was low for strain 58. The wild type and phosphorylation mutants had lower phosphocholine levels and higher CDP-choline levels than untransfected strain 58, which would be expected from their abilities to complement the genetic defect. There were, however, no consistent differences in metabolite levels between cells expressing the wild type enzyme and those expressing the phosphorylation site mutants (data not shown).

DISCUSSION

Experiments described in this paper used site-directed mutagenesis to determine the role of phosphorylation on the properties of cytidylyltransferase. Mutations of as many as all 16 of the phosphorylated residues did not dramatically alter enzymatic activity as judged by assay *in vitro* in the presence of activating lipids, suggesting that the COOH-terminal phosphorylation region does not interact closely with the more central catalytic core under these conditions. The mutations do alter the mobility of the enzyme in SDS gels, as would be expected from previous evidence showing that the slower mobility of the soluble enzyme is due to phosphorylation (18, 19). Conversion of all 16 Ser residues to Ala resulted in the same mobility as the fastest form of wild type cytidylyltransferase. Conversion of the same residues to Glu resulted in a slow moving enzyme, which is consistent with the mobility differences being due to a high degree of negative charge in this region.

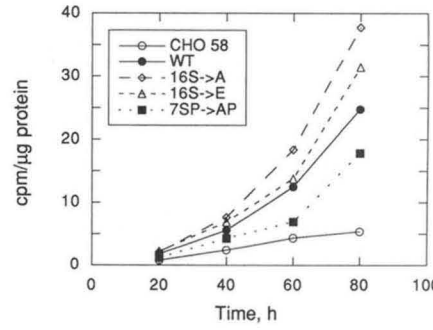

FIG. 5. **Incorporation of choline into lipids.** Cells were plated and left at 34 °C for 1 day. The cells were then washed twice with calcium- and magnesium-free phosphate-buffered saline and incubated with [^{3}H]choline at 40 °C for the indicated times. The cells were then harvested and extracted as described under "Experimental Procedures." *WT*, wild type; *CHO 58*, untransfected strain 58 cells.

Previous studies in which the phosphorylation sites of baculovirus-expressed cytidylyltransferase were determined by chemical sequencing failed to detect any phosphorylated residues other than the 16 Ser residues near the COOH terminus (17). Radiolabeling with ^{32}P of the 16S → A and 16S → E mutants confirmed the protein chemistry, indicating that no other residues are phosphorylated in these mammalian cells. Labeling of the mutants in which Ser-Pro sites were modified to Ala-Pro sites resulted in rather dramatic changes in labeling; loss of a third of the 16 phosphorylatable sites in the 5SP → AP mutant reduced labeling to only two major tryptic peptides, and loss of only two additional sites in the 7SP → AP mutant nearly eliminated labeling. These results suggest that modification by a Ser-Pro-directed protein kinase may be important for subsequent phosphorylation events by other protein kinases. This is supported by the observations of Jackowski (29), who has shown that the phosphorylation state of cytidylyltransferase varies with the cell cycle, in which several Ser-Pro-directed kinases function.

The subcellular location of the 16S → E mutant in control cells did not differ from that of the wild type enzyme, which is not surprising given that most of the wild type enzyme in control cells is soluble and highly phosphorylated. On the other hand, the subcellular location of all mutants in which Ser residues were changed to Ala was modified considerably. The specific activities of membrane-associated mutant enzymes assayed *in vitro* were 5–17-fold higher than that of wild type cytidylyltransferase in control cells, suggesting that dephosphorylation certainly promoted membrane association. The membrane-associated enzymes were not fully activated, however, as indicated by the lack of a dramatic increase in phosphatidylcholine biosynthesis. It is not likely that the membrane-associated 16S → A enzyme was actually 10-fold more active but that the rate of phosphatidylcholine degradation was also increased, because the experiments represented in Fig. 5 were measuring initial rates of phosphatidylcholine biosynthesis. Furthermore, there was no increase in glycerophosphocholine levels in the 16S → A cells, suggesting that increased

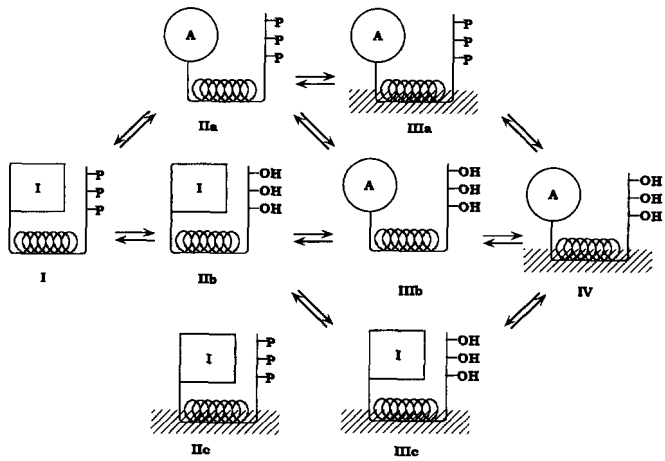

FIG. 6. **Possible intermediate steps in activation, dephosphorylation, and association of cytidylyltransferase with membranes.** *I*, inactive; *A*, active. The *coils* represents the membrane-binding domains (32), and the *hatched bars* represent the membranes.

turnover did not occur.

The amount of the 16S → A mutant that remained soluble ranged from about 40 to 60%, indicating that a completely dephosphorylated cytidylyltransferase can remain soluble. This suggests that dephosphorylation of cytidylyltransferase does not cause it to associate with the membrane but rather influences the extent of its association with the membrane. The lack of extensive phosphorylation of soluble cytidylyltransferase has previously been noted upon reversal of fatty acid treatment in HeLa cells (19). Within 1 or 2 min after removal of the fatty acid, cytidylyltransferase becomes predominantly soluble but migrates with the fast mobility of the membrane associated form, indicating that it is not highly phosphorylated. It was not clear from those studies, however, if the fast moving, soluble form is partially phosphorylated. In choline-starved HepG2 cells, forms of cytidylyltransferase with similarly low levels of phosphorylation are found in both soluble and membrane fractions (14).

The fact that more of the 16S → A mutant became membrane-bound with oleate treatment lends further support to the concept that a process distinct from dephosphorylation is involved in causing the enzyme to associate with membranes. In addition, the fact that the 16S → E mutant did not seem impaired in its ability to interact with membranes indicates that a high degree of negative charge in the phosphorylation region does not preclude cytidylyltransferase from associating with membranes. Although a Glu residue would not be as highly charged as phosphoserine, the evidence implies that phosphorylation per se does not keep cytidylyltransferase soluble. This is consistent with the observation that, upon treatment of rat hepatocytes with fatty acids or phospholipase C, phosphorylated cytidylyltransferase first becomes membrane-associated and subsequently is dephosphorylated (30).

Fig. 6 depicts the state of cytidylyltransferase in its various possible forms. Form I is soluble, relatively inactive, and highly phosphorylated. Form IV is membrane-associated, active, and extensively dephosphorylated. Both forms I and IV are well established from many previous studies. The forms in columns II and III are hypothetical intermediates in the transition between forms I and IV. Forms IIa–IIc would be produced in one step from form I, and forms IIIa–IIIc would be produced in one step from form IV. *A* in a *circle* and *I* in a *square* refer to the relative state of activation in the cell (active and inactive, respectively); all forms are proposed to be fully activable *in vitro*. The existence of the intermediate forms that are connected by *arrows* are supported by some evidence. Form IIa would correspond to a situation in which activation of the enzyme is not accompanied by dephosphorylation or appreciable translocation to membranes, such as in phorbol ester-treated HeLa cells (16). Form IIa may be active in a soluble form because it is activated by association with a lipoprotein complex (9). Form IIb would correspond to the 16S → A mutant in control cells where much of the enzyme remains soluble. Whether such a form exists for wild type cytidylyltransferase remains to be determined. The fact that the 16S → E mutant can readily associate with membranes in oleate-treated cells supports the existence of form IIIa. Moreover, form IIIa is strongly indicated by the translocation of phosphorylated cytidylyltransferase in rat hepatocytes discussed above (30). Form IIIb, which is soluble, active, and relatively dephosphorylated, is found in choline-starved HepG2 cells (14) and immediately after removal of oleate from HeLa cells (19). Form IIIc would correspond to the 16S → A mutant that is membrane-associated but relatively inactive in control cells. We are not aware of evidence supporting form IIc, in which inactive, phosphorylated cytidylyltransferase is membrane-associated, but such a form cannot yet be ruled out. Although evidence suggests that these various forms can exist, the preferred route of interconversion of forms I and IV is not yet clear. Multiple pathways may operate in some circumstances. For example, the route of formation for form IIIb in choline-starved cells may be I → IIa → IIIa → IV → IIIb.

The fact that membrane-associated 16S → A is not fully activated suggests that an additional component in the membrane is required to activate the enzyme. The question as to what causes activation of cytidylyltransferase *in vivo* is currently unresolved, but much evidence supports the concept that a suitably altered change in membrane lipid content can activate cytidylyltransferase (1, 31). What role, if any, does phosphorylation play? It seems evident that phosphorylation and dephosphorylation are not the triggers to inactivate and activate, respectively, cytidylyltransferase. The phosphorylation state of the enzyme, however, may be important for stabilizing it in either its soluble or membrane-associated forms. This in turn would shift the equilibrium between soluble and membrane forms, serving to promote the continued activation or inactivation of the enzyme.

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REFERENCES

1. Kent, C. (1990) *Prog. Lipid Res.* **29,** 87–105
2. Houweling, M., Tijburg, L. B., Vaartjes, W. J., Batenburg, J. J., Kalmar, G. B., Cornell, R. B., and Van Golde, L. M. (1993) *Eur. J. Biochem.* **214,** 927–933
3. Tessner, T. G., Rock, C. O., Kalmar, G. B., Cornell, R. B., and Jackowski, S. (1991) *J. Biol. Chem.* **266,** 16261–16264
4. Watkins, J. D., and Kent, C. (1992) *J. Biol. Chem.* **267,** 5686–5692
5. Wang, Y., Sweitzer, T. D., Weinhold, P. A., and Kent, C. (1993) *J. Biol. Chem.* **268,** 5899–5904
6. Morand, J. N., and Kent, C. (1989) *J. Biol. Chem.* **264,** 13785–13792
7. Weinhold, P. A., Charles, L., Rounsifer, M. E., and Feldman, D. A. (1991) *J. Biol. Chem.* **266,** 6093–6100
8. Feldman, D. A., Dietrich, J. W., and Weinhold, P. A. (1980) *Biochim. Biophys. Acta* **620,** 603–611
9. Weinhold, P. A., Rounsifer, M. E., Charles, L., and Feldman, D. A. (1989) *Biochim. Biophys. Acta* **1006,** 299–310
10. Weinhold, P. A., Rounsifer, M. E., Williams, S. E., Brubaker, P. G., and Feldman, D. A. (1984) *J. Biol. Chem.* **259,** 10315–10321
11. Pelech, S. L., Cook, H. W., Paddon, H. B., and Vance, D. E. (1984) *Biochim. Biophys. Acta* **795,** 433–440
12. Sleight, R., and Kent, C. (1980) *J. Biol. Chem.* **255,** 10644–10650
13. Sleight, R., and Kent, C. (1983) *J. Biol. Chem.* **258,** 831–835
14. Weinhold, P. A., Charles, L., and Feldman, D. A. (1994) *Biochim. Biophys. Acta* **1210,** 335–347
15. Sleight, R., and Kent, C. (1983) *J. Biol. Chem.* **258,** 836–839
16. Watkins, J. D., and Kent, C. (1990) *J. Biol. Chem.* **265,** 2190–2197
17. MacDonald, J. I., and Kent, C. (1994) *J. Biol. Chem.* **269,** 10529–10537
18. Watkins, J. D., and Kent, C. (1991) *J. Biol. Chem.* **266,** 21113–21117
19. Wang, Y., MacDonald, J. I., and Kent, C. (1993) *J. Biol. Chem.* **268,** 5512–5518
20. Esko, J. D., and Raetz, C. R. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5192–5196

21. Esko, J. D., Wermuth, M. M., and Raetz, C. R. (1981) *J. Biol. Chem.* **256,** 7388–7393
22. Sweitzer, T. D., and Kent, C. (1994) *Arch. Biochem. Biophys.* **311,** 107–116
23. Kalmar, G. B., Kay, R. J., Lachance, A., Aebersold, R., and Cornell, R. B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6029–6033
24. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
25. Weinhold, P. A., and Feldman, D. A. (1992) *Methods Enzymol.* **209,** 248–258
26. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
27. Pelech, S. L., Pritchard, P. H., Brindley, D. N., and Vance, D. E. (1983) *J. Biol. Chem.* **258,** 6782–6788
28. Whitlon, D. S., Anderson, K. E., and Mueller, G. C. (1985) *Biochim. Biophys. Acta* **835,** 369–377
29. Jackowski, S. (1994) *J. Biol. Chem.* **269,** 3858–3867
30. Houweling, M., Jamil, H., Hatch, G. M., and Vance, D. E. (1994) *J. Biol. Chem.* **269,** 7544–7551
31. Jamil, H., Hatch, G. M., and Vance, D. E. (1993) *Biochem. J.* **291,** 419–427
32. Craig, L., Johnson, J. E., and Cornell, R. B. (1994) *J. Biol. Chem.* **269,** 3311–3317