Neuropathy Target Esterase and Its Yeast Homologue Degrade Phosphatidylcholine to Glycerophosphocholine in Living Cells

Eukaryotic cells control the levels of their major membrane lipid, phosphatidylcholine (PtdCho), by balancing synthesis with degradation via deacylation to glycerophosphocholine (GroPCho). Here we present evidence that in both yeast and mammalian cells this deacylation is catalyzed by neuropathy target esterase (NTE), a protein originally identified by its reaction with organophosphates, which cause nerve axon degeneration. YML059c, a Saccharomyces cerevisiae protein with sequence homology to NTE, had similar catalytic properties to the mammalian enzyme in assays of microsome preparations and, like NTE, was localized to the endoplasmic reticulum. Yeast lacking YML059c were viable under all conditions examined but, unlike the wild-type strain, did not convert PtdCho to GroPCho. Despite the absence of the deacylation pathway, the net rate of [14C]choline incorporation into PtdCho in YML059c-null yeast was not greater than that in the wild type; this was because, in the null strain diminished net uptake of extracellular choline and decreased formation of the rate-limiting intermediate, CDP-choline, resulted in a reduced rate of PtdCho synthesis. In [14C]choline labeling experiments with cultured mammalian cell lines, production of [14C]GroPCho was enhanced by overexpression of catalytically active NTE and was diminished by reduction of endogenous NTE activity mediated either by RNA interference or organophosphatase treatment. We conclude that NTE and its homologues play a central role in membrane lipid homeostasis.

Levels of phosphatidylcholine (PtdCho), the major membrane lipid of eukaryotic cells, are tightly regulated by coordination of its synthesis and degradation. In both yeast (1) and mammalian cells (2), PtdCho synthesized by the CDP-choline pathway (see Fig. 1) is deacylated by as yet unidentified phospholipases to form glycerophosphocholine (GroPCho). In principle, this deacylation at both sn-2 and sn-1 positions of PtdCho could be mediated either by a single enzyme with phospholipase B activity or by sequential action of a phospholipase A2 and a lysophospholipase. We have reported that when mixed micelles of PtdCho with detergent were incubated with the recombinant catalytic domain of neuropathy target esterase (NTE), fatty acid was liberated very slowly from the sn-2 position followed by rapid deacylation of the resulting lysophospholipid (3). Because the rates and selectivities of bond cleavage observed in phospholipase assays in vitro are profoundly affected by the physicochemical nature of the substrate (4–6), it is possible that in vivo NTE could deacylate the sn-2 position of PtdCho more efficiently than observed in our experiments. Thus, NTE might be the phospholipase B responsible for converting PtdCho to GroPCho (see Fig. 1).

NTE was originally identified as the target site for those organophosphates that cause a paralyzing delayed neuropathy with degeneration of long nerve axons (7). In adult animals NTE is present in the nervous system and a variety of nonneuronal tissues (8). NTE is also widely expressed during fetal development (9). Studies on green fluorescent protein (GFP)-tagged NTE constructs expressed in COS cells indicate that NTE is anchored to the cytoplasmic face of the endoplasmic reticulum (10).

Definitive evidence that NTE can convert PtdCho to GroPCho could be obtained by comparing PtdCho metabolism in wild-type and NTE-null cells. However, mice lacking NTE die by mid-gestation (11, 12), and fibroblasts from day 8 embryos can be cultured for only limited periods (12). On the other hand, a gene, YML059c, in the yeast, Saccharomyces cerevisiae, encodes a putative protein with substantial sequence homology to NTE. The availability of a YML059c-null mutant strain from the Euroscarf collection suggested that this protein is not essential for yeast viability under all growth conditions. The aim of the present study was to determine whether YML059c has the same catalytic properties and subcellular location as NTE, whether YML059c-null mutant yeast can be used to investigate whether NTE mediates the same biochemical reaction in cultured mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**[Methyl-1-14C]Choline chloride (56 mCi/mmol) and [1-14C]methionine (59 mCi/mmol), cytidine 5’-diphospho[methyl-14C]choline (56 mCi/mmol), and phosphoryl[methyl-14C]choline (55 mCi/mmol) were from Amersham Biosciences. Lysophosphatidylcholine (Lysophosphatidylcholine; Cho) was obtained from egg yolk containing primarily palmitic and stearic acids (catalogue number L4129), choline, and methionine were purchased from Sigma. Double-stranded siRNA (with and without a 3’-fluorescein label) was synthesized by Xeragon, and the sequences used were 5’-AAGAUUAUUGCAGAAGUUGUCAATTT-3’ for NTE-siRNA and 5’-CAGAGUGUAGCUGCGUAGGUAAGTT-3’ as a control siRNA. The sources for all other reagents have been described previously (3, 10).
Generation of Yeast Strains—The basic yeast strains used in this study (Table I) were obtained from Euroscarf and were maintained on standard YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose). Unless stated otherwise, yeast strains were grown at 30 °C. The Euroscarf Yml059cΔ deletion strains carry an allele wherein the complete YML059c gene had been replaced by PCR-mediated insertion of the Gal1-1 promoter plus GFP tag and HIS3MX6 marker amplified by PCR from a pRF6a-HIS3MX6-Pgal-GFP plasmid (15); the PCR primers were designed so that the flanking sequence of the cassette would allow insertion by homologous recombination of the selectable marker, the Gal1 promoter, and the GFP tag upstream of YML059c. One of the resultant heterozygous diploid transformants was induced to undergo meiosis and sporulation to give the haploid progeny BY4741 Pgal-GFP-YML059c and BY4742 Pgal-GFP-YML059c, which were subsequently crossed to form the homostegous diploid BY4743 Pgal-GFP-YML059c Pgal-GFP-YML059c (hereafter called GFP-YML059c +/-) .

To allow overexpression of a plasmid-based construct (rather than a chromosomal one, as above), genomic DNA from BY4743 Pgal-GFP-YML059c was amplified by PCR, and the resulting product was ligated into the SacI and Xhol sites of the URA3 centromeric yeast cloning vector pRS416 (14). The codon for the active site serine (Ser-1406) in the plasmid pRS416-Pgal-GFP-YML059c was mutated to code for alanine by making a single T to G transversion of base 4216 using the Stratagene QuikChange mutagenesis kit. Yeast (BY4743 strains) were transformed to uracil prototrophy with these plasmids by heat shocking in 0.1 M lithium acetate (14).

Phenotypic Tests—A series of standard tests (17) was carried out to compare the haploid wild-type BY4741 and BY4741 yml059cΔ mutant response to a wide variety of nutrients, stress conditions, and inhibitors. Most of these involved spotting serial dilutions of the wild-type or mutant yeast onto YP (1% yeast extract, 2% bactopeptone medium) agar plates with a variety of carbon sources (glucose, galactose, sucrose, maltose, glycerol, acetate, and a 2-deoxyglucose/sucrose mixture) or on YPD agar with various concentrations of stressor/inhibitor agents (H2O2, sorbitol, acid, ethanol, calcofluor, caffeine, EDTA, phenanthroline, formamide, cycloheximide, sodium orthovanadate, and heavy metal ions). Growth on YPD-agar was assessed at various temperatures from 4 to 37 °C and at 30 °C after 55 °C heat shock (30–90 min). Growth was also assessed under anaerobic conditions on YPD-agar containing Tween 80 and ergosterol and under aerobic conditions on yeast nitrogen base agar with either ammonium sulfate or proline as the nitrogen source.

Growth of Yeast for Overexpression of YML059c and Isolation of Microsomes—Overnight cultures of yeast strains in YP medium containing 2% galactose were diluted to a standard concentration (A\text{600} ≈ 0.2) and then grown in YP, 2% galactose until A\text{600} = 1.0 (5–6 h). Yeast were harvested, washed twice with phosphate-buffered saline (PBS), and then stored at -20 °C. Frozen yeast cell pellets (equivalent to 30-ml culture with A\text{600} = 1.0) were thawed, resuspended in 0.5 ml of ice-cold TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0), and then vortexed (5 min; 4 °C) with 0.5 g of glass beads (425–600 μm diameter; Sigma). The resulting homogenate was made up to a volume of 1 ml with TE buffer, then centrifuged at 100,000 × g for 45 min at 4 °C. The pellet fraction was resuspended in 1 ml of ice-cold TE buffer by using a syringe with a 25-gauge needle, and then spun in an Eppendorf centrifuge (1 min; 300 × g). The resulting supernatant fraction was carefully recovered and used as a yeast microsomal preparation.

Localization of GFP-YML059c by Confocal and Immunoelectron Microscopy—Yeast overexpressing GFP-YML059c were grown as described above, harvested, and washed twice with PBS. For confocal microscopy, unfixed yeast were immobilized in agarose and examined using a Zeiss LSM 510 confocal microscope with a 488-nm argon laser. Confocal micrographs were deconvolved using the Huygens software system, and volume rendering of confocal image stacks was done using Imaris Velocity 2.5 software. Other samples were fixed with 2% glutaraldehyde and then treated with tannoyse (18) before being prepared for routine electron microscopy. Yeast for immunoelectron mi-
cAMP-binding proteins are marked with putative regulatory regions. Sequences with similarity to those of gray domain homology between NTE and YML059c. Homologous regions are shown in gray; within these regions, sequence identity of NTE and YML059c is 39% in the catalytic domain and (an average of) 30% in the putative regulatory domain. Sequences with similarity to those of cAMP-binding proteins are marked with dotted boxes, and the positions of the amino-terminal transmembrane helix (thick vertical line) and the active site serine are shown. The broken line indicates the approximate extent of the catalytic and regulatory domains. b, c, and d, esterase activities of NTE and YML059c are inhibited by a series of OPs with the same rank potency order. Microsomes isolated from COS cells transfected with NTE-GFP or GFP-YML059c/+/strain yeast were preincubated (37 °C; 20 min) with the indicated concentrations of OPs, and then substrate (phenyl valerate) was added, and esterase activities were determined in a 20-min (37 °C) incubation period (see “Experimental Procedures”). The OPs used were phenyl saligenin phosphate (PSP), phenylidipentyl phosphate (PDP), diisopropyl fluorophosphate (DFP), and paraoxon (PXN). Data are from duplicate determinations with values within <10% of the mean and are representative of two experiments. d, NTE and YML059c deacyleate exogenous LysoPtdCho. Microsomes from COS cells (transfected with either NTE-GFP or GFP alone) or yeast (wild type or overexpressing GFP-YML059c) were isolated and incubated with the indicated concentrations LysoPtdCho, and fatty acid liberated was determined as described under “Experimental Procedures.” Data are representative of five experiments.
supernatant, the pellet was resuspended in 1.0 ml of 1% SDS in TE buffer, and 0.5 ml (representing the membrane fraction) was saved. Total 14C radioactivities in the three 0.5-ml aliquots representing extracellular, soluble intracellular, and membrane fractions (see Ref. 1) were determined by scintillation counting. Aliquots (0.5 ml) of the resuspended membrane fraction were extracted with 1.0 ml chloroform-methanol-acetic acid (2:1:0.02), and distribution of radioactivity between the aqueous and organic phases was determined. TLC fractionation in chloroform, methanol, acetic acid, 0.9% NaCl, aqueous ammonia (1:1:1:0.1) (21) followed by phosphorimaging was used to show that essentially all the radioactivity in the organic phase comigrated with PtdCho.

To examine PtdCho synthesis via the CDP-choline pathway, yeast were grown for 18 h at 25°C in SD medium with 0.1 mM choline, then pelleted by centrifugation, resuspended to a density of A600 of 0.3–0.4 in fresh SD medium containing 0.1 mM choline supplemented with 1 µCi/ml [14C]choline, and grown at 37°C. Aliquots (1.0 ml) were removed at various times and pelleted in an Eppendorf centrifuge. The cells were washed twice with ice-cold water and then frozen. After thawing, yeast were vortexed for 5 min at 4°C in chloroform-methanol (1:1) with glass beads, and then water and chloroform were added to allow phase separation as described by Williams and McMaster (21). Radioactivity in aliquots of the aqueous and organic solvent phases was determined by scintillation counting. The remainder of the aqueous phase was dried in a Speed-Vac, then dissolved in water and fractionated by TLC in methanol, 0.6% NaCl, aqueous ammonia (1:1:0.1) (21). The distribution of [14C]-labeled metabolites on the TLC plate was determined by phosphorimaging.

RESULTS

YML059c, a Putative NTE Homologue, Is Not Essential for Yeast Viability under a Variety of Conditions—A battery of standard tests were performed to determine whether yeast lacking YML059c display a phenotype (see “Experimental Procedures”). However, no significant difference in growth between the wild-type and deletion mutant was detected under any of these conditions. Similarly, other laboratories have attempted global phenotypic analysis by examining large numbers of S. cerevisiae deletion mutants, some of which included YML059c-null mutants (20). Sequence similarity (see Table II) strongly suggest that the yeast protein may have serine esterase activity. However, when using phenyl valerate as a substrate, we were unable to detect a significant difference between the low levels of esterase activity in microsomes from wild-type or YML059c-null mutant yeast (Table II). A recent study determined levels of expression of >4000 open reading frames of S. cerevisiae by tagging with GFP and quantitative Western blotting; this revealed that YML059c is present at a level of only ~500 copies per cell (24). To permit detection of this protein’s putative esterase activity we overexpressed various GFP-tagged constructs (see “Experimental Procedures”). Microsomes from yeast overexpressing a chromosomal construct of GFP-YML059c displayed substantially greater esterase activity than the wild-type parent, and the diploid overexpressing strain had twice the activity of the haploid (Table II). Similarly, overexpression of YML059c from a plasmid also led to increased esterase activity, but this increase was abolished in a strain expressing an analogous construct in which the putative active site serine, Ser-1406, was mutated to alanine (Table II). However, no significant difference in activity could be detected between YML059c and NTE (39% identity in the catalytic domain; see Fig. 2a and alignments in Ref. 20) strongly suggest that the yeast protein may have serine esterase activity. However, using phenyl valerate as a substrate, we were unable to detect a significant difference between the low levels of esterase activity in microsomes from wild-type or YML059c-null mutant yeast (Table II). A recent study determined levels of expression of >4000 open reading frames of S. cerevisiae by tagging with GFP and quantitative Western blotting; this revealed that YML059c is present at a level of only ~500 copies per cell (24). To permit detection of this protein’s putative esterase activity we overexpressed various GFP-tagged constructs (see “Experimental Procedures”). Microsomes from yeast overexpressing a chromosomal construct of GFP-YML059c displayed substantially greater esterase activity than the wild-type parent, and the diploid overexpressing strain had twice the activity of the haploid (Table II). Similarly, overexpression of YML059c from a plasmid also led to increased esterase activity, but this increase was abolished in a strain expressing an analogous construct in which the putative active site serine, Ser-1406, was mutated to alanine (Table II).

NTE was originally identified in brain homogenates as an esterase activity inhibited by neuropathic OP compounds (7). Esterase activity in microsomes from COS cells overexpressing...
NTE Deacylates Phosphatidylcholine

Fig. 4. Degradation of PtdCho at 37 °C in wild-type and YML059c-null yeast and its inhibition by OPs. Wild-type (WT) and YML059cΔΔ strains were metabolically labeled (18 h; 25 °C) with either [14C]Cho or [methyl-14C]methionine (Met). Subsequently label was chased out in the presence of 10 mM unlabeled Cho or Met by growth at 37 °C for 4 h followed by growth at 37 °C for the times indicated. a, NTE-GFP and from yeast overexpressing GFP-YML059c was inhibited by a series of OPs, with both enzymes showing the same rank order of sensitivity; phenyl saligenin phosphate (PSP) > diisopropyl fluorophosphate (DFP) > paraoxon (Fig. 2, b and c). The potency of OPs toward YML059c in yeast microsomes was 5–10-fold less than against NTE in COS cell microsomes. Preincubation of OPs with yeast microsomal preparations did not reduce their potency to subsequently inhibit the esterase activity of the purified recombinant catalytic domain of NTE (data not shown); this indicates that OPs are not degraded by enzymes in the yeast preparation but, rather, that YML059c is intrinsically less sensitive than NTE to OP inhibition.

The purified recombinant catalytic domain of NTE has potential lysophospholipase activity (3). Microsomes from COS cells overexpressing NTE-GFP or yeast overexpressing GFP-YML059c showed substantially increased lysophospholipase activity against exogenous LysoPtdCho compared with control microsomes (Fig. 2d). Determination of activity at varying substrate concentrations indicated that both NTE and YML059c were half-maximally active at LysoPtdCho concentrations around 0.05 mM, comparable with the \( K_m \) value determined previously for the purified recombinant catalytic domain of NTE (3).

YML059c Localizes to Yeast Endoplasmic Reticulum—Confocal microscopy of yeast overexpressing GFP-tagged YML059c from the chromosomal construct or plasmids showed that fluorescence was confined to the cytoplasm, where it exhibited a reticular distribution with particular focal concentrations (Fig. 3a). Electron microscopy showed that the cytoplasm of most cells contained tubuloreticular complexes that were in continuity with the smooth endoplasmic reticulum (Fig. 3, b and c). Huh et al. (25) have recently localized >4000 GFP-tagged yeast proteins expressed at their endogenous levels and concluded that YML059c is associated with the endoplasmic reticulum. We have confirmed this localization by immunoelectron microscopy, which revealed that GFP-tagged YML059c was associated with the cytoplasmic complexes (Fig. 3d); these resembled, but were less pronounced and had many more associated mitochondria than complexes observed in mammalian cells overexpressing GFP-tagged NTE (10). Importantly, we did not detect immunogold labeling in mitochondria despite their close association with the endoplasmic reticulum (Fig. 3d) or in the Golgi apparatus.

YML059c Deacylates CDP-choline-derived Phosphatidylcholine—Dowd et al. (1) report that PtdCho synthesized by the CDP-choline pathway is rapidly deacylated by an unidentified phospholipase in wild-type S. cerevisiae grown at 37 °C. To determine whether YML059c mediates this deacylation, we labeled PtdCho in wild-type and YML059c-null strains by growing them at 25 °C in the presence of [14C]choline and then chased out the label by growing at 37 °C in the presence of excess unlabeled choline. We performed this experiment and

\[ \text{[14C]PtdCho remaining} = \frac{100\% \times \text{dpm}}{\text{dpm}} \]

Mean 100% values were 8.2 ± 2.1 \times 10^3 \text{ dpm (wild type)} and 8.1 ± 0.26 \times 10^3 \text{ dpm (ΔΔ)} for [14C]Cho labeling and 40.3 ± 1.4 \times 10^3 \text{ dpm (wild type)} and 25.9 ± 0.5 \times 10^3 \text{ dpm (ΔΔ)} for [14C]Met labeling. b, total intracellular water-soluble [14C]-labeled metabolites. c, total extracellular water-soluble [14C]-labeled metabolites were determined as described under "Experimental Procedures." Data are the means and S.D. of triplicate determinations and are representative of three experiments. d, wild-type yeast labeled with [14C]Cho as above were grown in the presence of the indicated OPs for the final 1 h of the 25 °C chase (see above) before shifting to growth at 37 °C for 1 h, after which the amount of [14C]PtdCho remaining was determined. Data are the means and S.D. of triplicate determinations and are representative of two experiments. PSP, phenyl saligenin phosphate; DFP, diisopropyl fluorophosphate; PXN, paraoxon.
analyzed the data essentially as described by Dowd et al. (1). Under these conditions, [14C]PtdCho was degraded by about 50% within 1 h in wild-type yeast but was much more stable in the YML059c-null strain (Fig. 4a). By contrast, PtdCho labeled by growing yeast in [methyl-14C]methionine (via methylation of phosphatidylethanolamine) was relatively stable in both wild-type and null strains during a subsequent 4-h chase at 37 °C in the presence of excess unlabeled methionine (Fig. 4a). Interestingly, the degradation of [14C]Cho-labeled PtdCho could be inhibited by incubating (1 h at 25 °C) yeast with OPs before starting the 37 °C chase (Fig. 4d); the rank potency of OPs in preventing PtdCho degradation was identical to that observed for inhibition of YML059c esterase activity in yeast microsome preparations (Fig. 2b).

Determination of total radioactivity in the intracellular water-soluble fraction of [14C]choline pulse-chase yeast (cf. Ref. 1) revealed a rapid initial increase in the wild-type strain but not in the YML059c-null mutant (Fig. 4b). This increase was essentially maximal by 1 h and remained steady for a further 3 h (Fig. 4b). Dowd et al. (1) report that, in wild-type yeast under these conditions, GroPCho is the major soluble 14C-labeled metabolite in the intracellular pool (see also Fig. 5b). Thus, the YML059c-null mutant is unable to deacetylate PtdCho to GroPCho. In both the wild-type and YML059c-null strains, extracellular radioactivity initially decreased at 1 h of chase followed by an increase over the next 3 h (Fig. 4c). Dowd et al. (1) show that the major extracellular-labeled metabolite under these conditions is [14C]choline, and we have confirmed this (data not shown).

![Fig. 5. GroPCho is absent, and CDP-choline production and Cho net uptake are diminished in YML059c-null yeast.](http://www.jbc.org/Downloaded from)
shown). Thus, the increase in extracellular [14C]choline between 1 and 4 h of chase mirrored the slow decline in [14C]Pt-
dCho over the same time period (Fig. 4a), suggesting that this part of PtdCho degradation was mediated by phospholipase D (see Ref. 26) to yield (unlabeled) phosphatidic acid and [14C]choline, which would be secreted into the extracellular medium (Fig. 1).

**Synthesis of CDP-choline-derived PtdCho Is Restricted in YML059c-null Yeast**—It might be predicted that at 37 °C the net rate of incorporation of [14C]choline into PtdCho would be faster in the YML059c-null strain than wild-type yeast since deacylation is not possible in the mutant. However, in the null strain, net formation of labeled PtdCho showed an initial lag of ~2 h, thereafter proceeded at essentially the same rate as the wild type, and between 4 and 8 h of labeling time attained levels that were ~60% of those in the wild type (Fig. 5a).

Measurement of A600 values over this time course indicated that both strains grew at approximately the same rate (data not shown). However, analysis of the water-soluble intracellular [14C]labeled fraction revealed that whereas GroPCho was by far the dominant metabolite in wild-type yeast, it was absent in the YML059c-null strain (Fig. 5b). Although [14C]Cho levels were similar or even somewhat greater in the null strain (Fig. 5c), intracellular [14C]Cho rapidly reached levels in the wild-type that were approximately twice those in the YML059c-null mutant (Fig. 5d). This suggests that, in the YML059c-null mutant, uptake of extracellular choline is reduced and/or choline efflux is increased (Fig. 1). By labeling yeast with higher specific radioactivity [14C]choline we showed that the minor soluble metabolite, CDP-choline, was present at substantially lower levels in the YML059c-null strain than the wild-type (Fig. 5e); this indicates that, in the null mutant, PtdCho synthesis is restricted at the rate-limiting step mediated by CTP-phosphocholine cytidylyltransferase (CCT) (Fig. 1).

**NTE Converts PtdCho to GroPCho in Mammalian Cells**—We performed [14C]choline labeling experiments to seek evidence that NTE enhances formation of GroPCho in mammalian cells. Between 6 and 24 h labeling times, cells transfected with NTE-GFP contained approximately twice as much [14C]GroPCho as those transfected with either GFP or the catalytically inactive active site serine mutant NTE(S966A)-GFP (Fig. 6a). The latter is an important control because overexpression of NTE in mammalian cells causes deformation and mild proliferation of the endoplasmic reticulum by a non-enzymatic mechanism (10). Indeed, in cells overexpressing either NTE-GFP or NTE(S966A)-GFP, the rate of incorporation of [14C]Cho into PtdCho (Fig. 6b) and into PCho (Fig. 6c) was 50–100% greater than that in vector-transfected cells. We did not detect any change in the levels of LysoPtdCho in cells overexpressing NTE-GFP after incubation with either [14C]-labeled Cho or palmitic acid (data not shown).

To examine the effect on GroPCho production of a reduction in NTE expression we used RNA interference by transfecting cultured cells with short double-stranded RNAs (27). Si-RNA encoding a 5'-sequence of NTE (see “Materials” under “Experimental Procedures”) caused a dose-dependent inhibition of both [14C]GroPCho production and endogenous NTE-esterase activity, whereas a control sequence siRNA had little or no significant effect (Fig. 7, a and b). Similarly, the addition of OPs to the [14C]choline-labeling medium also reduced the formation of [14C]GroPCho and inhibited NTE-esterase activity (Fig. 7, c-f) with similar potency for both processes, except for PDPP, which appeared rather more potent as an esterase inhibitor.

**DISCUSSION**

We have presented evidence that YML059c is the *S. cerevisiae* homologue of NTE. The yeast and mammalian proteins...
have conserved primary sequences, localize to the endoplasmic reticulum, and have very similar catalytic reactivities with ester substrates and OP inhibitors in vitro. In contrast to the embryonic lethal phenotype of NTE knock-out mice (11, 12), yeast lacking YML059c are viable under all the conditions tested in this report. The viability of YML059c-null yeast allowed us to test the hypothesis that YML059c is the enzyme responsible for deacylating PtdCho (Fig. 1). In [14C]choline-labeling experiments at 37 °C, [14C]GroPCho was the dominant (>85%) soluble intracellular metabolite in wild-type yeast but was absent from the YML059c-null mutant strain. In pulse-chase experiments, CDP-choline-derived [14C]PtdCho was rapidly degraded at 37 °C in wild-type yeast but was much more stable in the YML059c-null strain. In both strains PtdCho was also degraded by a second, slower pathway (probably by phospholipase D; cf. Ref. 26). We conclude that YML059c is the major, possibly the only, enzyme in S. cerevisiae responsible for the sequential double deacylation of PtdCho to yield GroPCho and free fatty acids (Fig. 1).

**Fig. 7. Reduction of endogenous NTE activity inhibits production of GroPCho in mammalian cells.** a and b, RNA interference inhibits both GroPCho production and NTE esterase activity. COS cells were transfected with (unlabeled) NTE-specific or control-siRNAs at the indicated concentrations and incubated with [14C]Cho for 24 h, and subsequently, [14C]GroPCho production or NTE-esterase activity was determined as described under "Experimental Procedures." c–f, OP treatment inhibits incubated with OP's at the indicated concentrations (with or without [14C]Cho) for 24 h, and subsequently, [14C]GroPCho formation and NTE-esterase activity were determined as described under "Experimental Procedures." In a–f, data are the means and S.D. from three independent experiments with three dishes of cells for both assays in each experiment. PSP, phenyl saligenin phosphate; PDPP, phenyldipentyl phosphinate; DFP, diisopropyl fluorophosphate; PXN, paraoxon.
more slowly in wild-type yeast grown at 30 °C than at 37 °C, whereas PtdCho made by methylation of phosphatidylethanolamine is relatively stable even at 37 °C; the stability of the latter pool appears to reflect the fact that its synthetic rate is barely changed between 30 and 37 °C, whereas that for the former increases by about 4-fold (1). Boumann et al. (28) show that, in wild-type yeast grown at 30 °C, PtdCho produced via CDP-choline has a more diverse acyl chain composition than that synthesized by the methylation pathway and that this reflects its deacylation and reacylation at both sn-1 and sn-2 positions (28). Thus, the major function for YML059c in yeast grown at 20–30 °C may be to allow remodeling of the acyl chain composition of PtdCho.

Despite the absence of the deacylation pathway, PtdCho levels in YML059c-null yeast were not greater than in the wild type. This reflected a reduction in the rate of PtdCho synthesis in the null strain brought about by diminished net uptake/retention of extracellular choline and by inhibition of the rate-limiting step catalyzed by CCT (Fig. 1). A complex of PtdCho bound to the phospholipid-transfer protein SEC14 somehow inhibits the activity of CCT (29, 30). Presumably, transiently increased PtdCho in the YML059c-null strain is bound by SEC14, and CCT activity is then appropriately reduced.

As the major membrane lipid in eukaryotic cells, synthesis and turnover of PtdCho is required for cell division and creation of new membranes (31–34). In yeast dividing every 1.5–2 h, PtdCho turnover is faster than in cultured mammalian cell lines dividing every 1–2 days. Walkey et al. show (35) that in cultured COS cells CDP-choline-deriv PtdCho turns over with a half-life of ~40 h. Similarly, we found only a ~15% loss in COS or HeLa cell [14C]Cholsabeled PtdCho during a 24-h chase (data not shown). However, despite the relatively slow turnover of PtdCho in mammalian cells compared with yeast, we showed by [14C]choline labeling that production of [14C]GroPCos is inhibited when NTE expression is reduced by RNA interference and is increased by overexpression of catalytically active NTE. The relatively modest degree (~2-fold) of increased GroPCos production in cells overexpressing NTE may, in part, reflect the inhibition of normal cell division in these cells; for example, we have been able to create stable cell lines expressing GFP alone but not expressing NTE-GFP.

Our data indicate that in mammalian cells NTE appears to mediate the same biochemical reaction as does YML059c in yeast. Overexpression of calcium-independent phospholipase A2 in COS cells has also been shown to cause increased production of GroPCos in cells overexpressing NTE in wild-type yeast, which was accompanied by an increase in LysoPtdCho (36). Similarly, inhibition of GroPCos production in HeLa cells by bromoënollactone has been taken to imply calcium-independent phospholipase A2 in PtdCho deacylation (2); however, this compound is also a potent inhibitor of NTE in vitro (3). Clearly, further work is required to evaluate the relative contributions of NTE and calcium-independent phospholipase A2 plus lysophospholipases to GroPCos production in various mammalian cells. It may be that, although YML059c is the only enzyme in S. cerevisiae responsible for production of GroPCos, some mammalian cells deacylate PtdCho by more than one pathway.

Incubation of COS and HeLa cells with OPs inhibited both GroPCos production and NTE-esterase activity. The OPs appeared much less potent in assays with intact cells than with microsomal preparations, and this reflects the presence in mammalian cells of various enzymes capable of degrading certain OPs (37). However, although diisopropyl fluorophosphate, phenyl saligenin phosphate, and paraxox showed roughly similar potency against GroPCos production and NTE-esterase activity, PDPP was rather more potent against the esterase activity. PDPP is the only organophosphinate in this series (that is, its phosphorous atom is linked directly to the carbon of the alkyl group rather than via oxygen in the organophosphates). Furthermore, although PDPP and other organophosphinates are potent inhibitors of brain NTE-esterase activity in animal-dosing experiments, unlike organophosphate inhibitors of NTE, they do not cause neuropathy (38).

It has been suggested that excess PtdCho is intrinsically toxic to yeast Golgi secretory function (39). Thus, reduction in the rate of PtdCho synthesis in yeast lacking YML059c to ensure that PtdCho levels do not increase to above those in the wild-type may be an essential homeostatic mechanism to allow survival of the null mutant. By contrast, in certain cells of some metazoan organisms, where homeostasis may differ from that in yeast, loss of the deacylating activity of NTE homologues could result in deleterious accumulation of PtdCho-containing membrane; the situation might be particularly severe in situations where large amounts of membrane are being synthesized. One example may be the excessive production of loosely wrapping plasma membrane by glial cells in the brain of Dro sophila with mutations in the gene for the NTE homologue, Swiss cheese protein (40). Last, although the mechanism of OP-induced neuropathy in adult vertebrates is still unclear, maintenance and repair of long nerves is a process requiring prodigious amounts of membrane synthesis; in rodent neural cultures, PtdCho appears to be synthesized locally within neuritic processes, partly independent of the neuronal cell body (41), and accumulation of excess axonal smooth reticulum is an early ultrastructural change in OP-poisoned nerves in vivo (42).

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Neuropathy Target Esterase and Its Yeast Homologue Degrade Phosphatidylcholine to Glycerophosphocholine in Living Cells

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