Localization-independent Regulation of Homocysteine Secretion by Phosphatidylethanolamine N-Methyltransferase*

Received for publication, April 28, 2005, and in revised form, May 27, 2005
Published, JBC Papers in Press, May 31, 2005, DOI 10.1074/jbc.M504568200

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Elevated Hcy1 levels are a risk factor for cardiovascular disease, in particular atherosclerosis and myocardial infarction (1, 2). Additionally, meta-analyses of clinical studies have revealed that hyperhomocysteinemia is positively associated with an increased risk of cardiovascular disease and stroke independently of other risk factors (3–6). Hyperhomocysteinemia is also an independent risk factor for Alzheimer disease and dementia (7). Although plasma Hcy levels are clearly of significant clinical interest, the molecular basis underlying Hcy homeostasis remains poorly understood.

Recently, we demonstrated that mice homozygous for a disrupted PEMT allele display a >50% reduction in circulating Hcy concentrations (8). Furthermore, primary hepatocytes cultured from PEMT null mice secrete ~50% less Hcy (8). Conversely, ectopic expression of PEMT in a cell line that lacks endogenous PEMT promotes Hcy secretion (8). In early studies, the synthesis of creatine by guanidinoacetate methyltransferase was proposed to be the major contributing reaction in homocysteine formation (9, 10). Our data reveal a key, yet previously unrecognized, role for PEMT in Hcy homeostasis.

PEMT is a liver-specific enzyme that catalyzes three sequential transmethylation reactions in the production of phosphatidylcholine (PC) (11). S-Adenosylmethionine (AdoMet) is the methyl donor for each reaction and S-adenosylhomocysteine (AdoHcy) is the demethylated product, which is subsequently hydrolyzed to adenosine and the non-protein amino acid, Hcy (11, 12). Hydrolysis of AdoHcy is carried out by the cytosolic enzyme, S-Adenosylhomocysteine hydrolase (AdoHcyase) (12, 13). Efficient hydrolysis of AdoHcy is essential, as an increase in AdoHcy levels, along with the associated decrease in the cellular AdoMet/AdoHcy ratio, inhibits AdoMet-dependent methyltransferases (14).

Approximately 40 different mammalian methyltransferases, including DNA, RNA, protein, lipid, and small molecule methyltransferases, use AdoMet as a methyl donor and consequently produce AdoHcy during the methylation reaction (15, 16). Because AdoHcy from each transmethylation reaction can potentially be used to produce Hcy, it is all the more striking that genetic ablation of just one methyltransferase, PEMT, suffices to depress circulating Hcy levels by half (8). The question thus arises: what molecular features of the PEMT pathway cause the preferential use of PEMT-derived AdoHcy as a substrate for AdoHcyase?

PEMT is localized in the ER and is present in a subfraction of ER membranes that co-fractionates biochemically with mitochondria, known as mitochondria-associated membranes (17, 18). The mitochondria-associated membranes represent a metabolically important region of the ER, characterized by a significant enrichment in lipid biosynthetic enzymes relative to other areas of the ER (19–21). Because the PEMT pathway is also a metabolically channeled process (22), we investigated whether precise subcellular localization might be essential to the role of PEMT in Hcy homeostasis.

To evaluate the importance of PEMT localization for Hcy homeostasis, we relocalized PEMT to a different cellular sub-

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* This research was supported in part by grants from the Canadian Institutes for Health Research (CIHR) and the Canadian Diabetes Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Hcy, homocysteine; AdoHcy, S-adenosylhomocysteine; AdoHcyase, S-adenosylhomocysteine hydrolase; AdoMet, S-adenosylmethionine; CBS, cystathionine β-synthase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; HA, hemagglutinin; Man II, mannosidase II; NRK, normal rat kidney; PC, phosphatidylcholine; PDI, protein disulfide isomerase; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; TGN-38, trans-Golgi network protein 38.
compartment by defining and ablating the ER retrieval motif. The canonical retrieval motif for ER membrane proteins features a lysine residue at the −4, −3 (KKXX) or −5, −3 (KKXXX) positions relative to the C-terminal end (23). This motif functions in mammals, yeast, and plants (24–26) through interactions with specific subunits of the Cop I complex (27). Here, we have validated the minimal ER targeting motif of PEMT and have presented biochemical evidence that PEMT can promote Hcy secretion through an ER localization-independent mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, restriction endonucleases, and Platinum™ Pfx DNA polymerase were from Invitrogen. Oligonucleotides were synthesized at the Institute of Biomolecular Design, University of Alberta. S-adenosyl-L-[methyl-3H](methionine (15 Ci/mmol) was obtained from Amersham Biosciences. Non-radioabeled S-adenosyl-l-methionine and anti-FA monoclonal antibody were from Sigma. FuGENE 6™ transfection reagent was from Roche Applied Science.

Phosphatidylinomethanolamine was from Avanti Polar Lipids. Anti-protein disulfide isomerase (PDI) antibody was from Stressgen Biotech. Mannosidase II (Man II) and TGN-38 antibodies were kindly provided by Dr. Paul Melancon, University of Alberta. Goat anti-rabbit and goat anti-mouse secondary antibodies were from Pierce. Texas Red-X goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit, and Prolong Antifade™ reagent were from Molecular Probes.

**Recombinant Plasmid Construction**

Plasmids encoding PEMT localization mutants were generated by PCR-based mutagenesis using the hemagglutinin (HA)-hPEMT-pCI plasmid as template (18). This plasmid encodes a previously described HA-tagged derivative of the human PEMT protein, which is enzymatically active (18). To mutate the residues of the putative ER retrieval motif, a common HA-PEMT-specific sense primer and a different antisense primer for each mutant were used. Primer sequences are available upon request. Mutant PCR products were subcloned into pCI (Promega) using Xhol and XbaI restriction sites. Transcription from the pCI vector is under the control of a CMV promoter. All constructs were sequenced at the Molec-

**Cell Culture, Transient Transfections, and Generation of Stable Cell Lines**

Cell lines were obtained from the American Type Culture Collection repository. NRK (normal rat kidney) cells were maintained in DMEM, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate at 37 °C, 5% CO2. McArdle RH-7777 hepa-
toma cells were similarly maintained, except the culture media also included 10% horse serum. Transient transfection of NRK cells was performed using the FuGENE 6™ reagent according to the manufacturer’s instructions. McArdle RH-7777 stable transfecants were generated using the FuGENE 6™ reagent or by the calcium phosphate precipitation method (28).

**Confocal Microscopy Analysis**

NRK cells were seeded onto 22-mm coverslips in 6-well plates at a density of 1 x 10⁵ cells/well. Following overnight incubation at 37 °C, 5% CO2, the cells were transfected with plasmids encoding mutant PEMT derivatives as described above. 24 h post-transfection, cells were serum-starved for 1 h and fixed in 4% paraformaldehyde/phosphate-buffered saline, pH 7, for 10 min. Following permeabilization with 0.2% Triton X-100/phosphate-buffered saline for 2 min, cells were incubated with the first primary polyclonal antibody at 37 °C for 1 h at the indicated dilution: PDI (1:100), Man II (1:300), or TGN-38 (1:200). Cells were then incubated with the secondary antibody (monoclonal anti-FA, 1:150) for 1 h at 37 °C. Cells were subsequently incubated for 1 h at 37 °C with both secondary antibodies: Texas Red-X goat anti-
mouse (1:200) and Alexa Fluor 488 goat anti-rabbit (1:200). Coverslips were mounted onto slides with Prolong Antifade™ reagent and immu

**PEMT Enzymatic Assays**

In Vitro PEMT Assays—These assays were performed as described previously (29). Briefly, cells were washed and harvested into phosphate-buffered saline, pelleted at 1000 × g, and resuspended in buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA). Following homogenization by sonication, protein homogenates (50 μg) were assayed for PEMT activity using phosphatidylinomethanolamine as a methyl acceptor and S-adenosyl-L-[methyl-3H](methionine as the methyl group donor.

Assays of PEMT Activity in Cells—cells were plated at a density of 1.5 x 10⁵ cells/60-mm dish. After 24 h, cells were washed with DMEM and incubated for 2 h with 1.5 ml of DMEM containing 0.4 mM oleate, [1-C14]glycercer 0.5 μCi/dish), and [3H]ethanolamine or [3H]methionine (5 μCi/dish). Radiolabeled lipids were extracted and quantitated (30).

**Immunoblot Analysis**

Proteins from cell homogenate (5 μg) were separated by Tris-glycine SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels calibrated with prestained molecular weight standards (Bio-Rad). Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with monoclonal anti-FA antibody (1:5000 dilution). Protein-antibody complexes were detected by enhanced chemiluminescence with horseradish peroxidase-conjugated secondary antibody using the ECL reagent (Amersham Pharmacia Bio-
sciences) as directed (1:5000 dilution). Membranes were exposed to Biomax MR film (Kodak) at room temperature.

**Homocysteine Secretion**

Total media Hcy content was assayed by reverse phase high-performance liquid chromatography and fluorescence detection of ammonium 7-fluoroc-2-oxa-1,3-diazole-4-sulfonate thiaducts (31).

**Statistical Analysis**

Statistical differences between the means of two groups were determined using a Student’s t test. Mean values of more than two groups were compared using a one-way analysis of variance followed by a Student-Newman-Keuls post-hoc test.

**RESULTS**

**Identification of an ER Retrieval Motif**—To identify a puta-
tive ER retrieval motif, comparative analysis of known PEMT primary sequences was performed. A classical C-terminal ER retrieval motif (KKXX) is present in yeast and neurospora PEMT orthologs (Fig. 1A). Although the functional relevance of this motif is unknown, only the lysine residue at the −3 position (Lys-197 in humans) is conserved in higher species (Fig. 1A). Either lysine in a di-lysine motif can be mutated to an arginine or histidine residue without detriment to ER targeting (32). Because Lys-197 is flanked by histidine and arginine residues (HKRX), one of several combinations of basic residues could target PEMT to the ER (Fig. 1A). To serve as a functional ER retrieval signal, the C-terminal motif should be exposed to the cytosol (24, 25). PEMT adopts a topology within the ER membrane that positions both termini in the cytosol (Fig. 1B) (18). Thus, Lys-197 of human PEMT is optimally positioned to interact with the ER retrieval machinery.

**Mutation of Lys-197 Prevents Targeting of PEMT to the ER**—To investigate the role of the putative retrieval motif in targeting PEMT to the ER, we generated recombinant constructs in which Lys-197 and the flanking basic residues, His-196 and Arg-198, were mutated to serine residues. Each of the constructs included a N-terminal HA tag, because the epitope recognized by the PEMT antibody spans the region that includes the putative retrieval motif (17) and mutations in this region abolish epitope recognition.2 HA-tagged PEMT derivatives have been characterized, and the tagged enzyme retains significant transmethylation activity compared with the unmodified enzyme (18).

2 D. J. Shields, S. Lingrell, L. B. Agellon, J. T. Brosnan, and D. E. Vance, unpublished results.
To assess the role of specific residues in targeting PEMT to the ER, constructs encoding the mutant PEMT proteins were transfected into NRK cells, and colocalization studies were performed using the ER marker, PDI. NRK cells, which do not express endogenous PEMT protein, are commonly used for subcellular localization studies (33, 34). As expected, the unmodified PEMT enzyme co-localized with the ER protein, PDI (Fig. 2A). In contrast, a PEMT derivative in which both Lys-197 and Arg-198 were mutated to serine residues (K197S/R198S) failed to colocalize with PDI; instead, the double mutant displayed a juxtanuclear localization (Fig. 2B). Mutation of His-196 resulted in a PEMT derivative that was either poorly expressed or rapidly degraded, as H196 transfectants were very rarely detected. Thus, the role of His-196 in targeting PEMT to the ER could not be determined.

To determine whether Lys-197 and Arg-198 are both required for retrieval of PEMT to the ER, each residue was individually mutated to serine residues, generating the constructs K197S and R198S. Confocal analysis demonstrated that the R198S mutant co-localized with PDI, suggesting that Arg-198 is not essential for ER localization (Fig. 2C). In contrast, mutation of Lys-197 caused a loss of colocalization with PDI (Fig. 2D). Moreover, the K197S (Fig. 2B) mutant adopted a juxtanuclear localization similar to that of the K197S/R198S double mutant (Fig. 2B). Therefore, mutation of Lys-197 is sufficient to abrogate ER targeting of PEMT, supporting the notion that Lys-197 is essential for retrieval of PEMT to the ER.

PEMT-K197S Mutants Are Localized to the Golgi—PEMT derivatives that harbor a mutation of Lys-197 (K197S/R198S or K197S) adopt a juxtanuclear localization that resembles the pattern of Golgi. To define the localization of the displaced K197S mutants, we performed co-localization analysis with the Golgi enzyme Man II. Extensive co-localization between the Lys-197 mutants and Man II was observed, suggesting that mutation of Lys-197 relocates PEMT to the Golgi (Fig. 3, B and D). Conversely, the unmodified enzyme and the R198S mutant, each of which colocalized with PDI, did not colocalize with Man II (Fig. 3, A and C). Thus, Lys-197 is essential for targeting PEMT to the ER, as mutagenesis causes relocation to the Golgi.

Golgi-localized PEMT Catalyzes PC Biosynthesis—Identification of Lys-197 as a key residue in targeting PEMT to the ER enabled us to engineer PEMT variants that are localized to distinct regions of the cell. By modulating the subcellular localization of the enzyme, the significance of PEMT localization in Hcy homeostasis could thus be investigated. To directly assess the functional significance of PEMT localization, McArdle RH-7777 cell lines were generated that stably express either ER- or Golgi-localized PEMT. Mutation of Lys-197 also caused the relocation of PEMT from the ER to the Golgi in McArdle RH-7777 cells. McArdle hepatoma cells are a frequently used model for the study of lipid metabolism and energy homeostasis and do not express endogenous PEMT (8, 35, 36).

PEMT catalyzes the AChoMet-dependent methylation of PE to produce PC (11). To determine whether the Golgi-localized enzymes are active, PEMT enzymatic assays were performed. Cells expressing PEMT localization mutants were radiolabeled with metabolic precursors, and the radiolabeling of PE and PC was quantitated. As expected, stable expression of the unmodified enzyme caused a significant increase in the PC/PE ratio (Fig. 4A). Similarly, expression of the ER-localized R198S mutant resulted in a marked increase in the PC/PE ratio, demonstrating that mutation of Arg-198 is not inhibitory for PEMT activity (Fig. 4A). Surprisingly, stable expression of each of the Golgi-localized PEMT mutants (K197S/R198S and K197S) also caused a significant increase in the PC/PE ratio compared with

**Fig. 2.** Mutagenesis of Lys-197 displaces PEMT from the ER. NRK cells were transiently transfected with plasmids (2 μg) containing wild-type PEMT (A) or localization mutants, PEMT-K197S/R198S (B), PEMT-R198S (C), or PEMT-K197S (D). Cells were fixed onto coverslips and incubated with anti-PDI and anti-HA antibodies. Endogenous PDI and HA-tagged PEMT derivatives were visualized with Alexa Fluor 488-conjugated goat anti-rabbit and Texas Red-X-conjugated goat anti-mouse secondary antibodies, respectively. Merged confocal images are displayed. Co-localization of PDI and PEMT derivatives is observed as a yellow signal. Magnification, ×63.
cells stably transfected with empty vector (Fig. 4A). Thus, displacement of PEMT from the ER does not abolish the methyltransferase activity in cells. Furthermore, localization of PEMT in the Golgi does not preclude the methylation-based synthesis of PC. Although the ER- and Golgi-localized PEMT derivatives displayed similar activity levels in cells (Fig. 4A), immunoblot analysis revealed that amounts of the Golgi-localized enzymes were in fact significantly lower than the ER-localized PEMT derivatives (Fig. 4B). These data suggest that Golgi-localized PEMT displays higher specific activity than the corresponding ER-localized enzyme. Alternatively, PC biosynthesis may be limited either through product-based feedback inhibition or via an unknown PC-sensing regulatory system.

**PEMT Promotes Hcy Secretion Independently of Subcellular Localization**—PEMT catalyzes three sequential methylation reactions in the biosynthesis of PC (Fig. 5A) (11). Generation of a PC molecule results in the concomitant production of three molecules of AdoHcy, each of which can be hydrolyzed to yield Hcy (Fig. 5A) (12). In the next series of experiments we investigated whether PEMT promotes Hcy secretion through a localization-dependent mechanism. Media were collected from cells that stably expressed ER- or Golgi-localized PEMT, and Hcy levels were measured. As predicted, stable expression of the ER-localized enzymes (PEMT and R198S) resulted in a significant increase in media Hcy content (Fig. 5B). However, stable expression of the Golgi-localized PEMT mutants (K197S/R198S and K197S), also significantly increased the secretion of Hcy compared with cells that were stably transfected with empty vector (Fig. 5B). Thus, these data suggest that PEMT promotes Hcy secretion through a localization-independent mechanism.

**Hcy Secretion Increases Concomitantly with PEMT Activity**—To further define the molecular determinants of PEMT-dependent Hcy homeostasis, we investigated whether Hcy secretion is modulated coordinately with PEMT activity. Cell lines that stably expressed various levels of ER-localized PEMT or the Golgi-localized derivative, K197S, were identified by in vitro PEMT assays (Fig. 6A). To test the hypothesis that Hcy secretion is regulated coordinately with PEMT activity, media Hcy content from the different cell lines was measured.

As before, expression of either the ER- or Golgi-localized PEMT enzymes increased the media Hcy content (Fig. 6B). Because a PEMT-dependent increase in Hcy secretion was evident in all stable cell lines (each represents a distinct clone), it is unlikely that the promotion of Hcy secretion by ER- or Golgi-localized PEMT is due to genomic integration effects. Notably, Hcy secretion was augmented with increasing PEMT activity. Moreover, Hcy secretion was strongly correlated with methyl-
Localized (R and 40-fold elevation in plasma Hcy levels respectively (38). A localized PEMT-K197S.

ER- or Golgi-localized PEMT. McArdle RH-7777 hepatoma cells transferase activity of both the ER (R² = 0.7637)- and Golgi-localized (R² = 0.9364) enzymes (Fig. 6C). Therefore, PEMT promotes Hcy secretion through a localization-independent mechanism, and Hcy secretion correlates with PEMT activity.

DISCUSSION

Plasma Hcy concentrations elicit significant clinical interest because elevated levels are associated with coronary heart disease, stroke, peripheral arterial occlusive disease, Alzheimer disease, and dementia (3, 5–7). A number of animal models have been engineered that display mild or severe hyperhomocysteinemia; for example, mice that are heterozygous or homozygous for a disrupted Lys-197 residue (47). The role of this lysine residue in targeting the enzyme to the Golgi. Approximately 23% of ER membrane proteins are distributed throughout the cytosol (45). Furthermore, AdoHcyase, which hydrolyzes PEMT-derived AdoHcy to supplement the circulating Hcy pool, is also present throughout the cytosol (46). Because the key elements in the PEMT-dependent production of Hcy are not spatially delimited, we rationalized that PEMT might modulate Hcy homeostasis irrespective of subcellular localization.

In this study, we have demonstrated that the conserved C-terminal Lys-197 residue (−3 position) is essential for targeting PEMT to the ER, as mutation causes the mislocalization of the enzyme to the Golgi. Approximately 23% of ER membrane proteins are predicted to be targeted to the ER by a C-terminal di-lysine motif, but only 3.5% of ER membrane proteins feature a single lysine residue at the −3 position (47). The role of this lysine residue in targeting proteins other than PEMT to the ER remains to be determined.

As predicted, Golgi-localized PEMT retains methyltransferase activity, supporting our hypothesis that a correctly folded enzyme catalyzes the reactions of the PEMT pathway irrespective of localization. Furthermore, expression of PEMT in either the ER or the Golgi promoted Hcy secretion. This result is significant insofar as it suggests that it is PEMT, and not a PEMT-dependent ancillary factor in the ER, that causes the altered plasma Hcy levels in PEMT knock-out mice; mutation of PEMT Lys-197 would not be expected to relocalize such an ancillary factor to the Golgi.

Large quantities of PC are produced in the liver to replenish the hepatic pools used for production of bile and very low density lipoproteins (48). PEMT accounts for 30% of hepatic PC biosynthesis (22, 49, 50), which translates to significant levels of PC synthesis. We cannot rule out the possibility that a perturbation of hepatic membrane lipid composition alters the secretion of Hcy or the reuptake of an intermediary metabolite such as methionine, in PEMT knock-out

FIG. 6. Hcy secretion correlates with the enzymatic activity of ER- or Golgi-localized PEMT. McArdle RH-7777 hepatoma cells were stably transfected with cDNAs encoding wild-type PEMT or Golgi-localized PEMT-K197S. A. PEMT activity in cell homogenates (50 μg protein) of different stable clones: PEMT (A–C) and K197S (D–F). The results are expressed as the means of three separate experiments (each performed in duplicate) ± S.E. B, total media Hcy content of each stable cell line (PEMT (A–C) and K197S (D–F)) was measured after 18 h of incubation. C, correlation between Hcy secretion and PEMT-specific activity. Filled diamonds represent data from three separate experiments with three different cell lines that stably express wild-type PEMT. Filled triangles represent data from three separate experiments with three different cell lines that stably express PEMT-K197S.
mice. Nevertheless, it is likely that plasma Hcy levels are decreased because of the drastic reduction in levels of PEMT-derived Hcy. This notion is further supported by our finding that Hcy secretion is strongly correlated with PEMT activity and hence with PC biosynthesis.

Previously, we identified key motifs in PEMT that bind AdoMet/AdoHcy and resolved the topography of the enzyme (51). Knowledge of the membrane topography of PEMT enabled us to map the AdoMet/AdoHcy binding sites to the cytosolic portion of the enzyme. Golgi-localized PEMT displays methyltransferase activity, indicating that the mislocalized enzyme likely adopts the correct topology within the membrane. Such a topographical organization may prove central to the role of PEMT in Hcy homeostasis, as it facilitates access to both the cytosolic pool of AdoMet and the soluble enzyme AdoHcyase (45, 46). Thus, a key determinant of PEMT-mediated Hcy secretion may be the spatial organization of PEMT within the ER membrane rather than the specific subcellular localization of PEMT. Furthermore, although the reactions of the PEMT pathway are metabolically channeled, orientation of the PEMT active site in the cytosol with AdoHcyase may represent an additional level of efficiency in Hcy production. Intriguingly, recent findings suggest that the yeast genes encoding phospholipid biosynthesis enzymes and AdoHcyase are coordinately regulated at the transcriptional level (52). Thus, coordinate regulation of PEMT and AdoHcyase, whether at the transcriptional level or through spatial juxtaposition of enzymatic activities, may be an evolutionary conserved facet of Hcy homeostasis.

In summary, we have demonstrated that Lys-197 is essential for targeting PEMT to the ER, as mutation of this conserved residue relocated the methyltransferase to the Golgi. Golgi-localized PEMT not only catalyzes the reactions of the PEMT pathway but also promotes secretion of Hcy. Moreover, Hcy secretion correlates with the activity of both ER- and Golgi-localized PEMT. Because PEMT modulates plasma Hcy homeostasis, the enzyme represents a novel target for therapeutic intervention in patients with hyperhomocysteinemia. Resolution of the molecular basis of PEMT-dependent Hcy homeostasis may aid in the development of such novel approaches and therapeutic agents.

Acknowledgments—We thank Drs. Richard Lehner, Judith Altarcejos, Jean Vance, and René Jacobs for helpful discussions and Gerry Barron for assistance with confocal analysis.

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J. Biol. Chem. 2005, 280:27339-27344.
doi: 10.1074/jbc.M504658200 originally published online May 31, 2005

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