Cholesteryl ester transfer protein and phospholipid transfer protein have non-overlapping functions in vivo

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1. Abbreviations used in this paper: AI, apolipoprotein AI; CETP, cholesteryl ester transfer protein; FC, free cholesterol; FPLC, fast protein liquid chromatography; KO, gene knock-out; PL, phospholipid; PLTP, phospholipid transfer protein; TRL, triglyceride-rich lipoproteins.

**Acknowledgments**

This work was supported by HL-54591
Abstract

Plasma phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) are homologous molecules which mediate neutral lipid and phospholipid exchange between plasma lipoproteins. Biochemical experiments suggest that only CETP can transfer neutral lipids, but that there could be overlap in the ability of PLTP and CETP to transfer or exchange phospholipids. Recently developed PLTP gene knock-out (PLTP0) mice have complete deficiency of plasma phospholipid transfer activity, and markedly reduced HDL levels. To see if CETP can compensate for PLTP deficiency in vivo, we bred the CETP transgene (CETPTg) into the PLTP0 background. Using an in vivo assay, to measure the transfer of $[\text{3H}]$PC from VLDL into HDL, or an in vitro assay, which determined $[\text{3H}]$PC transfer from vesicles into HDL, we could detect no phospholipid transfer activity in either PLTP0 or CETPTg/PLTP0 mice. On a chow diet, HDL-PL, HDL-CE and HDL-apoAI in CETPTg/PLTP0 mice were significantly lower than in PLTP0 mice (45\pm7 vs. 79\pm9 mg/dl; 9\pm2 vs. 16\pm5 mg/dl; and 51\pm6\% vs. 100\pm9, arbitrary units, respectively). Similar results were obtained on a high fat, high cholesterol diet. These results indicate that 1) there is no redundancy in function of PLTP and CETP in vivo and 2) The combination of
the CETP transgene with PLTP deficiency results in an additive lowering of HDL levels, suggesting that the phenotype of a human PLTP deficiency state would include reduced HDL levels.
Introduction

The plasma lipid transfer proteins, phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP), play a central role in high density lipoprotein (HDL) metabolism. PLTP and CETP belong to a family of lipid transfer/lipopolysaccharide binding proteins and show sequence homology, related gene structures and probably share a similar protein fold (1). Genetic models have been central to elucidating the role of PLTP and CETP in mouse and human physiology. The importance of CETP in HDL metabolism and reverse cholesterol transport has been elucidated by human genetic deficiency of CETP (2-4), as well as by the introduction of CETP, normally absent from mouse plasma, into mice by transgenesis (5-8). In human CETP deficiency, HDL levels are markedly elevated, while the opposite effect was observed in CETP transgenic mice.

Markedly reduced plasma HDL levels in PLTP gene knock-out (PLTP KO) mice have recently provided the first in vivo evidence for a crucial role of PLTP-mediated lipid transfer in the maintenance of HDL levels (9). PLTP also facilitates free cholesterol influx into HDL (9). This is followed by CE formation by lecithin:cholesterol acyltransferase, and in species such as humans,
redistribution of CE to triglyceride-rich lipoproteins by CETP.

Although biochemical experiments indicate that PLTP and CETP have different abilities to transfer neutral lipids between lipoproteins, they also suggest overlap in their ability to transfer or exchange phospholipids. Both lipid transfer proteins have been reported to facilitate phospholipid exchange between the plasma lipoproteins (10,11). In humans with genetic deficiency of CETP, plasma phospholipid exchange activities are about 50% of normal, suggesting that CETP and PLTP might contribute equally to this activity in human plasma (12). However, PLTP enhances net transfer of phospholipids from phosphatidylcholine (PC) vesicles into HDL, while CETP does not (10,11). This is thought to be analogous to the transfer of very low density lipoprotein (VLDL) phospholipids into HDL, but the ability of CETP to transfer phospholipids from VLDL into HDL has never been tested in vivo. Thus it is possible that there is in vivo redundancy in function. In the present study, this was evaluated by crossing the CETP transgene into the PLTP0 background. If there is redundancy of phospholipid transfer activities, CETP would be expected to ameliorate the low HDL state of PLTP deficiency.
Materials and Methods

Animals and diets used in this study. All phenotypic characterization was performed with female wild-type (Wt), PLTP0, CETPTg, and CETPTg/PLTP0 mice (in the C57BL/6 background), 10-12 weeks old. Two diets were used: Chow (Purina Laboratory Rodent Chow 5001), or chow diet plus 20% hydrogenated coconut oil and 0.15% cholesterol (Research Diets Inc. New Brunswick, New Jersey).

Lipids and lipoprotein measurements. Fasted blood (food was removed at 9:00am and blood was collected at 4:00pm) was collected for lipoprotein isolation and lipid measurement. Total cholesterol, free cholesterol (FC) and phospholipid (PL) in plasma and lipoproteins were assayed by enzymatic methods (Wako Pure Chemical Industries Ltd., Osaka, Japan). Cholesteryl ester concentration was calculated by subtracting the amount of free cholesterol from the total plasma cholesterol. Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC) using a Sepharose 6B column as described previously (9). A 200 µl aliquot of pooled plasma (from 7 animals) was loaded onto the column, and eluted with TS buffer (50 mM Tris, 0.15 M NaCl, pH 7.5) at a constant flow rate of 0.35 ml/min. An aliquot of 80 µl
from each fraction (1 ml) was used for the determination of total cholesterol and phospholipid.

PLTP Activity Assays. Ten µmol of egg phosphatidylcholine containing 10 nmol of ³H-phosphatidylcholine (L-α-dipalmitoyl [2-palmitoyl-9,10-³H(N)]-phosphatidylcholine) was dried under a stream of nitrogen, resuspended in 1 ml of a solution of 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, probe-sonicated and centrifuged. Transfer of radiolabeled phospholipid was measured by incubating 3 µl of plasma with radiolabeled phospholipid vesicles (125 nmol PC) and HDL (250 µg protein) in a final volume of 400 µl at 37°C for 1 hour. Vesicles were subsequently precipitated by the addition of 300 µl of a solution of 500 mM NaCl, 215 mM MnCl₂, 445 U/ml heparin, and the radioactivity of a 500 µl aliquot of the supernatant was measured.

Lipoprotein Turnover Study. Plasma VLDL was labeled with [³H]-dipalmitoylphosphatidylcholine (DPPC) as described previously (13). Mice were injected intravenously (femoral vein) with [³H]-VLDL, 7.0 x 10⁵ cpm. Blood (70 µl) was taken from the tail vein at different times. VLDL and LDL were precipitated using an HDL cholesterol reagent (Sigma
Chemical Co., St. Louis, Missouri) and the radioactivity in the supernatant, representing [3H]-PC transferred from VLDL to HDL, was measured.

CETP Activity Assay. HDL containing [3H]-cholesteryl ester and LDL were prepared as described previously (14). CE transfer activity was measured by incubating 3 µl of plasma with radiolabeled CE-containing HDL and unlabeled LDL for 4 hours, in 30 µl of 50 mM Tris, 159 mM NaCl, and 2 mM EDTA, pH 7.5, then precipitating the LDL by the heparin/Mn²⁺ method (15), and counting an aliquot of the supernatant.

CETP Mass Detection. Ten µl of mouse plasma together with 4 µl of 40% sucrose and 3 µl of Sudan Black (0.04%) was loaded on an agarose gel containing 0.8 g agarose, 5% sucrose, 50mM sodium barbital and 10mM barbital. The gel was run in a 50mM sodium barbital and 10mM barbital buffer at 80 V for 50 min. The gel was then blotted onto a nitrocellulose membrane overnight. CETP was detected by incubating the membrane with ¹²⁵I-labeled anti-CETP mAb (TP2) as described previously (16). The relative CETP concentration was determined by quantitative scanning using a Phosphorimager.
SDS-PAGE Analysis. To analyze the apolipoprotein composition, the ultracentrifugally isolated plasma lipoproteins (VLDL+LDL, d<1.063 g/ml, and HDL d=1.063-1.21 g/ml) were run on a 4-20% gradient SDS-PAGE gel under reducing conditions. The apolipoproteins then were stained by Coomassie brilliant blue as described. Apolipoprotein was quantitated by gel scanning software (NIH Image).

Statistical analysis. Differences between groups were tested by Student’s t test. Data are presented as mean+S.D.
Results

Plasma Lipid Transfer Activities

To evaluate possible redundancy in the in vivo functions of PLTP and CETP, we crossed CETPTg and PLTP0 mice, both in the C57BL/6 background. Plasma PLTP activity analysis using $^3$H-phosphatidylcholine (PC) vesicles as donor and HDL as acceptor, showed no PC transfer activity in either PLTP0 or CETPTg/PLTP0 mice (Fig. 1). This activity was detected at similar levels in Wt and CETPTg mice (Fig. 1). To evaluate the in vivo transfer of phospholipid between plasma lipoproteins, $[^3]$H-PC-VLDL was injected intravenously into mice of different genotypes. In CETPTg mice, there was rapid transfer of a substantial portion of PC radioactivity into HDL. In contrast, $^3$H-PC transfer from VLDL to HDL was almost absent in CETPTg/PLTP0 mice (Fig. 2), similar to results obtained in PLTP0 mice (9). This suggests that in vivo CETP has no PLTP-like phospholipid transfer function, at least based on these assays.

Plasma CETP activity was also measured in these mice, using $[^3]$HCE-HDL as a donor and excess unlabeled LDL as acceptor. This showed significantly lower activity in CETPTg/PLTP0 mice, compared to CETPTg mice (Fig. 3, 52$\pm$6 vs. 100$\pm$9, arbitrary units, p<0.001). As expected, CE transfer activity was not detected in either Wt or PLTP0
mice (Fig. 3). To confirm the result, we quantitated CETP mass in CETPTg and CETPTg/PLTP0 mouse plasma by agarose electrophoresis and immunoblot analysis. By this assay, CETP levels in CETPTg/PLTP0 mice were 25% (p<0.05) lower than in CETPTg mice (Fig. 4).

**Plasma Lipoprotein Analysis**

The distribution of plasma lipoprotein lipids was determined by fast protein liquid chromatography (FPLC) of pooled plasma samples (Fig. 5). In PLTP0 mice, all HDL lipids were markedly reduced compared to Wt mice. Importantly, in CETPTg/PLTP0 mice, HDL-PL and HDL-CE were significantly decreased compared to PLTP0 mice. Plasma lipoprotein analysis by precipitation showed a significant reduction in total phospholipid (PL), HDL-PL and cholesteryl ester (HDL-CE) (31%, 43% and 44%, respectively) in CETPTg/PLTP0 mice versus PLTP0 littermates (Table 1). Non-HDL lipids were not significantly altered. All lipid levels, except non-HDL-FC, were significantly decreased in both PLTP0 and CETPTg/PLTP0 mice compared to Wt and CETPTg mice (Table 1). Assessment of apolipoprotein composition of centrifugally isolated lipoproteins by reducing SDS-PAGE gels revealed a significant decrease of apoAI in HDL (51±6 vs. 100±9, arbitrary units, p<0.01) (Fig. 6). We noted that apoB100 was decreased in non-HDL from PLTP0 mice compared
to Wt mice, as suggested previously (9). Also apoB100 was increased in non-HDL from CETPTg mice compared to Wt mice (Fig.6), which is probably due to the downregulation of the LDL receptor (17).

Previously, we found that there is a dramatic accumulation of FC/PL-rich vesicular particles in PLTP0 mouse mice on a coconut oil-based high fat diet (0.15% cholesterol, 20% hydrogenated coconut oil) (9). In order to determine whether CETP expression has an impact on the accumulation of these particles, three months old female mice were challenged with the high fat diet for two weeks. HDL-PL and HDL-CE were significantly decreased in CETPTg/PLTP0 mice compared to PLTP0 mice (18% and 48%, respectively) (Table 1). Like the PLTP0 mice, the double-mutant mice also showed a dramatic increase in non-HDL-FC and non-HDL-PL levels compared to Wt and CETPTg mice (Table 1). However, there was no significant difference between CETPtg/PLTP0 and PLTP0 mice, in terms of the accumulation of FC/PL-rich particles. These results suggest that the increased flux of lipid through the plasma compartment on the coconut oil-based high fat diet leads to accumulation of FC and PL enriched surface components of triglyceride rich lipoprotein in the non-HDL fraction in PLTP deficient mice. However, the expression of the human CETP gene in
these mice does not influence the accumulation of such particles.
Discussion

Related members of the same gene family often show in vivo redundancy in their functions. As a result, knock-out of one gene may not result in an in vivo phenotype, until combined with deficiency of the related compensating gene. LDL receptor related protein (LRP) is an example, where the lipoprotein phenotype only becomes evident after combination with a knock-out of the LDL receptor (LDLR) (18). Even though CETP and PLTP show moderate homology of sequence (19), and similar structural features (1,20), they show no overlap in their in vivo functions. This was demonstrated in the present study by preparing CETP Tg/PLTP0 mice: the expression of CETP did not rescue the low HDL phenotype of PLTP deficiency. In fact the phenotypes were additive, resulting in markedly reduced HDL levels in the CETP Tg/PLTP0 mouse. Similarly, the related plasma lipopolysaccharide binding protein (LBP), even though it has some ability to transfer phospholipids in vitro (21), does not compensate for deficiency of PLTP in vivo (9). Thus, these different members of the lipid transfer/lipopolysaccharide binding gene family have evolved diversified, non-overlapping functions.

PLTP and CETP were originally shown to have distinct abilities to mediate net transfer of phospholipids from PC
vesicles into HDL, even though both could stimulate phospholipid exchange between HDL and LDL or HDL and VLDL (10). Both exchange and net transfer activities were stimulated by lipolysis of VLDL (22). The present findings support the original view that the net transfer of phospholipids from vesicles into HDL could be regarded as a model of the transfer of phospholipids from VLDL to HDL during lipolysis in vivo (10). Recent studies in PLTP0 mice have shown that low HDL primarily results from increased catabolism of HDL lipid and protein (23). Thus, the net transfer of phospholipids from VLDL to HDL serves to metabolically stabilize HDL in the circulation. In PLTP0 mice, the HDL is specifically depleted in PC molecules (23). This might account for the reduced levels of CETP in CETPTg/PLTP0 mice, compared to CETPTg mice, since HDL PC has been shown to represent the primary binding site for CETP in HDL (24). In the present study we did not measure phospholipid exchange activity between the plasma lipoproteins, so it is possible that this activity, and resultant changes in lipoprotein phospholipid molecular species composition, was altered in CETP Tg/PLTP0 mice.

CETP and PLTP are likely to have substantial structural similarities. Three dimensional structural modeling, based on the crystal structure of the related BPI
(25), shows that both molecules have an extended, boomerang-shaped conformation (1). There are two lipid binding pockets, one in the N-terminus and the other in the C-terminus. The inability of PLTP to transfer CE and TG could perhaps be related to the distinctive C-terminal helical peptide of CETP, which plays an essential role in neutral lipid transfer activity (26). This peptide may perturb the surface of HDL in a way that facilitates entry of CE molecules into the N-terminal pocket. Recent site-directed mutagenesis studies indicate that the N-terminal lipid binding pocket of both CETP and PLTP is involved in phospholipid exchange activity (20) (C. Bruce and A. Tall, unpublished observation). Thus, the distinct functions of PLTP and CETP in mediating net phospholipid movement are probably not related to the properties of the lipid binding pockets. Rather this could reflect different abilities of PLTP and CETP to bind to phospholipid donors in the circulation, or to a distinct ability of PLTP to mediate fusion of lipoproteins or lipoproteins and vesicles (27). This latter property is poorly understood, but could perhaps involve ternary complex formation.

Crossing human apoAITg mice with either human CETPTg or human LCATTg mice, the phenotypic effect of CETP and LCAT becomes much more pronounced, indicating that human
CETP and LCAT work much better when they use human-like HDL as their substrate in mice (28,29). This preference is also shared by PLTP (30). There is a possibility that the inability of CETP to reverse the phenotype of PLTP0 mice may be due to the fact that the HDL in these mice are a sub-optimal lipoprotein substrate for CETP. However, it is not likely, because HDL levels in CETPTg/PLTP0 mice are further decreased compared to PLTP0 mice, indicating that HDL from PLTP0 mice still can serve as a CETP substrate.

Several genetically determined low HDL states are now known, including rare apoA-I mutations (31), common missense or truncation variants in lipoprotein lipase (32), and, most recently, mutations in ABC1 (homozygous in the rare Tangier Disease and heterozygous in more common familial hypoalphalipoproteinemia) (33-35). Although a human deficiency state of PLTP has not yet been described, the present study suggests that the phenotype would include markedly reduced HDL levels, analogous to the CETP Tg/PLTP0 mice. Recent studies in ABC1 knock-out mouse liver, using gene expression profiling approaches, have shown that the largest change in any hepatic mRNA is up-regulation of PLTP (36). This suggests overlap in the functions of PLTP and ABC1. Thus, ABC1 may initiate nascent HDL formation by interacting with free apoA-I at the cell surface, and the
activity of PLTP (transfer of PL from TRL) may lead to further increase in the size of HDL particles (23). It is interesting to speculate that compound deficiency states involving low activity variants of PLTP combined with common low activity variants of LPL or ABC1 could result in marked hypoalphalipoproteinemia.
Figure legend

Fig. 1. PLTP activity assay. Transfer of $[^3\text{H}]PC$ was measured by mouse plasma incubating (3 μl) with $[^3\text{H}]PC$-vesicles (125 nmol PC) and HDL (250 μg protein) in a final volume of 400 μl at 37°C for 1 hour. Vesicles were subsequently precipitated by heparin-Mn solution as described in “Method”. The radioactivity in the supernatant, representing $[^3\text{H}]PC$ transferred from PC vesicle to HDL, was measured. Results are shown for female Wt mice (n=8), CETPTg mice (n=6), PLTP0 mice (n=7) and CETPTg/PLTP0 mice (n=6).

Fig. 2. In vivo transfer of PC from VLDL into HDL. Mice were injected intravenously (femoral vein) with $[^3\text{H}]-\text{DPPC-VDL}$, $7.0 \times 10^5$ cpm. Blood (70 μl) was taken from the tail vein at different times. VLDL and LDL were precipitated (see “Method”). The radioactivity in supernatant, representing $[^3\text{H}]$-PC transferred from VLDL to HDL, was measured. Results are shown for CETPTg mice (n=5), and PLTP0/CETPTg mice (n=5).
Fig. 3. CETP Activity Assay. CE transfer activity was measured by incubating 3 µl of plasma from different mice with [3H]-CE-containing HDL and unlabeled LDL for 4 hours, and then precipitating the LDL by the heparin/Mn method and counting an aliquot of the supernatant. Results are shown for Wt mice (n=8), CETPTg mice (n=6), PLTP0 mice (n=7) and CETPTg/PLTP0 mice (n=6).

Fig. 4. Detection of CETP in CETPTg and CETPTg/PLTP0 mouse plasma. Ten µl of mouse plasma was run on a sodium barbital/barbital buffered agarose gel and blotted onto a nitrocellulose membrane as described in "Materials and Methods". CETP was detected by blotting with 125I-labeled anti-CETP mAb (TP2). The relative CETP concentration was determined by quantitative scanning using a Phosphorimager.

Fig. 5. Plasma lipoprotein analysis by FPLC. An aliquot (200 µl) of pooled plasma (from 6-8 animals) was loaded onto a Superose 6B column and eluted with TS buffer (50 mM Tris, 0.15 M NaCl, pH 7.5). An aliquot of 80 µl from each fraction (1 ml) was used for the determination of free cholesterol, cholesteryl ester and phospholipid.
Fig. 6. SDS-PAGE Analysis of apolipoproteins from ultracentrifugally isolated plasma lipoproteins. The ultracentrifugally isolated plasma (pooled sample from 6 animals) lipoproteins (VLDL+LDL, d<1.063 g/ml, and HDL d=1.063-1.21 g/ml) were run on a 4-20% gradient SDS-PAGE gel under reducing condition. The apolipoproteins then were stained by Coomassie brilliant blue as described.
Table 1 Plasma and lipoprotein lipid analysis in Wt, CETPTg, PLTP0, and CETPTg/PLTP0 mice on a chow diet and a high fat high cholesterol diet

<table>
<thead>
<tr>
<th>Mice</th>
<th>Plasma PL</th>
<th>HDL PL</th>
<th>Non-HDL PL</th>
<th>Plasma Chol CE</th>
<th>HDL CE</th>
<th>HDL FC</th>
<th>NON-HDL CE</th>
<th>Non-HDL FC</th>
</tr>
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<tr>
<td><strong>Chow diet</strong></td>
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<td></td>
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<td></td>
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<tr>
<td>Wt</td>
<td>171±16</td>
<td>130±4</td>
<td>41±9</td>
<td>78±8</td>
<td>44±6</td>
<td>16±3</td>
<td>15±6</td>
<td>4±1</td>
</tr>
<tr>
<td>CETPTg</td>
<td>186±28</td>
<td>139±12</td>
<td>47±6</td>
<td>72±9</td>
<td>36±7a</td>
<td>14±2</td>
<td>16±7</td>
<td>3±2</td>
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<tr>
<td>PLTP0</td>
<td>124±16</td>
<td>79±9</td>
<td>45±7</td>
<td>40±5</td>
<td>16±5</td>
<td>6±3</td>
<td>14±4</td>
<td>3±1</td>
</tr>
<tr>
<td>CETPTg/PLTP0</td>
<td>85±11b</td>
<td>45±7c</td>
<td>40±5</td>
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<td>9±2b</td>
<td>5±2</td>
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<tr>
<td>Wt</td>
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<td>165±16</td>
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<tr>
<td>CETPTg</td>
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<td>80±7</td>
<td>111±26</td>
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<tr>
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<td>133±22</td>
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<td>23±8</td>
<td>8±3</td>
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<tr>
<td>CETPTg/PLTP0</td>
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<td>139±33</td>
<td>115±12</td>
<td>12±2b</td>
<td>6±2</td>
<td>39±7</td>
<td>59±9</td>
</tr>
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</table>

HDL was separated from Non-HDL by precipitation with HDL reagent (Sigma). The total cholesterol, free cholesterol and phospholipid concentrations were determined by enzymatic methods. Wt mice versus CETPTg mice, \(^{a}p<0.05\). PLTP0 mice versus CETPTg/PLTP0 mice, \(^{b}p<0.01\), \(^{c}p<0.05\). Values are mean±SD based on analyses of individual mouse plasma and represent 6-10 animals per group. PL, phospholipid; CE, cholesteryl ester; FC, free cholesterol.
Reference


Kawano et al., Fig. 1
Kawano et al., Fig. 2

% of Radioactivity on HDL

- CETPTg
- CETPTg/PLTP0

Time (min)
Figure 3, Kowano et al.
Figure 4, Kowano et al.
Kawano et al., Fig. 5

Phospholipid (mg/dL)

Free Cholesterol (mg/dL)

Cholesterol ester (mg/dL)

- Wt
- CETPTg
- PLTPO
- CETPTg/PLTPO

Fractions
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J. Biol. Chem. published online July 12, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003523200

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