Thioltransferase Mediated Ascorbate Recycling in Human Lens Epithelial Cells

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PURPOSE. This study was undertaken to investigate whether thioltransferase (TTase) exhibits dehydroascorbate (DHA) reductase activity in human lens epithelial cells.

METHODS. TTase was investigated for DHA reductase activity in vitro by the method of glutathione reductase-coupled spectrophotometric assay. DHA reductase activities of human lens epithelial (HLE-B3) cell lysate and TTase-depleted HLE-B3 cell lysate were determined with a 6-deoxy-6-fluoro-DHA probe and 19F-NMR spectroscopy. TTase-overexpressing and -depleted HLE-B3 cells were investigated for DHA reductase activity.

RESULTS. TTase showed DHA reductase activity at a Kₐ of 0.15 mM and Vₚmax of 35 nmol/min. Investigation of the DHA reductase activity in human lens epithelial (HLE-B3) cell lysate, by using a 6-deoxy-6-fluoro-DHA probe and 19F-NMR spectroscopy, revealed that cell lysate possesses significant DHA reductase activity. This activity decreased extensively when TTase was depleted from the cell lysate by immunoprecipitation. In a cell-free system with externally added DHA, nearly 70% of the recycling ability was diminished when TTase was removed from the lysate. The TTase-overexpressing cells increased DHA reductase activity twofold. HLE-B3 cells showed an ability to take up and recycle DHA, and this ability was increased approximately twofold in the TTase-transfected cells. Suppression of TTase in HLE-B3 cells by an antisense CDNA strategy resulted in a 77% decrease in DHA reductase activity.

CONCLUSIONS. The data provide evidence that TTase plays a major role in ascorbic acid recycling in human lens epithelial cells. (Invest Ophthalmol Vis Sci. 2004;45:250–257) DOI: 10.1167/iovs.03-04545

The ocular tissue, especially the eye lens is constantly at risk of oxidative stress from exogenously and endogenously generated reactive oxygen species (ROS). Hence, the lens is equipped with a variety of antioxidant systems to handle these oxidative challenges, as well as some mechanisms to repair the oxidatively damaged biologically important proteins and enzymes. The antioxidant defense system in the lens includes ascorbic acid, glutathione (GSH), and vitamin E, which can quench free radicals and enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase, which can decompose the oxidants. The concentration of GSH (2–5 mM) and ascorbate (1–2 mM) in the lens is several times higher than in other tissues.1,2 GSH is believed to protect protein thiols in the cells against oxidative damage and is itself oxidized. Regeneration of oxidized glutathione (GSSG) to GSH by the reducing system of glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) has been extensively studied.3,4 Ascorbate is essential in cells for its antioxidant capacity and its role as a cofactor in many enzyme systems. In addition, ascorbate is involved in regenerating oxidized vitamin E to its reduced form.5 Because vitamin E plays a crucial role in keeping cell membrane integrity, this further substantiates the importance of ascorbic acid. Although ascorbate is a potent antioxidant, recent studies have shown that its main oxidized product, DHA, is highly toxic to mammalian cells. It has been shown that DHA causes neuronal damage and triggers apoptosis in an in vitro system,6 and in cultured PC12 cells, where it induces apoptotic cell death through oxidative stress.7 Recent evidence has shown that DHA generates oxidative stress in pancreatic tissues8 and it is also known to form glycated protein conjugates, that contribute to lens opacification and pigmentation.9 Therefore, it is reasonable to believe that a healthy lens may have an efficient mechanism or mechanisms to recycle DHA to ascorbic acid. Several studies indicate that DHA is reduced nonenzymatically by GSH alone, specially in lens epithelial cells.10–12 However, when cells are challenged with oxidative stress, the cellular GSH level may be drastically reduced, and the lowered GSH may not perform the nonenzymatic reduction of DHA efficiently. Therefore we speculate that the lens cells may have enzymatic mechanisms that reduce DHA effectively, similar to plant systems in which a GSH-utilizing DHA reductase system is present.13

Thioltransferase (TTase) is a small (molecular mass 11.8 kDa) heat-stable, ubiquitous thiol/disulfide exchange enzyme (EC 1.8.4.3) present in both prokaryotes and eukaryotes.14 It is a multifunctional enzyme, and its functions include the reduction of important enzyme ribonucleotide reductase,15 catalysis of the dethiolation of protein-thiol mixed disulfide,16,17 sulfate reduction,18 and the enzymatic deiodination of thyroid hormone.19 This enzyme has been purified, characterized, and cloned from various tissues,20–25 including human lens.25 It has been shown that TTase efficiently dethiolates S-thiolated proteins in lens cells and thus may protect the lens from opacification or cataract formation.17 In addition to these functions, TTase has been implicated in the catalytic reduction of DHA to ascorbic acid in vitro26 similar to the catalytic actions of protein disulfide isomerase (PD1) and liver albumin.26,27 To investigate whether TTase has the capacity as a DHA reductase in vivo, we studied the ascorbate regeneration system by using human lens epithelial cells as a model. This study provided evidence that TTase is a major enzyme involved in regenerating ascorbate in human lens epithelial cells.
**Role of Thioltransferase in Ascorbate Recycling in the Lens**

**Materials and Methods**

**Cell Culture and Materials**

Human lens epithelial cell line (HLE-B3), immortalized by infecting with adenovirus 12-SV40, was generously provided by Usha Andley (Washington University, St. Louis, MO). These cells were grown in minimum essential medium (MEM) supplemented with 20% fetal calf serum and 50 μg/mL gentamicin in 100 X 20-mm tissue culture plates in humid atmosphere of 5% CO\textsubscript{2} at 37°C. Sodium-furoyl-deoxy-ascorbic acid (F-ASA) was synthesized in the laboratory of Vincent Monnier at the Case Western Reserve University, as previously described.\textsuperscript{29} All the other chemicals and reagents were standard commercial products of analytical grade.

**TTase and Protein Assays**

TTase activity was assayed according to a previously described method.\textsuperscript{25} Protein concentrations in cell lysates were determined by the bicinchoninic acid (BCA) method according to the manufacturer’s protocol (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard.

**Purification of Recombinant Human Lens TTase**

Human lens TTase cDNA was cloned as previously described and was expressed in *Escherichia coli*, using the pET expression system from Novagen (Madison, WI), according to Qiao et al.\textsuperscript{25} The recombinant TTase was purified using Sephacry G-7s and Q-Sepharose FF (Pierce/Pharmacia, Groton, CT) columns according to procedures described previously.\textsuperscript{25}

**Preparation of Anti-TTase Antibody**

Affinity-purified rabbit anti-TTase antibody was prepared with purified recombinant human TTase by Bethyl Laboratories, Inc. (Montgomery, TX). Antiserum was made by immunization of albino rabbit. For primary immunization, 1 mg of recombinant human TTase was emulsified with Freund’s complete adjuvant and injected subcutaneously. Eight weeks later, the initial injection was boosted with 1 mg recombinant human TTase emulsified with Freund’s incomplete adjuvant; antiserum was collected 10 days after the booster. Antiserum was affinity purified by using a TTase affinity column, which was prepared by linking TTase to agarose using the cyanogen bromide method.

**DHA Reductase Assay**

The DHA reductase activity of recombinant human TTase, bovine liver PDI, and albumin was assayed at 25°C by a GSH-reductase-coupled spectrophotometric assay as described by Kan and Wells.\textsuperscript{29} The reaction mixture contained Na phosphate buffer (100 mM, pH 7.5), EDTA (1 mM), GSH (0.5 mM), 2 U GSH reductase (Sigma-Aldrich) and NADPH (0.5 mM). The reaction was started by adding 1 mM freshly prepared DHA in the mixture and followed the decline of absorbance at 340 nm for 5 minutes. One unit was defined as the catalytically oxidized 1 μM/min NADPH.

**Determination of Ascorbate Concentration and DHA Reductase Activity of HLE-B3 Cell Lysate by 19F-NMR Spectroscopy**

Fluorosascorbic acid (6-deoxy-6-fluoro-ascorbic acid) was synthesized as previously described.\textsuperscript{29} For the production of fluoro-DHA (F-DHA), fluorosascorbic acid (FASA) was dissolved in ice-cold water (Milli Q; Millipore, Bedford, MA) and 10 μL bromine (catalog no. B8548, minimum 99.5%, Sigma-Aldrich) was added. The reaction mixture was kept on ice, and N\textsubscript{2} gas was bubbled through the solution until it changed from yellow to colorless. DHA reductase assay was assayed at 25°C in a mixture containing Na phosphate buffer (137 mM, pH 6.8), EDTA (1 mM), GSH (0.5 mM), and either water (control) or HLE-B3 cell lysate (150 μg of protein for a reaction mixture). This mixture was incubated for 5 minutes at 25°C, followed by addition of F-DHA (1 mM) and incubation was continued for the indicated times (0, 0.5, 1, 2, 5, 7.5 min). At the end of the each incubation period ice-cold metaphosphoric acid (4%), containing 1 mM of 6-fluoro-6-deoxy-6-glucose (Sigma-Aldrich) was added to each sample as an internal standard. The precipitated proteins were removed by centrifugation at 15,000 rpm at 4°C for 30 minutes. The supernatant was saved and used to detect the fluoro-ascorbic acid produced during the reaction by 19F-nuclear magnetic resonance (NMR) spectroscopy. 19F 705.5-MHz NMR spectra were obtained at the University of Akron (Akron, Ohio) with a 750-MHz spectrometer (Unity Plus; Varian Analytical Instruments, Sunnyvale, CA) equipped with a 5-mm H/1H/C/3F PP FG triple-resonance probe (hardware configured for 19F detection and 1H/13C decoupling; Varian). The data were described.\textsuperscript{30} 19F spectra were acquired at 25°C with a 16.8-μs (~90°) 19F pulse width, 0.8-second acquisition time, 9.1-kHz spectral window, gated 1H decoupling to suppress the nuclear Overhauser effect, using a 1.55-kHz decoupling field during the reaction. A standard consisting of F-o-glucose in D\textsubscript{2}O was added immediately before the measurement. For calibration, the chemical shift of the downfield furanose conformer of F-glucose was set at −219 parts per million (ppm) based on the shift of C\textsubscript{C2}F, (δ\textsubscript{p} = 76 ppm) as the external standard. The other F-glucose signal at −218.3 ppm likely corresponds to the pyranose conformer as judged by the 60% to 40% ratio of these two signals. For quantitation, the sum of the two signals was assumed to represent 1.0 mM of fluorocarbons. An example of the proton-decoupled spectrum depicting the relative position of FASA and F-DHA compared with the doublet signals of the internal standards is shown in Figure 3.

**Inhibition of DHA Reductase Activity of HLE-B3 Cell Lysate by Anti-TTase Antibody**

HLE-B3 cells were harvested and lysed in a buffer, which contained 50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% IgG (Sigma-Aldrich), and protease inhibitor (Cocktail Set III; Calbiochem, La Jolla, CA) and incubated on ice for 15 minutes. The lysate was divided into two equal volumes. To one volume, 10 μg anti-TTase antibody was added and, to the other, 10 μg human IgG was added. Both tubes were incubated for 2 hours at 4°C with head-to-tail shaking. After the incubation period, 40 μL of protein A Sepharose (KPL; Gaithersburg, MD) was added to each tube. The tubes were then centrifuged for 1 minute at 10,000 rpm, and supernatants were collected and aliquots containing 150 μg of protein from both tubes were used to prepare incubation mixtures as described earlier. These mixtures were incubated at 25°C for 5 minutes, and F-DHA was added and incubated at 25°C for the indicated times (0, 0.5, 2, 5, 10 min). At the end of the experiment, the FASA concentration was assayed as described earlier. Estimation of F-DHA degradation products, −214 ppm and −213.4 ppm in reactions catalyzed by TTase-depleted and intact HLE-B3 cell lysate, was by 19F-NMR spectroscopy. HLE-B3 cell lysates were prepared and TTase was depleted as described earlier. Two sets of reaction mixtures were prepared, one with intact HLE-B3 cell lysate and the other with TTase-depleted HLE-B3 cell lysate, essentially as described earlier. The reaction was started by adding ice-cold F-DHA (1 mM) and incubating at 25°C for the indicated times (0, 0.5, 1, 2, 5, 10 min). Samples were then prepared for 19F-NMR spectroscopy.

**Quantification of Ascorbate Concentration in HLE-B3 Cell Lysate**

Ascorbic acid concentration was quantified using the 2,6-dichlorophenol-indophenol (DCIP) method of Omaye et al.\textsuperscript{31} Cells were lysed in M-PER lysis buffer (Pierce Biotechnology) and an equal volume of 10% metaphosphoric acid was added, mixed, and centrifuged for 20 minutes at 3500g. An aliquot of the supernatant was mixed with 0.3 mL citrate-
IgG (100/H9262/H11001) bated for 1 week, incubated in 40% FBS.

were grown under 400°C according to the manufacturer.

were grown for 2 days before they were transferred to fresh medium containing 40% FBS plus 200g/mL geneticin for selection and were fed every 4 days.

The cells were harvested and lysed (0.1 g wet cell pellet per milliliter buffer) using M-PER lysis buffer (Pierce Biotechnology), which was supplemented with 150 mM NaCl, 5 mM EDTA and 30 µL of protease inhibitor (Cocktail Set III; Calbiochem, Inc.). The lysate was then divided into three equal fractions. The first fraction was treated with anti-TTase antibody to deplete TTase, and the second and third fractions were untreated. The reaction was performed by supplementing the first and the second fractions with DHA and NADPH to reach a final concentration of 6.25 and 1.875 mM, respectively. The third fraction without any addition served as the control. All three fractions were incubated at 25°C for 2 hours, and the level of ascorbic acid was assayed at 30-minute intervals, according to the method of Omaye et al.31

**Effect of Anti-TTase Antibody on the Ascorbic Acid Recycling Ability of HLE-B3 Cells**

HLE-B3 cells were harvested and lysed (0.1 g wet cell pellet per milliliter buffer) using M-PER lysis buffer (Pierce Biotechnology), which was supplemented with 150 mM NaCl, 5 mM EDTA and 30 µL of protease inhibitor (Cocktail Set III; Calbiochem, Inc.). The lysate was then divided into three equal fractions. The first fraction was treated with anti-TTase antibody to deplete TTase, and the second and third fractions were untreated. The reaction was performed by supplementing the first and the second fractions with DHA and NADPH to reach a final concentration of 6.25 and 1.875 mM, respectively. The third fraction without any addition served as the control. All three fractions were incubated at 25°C for 2 hours, and the level of ascorbic acid was assayed at 30-minute intervals, according to the method of Omaye et al.31

**DHA Recycling in Normal and TTase Overexpressing HLE-B3 Cells**

Normal and TTase-overexpressing HLE-B3 cells were each grown in 10-cm plates. After cells reached confluence, five plates were taken from each group and cells were washed thoroughly with PBS. The cells were incubated in serum-free MEM in the presence of DHA at the concentrations of 0, 0.5, 1, 1.5, and 2 mM in each plate, respectively, at 20°C for 30 minutes. The cells were lysed in lysis buffer (0.1 g wet cell pellet per milliliter buffer) after thorough washing with PBS solution. Ascorbic acid concentrations were quantified by the method of Omaye et al.31

**DHA Reductase Activity of TTase- Suppressing Cells**

TTase antisense transfected and the vector transfected HLE-B3 cells were grown in MEM supplemented with 40% FBS and 400 µg/mL genetin. Cells lysates were prepared from both groups (0.1 g wet cell pellet per milliliter buffer) and measured for DHA reductase activity.

**RESULTS**

**DHA Reductase Activity of Selected Enzymes and Proteins In Vitro**

As a preliminary step toward elucidating the DHA reduction in human eye lens, we studied the in vitro DHA reductase activity of humans.

**Overexpression and Suppression of TTase in HLE-B3 Cells**

Sense or antisense cDNA for thioltransferase was introduced into the multicloning site of a geneticin (G418 sulfate)-resistant mammalian expression vector (pCR3.1-Uni) to construct sense or antisense plasmids. The sense and antisense plasmids were then each transfected into HLE-B3 cells (Lipofectamine Plus reagent; Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. For the preparation of TTase-overexpressing cells, cells transfected with the sense cDNA were incubated with transfection medium for 1 day, and the cells were passed into new plates with fresh culture medium. The cells were grown for 2 days before they were transferred to fresh medium containing 1 mg/mL geneticin for selection and were fed every 4 days. After 4 weeks of selection, the cells were passed into new plates containing fresh medium with 400 µg/mL genetin and grown to confluence before use. For the preparation of TTase-suppressing cells, the procedure was similar to that of the sense-transfected cells, except the cells were incubated for 4 days in medium containing 40% FBS instead of 20% to increase cell viability.

The cells were then transferred to medium containing 40% FBS plus 200 µg/mL genetin and incubated for 1 week, incubated in 40% FBS + 400 µg/mL genetin for a second week, and cultured in 40% FBS + 800 µg/mL genetin for a third week. Finally, the cells were passed into new plates and maintained with 40% FBS plus 400 µg/mL genetin until use.

**DHA Reductase Activity in TTase-Overexpressing HLE-B3 Cells**

TTase-overexpressing HLE-B3 cells and control cells carrying only the pCR3.1-Uni vector were prepared as described. Both groups of cells were grown under 400 µg/mL genetin selection pressure, and cell lysates were prepared. Various amounts of proteins were taken from both cell lysates, and DHA reductase activity was assayed by measuring the decrease in absorbance of NADPH at 340 nm.

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932922/)
of purified recombinant human lens TTase, bovine liver PDI, and rat liver albumin using a GSH reductase-coupled spectrophotometric method. TTase, PDI, and albumin exhibited significant DHA reductase activity with TTase being the most effective enzyme. Kinetic analysis of DHA reductase activity of the three active enzymes-proteins showed that TTase (0.79 \mu g/mL) had the lowest $K_m$ (0.15 ± 0.4 mM) and the highest $V_{max}$ (35 ± 1.5 nanomoles/min), followed by bovine liver PDI (25 \mu g/mL), which had $K_m$ and $V_{max}$ of 17.5 ± 3.8 mM and 8 ± 2.2 nanomoles/min, respectively. The rat liver albumin (750 \mu g/mL) showed a $K_m$ of 43 ± 2.5 mM and a $V_{max}$ of 0.85 ± 0.3 nanomoles/min.

**FIGURE 2.** $^{19}$F-NMR spectroscopic detection of F-DHA degradation products in reactions catalyzed by HLE-B3 cell lysates, with and without TTase depletion by immunoprecipitation. Two types of reaction mixtures were prepared essentially the same as described in Figure 1B. Reactions were started by adding ice cold F-DHA (1 mM) and incubated at 25°C for indicated times and samples were prepared for $^{19}$F-NMR spectroscopy. (A) $^{19}$F-NMR signals of F-ASA plus F-DHA and their degradation products at −213.4 ppm and −214 ppm (F-DKG). The identity of the compound at −213.4 ppm is unknown. (B) The kinetics of the formation of F-DKG in TTase depleted (▲) and control cell lysate pretreated with human IgG (■), respectively. Each value represents a single determination. (C) The kinetics of the formation of −213.4 ppm compound in TTase-depleted (▲) and control cell lysate pretreated with human IgG (■), respectively. Each data point represents a single determination.
Comparison of DHA Reduction by GSH with and without the Presence of HLE-B3 Cell Lysate Using 19F-NMR Spectroscopy

This experiment was designed to investigate the hypothesis that cultured human lens epithelial cells have intrinsic enzymes that can catalyze the reduction of DHA to ascorbic acid in situ and to compare such enzymatic reduction with chemical reduction by GSH. By using the spectrophotometric method, we found that HLE-B3 cell lysate in the presence of 0.5 mM GSH reduced DHA more efficiently than the chemical reduction of DHA by GSH alone (data not shown). Reduction of DHA to ascorbate was confirmed with a highly specific assay system that detected the production of F-ASA from F-DHA by 19F-NMR spectroscopy. Figure 1A shows the levels of F-ASA detected in human IgG (10 g) by using 19F-NMR spectroscopy. As shown in Figure 2A, the intact cell lysate with DHA and NADPH supplementation did not result in ascorbate production in comparison to GSH alone. Little difference was seen during the first 2 minutes of the reaction, but after 120 minutes, approximately 60% increase of F-ASA was observed at 5 minutes, and nearly double the amount was detected at 7.5 minutes, when the incubation mixture included the cell lysate.

Inhibition of DHA Reductase Activity of HLE-B3 Cell Lysate by Anti-thioltransferase Antibody

To show that the DHA reduction observed in Figure 1A is caused by the enzyme action of TTase, we used an anti-TTase antibody to neutralize the TTase function. The rabbit antibody against recombinant human TTase detected a single band at 11.8 kDa in the cell lysate that corresponded to pure recombinant human thioltransferase in a concentration dependent manner (data not shown). Nonrelevant antibodies (human IgG; Sigma-Aldrich, St. Louis, MO) had no effect on TTase activity. No immunoreactive band was detected with human IgG. Therefore, we treated the cell lysate with this antibody to immunoprecipitate and remove the cellular TTase from the lysate. The TTase-free lysate was then compared for its ability to reduce DHA with the intact cell lysate (150 µg of total protein) pretreated with human IgG (10 µg) by using 19F-NMR spectroscopy. As shown in Figure 1B, removal of TTase from the cell lysate caused an extensive reduction in the production of F-ASA in the reaction mixture, similar to the pattern of F-ASA production by GSH alone, as observed in Figure 1A, indicating that the F-ASA production in this reaction mixture using the anti-TTase antibody immunoprecipitated cell lysate may be due mainly to GSH reduction.

19F-NMR Spectroscopic Detection of F-DHA Degradation Products in Reactions Catalyzed by HLE-B3 Cell Lysates with and without TTase Immunoprecipitation

19F-NMR spectroscopic analysis of the reaction mixtures containing cell lysate with and without immunoprecipitation revealed, besides F-ASA and F-DHA, two additional peaks representing DHA degradation products (Fig. 2A). The product at −214 ppm was identified as 6-fluoro-2,5-diketo-gulonic acid (FDKG) but the product at −213.4 ppm has not yet been identified. The formation of both F-DKG (Fig. 2B) and the −213.4-ppm compound (Fig. 2C) were very rapid but formed faster and in larger quantities when TTase was absent in the cell lysate. These data indicate that TTase can efficiently catalyze the reduction of DHA and minimize degradation product formation in HLE-B3 cells.

Time-Dependent Regeneration of Ascorbate and Its Inhibition by Anti-TTase Antibody in a Cell-Free System

A cell-free system was prepared to investigate the time dependent regeneration of ascorbate and its inhibition by anti-TTase antibody. Cell lysate prepared from HLE-B3 cells was divided into three equal fractions. One fraction was used as the control without any addition. First fraction was used as the control without any addition (●). TTase was removed from one fraction by immunoprecipitation. The TTase-depleted fraction (▲) and the remaining fraction (▼) were supplemented with DHA and NADPH to concentrations of 6.25 mM and 1.875 mM, respectively. All fractions were incubated at 25°C for 150 minutes and ascorbic acid concentration was assayed at 30 minutes interval according to the method of Omaye et al.31 The results are based on the average of three determinations. Error bars, SEM. *Significantly different from values for the TTase-depleted lysate at **P < 0.025 and *P > 0.005 as determined by Student’s t-test.

Effect of Overexpression of TTase on DHA Reductase Activity in HLE-B3 Cells

This experiment was performed to test the hypothesis that increased cellular TTase concentration can simultaneously enhance cellular DHA reductase activity. Confluent HLE-B3 cells stably transfected with sense cDNA and vector transfected control cells were lysed, and TTase activity was compared. There was an approximate twofold increase in TTase activity in TTase cDNA-transfected cells over the control (data not shown). This was further confirmed by immunoblot analysis of both lysates (Fig. 4A), in which approximately double the amount of TTase was noted in the TTase-overexpressing cells (lane 2) over the vector-transfected control (lane 3). Figure 4B shows that the DHA reductase activities in the lysates of TTase-overexpressing cells and control cells were proportionally increased as a function of the concentration of the proteins used.
in the assay. TTase-overexpressing cells showed more than a twofold increase in DHA reductase activity over the control.

**DHA Reductase Activity in the TTase-Suppressed HLE-B3 Cells**

By using the antisense method, we were able to suppress TTase expression in the HLE B3 cells successfully, indicated by the disappearance of the TTase band in the Western blot (Fig. 4A, lane 4). The dethiolase activity in the TTase-suppressed cells was 70% to 80% lower than in the vector-transfected control cells (data not shown). The DHA reductase activity in the TTase-suppressed HLE-B3 cells was also lowered to 70% to 80%, corresponding to the amount of protein used in the assay (Fig. 4C).

**Dehydroascorbate Recycling in Normal and TTase Enriched HLE-B3 Cells**

It has been documented that leukocytes and erythrocytes can take up DHA and recycle it efficiently.\textsuperscript{32-35} We investigated whether HLE-B3 cells could also take up and recycle DHA similar to erythrocytes. Figure 5 shows that normal HLE-B3 cells took up DHA in the tested range of 0.5 to 2.0 mM and recycled it efficiently. However, cells enriched with TTase further enhanced this ability approximately twofold over the control (the TTase unenhanced group).

**DISCUSSION**

Most of the work on mammalian enzymes with DHA reductase activity has been performed using in vitro systems. We investigated some of the mammalian enzymes and proteins that have been thought to possess DHA reductase activity in vitro, and our results showed that TTase had the highest DHA reductase activity in comparison to rat liver albumin and bovine liver PDI, confirming the earlier reports of Wells et al.\textsuperscript{26} Sasaki et al.\textsuperscript{12} found no evidence for the presence of DHA reductase activity in canine or rabbit lens epithelium, the whole lens, ciliary body, or retina, thus suggesting that DHA is reduced solely by GSH through chemical reduction.\textsuperscript{34,35} These investigators have assayed the DHA reductase activity by observing the increase in absorbance at 265 nm as a result of GSH-dependent production of ascorbate. Their assay mixture contained 2.5 mM GSH and 0.2 mM DHA in phosphate buffer, which we found unsuitable in our assay system, because of the high background
results are based on the average of three determinations. Error bars, copy.30 The spectroscopic data (Fig. 1A) not only demon-

strated that HLE-B3 cell lysate was far more effective in reduc-
ing DHA than GSH alone but also con-

cluded that F-DHA is very unstable, and that HLE-B3 cell lysate (containing TTase; Fig. 1B). This indicates that TTase

plays a major role in DHA reduction in lens epithelial cells.

It has been well documented that the DHA is very unstable, and, if it is not reduced to ascorbate, it can be irreversibly oxidized to L-erythroascorbic acid based on observations made by Jung and Wells39 who demonstrated that L-dehydroascorbic acid spontaneous

lyzes to L-erythroascorbic acid at physiological pH in addition to the well-established delactonization to 2,3-DKG.37 Because the latter behaves chemically like ascorbic acid, there is potential for overestimation of ascorbic acid production by the 2,6-dichloroindophenol assay. However, although the absolute amount of estimated ascorbic acid may be high in Figures 3 and 5, the differences between depletion or overexpression of TTase can be attributed only to the presence or absence of the enzyme itself. Furthermore, recent studies with F-ASA and HLE-B3 cells have clearly shown that more than 95% of F-DHA is intracellularly converted to F-ASA,30 erasing any concerns about the ascorbic acid concentrations in Figure 5.

Formation of DHA degradation products can be consider-
ably slowed down and avoided if TTase is present in the cells (Figs. 2B, 2C). The incomplete prevention of the degradation of F-DHA by TTase may in part relate to the fact that the half-life of F-DHA is twice as fast as that of native DHA.28

Removal of TTase from HLE-B3 cell lysate also significantly impairs the cell’s ability to recycle ascorbic acid. In TTase-free lysate (Fig. 3), there was no recycling of ascorbate up to 60 minutes. After that, there was a recycling process, but it was much less than that of the whole lysate. This indicates that apart from TTase, there may be some other systems like PDI, a membrane-bound enzyme, glutaredoxin 2, a mitochondrial TTase,40 a 31-kDa protein isolated from rat liver with GSH-dependent DHA reductase activity,41 and a 32-kDa protein from human erythrocytes with GSH-dependent DHA reductase activity,42 all of which can reccycle ascorbate in HLE-B3 cells and may account for approximately 30% of the recycling ac-

tivity, as shown in Figure 3. Because we have used 25°C in the assay system set for TTase function, the catalytic activities of these enzymes may be delayed in this low temperature until sometime later (60 minutes). Alternatively, the delayed reduc-
tion of DHA in the TTase-depleted lysate may be the result of a spontaneous conversion of DHA to ascorbate and erythroascorbate39 or a combination of both enzymatic and nonen-
zymatic processes.

When TTase was overexpressed in HLE-B3 cells, TTase activity increased twofold. According to Figure 4B, there was a parallel increase in the DHA reductase activity in the trans-
fected cells, indicating that TTase provides the major DHA reductase enzyme in vivo. Data presented in Figure 5 provide strong evidence for the ability of TTase to recycle ascorbate in vitro. TTase-overexpressing cells have twice as much ascorbate-recycl-
ing ability as normal-expressing HLE-B3 cells.

It has been reported that depletion of TTase by transfecting cells with antisense cDNA of thioltransferase is lethal to cells.43 We also observed the same phenomenon. Most of the cells died while selecting stable transfections under geneticin selection pressure. Therefore, to increase cell viability, we increased the PBS level in growth medium from 20% to 40%. When TTase was suppressed in HLE-B3 cells by an antisense strategy, there was very little remaining DHA reductase activity in HLE-B3 cells, indicating that TTase provides the major DHA reductase activity in lens epithelial cells. The remaining DHA reductase activity may be contributed by the other DHA reductase en-
zymes mentioned herein.

In summary, the present work provides strong evidence for the enzymatic reduction of DHA by TTase in lens epithelial cells, cell-free systems, and in vivo experiments. We have demonstrated that TTase had the highest DHA reductase activity of the enzymes and proteins tested. We have also shown that HLE-B3 cells have intrinsic DHA reductase activity, approxi-

mately 70% of which can be inhibited by anti-TTase antibody. Overexpression of TTase increased the DHA reductase activity of HLE-B3 cells, and suppression of it caused a substantial decrease in intrinsic DHA reductase activity. These data pro-
vide supportive evidence for the hypothesis that TTase is a participant in a major ascorbate recycling system in lens epithe-

lial cells.

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