Colocalization of leukotriene C synthase and microsomal glutathione S-transferase elucidated by indirect immunofluorescence analysis

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Abstract We have previously shown that the two membrane bound enzymes leukotriene C synthase and microsomal glutathione S-transferase interact in vitro and in vivo. Rat basophilic leukemia cells and murine mastocytoma cells, two well-known sources of leukotriene C synthase, both expressed microsomal glutathione S-transferase as determined by Western blot analyses. Several human tissues were found to contain both leukotriene C synthase and microsomal glutathione S-transferase mRNA. These data suggest that the interaction may be physiologically important. To study this further, expression vectors encoding the two enzymes were cotransfected into mammalian cells and the subcellular localization of the enzymes was determined by indirect immunofluorescence using confocal laser scanning microscopy. The results showed that leukotriene C synthase and microsomal glutathione S-transferase were both localized on the nuclear envelope and adjacent parts of the endoplasmic reticulum. Image overlay demonstrated virtually identical localization. We also observed that coexpression substantially reduced the catalytic activity of each enzyme suggesting that a mechanism involving protein–protein interaction may contribute to the regulation of LTC_4 production. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Leukotrienes (LTs) are biologically active compounds derived from arachidonic acid with important functions in inflammation [1,2]. The cysteinyl LT LTC_4 is produced by addition of a glutathione (GSH) moiety to LTA_4 catalyzed by LTC_4 synthase (LTCS) [3], a 17 kDa homodimeric microsomal protein [4,5]. LTC_4 stimulates bronchoconstriction, airway mucous production and edema formation in nearly every vascular bed investigated [6].

Microsomal GSH S-transferase (MGST) is a homotrimeric 18 kDa protein and like other GSH transferases it is involved in detoxification and metabolism of xenobiotics [7]. This enzyme was shown to bind LTC_4 [8] and we have previously reported a direct protein–protein interaction between MGST and LTCS in vitro and developed an affinity purification of LTCS based on this interaction [9]. We also demonstrated in vivo interaction between LTCS and MGST using the yeast two-hybrid system [10]. LTCS and MGST both belong to a gene family of membrane-associated proteins in eicosanoid and GSH metabolism (MAPEG) [11].

This paper reports further studies concerning the physiological significance of the interaction between LTCS and MGST.

2. Materials and methods

2.1. Materials

Human RNA Master Blot and express hybridizing solution were purchased from Clontech. Dulbecco's modified Eagle's medium, fetal calf serum, antibiotics and competent *Escherichia coli* DH5α cells were from Gibco BRL. Restriction enzymes, deep vent DNA polymerase and T4 DNA ligase were from New England Biolabs. LTC_4 and LTA_4 were purchased from Cayman Chemicals Inc. All other chemicals used were from Sigma. Dr. David Tu kindly provided human MGST cDNA.

2.2. Antibodies

A monoclonal antibody (M2) raised against the FLAG epitope was purchased from Kodak, New Haven, CT, USA. Dr. Ralf Morgenstern kindly provided rabbit anti-rat MGST antiserum. Fluorochrome-conjugated secondary antibodies, anti-mouse fluorescein isothiocyanate (FITC) affinipure F(ab')$_2$ goat IgG, was from Dakopatts and anti-rabbit Cy3 affinipure F(ab')$_2$ sheep IgG was from Sigma. A mouse monoclonal antibody, anti-His$_6$, was from Roche Molecular Biochemicals and peroxidase-conjugated goat anti-mouse IgG was from Dakopatts. Affinity-purified [^{125}I]protein A was from Amersham Pharmacia Biotech.

2.3. Plasmids

Human LTCS cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) in frame with an upstream FLAG epitope. MGST cDNA was subcloned into pcDNA3 without the FLAG epitope.

2.4. Northern dot blot analysis

LTCS cDNA was labeled with [α-^{32}P]dATP (3 Ci/mmol) by a random prime technique. The membrane was prehybridized with express hybridized solution containing heat-denatured sheared salmon sperm DNA for 30 min at 65°C. Approximately 7.5 μCi of denatured probe was hybridized with the membrane overnight at 65°C. The membrane was stringently washed and exposed to X-ray film for 5 days. The blot was then stripped by boiling in 0.1% sodium dodecyl sulphate (SDS)

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Abbreviations: BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitro benzene; FITC, fluorescein isothiocyanate; GSH, glutathione; LTCS, leukotriene C synthase; MAPEG, membrane-associated proteins in eicosanoid and glutathione metabolism; MGST, microsomal glutathione transferase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate

for 10 min and reprobed with MGST cDNA probe prepared in the same way.

2.5. Heterologous expression of LTCS and MGST in CV-1 cells

20 μg of recombinant pcDNA3-LTCS or pcDNA3-MGST plus 20 μg of empty vector DNA were used to transfect CV-1 cells, grown in 10 cm Petri dishes, using the calcium phosphate precipitation method [12]. For double transfected cells, 20 μg of each vector was used making the total amount of DNA equal in all experiments. Transfected cells were harvested after 48 h and microsomes prepared, resuspended in phosphate-buffered saline (PBS) and solubilized with 1% CHAPS. The solubilized membrane fractions were assayed for enzymatic activities.

2.6. Western blotting

CV-1 cells transfected with vectors expressing LTCS or MGST fused to a His_6 C-terminal epitope. Solubilized membrane fractions were passed through a Ni-NTA-affinity matrix and retained material was eluted and separated by SDS–PAGE on a 15% slab gel followed by electrophoretic transfer onto nitrocellulose filter. The nitrocellulose filter was blocked using 3% bovine serum albumin (BSA) in PBS and incubated with a monoclonal anti-His antibody followed by incubation with a peroxidase-conjugated anti-mouse goat IgG antibody. Immunoreactive bands were visualized with chemiluminescence.

Alternatively, CV-1 cells were transfected with vectors expressing LTCS fused to a C-terminal FLAG epitope or MGST fused to a C-terminal V5 epitope. Membrane fractions prepared from sonicated cells by centrifugation at $100\,000 \times g$ for 60 min were resuspended in 10 mM Tris–HCl pH 7.6 and separated by SDS–PAGE (15% slab gels) followed by electrophoretic transfer onto nitrocellulose filter. The nitrocellulose filter was blocked using 3% BSA in PBS and in-

cubated with monoclonal anti-FLAG antibody followed by peroxidase-conjugated goat anti-mouse IgG. Immunoreactive bands were visualized by chemiluminescence.

Murine mastocytoma cells (CXBGABMCT-1) were propagated in CB6F1 mice as described [13]. A membrane fraction was isolated from homogenized tumors by centrifugation at $100\,000 \times g$ for 60 min, resuspended in 0.1 M $NaPO_4$ buffer, pH 8.06, and separated by SDS–PAGE (15% slab gel) followed by electrophoretic transfer onto nitrocellulose filter. The nitrocellulose filter was blocked using 3% BSA in PBS and incubated with polyclonal antibody against MGST followed by affinity-purified [^{125}I]protein A. Immunoreactive bands were visualized by autoradiography.

Rat basophilic leukemia (RBL-1) cells were cultured and harvested as described [14] and a membrane fraction was isolated and subjected to Western blot analyses as described above for murine mastocytoma cells.

2.7. Protein concentrations

Protein concentrations were determined using the method described by Bradford [15].

2.8. Assay of MGST activity

MGST enzymatic activity was measured spectrophotometrically at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced GSH as substrates [16].

2.9. Assay of LTCS activity

LTCS enzymatic activity was measured in microsome preparations preincubated in 0.1 M sodium phosphate pH 7.0 buffer for 1 min followed by 5 mM GSH for 1 min at 30°C in 100 μl reaction volume. The reaction was started by addition of LTA_4 (free acid) to a final
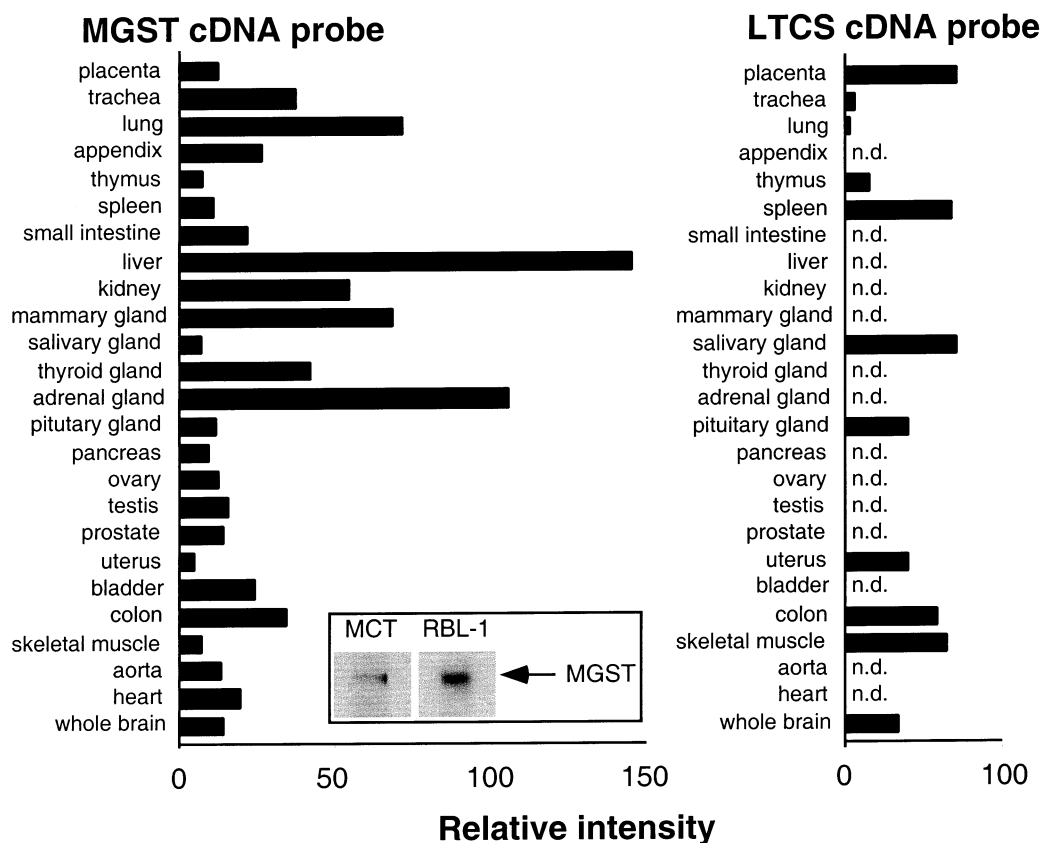

Fig. 1. Tissue distribution of MGST and LTCS mRNA. The expression pattern of MGST mRNA and LTCS mRNA was studied using Northern dot blot analysis. A human RNA Master Blot (Clontech) was prehybridized for 30 min at 65°C. Approximately 7.5 μCi of denatured random ^{32}P-labeled LTCS cDNA probe was hybridized with the membrane overnight at 65°C. The membrane was stringently washed and subsequently subjected to autoradiography. The blot was then stripped by boiling in 0.1% SDS for 10 min and reprobed with MGST cDNA probe prepared in the same way. The inset shows the presence of MGST in murine mastocytoma (MCT) and RBL-1 cells detected by immunoblotting as described in Section 2.
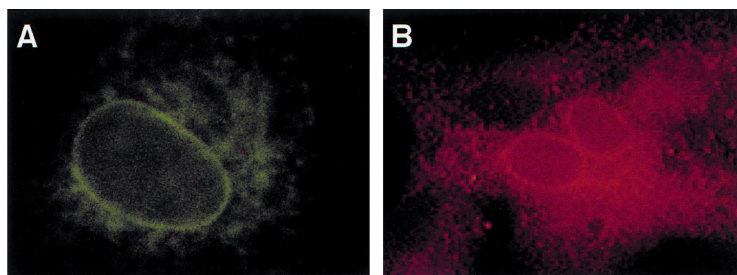
Fig. 2. Subcellular distribution of LTCS and MGST protein in CV-1 cells. CV cells were plated on glass coverslips and transfected with vectors directing the expression of epitope-tagged LTCS (A) or MGST. 24 h later, the cells were fixed and prepared for indirect immunofluorescence labeling using either an anti-epitope antibody (A) or an anti-IgG antibody (B) followed by chromophore-conjugated secondary antibodies (A: FITC anti-mouse goat IgG; B: Cy3 anti-rabbit sheep IgG) as described in Section 2. A shows a picture of a representative transfected cell expressing high levels of LTCS. B shows a picture of representative cells expressing transfected MGST. The immunostaining in A is concentrated to the nuclear envelope. B shows a similar staining pattern but in addition staining is observed throughout membrane structures throughout the cytoplasm.

concentration of 40 µM and allowed to continue for 15 min at 30°C. Addition of 100 µl of acidified cold methanol terminated the reaction. Proteins were precipitated at −20°C for 1 h and removed by $5000 \times g$ centrifugation for 10 min. A 150 µl aliquot of the supernatant was high performance liquid chromatography-separated on a C_{18} Nucleosil reverse phase column (4.6×150 mm, 5 µm particles) using a Hewlett Packard model 1090 instrument equipped with a model 1040 diode array detector. The solvent used was methanol, water, acetic acid and orthophosphoric acid (70:30:0.07:0.03, v/v/v/v) adjusted to pH 5.6; the flow rate was 1.0 ml/min. LTC_4 formation was quantified by the area of the peak at 280 nm in relation to the area of the injected standard LTC_4.

2.10. Indirect immunofluorescence

CV-1 cells grown in six-well dishes were transiently transfected with pcDNA3-LTCS cDNA and pcDNA3-MGST cDNA (5 µg of each plasmid). After 12 h, the cells were washed and fresh medium was added. The cells were incubated for an additional 24 h and then fixed in 2% paraformaldehyde in PBS for 45 min. Fixed cells were washed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. Rabbit anti-rat MGST antiserum plus monoclonal anti-FLAG antibody, M2 (1:50 dilutions in PBS, 1% BSA), were applied for 60 min, followed by five washes in PBS, 0.1% BSA. Fluorochrome-conjugated secondary antibodies (Cy3-labeled sheep anti-rabbit IgG diluted 1:150 plus FITC-labeled goat anti-mouse IgG diluted 1:150) were applied for 60 min followed by five more washes.

2.11. Confocal laser scanning microscopy

Fluorescent sections were imaged at an optical magnification of 60× using a Sarastro confocal microscope (Molecular Dynamics, Sunnyvale, CA, USA) equipped with an argon laser coupled to a Nikon microscope (Tokyo, Japan).

3. Results

3.1. Tissue distribution of LTCS and MGST

To evaluate the tissue distribution of the two enzymes, we performed Northern dot blot analyses. The expression of LTCS mRNA was substantially lower than that of MGST mRNA (Fig. 1). It was predominantly observed in placenta, salivary gland, spleen, skeletal muscle, colon, pituitary gland, uterus, whole brain, thymus and trachea. MGST mRNA expression was similar to recently published data [17].

3.2. Subcellular distribution of MGST and LTCS

In order to determine the subcellular distribution of LTCS and MGST, we performed indirect immunofluorescence analyses on transfected CV-1 cells. Both LTCS and MGST were mainly membrane bound and the staining in both cases was especially strong in the nuclear envelope. Fig. 2A shows a representative cell expressing high levels of transfected LTCS. Similarly, in Fig. 2B, a representative cell expressing transfected MGST is shown. When the fluorescence representing LTCS and MGST, respectively, was superimposed, colocalization on the nuclear envelope was evident (Fig. 3C).

3.3. Enzymatic activities of heterologously expressed MGST and LTCS

In order to investigate if coexpression of LTCS and MGST would influence their ability to catalyze the conjugation of GSH with xenobiotic compounds (MGST) or LTA_4 (LTCS), CV-1 cells were transiently transfected with vectors
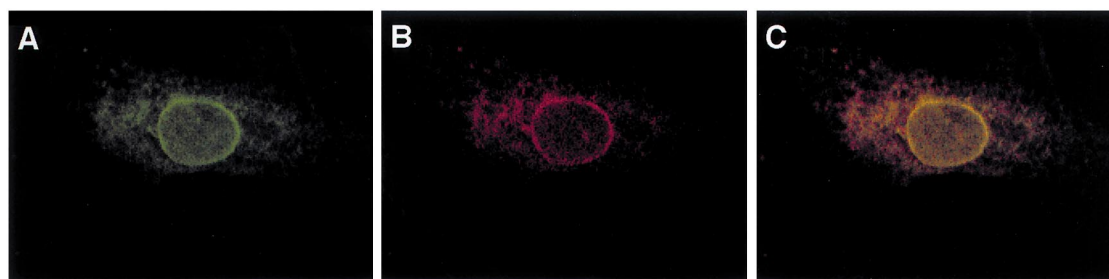

Fig. 3. Colocalization of LTCS and MGST on the nuclear envelope. CV-1 cells were plated on glass coverslips and transfected with epitope-tagged LTCS and MGST. Cells were harvested 24 h later and immunostained with anti-MGST antiserum followed by Cy3-conjugated sheep anti-rabbit antibody and monoclonal anti-FLAG antibody (M2) followed by FITC-conjugated goat anti-mouse antibody. Images were collected using a Sarastro 2000 confocal imaging system linked to a Nikon microscope. The LTCS signal alone is illustrated in A. The MGST signal from the same optical section is illustrated in B. The sections are merged in C. Regions in which LTCS and MGST colocalize appear yellow.
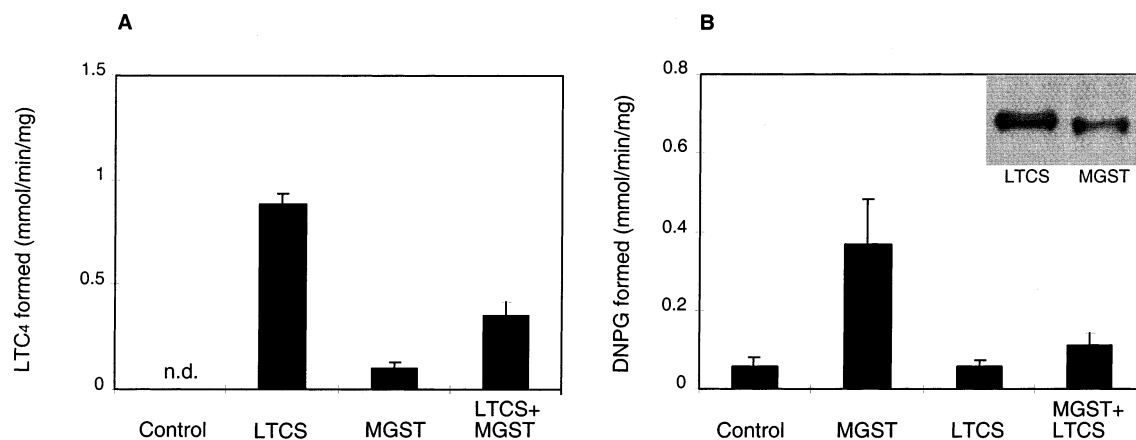
A

B

Fig. 4. Effect of cotransfection of LTCS and MGST on their enzymatic activities. LTCS and MGST activities were measured in a membrane fraction isolated from homogenates of cells, transiently transfected with vectors directing the expression of LTCS and MGST as indicated. LTCS activity was markedly decreased when the cells were transfected with both LTCS and MGST as compared with LTCS alone (A). A similar effect was observed when measuring MGST enzymatic activity (B). The inset shows Western blots of the heterologously expressed LTCS and MGST in transfected CV-1 cells.

directing the expression of LTCS and MGST, respectively. Homogenate was prepared from these cells and LTCS enzymatic activity was measured in microsomes. MGST enzymatic activity was also assayed in these homogenates. There was a greater than 50% decrease in LTCS activity when the cells were transfected with both LTCS and MGST as compared with LTCS alone (Fig. 4A). A similar effect was observed when measuring MGST enzymatic activity instead (Fig. 4B). The diminished LTCS activity was not caused by decreased enzyme protein expression (Fig. 5).

4. Discussion

In the present study, we compared the tissue distribution of MGST and LTCS mRNA in order to determine if the earlier described interaction between these enzymes in vitro and in vivo has physiological relevance. The distribution of human MGST mRNA was recently reported [17] and our results were in agreement with those except for pancreas (which in our

Fig. 5. Expression of LTCS in CV-1 cells cotransfected with MGST. CV-1 cells were transfected at 50% confluency with 20 μg of a vector encoding C-terminal FLAG epitope tagged LTCS (pcDNA3-LTCS) plus 20 μg of empty vector (pcDNA3), or with 20 μg of a vector encoding C-terminal V5 epitope tagged MGST (pcDNA3-MGST) plus 20 μg of empty vector (pcDNA3) or with 20 μg pcDNA3-LTCS plus 20 μg pcDNA3-MGST. Cells were harvested after 36 h and membrane fractions were prepared. Equal amounts of protein were loaded in each well and separated by SDS–PAGE followed by electrophoretic transfer onto nitrocellulose filter. The nitrocellulose filter was blocked using 3% BSA in PBS and incubated with monoclonal anti-FLAG antibody followed by incubation with peroxidase-conjugated goat anti-mouse IgG. Immunoreactive bands corresponding to LTCS were visualized by chemiluminescence.

case was < 10% of liver compared to 103% in [17]), prostate (~10% of liver compared to 45% in [17]), lung (~50% of liver compared to 15% in [17]). Human LTCS mRNA levels were considerably lower than corresponding MGST mRNA. The highest expression was detected in placenta, spleen, salivary gland, colon and skeletal muscle. Somewhat surprisingly, LTCS was expressed in comparably low amounts in lung. Both enzymes were expressed in placenta, colon and brain in relatively high amounts, and in lower amounts in pituitary gland, thymus, uterus, lung and trachea.

The subcellular distribution has previously been determined for cyclooxygenase I (COX1), COX2, 5-lipoxygenase (5-LOX) and 5-LOX activating protein (FLAP) [18–21]. Often, these enzymes were found to be located on the nuclear envelope or in the nuclear matrix [18–21]. The physiological function of this is not known but recently several eicosanoids have been recognized as ligands for nuclear receptors [22–24]. In order to determine the subcellular distribution of LTCS, we performed indirect immunofluorescence analyses on transfected epitope-tagged LTCS in CV-1 cells. The cells showed distinct staining of the nuclear membrane and less distinct staining on surrounding endoplasmic reticulum, indicating that LTCS is located on the nuclear membrane. We also performed indirect immunostaining of MGST in transfected CV-1 cells. In this case the nuclear envelope was stained but the staining pattern was not limited to the nuclear membrane, intense staining was observed in membrane structures throughout the cells. When the staining patterns of LTCS and MGST in doubly stained cells were superimposed using laser-assisted confocal microscopy, there was a clear indication of colocalization of LTCS and MGST on the nuclear envelope.

LTC$_4$, the product of LTCS catalysis, is a strong inhibitor of MGST [25]. Our finding that LTCS and MGST colocalized intracellularly led us to investigate the effects of coexistence of the two enzymes on their catalytic activity. We performed enzymatic measurements on transfected cells and determined the effects of coexpressing the other enzyme. When MGST was cotransfected with LTCS, there was a marked effect on LTCS resulting in a 50% loss of enzymatic activity. Similarly, the MGST enzymatic activity as measured with CDNB decreased approximately 50% when MGST was coexpressed
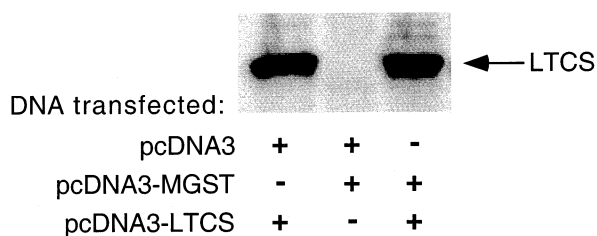
with LTCS. The observations that a number of tissues express both MGST and LTCS, that the two enzymes have virtually indistinguishable subcellular distribution and inhibit each others catalytic activity strongly suggest that the protein–protein interaction between these two GSH conjugating enzymes contributes to the function of these medically important proteins.

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