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Photoaffinity labelling of human leukotriene C_4 synthase in THP-1 cell membranes

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Human leukotriene C_4 synthase specific activity in the human monocytic leukemia cell line THP-1 (0.302 ± 0.062 nmol LTC_4 formed $\cdot$ $min^{-1} \cdot mg^{-1}$) was 7.6-fold higher than in U937 cells (0.040 ± 0.017 nmol LTC_4 formed $\cdot$ $min^{-1} \cdot mg^{-1}$) and comparable to dimethylsulfoxide-differentiated U937 cells (0.399 ± 0.084 nmol LTC_4 formed $\cdot$ $min^{-1} \cdot mg^{-1}$). Using the photoaffinity probe, azido[^{125}I]-LTC_4, a single polypeptide with a molecular mass of 18 kDa was specifically labelled in THP-1 microsomal membranes. The rank order of potencies for competition of azido[^{125}I]-LTC_4 photolabelling of the 18 kDa protein by glutathione, leukotrienes and their analogs was found to be LTC_2 > (azido[^{127}I]-$LTC_4 \approx LTC_4$) > ($LTD_4 \approx LTE_4$) > ($LTA_4 \approx LTB_4$) > *S*-hexyl glutathione > glutathione, corresponded with the rank order of potencies for inhibition of LTC_4 synthase activity but not inhibition of microsomal glutathione *S*-transferase activity. The 18 kDa protein specifically labelled by azido[^{125}I]-LTC_4 had high specificity for LTC_4 and closely related leukotrienes and was separable from microsomal glutathione *S*-transferase. We conclude that azido[^{125}I]-LTC_4 specifically photolabels LTC_4 synthase which is an 18 kDa polypeptide or contains an 18 kDa subunit.

Leukotriene; LTC_4 synthase; Glutathione *S*-transferase; Membrane photolabel

1. INTRODUCTION

The leukotrienes are a group of arachidonic acid metabolites which have been implicated in immediate hypersensitivity and inflammatory reactions (for reviews see [1–4]). They are formed through the oxygenation of free arachidonic acid and subsequent dehydration to form the epoxide intermediate, leukotriene A_4 (LTA_4) through the action of 5-lipoxygenase. LTA_4 can be conjugated with reduced glutathione by LTC_4 synthase to form the sulfidopeptide leukotriene, LTC_4. This enzyme is distinct from other known glutathione *S*-transferases being responsible specifically for the biosynthesis of LTC_4 [5]. The complete purification and characterization of LTC_4 synthase has been hindered owing to the apparent instability of the enzyme in the semi-purified state [6], and the lack of an abundant source of the enzyme. However, partial purification and characterization of LTC_4 synthase from guinea pig lung (91-fold; [6–8]), rat basophilic leukemia cells (10-fold; [9]), mouse mastocytoma cells (4-fold; [10]) and human dimethylsulfoxide-differentiated U937 cells (10,000-fold; [11]) has been reported.

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Abbreviations: LT, leukotriene; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

We have recently described that differentiation of the human promonocytic leukemia cell line U937 into monocyte-like cells by growth in the presence of dimethylsulfoxide results in a 10-fold increase in LTC_4 synthase specific activity [5]. In initial experiments, two polypeptides (18 kDa and 27 kDa) were specifically photolabelled in differentiated U937 cell microsomal membranes with the use of a novel radioiodinated photoaffinity ligand based on the structure of LTC_4. The 18 kDa polypeptide in particular was identified as a candidate for being LTC_4 synthase, although it was not possible to exclude other proteins that might bind LTC_4, especially microsomal glutathione *S*-transferase. The intrinsic LTC_4 synthase specific activity in the related human monocytic cell line, THP-1, is equivalent to dimethylsulfoxide-differentiated U937 cells. We now demonstrate the specific labelling of a single polypeptide with a molecular mass of 18 kDa in the microsomal membranes of THP-1 cells. This 18 kDa polypeptide has high specificity for LTC_4 and closely related leukotrienes and is distinct from microsomal glutathione *S*-transferase.

2. MATERIALS AND METHODS

2.1. *Materials*

THP-1 cells (TIB 202) were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media, antibiotics, fetal bovine serum and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO). LTA_4-methyl ester, LTB_4, LTC_4, LTC_2, LTD_4, and LTE_4 were synthesized by the Department of Me-

dicinal Chemistry at the Merck Frosst Centre for Therapeutic Research.

2.2. *Cell growth and subcellular fractionation*

2.2.1. THP-1 cell growth

Cells from the human monocytic leukemic cell line THP-1 [12] were cultured in sterile RPMI-1640 medium (supplemented with 0.2% (w/v) $NaHCO_3$, 0.05 mM 2-mercaptoethanol and 0.03% (w/v) L-glutamine) containing 50 U penicillin/ml, 50 μg streptomycin/ml and 10% (v/v) fetal bovine serum (Sigma Hybri-Max, not heat-inactivated) at 37°C in a humidified atmosphere containing 6% CO_2. Cells were isolated by continuous-flow centrifugation, ruptured by nitrogen cavitation and the microsomal-membrane fraction was isolated as described previously for U937 cells [11].

2.2.2. Taurocholate solubilization

THP-1 cell microsomal membranes (100,000 × *g* pellet) at a protein concentration of 15 mg/ml were combined with an equal volume of 4% (w/v) taurocholate (Calbiochem) in PBS (pH 7.4), 2 mM EDTA. The mixture was shaken at 4°C for 30 min and subsequently spun at 200,000 × *g* for 60 min. The resulting supernatant (post-taurocholate supernatant) was recovered and the remaining pellet (post-taurocholate pellet) was resuspended in PBS (pH 7.4), 2 mM EDTA in the same volume as the original microsomal membranes by Dounce homogenization ('A' clearance pestle, 10 strokes).

2.3. *Measurement of LTC_4 synthase and glutathione* S-*transferase activities*

LTC_4 synthase activity was measured by monitoring the formation of LTC_4 (as determined by reverse-phase HPLC) in the presence of reduced glutathione and LTA_4 (free acid) essentially as described before [5,11]. Microsomal glutathione *S*-transferase activity was measured following pretreatment of Triton X-100 lysed samples by *N*-ethylmaleimide [13,14] then monitoring glutathione conjugation in the presence of 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM reduced glutathione ($\Delta OD_{340\ nm}, \varepsilon_{340\ nm}$ ($mM^{-1} \cdot cm^{-1}$) = 9.6; [15]). Protein was determined as described by Bradford [16] using bovine γ-globulin as standard.

2.4. *Photoaffinity labelling in THP-1 microsomes*

Azido[^{127}I]-LTC_4 and azido[^{125}I]-LTC_4 (2,200 Ci/mmol) were synthesized as described previously [5]. THP-1 cell microsomes were suspended at a concentration of 0.3 mg/ml (unless otherwise indicated) in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA and 50 mM serine-borate in the presence and absence of varying concentrations of competing ligands as indicated. Azido[^{125}I]-LTC_4 was then added to a final concentration of 20 pM and the mixture was incubated in the dark at 25°C for 30 min, followed by incubation at 4°C for 5 min. The samples were transferred to 6-well tissue culture cluster plates, photolysed under a UV light source (Philips, λ_{max} = 350 nm) for 2 min at 4°C at a distance of 14 cm. Membranes were re-harvested by centrifugation at 200,000 × *g* for 15 min at 4°C, solubilized in SDS-containing sample buffer, and resolved by SDS gel electrophoresis [17]. The polyacrylamide gel was dried under vacuum and used to expose X-Omat AR film (Eastman Kodak Co., Rochester, NY) for 2–3 weeks at −80°C. Band intensities were quantified by laser densitometry (Molecular Dynamics Computing Densitometer).

3. RESULTS

THP-1 cells are a suitable source for the purification and characterization of human LTC_4 synthase because their intrinsic LTC_4 synthase activity (0.302 ± 0.062 (n = 9) nmol LTC_4 formed · min^{-1} · mg^{-1}) is equivalent to dimethylsulfoxide-differentiated U937 cells [5] (0.399 ± 0.084 (n = 47) nmol LTC_4 formed · min^{-1} · mg^{-1}). Previous experiments using U937 cell microsomal membranes have shown an 18 kDa polypeptide to be specifically labelled with the radioiodinated, photolabile probe, azido[^{125}I]-LTC_4. Thus similar experiments were carried out with THP-1 cell microsomal membranes.

3.1. *Photoaffinity labelling of THP-1 cell microsomal membranes by azido[^{125}I]-LTC_4: competition by LTC_4 and reduced glutathione*

THP-1 cell microsomal membranes were probed with azido[^{125}I]-LTC_4 (ca. 2,200 Ci/mmol) to identify potential LTC_4 synthase candidates or subunits of LTC_4 synthase (Fig. 1). Only one polypeptide, with a molecular mass of 18 kDa, was specifically labelled. Photolabelling of the 18 kDa polypeptide was inhibited by 50% in the presence of 0.1 μM LTC_4 (Fig. 1, lane 4 vs. 2) but was not competed for at all by the presence of even 1.0 mM reduced glutathione (Fig. 1, lane 8).

3.2. *Competition of azido[^{125}I]-LTC_4 photolabelling of an 18 kDa membrane polypeptide corresponds to inhibition of LTC_4 synthase biosynthetic activity*

To assess the relative potencies of leukotrienes structurally related to LTC_4 as competing ligands for photolabelling of the 18 kDa polypeptide, THP-1 cell microsomal membranes were incubated with azido[^{125}I]-LTC_4 in the presence of varying concentrations of either LTC_4, LTC_2, LTD_4, LTE_4, LTB_4, LTA_4, reduced glutathione, *S*-hexyl glutathione or azido[^{127}I]-LTC_4 (Fig. 2). Using laser densitometry, the relative potencies of the various competing ligands were quantified. The rank order of potencies for the inhibition of labelling of the 18 kDa polypeptide was LTC_2 > (azido[^{127}I]-LTC_4 ≈ LTC_4) > (LTD_4 ≈ LTE_4) > (LTB_4 ≈ LTA_4) > *S*-hexyl glutathione > glutathione.

Next, to determine if the relative potencies of the various leukotrienes and *S*-hexyl glutathione as competing ligands for the photoaffinity labelling of the 18 kDa polypeptide were reflected by their ability to inhibit enzymic activity, LTC_2, LTD_4, LTE_4, LTB_4, azido[^{127}I]-LTC_4 and *S*-hexyl glutathione at varying concentrations were included in LTC_4 synthase enzyme incubation mixtures. (LTC_4 was not tested for its ability to inhibit LTC_4 synthase activity since the retention time on reverse-phase HPLC of enzymatically produced LTC_4 is indistinguishable from LTC_4 added to the incubation mixture. Similarly, neither LTA_4 nor glutathione were tested since they are the substrates of LTC_4 synthase.) The IC_{50} values for LTC_2 and azido[^{127}I]-LTC_4 were determined to be 2.6 μM and 7.0 μM, respectively, indicating that azido[^{127}I]-LTC_4 was specifically recognized by and therefore inhibited LTC_4 synthase activity. The rank order of potencies for the inhibition of LTC_4 synthase activity was LTC_2 > azido[^{125}I]-LTC_4 > (LTD_4 ≈ LTE_4) > LTB_4 > *S*-hexyl glutathione (Fig. 3). This rank order of potencies for the inhibition of LTC_4 synthase activity mirrors the rank order of potencies for the inhibition of labelling of the 18 kDa polypeptide, and

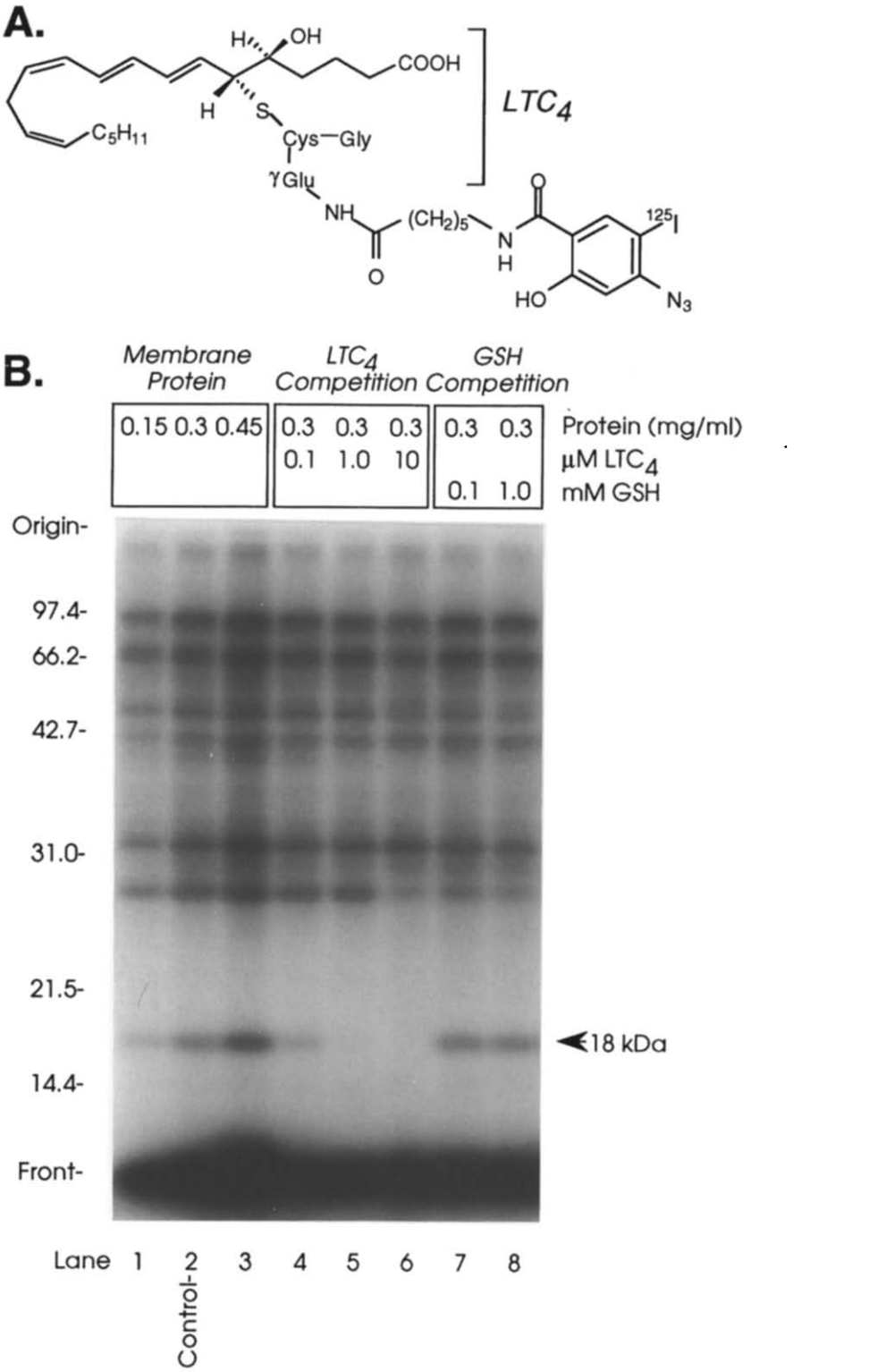


Fig. 1. Photoaffinity labelling of THP-1 cell microsomal membranes by azido[^{125}I]-LTC$_4$. (A) Structure of azido[^{125}I]-LTC$_4$. (B) THP-1 cell microsomal membranes (0.3 mg protein, except lanes 1–3 as indicated) were incubated with 20 pM azido[^{125}I]-LTC$_4$ in either the absence (lanes 1–3) or presence of 0.1, 1.0, 10 μM LTC$_4$ (lanes 4–6) or 0.1, 1.0 mM reduced glutathione (GSH; lanes 7–8) for 30 min at 25°C, cooled then photolysed as described in section 2. Labelled proteins were resolved by SDS gel electrophoresis and visualized by autoradiography. A representative experiment is shown (n = 4). The migration of molecular weight standards ($\times 10^{-3}$) is indicated on the left. A specifically labelled 18 kDa band is indicated by an arrowhead on the right.

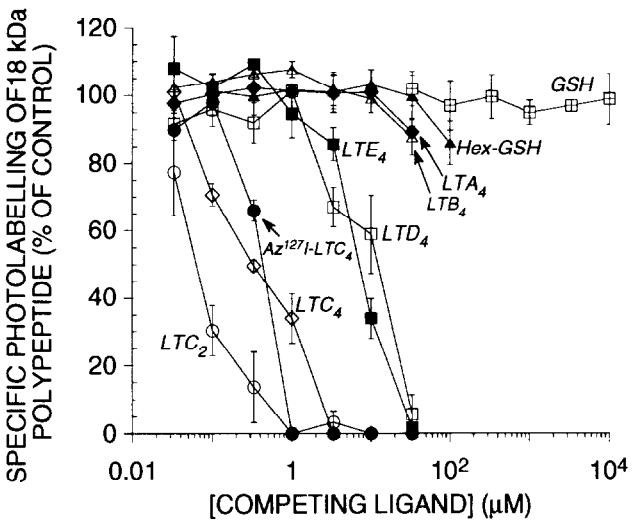


Fig. 2. Competition by leukotrienes and glutathiones for photoaffinity labelling of the 18 kDa microsomal membrane protein by azido[^{125}I]-LTC_4 is dependent on structural relatedness to LTC_4. Incubation mixtures (1 ml each) were prepared containing 0.3 mg of microsomal membrane protein from THP-1 cells, 20 pM azido[^{125}I]-LTC_4 plus varying concentrations of either the indicated leukotrienes, *S*-hexyl glutathione or glutathione. The mixtures were incubated for 30 min at 25°C, cooled then photolysed as described in section 2. The membranes were re-isolated by ultracentrifugation, dissociated in SDS-containing sample buffer and resolved by SDS-gel electrophoresis. Radioactive bands were visualized by autoradiography of the dried gels and the intensities of the photolabelled 18 kDa polypeptide were quantified by laser densitometry. Data are expressed as a percentage of the controls to which no competing ligand was added. Each point represents the mean ± S.E.M. of three separate experiments.

therefore supports the hypothesis that this specifically labelled polypeptide is LTC_4 synthase. The IC_{50} values for inhibition of labelling of the 18 kDa polypeptide were lower overall than the IC_{50} values for inhibition of LTC_4 synthase activity. This was due to the fact that in the former experiment (photolabelling) the added ligand was competing with 20 pM azido[^{125}I]-LTC_4, whereas in the latter experiment (LTC_4 synthase) the added ligand had to compete for binding to LTC_4 synthase in the presence of 40 μM LTA_4 and 10 mM glutathione. Therefore, the rank order of potencies and not the IC_{50} values of the various competitors was compared.

3.3. *Competition of azido[^{125}I]-LTC_4 photolabelling of an 18 kDa membrane polypeptide does not correspond to inhibition of microsomal glutathione S-transferase activity*

THP-1 cell microsomal membranes contain a 17.2 kDa *N*-ethylmaleimide-insensitive glutathione *S*-transferase (microsomal glutathione *S*-transferase) [18]. In order to distinguish the labelled 18 kDa polypeptide from this enzyme, the inhibition profiles of LTC_4, LTC_2, azido[^{127}I]-LTC_4, LTD_4, LTE_4, LTB_4 and *S*-hexyl glutathione for microsomal glutathione *S*-transferase activity were determined. The rank order of potencies was: LTC_2 > (LTC_4 ≈ azido[^{127}I]-LTC_4 ≈ *S*-hexyl glutathione) > LTD_4 > LTE_4 > LTB_4 (Fig. 4). This order was not reflected by the rank order of potencies of these competing ligands for the inhibition of photolabelling of the 18 kDa polypeptide. In particular, *S*-hexyl glutathione was as potent as LTC_4 in inhibiting microsomal glutathione *S*-transferase activity, but was a much less potent competitor than LTC_4 for photolabelling of the 18 kDa polypeptide. In the case of LTC_4 synthase, *S*-hexyl glutathione was also a much less potent inhibitor (IC_{50} value = 10 mM) than the LTC_4 analog, LTC_2 (IC_{50} value = 2.6 μM).

3.4. *LTC_4 synthase activity and a specifically photolabelled 18 kDa polypeptide are both selectively solubilized by the anionic detergent taurocholate, whereas microsomal glutathione S-transferase is not*

Taurocholate is an anionic detergent that has previously been shown to solubilize approximately 80–100% of total LTC_4 synthase activity but not microsomal glutathione *S*-transferase activity from U937 cell microsomal membranes [5]. THP-1 cell microsomal membranes were similarly solubilized with 2% (w/v) taurocholate. Following centrifugation at 200,000 × *g* both the post-taurocholate pellet and the supernatant were assayed for LTC_4 synthase and microsomal glutathione *S*-transferase activity (Fig. 5). Taurocholate effectively solubilized LTC_4 synthase activity from THP-1 cell microsomal membranes but not microsomal glutathione

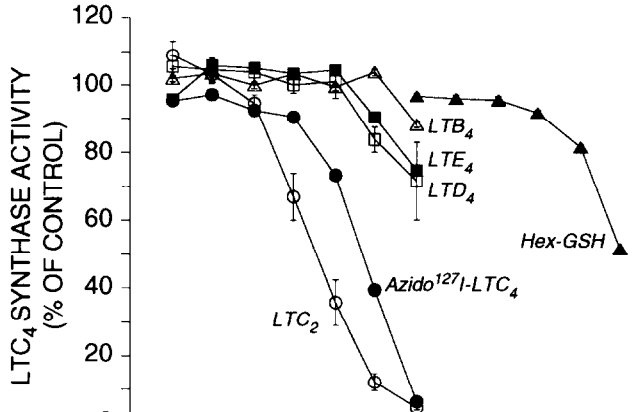


Fig. 3. Inhibition of LTC_4 synthase activity by leukotrienes and *S*-hexyl glutathione. LTC_4 synthase activity in THP-1 cell microsomal membranes was measured in the presence of varying concentrations of LTC_2 (○), azido[^{127}I]-LTC_4 (●), LTD_4 (□), LTE_4 (■), LTB_4 (△) or *S*-hexyl-glutathione (Hex-GSH, ▲) in standard LTC_4 synthase incubation mixtures as described in section 2. Activity is expressed as a percentage of the control to which no competing ligand was added. Each point represents the mean ± S.E.M. of three separate experiments.

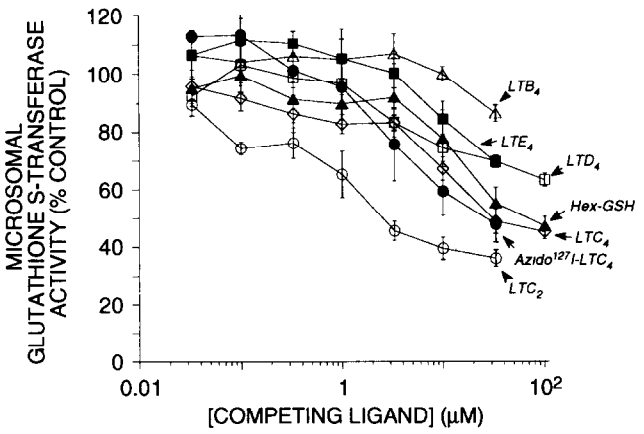


Fig. 4. Inhibition of microsomal glutathione *S*-transferase activity by leukotrienes and *S*-hexyl glutathione. Microsomal glutathione *S*-transferase activity was measured in incubation mixtures containing 0.75 mg of *N*-ethylmaleimide-treated THP-1 cell microsomal membranes and varying concentrations of LTC_2 (○), azido[^{127}I]-LTC_4 (●), LTC_4 (◇), LTD_4 (□), LTE_4 (■), LTB_4 (△) or *S*-hexyl glutathione (Hex-GSH, ▲) as described in section 2. Activity is expressed as a percentage of the controls to which no competing ligand was added. Each point represents the mean ± S.E.M. of three separate experiments.

S-transferase activity. In accordance with this specific labelling of the 18 kDa polypeptide was observed in the 100,000 × *g* THP-1 microsomal fraction (Fig. 6) and 0.1, 1.0 and 10 μM LTC_4 competed for the labelling of the 18 kDa polypeptide (Fig. 6A, lanes 1–4). The specifically-labelled 18 kDa polypeptide, however, was not present in the post-taurocholate pellet (Fig. 6A, lanes 5–8). Instead, a 20 kDa polypeptide was labelled in the post-taurocholate pellet but was not completely competed for by up to 10 μM LTC_4. In an alternative approach, THP-1 cell microsomal membranes were first photolabelled by azido[^{125}I]-LTC_4 then subsequently solubilized with taurocholate (Fig. 6B). The specifically labelled 18 kDa polypeptide was found exclusively in the resulting supernatant fraction, co-fractionating with LTC_4 synthase.

4. DISCUSSION

Recently, we have presented evidence supporting the hypothesis that LTC_4 synthase, an enzyme specifically dedicated to the production of LTC_4 from LTA_4 and reduced glutathione, is a unique enzyme distinct from other known glutathione *S*-transferases [5]. In an attempt to identify putative polypeptides as candidates for being LTC_4 synthase, a novel radioiodinated photoreactive affinity ligand with a structure based on LTC_4 was synthesized. LTC_4 was chosen as the basis of the photoaffinity ligand since it was likely to have high affinity for LTC_4 synthase, as evidenced by the high potency of LTC_2, a structural analogue of LTC_4, for inhibiting LTC_4 synthase activity (IC_{50} = 2.6 μM). Photoreactive derivatives of the substrates of LTC_4 synthase, LTA_4 and glutathione were not synthesized due to their high instability and low specificity, respectively.

In an initial experiment, we had demonstrated that the photoaffinity ligand, azido[^{125}I]-LTC_4, specifically labelled an 18 kDa polypeptide and a 27 kDa polypeptide in U937 cell microsomal membranes [5]. Photolabelling of the 27 kDa polypeptide, which was competed for by both LTC_4 and glutathione, was not observed in THP-1 cell microsomal membranes and this polypeptide therefore does not appear to be involved in LTC_4 biosynthesis. Specific labelling of an 18 kDa polypeptide, however, did occur in THP-1 cell microsomal membranes and was strongly competed for by LTC_4 (>50% at 0.1 μM) but not at all by up to 1 mM glutathione, indicating that this polypeptide had a high affinity for LTC_4 and thus could be LTC_4 synthase or a subunit thereof. This, however, does not exclude the possibility that this polypeptide may be either microsomal glutathione *S*-transferase, a putative LTC_4 receptor or transport protein or γ-glutamyl transpeptidase. To further address the possibility that this polypeptide may be LTC_4 synthase, two experimental approaches were taken.

First, we compared the rank order of potencies of leukotrienes and *S*-hexyl glutathione at competing for the specific labelling of the 18 kDa polypeptide with their ability to inhibit LTC_4 synthase and microsomal glutathione *S*-transferase activities. The rank order of potencies of the various competing ligands for inhibition of the photolabelling of the 18 kDa polypeptide was mirrored exactly by their ability to inhibit LTC_4

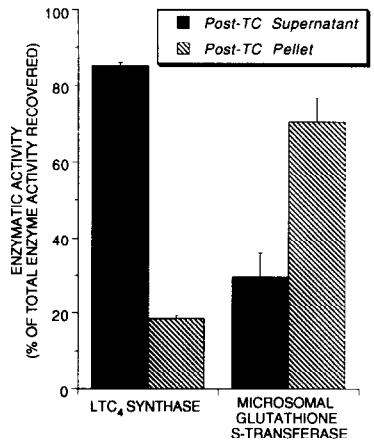
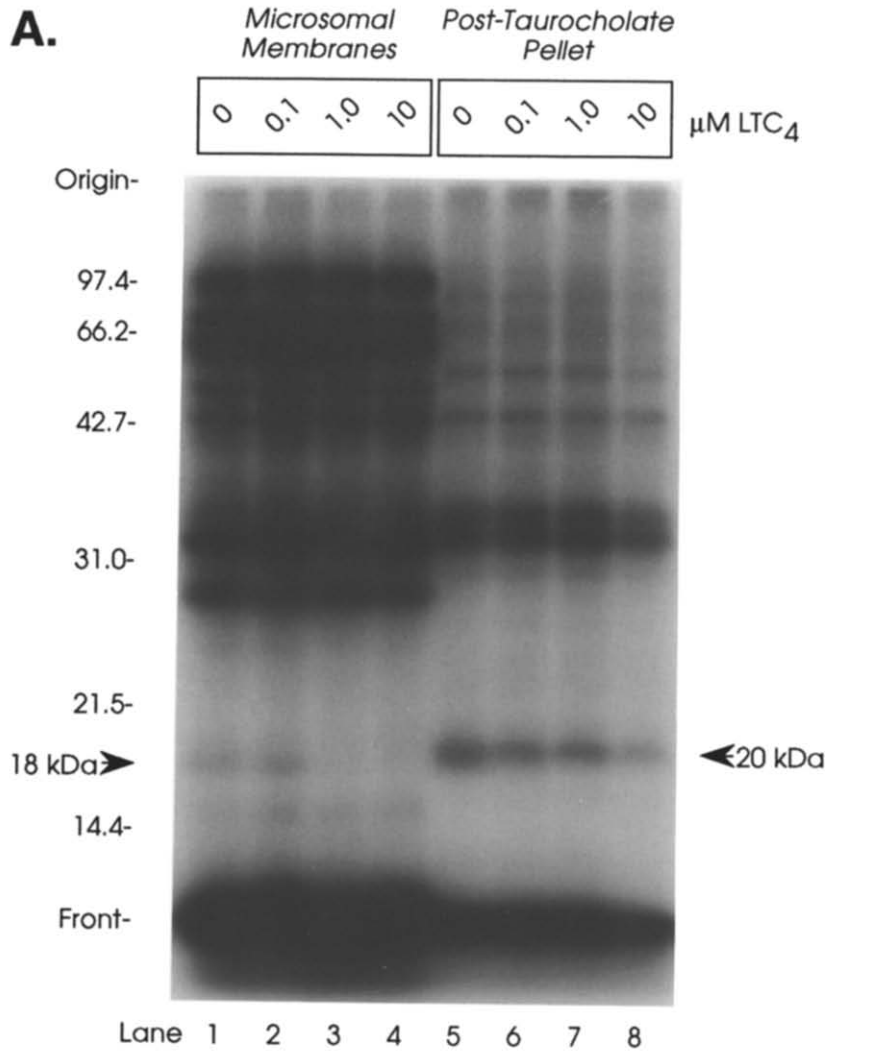


Fig. 5. Differential taurocholate solubilization of microsomal glutathione *S*-transferase and LTC_4 synthase activities from THP-1 cell microsomal membranes. THP-1 cell microsomal membranes at a concentration of 15 mg protein/ml were solubilized by combining them with an equal volume of 4% (w/v) taurocholate and shaking for 30 min at 4°C. Following centrifugation at 200,000 × *g* both the post-taurocholate pellet and the post-taurocholate supernatant were assayed for LTC_4 synthase activity and microsomal glutathione *S*-transferase activity as described in section 2. Activity is expressed as a percentage of total activity recovered. Each bar represents the mean ± S.E.M. of three separate experiments.


A.
Microsomal Membranes
Post-Taurocholate Pellet
0 0.1 1.0 10 0 0.1 1.0 10
μM LTC4
Origin-
97.4-
66.2-
42.7-
31.0-
21.5-
18 kDa
20 kDa
14.4-
Front-
Lane 1 2 3 4 5 6 7 8

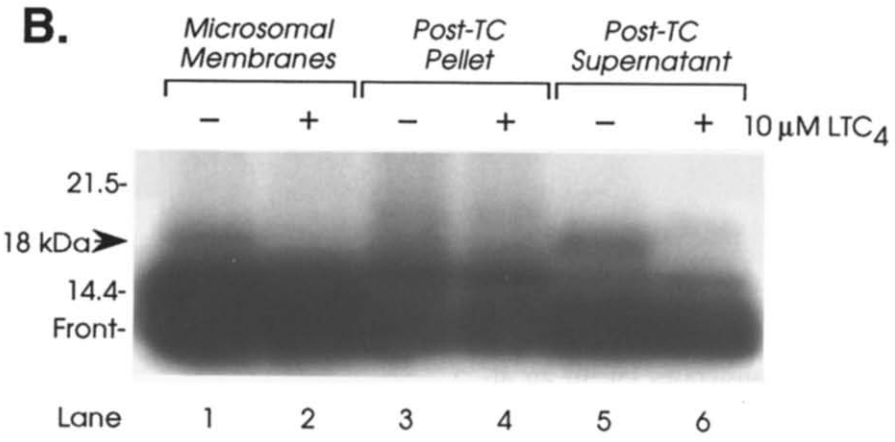

B.
Microsomal Membranes
Post-TC Pellet
Post-TC Supernatant
– + – + – +
10 μM LTC4
21.5-
18 kDa
14.4-
Front-
Lane 1 2 3 4 5 6

Fig. 6. (A) Photoaffinity labelling of THP-1 cell microsomal membranes and the post-taurocholate pellet fraction with azido[^{125}I]-LTC_4. THP-1 cell microsomal membranes (0.3 mg protein/ml; lanes 1–4) and the 200,000 × *g* post-taurocholate pellet fraction (an amount equivalent to 0.3 mg/ml of original microsomal-membrane protein; lanes 5–8) were photoaffinity labelled with 20 pM azido[^{125}I]-LTC_4 in the presence of the indicated concentrations of LTC_4 as described in section 2. The migration of molecular weight standards ($\times 10^{-3}$) are indicated on the left. (B) Taurocholate solubilization of a specifically photolabelled 18 kDa membrane polypeptide. THP-1 cell microsomal membranes were photoaffinity labelled in the absence (lane 1) or presence (lane 2) of 10 μM LTC_4 as described for panel A. The membranes from half the samples (the other halves having been used for lanes 1 and 2) were re-isolated by centrifugation (30 min at 200,000 × *g*) then solubilized with taurocholate. Following further centrifugation (30 min at 200,000 × *g*), the resulting pellet (Post-TC Pellet; lanes 3 and 4) and supernatant (Post-TC Supernatant; lanes 5 and 6) were resolved by SDS-gel electrophoresis and visualized by autoradiography (proteins in the supernatant following taurocholate solubilization were precipitated overnight by acetone at −20°C prior to gel electrophoresis). The relevant section of the resulting autoradiograph, containing the 18 kDa polypeptide (arrowhead), is shown.

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synthase activity, but not microsomal glutathione *S*-transferase. To further support the hypothesis that the 18 kDa photolabelled polypeptide is LTC_4 synthase we have exploited the ability of the anionic detergent taurocholate to solubilize LTC_4 synthase from THP-1 cell microsomal membranes but not microsomal glutathione *S*-transferase. As expected, the specific labelling of the 18 kDa polypeptide was observed in the THP-1 cell microsomal membranes before but not after solubilization with taurocholate. Similarly, the photolabelled 18 kDa polypeptide could be solubilized with taurocholate, consistent with the solubilization of LTC_4 synthase by this detergent.

We therefore conclude that the 18 kDa polypeptide specifically labelled in THP-1 cell microsomal membranes by azido[^{125}I]-LTC_4 is LTC_4 synthase or a subunit of LTC_4 synthase and that this polypeptide is distinct from microsomal glutathione *S*-transferase.

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