Leukotriene C₄ synthase (LTC4S) catalyzes the formation of the proinflammatory lipid mediator leukotriene C₄ (LTC₄). LTC₄ is the parent molecule of the cysteinyl leukotrienes, which are recognized for their pathogenic role in asthma and allergic diseases. Cellular LTC₄S activity is suppressed by PKC-mediated phosphorylation, and recently a downstream p70S6k was shown to play an important role in this process. Here, we identified Ser³⁶ as the major p70S6k phosphorylation site, along with a low frequency site at Thr⁴⁹, using an in vitro phosphorylation assay combined with mass spectrometry. The functional consequences of p70S6k phosphorylation were tested with the phosphomimetic mutant S36E, which displayed only about 20% (20 mol/min/mg) of the activity of WT enzyme (95 mol/min/mg), whereas the enzyme activity of T40E was not significantly affected. The enzyme activity of S36E increased linearly with increasing LT4 concentrations during the steady-state kinetics analysis, indicating poor lipid substrate binding. The Ser³⁶ is located in a loop region close to the entrance of the proposed substrate binding pocket. Comparative molecular dynamics indicated that Ser³⁶ upon phosphorylation will pull the first luminal loop of LTC₄S toward the neighboring subunit of the functional homotrimer, thereby forming hydrogen bonds with Arg¹⁰⁴ in the adjacent subunit. Because Arg¹⁰⁴ is a key catalytic residue responsible for stabilization of the glutathione thiolate anion, this phosphorylation-induced interaction leads to a reduction of the catalytic activity. In addition, the positional shift of the loop and its interaction with the neighboring subunit affect active site access. Thus, our mutational and kinetic data, together with molecular simulations, suggest that phosphorylation of Ser³⁶ inhibits the catalytic function of LTC₄S by interference with the catalytic machinery.

Leukotriene (LT)² C₄ synthase (LTC₄S) catalyzes the formation of LTC₄ by conjugating the unstable allylic epoxide intermediate LT₄A with reduced glutathione (GSH) (1). LTC₄ and its metabolites LTD₄ and LTE₄ are known as cysteinyl leukotrienes (cys-LTs), which are involved in bronchial asthma and allergic inflammatory disorders (1–3). The cys-LTs signal through two G-protein-coupled receptors, denoted CysLT₁ and CysLT₂, to exert their biological functions such as smooth muscle contraction and increased vascular permeability. Several drugs, typified by montelukast, have been developed that specifically target the CysLT₁ receptor (4). Recently, additional G-protein-coupled receptors that recognize cys-LTs have been identified, in particular gpr17 and CysLT3 (5, 6). The increasing complexity of cys-LT signaling has promoted research and drug development efforts targeting the upstream LTC₄S as it catalyzes the committed step in cys-LT biosynthesis (7).

The leukotrienes are derived from arachidonic acid through the 5-lipoxygenase pathway where cytosolic phospholipase A₂, 5-lipoxygenase, and 5-lipoxygenase-activating protein play important roles (7). Protein phosphorylation/dephosphorylation appear to be important regulatory mechanisms for cellular LT biosynthesis. The two key upstream enzymes, cytosolic phospholipase A₂ and 5-lipoxygenase, are regulated through phosphorylation events, apparently for activation (8) and translocation (9) to the nuclear membrane. LTC₄S is yet another enzyme in the 5-lipoxygenase pathway that is regulated by intracellular phosphorylation (10, 11).

LTC₄S is an integral membrane protein that belongs to the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) superfamily whose six human members share structural similarity and form homotrimeric enzymes involved in arachidonic acid metabolism and detoxification (12). For 5-lipoxygenase-activating protein, LTC₄S, and microsomal prostaglandin E synthase-1, crystal structures have been determined (13–16). Besides LTC₄S, no other MAPEG member has been reported to be regulated by phosphorylation. Phosphoregulation of LTC₄S was first recognized when protein kinase C (PKC) activation of leukocytes was found to down-regulate LTC₄S enzyme activity and attenuate cys-LT production (10). When LTC₄S was cloned and sequenced, two putative PKC consensus sites (Ser-Ala-Arg) were found at positions 28 and 111 but were not investigated experimentally (17). In a recent study, it was demonstrated that a ribosomal S6 kinase (p70S6k) is responsible for the observed phosphoregulation of LTC₄S in monocytes (18).

Received for publication, May 3, 2016, and in revised form, June 14, 2016. Published, JBC Papers in Press, June 30, 2016, DOI 10.1074/jbc.M116.735647
The aim of our study was to identify the site(s) on LTC4S that is phosphorylated by p70S6k and investigate the molecular mechanism for suppression of enzyme activity. We identified the sites with an in vitro phosphorylation assay, autoradiography, and mass spectrometry. The effects of phosphorylation on enzyme activity and kinetic properties were investigated using LTC4S with phosphorylation-mimicking mutations. To obtain a mechanistic explanation for our experimental results, we analyzed phosphorylated and unphosphorylated forms of LTC4S by comparative molecular dynamics (MD) and determined the crystal structure of the phosphomimetic S36E mutant.

Results and Discussion

Protein phosphorylation constitutes an essential part of the regulation of almost every aspect of cellular function (19). It has previously been shown that LTC4S is also regulated by phosphorylation, and p70S6k was found to be one of the key players in this process (10, 18). The p70S6k is a serine/threonine-specific kinase localized both in the cytosol and nucleus (20). In this study, we show that the predominant p70S6k phosphorylation site on LTC4S is Ser36.

Prediction of Candidate Phosphorylation Site(s)—Initially, we used an online phosphorylation prediction tool, NetPhos 2.0 Server, to identify the potential phosphorylation site(s) based on sequence information using an artificial neural network method (21). The prediction identified six Ser, two Thr, and one Tyr residue (Fig. 1A). The major inclusion criteria for further characterization of individual residues were their predictive score and location in the structure. Thus, Ser23, Ser28, and Ser57 had the highest probability scores of 0.810 and 0.987, respectively, whereas serine residues at positions 23, 57, 100, and 111 exhibited very low probability scores ranging from 0.002 to 0.159. Because Ser23, Ser28, Ser37, Tyr97, and Ser111 are located within the membrane lipid bilayer (Fig. 1, B and C) with poor accessibility for kinases, we reasoned that the probability that these residues will be phosphorylated is very low. It is common that phosphorylation sites on membrane proteins are located in extramembrane loop regions as in human cardiac Na+ channel Na1.5 (22). In LTC4S, Ser36 is located in a loop region, and Ser100 is found just above the membrane-spanning region within the active site (Fig. 1C). The server also predicted two neighboring Thr residues at positions 40 and 41 with relatively high probability scores and located in the same loop as Ser36 but with their side chains pointing toward the LTC4S trimer interface (Fig. 1C). It should also be noted that a serine/threonine-specific kinase was used for this study, and the relative abundance of the phosphorylated form of Ser, Thr, and Tyr has been reported as a ratio of 1800:200:1 in vertebrate cells (23). Hence, four of nine predicted residues were selected for further analysis, viz. Ser36, Ser100, Thr40, and Thr41.

Identification of Phosphorylation Site(s) on LTC4S by MS/MS Analysis—To identify the phosphorylation sites, in vitro phosphorylated proteins were analyzed by mass spectrometry. The MS analyses of LTC4S achieved up to 32% sequence coverage. The peptides containing Ser23, Thr40, Thr41, and Ser111 could be identified in WT and S36A samples. The peptide covering Ser28, 3DE4VALLAATLGLQAYFSlQISARg, was most likely too large and hydrophobic, whereas the peptide covering Ser100, 100LARPLLYASARhWllVAALGALhFAALhLaALhAAAAAL, was too short and hydrophilic. The peptides covering Ser23, Thr40, and Thr41 were repeatedly found to be phosphorylated. Only singly phosphorylated peptides were detected. In all but two cases, the peptides were phosphorylated at Ser36 and in the other two cases, peptides were phosphorylated at Thr40 (Fig. 2), suggesting that Ser36 is the predominant phosphorylation site. No evidence was found for phosphorylation of Thr41 (supplemental Fig. S1).

To increase the chance of identifying phosphorylation at Ser100, two mutants were used in which the tryptic cleavage site immediately prior to or immediately after the Ser100 had been mutated. The R99H was specifically generated for this study, whereas the mutation R104A was published previously (24). MS analyses identified 39YFQGYHAASLQR104 and 32AFRvPSPLTTGPEFER146 and 32AFRvPSPLTTGPEFER146 where pS is phosphoserine, were repeatedly found to be phosphorylated. Only singly phosphorylated peptides were detected. Hence, we could not find any mass spectrometric evidence for phosphorylation of Ser100.

Analysis of LTC4S Phosphorylation by Autoradiography—It has been shown previously that LTC4S can be phosphorylated

![Phosphorylation of Leukotriene C4 Synthase](image-url)

**FIGURE 1.** Phosphorylation sites in LTC4S predicted with an online tool, NetPhos 2.0 Server. A, the probability scores for each predicted residue. B, the predicted sites are marked in the LTC4S primary structure with the membrane-spanning regions underlined in green. Predicted phosphorylation sites located within the membrane are marked in blue, and the four residues that were selected for further characterization are marked in red. C, predicted serine phosphorylation sites are indicated in the LTC4S trimeric structure (Protein Data Bank code 2UJH). DDM, a detergents molecule that is supposed to bind to the same site as LTα, is indicated in red, and the second substrate GSH is shown in blue within the active site. Residues within the membrane-spanning region are labeled in blue. A magnified view of the active site of LTC4S within the dimer interface is shown to the right in which the DDM molecule has been removed for clarity. The predicted phosphorylation sites (Ser23, Ser100, Thr40, and Thr41) are shown in that region together with the catalytic Arg104 and a bound GSH (blue) molecule.
Phosphorylation of Leukotriene C₄ Synthase

**FIGURE 2.** MS/MS (higher energy C-trap dissociation) analysis of phosphorylated LTC4S. The protein sample was prepared after in vitro phosphorylation with p70S6k followed by trypsin digestion. The figure shows the two identified phosphorylation sites. A, the annotated MS/MS of the peptide [AFRvSPPLTIGPPEFER] with the phosphorylation assigned to Ser36. B, the annotated MS/MS of the peptide [VSPPTIGPPEFER] with the phosphorylation assigned to Thr40. B- and y-ions are indicated in red and blue, respectively, together with neutral loss of the phosphate group. Red bars in the peptide sequence indicate cleavage of the peptide backbone. Neutral loss is indicated by *, pS, phosphoserine; pT, phosphothreonine.

**FIGURE 3.** Effect of DDM, a lipid substrate mimic, on the phosphorylation of LTC4S. Enzyme was subjected to in vitro phosphorylation and analyzed by autoradiography as described under “Experimental Procedures.” The first lane shows the band for p70S6k at around 60 kDa as a control, the second lane shows that phosphorylated LTC4S purified using Triton X-100 as the detergent was detected with a size of 18 kDa, and the third lane shows that phosphorylation was inhibited when LTC4S was purified with DDM as the detergent.

**TABLE 1.** Steady-state kinetic parameters for LTC4S and T40E mutants using LTA₄ and GSH as substrates

| Substrate | LTC4S | T40E |
|-----------|-------|------|
| LTA₄      | 95    | 67   |
| GSH       | 95    | 67   |

**A** and **B** represent the relative abundance of identified phosphorylation sites. Red bars indicate cleavage sites in the peptide sequence. Neutral loss is indicated by *.

**pS**, phosphoserine; **pT**, phosphothreonine.

In vitro and analyzed by autoradiography. A recombinant p70S6k was used for the in vitro phosphorylation assay as described (18). WT LTC4S was incubated with p70S6k in the presence of radioactive [γ-³²P]ATP in a reaction mixture as described under “Experimental Procedures.” The phosphorylation of LTC4S was found to be detergent-specific. Enzyme purified with Triton X-100 as a detergent displayed a radioactive band with an Mr of about 18,000, corresponding to phosphor-ylated LTC4S, whereas protein purified with the detergent n-dodecyl β-D-maltopyranoside (DDM) was not phosphorylated as judged by the faint radioactive band in the autoradiography (Fig. 3). In the crystal structure of LTC4S, a DDM molecule is bound in an intermonomeric, hydrophobic crevice believed to accommodate the substrate LTA₄ (Protein Data Bank code 2UUH; Fig. 1C). Thus, a possible explanation for the prevention of phosphorylation by DDM could be that the detergent molecule blocks the access of the kinases to the phosphorylation site(s). Based on the identification of Ser³⁶ as a phosphorylation site in LTC4S, we generated the mutant S36A and incubated it with p70S6k in the presence of radioactive [γ-³²P]ATP as with WT LTC4S. However, the results from autoradiography were not consistent and did not provide conclusive evidence to show that S36A mutant was not phosphorylated (data not shown). Thus, a radioactive band was consistently observed that could be due to the presence of an additional site(s), possibly Thr⁴⁰, as indicated by MS analysis.

The Mutant S36E Displays Significantly Reduced LTC4S Activity—We next investigated the functional consequences of phosphorylation at Ser³⁶ by replacing this residue with a Glu (S36E), a common mimic of phosphoserine (25). The effects of mutation on LTC4S activity and kinetic parameters with its physiological substrates LTA₄ and GSH were assessed. Likewise, the mutant T40E was constructed and functionally characterized. The specific activity of S36E LTC4S was reduced by nearly 80% (20 µmol/min/mg) as compared with WT LTC4S (95 µmol/min/mg), whereas for the T40E mutant (67 µmol/min/mg), the activity was reduced by 30% (Fig. 4A). The loss of activity of S36E agrees well with the previous observations of down-regulation of LTC4S activity by protein kinases (10, 11). The steady-state kinetic parameters were determined for S36E and T40E mutants using LTA₄ as a substrate to test the effects on substrate binding and catalytic efficiencies (Table 1 and Fig. 4B). Interestingly, the catalytic constants $k_{cat}$ LTA₄ and $K_M$ LTA₄...
due to the lower solubility and stability of LTA₄ compared with that of GSH. The values of $K_m^\text{GSH}$ for S36E and T40E were a bit higher (120 and 220 $\mu M$, respectively) compared with WT enzyme (70 $\mu M$) (Table 1). For T40E, the 3-fold increase in $K_m^\text{GSH}$ may be explained by the results of MD simulations (see below).

**Comparative Molecular Dynamics Suggests That a Phosphorylated Ser³⁶ Interacts with the Catalytic Arg^{104}**—We performed 100-ns molecular dynamics simulations, with snapshots taken every nanosecond, of LTC4S embedded in a lipid bilayer with and without phosphorylation at Ser³⁶ (Ser(P)²⁶). Analysis of the simulation snapshots indicated that the simulated systems generally appeared stable with energies and root mean square deviation values stabilizing after $\sim$5 ns of simulation time (supplemental Fig. S2). The largest movements observed were in terminal regions distant from the site of phosphorylation and active site residues and were thus of limited functional relevance.

To identify functionally relevant changes in the motions of LTC4S upon phosphorylation, the dynamic cross-correlation matrix for each simulation was derived (26). To highlight phosphorylation-related differences in correlated movements, the dynamic cross-correlation matrix of native LTC4S was subtracted from the dynamic cross-correlation matrix of phosphorylated LTC4S and plotted as a heat map (supplemental Fig. S3). Two regions stood out in this map: the intersection between the first luminal loop (Arg³⁴–Pro³¹, including the phosphorylation site) and residues located around the second luminal loop (Ala⁹⁰–Ala¹¹⁰, including the end of helix 3 and beginning of helix 4) of the neighboring subunit and the intersection between the first luminal loop and residues in the beginning of helix 2 (Pro³³–Phe⁶⁰, i.e. right after the first luminal loop) of the neighboring subunit.

The obvious explanation for the changes highlighted by the dynamic cross-correlation matrix analysis is that phosphorylation of Ser³⁶ will pull this region toward spatially neighboring residues that provide complementary binding partners. Not all three subunits of LTC4S behaved exactly the same in this aspect, and the cross-subunit contacts were different for the three interfaces. This could be a real difference or just an effect of the limited time span (100 ns) of the simulation, but it is possible that a longer time course would equilibrate all structures at a more uniform state.

A detailed analysis of all simulation snapshots was performed to identify hydrogen bond (H-bond) partners of Ser³⁶/Ser(P)²⁶ throughout the simulation time course (supplemental Table S2). The most striking interactions were the H-bonds formed between Ser(P)²⁶ and Arg¹⁰⁴ (Fig. 5) that were present in 15–88% (depending on subunit interface) of all snapshots, which should be compared with <0.4% in the unphosphorylated model (Table 2). This implies a high probability for formation of H-bonds between these residues upon phosphorylation. Importantly, Arg¹⁰⁴ is the key catalytic residue responsible for formation of the GSH thiolate anion (24). In addition, phosphorylation led to an increased frequency of H-bonding with Arg³⁶ in the same subunit. However, because Ser³⁶ and Arg³⁶ are very close (both spatially and in the primary sequence), this interaction is not likely to have significant functional effects. In
**Phosphorylation of Leukotriene C4 Synthase**

**TABLE 1**

| Enzyme   | $K_m$ (μM) | $K_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) |
|----------|------------|----------------------|----------------------------------|
| WT       | 70 ± 18    | 19 ± 1.0             | (2.7 ± 0.6) × 10$^2$             |
| T40E     | 220 ± 74   | 12 ± 1.1             | (0.5 ± 0.1) × 10$^2$             |
| S36E     | 120 ± 20   | 3 ± 0.1              | (0.26 ± 0.03) × 10$^2$           |

---

**TABLE 2**

| Interface  | LTC4S + GSH | [Ser(P)$^{36}$]LTC4S + GSH | Probability of H-bond between Ser$^{36}$/Ser(P)$^{36}$ and Arg$^{104}$ |
|------------|-------------|-----------------------------|--------------------------------------------------|
| A-B        | 0.1         | 87.7                        | %                                               |
| B-C        | 0.4         | 17.1                        |                                                 |
| C-A        | 0           | 14.5                        |                                                 |

---

**FIGURE 5.** MD simulation of LTC4S and [Ser(P)$^{36}$]LTC4S. Shown is a representation of 45 snapshots of MD simulation performed with LTC4S (left) and [Ser(P)$^{36}$]LTC4S (right). Left, a ribbon representation of subunits A and B with Ser$^{36}$ (red) of subunit A and Arg$^{104}$ (blue) of subunit B shown for LTC4S. Right, the same representation for phosphorylated LTC4S with Ser(P)$^{36}$ (yellow-red) of subunit A interacting with Arg$^{104}$ (blue) of subunit B.

---

addition, Arg$^{34}$ is not known to be important for catalysis or substrate binding.

Molecular dynamic simulation was also performed with a phosphorylated Thr$^{40}$ (Thr(P)$^{40}$) of LTC4S, which revealed a markedly pronounced interaction between Thr(P)$^{40}$ and Arg$^{51}$ of the neighboring subunit (data not shown). Arg$^{51}$ is an anchoring residue for the carboxyl group of the GSH (27) and was found to be not essential for catalysis (24), and its interaction with Thr(P)$^{40}$ may explain the increased $K_m$ of T40E LTC4S as compared with WT enzyme (Table 1).

**Crystal Structure of S36E LTC4S**—A crystal structure of the mutant S36E was determined at 3-Å resolution (Protein Data Bank code 5HV9; supplemental Table S3), which indicates that the mutant was correctly folded and that the loss of activity is not due to the misfolded protein. The overall structure was similar to native LTC4S (Protein Data Bank code 2UUI) with one monomer in the asymmetric unit. Nine residues (Met$^{-5}$ to Glu$^{4}$) and three residues (Pro$^{148}$ to Ala$^{150}$) were removed in the present structure at the N and C termini, respectively, because of the lack of density. Strong positive density was observed for the thiol group of GSH in the $F_o - F_s$ difference map at 3σ after initial refinement. Contouring $F_o - F_s$ map to 2.5σ resulted in a positive curved density representing GSH at the active site. The GSH molecule was fitted, and complete density was achieved at 1σ of $2F_o - F_s$ map. A minor positional shift for the thiol group of GSH accompanied by an increased distance (0.7 Å) to Arg$^{104}$ was observed at the active site (Fig. 6) compared with the structure of WT LTC4S with bound GSH (Protein Data Bank code 2UUH), which corroborates the results of MD simulations and provides a structural basis for the reduced catalytic activity displayed by S36E. Moreover, no positive density for a DDM molecule (mimics LTA$_4$ in 2UUH) was detected in the putative active site of the S36E structure, suggesting that lipid substrate binding is compromised.

**Phosphomimetic Mutant S36E Is Less Sensitive to a Synthetic Inhibitor**—A recent study suggests that protein phosphorylation may affect drug inhibitor binding to target proteins (28). They classified two types of mechanisms by which phosphorylation affects drug efficacy. In one type, phosphorylation inhibits both drug binding and target activity, whereas in the other type, phosphorylation inhibits drug binding while increasing target activity (28). Here, we used the phosphomimetic mutant S36E to test the effect of phosphorylation on inhibitor binding to LTC4S. In a previous study, a nanomolar inhibitor, TK04, was used to probe the inhibition of human and mouse LTC4S (29). The inhibitor (0.05–15 μM) was incubated with the same amount of WT and S36E enzyme (0.1 μg) at a fixed concentration of LTA$_4$ (20 μM) and GSH (5 mM). TK04 was less efficient in inhibiting the S36E mutant ($IC_{50}$ = 389 ± 69 nm) at low inhibitor concentrations (<IC$_{50}$) compared with WT enzyme ($IC_{50}$ = 211 ± 52 nm), whereas at higher concentrations there were no significant differences (Fig. 7). The observed behavior with the S36E mutant indicates that phosphorylation at Ser$^{36}$ may interfere with TK04 binding, thus effecting the inhibitor potency. TK04 was proposed to occupy the LTA$_4$ binding site and interact with Arg$^{104}$ based on molecular docking results (30). The efficiency of the TK04 inhibitor at concentrations around or below IC$_{50}$ seems reduced with the phosphomimetic S36E mutant, which is yet an indication that phosphorylation affects lipid substrate binding.

**Concluding Remarks**—We have used MS/MS analysis and site-directed mutagenesis to identify Ser$^{36}$ as a predominant and functionally important p70S6k phosphorylation site in LTC4S. An alternative site, Thr$^{40}$, was also identified at low frequency and was found to be of marginal functional relevance. Results of comparative MD simulations and x-ray crystallography indicate that phosphorylation of Ser$^{36}$ acts by disturbing the catalytic action of Arg$^{104}$ and reducing substrate access to the active site (Fig. 8). P70S6K-dependent LTC4S phosphorylation was demonstrated in monocytes (18). Given the complexity of PKC-mediated protein phosphory-
Phosphorylation of Leukotriene C₄ Synthase

Experimental Procedures

Chemicals, Reagents, and Enzymes—GSH, 2-mercaptoethanol, imidazole, Tris base, NaCl, Triton X-100, and sodium deoxycholate were obtained from Sigma. DDM was purchased from Anatrace. [γ-³²P]ATP was ordered from PerkinElmer Life Sciences. p70S6k, Mg-ATP mixture, and kinase assay dilution buffer were purchased from Merck-Millipore. Pepsin, trypsin, and chymotrypsin were obtained from Promega (Madison, WI). Protease and phosphatase inhibitor mixtures were from Sigma and Thermo Scientific, respectively. LTA₄ was purchased from Cayman as LTA₄ methyl ester and further converted to LTA₄ by saponification as described previously (31).

Site-directed Mutagenesis—Site-directed mutagenesis was performed according to the QuikChange protocol (Stratagene, La Jolla, CA). WT LTC4S cDNA with an additional N-terminal His₆ tag was subcloned into pPICZA (Invitrogen) vector and used as a template to generate all other mutants using the primers listed in supplemental Table S1. To check the mutations and other nonspecific changes, the protein-coding part of the plasmid vectors was verified by DNA sequencing from SEQLAB, Göttingen, Germany.

Protein Expression and Purification—WT LTC4S and all mutants were expressed in yeast Pichia pastoris, and the purification was performed in a single step on an S-hexylglutathione-agarose column (24). The protein used for the in vitro phosphorylation assay was desalted on a PD-10 column to exchange buffer with 20 mM HEPES (pH 7.4) containing 0.1 mM dithiothreitol (DTT) and 0.05% Triton X-100. Conversely, the protein prepared for crystallization was further purified on a Superdex 200 16/60 (GE Healthcare) with 20 mM Tris (pH 8.0), 100 mM NaCl, 0.03% DDM (w/v), and 0.5 mM tris(2-carboxyethyl)phosphine. Protein concentration was determined by the Lowry method (32) or the Pierce™ BCA Protein Assay kit followed by SDS-PAGE on a Phast system (GE Healthcare).

In Vitro Phosphorylation Assay and Autoradiography—The in vitro phosphorylation assay was performed as described (18). Briefly, purified LTC4S (2 μg) was incubated with [γ-³²P]ATP (0.8 μCi) or 0.5 mM cold ATP premixed in magnesium acetate/ATP mixture (2.5 mM HEPES (pH 7.4), 0.5 mM ATP, and 50 mM magnesium acetate) together with 0.1 μg of p70S6k (T412E; Millipore) in a 5× reaction buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT; Upstate, Millipore) for 30 min at 30 °C. The proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes. The in vitro phosphorylated bands were detected by autoradiography using high performance films (Amersham Biosciences Hyperfilm™ MP, GE Healthcare).

Identification of Phosphorylation Site(s) by Mass Spectrometry—In vitro phosphorylated WT LTC4S was separated using one-dimensional PAGE and stained with Coomassie Brilliant Blue. The protein band was excised manually and digested in gel using a MassPREP robotic protein-handling system (Waters, Millford, MA) according to the manufacturer’s instructions. After reduction with DTT and alkylation with iodoacetamide, the proteins were digested with 0.3 μg of trypsin (modified; Promega) in 50 mM ammonium bicarbonate for 5 h at 40 °C. The tryptic peptides were extracted with 1% formic acid and 2% acetonitrile followed by 50% acetonitrile twice. Phosphopeptides were further enriched on a PhosphoCatch™ microspin column (Promega) loaded with a combination of zirconium and titanium oxide resins.

The in vitro phosphorylated samples were digested in solution as described previously (33). The proteins were digested overnight in 50 mM ammonium bicarbonate, 30% DMSO, and trypsin (at a ratio of 1:20 trypsin:protein; modified) at 37 °C.

Peptides from both in-gel and in-solution digestion were desalted using ZipTips (C₁₈; Merck Millipore Ltd., Ireland) followed by separation using online nano-scale LC-MS/MS (reversed phase C₁₈) and analyzed on an LTQ Velos Orbitrap electron transfer dissociation mass spectrometer (Thermo Fisher Scientific, Germany). A 40-min gradient of buffer A and B (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) was used for the separation as follows: 5–30% B in 35 min followed by 30–95% B in 5 min. The flow rate was 300 nL/min. MS spectra were acquired at a resolution of 60,000 followed by fragmentation of the five most intense peaks. The peptides were
either fragmented by only collision-induced dissociation or by higher energy C-trap dissociation first followed by electron transfer dissociation of the same precursor.

Mass lists extracted by Raw2MGF v2.1.3 (34) were searched against the Swiss-Prot database (downloaded February 7, 2014) using Mascot search engine v2.3.02 (Matrix Science Ltd., London, UK). The WT and mutant constructs used in this study were also added to the database. The following parameters were used for the database searching: tryptic digestion (with a maximum of two miscleavages); carbamidomethylation (Cys) as fixed modification; oxidation (Met), pyroglutamate (Gln), deamidation (Asn/Gln), and phosphorylation (Ser/Thr/Tyr) as variable modifications; 10 ppm as precursor tolerance; and 0.25 Da as fragment tolerance.

Enzyme Activity Assay—Formation of the enzyme product LTC₄ was measured by UV absorbance at 280 nm using high performance liquid chromatography (HPLC) as described earlier (24). Enzyme (0.1 g) together with GSH (5 mM) was incubated in the presence of LTA₄ (30 μM) for 15 s at room temperature in a 100-μl reaction volume and terminated by adding 200 μl of methanol to the reaction mixture followed by the addition of prostaglandin B₂ as an internal standard. The reaction buffer contained 25 mM Tris-HCl (pH 7.8), 0.05% Triton X-100, and 5 mM 2-mercaptoethanol. The steady-state kinetic parameters were determined by varying the LTA₄ concentration from 10 to 120 μM while keeping the GSH concentration at 5 mM. Alternatively, the GSH concentration was varied between 0 and 15 μM while keeping the LTA₄ concentration constant at 20 μM in the presence of 5 mM GSH and 0.1 μg of enzyme. The data were analyzed with non-linear regression using GraphPad Prism to extract all the kinetic parameters. The k₅₆/Kₘ was determined using RFFIT in SIMFIT.

Enzyme Inhibition Assay—Inhibition studies of WT and S36E LTC₄S with the inhibitor TK04 were performed using an assay in a 96-well format as described earlier (29) to determine the inhibition parameters. The TK04 concentration was varied between 0 and 15 μM while keeping the LTA₄ concentration constant at 20 μM in the presence of 5 mM GSH and 0.1 μg of enzyme. The data were analyzed with non-linear regression using GraphPad Prism to calculate the IC₅₀ values.

Molecular Dynamics—For molecular dynamics, the software YASARA Structure was used (35). The crystal structures of human LTC₄S with bound GSH (Protein Data Bank code 2UUH with His tag removed and stripped of all water and ligands other than GSH) and with modeled hydrogens in H-bond-optimized positions (36) were used in the simulations. The trimeric form of the enzyme was generated from the information in the Protein Data Bank file. Phosphorylation of Ser³⁶ and Thr⁴⁰ was manually built. The AMBER03 force field (37) was used, and force field parameters for non-standard protein residues were generated with YASARA’S built-in AutoSMILES algorithm (38, 39).

By using the default protocol for simulation of membrane proteins in YASARA (YASARA macro for running a molecular dynamics simulation of a membrane protein with normal or fast speed), the protein was embedded in a phosphatidylethanolamin lipid bilayer and slowly adapted (including deletion of clashing protein-membrane residues and energy minimization) to accommodate the inserted protein. Subsequently, water molecules were added, pKa values were assigned, the whole simulation cell was neutralized by addition of sodium (at locations of lowest electrostatic potential) and chloride (at locations of highest electrostatic potential) ions to a final concentration of 154 mM, and the system was energy-minimized (40).
The final simulation cell extended 15 Å from the protein in the plane of the membrane and 10 Å in the perpendicular direction. The final simulation cell had dimensions of 105.14 × 91.61 × 98.52 Å and contained 432 protein residues, three GSH residues, 266 lipid residues, and 17,908 solvent residues (water, Na⁺, and Cl⁻).

Simulations were run for 100 ns at constant pressure (1 bar) at 298 K (isothermal-isobaric ensemble), and snapshots were taken every 100 ps. To allow the membrane to equilibrate, water molecules were prevented entry to the lipid bilayer during the initial 250 ps of simulation.

Temperature control was achieved during simulation by a Berendsen thermostat with the scaling factor calculated from the time-averaged temperature (41, 42). For pressure control, the simulation cell was rescaled during simulation using the YASARA option with combined, time-averaged, density and manometer control (41). The integration time step was 4 fs for intermolecular forces and 2 fs for intramolecular forces. Covalent bonds and angles involving hydrogens were constrained using the YASARA version of the LINCS algorithm (41, 43). An 8-Å cutoff was used for van der Waals and long range electrostatic interactions, which were calculated using the particle mesh Ewald method (44).

Crystallization—S36E protein was supplemented with 1 mM GSH and concentrated to 4.5 mg/ml. Crystallization of the mutant was carried out as described (45). Briefly, the concentrated protein was mixed with reservoir solution containing 0.1 M sodium cacodylate (pH 6.5), 0.2 M NaCl, and 2 M ammonium sulfate. The sitting drop vapor diffusion method was performed at room temperature with a protein to reservoir ratio of 1:1. The matured crystals were harvested after 5 days and cryoprotected with reservoir solution containing 15% (v/v) glycerol.

Data Collection and Structure Determination—Cryoprotected S36E crystals were exposed to x-ray for data collection at 100K. 3000 frames were collected with 0.5° oscillation and 20-s exposure per frame. The data set was processed, and reflection output was converted using the XDS package. Molecular replacement was performed using PHASER with WT structure (Protein Data Bank code 2UUI) after removing the coordinates corresponding to heteroatoms. The output coordinates were refined initially with 20 cycles of rigid body refinement followed by restrained refinement in REFMAC. The Coot program was used for model building, and the figures were generated using PyMOL software.

Author Contributions—S. A. performed the experiments and prepared the preliminary version of the manuscript. A. J. Y. performed the MS/MS analysis and contributed to the manuscript. M. T. performed the structure determination and contributed to the manuscript. F. T. performed the MD simulations and contributed to the manuscript. T. B., R. Z., A. W., and A. R.-M. supervised and contributed to the manuscript preparation. J. Z. H. directed the research and contributed to the manuscript. All authors contributed to the analysis of the results and approved the final version of the manuscript.

Acknowledgments—We thank Julia Esser for providing help with the phosphorylation assay, Carina Palmberg for running preliminary MS/MS experiments, and Michaela Mårback for assistance in the kinetic experiments. We thank Diamond Light Source, UK for providing synchrotron radiation facility and assistance at beam line i04.

References
1. Lam, B. K. (2003) Leukotriene C₄ synthase. Prostaglandins Leukot. Essent. Fatty Acids 69, 111–116
2. Austen, K. F. (2008) The cysteinyl leukotrienes: where do they come from? What are they? Where are they going? Nat. Immunol. 9, 113–115
3. Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 220, 568–575
4. Drazen, J. M., Israel, E., and O’Byrne, P. M. (1999) Treatment of asthma with drugs modifying the leukotriene pathway. N. Engl. J. Med. 340, 197–206
5. Maekawa, A., Balestrieri, B., Austen, K. F., and Kanaoka, Y. (2009) GPR17 is a negative regulator of the cysteinyl leukotriene 1 receptor response to leukotriene D₄. Proc. Natl. Acad. Sci. U.S.A. 106, 11685–11690
6. Kanaoka, Y., Maekawa, A., and Austen, K. F. (2013) Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E₄ ligand. J. Biol. Chem. 288, 10967–10972
7. Haeggström, J. Z. and Funk, C. D. (2011) Lipoxigenase and leukotriene pathways: biochemistry, biology, and roles in disease. Chem. Rev. 111, 5866–5898
8. Gijon, M. A., Spencer, D. M., Kaiser, A. L., and Leslie, C. C. (1999) Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A2. J. Cell Biol. 145, 1219–1232
9. Lepley, R. A., Muskardin, D. T., and Fitzpatrick, F. A. (1996) Tyrosine kinase activity modulates catalysis and translocation of cellular 5-lipoxygenase. J. Biol. Chem. 271, 6179–6184
10. Ali, A., Ford-Hutchinson, A. W., and Nicholson, D. W. (1994) Activation of protein kinase C down-regulates leukotriene C₄ synthase activity and attenuates cysteinyl leukotriene production in an eosinophilic substrain of HL-60 cells. J. Immunol. 153, 776–788
11. Gupta, N., Nicholson, D. W., and Ford-Hutchinson, A. W. (1999) Demonstration of cell-specific phosphorylation of LTC₄ synthase. FEBS Lett. 449, 66–70
12. Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., and Perras, B. (1999) Common structural features of MAPEG—a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. Protein Sci. 8, 689–692
13. Martinez Molina, D., Wetterholm, A., Kohl, A., McCarthy, A. A., Niegowski, D., Ohlson, E., Hammarberg, T., Eshagi, H., Haeggström, J. Z., and Nordlund, P. (2007) Structural basis for synthesis of inflammatory mediators by human leukotriene C₄ synthase. Nature 448, 613–616
14. Sjögren, T., Nord, J., Ek, M., Johansson, P., Liu, G., and Geschwindner, S. (2013) Crystal structure of microsomal prostaglandin E₂ synthase provides insight into diversity in the MAPEG superfamily. Proc. Natl. Acad. Sci. U.S.A. 110, 3806–3811
15. Ferguson, A. D., McKeever, B. M., Xu, S., Wisniewski, D., Miller, D. K., Yamin, T. T., Spencer, R. H., Chu, L., Ujjainwalla, F., Cunningham, B. R., Evans, J. F., and Becker, J. W. (2007) Crystal structure of inhibitor-bound human 5-lipoxygenase-activating protein. Science 317, 510–512
16. Ago, H., Kanaoka, Y., Irikura, D., Lam, B. K., Shimamura, T., Austen, K. F., and Miyano, M. (2007) Crystal structure of a human membrane protein involved in cysteinyl leukotriene biosynthesis. Nature 448, 609–612
17. Lam, B. K., Penrose, J. F., Freeman, G. J., and Austen, K. F. (1994) Expression cloning of a cDNA for human leukotriene C₄ synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A₄. Proc. Natl. Acad. Sci. U.S.A. 91, 7663–7667
18. Esser, J., Gehrman, U., Salvador, M. D., Wetterholm, A., Haeggström, J. Z., Samuelsson, B., Gabrielson, S., Scheunias, A., and Rädmark, O. (2011) Zymosan suppresses leukotriene C₄ synthase activity in differentiating monocytes: antagonism by aspirin and protein kinase inhibitors. FASEB J. 25, 1417–1427
19. Cohen, P. (2002) The origins of protein phosphorylation. Nat. Cell Biol. 4, E127–E130
20. Fleckenstein, D. S., Dirks, W. G., Drexl, H. G., and Quentmeier, H. (2003) Tumor necrosis factor receptor-associated factor (TRAF) 4 is a new binding partner for the p70S6 kinase. Leuk. Res. 27, 687–694
21. Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-
Phosphorylation of Leukotriene C₄ Synthase

Based prediction of eukaryotic protein phosphorylation sites. J. Mol. Biol. 294, 1351–1362

Marionneau, C., and Abriel, H. (2015) Regulation of the cardiac Na⁺ channel NaV1.5 by post-translational modifications. J. Mol. Cell. Cardiol. 82, 36–47

Mann, M., Ong, S. E., Gernberg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol. 20, 261–268

Rinaldo-Matthis, A., Wetterholm, A., Martinez Molina, D., Holm, J., Niegowski, D., Ohlson, E., Nordlund, P., Morgenstern, R., and Haeggström, J. Z. (2010) Arginine 104 is a key catalytic residue in leukotriene C4 synthase. J. Biol. Chem. 285, 40771–40776

McSorley, T., Ort, S., Hazra, S., Lavié, A., and Konrad, M. (2008) Mimicking phosphorylation of Ser-74 on human deoxycytidine kinase selectively increases catalytic activity for dC and dC analogues. FEBS Lett. 582, 720–724

Ichiye, T., and Karplus, M. (1991) Collective motions in proteins: a covariance analysis of atomic fluctuations in molecular dynamics and normal mode simulations. Proteins 11, 205–217

Rinaldo-Matthis, A., Ahmad, S., Wetterholm, A., Lachmann, P., Morgenstern, R., and Haeggström, J. Z. (2012) Pre-steady-state kinetic characterization of thiolate ion formation in human leukotriene C₄ synthase. Biochemistry 51, 848–856

Smith, K. P., Gifford, K. M., Waitzman, J. S., and Rice, S. E. (2015) Survey of phosphorylation near drug binding sites in the Protein Data Bank (PDB) and their effects. Proteins 83, 25–36

Niegowski, D., Kleinschmidt, T., Ahmad, S., Qureshi, A. A., Màrback, M., Rinaldo-Matthis, A., and Haeggström, J. Z. (2014) Structure and inhibition of mouse leukotriene C₄ synthase. PLoS One 9, e96763

Kleinschmidt, T. K., Haraldsson, M., Basavarajappa, D., Lundeberg, E., Thulasingam, M., Ekoff, M., Fauland, A., Lehmann, C., Kahnt, A. S., Lindbom, L., and Haeggström, J. Z. (2015) Tandem benzophenone amino pyridines, potent and selective inhibitors of human leukotriene C₄ synthase. J. Pharmaceut. Exp. Ther. 355, 108–116

Wetterholm, A., Medina, J. F., Rådmark, O., Shapiro, R., Haeggström, J. Z., Vallee, B. L., and Samuelsson, B. (1991) Recombinant mouse leukotriene A₄ hydrolase: a zinc metalloenzyme with dual enzymatic activities. Biochim. Biophys. Acta 1080, 96–102

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275

Ytterberg, A. J., Peltier, J. B., and van Wijk, K. J. (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiol. 140, 984–997

Lyuvinisky, Y., Yang, H., Rutishauser, D., and Zubarev, R. A. (2013) In silico instrumental response correction improves precision of label-free proteomics and accuracy of proteomics-based predictive models. Mol. Cell. Proteomics 12, 2324–2331

Krieger, E., Darden, T., Nabuurs, S. B., Finkelstein, A., and Vriend, G. (2004) Making optimal use of empirical energy functions: force-field parameterization in crystal space. Proteins 57, 678–683

Krieger, E., Dunbrack, R. L., Jr., Hooft, R. W., and Krieger, B. (2012) Assignment of protonation states in proteins and ligands: combining pKₐ prediction with hydrogen bonding network optimization. Methods Mol. Biol. 819, 405–421

Duan, Y., Wu, C., Chowdhury, S., Lee, M. C., Xiong, G., Zhang, W., Yang, R., Cieplak, P., Luo, R., Lee, T., Caldwell, J., Wang, J., and Kollman, P. (2003) A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. J. Comput. Chem. 24, 1999–2012

Wang, J., Wolf, R. M., Caldwell, J. W., Kollman, P. A., and Case, D. A. (2004) Development and testing of a general amber force field. J. Comput. Chem. 25, 1157–1174

Jakalian, A., Jack, D. B., and Bayly, C. I. (2002) Fast, efficient generation of high-quality atomic charges. AM1-BCC model: II. Parameterization and validation. J. Comput. Chem. 23, 1623–1641

Krieger, E., Nielsen, J. E., Spronk, C. A., and Vriend, G. (2006) Fast empirical pKₐ prediction by Ewald summation. J. Mol. Graph. Model. 25, 481–486

Krieger, E., and Vriend, G. (2015) New ways to boost molecular dynamics simulations. J. Comput. Chem. 36, 996–1007

Berendsen, H. J. C., Postma, J. P. M., Van Gunsteren, W. F., DiNola, A., and Haak, J. R. (1984) Molecular dynamics with coupling to an external bath. J. Chem. Phys. 81, 3684–3690

Hess, B., Bekker, H., Berendsen, H. J. C., and Fraaije, J. G. E. M. (1997) LINCS: a linear constraint solver for molecular simulations. J. Comput. Chem. 18, 1463–1472

Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) A smooth particle mesh Ewald method. J. Chem. Phys. 103, 8577–8593

Niegowski, D., Kleinschmidt, T., Olsson, U., Ahmad, S., Rinaldo-Matthis, A., and Haeggström, J. Z. (2014) Crystal structures of leukotriene C₄ synthase in complex with product analogs: implications for the enzyme mechanism. J. Biol. Chem. 289, 5199–5207