Regulation of Leukotriene A₄ Hydrolase Activity in Endothelial Cells by Phosphorylation

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Endothelial cells contain leukotriene (LT) A₄ hydrolase (LTA-H) as detected by Northern and Western blotting, but several studies have been unable to detect the activity of this enzyme. Since LTA-H could play a key role in determining what biologically active lipids are generated by activated endothelium during the inflammatory process, we studied possible mechanisms by which this enzyme may be regulated. We find that LTA-H is phosphorylated under basal conditions in human endothelial cells and in this state does not exhibit epoxide hydrolase activity (i.e. conversion of LTA₄ to LTB₄). LTA-H purified from endothelial cells is efficiently dephosphorylated by incubation with protein phosphatase-1 in the presence of an LTA-H peptide substrate and not at all in the absence of substrate. Under conditions that lead to dephosphorylation, protein phosphatase-1 activates the epoxide hydrolase activity of LTA-H. Using peptide mapping and site-directed mutagenesis, we have identified serine 415 as the site of phosphorylation of LTA-H by a kinase found in endothelial cell cytosol. In parallel, we have studied a human lung carcinoma cell line that expresses active LTA-H. Although these cells have cytosolic kinases that phosphorylate recombinant LTA-H, they do not target serine 415 and thus do not inhibit LTA-H activity. We believe that LTA-H is regulated in intact cells by a kinase/phosphatase cycle and further that the kinase in endothelial cells specifically recognizes and phosphorylates a regulatory site in the LTA-H.

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The abbreviations used are: LT, leukotriene; LTA₄ and LTB₄, leukotriene A₄ and B₄, respectively; LTA-H, leukotriene A₄ hydrolase; PMNL, polymorphonuclear leukocyte; EC, endothelial cell(s); HUVEC, human umbilical vein endothelial cell(s); DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase HPLC; PCR, polymerase chain reaction; Bia-Tris, bia[2-hydroxyethyl]iminotris(hydroxymethyl)methane; PGB₂, prostaglandin B₂.

Immunohistostoechymal studies have suggested that the enzyme is widely spread, if not ubiquitous (11, 12). It is interesting to note, however, that 5-lipoxygenase, the enzyme that generates LTA₄, has a much more restricted distribution than LTA-H, and thus, many more cell types appear to have the ability to metabolize LTA₄ than have the capacity to synthesize it. This has led several groups to study the transfer of LTA₄ from PMNL to cells that lack the 5-lipoxygenase including erythrocytes (13, 14), platelets (15, 16), endothelial cells (16–19), and vascular smooth muscle (20). Similar transcellular metabolism has been demonstrated in perfused organ studies, suggesting a role in cardiac and pulmonary function (21–24).

We have focused on endothelial cells (EC), because the interaction of PMNL with the endothelium is the critical initiating step of the inflammatory process and because LTB₄ induces PMNL accumulation in the extravascular space and initiates the PMNL-dependent component of edema formation. Our earliest studies demonstrated that porcine aortic endothelial cells could metabolize LTA₄ to LTC₄ but not to LTB₄ (17), and later work extended these observations to other endothelial cell types including human umbilical vein endothelial cells (HUVEC) (25). If EC contain active LTA-H, the presentation of LTA₄ by activated, marginal PMNL could provide an important source of substrate for EC LTB₄ synthesis. Unregulated, this pathway could lead to uncontrolled synthesis of this bioactive lipid and result in inappropriate accumulation of PMNL and other manifestations of inflammation. Therefore, it has been proposed that EC may have a mechanism for regulating the activity of LTA-H (19). It is this question that we address in the present report.

EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells were cultured as previously reported (25). Cells were maintained in M-199 supplemented with 20% fetal bovine serum, endothelial cell growth supplement (3 µg/ml), and heparin (2 units/ml) in Petri dishes coated with 0.2% gelatin. Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. HUVEC were used in first to third passages.

EA.hy 926 and A549 cells were kindly provided by Dr. Cora Jean Edgell (University of North Carolina, Chapel Hill, NC). EA.hy 926 is a hybridoma line formed by the fusion of HUVEC with the human lung carcinoma cell line, A549. These cells have proven to be a good model for HUVEC in many studies (26, 27). EA.hy 926 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (28). A549 cells were cultured in the presence of 6-thioguanine (0.1 µM) in DMEM supplemented with 5% fetal bovine serum as described earlier (28).

Northern and Western Blots—Proteins, separated on 7.5% gels by SDSPAGE, were transferred to nitrocellulose according to the method of Towbin (29). Immunostaining of the blots was done with commercially available alkaline phosphatase-linked reagents. The primary antibodies were rabbit anti-human LTA-H (raised against recombinant LTA-H) or the monoclonal anti-FLAG antibody, M2 (Eastman Kodak Co.).

Total RNA (20 µg) extracted from cultured HUVEC, EA.hy 926 cells, and A549 cells according to the acid guanidinium isothiocyanate-phenol-chloroform method (30) was fractionated on a 1% denaturing agarose gel and transferred to nitrocellulose (31). Hybridization with [α-32P]dCTP-labeled cDNA LTA-H probe (32) was carried out overnight at 42 °C in a solution containing 5× SSPE, 5× Denhardt’s solution, 0.1% SDS, and 20% dextran sulfate. Membranes were washed twice with 2× SSC/0.1% SDS, and the DNA was visualized by autoradiography.
Phosphorylation Regulates LTA₄ Hydrolyase

rose gel, blotted to Hybond-N (Amersham Corp.) by capillary action, and cross-linked with UV light.

The blots were probed with an ECLRI expression vector, pEXS5 (31). The probes were labeled by random priming according to the manufacturer’s instructions (Boehringer Mannheim). Radiolabeled probes were excised from the gel and run in denaturing solution to a final concentration of approximately 10⁶ cpm/ml and incubated with the blot overnight at 42 °C. The membrane was washed once with 2 × SSC (0.15 M NaCl, 15 mM sodium citrate) with 0.5% SDS for 30 min at room temperature and three times for 20 min at 65 °C in 1 × SSC with 0.1% SDS. After drying, the blot was exposed to x-ray film overnight.

LTA₄ Hydrolysis in Intact Cells—EC or A549 monolayers were rinsed twice with phosphate-buffered saline and bathed in the same buffer containing 0.5% human serum albumin (2 ml). LTA₄ (5 mM; prepared as the lithium salt by saponification of LTA₄ methyl ester (32); Biomol) was added, and each dish was gently rocked for 30 min at 37 °C in a 5% CO₂ incubator. The reaction was terminated with ice-cold ethanol (10 ml) containing PGB₂ as internal standard (0.5 nmol), and the samples were kept at −20 °C for at least 30 min. Any precipitate was removed by centrifugation, and the sample was evaporated under reduced pressure. The residue was dissolved in mobile phase and analyzed by reverse phase HPLC (RP-HPLC) using a Nucleosil C₁₈ column (3 µm; 3 × 100 mm) eluted at 0.4 ml/min with methanol/water/acetic acid (700:29.9:0.1, v/v/v). Metabolites of LTA₄ were detected by UV absorbance at 270 nm.

Purification of LTA₄ Hydrolysis from EC—EC were scraped from culture dishes with a rubber policeman and suspended in buffer A (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 5 mM 2-mercaptoethanol, 2 µg/ml leupeptin) at 10⁶ cells/ml. The cells were disrupted by sonication with a Branson sonifier model 250 (three times, 1 min each; duty cycle 70%, output 3). The cell supernatant was obtained by centrifuging the sonicate at 10,000 × g for 15 min at 4 °C. The enzyme was partially purified by ammonium sulfate fractionation (40–70% saturation). The resulting precipitate was resuspended in buffer A and dialyzed against several changes of the same buffer overnight at 4 °C. The dialyzed sample was filtered (0.45 µm) and applied to an anion exchange column (Mono Q) equilibrated with 20 mM Tris-Cl, pH 8.0, on a Pharmacia FPLC system. Proteins were eluted with a linear gradient of KCl from 0 to 0.15 M over 60 min at a flow rate of 2 ml/min. Fractions containing LTA₄-H were identified by measuring the aminopeptidase activity of the enzyme as described below. Typically, LTA₄-H was found in fractions 36–41 (approximately 65–85 mM KCl). Active fractions were pooled, and the buffer was changed to 50 mM Tris-Cl, pH 8.0, by ultrafiltration (50,000 molecular weight cutoff; Centricon). LTA₄-H was purified further from the Mono Q fractions by chromatofocusing on a Mono P column (HR 5/5, Pharmacia Biotech Inc.). Samples were applied in 25 mM Bis-Tris, pH 6.7, and eluted with a linear gradient of Polybuffer 74 (pH 3.7; 10%, v/v) at a flow rate of 0.5 ml/min. Fractions were tested for aminopeptidase activity, and active fractions were pooled, concentrated by ultrafiltration, and reconstituted in 50 mM Tris-Cl, pH 8.0.

Purification of Recombinant LTA₄ Hydrolyase—Recombinant LTA₄ was expressed in bacteria as described elsewhere (31). The enzyme was purified from sonicated bacterial cultures essentially as described above. LTA₄-H Assay with Purified Enzyme—Recombinant LTA₄-H (1–2 µg) was incubated in 50 mM Tris-Cl, pH 8.0, with human serum albumin (2 mg/ml; 50 µl) and LTA₄ (25–100 µM) at 37 °C for 1 min. The reaction was terminated by adding ice-cold methanol (150 µl) containing PGB₂ (3.3 nmol) as internal standard. The sample was stored at −20 °C for at least 30 min, and precipitated protein was removed by centrifugation. LTB₂ was quantified by RP-HPLC on a Nucleosil C₁₈ column eluted with methanol/water/acetic acid (65:35:0.01, v/v/v) at a flow rate of 0.4 ml/min. The eluant was monitored at 270 nm, and LTB₂ was quantified by comparison with a standard curve generated from known amounts of LTB₂ and PGB₂. The identity of LT peaks was verified by parallel analysis of synthetic standards.

Aminopeptidase Activity Assay—The aminopeptidase activity was determined by incubating recombinant LTA₄-H (1–2 µg) with alanine-p-nitroanilide (1 mM) in 50 mM Tris-Cl, pH 8.0, with bovine serum albumin (1 mg/ml) in 96-well microtiter plates at 37 °C for 30 min. The reaction was stopped by adding a final concentration of 2.2% formic acid, 7.8% glacial acetic acid (v/v) containing unlabeled phosphonoacetic acid standards (0.5 µg each), and the phosphonoacids were separated by thin layer electrophoresis according to the method of Hunter (33). The first dimension was run for 20 min at 1.5 kV in pH 1.9 buffer. After thoroughly drying and rotating the plate, a second dimension was run in pH 3.5 buffer (5% glacial acetic acid, 0.5% pyridine (v/v)) for 20 min at 1.3 kV. The plate was dried, sprayed with ninhydrin solution (0.25% in acetone), and developed at 65 °C for 15 min. The location of the standards was noted, and the plate was exposed to x-ray film at −70 °C overnight.

In Vivo Phosphorylation of LTA₄-H by EC Lysate and Peptide Mapping—EC extracts were prepared from confluent EA.hy 926 cell cultures. The cells were washed three times with phosphate-buffered saline and scraped off the plates with a rubber policeman. EC were recovered in a pellet after centrifugation at 4 °C and were resuspended in kinase buffer (100 µl/100-mm plate of 20 mM HEPES, pH 7.5, 0.34 mM EDTA, 0.34 mM EGTA, 1.67 mM CaCl₂, 1.0 mM DTT, 10 mM MgCl₂, 1% phosphoethanolamine, 1% bovine serum albumin, and 0.1% Triton X-100). The cells were sonicated on ice for 15 s each time (duty cycle 30%, output 4), and a cytosolic extract was obtained by centrifugation at 10,000 × g for 4 min at 4 °C. The digest was separated by SDS-PAGE, blotted to nitrocellulose, and used for peptide mapping (34).

The band corresponding to LTA₄-H was identified, excised from the membrane, and cut into smaller pieces. The membrane pieces were incubated with polyvinylpyrrolidone (0.5%, w/v) in acetic acid (100 mM) for 30 min at 37 °C to block nonspecific binding. The acid was aspirated, and the membrane pieces were washed five times with water. The immobilized proteins were then carboxymethylated with iodoacetate (100 mM) for 10 min at room temperature in the dark. After the membranes were washed three times with water, the proteins were denatured with urea (1.5 M) in borate buffer (100 µl; 50 µM, pH 8.0), and the protein was digested with sequencing grade trypsin or endoproteinase Lys-C (10 µg; Boehringer Mannheim) at 37 °C overnight. The digested peptides were eluted from the membrane by sonication, transferred to a clean tube, and lyophilized. The residue was reconstituted in 0.1% trifluoroacetic acid, and the digest was fractionated by RP-HPLC on a Vydac semimicro C₁₈ column (2.1 × 250 mm). Peptides were eluted with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 140 min at a flow rate of 1 ml/min. The eluant was monitored at 220 nm, and 30-ml fractions were collected. Radioactivity in each fraction was determined by Cerenkov counting.

Creation of FLAG-LTA₄-H and Site-Directed Mutants—pEXS5, the bacterial expression vector that contains the cDNA for human LTA₄-H, was used as a template to amplify the 2-kilobase insert by polymerase chain reaction (PCR). The original vector encoded a fusion protein with 10 extraneous amino acids at the N terminus. Primers were designed to replace this section with the FLAG epitope (DYKDDDDK) and were synthesized in the Columbia University DNA Core. The sense primer,
ACCATGGAATCAAGGAGCTGATGCAAGCCTGGTAGTGGG,
contains a Rozak translation initiation consensus sequence followed by the
coding sequence for the FLAG epitope and then a stretch of LTA-H
sequence. The antisense sequence was 5′-TCAATACGAGCCTTTTTYA-
TCACTTTTGTATCC-3′. The sense primer was phosphory-
lated at its 5′-end by T4 polynucleotide kinase.

A 20-cycle PCR reaction was run containing deoxynucleotides (25 μM
each), pEX58 (100 ng), sense and antisense primers (50 pmol each), and
Tag DNA polymerase (5 units) in PCR buffer (50 μl of 10 mM Tris-Cl, pH
8.3, 50 mM KCl, 2.5 mM MgCl2, 0.001% gelatin). The initial denaturing
step was 2 min at 94 °C followed by 19 cycles (94 °C for 1 min, 55 °C for 1
min, and 72 °C for 1 min). The final extension reaction was 7 min at 72 °C.
The PCR product was directly ligated to the PCR3.1 vector at 14 °C
overnight, and a portion of the ligation mixture (2 μl) was used to
transform Escherichia coli. Transformants were selected on LB plates
containing kanamycin (50 μg/ml). Several clones were picked and ex-
panded, and the plasmid DNA was purified with Magic minipreps
(Promega). The orientation of the insert was confirmed by restriction
digestion of the plasmid, and positive clones were sequenced to ensure
that the insert had no mutation.

FLAG-LTA-H cDNA was subcloned into a pET vector (Promega) for
expression in bacteria and for site-directed mutagenesis. The mutation
of serine 415 to alanine was accomplished with the PCR-based
QuickChange system according to the manufacturer’s instructions
(Stratagene), and the wild-type and mutant proteins were expressed in
Escherichia coli. The bacteria were grown in LB/AMP medium to a density of 0.6
at 600 nm and were induced by the addition of isopropyl-1-thio-
galactopyranoside (0.4 mM). FLAG-LTA-H and S415A-FLAG-LTA-H were
purified from the 10,000 × g supernatants of sonicated E. coli by
ammonium sulfate precipitation and anion exchange chromatogra-
phy as described above, followed by affinity chromatography over an M2
column (Kodak). The column was washed with three aliquots of glycine,
(5 ml of 50 mM Tris, pH 7.4, 150 mM NaCl). Partially purified enzyme was
applied to the column, which was then washed three times with Tris-
buffered saline (12 ml wash). The FLAG-tagged proteins were then eluted
by the application of excess FLAG peptide (five 1-ml aliquots at 100
μg/ml), and for quantification, aliquots were taken for aminopeptidase activity as de-
scribed above. The active fractions were pooled, and the buffer was
changed to 50 mM Tris-Cl, pH 8.0, by ultrafiltration. The purity of the
protein was checked by SDS-PAGE stained with Coomassie Blue.
In some experiments, the identity of the FLAG-tagged protein as LTA-H
was verified by immunostaining with anti-LTA-H antibody.

**RESULTS**

**Presence of LTA-H in Endothelial Cells**—Despite the fact
that LTA-H activity was not detected in various endothelial
cells, we tested for the presence of messenger RNA and enzyme
protein in these cells. Both Northern and Western blots dem-
strated the presence of LTA-H in HUVEC and in the endo-
theelial cell line, EA.hy 926. Similarly, the human lung carci-
noma line, A549, that served as the fusion partner for the
EA.hy 926 was positive for LTA-H message and protein (Fig. 1).

We used RNA from these cells as the source of LTA-H activity in
an assay that measures the production of LTB4 from exoge-
nous LTA4. Neither HUVEC (data not shown; see Ref. 25) nor
EA.hy 926 cultures (Fig. 2B) converted LTA4 to LTB4, A549,
on the other hand, readily converted LTA4 to LTB4 (Fig. 2C).

To be sure that the apparent absence of LTA-H activity was
due to some inability of the cells to transport LTA4 across
the cell membrane, we repeated these assays in cell lysates and
on purified enzyme obtained from these cells. The results
confirmed our findings in the intact cells. LTA-H purified from
HUVEC and EA.hy 926 was inactive, while the same enzyme
obtained from A549 cells readily converted LTA4 to LTB4 (data not shown).

**Phosphorylation of the Native Endothelial Cell LTA-H—A
brief examination of the deduced amino acid sequence of
LTA-H showed that the enzyme contains multiple phosphoryl-
ation sites. To determine if the inactivity of the purified EC
enzyme might be due to a post-translational modification by a
kinase, we incubated HUVEC cultures with [32P]orthophos-
phate, lysed the cells, and recovered the endogenous LTA-H by
immunoprecipitation. The precipitate was fractionated by
SDS-PAGE, blotted to nitrocellulose, and exposed to x-ray film.
LTA-H incorporated radioactive phosphate under these condi-
tions, confirming that the native, inactive enzyme is phospho-
ylated in intact HUVEC (data not shown). Enzyme purified from
[32P]orthophosphate-loaded HUVEC was hydrolyzed in 6
× HCl for phosphoamino acid analysis. The only radiolabeled
amino acid in this preparation was phosphoserine (Fig. 3).
Identical results were obtained with EA.hy 926 cultures (data not shown).

**Peptide Mapping of Phosphorylated LTA-H—To identify the
site(s) of LTA-H phosphorylation, we analyzed the peptide map of
recombinant LTA-H phosphorylated in vitro by the kinase(s)
in cell lysates prepared from EA.hy 926 cell cultures. Phospho-
ylated LTA-H was digested either with trypsin or Lys-C as
detailed under “Experimental Procedures,” and the resulting
peptides were fractionated by RP-HPLC. Digestion with either
proteinase yielded one major phosphopeptide (Fig. 4, A and B).
These peptides were indistinguishable by HPLC analysis.
However, the Lys-C digest of LTA-H phosphorylated by A549
lysate was clearly different (Fig. 4, B and C). Although
phosphorylation was always on a serine (data not shown), these
data indicate that the kinases of the two cell types targeted
different sites in the LTA-H.

We focused our attention on the 14 peptides that overlap in
the trypsin and Lys-C peptide maps and contain a serine resi-
due. The radiolabeled peptide obtained from enzymatic digests
of LTA-H phosphorylated by EC lysate was identified by mi-

**FIG. 1. Northern and Western analysis from cultured HUVEC,**
**EA.hy 926, and A549 cells.** A, Northern blot. Total RNA was extracted from
cultures as described under “Experimental Procedures.” 20 μg of RNA from each cell type was fractionated in a 1% formaldehyde-agarose
gel, transferred by capillary action to a nylon membrane, and fixed by
UV irradiation. The blot was hybridized with an EcoRI fragment from pEX58 (nucleotides 1055–1880) labeled by random priming with
[32P]dCTP. After washing, the membrane was dried and exposed to x-ray film. The positions of the 28 and 18 S ribosomal RNA bands are
marked. The LTA-H transcript is approximately 2.2 kb as reported by
others (44–46). Lane 1, HUVEC; lane 2, EA.hy 926; lane 3, A549. B,
Western blot. LTA-H was purified from HUVEC, EA.hy 926, and A549
cultures by ammonium sulfate fractionation, ion exchange chromatog-
raphy (Mono Q), and chromatofocusing (Mono P) as described under
“Experimental Procedures.” Purified proteins (100 ng) were run on 7.5%
SDS-PAGE and transferred to nitrocellulose, and LTA-H was detected
with a primary anti-LTA-H antibody and a secondary alkaline phos-
phatase-linked anti-rabbit IgG. The positions of molecular weight standards on the gel are marked. The apparent molecular mass of
LTA-H is 68 kDa as reported by others (47, 48). Lane 1, EA.hy 926; lane 2,
A549; lane 3, HUVEC.
Phosphorylation Regulates LTA₄ Hydrolase

Phosphorylation of LTA-H Inhibits LTB₄ Synthesis—To test whether phosphorylation of FLAG-LTA-H alters the metabolism of LTA₄ to LTB₄, we incubated LTA-H with cell lysate from EA.hy 926, stopping the reaction by dilution. The FLAG-LTA-H protein was rapidly recovered by immunoaffinity col-

Using a PCR-based mutagenesis, the serine corresponding to residue 415 of the wild-type enzyme was converted to alanine, and the sequence of the resulting expression vector was verified by cDNA sequencing. Mutant LTA-H (S415A-FLAG-LTA-H) was expressed in bacteria and purified over an anti-FLAG immunoaffinity column. The addition of the FLAG tag and the S415A mutation slightly reduced the specific activity of the recombinant LTA-H in the LTB₄ synthesis assay and in the aminopeptidase assay of this enzyme (Table 1), but these changes were not significant.

Both the FLAG-LTA-H and S415A-FLAG-LTA-H were incubated with cell lysates from EA.hy 926 or A549 cultures in an in vitro phosphorylation assay in the presence of [γ-³²P]ATP. FLAG-tagged proteins were recovered from an immunoaffinity column and fractionated by SDS-PAGE. Wild-type LTA-H was phosphorylated by EA.hy 926 lysate (Fig. 5, lane 1), whereas the S415A mutant was not a substrate for the kinase in this lysate (Fig. 5, lane 2). A549 lysate phosphorylated LTA-H at a different site and, as expected, catalyzed the incorporation of phosphate into both wild-type (Fig. 5, lane 3) and mutant LTA-H (Fig. 5, lane 4).

Studies of the EA.hy 926 Kinase-mediated Phosphorylation of LTA-H—Despite the fact that the kinase source for these experiments is a crude cell lysate, we determined some of the characteristics of this enzyme preparation. Peak phosphorylation occurred at 3 min and then fell off slightly over the next 10 min, indicating that the lysate probably contained a phospa-

case activity (Western analysis shows the presence of protein phosphatase-1 in these lysates; data not shown). The reaction appeared to have maximal activity with 50 μM ATP and did not require the addition of either calcium or phospholipid. Under these conditions, LTA-H kinase in EA.hy 926 lysate catalyzed the incorporation of approximately 0.1 mol of phosphate/mol of LTA-H.

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Results were obtained with EA.hy 926 or A549 cell cultures. Cultures were washed with phosphate-buffered saline and incubated at 37 °C in phosphate-buffered saline containing human serum albumin (0.5%). LTA₄ (5 μM) was added, and the incubation was continued for 30 min on a rocker table in a CO₂ incubator. LTB₄ and its isomers were isolated and fractionated by RP-HPLC with an ultraviolet absorbance detector set at 270 nm as described under “Experimental Procedures.” The absorbance was normalized to the peak height of the internal standard, PGB₂ (labeled in the figure).

FIG. 2. HPLC analysis of LTA-H activity in EA.hy 926 and A549 cell cultures. Cultures were washed with phosphate-buffered saline and incubated at 37 °C in phosphate-buffered saline containing human serum albumin (0.5%). LTA₄ (5 μM) was added, and the incubation was continued for 30 min on a rocker table in a CO₂ incubator. LTB₄ and its isomers were isolated and fractionated by RP-HPLC with an ultraviolet absorbance detector set at 270 nm as described under “Experimental Procedures.” The absorbance was normalized to the peak height of the internal standard, PGB₂ (labeled I.S. in the figure). Panel A, nonenzymatic decomposition of LTA₄ incubated in buffer alone (peaks at 12 and 14 min are 6-trans-LTB₂ and 12-epi-6-trans-LTB₂). Panel B, incubation of LTA₄ with an EA.hy 926 monolayer culture. Panel C, incubation of LTA₄ with an A549 culture. Retention time of standard LTB₂ is noted by the arrow. This result is typical of at least three experiments.

Crosssequencing to be the tetrapeptide FSYK, and thus the phosphorylated residue was serine 415.

Site-directed Mutagenesis of the Putative Phosphorylation Site—The recombinant LTA-H used in our earliest studies was expressed in E. coli as a fusion protein that contained 10 extraneous amino acids on the N terminus (31). Prior to initiating site-directed mutagenesis studies, the expression vector for this recombinant LTA-H, pEX85, was reengineered to replace this extraneous sequence with the FLAG epitope. FLAG-tagged recombinant LTA₄ hydrolase (FLAG-LTA-H) was expressed and purified over an anti-FLAG immunoaffinity column.

The recombinant LTA-H used in our earliest studies was expressed in E. coli and purified over an anti-FLAG immunoaffinity column. The addition of the FLAG tag and the sequence of the resulting expression vector was verified by cDNA sequencing. Mutant LTA-H (S415A-FLAG-LTA-H) was expressed in bacteria and purified over an anti-FLAG immunoaffinity column. The addition of the FLAG tag and the S415A mutation slightly reduced the specific activity of the recombinant LTA-H in the LTB₄ synthesis assay and in the aminopeptidase assay of this enzyme (Table 1), but these changes were not significant.

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Fig. 4. Peptide mapping of phosphorylated LTA-H. Recombinant LTA-H was phosphorylated by incubation with lysate prepared from cultures of EA.hy 926 or A549 cells and [γ-32P]ATP as described under “Experimental Procedures.” Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Radiolabeled LTA-H was identified by exposing the blot to x-ray film, and the band was excised from the blot. Filter pieces were incubated overnight at 37 °C with trypsin (1:20, w/w) or endoproteinase Lys-C (1:20, w/w), and the resulting peptides were fractionated by RP-HPLC (Vydec C18 column eluted at 1 ml/min with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 140 min). Thirty-second fractions were collected, and the radioactivity in each fraction was measured by Cerenkov counting. Thirty-second fractions were collected, and the radioactivity in each fraction was measured by Cerenkov counting.桌

DISCUSSION

Several studies of LT metabolism in EC have failed to demonstrate the conversion of LTA₄ to LTBr (16–19). In startling disagreement, experiments by Claesson and Haegeström (35) found evidence of active LTA-H in primary HUVEC cultures. Our findings confirm that EC contain LTA-H as assessed by Northern and Western blotting; however, LTA-H purified from EC was unable to catalyze the metabolism of LTA₄. Immunoassays (12, 36) and enzymatic assays (11, 37) have shown that LTA-H is a widely distributed protein, but enzyme activity measurements detected a considerable tissue-dependent variation in specific activity (11). These data lead us to speculate that a system for the cellular regulation of LTA-H activity may exist (19). Until now, only indirect evidence has been generated to address this hypothesis.

What is the significance of cellular regulation of LTA-H, especially in EC that do not synthesize LTA₄? It is clear that EC can utilize LTA₄ generated by PMNL in a process known as transcellular metabolism (17). In fact, PMNL, which synthesize both LTA₄ and LTBr, have been shown to export as much as 50% of the LTA₄ generated after activation by the calcium ionophore, A23187 (38, 39). Thus, the capacity of EC to take up and metabolize LTA₄ generated by PMNL, especially when the two cell types are in intimate contact, can lead to physiologically significant quantitative or qualitative changes in LT production by the system. Evidence for EC production of LTBr via transcellular metabolism has been presented and discussed elsewhere (16–19). What if EC could also generate LTBr when in contact with activated PMNL? LTBr is a potent stimulus for PMNL adhesion to EC (5, 40) and transendothelial migration in vitro (42). Similar observations of the biological activity of LTBr have been reported and reviewed (43), but all indicate that the production of LTBr by EC could be an important feed-forward signal during the development of inflammation by generating an intense local gradient of this chemotactic, PMNL-activating
Phosphorylation Regulates LTA₄ Hydrolase

Fig. 6. Phosphorylation of wild-type LTA-H by EA.hy 926 lysate inhibits its activity but does not affect the activity of the S415A mutant. Recombinant FLAG-LTA-H (1 µg) or S415A-FLAG-LTA-H (1 µg) was incubated with cell lysate from EA.hy 926 cell cultures (equivalent to the 14,000 × g supernatant from a confluent 100-mm dish) with (or without) ATP (150 µM) for 3 min at 37 °C. Bovine serum albumin (1 mg/ml, final concentration) was added, and the LTA-H reaction was initiated by the addition of LTA₄ (50 µM). The reaction was run for an additional 1 min at 37 °C and then stopped with ice-cold methanol containing the internal standard (PGB2; 3.3 nmol). Samples were analyzed by RP-HPLC with a UV absorbance detector as described in the legend to Fig. 2. Each run was normalized to the internal standard (I.S.). The retention time of LTB₄ in the presence (I.S.) or absence (A) of protein phosphatase-1 (0.2 units) at 37 °C for 30 min. Both incubations contained p-nitroaniline (the aminopeptidase reaction product, 1 mM), which had been shown earlier to enhance the ability of protein phosphatase-1 to dephosphorylate LTA-H. After this, LTA₄ (50 µM) was added, and the reaction was run for an additional 1 min at 37 °C. The reaction was stopped with ice-cold methanol containing the internal standard (PGB₂; 3.3 nmol), and the samples were analyzed as described in the legend to Fig. 2. The scale is normalized to the peak height of the internal standard (I.S.), and the retention time of LTB₄ is noted. This result is typical of three experiments.

We find that LTA-H is a substrate for one or more kinase found in the cytosol of at least two cell types, A549, a human lung carcinoma cell line, and EA.hy 926, an endothelial cell-like hybridoma line derived from the fusion of A549 with HUVEC. However, the phosphorylation in these cases occurs at distinct sites, and it is only the EC kinase that inhibits LTA-H activity. In addition, A549 cells contain active LTA-H, suggesting that the LTA-H kinase is differentially distributed. This may account for the variations in specific activity of this enzyme seen in various tissues.

The kinase in EC that phosphorylates LTA-H? Current data leads us to predict that this enzyme is not a known kinase and suggest that it may be a previously undescribed enzyme. For example, by the comparison of peptide maps, it is
clear that the EC LTA-H kinase is not one of the α-β- or γ-isomers of protein kinase C. By this criterion, the kinase identified in A549 lysates is probably one of these isoforms, since it phosphorylates the LTA-H at the same site as commercially prepared protein kinase C.\(^3\) In addition, LTA-H kinase is stable at 4 °C for weeks and is not dependent upon calcium or added phosphohistidin. This tends to rule out the protein kinase C family as candidates. We have observed that LTA-H is not a substrate for PKA in vitro, and although LTA-H is phosphorylated by casein kinase and the insulin receptor kinase, both produce a very weak signal and have no effect on LTA-H activity.\(^3\) Finally, the sequence at which the LTA-H is phosphorylated does not fit with any known consensus kinase sequence. Although this analysis is limited, we are proceeding with our effort to purify the EC kinase under the assumption that it is a novel protein kinase.

Finally, by site-directed mutagenesis, we have unequivocally identified the site at which the EC LTA-H kinase phosphorylates and thereby regulates the enzyme. The change of serine 415 to alanine does little to alter the activity of the enzyme; therefore, it is not clear what role, if any, this residue may play in the catalysis of epoxide hydrolysis. However, the mutant LTA-H is not a substrate for the EC LTA-H kinase, and its activity is not altered by incubation with the cell lysate under conditions known to result in phosphorylation and inhibition of the wild-type protein.

We have consistently obtained modest levels of inhibition of LTA-H in the studies described in this report, and our phosphorylation conditions yield a product that incorporates only about 0.1 mol of phosphate/mol of enzyme. In a limited number of trials, we have observed that the inhibition of LTA-H synthesis doubled with a doubling of the incorporation of phosphate (n = 3).\(^5\) It seems likely that there are at least two independent reasons for the low level of phosphate incorporation in these experiments. First, our studies of phosphate incorporation versus time indicate a peak at 3 min that falls slowly after 10 min. This result is probably caused by the action of a protein phosphatase in the kinase preparation. This is further supported by studies with a more purified kinase preparation isolated from EC supernatant (and able to phosphorylate wild type but not S415A-LTA-H). These studies have produced a somewhat higher stoichiometry of phosphate incorporation (0.5 mol/mol).\(^3\) However, it is clear from the protein phosphatase experiments that the phosphorylation site is somehow protected within the three-dimensional structure of the enzyme. Thus, protein phosphatase-1 alone will not strip radiolabeled phosphate from LTA-H. The phosphatase is active only in the presence of substrate (i.e., the presence of substrate) is also required.

The inhibition of the phosphorylation site from the action of phosphatase implies that this site may also be protected from the LTA-H kinase. This may explain our routine finding of 30–40% recombination of inactivating enzyme phosphorylated by cell lysate. The native EC enzyme, on the other hand, may be phosphorylated as a nascent protein and thus would be synthesized in an inactive state. These data suggest that some mechanism exists to regulate LTA-H in endothelial cells and presumably in other tissues as well. Based on our findings, we believe that LTA-H is tightly regulated by phosphorylation by a specific LTA-H kinase.

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\(^2\) I. V. Rybina and S. J. Feinmark, unpublished data.

\(^3\) I. V. Rybina, D. H. Solomon, and S. J. Feinmark, unpublished observation.