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Demonstration of cell-specific phosphorylation of LTC_4 synthase

Namrata Gupta[a,b,*], Donald W. Nicholson[a], Anthony W. Ford-Hutchinson[a]

[a] *Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Que. H9R 4P8, Canada*
[b] *Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond, Montreal, Que. H3G 1Y6, Canada*

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Abstract PMA-induced leukotriene C_4 synthase (LTC_4S) phosphorylation was investigated over a period of 8 h in a monocytic cell line (THP-1). The level of LTC_4S phosphorylation was increased 3–5 fold over a 4 h period decreasing to basal levels after 8 h. This phosphorylation event was found to be specific to THP-1 cells as there was a lack of LTC_4S phosphorylation in both COS-7 and K-562 cells, and was also found to be dependent on the cellular confluency. In the presence of specific protein kinase C (PKC) inhibitors, a dose-dependent inhibition of the phosphorylation of LTC_4S became evident, an effect not seen with PKA and tyrosine kinase inhibitors. This represents the first direct demonstration of LTC_4S phosphorylation in whole cells.

Key words: Leukotriene C_4 synthase; Protein kinase C; Phorbol ester; THP-1 cell; Protein phosphorylation

1. Introduction

Cysteinyl leukotrienes (LTs), LTC_4, LTD_4, and LTE_4 comprise the slow-reacting substance of anaphylaxis (SRS-A) and are smooth muscle contractile lipid mediators with important biological effects elicited through G-protein coupled receptors [1–4]. The formation of cysteinyl LTs is initiated by the activation of cytosolic phospholipase A_2 ($cPLA_2$) which liberates arachidonic acid (AA) from phospholipids [5,6]. In the lipoxygenase pathway, AA binds to an integral membrane protein, 5-lipoxygenase-activating protein (FLAP), which is then presented to the soluble enzyme 5-lipoxygenase (5-LO). Via the action of 5-LO, AA is converted to the unstable epoxide, LTA_4 [7]. At this point, LTA_4 can follow one of two divergent pathways within the cell of synthesis. Hydrolysis of LTA_4 through the action of cytosolic LTA_4 hydrolase yields the pro-inflammatory mediator LTB_4 [8]. Conjugation with reduced glutathione (GSH) converts LTA_4 to biologically active LTC_4, a stereospecific reaction catalyzed by leukotriene C_4 synthase (LTC_4S), an integral perinuclear membrane protein active as a homodimer [9]. LTC_4 is then either exported into the extracellular milieu directly, or is sequentially metabolized by the successive cleavage of glutamic acid and glycine to generate LTD_4 and LTE_4 via a transpeptidase and a peptidase, respectively [10,11].

Proteins in the leukotriene biosynthetic pathway are tightly regulated and many require various co-factors for function and full activity. Cytosolic PLA_2 requires increased Ca^{2+} concentrations in the μM range in conjunction with a serine phosphorylation by MAP kinase at position 505 of $cPLA_2$ for activation and translocation [12]. The reaction, catalyzed by 5-LO, requires not only increases in Ca^{2+} levels but also ATP [13]. In whole cells, FLAP is an essential component for 5-LO activity [14–17]. Cytosolic LTA_4 hydrolase has recently been shown to be regulated by phosphorylation in endothelial cells where under basal conditions the protein is phosphorylated at serine-415, leading to an inactive state of the enzyme. Incubation with phosphatases reverts the protein to an active state possessing epoxide hydrolase activity, thereby showing that LTA_4 hydrolase may be regulated by a kinase/phosphatase cycle [18].

The alternative pathway taken by LTA_4 through the action of LTC_4S is its conjugation to GSH. LTC_4S is an 18-kDa protein which is neither dependent on any co-factors for activity, requires post-translational modification by glycosylation nor formed as a pro-enzyme. Of interest are two putative protein kinase C (PKC) consensus sites, Ser-Ala-Arg, found on the protein at positions 28 and 111 [9,19]. These are suggestive of LTC_4S being subject to regulation by phosphorylation. Studies on the activity of LTC_4S have been conducted in our laboratory [20,21], and by other investigators [22], which have also led to the same conclusions showing that the activity of LTC_4S was attenuated upon activation of PKC by the phorbol ester, PMA. This suggests the possibility of LTC_4S enzymatic activity being directly regulated by phosphorylation of the enzyme.

2. Materials and methods

2.1. Cell culture

Cells from the human monocytic leukemic cell line THP-1 (ATCC), and K-562 (ATCC), a lymphoid cell line, were both cultured in a similar manner. Cells were maintained in sterile RPMI 1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM glutamine and 25 mM HEPES buffer at pH 7.4. COS-7 cells (ATCC), a fibroblast-like cell line, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere of 94% air and 6% CO_2.

2.2. Creation of LTC_4S-flag

Human LTC_4S cDNA (in pBluescript II KS^+, Invitrogen) was used as a template for the introduction of a 5′ Kozak translation initiation consensus sequence (CCACC) and a flag epitope (DYKDDDDK) at the carboxyl terminus of LTC_4S via PCR with the following oligonucleotides: 5′ CCG CTC GAG CGG CCG CGG ATC CAC CAT GAA GGA CGA GGT AGC TCT ACT GGC TGC 3′ and 5′ GCT CTA GAG CGG CCG CGG ATC CTA TTA TTT ATC ATC ATC ATC TTT ATA ATC GGC CCA CGG CAG CAG CGT CCG GAG CCG TC 3′.

A 25-cycle PCR reaction was run containing deoxynucleotides (250 μM), cDNA template (1/100 of mini-prep), sense and antisense primers (5 μM each), GC melt (Clontech) (1:5 by volume), and Pwo DNA polymerase (2.5 units) in PCR buffer (10 μl of 100 mM Tris-HCl, pH 8.85 (20°C), 250 mM KCl, 50 mM $(NH_4)_2SO_4$, and 20 mM $MgSO_4$). The initial denaturing step was 2 min at 94°C followed by

*Corresponding author. Fax: (1) (514) 428 4900.
E-mail: namrata_gupta@merck.com

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25 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min). The PCR product was PCR purified (Qiagen), digested with *Xho*I and *Bam*HI, gel purified (Qiagen), ligated into pBluescript II KS$^+$ (Rapid DNA Ligation Kit from Boehringer Mannheim), and transformed into Epicurian Coli XL2 Blue Ultracompetent cells (Stratagene). Transformants were selected on LB plates containing X-galactosidase (40 μg/ml). Several clones were picked, grown in culture, and the plasmid DNA was prepared by miniprep (Qiagen). The orientation of the insert (LTC_4S-flag) was confirmed by restriction digestion of the plasmid, and the lack of any inadvertently incorporated mutations was ensured with bi-directional sequencing using the ABI automated sequencer. LTC_4S-flag/pBluescript was then subcloned into pcDNA3.1(+) (Invitrogen) for transfections into cell lines.

2.3. Transient transfection of COS-7 and K-562 cells

COS-7 cells were seeded a day prior to reach a confluency of 50–75% for transfections in 60-mm plates. Transfections of COS-7 cells were performed using the Lipofectamine Plus method (Life Technologies). 10 μg of LTC_4S DNA was incubated for 15 min with 300 μl of Opti-MEM media and 5 μl of Plus reagent followed by the addition of Lipofectamine-Opti-MEM media (12 μl Lipofectamine+300 μl Opti-MEM) which was further incubated for another 15 min. Culture media was removed from the plates and in place 2.4 ml of Opti-MEM was added to the cells. DNA-Lipofectamine solution was then added to these plates. Transfections were performed for 3 h at 37°C in a 6% CO_2 atmosphere. DNA-Lipofectamine-Opti-MEM solution was then aspirated and replaced with complete DMEM media and the cells were then cultured at 37°C with 6% CO_2. Cells were then either harvested after 40–48 h of incubation, assayed for protein concentration as per Bio-Rad, and subject to Western Blot analysis to detect transfection efficiency, or transfected cells were directly subjected to ^{32}P-metabolic labeling followed by protein determination and Western blot analysis.

Transfection into K-562 cells followed the same protocol as for COS-7 cells with the following exceptions. DMEM media was replaced with RPMI 1640 and Opti-MEM was used to wash the cells prior to transfection. The number of K-562 cells used per transfection was 1×10^6 cells.

2.4. Phosphorylation of LTC_4S into transfected cells

After transfections were complete, cells were washed twice with phosphate-buffered saline and then starved for 1 h at 37°C, 6% CO_2 in 2 ml of phosphate-free DMEM medium. Cells were then labeled with 0.5 mCi/ml of aqueous [^{32}P]orthophosphate (10 mCi/ml from Amersham) for 4 h and treated with or without 50 nM PMA for appropriate activation times. Cells were subsequently harvested by a wash with phosphate-buffered saline followed by a spin at 4°C. The pellets were then resuspended in 1 ml of solubilization buffer (50 mM Tris-HCl, pH 7.7, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 100 mM NaCl, 2.5 mM EGTA, 10 mM NaF, 0.2 mM vanadate and a cocktail of protease inhibitors from Boehringer Mannheim) and vortexed to ensure lysis. After a 10-min incubation on ice, the lysates were spun down at 4°C and the supernatant either frozen at −80°C or used immediately for Western blot analysis and immunoprecipitation of LTC_4S.

2.5. Endogenous phosphorylation of LTC_4S in THP-1 cells

THP-1 cells grown to confluency were washed once with phosphate-buffered saline and then resuspended at 2×10^6 cells/ml in phosphate-free RPMI 1640, supplemented with 10% (v/v) dialyzed fetal bovine serum for a 0.5 h starvation period at 37°C and 6% CO_2. Aqueous [^{32}P]orthophosphate (10 mCi/ml) was added to 1 mCi/ml, and treated with or without 50 nM PMA for appropriate activation times in the presence or absence of kinase inhibitors, and the cells were labeled for a period of 4 h. Cells were subsequently harvested by centrifugation, washed in phosphate-buffered saline and lysed by vigorous vortexing in 1 ml of solubilization buffer (components already described). The lysates were then spun down and the supernatants were either stored at −80°C or processed immediately for level of LTC_4S phosphorylation by immunoprecipitation and protein determination by Western blot analysis.

2.6. Western blot analysis of cell lysates using anti-LTC_4S antibody

SDS-containing Laemmli sample buffer was added to 100 μg of protein as measured by the Bio-Rad assay and the volume was adjusted equally with dH_2O. The samples were subsequently heated for 5 min at 95°C, electrophoresed through SDS-polyacrylamide gels (Novex), and electroblotted onto nitrocellulose membranes (Novex). Ponceau S staining was used to visualize the efficiency of transfer. Membranes were then soaked for 1 h at 25°C in Tris-buffered saline containing 0.1% (v/v) Tween 20 (0.1% T TBS; 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) containing 5% (w/v) Bio-Rad Blotting Grade Blocker non-fat dry milk. Blots were then washed twice for 5 min each with 0.1% T TBS and subsequently treated for 1 h at 25°C with anti-LTC_4S antibody (dilution 1:2000) in 0.05% T TBS containing 5% milk. After washing the blots three times for 5 min each with 0.1% T TBS the membranes were incubated for 1 h at 25°C with a horseradish peroxidase-linked donkey anti-rabbit antibody (Amersham) (dilution 1:3000) in 0.05% T TBS containing 1% milk. The blots were washed three times for 5 min each with 0.3% T TBS and then three times for 5 min each with 0.1% T TBS and subsequently developed using enhanced chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent, DuPont NEN), according to the manufacturer's instructions.

2.7. Immunoprecipitation of LTC_4S from labeled cells

Equivalent amounts of protein as measured by the Bio-Rad assay were mixed with 500 μl of immunoprecipitation buffer (10 mM Tris-HCl pH 7.2, 1% (v/v) Triton X-100 and 300 mM NaCl) and incubated on a rotator for 0.5 h at 4°C followed by a 15 min spin at $14\,000\times g$ in an Eppendorf microfuge. LTC_4S was then isolated from the supernatants by immunoprecipitation using 20 μl of anti-LTC_4S antibody prebound to protein A-Sepharose (Pharmacia). The immune complex was then further incubated on a rotator for 1 h at 4°C. The immune complexes were collected by centrifugation for 5 min at $14\,000\times g$ in an Eppendorf microfuge, and the pellets were washed twice with 0.5 ml of solubilization buffer (components described above) and twice with 0.5 ml of a more stringent wash buffer (50 mM NaF, 30 mM Na-pyrophosphate, 0.2 mM vanadate). After the final wash the pellet was aspirated to dryness and LTC_4S was then eluted with 40 μl of 2× Laemmli's buffer. Samples were denatured by boiling for 5 min, and following centrifugation the eluants were subject to SDS-PAGE.

2.8. SDS-PAGE and analysis of LTC_4S

Aliquots of immunoprecipitated proteins were separated by SDS-PAGE on 10–20% polyacrylamide gels. For autoradiography, gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid for 15 min at room temperature, soaked in Enlightening for 20 min at room temperature, dried at 80°C at a ramp setting for a period of 2 h and exposed to Kodak MR film for 2–3 days at room temperature. Quantitation of proteins was done using a PhosphorImager (Molecular Dynamics) with the phosphorscreen scanned after 1–2 days exposure. For each experiment the area under the peak of the scans which corresponded to the LTC_4S band was used as a measure of the amount of LTC_4S in the sample. Although this determination is not a measure of the absolute quantity of the protein in the samples, these data provide a reliable estimate of relative differences of levels of LTC_4S among the various samples.

3. Results

3.1. Evidence for phosphorylation of endogenous LTC_4S and its specificity to THP-1 cells

Examination of the deduced amino acid sequence of LTC_4S showed that the enzyme contains two PKC serine phosphorylation consensus sites at positions 28 and 111 [9,19]. It has also been shown that activation of PKC by PMA leads to an attenuation of LTC_4S enzymatic activity [20–22]. To determine whether the inactivity of the enzyme might be due to a post-translational modification by a protein kinase, we labeled THP-1 cells with [^{32}P]orthophosphate for a period of 4 h and activated the cells with 50 nM PMA. The cells were then lysed in a buffer containing protease and phosphatase inhibitors, and endogenous LTC_4S was recovered by immunoprecipitation using a specific polyclonal antibody raised against peptides of LTC_4S. The precipitate was then separated by SDS-

PAGE, dried down, and exposed to X-ray film. Under these conditions, incorporation of radioactive phosphate would indicate that the native protein is phosphorylated.

LTC_4S was found to be directly phosphorylated in THP-1 cells. The level of phosphorylation in THP-1 cells was seen to increase by a factor of 3–5 fold as a result of PMA activation for 4 h in comparison to the DMSO control, as measured by autoradiography (Fig. 1).

In an attempt to determine whether the observed phosphorylation of LTC_4S is a post-translational modification common to other cell types other than the human monocytic-like THP-1 cell type, recombinant LTC_4S was transfected into two other cell types, namely COS-7, and K-562. Transfected cells were then subjected to metabolic labeling with [^{32}P]orthophosphate, activation by PMA, followed by immunoprecipitation with anti-LTC_4S antibody and detection by SDS-PAGE-autoradiography. Transfections into both cell lines were successful in view of the Western blotting results in the presence of LTC_4S DNA (Fig. 1). Both of these cell lines were found to lack endogenous LTC_4S as detected by Western blotting of the mock transfections (data not shown). As can be seen by the autoradiography results, LTC_4S was not phosphorylated in COS-7 cells nor in K-562 cells although LTC_4S protein was present. In contrast, endogenous LTC_4S in THP-1 cells was detected at the protein level and was found to be phosphorylated (Fig. 1). This is indicative of the fact that phorbol-ester induced PKC activity requires downstream effectors leading to the phosphorylation of LTC_4S in THP-1 cells, which are absent in COS-7 and K-562 cell lines but present in THP-1 cells. Therefore, phosphorylation of LTC_4S was observed only in THP-1 cells and thus was found to be cell-specific, requiring specific effector(s) (kinases and phosphatases) which impart the cell-specificity. Since phosphorylation of LTC_4S was only evident in THP-1 cells, all further studies were conducted using this system.

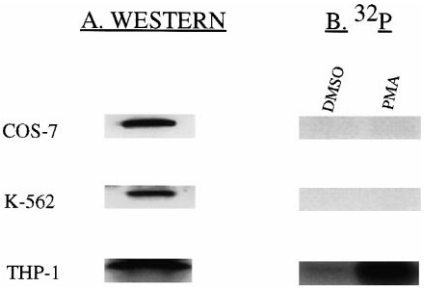

Fig. 1. Endogenous LTC_4S is directly phosphorylated upon activation with PMA and is specific to THP-1 cells. A: Western blot. LTC_4S was solubilized from transfected cells (COS-7 and K-562) and from THP-1 cells as described in Section 2. Proteins (100 μg) in the presence of SDS-containing buffer were run on a 10–20% SDS-PAGE and transferred to nitrocellulose, and LTC_4S was detected with a primary anti-LTC_4S antibody and a secondary horseradish peroxidase-linked donkey anti-rabbit antibody. B: Autoradiography. Equivalent numbers of LTC_4S transfected cells and THP-1 cells were metabolically labeled with 0.5 mCi/ml aqueous [^{32}P]orthophosphate for 4 h and activated with or without 50 nM PMA. Cells were then harvested, solubilized, followed by immunoprecipitation of LTC_4S with an anti-LTC_4S antibody pre-bound to Protein A Sepharose, washed, separated by a 10–20% SDS-PAGE and visualized by autoradiography. These data are typical of three independent experiments.

3.2. *Phosphorylation of LTC_4S is dependent on cell confluency*

Hyperphosphorylation of basal levels of endogenous LTC_4S was evident in certain experiments performed in the process of assessing levels of phosphorylation in the absence of any given stimuli (data not shown). To establish the nature and possible cause of the observed hyperphosphorylation, it was hypothesized that perhaps the cell confluency had an influence on the state of the cell, thereby affecting signal transduction processes. The level of phosphorylation of LTC_4S was tested at various starting THP-1 cell confluencies from which equal number of cells were used to metabolically label and immunoprecipitate LTC_4S. A pattern became evident where the basal level of phosphorylation was found to increase as the confluency of the cell decreased from 1:5 to a 1:20 dilution without activation of PKC, as observed in the DMSO control samples. The level of LTC_4S phosphorylation in the presence of PMA was not affected with respect to the varying cell confluencies (Fig. 2). At a dilution of 1:50, cells were found to aggregate and their growth hindered due to cell clumping making it difficult to assess their phosphorylation state. This is suggestive of possible cell-cell interactions having an effect on the signaling of the cell. That is, it may be possible that such interactions may initiate a cascade of events which leads to phosphorylation, perhaps via the activation of a kinase(s) or a phosphatase(s). It is interesting that the PMA-induced LTC_4S phosphorylation was not affected by the cell confluency. This is suggestive of the fact that these events may be either independent of the PMA-induced phosphorylation of LTC_4S or perhaps the activation of PMA may overpower the effects due to cell-cell interaction. It should be noted that all subsequent experiments were carried out with the cellular dilution at 1:5, in order to minimize basal levels of phosphorylation.

3.3. *Time-course of phosphorylation of endogenous LTC_4S in THP-1 cells*

A time-course of the phosphorylation event was carried out where cells were labeled for a period of 4 h and activated with 50 nM PMA for either 30 min, 1.5 h, 4 h, or 8 h (in which case the cells were labeled for 8 h). Endogenous phospho-

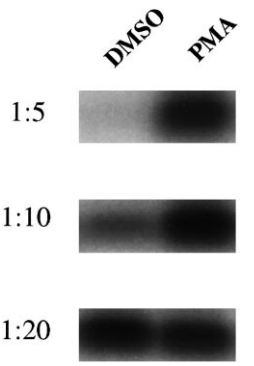

Fig. 2. The level of phosphorylation of LTC_4S is dependent on the cell confluency. THP-1 cells at 1×10^6 cells/ml were seeded a week prior to metabolic labeling at the following dilutions: 1:5, 1:10, and 1:20. Equivalent number of cells from the varied dilutions were labeled with 0.5 mCi/ml aqueous [^{32}P]orthophosphate for 4 h and activated with or without 50 nM PMA. Cells were then harvested, solubilized, followed by immunoprecipitation of LTC_4S with an anti-LTC_4S antibody pre-bound to protein A-Sepharose, washed, separated by a 10–20% SDS-PAGE and visualized by autoradiography. These data are a representation of three typical independent experiments.

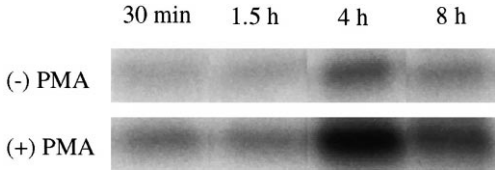

Fig. 3. Time course of the phosphorylation of endogenous LTC_4S in THP-1 cells. Confluent THP-1 cells were labeled for a period of 4 h with 0.5 mCi/ml aqueous [^{32}P]orthophosphate and activated with or without 50 nM PMA for either 30 min, 1.5 h, 4 h, or 8 h (in which case the cells were labeled for 8 h). Cells were then harvested, solubilized, followed by immunoprecipitation of LTC_4S with an anti-LTC_4S antibody pre-bound to protein A-Sepharose, washed, separated by a 10–20% SDS-PAGE and visualized by autoradiography. Four independent experiments were performed and represented here is one typical one.

rylation was found to peak at 4 h and then decrease to basal levels for both PMA activated cells and DMSO controls (Fig. 3).

Of interest was the trend observed with the DMSO vehicle controls where the level of phosphorylation was also seen to increase up to 4 h and then return to basal levels at 8 h. This confirms that the observed phosphorylation event was not only a consequence of induction by PMA but is also significant physiologically under no stimulus and follows a particular kinetic profile. The same trend was evident in the absence of DMSO thereby confirming that the trend was not an artifact due to DMSO (data not shown).

3.4. Inhibition of LTC_4S phosphorylation by PKC-specific inhibitors

In order to shed light on the protein kinase responsible for the phosphorylation of LTC_4S, inhibitors of PKC, PKA, and of tyrosine kinase were tested for their ability to block the observed PMA-mediated phosphorylation. Staurosporine, bisindolylmaleimide, and GF109203X, all specific inhibitors of PKC, dose-dependently attenuated the phosphorylation of LTC_4S evoked by PMA (Fig. 4). The effects of staurosporine were apparent at a concentration of 0.1 μM, bisindolylmaleimide was effective at 1 μM, and GF109203X at 7.5 μM. These PKC inhibitors were found to be capable of attenuating the level of phosphorylation to basal levels and below at higher inhibitor concentrations. In contrast, inhibitors of tyrosine kinase, namely, herbimycin and tyrophostin and of PKA, KT5270, were found to produce no inhibition. This evidence suggests a PKC-mediated cascade of events either directly or via other proteins, leading to the phosphorylation of LTC_4S, and is in accordance with previous studies on PMA-mediated attenuation of LTC_4S activity being restored upon treatment with inhibitors of PKC.

4. Discussion

Several independent studies have demonstrated downregulation of LTC_4S enzymatic activity by phosphorylation, initiated by a cascade of kinases, in particular PKC [20–22]. LTC_4S activity measured in neutrophilic [21], and eosinophilic [20] cell lines and in human granulocytes [22], challenged with PMA have demonstrated that the activity was inhibited resulting in an attenuation of cysteinyl LTs without affecting non-cysteinyl LT biosynthesis. Moreover, the attenuation of the activity was completely prevented in the presence of inhibitors

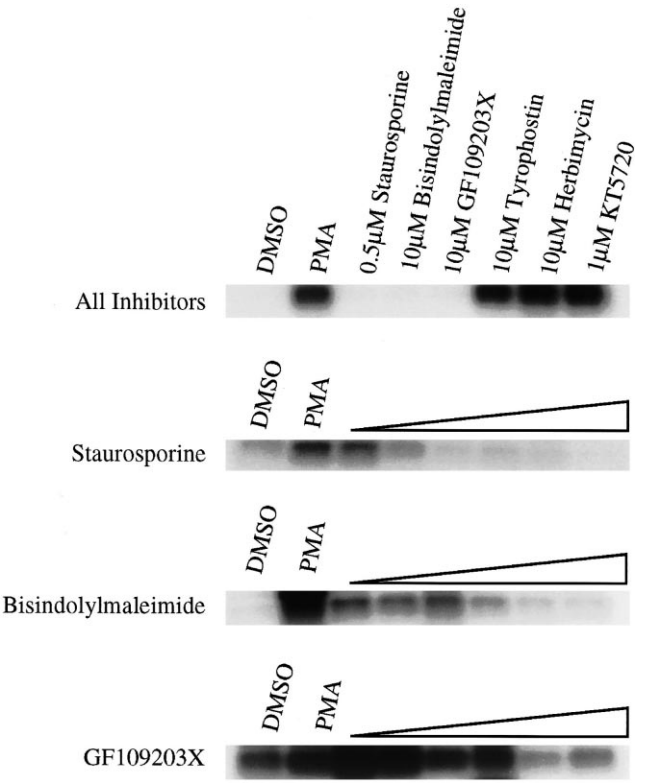

Fig. 4. Phosphorylation of LTC_4S is a PKC-mediated event. THP-1 cells were grown to confluency and labeled with 0.5 mCi/ml aqueous [^{32}P]orthophosphate for a period of 4 h. During labeling, cells were treated with or without 50 nM PMA in the presence or absence of a range of kinase inhibitor concentrations. Staurosporine, 0.05–10 μM; bisindolylmaleimide, 0.5–10 μM; GF109203X, 0.5–10 μM; tyrophostin, 10 μM; herbimycin, 10 μM; and KT5720, 1 μM. Cells were then harvested, solubilized, followed by immunoprecipitation of LTC_4S with an anti-LTC_4S antibody pre-bound to protein A-Sepharose, washed, separated by a 10–20% SDS-PAGE and visualized by autoradiography. Represented data is one of three typical independent experiments.

of PKC but not by inhibitors of tyrosine kinase nor protein kinase A. The presence of two putative PKC consensus sites on LTC_4S was another indicator for the potential for LTC_4S phosphoregulation [9,19]. Prior to the present study, there was no evidence of a direct phosphorylation event upon LTC_4S. This study clearly indicates that LTC_4S is phosphorylated at the protein level in THP-1 cells, not only in response to PMA activation, but also at a lower basal level which may be attributed to a pool of LTC_4S which consists of both phosphorylated and non-phosphorylated forms of the enzyme. Therefore in our study, the levels of phosphorylation were compared with basal phosphorylation levels.

The lack of LTC_4S phosphorylation in COS-7 and K-562 cells indicates THP-1 cell-specificity. Numerous reasons may explain this observed specificity, including the lack of an appropriate target for PMA, downstream effectors, and the localization of LTC_4S in these cells as compared to THP-1 cells.

An interesting observation was made from the time-course of activation which revealed that the phosphorylation event followed a particular kinetic profile. The level of LTC_4S phosphorylation was seen to increase with time of PMA activation up to 4 h, after which the enzyme became dephosphorylated to basal levels after 8 h of activation, suggesting a negative

feedback mechanism. This feedback loop may generate signals which may allow the enzyme to dephosphorylate itself, returning the enzyme to its active state. The slight increase seen in the level of LTC_4S phosphorylation in the DMSO control samples (Fig. 3) can be attributed to the exchange of endogenous ^{31}P for ^{32}P.

The level of phosphorylation was markedly influenced by the cellular density. A recent study in NIH 3T3 mouse fibroblasts showed that as cells became confluent the phosphorylation of c-Jun following UVC irradiation or growth factor stimulation was strongly inhibited. It was also demonstrated that this inhibition was due to the inhibition of the activation of the stress kinase pathways (JNK/SAPKs and p38) whereas classical MAPK activation was not affected by cell density [23]. Other than possible cell-cell interactions, a difference in cell density can also result in a change in numerous other parameters. For example, the pH and the acidity of the media may be influenced by the confluency leading to a change in the environment of the cell. These changes may in turn effect the behavior of the cell and its state of basal levels of phosphorylation. In order to avoid artifactual interferences as a result of this effect, the confluency at which the cells were used throughout the study was kept constant.

The inhibitor profile of this and previous studies indicates that protein kinase C is either directly or indirectly responsible for LTC_4S phosphorylation. There have been reported to be up to 11 isoforms of PKC present in either cytosolic or nuclear pools [24]. THP-1 cells have been examined for the presence of α, β, γ, δ and ε classes of PKC and only α and δ are expressed in this cell line [25], which suggests that one of the two may be the kinase which regulates LTC_4S.

In summary, we have demonstrated for the first time that LTC_4S is directly phosphorylated in THP-1 cells at the protein level, an event which is initiated specifically by protein kinase C. Mutagenic analyses of LTC_4S has recently shown the involvement of residues arginine-51 and tyrosine-93 for the opening of the epoxide ring of LTA_4 and the activation of GSH, respectively [26]. Determination of the site(s) of phosphorylation would shed light on the mechanism by which the enzyme loses its activity. Phosphorylation may cause a change in the 3-dimensional structure of the enzyme, sterically hinder the substrate binding sites, directly prevent substrate interaction or activation, or perhaps allow for another inhibitory entity to interact with LTC_4S, all leading to the down-regulation of its activity.

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