Leukotriene D₄ Mediates Survival and Proliferation via Separate but Parallel Pathways in the Human Intestinal Epithelial Cell Line Int 407

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RUNNING TITLE: LTD₄ regulates proliferation and survival via two pathways
SUMMARY

We previously demonstrated that leukotriene D₄ (LTD₄) regulates proliferation of intestinal epithelial cells through a CysLT receptor by PKCε-dependent stimulation of the mitogen-activated protein kinase Erk-1/2. Our current study provides the first evidence that LTD₄ can activate 90-kDa ribosomal S6 kinase (p90RSK) and cAMP response element-binding protein (CREB) via pertussis-toxin-sensitive Gi protein pathways. Transfection and inhibitor experiments revealed that activation of p90RSK, but not CREB, is a PKCε/Raf-1/Erk-1/2-dependent process. LTD₄-mediated CREB activation was not affected by expression of kinase-dead p90RSK but was abolished by transfection with the regulatory domain of PKCα (a specific dominant inhibitor of PKCα). Kinase-negative mutants of p90RSK and CREB (K⁻p90RSK and K⁻CREB) blocked the LTD₄-induced increase in cell number and DNA synthesis (thymidine incorporation). Compatible with these results, flow cytometry showed that LTD₄ caused transition from the G₀/G₁ to the S+G₂/M cell cycle phase, indicating increased proliferation. Similar treatment of cells transfected with K⁻p90RSK resulted in cell cycle arrest in the G₀/G₁ phase, consistent with a role of p90RSK in LTD₄-induced proliferation. On the other hand, expression of K⁻CREB caused a substantial build up in the sub-G₀/G₁ phase, suggesting a role for CREB in mediating LTD₄-mediated survival in intestinal epithelial cells. Our results show that LTD₄ regulates proliferation and survival via distinct intracellular signaling pathways in intestinal epithelial cells.
INTRODUCTION

Cysteinyl leukotrienes (LTC\textsubscript{4}, LTD\textsubscript{4}, and LTE\textsubscript{4}) belong to an important group of pro-inflammatory mediators that are derived from arachidonic acid via the 5-lipoxygenase pathway that have a number of pathophysiological functions in the inflammatory process, such as induction of proliferation and contraction of smooth muscle cells, promotion of eosinophil migration, and amplification of vascular permeability (1, 2, 3). LTD\textsubscript{4} is the most potent of the cysteinyl leukotrienes, and its effects are mediated through a seven-transmembrane G-protein-coupled receptor type. Two such receptors have been cloned and characterized as CysLT\textsubscript{1} and CysLT\textsubscript{2} (4, 5, 6). In binding studies, the CysLT\textsubscript{1} receptor has been shown to have the highest affinity for LTD\textsubscript{4} and is generally believed to be the most important receptor in mediating the functional effects of this cysteinyl lekotriene (4). The CysLT\textsubscript{2} receptor, on the other hand, exhibit equal binding affinities for LTD\textsubscript{4} and LTC\textsubscript{4} (7). Some of the G proteins to which CysLT receptors are linked are sensitive to pertussis toxin (PTX), whereas others are not. This indicates that different signal transduction pathways can be initiated by CysLT receptors, thus enabling this receptor type to mediate a variety of functional activities in the same cell (8, 9).

Sheng et al. (10) have suggested that there is a connection between inflammation and the development of cancer, which is also supported by results showing that ulcerative colitis is associated with an increased incidence of neoplastic transformation (11) and the observation that colon cancer is underrepresented in populations treated with non-steroidal anti-inflammatory drugs (12). Furthermore, in previous experiments using non-transformed intestinal epithelial cells (13), we found that prolonged exposure to LTD\textsubscript{4} resulted in upregulation of several proteins associated with colon carcinogenesis, among others.
cyclooxygenase-2 (COX-2), β-catenin, and the cell-survival protein Bcl-2 (13, 14). We have also recently shown that the CysLT₁ receptor is upregulated in colon cancer tissues and that LTD₄ signaling facilitates survival of colon cancer cells (15) and non-transformed epithelial cells (13). These findings are interesting, since both COX-2 and CysLT₁ are accessible targets for drug therapy. In addition to its effect on survival, LTD₄ can induce a proliferative response in intestinal epithelial cells via a signaling pathway that includes activation of a PTX-sensitive G protein, PKCε, Raf-1, and the mitogen-activated protein kinase (MAPK) Erk-1/2 (16). These data indicate that the inflammatory mediator LTD₄ can contribute to increased survival and growth of intestinal epithelial cells in pathological inflammatory conditions, although the effectors situated downstream of Erk-1/2 have not been identified.

Considering the ways in which Erk-1/2 can mediate its effect on proliferation and survival in intestinal epithelial cells, two major downstream targets/substrates are of particular interest: the p90-kDa ribosomal S6 kinase (p90RSK) and the cAMP response element-binding protein (CREB). Experiments performed in vitro and in vivo have revealed that Erk-1/2 can interact and directly phosphorylate p90RSK on several different serine and threonine residues (17, 18). The protein p90RSK is unique among serine-threonine kinases in that it contains two functional kinase domains (19), and it has been suggested that regulation of the N-terminal catalytic domain of p90RSK is mediated by activation of the C-terminal catalytic domain that occurs through MAPK-induced phosphorylation (17). The other potential target/substrate of Erk-1/2 that might mediate the effect of this protein on intestinal cell proliferation is CREB. The carboxyl terminus of CREB contains a basic DNA-binding domain and an adjacent leucine zipper domain, the latter of which is required for dimerization of CREB (20). CREB also has a transactivation domain that contains several
independent regions, including one identified as the kinase-inducible domain, which comprises consensus phosphorylation sites for several kinases, among them protein kinase A (21). Kinase-induced phosphorylation of Ser\textsuperscript{133} on CREB facilitates attachment of this protein to the 256-kDa CREB-binding protein (CBP). The CREB-CBP complex can, in turn, interact with and activate the basal transcription machinery (21). Previous studies have demonstrated that multiple signaling pathways can mediate the phosphorylation and activation of CREB in different cell lines. Transcriptional activation of CREB, through phosphorylation of the Ser\textsuperscript{133} residue, can obviously be induced by the catalytic subunit of the cAMP-activated protein kinase A (21). However, additional serine/threonine kinases, such as protein kinase C (PKC; 22), p38 MAPK (23), Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CAMK; 24), a Ras-dependent p105 kinase (25), and, interestingly enough, also p90\textsuperscript{RSK} (22), have been shown to phosphorylate and activate CREB. In some cases, several of these kinases can participate in the same agonist-induced signaling pathway; for example, engagement of the T cell receptor leads to a rapid phosphorylation and activation of CREB via a signaling cascade that involves stimulation of the tyrosine kinase p56\textsuperscript{ck}, PKC, Ras, Raf-1, Erk-1/2, and p90\textsuperscript{RSK2} (26).

The participation of PKC isoforms in activation of CREB is particularly interesting, because we have previously shown that the LTD\textsubscript{4}-induced proliferative response in intestinal epithelial cells is mediated via activation of PKC\textgreek{e} (16). PKCs are members of a family of serine/threonine kinases that have been shown to modulate diverse cellular functions, including proliferation, differentiation, and gene activation (27). There are at least eleven isoforms of PKC, which are classified as conventional (\alpha, \beta\textsl{I}, \beta\textsl{II}, and \gamma), novel (\delta, \epsilon, \theta, \eta), and atypical (\zeta, \upsilon/\lambda), and also PKC\textgreek{m}. Upon activation, the individual isoforms can exert
unique effects in the cell. In NIH3T3 cells, overexpression of PKCε has been found to increase the growth rate, whereas PKCδ has the opposite effect (28), and PKCα has been implicated in an anti-apoptotic response in COS-1 cells (29).

The present results show that LTD₄ activates both CREB and p90RSK, but these effects occur via separate but parallel signaling pathways: for CREB a PKCα-dependent pathway, and for p90RSK a PKCε–Raf-1–Erk-1/2 signaling cascade. Based on our observations in cells transfected with dominant inhibitory mutant signaling proteins and in cells treated with pharmacological inhibitors, we conclude that, in intestinal epithelial cells, LTD₄-induced activation of the PKCα-CREB signaling pathway favors survival, whereas simultaneous activation of the Erk-1/2–p90RSK pathway mediates a proliferative response.
EXPERIMENTAL PROCEDURES

Materials—Phosphospecific antibodies and total antibodies to p90RSK, CREB, p38 MAPK, and the inhibitors SB203580 and PD98059 were purchased from New England BioLabs, Inc. (Beverly, MA). CysLT2 antibody, LTB4 and LTD4 were obtained from Cayman Chemical Company (Ann Arbour, MI), and CysLT1 (N-term) were purchased from Innovagen (Lund, Sweden). ECL Western blot detection reagents, hyperfilm and methyl-[3H] thymidine were from Amersham International (Buckinghamshire, UK). Wortmannin, LY294002, forskolin, KN-62, Gö6976, GF109203X, phorbol 12-myristate 13-acetate (TPA), Rp-cAMPs, and FTI-277 were acquired from Calbiochem (San Diego, CA). HA and Myc antibodies originated from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), pertussis toxin was from Speywood Pharma Ltd. (Maidenhead, UK), and peroxidase-linked goat anti-rabbit and mouse IgG were from Dako A/S (Copenhagen, Denmark). PP1 was purchased from Alexis (San Diego, CA). ZM-198,615 (ICI-198,615) was a gift from Dr. R. Metcalf (Zeneca Pharmaceuticals, Macclesfield, Cheshire, England). All other chemicals were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture—Human intestinal epithelial cells (Int 407; 30) were used in all experiments. These cells, which exhibit typical epithelial morphology and growth, were cultured as a monolayer to approximately 80% confluence for 5 days. Cell cultures were kept at 37°C in a humidified atmosphere of 5% CO2 and 95% air in Eagle's basal medium supplemented with 15% new-born calf serum, 55 IU/ml penicillin, and 55 µg/ml streptomycin. The cells were regularly tested to ensure the absence of mycoplasma contamination.
cDNAs and Transfections—Cells were transfected with different signaling protein constructs for 6 h and were then allowed to grow in medium supplemented with serum for another 24 or 48 h as indicated. The constructs we used were generously provided by the cited investigators: a full-length human HA-tagged dominant-negative Ras construct (N17 Ras; 31), EGFP- or myc-tagged regulatory domains (RDs) of PKCe or α constructs (32), or a W437 kinase-dead flag-tagged PKCe construct (KPKCe), from Dr. Arthur Mercurio (Beth Israel Deaconess Medical Center, Boston, MA); HA-tagged kinase inactive c-Raf construct (K’Raf-1), from Dr. Larry Karnitz (Mayo Clinic, Rochester, MN); a kinase-dead HA-tagged p90RSK (K’p90RSK), from Dr. John Blenis (Harvard Medical School, Boston, MA); a kinase-dead CREB construct (K’CREB), from Dr. Richard H. Goodman (Oregon Health Sciences University, Portland, OR). Transient transfections of the cells were achieved using 3.5 μl of Lipofectamine (Invitrogen life technologies) and 1.8 μg of plasmid DNA/ml of medium, and were performed in serum-free medium, essentially according to the protocol provided by the supplier. In all transfection experiments, it was routinely confirmed that the empty vector had no effect, and the efficiency of transfection was determined by control co-transfections with an empty pEGFP-N1 vector (Clontech), except for the transfections with an EGFP-tagged regulatory domain (RD) of PKCe or α.

Incubations and Lysis of the Cells—Cells in tissue culture flasks were pre-incubated with one of the following: the CysLT1 receptor antagonist ZM-198,615 (50 μM, 15 min); the Gi/o protein inhibitor pertussis toxin (500 ng/ml, 2 h); the MEK inhibitor PD98059 (50 μM, 30 min); the p38 MAPK inhibitor SB203580 (20 μM, 30 min); the phosphoinositide-3 kinase (PI3K) inhibitor LY294002 (50 μM, 30 min); and then the farnesyl transferase inhibitor FTI-
277 (20 μM, 48 h); the CaMKII inhibitor KN-62 (10 μM, 30 min); the two PKC inhibitors GF109203X and Gö6976 (both 2 μM, 30 min). The cells in each of the flasks were subsequently stimulated with 80 nM LTD₄ or 100 nM TPA in the absence or presence of the respective inhibitor for the indicated periods of time. The stimulation was terminated by adding ice-cold lysis buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 4 μg/ml leupeptin, and 30 μg/ml phenylmethanesulfonyl fluoride). Thereafter, the cells were kept on ice for 30 min in the lysis buffer, and the remaining cell debris was scraped loose from the bottom of the flasks into the buffer. The lysates were homogenized 10 times on ice with a glass tissue grinder (Dounce) and then centrifuged at 10,000 x g for 15 min. The supernatants were collected, the protein content was measured and compensated for, and further processed for protein separation by gel electrophoresis.

_Gel electrophoresis_—Cell lysates were solubilized by boiling at 100°C for 5 min in a sample buffer (final concentrations of the components: 62 mM Tris [pH 6.8], 1.0% SDS, 10% glycerol, 15 mg/ml dithiothreitol, and 0.05% bromophenol blue), and the solubilized proteins were separated by electrophoresis on a 10–12% homogeneous polyacrylamide gel in the presence of SDS.

_Immunoblotting_—The separated proteins were electrophoretically transferred to PVDF membranes. The membranes were blocked for 1 h with 5% non-fat dried milk at room temperature and then incubated with a primary antibody (diluted 1:1,000) for 1 h at room temperature or overnight at 4°C. The membranes were subsequently washed extensively and
incubated with a horseradish peroxidase-linked goat anti-rabbit, anti-sheep, or anti-mouse antibody (1:5,000) for 1 h at room temperature. Thereafter, the membranes were extensively washed, incubated with ECL Western blot detection reagents, and finally exposed to hyperfilm-ECL to visualize immunoreactive proteins. The phospho-p90<sup>RSK</sup> and phospho-CREB blots were routinely stripped and reprobed to determine the total amounts of p90<sup>RSK</sup> and CREB.

**Immunofluorescence**—The cells were seeded on glass coverslips and grown for five days. During the last 24 h of the incubation, the cells were cotransfected with dominant-negative K-p90<sup>RSK</sup> and EGFP vector or transfected with EGFP-tagged RD-PKCα as described above. Thereafter, the cells were serum-starved for 2 h and stimulated with LTD₄. The stimulation was terminated by fixing for 10 min at room temperature in a 3.7% paraformaldehyde/PBS solution, after which the cells were permeabilized in a 0.5% Triton X-100/PBS solution for 15 min. The coverslips were subsequently washed twice in PBS and incubated at room temperature in a 3% BSA/PBS solution for 15 min, and the cells were stained for 1 h with a phosphospecific antibody against CREB. The coverslips were then washed six times in PBS and incubated with Alexa Fluor 568 goat anti-rabbit secondary antibody (diluted 1:200 in blocking buffer). The coverslips were thereafter washed six times in PBS and mounted in fluorescent mounting medium (DAKO A/S). Samples were examined and photographed with a Nikon Eclipse 800 microscope, using a Plan-Apo 60 X objective. Images were recorded with a scientific-grade, charge-coupled device (CCD) camera (Hamamatsu, Japan) and subsequently analyzed with HazeBuster deconvolution software (Vay Tek, Inc., Fairland, CT).
Cell Counting and Thymidine Incorporation—The cells were cotransfected with dominant-negative K−p90RSK or dominant-negative KCREB and EGFP vector or transfected with EGFP vector alone and allowed to grow for another 24 h in medium supplemented with serum as described above. Thereafter, the cells were serum-starved and stimulated with LTD$_4$ and or inhibitors as indicated in fig. 6A. During the course of the experiment, fresh media, LTD$_4$ and inhibitors were added every 24 h. To determine the number of viable cells, counting was done in the presence of 0.2% trypan blue after treatment for the periods of time indicated in fig. 6A. For thymidine incorporation, cells were cultured in 24-well plates and cotransfected as described above or incubated with the indicated inhibitors. In case of transfected samples, cells were either transfected with empty vector or with the vector indicated for 6 h and were then allowed to grow for an additional 24 h in medium supplemented with serum. Cells were then stimulated with LTD$_4$ for 48 h and cellular DNA synthesis was assayed by adding 0.5 µCi [$^3$H]-labeled thymidine during the last 24 h of stimulation. Cells were washed twice with PBS, treated with 10% trichloroacetic acid for 30 min, and then lysed in 1 M NaOH. The level of radioactivity, indicating the incorporation of [$^3$H]thymidine into DNA, was measured using a beta liquid scintillation counter (LKB RackBeta, Wallac).

Flow Cytometry—The cells were cotransfected with dominant-negative K−p90RSK or dominant-negative KCREB and EGFP vector or transfected with EGFP vector alone and allowed to grow for another 24 h in medium supplemented with serum as described above. Thereafter, the cells were serum-starved and stimulated with 80 nM LTD$_4$ for 48 h in the absence or presence of the indicated inhibitors. During the course of the experiment, fresh
media, LTD₄ and inhibitors were added after 24 h. Cells that had spontaneously detached from the monolayer and were floating in the medium were collected by centrifugation and mixed with cells that had been detached from the same monolayer by the addition of trypsin-EDTA. These cells were slowly fixed by adding ice-cold ethanol while gently vortexing the sample. The DNA content of each sample (approximately 2 x 10⁵ cells) was estimated by staining with propidium iodide using the Vindelöv reagent (33) and then filtering (40 μm) the samples and quantifying the fluorescence by use of a Cell Quest FACScan flow cytometer (Becton Dickinson). Each measurement was based on analysis of 10,000 cells (events).
RESULTS

*LTD₄ Induces Phosphorylations of p90RSK and CREB in a Time-dependent Manner—*

To examine the ability of LTD₄ to activate p90RSK and/or CREB, we studied the effects of this leukotriene on the phosphorylation status of these two proteins. We found that LTD₄ significantly increased the level of phosphorylation of both p90RSK and CREB. For p90RSK, the phosphorylation was rapid and transient (Fig. 1A), reaching a maximum (3.5-fold increase) after only 5 min and approaching the initial basal level after 90 min. By comparison, the LTD₄-induced phosphorylation of CREB was also transient but had a slower onset, reaching a maximum level after 30 min and starting to decline after 90 min of exposure to the leukotriene (Fig. 1B). In our investigation of the intracellular signaling pathways involved in LTD₄-induced activation of p90RSK and CREB, our first objective was to confirm the participation of a PTX-sensitive G protein in the initial signaling of LTD₄. We incubated cells with 500 ng/ml PTX for 2 h and then stimulated them with LTD₄ for 5 min (p90RSK) or 30 min (CREB). These periods of time were chosen because they represent the time required to reach maximum levels in p90RSK and CREB activities (Figs. 1A, B) and were subsequently used throughout this study when testing the effects of transfections and inhibitors on their activities. PTX inhibited the LTD₄-provoked activation of both p90RSK and CREB (Fig. 1C), which agrees well with our previous observation that the LTD₄-induced proliferative response in intestinal epithelial cells is mediated via a PTX-sensitive G protein (16). Western blot analysis of cell lysates show expression of both CysLT₁ and CysLT₂ receptors in these cells (Fig. 1D). We therefore tested the sensitivity of the LTD₄-induced p90RSK and CREB activity for the CysLT₁ receptor antagonist ZM-198,615. Our data show that the LTD₄–induced activation of both p90RSK and CREB could be blocked by this
antagonist, although at a relatively high concentration, 50 μM (Fig. 1E). Consequently, we cannot in the present study conclude if the LTD₄ effects are mediated via either or both the CysLT₁ and the CysLT₂ receptor. For comparison, we also examined the ability of LTB₄ to activate p90RSK and CREB. It is clear that LTB₄ induces phosphorylation of both p90RSK and CREB to a similar degree as LTD₄ (Fig. 1F). This finding is in good agreement with our previous observation that LTB₄ also triggers a proliferative response in intestinal epithelial cells (34). The total levels of p90RSK and CREB during the LTB₄- and LTD₄-induced stimulations were unchanged (Fig. 1 A–E).

**LTD₄-induced Activation of p90RSK Is Mediated by an Erk-1/2 Signaling Pathway**—

We have previously found that, in addition to and downstream of G-protein activity, the LTD₄-induced proliferative response requires the sequential activation of PKCe, Raf-1, and Erk-1/2 (16). To ascertain whether the LTD₄-elicited activation of p90RSK is a downstream target/substrate of this signaling pathway, we first examined the ability of this leukotriene to activate p90RSK in cells transfected with kinase-dead PKCe (K–PKCe). The results showed that the expression of K–PKCe completely blocked LTD₄-induced stimulation of p90RSK (Fig. 2A). In subsequent experiments, we also found that such activation of p90RSK was inhibited in cells transfected with an HA-tagged kinase-dead Raf-1-expressing vector (K–Raf-1) (Fig. 2B). Thereafter, we pre-incubated non-transfected cells with PD98059, which is a MEK inhibitor that prevents the activation of Erk-1/2, and observed that this treatment completely blocked the LTD₄-mediated activation of p90RSK (Fig. 2C). Our data indicate that p90RSK is a downstream substrate/target of the LTD₄-induced PKCe, Raf-1 and Erk-1/2 signaling cascade.
LTD4-induced Activation of CREB Is Not Mediated by PKA, p90RSK, p38 MAPK, PI3K, Ras or CAMK Signaling Pathways—An intracellular increase in cAMP is known to cause activation of CREB via an activation of protein kinase A (PKA; 21). In agreement with this, our control experiments revealed that forskolin-induced activation of adenylate cyclase caused significant activation of CREB also in intestinal epithelial cells (Fig. 3A). However, the PKA inhibitor Rp-cAMPs did not block the LTD4-mediated phosphorylation of CREB (Fig. 3A). G-protein-coupled receptors have been shown to propagate membrane receptor signals to the nucleus through Erk-1/2-induced activation of CREB (22). Consequently, we next performed experiments to determine whether the signaling cascade by which LTD4 induces activation of p90RSK (Fig. 2) also leads to stimulation of CREB. However, neither transfection of the regulatory domain (RD) of PKCε nor use of the kinase-dead mutant of PKCε had any effect on the LTD4-induced activation of CREB (Fig. 3B). It has previously been shown that RDs can act as specific dominant-negative inhibitors of their PKC isoforms (16, 35). Furthermore, we noted that neither transfection of cells with kinase-dead Raf-1 nor inhibition of Erk-1/2 activation had any effect on the LTD4-mediated phosphorylation of CREB (Fig. 3B and C). Likewise, activation of CREB by LTD4 was not influenced by transfection of cells with HA-tagged kinase-dead p90RSK (Fig. 3D, lane 3, 4). Together these data indicate that LTD4-induced stimulation of CREB does not involve the PKCε–Raf-1–and Erk-1/2 signaling cascade or activation of p90RSK, which suggests that this leukotriene activates p90RSK and CREB via independent but parallel signaling pathways.

In an attempt to identify signaling molecules that participate in LTD4-induced activation of CREB, we studied regulatory proteins that have previously been found to be
involved in stimulation of CREB, among others the p38 MAPK (23). We found that LTD₄ caused a significant (threefold) activation of p38 MAPK (Fig. 3E). However, SB203580 had no effect on the LTD₄-generated phosphorylation of CREB although it inhibited the activation of p38 MAPK (Fig. 3E). In light of our earlier results showing that LTD₄ also activates Ras (16) and PI 3-kinase (36) in intestinal epithelial cells, and also a report implicating a Ras-dependent kinase in the activation of CREB (25), we conducted further work to investigate possible participation of a Ras and/or PI3K signaling pathway(s) in LTD₄-mediated phosphorylation of CREB. We found that activation of CREB was not prevented by the following: pre-incubation with the PI3K inhibitor LY294002 (Fig. 3F), exposure to the farnesyltransferase inhibitor FTI-277, or transfection with the dominant-negative mutant N17 Ras (Fig. 3G). Even though inhibition of PI3K and Ras did not reduce the CREB phosphorylation, our data might instead suggest that these signals negatively regulate CREB (Fig. 3F, G). But a great deal of work is obviously needed to confirm such roles for these signaling molecules. Also of interest in this context, CAM kinases, especially CAMK II, have been reported to be involved in phosphorylation of CREB in other types of cells (24), but we found that the CAMK inhibitor KN-62 had no effect on LTD₄-stimulated CREB phosphorylation (Fig. 3H).

*Classical PKC Isoform(s) Are Involved in LTD₄-induced Activation of CREB*—Other investigators have described PKC-mediated activation of CREB in different kinds of cells (22, 24), whereas we found that neither kinase-dead PKCe nor the RD of PKGε had an impact on LTD₄-induced activation of CREB (Fig. 3B). However, since several isoforms of PKC are expressed in intestinal epithelial cells, and LTD₄ causes activation not only of
PKCε but also of the α and δ isoforms, we examined the influence of the phorbol ester TPA on CREB phosphorylation. TPA (100 nM, 15 min) treatment led to an unambiguous activation of CREB which was reduced by Gö6976 (2 μM, 30 min; Fig. 4A). The LTD₄-induced (80 nM, 30 min) phosphorylation of CREB was blocked by both GF109203X (2 μM, 30 min) and Gö6976 (Fig. 4B). Notably, we used these two inhibitors at concentrations that are known to prevent activation of the classical isoforms of PKC, which suggests that such PKCs play a role in the LTD₄-mediated activation of CREB. Under similar conditions, these classical PKC inhibitors had no effect on the LTD₄-mediated p90RSK activation (80 nM LTD₄ for 5 min; Fig. 4B). We have previously shown that (16, 37) different PKC isoforms vary in regard to their sensitivity to prolonged TPA-induced downregulation. Cells exposed to TPA (1 μM) for 16 h completely downregulated their expression of PKCα, whereas their expression of another classical PKC isoform, βII, was not affected (Fig. 4C). That finding, together with our observation that pretreatment with TPA significantly decreased a subsequent LTD₄-mediated phosphorylation of CREB, suggests that PKCα is involved in activation of CREB (80 nM LTD₄ for 30 min; Fig. 4D). Accordingly, we transfected the cells with the myc-tagged RD of PKCα and found that expression of PKCα-RD blocked LTD₄-induced phosphorylation of CREB (Fig. 4E, lane 3, 4). These data confirm the signaling function of PKCα in LTD₄–induced activation of CREB.

To gain further evidence that PKCα is involved in LTD₄-induced activation of CREB, we used deconvolution microscopy to examine cells transfected with EGFP-tagged PKCα-RD or Kp90RSK. These cells were stimulated with LTD₄ and then immunostained with a phospho-CREB antibody (Fig. 5). The antibody staining was weak in untreated control cells but was strong in the nuclei of the LTD₄-stimulated cells (Fig. 5A). This LTD₄-
induced CREB phosphorylation was not impaired by expression of K–p90RSK, which again indicates that such phosphorylation does not require an activation of p90RSK (Fig. 5A). On the other hand, expression of an EGFP-tagged RD of PKCα completely abolished the LTD4-mediated phosphorylation of CREB (Fig. 5B), which further implies that PKCα is involved in the LTD4-induced signal that leads to CREB phosphorylation.

We have previously observed that LTD4 increases the survival (13) and proliferation (16) of intestinal epithelial cells, and in the current study we therefore evaluated the roles of LTD4-induced activation of p90RSK and CREB in those processes. The growth rate of the intestinal epithelial cells was elevated in the presence of LTD4, the results suggest that the LTD4-induced increase in growth rate was limited to the first two days (Fig. 6A). This conclusion is also supported by the LTD4-induced increase in thymidine incorporation, evaluated between 24 and 48 hours (Fig. 6B). Cells expressing either K–p90RSK or K–CREB and subsequently stimulated with LTD4, the growth rate was even less than that exhibited by unstimulated control cells (Fig. 6A). Inasmuch as the transfection efficiency was not 100% in those experiments, we also tested inhibitors of Erk-1/2 (PD98059) and PKCα (Gö6976), which respectively blocked the LTD4-induced activation of p90RSK and CREB (Fig. 6A). As expected, these inhibitors counteracted the LTD4-induced increase in growth rate even more than that obtained by transfection with K–p90RSK or K–CREB (Fig. 6A). Similar results were obtained when cell growth was assayed by thymidine incorporation. Cells stimulated with LTD4 (80 nM, 48 h) exhibited a 164% increased uptake of thymidine that was reduced to 78 and 68% by Gö6976 and PD98059, respectively (Fig. 6B). In cells transfected with an empty vector LTD4 stimulation caused a 159% increased uptake of thymidine that was
reduced to 107 and 100% when the cells were transfected with K-p90RSK and K-CREB, respectively (Fig. 6B).

The effects of inhibition of p90RSK and CREB on LTD4-induced cell growth were further examined by flow cytometry (Fig. 6C, D). In these experiments, the cells were stained with propidium iodide, which becomes fluorescent when it binds to DNA, thus the level of fluorescence is directly proportional to the amount of cellular DNA. Cells containing an unreplicated complement of DNA are in G0/G1 phase, and those that have a fully replicated complement of DNA are in G2/M phase. Compared to unstimulated control cells, cells stimulated with LTD4 exhibited a significantly lower G0/G1 peak but a higher S + G2/M peak, which indicates elevated synthesis of DNA (Fig. 6C, D). Cells transfected with K-CREB or treated with the PKC inhibitor Gö6976 and then exposed to LTD4 accumulated in the sub-G0/G1 phase (Fig. 6C, D), which suggests an elevated level of cell death. In contrast, examining cells transfected with K-p90RSK or treated with the Erk-1/2 inhibitor PD98059, we found that exposure to LTD4 caused a significant arrest in the G0/G1 phase, a totally reverted S + G2/M transition phase, and only a slight change on the sub-G0/G1 peak (Fig. 6C, D), which indicates that p90RSK is involved in progression of the cell cycle.

Our data is summarized in the schematic model outlined in fig. 7. This model reveals our present knowledge of the LTD4-induced signaling pathways involved in the activation of CREB and p90RSK and how these kinases affect the cellular regulation of survival and proliferation.
DISCUSSION

We have previously found that the pro-inflammatory mediator LTD$_4$ induces proliferation of intestinal epithelial cells, Int 407, by $G_i$–protein initiated dual pathways: the Ras-independent PKC$_\varepsilon$–Raf-1–Erk-1/2 cascade and the Ras-dependent pathway (16). In the present study, we again used Int 407 cells and investigated the signaling routes responsible for activation of two downstream proteins implicated in cell proliferation, p$^{90}_{RSK}$ and CREB. Our results are the first to show that LTD$_4$ mediates the phosphorylation and thus activation of p$^{90}_{RSK}$ and CREB in a transient and time-dependent manner. Moreover, LTB$_4$ and LTD$_4$ had almost identical effects on p$^{90}_{RSK}$ and CREB phosphorylation, suggesting that several inflammatory mediators could have stimulatory effects on survival and proliferation of intestinal epithelial cells.

It is well known that p$^{90}_{RSK}$ is a substrate of Erk-1/2, and several reports have suggested that, when activated, both Erk-1/2 and p$^{90}_{RSK}$ are translocated to the nucleus, where p$^{90}_{RSK}$ phosphorylates CREB at Ser$^{133}$ (38, 39). In accordance, we found that activation of these two proteins by LTD$_4$ is stimulated via a $G_i$ pathway and that LTD$_4$ induced phosphorylation of CREB in the nuclei of intestinal cells. We found that expression of kinase-dead K$^{-}$Raf-1 and K$^{-}$PKC$_\varepsilon$ constructs blocked LTD$_4$-mediated phosphorylation of both Erk-1/2 and p$^{90}_{RSK}$ as did pre-incubation with the MEK inhibitor PD98059. Furthermore, our observation that p$^{90}_{RSK}$ was activated more rapidly than CREB by LTD$_4$, and earlier investigations demonstrating that CREB can be phosphorylated via an Erk signaling pathway (18, 22), makes it plausible to assume that LTD$_4$ regulates activation of CREB through p$^{90}_{RSK}$ phosphorylation. However, both overexpression of K$^{-}$PKC$_\varepsilon$/PKC$_\varepsilon$-RD constructs and pre-incubation with the MEK inhibitor PD98059 blocked LTD$_4$-mediated...
activations of Erk-1/2 and p90<sup>RSK</sup> but did not affect the phosphorylation of CREB induced by this leukotriene. In addition, transfection of cells with K-p90<sup>RSK</sup> constructs had no effect on CREB phosphorylation. These results indicate that LTD<sub>4</sub> activates p90<sup>RSK</sup> and CREB by different mechanisms.

The members of the CREB family have been extensively characterized as nuclear substrates of PKA. We found that a strong activation of the PKA signaling pathway in intestinal epithelial cells by forskolin caused phosphorylation of CREB. However, the present LTD<sub>4</sub>-mediated CREB phosphorylation was not mediated by such a PKA signal. We could also rule out the participation of other potential signals previously suggested to mediate CREB phosphorylation such as p38 MAPK (23), PI3K (40) and Ras (25) in the present LTD<sub>4</sub>-induced CREB phosphorylation.

CREB proteins have also been shown to in certain situations be phosphorylated by PKCs at multiple sites, including Ser<sup>133</sup> (22). A role for PKCs is further implied by observations demonstrating that the phorbol ester and PKC activator TPA stimulates CREB in several cell systems (22), which agrees with our finding that TPA caused substantial phosphorylation of CREB in Int 407 cells. Experiments on the human erythroleukemia cell line TF-1 have shown that PKC<sub>e</sub> stimulates a CREB-dependent transcription in response to βcR cytokines (41). However, our experiments with PKC inhibitors suggested that a classical PKC(s) is involved in the LTD<sub>4</sub>-induced activation of CREB. In accordance, downregulation of PKC<sub>α</sub> followed by exposure to LTD<sub>4</sub> completely blocked the activation of CREB. We have previously observed that treatment with TPA for 16 h also downregulates PKC<sub>ε</sub> (16). However, LTD<sub>4</sub>-mediated CREB phosphorylation was not affected by transfection with either kinase-dead K-PKC<sub>ε</sub> or the regulatory domain of PKC<sub>ε</sub> (PKC<sub>ε</sub>-RD) in our present
experiments, thus PKCε can not mediate this phosphorylation. Finally, transfection of cells with PKCα-RD inhibited the LTD₄-induced CREB phosphorylation, which also indicate that the PKCα isoform mediates the LTD₄-induced activation of CREB. Additional work is needed to determine whether LTD₄ activates CREB through direct phosphorylation by PKCs, or if other kinases are involved.

LTD₄ induced significantly an increase in cell proliferation compared to untreated controls, as we had also seen in an earlier study (16). Expression of the kinase-negative mutants of both p90RSK and CREB, in particular the former, significantly inhibited the LTD₄-mediated proliferative response, which supports the notion that the Erk–p90RSK and PKCα–CREB pathways play an active role in the proliferative response of Int 407 cells. The effects of the p90RSK and CREB signaling pathways, induced by LTD₄, on cell proliferation were confirmed by three different methods (cell counting, thymidine uptake and flow cytometry). Furthermore, we have complemented our experimental approach to transfet the cells with the use of relevant and specific inhibitors, since in the latter case all cells are affected. Interestingly enough, a number of studies have shown that CREB regulates the transcription and expression of several proteins involved in cell proliferation, including cyclin A and D₁, proliferating cell nuclear antigen and COX-2 (42, 43, 44). In good agreement, we have previously shown that LTD₄ triggers an increased transcription and expression of COX-2 in intestinal epithelial cells through a Erk-1/2 dependent pathway, as revealed by a COX-2 reporter gene assay and by Western blotting (13, 14, 15). The clinical relevance of these experimental findings is underlined by our recent finding of an increased expression of both COX-2 and the CysLT₁ receptor in colon cancer tissue (15).
To determine whether activation of p90\textsuperscript{RSK} and CREB is involved in cell survival or regulation of the cell cycle, we used these dominant-negative mutants to analyze these two processes in the presence of LTD\textsubscript{4}. Interestingly, we found with flow cytometry that cells treated with LTD\textsubscript{4} exhibited a significantly reduced G\textsubscript{0}/G\textsubscript{1} phase, and they also showed a build up in the S + G\textsubscript{2}/M phases, which indicates proliferation. Furthermore, the sub-G\textsubscript{0}/G\textsubscript{1} phase was lowered to some extent in LTD\textsubscript{4}-treated cells compared untreated control cells. An increase in the sub-G\textsubscript{0}/G\textsubscript{1} phase, indicating cell death, occurred after inhibition of PKC\textalpha\textsubscript{a}-induced CREB activation or overexpression of K-CREB and subsequent exposure to LTD\textsubscript{4}. These data indicate that CREB is related to LTD\textsubscript{4}-mediated cell survival. In support of such a concept, expression of dominant negative PKCs induced apoptosis in COS cells. However, co-expression of wild-type PKC\textalpha\textsubscript{a} rescued these cells from apoptosis (29). Furthermore, studies in different cell types have also shown that CREB promotes cell survival and protects against apoptosis provoked by diverse stimuli (45, 46, 47), whereas expression of dominant-negative CREB leads to increased susceptibility to apoptosis (46). Notably, induction of Bcl-2 has been reported to occur in a CREB-dependent manner (48, 49). In prostrate cancer cell lines expression of CREB repressed inhibition of Bcl-2 promoter activity caused by the tumor suppressor gene PTEN (50). These investigators also noted that expression of Bcl-2 saved cells from PTEN-induced death but did not stop the cycle in the G\textsubscript{1} phase, suggesting that CREB and Bcl-2 play an active role in survival rather than cell cycle transition in prostrate cancer cell lines. Moreover, earlier investigations in our laboratory revealed that treatment of Int 407 and Caco-2 cells with LTD\textsubscript{4} leads to significant increased expression of Bcl-2 protein (13, 14). Nonetheless, further research is needed to evaluate whether LTD\textsubscript{4}-
induced expression of Bcl-2 in these cells is actually mediated by CREB, and also to
determine whether the survival effect of CREB is transduced via Bcl-2 protein.

In contrast, we found that the arrest in G₀/G₁ phase and the increase in S phase caused
by LTD₄ was significantly inhibited in cells in which the activation of p90RSK was impaired.
In addition, such treatments also caused a relatively low rate of dead cells. It is well known
that Erk mediates cell cycle progression by activating the c-Fos transcription factor through
phosphorylation of Elk-1 (51). In addition, Erk has also been shown to phosphorylate several
other nuclear transcription factors such as JUN, MYC, p62TCF, and FOS, and also regulate
the expression of their downstream genes (38, 51, 52). In studies of the human myelo-
erthroid CD34+ leukemia cell line it was observed that ionomycin-induced MEK-dependent
biphasic activation of Erk-1/2 was sufficient to cause the G₀/G₁ to S/M phase transformation
(53). Involvement of LTD₄ in proliferation has been established in several cell systems (54,
55), but the actual LTD₄ signaling pathways that regulate cell proliferation have not been
thoroughly elucidated. Our results show that LTD₄ increase proliferation by a PKCε–Raf-
1–Erk-1/2 signaling cascade that leads to the activation of p90RSK.

Our present findings are the first to show that LTD₄ can activate p90RSK and CREB
by two distinct signaling pathways and that the CREB pathway is more important for cell
survival, whereas the Erk–p90RSK pathway play a significant role in cell proliferation.
ACKNOWLEDGMENTS

The authors are grateful to Ms. Patricia Ödman for linguistic revision of the manuscript and to Dr. R. Metcalf, Zeneca Pharmaceuticals, Macclesfield, UK, for providing ZM198,615. This work was supported by grants to A.S. from the Swedish Medical Research Council, the Magnus Bergvall Foundation, the Crafoord Foundation, the Foundations at Malmö University Hospital, the Kock Foundation, the Zoega Fondation; by grants to S.P. from the Royal Physiographic Society in Lund.
REFERENCES


7. Heise, C. E., Odowd, B. F., Figueroa, D. J., Sawyer, N., Nguyen, T., Im, D. S.,
Stocco, R., Bellefeuille, J. N., Abramovitz, M., Cheng, R., Williams, D. L., Zeng, Z., Liu, Q.,
Ma, L., Clements, M. K., Coulombe, N., Liu Y., Austin, C. P., George, S. R., Oneill, G. P.,

Chem.*** **265**, 20976-20981


10. Sheng, H., Shao, J., Kirkland, S. C., Isakson, P., Coffey, R. J., Morrow, J.,


54. Braccioni, F., Dorman, S. C., O'byrne, P. M., Inman, M. D., Denburg, J. A.,
   Immunol.* 110, 96-101

   (2002) *FASE J.* 16, 1817-1819
FIGURE LEGENDS

FIG. 1. Time course of LTD₄-induced activation of p⁹⁰RSK and CREB.

Int 407 cells were incubated in the absence or presence of 80 nM LTD₄ for the indicated periods of time and then lysed. The proteins in the lysates were separated by SDS-PAGE and immunoblotted with antibodies specific for phosphorylated p⁹⁰RSK or CREB. Thereafter, the blots were stripped and reprobed for total p⁹⁰RSK or CREB. Representative blots and the accumulated results of densitometric analysis of LTD₄-induced p⁹⁰RSK phosphorylation (A) and CREB phosphorylation (B) are shown. In C, cells were pre-incubated for 2 h in the absence (lanes 1 and 2) or presence of pertussis toxin (500 ng/ml; lane 3) and subsequently stimulated with 80 nM LTD₄ (lanes 2 and 3) for 5 min (p⁹⁰RSK) or 30 min (CREB) and thereafter cell samples were analyzed as in A and B. In D, cells were lysed, two samples were taken from the same lysate and run in parallel until the membrane was cut to enable incubation with antibodies against the CysLT₁ and CysLT₂ protein, respectively. The blots were developed together on the same film. In E, cells were pre-incubated in the absence (lanes 1 and 2) or presence of the CysLT₁ receptor antagonist ZM198,615 (50 μM; lane 3) for 15 min and thereafter stimulated and analyzed as in C. In F, cells were incubated in the absence (lane 1) or presence of either 80 nM LTB₄ (lane 2) or LTD₄ (lane 3) and thereafter analysed as in C. The phosphorylation values were calculated as percentages of unstimulated cells, and they are means ± S.D. of four separate experiments. The illustrated blots are representative of at least four separate experiments.

FIG. 2. Characterization of the signals involved in LTD₄-induced activation of p⁹⁰RSK.

A, the cells were transfected with either empty vectors (lanes 1 and 2) or KPKCε vectors
(kinase dead PKCε, lane 3) and thereafter incubated in the absence (lane 1) or presence of 80 nM LTD₄ for 5 min (lanes 2 and 3). B, the cells were transfected with empty vectors (lanes 1 and 2) or K Raf-1 vectors (kinase dead Raf-1, lane 3) and thereafter incubated in the absence (lane 1) or presence of 80 nM LTD₄ for 5 min (lanes 2 and 3). C, the cells were incubated in the absence (lanes 1 and 2) or presence of the MEK inhibitor PD98059 (50 μM, lane 3) for 30 min and were then incubated with vehicle alone (lane 1) or with 80 nM LTD₄ (lanes 2 and 3) for 5 min. After all incubations, the cells were lysed, the proteins were separated by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated p90RSK, and the blots were subsequently reprobed with an antibody specific for total p90RSK. The blots shown are representative of four separate experiments.

**FIG. 3.** **Characterization of the signals involved in LTD₄-induced activation of CREB.**

A, Int 407 cells were pretreated with the PKA inhibitor Rp-cAMPs (50 μM, 30 min, lane 3) or were not (lanes 1 and 2) or the adenylate cyclase activator forskolin (100 μM, 15 min, lane 4). In B, the cells were transfected with either empty vectors (lanes 1 and 2), RD-PKCε vectors (lane 3), K PKCε vectors (lane 4), or K Raf-1 vectors (lane 5) and then incubated in the absence (lane 1) or presence (lanes 2-5) of 80 nM LTD₄ for 30 min. C, the cells were pre-incubated in the absence (lanes 1 and 2) or presence (lane 3) of the MEK inhibitor PD98059 (50 μM) for 30 min and then incubated in the absence (lane 1) or presence (lanes 2 and 3) of 80 nM LTD₄ for 30 min. D, the cells were transfected with either empty vectors (lanes 1 and 2) or K p90RSK vectors (lanes 3 and 4) and then incubated with 80 nM LTD₄ for 30 min (lanes 2 and 4). E-H, the cells were or were not (as indicated in the figure) pretreated with the p38 MAPK inhibitor SB203580 (20 μM, 30 min; E), the PI 3-kinase inhibitor LY294002 (50 μM, 30 min; F), the Src kinase inhibitor PP2 (50 μM, 30 min; G), or the PI 3-kinase activator PMA (100 nM, 15 min; H).
(50 μM, 30 min; F), the farnesyl transferase inhibitor FTI-277 (20 μM, 48 h; G), or the CAMK-II inhibitor KN-62 (10 μM, 30 min; H). The cells analysed in lane 3 in panel G were transfected with dominant-negative N17 Ras vectors. After the pre-treatments, all cells were, as indicated in the figure, incubated in the absence or presence of 80 nM LTD₄ for 30 min, and they were subsequently lysed. The proteins were separated by SDS-PAGE and immunoblotted, as indicated in the figure, with a specific anti-phospho-CREB antibody (all except E) or with a specific anti-phospho-p38 MAPK antibody (E). The blots were then stripped and reprobed, as indicated in the figure, with either an anti-total-CREB (A-D and F-H) or an anti-total p38 MAPK (E) antibody, and in addition with an anti-HA antibody (D) for the detection of HA-tagged K-p90RSK. The illustrated blots are representative of at least three separate experiments.

Fig. 4. Identification of a possible PKC isoform(s) involved in LTD₄-induced activation of CREB. A, the cells were pretreated in the absence (lanes 1 and 2) or presence of the PKC inhibitor Gö6976 (2 μM, lane 3) for 30 min; and were then incubated in the absence (lane 1) or presence of TPA (100 nM, lanes 2 and 3) for 15 min. B, the cells were pretreated in the absence (lanes 1 and 2) or presence of the general PKC inhibitor GF109203X (2 μM, lane 3) or the inhibitor of classical PKC isoforms Gö6976 (2 μM, lane 4) for 30 min. The cells were subsequently incubated in the absence (lane 1) or presence (lanes 2-4) of 80 nM LTD₄ for either 30 min (CREB) or 5 min (p90RSK). C, the cells were pre-incubated in the absence (lane 1) or presence (lane 2) of 1 μM TPA (down-regulation) for 16 h, after which whole-cell lysates were analyzed by immunoblotting for the presence of the PKC isoforms α and βII, as indicated in the figure. D, the cells were pre-incubated in the absence (lanes 1 and 2) or
presence (lane 3) of 1 μM TPA (down-regulation) for 16 h and then incubated in the absence (lane 1) or presence (lanes 2 and 3) of 80 nM LTD₄ for 30 min. E, the cells were transfected with either empty vectors (lanes 1 and 2) or PKCα-RD vectors (lanes 3 and 4) and then incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 80 nM LTD₄ for 30 min. The stimulations in A, B, D, and E were terminated by lysis of the cells. The proteins were separated by SDS-PAGE and immunoblotted with an anti-phospho-CREB (A, B, D and E) and in panel B also with an anti-p90RSK antibody, after which the blots were stripped and reprobed with an anti-total-CREB (A, B, D, E), an anti-total-p90RSK (B) or an anti-myc (E) antibody for the detection of myc-tagged RD-PKCα. In panel C, the membranes were immunoblotted with either an anti-PKCα or an anti-PKCβII antibody. The illustrated blots are representative of at least three separate experiments.

**Fig. 5. Fluorescence microscopy of the effects of K–p90RSK and PKCα-RD on LTD₄-induced phosphorylation of CREB.** The cells were co-transfected with dominant-negative K–p90RSK vectors along with empty EGFP vectors (A) or with EGFP-tagged PKCα-RD vectors (B), and they were subsequently incubated in the absence or presence of LTD₄ (80 nM) for 30 min, as indicated in the figure. Thereafter, the cells were fixed, permeabilized, and stained with a specific phospho-CREB antibody as described in “Experimental Procedures”. In both A and B, the top three images show (from left to right) EGFP expression, phospho-CREB staining, and an overlay image of non-stimulated control cells; the bottom three images depict the same analysis of cells stimulated with LTD₄. The illustrated results are representative of three separate experiments.
FIG. 6. **LTD₄ mediates both the proliferation and survival of intestinal epithelial cells.**

All cells were either pre-incubated in the absence or presence of the PKC inhibitor Gö6976 (2 μM) or the Erk-1/2 inhibitor PD98059 (50 μM) for 30 min, or they were transfected with either empty (tr), K⁻p90RSK or K⁻CREB vectors. The cells in A were subsequently incubated in the absence or presence of 80 nM LTD₄, the specified inhibitors or vectors, as indicated in panel A, for up to five days. The proliferative responses were measured each day by counting the cells in the presence of trypan blue. B, thymidine uptake analysis of cells pre-treated as in A, but incubated in the absence or presence of 80 nM LTD₄ for 48 h during which methyl-[³H]thymidine (0.5 μCi per well) was added for the last 24 h. Then, duplicates of each lysate were mixed with scintillation liquid and their radioactivities were measured in a LKB Wallace, 1209 RackBeta counter. The results shown in B are based on four independent experiments and are given as means ± S.D. C, a representative flow cytometric analysis of cells that were pre-treated as in A and then labeled with propidium iodide after incubation in the absence or presence of 80 nM LTD₄ for 48 h. D, statistical analysis of the flow cytometric data outlined in panel C. Values given represent percentages of cells in sub-G₀/G₁, G₀/G₁, and S + G₂/M phases and they are means ± S.D. of four experiments.

FIG. 7 **A schematic model of the LTD₄-induced signaling pathways involved in the activations of CREB and p90RSK, respectively.**
Fig. 1
Fig. 2
Fig. 4
Fig. 5
Fig. 7
Leukotriene D4 mediates survival and proliferation via separate but parallel pathways in the human intestinal Epithelial cell line Int 407
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J. Biol. Chem. published online August 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302881200

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