Leukotriene D_4 activates MAPK through a Ras-independent but PKCε-dependent pathway in intestinal epithelial cells

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Summary

We have recently shown that leukotriene D_4 (LTD_4) increases cell survival in intestinal epithelial cells. Here we report and explore the complementary finding that LTD_4 also enhances proliferation in these cells. This proliferative response was approximately half of that induced by epidermal growth factor (EGF) and its required activation of protein kinase C (PKC), Ras and the mitogen-activated protein kinase (MAPK) Erk-1/2. EGF also activated Erk-1/2 in these cells; however the EGF-receptor inhibitor PD153035 did not affect the LTD_4-induced activation of Erk-1/2. In addition, LTD_4 did not induce phosphorylation of the EGF receptor, nor did pertussis toxin (PTX) block EGF-induced activation of Erk-1/2, thus refuting a possible crosstalk between the receptors. Furthermore, LTD_4-induced, but not EGF-induced, activation of Erk-1/2 was sensitive to PTX, PKC inhibitors and downregulation of PKCε. A definite role for PKCε in LTD_4-induced stimulation of Erk-1/2 was documented by the inability of LTD_4 to activate Erk-1/2 in cells transfected with either the regulatory domain of PKCε (an isoform specific dominant-negative inhibitor) or a kinase-dead PKCε. Although Ras and Raf-1 were both transiently activated by LTD_4, only Raf-1 activation was abolished by abrogation of the PKC signal. Furthermore, the LTD_4-induced activation of Erk-1/2 was unaffected by transfection with dominant-negative N17 Ras but blocked by transfection with kinase-dead Raf-1. Consequently, LTD_4 regulates the proliferative response by a distinct Ras-independent, PKCε-dependent activation of Erk-1/2 and a parallel Ras-dependent signaling pathway.

Key words: Leukotriene D_4, Ras; Protein kinase C, Raf-1, MAPK, Intestinal epithelial cell proliferation

Introduction

The powerful pro-inflammatory mediator LTD_4 has been implicated in the pathophysiology of several inflammatory disorders, particularly asthma and inflammatory bowel diseases (Samuelsson, 1983; Samuelsson, 2000; Horwitz et al., 1998). LTD_4 mediates its effects through the $CysLT_1$ receptor, which has been cloned and characterized as a seven transmembrane-spanning receptor (Lynch et al., 1999; Sarau et al., 1999). These findings confirm earlier observations made by our research group (Sjölander et al., 1990; Adolfsson et al., 1996) and by other investigators (Watanabe et al., 1990) showing that LTD_4 signaling occurs through heterotrimeric G-proteins. We have also found that the LTD_4-induced calcium signal is regulated by at least two different G-proteins in intestinal epithelial cells (Sjölander et al., 1990; Adolfsson et al., 1996; Grönroos et al., 1996). Moreover, we recently demonstrated that Gβγ subunits of heterotrimeric G-proteins function as 'docking proteins' in the LTD_4-induced activation of PLC-γ1 in intestinal epithelial cells; we also demonstrated that activation of c-Src is essential for this interaction and signal (Thodeti et al., 2000).

Furthermore, ulcerative colitis is associated with an increased incidence of neoplastic transformation (Ekbom et al., 1990), and several studies have shown that colon cancer is under-represented in populations treated with non-steroidal anti-inflammatory drugs (Smalley and DuBois, 1997). A possible link between inflammation and the occurrence of cancer has been suggested (Sheng et al., 1997). To determine whether LTD_4 is involved in the coupling between inflammatory bowel conditions and an increased risk of cancer, we have previously exposed non-transformed intestinal epithelial cells to LTD_4 for prolonged periods of time (Öhd et al., 2000). Such exposure caused an upregulation of the cancer-associated proteins COX-2 and β-catenin, as well as the anti-apoptotic protein Bcl-2. Furthermore, LTD_4 also caused a PKC-dependent upregulation of active β1 integrins and an enhanced β1-integrin-dependent adhesion of intestinal epithelial cells (Massoumi and Sjölander, 2001). Taken together, these results suggest that LTD_4 signal a switch from cell death to cell survival.

MAPKs belong to a group of serine threonine kinases, and the MAPK family in mammalian cells includes extracellular signal-regulated kinase-1 and -2 (Erk-1/2), the c-Jun NH2-terminal kinases (JNK) and p38 MAPK (Garrington and Johnson, 1999). These MAPKs integrate multiple signals from various receptors and second messengers and are involved in the regulation of cellular proliferation and differentiation (Garrington and Johnson, 1999). Once activated, a MAPK can translocate to the nucleus, where it presumably regulates the expression of different transcription factors (Garrington and

Johnson, 1999; Velarde et al., 1999). It has been shown that Erk-1/2 is activated by a variety of receptor tyrosine kinases and G-protein-coupled receptors. The mechanism underlying activation of Erk-1/2 seems to be highly receptor and cell specific (Daulhac et al., 1999), and, for many different types of receptors and cells, such activation is induced by a PKC- and/or a Ras-dependent signaling pathway (Hawes et al., 1995). It has been shown that different PKC isoforms stimulate Erk-1/2 along Ras-dependent or Ras-independent signaling pathways (Li et al., 1998), suggesting that PKC acts upstream of Ras and Raf-1 (Miranti et al., 1999) or directly upstream of Raf-1 (Kolch et al., 1993; Cheng et al., 2001). Activation of the serine/threonine kinase Raf-1 is a complicated and not fully elucidated event that includes association with the active GTP-bound form of Ras at the membrane, Ras-dependent phosphorylation of Ser338 and most probably an additional phosphorylation of Tyr341 (Mason et al., 1999). Different second messengers converge at Raf for subsequent downstream activation of MAPKs.

It has also been proposed that activation of Erk-1/2 by G-protein-coupled receptors occurs through transactivation of receptor tyrosine kinases (Daub et al., 1996; Rao et al., 1995). In Rat-1 fibroblasts and COS-7 cells, inhibition of EGF receptor function abrogates tyrosine phosphorylation of Shc and the subsequent activation of Erk-1/2 in response to LPA, endothelin-1 and thrombin, all of which bind to G-protein-coupled receptors (Daub et al., 1996; Daub et al., 1997). Such crosstalk between EGF and LTD_4 receptors has been demonstrated in experiments showing that EGF-induced modulations of the cytoskeleton in fibroblasts are mediated by the $CysLT_1$ receptor (Peppelenbosch et al., 1995).

In light of our previous study of intestinal epithelial cells, showing that LTD_4 can reduce apoptosis and upregulate distinct proteins such as COX, we conducted the present investigation to determine if and how LTD_4 affects proliferation in these cells.

Materials and Methods

Materials

Phosphospecific antibodies to MAPK Erk-1/2 (p42/44) and MEK inhibitor PD98059 were purchased from New England BioLabs, Inc. (Beverly, MA); protein tyrosine kinase inhibitor genistein and antibodies to anti-MAPK were from Calbiochem (San Diego, CA). Isoform-specific PKC antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-EGF receptor antibodies and EGF were from Upstate Biotechnology Inc. (Lake Placid, NY); the activated form of EGF receptor antibody and the Ras antibody (RO2120) were from Transduction Laboratories (Lexington, KY). LTD_4 was purchased from Cayman Chemical Company (Ann Arbour, MI). ECL Western blot detection reagents and hyperfilm were from Amersham International (Buckinghamshire, UK). Ionomycin, MAPTAM, EGF-receptor inhibitor PD153035, PI-3Kinase inhibitors wortmannin, LY294002 and farnesyltransferase inhibitor FTI-277 were from Calbiochem (San Diego, CA). Pertussis toxin (PTX) was obtained from Speywood Pharma Ltd. (Maidenhead, UK). Peroxidase-linked goat anti-rabbit and mouse IgG originated from Dako A/S (Copenhagen, Denmark). Src family kinase inhibitor PP1 was from Alexis (San Diego, CA). All other chemicals were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture

Human embryonic intestinal epithelial cells [Intestine 407 (Henle and Dienhardt, 1957)], 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% CO_2 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.

MTS assay

The cells were cultured on Nunclon (Nalge Nunc International, Denmark) 96-well plates ($5\text{-}10\times10^3$ cells per well) for three days. They were subsequently pre-incubated in the absence or presence of the MEK inhibitor PD98059 (50 μM for 30 minutes), the PKC inhibitor GF109203X (30 μM for 30 minutes) or the farnesyltransferase inhibitor FTI-277 [20 μM for 48 hours (Lerner et al., 1995)]. The cells were then allowed to grow in fresh media for another two days in the absence or presence of 80 nM LTD_4 or 100 ng/ml EGF and the above inhibitors. The control cells were allowed to grow in the absence of LTD_4, EGF and any inhibitor for the same period of time as the treated cells. The MTS assay (Promega, Madison, WI) was carried out according to the protocol provided by the manufacturer. Briefly, the cells were incubated in 20 μl of MTS/PMS solution for 2 hours, after which soluble formazan (reduced MTS tetrazolium) was measured at 490 nm in a BMG plate reader (Offenburg, Germany).

Cell counting

The cells were cultured for three days in 35×10 mm Petri dishes. The cells were subsequently pre-incubated with or without pertussis toxin, PTX (500 ng/ml for 2 hours) or FTI-277 (20 μM for 48 hours). Then the cells were allowed to grow in fresh media for 2 days in the absence or presence of 80 nM LTD_4 or 100 ng/ml EGF and the above inhibitors. The effects of 80 nM LTD_4 or 100 ng/ml EGF were also tested in cells transfected with N17 Ras or the empty vector. To determine the number of viable cells, all cell counts were performed in the presence of 0.2% trypan blue.

Expression of N17 Ras, K⁻PKCε, K⁻Raf-1 and RD-PKC in Int 407 cells

Cells were transfected for 24 or 48 hours with a full-length human HA-tagged N17 Ras construct (Odajima et al., 2000), GFP-tagged RD-PKCε, RD-PKCδ (Zeidman et al., 1999) or a W437 kinase-dead PKCε construct (K⁻PKCε), which was generously provided by Arthur Mercurio (Beth Israel Deaconess Medical Center, Boston, MA, USA) or HA-tagged kinase inactive c-Raf construct (K⁻Raf-1), generously provided by Larry Karnitz (Mayo Clinic, Rochester, MN, USA). Control cells were transfected with empty pEGFP-N1 vector from Clontech. Transient transfections of the cells were achieved using 3.5 μl of Lipofectamine (Gibco) and 1.8 μg of plasmid DNA/ml and were performed in serum-free medium, essentially according to the protocol provided by the supplier.

Cell lysis

Cells were serum-starved for 2 hours, pre-incubated with inhibitors for the indicated periods of time and stimulations were terminated by adding ice-cold lysis buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 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, PMSF). Thereafter, the cells were kept on ice for 30 minutes in the lysis buffer, and the remaining cell debris was scraped loose into the buffer. The lysates were homogenized 10 times on ice in a glass tissue grinder (Dounce) and then centrifuged at 10,000 ***g*** for 15 minutes. The protein

content of each supernatant was measured and compensated for prior to electrophoresis.

Cell fractionation

Cell stimulations were terminated by adding ice-cold buffer A containing 20 mM NaHepes (pH 8), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM Na_3VO_4, 4 μg/ml leupeptin and 30 μg/ml PMSF. Thereafter, the cells were scraped loose into the cold buffer, homogenized 10 times on ice in a glass tissue grinder (Dounce) and then centrifuged at 200 ***g*** for 10 minutes. The protein content of the supernatant was measured and compensated for, and the supernatant was subsequently centrifuged at 1000 ***g*** for 5 minutes. The supernatant of the 1000 ***g*** fraction was further centrifuged at 200,000 ***g*** for 30 minutes. The resulting membrane-rich pellet was suspended in 150 μl of buffer A.

GST fusion proteins and binding assays

The cDNA clone encoding the GST fusion protein of the Raf minimal binding domain (RBD) of Ras in pGEX vector was transformed into *Escherichia coli* and cultured at 30°C (Hallberg et al., 1994). Expression of the GST fusion proteins was induced with 1 mM isopropyl-1-thio-D-galactopyranoside, and the *E. coli* were subsequently collected by centrifugation at 3500 ***g*** for 15 minutes followed by sonication in phosphate-buffered saline. Triton X-100 was added to the lysate (final concentration 1%), and particulate matter was removed by centrifuging at 5000 ***g*** for 15 minutes. The cleared lysate was incubated with glutathione-agarose beads (Sigma) for 1 hour at 4°C, and the beads were subsequently washed three times with ice-cold PBS. Lysates of unstimulated or stimulated Int 407 cells were prepared in 1.0 ml of the lysis buffer supplemented with 10 mM $MgCl_2$. GST fusion protein (5-10 μg) or GST alone was pre-bound to agarose beads and incubated with 1.0 ml of one of the cell lysates (1 mg/ml total cell protein) for 2 hours at 4°C. Thereafter, the beads were washed once with ice-cold lysis buffer supplemented with 0.5 M NaCl and twice with buffer A.

Gel electrophoresis

Cell lysates, membrane fractions or precipitated proteins were solubilized by boiling at 100°C for 5 minutes in a sample buffer [62 mM Tris (pH 6.8), 1.0% SDS, 10% glycerol, 15 mg/ml dithiothreitol, and 0.05% bromophenol blue]. The solubilized proteins were subjected to electrophoresis on 10-12% homogeneous polyacrylamide gels in the presence of SDS.

Immunoblotting

The separated proteins were electrophoretically transferred to a PVDF membrane. All membranes were blocked for 1 hour with 5% non-fat dried milk at room temperature and then incubated with a primary antibody for 1 hour at room temperature or overnight at 4°C. A 1:500 dilution was used for the anti-Ras (RO2120) antibody, whereas 1:1000 dilutions were used for all other antibodies. Subsequently all membranes were washed extensively and incubated with a horseradish-peroxidase-linked goat anti-rabbit, anti-sheep or anti-mouse antibody (1:5000) for 1 hour at room temperature. Thereafter, the membrane was again washed extensively, incubated with ECL western blot detection reagents and finally exposed to hyperfilm-ECL to visualize immunoreactive proteins. The phospho-MAPK blots were stripped and reprobed to detect total MAPK.

MEK-1, Raf-1 and B-Raf kinase assays

MEK-1, Raf-1 and B-Raf kinase were assayed using commercial kits from Upstate Biotechnology. The cells were first pre-incubated in the absence or presence of the MEK inhibitor PD98059 (50 μM for 30 minutes), the Src tyrosine kinase family inhibitor PP1 (10 μM for 15 minutes), PTX (500 ng/ml for 2 hours) or the PKC inhibitor GF109203X (30 μM for 30 minutes). Alternatively, the cells were depleted of PKC by incubation with 1 μM TPA (12-O-Tetradecanoylphorbol 13 acetate) for 24 hours. Thereafter, the cells were incubated in the absence or presence of 80 nM LTD_4 for 3 minutes and lysed. The cell lysates (1 mg aliquots) were then used for immunoprecipitation (1 hour at 4°C) with either 2 μg of an anti-MEK-1, 2 μg of an anti-Raf-1 or 4 μg of an anti-B-Raf antibody. Thereafter, 30 μl of a 3 mg/ml solution of protein G-agarose beads was added, and the mixture was allowed to stand for an additional hour at 4°C. The immunoprecipitates were washed twice with lysis buffer, after which kinase activities were measured with a coupled-enzyme assay.

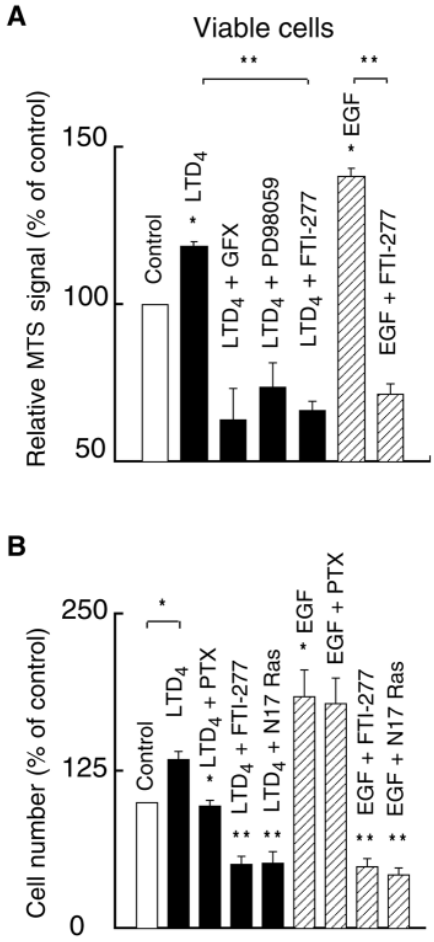

Fig. 1. LTD_4-induced proliferation of Int 407 cells. Cells were pre-incubated in the absence or presence of GF109203X (GFX; 30 μM for 30 minutes), PD98059 (50 μM for 30 minutes), FTI-277 (20 μM for 48 hours), PTX (500 ng/ml for 2 hours) or transfected with N17 Ras. Thereafter, the cells were incubated in the absence or presence of 80 nM LTD_4 or 100 ng/ml EGF in the absence or presence of the indicated inhibitor for 48 hours. After 48 hours, the proliferative responses were determined. In (A), the conversion of tetrazolium into formazan was measured by analyzing the absorbance of formazan at 490 nm. The absorbance values are given as percentages relative to untreated cells. (B) outlines the results of the cell count, and the values are given as percentages relative to untreated cells. The data in both panels represent means±s.e.m. of four separate experiments. Statistical significant effects of the inhibitors (compared with untreated cells) were evaluated using the unpaired Student's *t*-test, **P*<0.05 and ***P*<0.01.

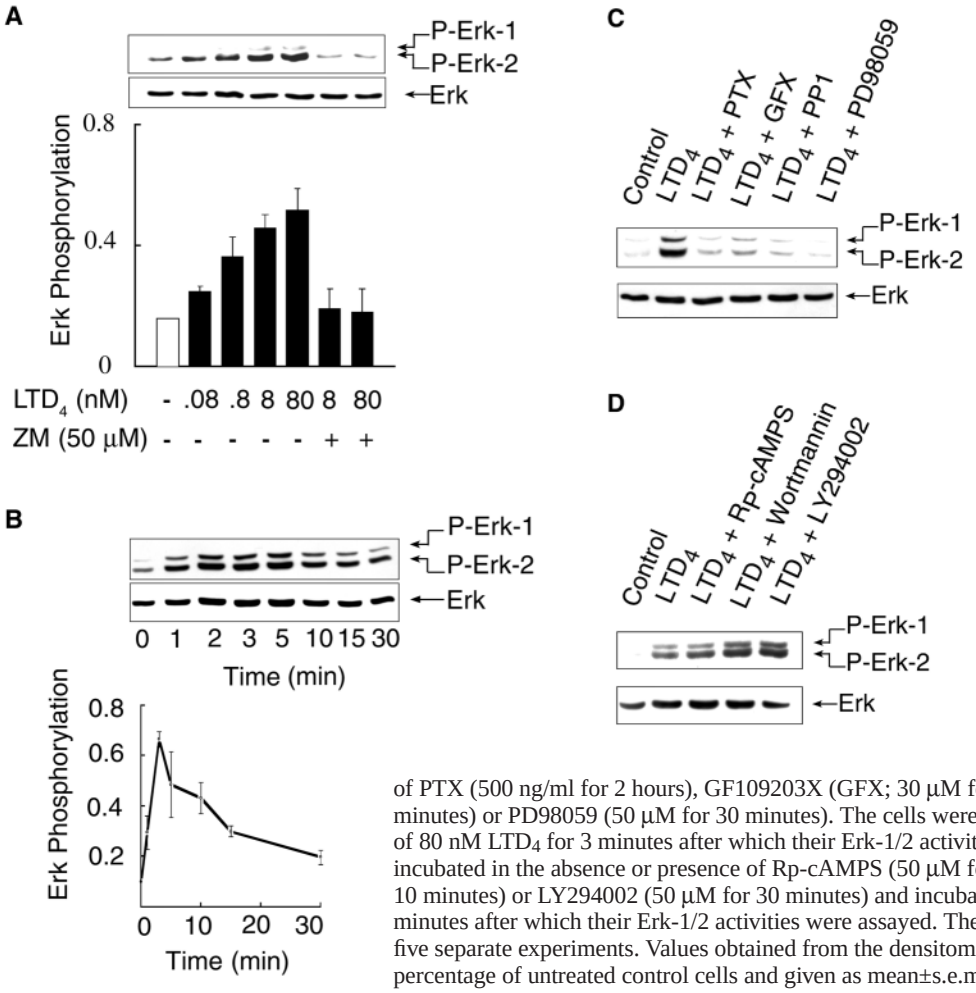

Fig. 2. Characterization of LTD_4-induced activation of Erk-1/2. Cells were incubated in the absence or presence of 80 nM LTD_4 and the indicated inhibitors. Cell lysates were separated by SDS-PAGE and immunoblotted with antibodies specific for phosphorylated Erk-1/2. Thereafter, the blots were reprobed for total Erk-1/2. (A) outlines a representative blot and the accumulated concentration curve of LTD_4-induced Erk-1/2 phosphorylation. Cells were treated with the indicated concentrations of LTD_4 for 3 minutes with or without ZM-198,615 (ZM; 50 μM, 15 minutes) after which their degrees of Erk-1/2 phosphorylation were determined. (B) shows a representative blot and graph of the accumulated time course of LTD_4-induced Erk-1/2 phosphorylation. Cells were treated with 80 nM LTD_4 for the indicated periods of time, after which Erk-1/2 activities were assayed as described in the Materials and Methods. (C) Cells were pre-incubated in the absence or presence of PTX (500 ng/ml for 2 hours), GF109203X (GFX; 30 μM for 30 minutes), PP1 (10 μM for 15 minutes) or PD98059 (50 μM for 30 minutes). The cells were then incubated in the absence or presence of 80 nM LTD_4 for 3 minutes after which their Erk-1/2 activities were assayed. In (D), cells were pre-incubated in the absence or presence of Rp-cAMPS (50 μM for 30 minutes), wortmannin (100 nM for 10 minutes) or LY294002 (50 μM for 30 minutes) and incubated with or without 80 nM LTD_4 for 3 minutes after which their Erk-1/2 activities were assayed. The blots shown are representative of at least five separate experiments. Values obtained from the densitometric analyses are calculated as a percentage of untreated control cells and given as mean±s.e.m. of five separate experiments.

In short, the immune complexes were incubated for 30 minutes at 30°C with 10 μl of cold Mg-ATP buffer (Upstate Biotechnology) and the specified substrates (0.4 μg of inactive MEK-1 and 1 μg of inactive Erk-2 for Raf assays and MEK assays). This mixture (4 μl) was incubated for 10 minutes at 30°C with 10 μl of 2 mg/ml MBP substrate and 10 μl of a 1:10 dilution of [γ-^{32}P]ATP (1 mCi/100 μl) diluted with the cold Mg-ATP buffer. The reaction mixture (25 μl) was spotted onto the center of a P81 phosphocellulose paper square and washed thoroughly several times with 0.75% phosphoric acid and then once with acetone and thereafter subjected to liquid scintillation counting.

Immunofluorescence

The cells were seeded onto glass coverslips and grown for 5 days, during the last 24 hours they were cotransfected with N17 Ras and EGFP (empty vector). Thereafter, the cells were serum-starved for 2 hours and stimulated with 80 nM LTD_4 for 3 minutes or 100 ng/ml EGF for 5 minutes at 37°C. The stimulations were terminated by fixation of the cells for 10 minutes at room temperature in a 3.7% paraformaldehyde/PBS solution, after which the cells were permeabilised in a 0.5% Triton X-100/PBS solution for 5 minutes. The coverslips were subsequently washed twice in PBS and incubated at room temperature in a 3% BSA/PBS solution for 15 minutes. The cells were stained for 1 hour with a phospho-specific antibody against Erk-1/2. Thereafter, the coverslips were washed six times in PBS and incubated with a 1:200 dilution (in blocking buffer) of Alexa Fluor 568 goat anti-rabbit secondary antibody. The coverslips were finally washed six times in PBS and mounted in fluorescent mounting medium (DAKO A/S). Samples were examined and photographed in a Nikon Eclipse 800 microscope, using a 60× objective. Images were recorded with a scientific-grade, charge-coupled device (CCD) camera (Hamamatsu, Japan) and subsequently analysed with HazeBuster deconvolution software (VayTek, Inc., Fairland, CT, USA).

Results

LTD_4 increases epithelial cell proliferation via a pathway sensitive to inhibitors of PKC, Ras and MEK

LTD_4-induced proliferation in Int 407 intestinal epithelial cells (Fig. 1), as determined by the MTT assay (Cory et al., 1991) and cell counting. The former is based on the following: tetrazolium salts are reduced to formazan compounds by dehydrogenase enzymes in metabolically active cells. The LTD_4-mediated increase in cell proliferation was effectively abolished by PTX (500 ng/ml; Fig. 1B**)**, the PKC inhibitor GF109203X (30 μM; Fig. 1A), the MEK inhibitor PD98059 (50 μM; Fig. 1A), and the Ras farnesyltransferase inhibitor FTI-277 (20 μM; Fig. 1). Transfecting the cells with N17 Ras also blocked this proliferative response (Fig. 1B). As a comparison we show that the EGF-induced proliferative response (striped bars) was also effectively blocked by Ras inhibition (Fig. 1), supporting an active role for Ras in the proliferative response in Int 407 cells. However, EGF induced

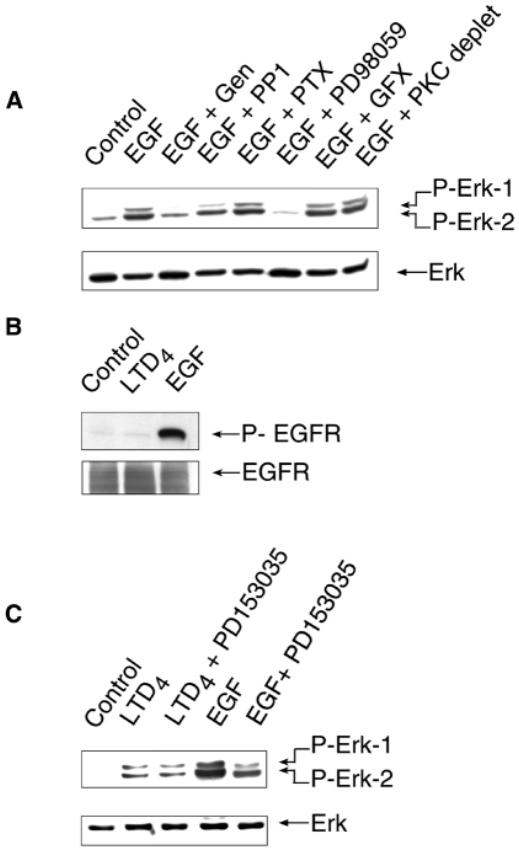

Fig. 3. Characterization of EGF-induced Erk-1/2 activation and LTD_4-induced EGF receptor phosphorylation in Int 407 cells. The cells were pre-incubated in the absence or presence of genistein (Gen; 50 μg/ml for 30 minutes), PP1 (10 μM for 15 minutes), PTX (500 ng/ml for 2 hours), PD98059 (50 μM for 30 minutes), GF109203X (GFX; 30 μM for 30 minutes) or TPA (1 μM for 24 hours, i.e. PKC depletion). Then the cells were stimulated with 100 ng/ml EGF (5 minutes), lysed, and the lysates were separated by SDS-PAGE as described in the Materials and Methods. (A) shows a representative immunoblot with a specific anti-phospho-Erk-1/2 antibody that was then reprobed with an anti-total-Erk-1/2 antibody. (B) illustrates results from cells that were stimulated with either 80 nM LTD_4 for 3 minutes, 100 ng/ml EGF for 5 minutes or not stimulated at all (control). Lysates of these cells were separated by SDS-PAGE, immunoblotted with an anti-phospho-EGF receptor antibody and then reprobed with an anti-total-EGF receptor antibody. (C) illustrates results from cells pre-incubated with or without 2 μM PD153035 for 30 minutes and thereafter stimulated as above. Lysates of these cells were separated by SDS-PAGE, immunoblotted with a specific anti-phospho-Erk-1/2 antibody and then reprobed with an anti-total-Erk-1/2 antibody. The blots shown are representative of five separate experiments.

proliferation was unaffected by PTX treatment (Fig. 1B). The control cells (white bars) were allowed to grow in the absence of LTD_4, EGF and any inhibitor for the same period of time as the treated cells.

LTD_4 activates Erk-1/2 in intestinal epithelial cells

We observed a significant activation of Erk-1/2 in cells stimulated with LTD_4 (Fig. 2), although this was not as pronounced as the response induced by EGF (Fig. 3C). A concentration of 0.8 nM LTD_4 was sufficient to induce activation of Erk-1/2, and the response at that level was half of that noted at 80 nM LTD_4 (Fig. 2A). We refrained from using higher and non-physiological concentrations. The specific LTD_4 receptor $CysLT_1$ antagonist ZM198,615 (ICI-198,615, 50 μM for 15 minutes) abolished Erk-1/2 activation induced by 80 nM LTD_4, indicating that the effect is mediated by the $CysLT_1$ receptor (Fig. 2A). The response to LTD_4 was rapid, reached a peak after 3 minutes and returned to basal level after about 30 minutes (Fig. 2B).

LTD_4-induced Erk-1/2 activation is mediated via a pathway sensitive to PTX, GF109203X, PP1 and PD98059

As mentioned above, G-protein-coupled receptors are known to activate Erk-1/2 through different second messenger pathways or by transactivation of EGF receptors. To identify the signals involved in LTD_4-induced activation of Erk-1/2 and to compare them with those involved in LTD_4-effected cell proliferation (Fig. 1), we initially used compounds that inhibit various signaling molecules. We found that pre-incubation with 500 ng/ml PTX for 2 hours, 30 μM GF109203X for 30 minutes, 10 μM PP1 for 15 minutes **(**a Src family kinase inhibitor) and 50 μM PD98059 for 30 minutes blocked LTD_4-induced activation of Erk-1/2 (Fig. 2C). Neither the protein kinase A inhibitor Rp-cAMPS **(**50 μM for 30 minutes**)** nor the PI 3-kinase inhibitors wortmannin **(**100 nM for 10 minutes**)** and LY294002 **(**50 μM for 30 minutes**)** had any inhibitory effect on the LTD_4-induced activation of Erk-1/2 (Fig. 2D). These results suggest that a heterotrimeric G-protein, PKC, and MEK are involved in LTD_4-mediated activation of Erk-1/2. Inhibition of LTD_4-induced Erk-1/2 activation by the PP1 can readily be explained by the involvement of a Src-like kinase(s), although it could also imply crosstalk between the LTD_4 receptor and the EGF receptor.

Transactivation of the EGF receptor is not involved in LTD_4-mediated activation of Erk-1/2

To determine whether crosstalk occurs between LTD_4 and EGF receptors, we first investigated possible signals involved in EGF-induced Erk-1/2 activation. As expected, EGF-induced activation of Erk-1/2 was inhibited by genistein (a protein tyrosine kinase inhibitor; 50 μg/ml for 30 minutes**)**, PD153035 (an EGF-receptor inhibitor; 2 μM for 30 minutes**)**, PD98059 **(**50 μM for 30 minutes**)** and to a lesser extent by PP1 (10 μM for 15 minutes**)**, but not by 500 ng/ml PTX (for 2 hours), GF109203X **(**30 μM for 30 minutes**)** or PKC depletion by 1 μM TPA for 24 hours (Fig. 3A,C). The lack of effect of PTX indicates that EGF-mediated Erk-1/2 activation in intestinal epithelial cells does not occur via the LTD_4 receptor, which has been suggested for the effects of EGF on the cytoskeleton in fibroblasts (Peppelenbosch et al., 1995). We also used an antibody that recognizes the phosphorylated form of the EGF receptor to determine whether LTD_4 participates in activation of the EGF receptor. As shown in Fig. 3B, stimulation with LTD_4 did not lead to any detectable phosphorylation of the EGF receptor, whereas treatment with EGF caused a 40-fold increase in EGF receptor phosphorylation. Furthermore, the LTD_4-induced activation of Erk-1/2 was not affected by the

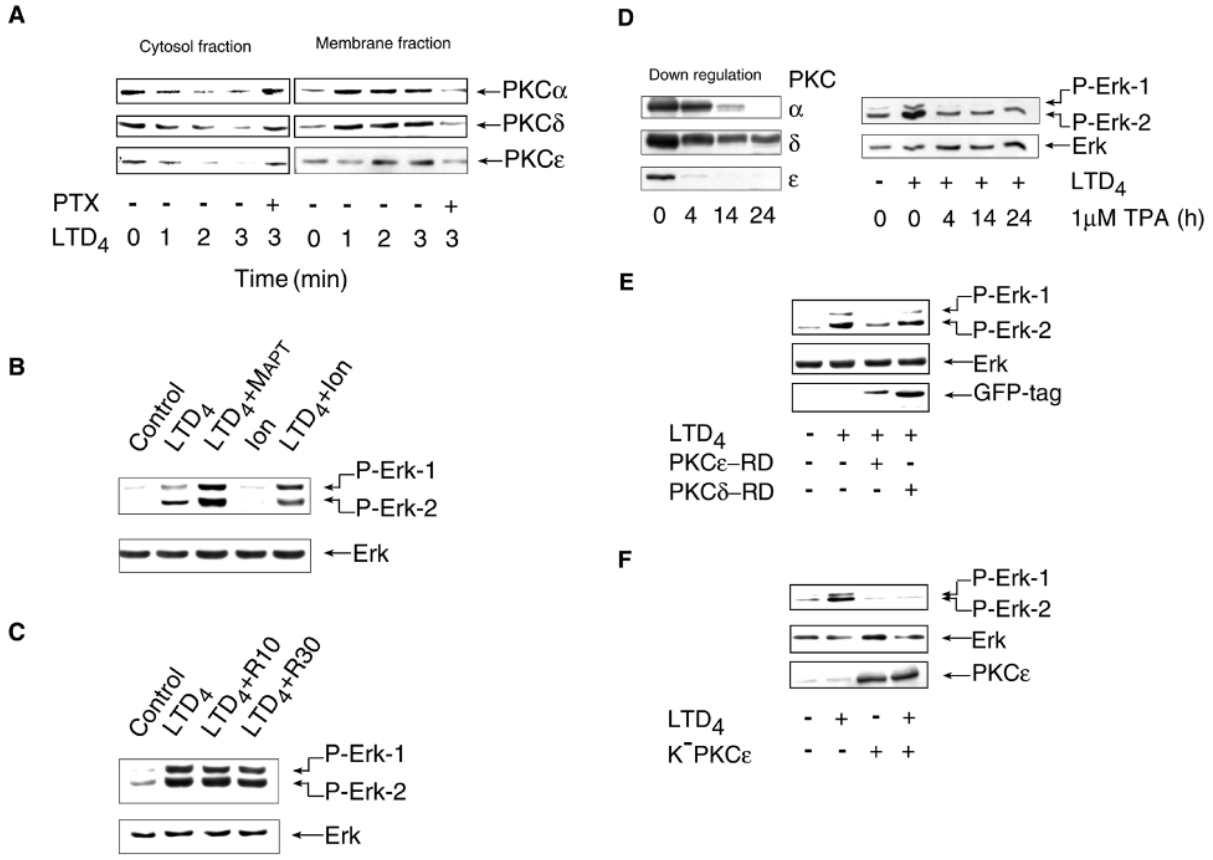

Fig. 4. Identification of possible PKC isoform(s) involved in LTD_4-induced Erk-1/2 activation. In (A), cells were pre-incubated in the absence or presence of PTX (500 ng/ml for 2 hours) and then stimulated with or without 80 nM LTD_4 for the indicated periods of time. Membrane and cytosolic fractions were isolated, separated on SDS-PAGE and immunoblotted with the indicated specific anti-PKC isoform antibodies as described in the Materials and Methods. In (B), cells were pre-incubated in the absence or presence of 10 μM MAPTAM for 1 hour or 1 μM ionomycin for 5 minutes and thereafter stimulated or not with 80 nM LTD_4 for 3 minutes after which they were lysed. The cell lysates were then separated by SDS-PAGE and immunoblotted with an anti-phospho-Erk-1/2 antibody and then reprobed with an anti-total-Erk-1/2 antibody. In (C), cells were pre-incubated in the absence or presence of rottlerin (10 or 30 μM for 30 minutes), stimulated or not with 80 nM LTD_4 for 3 minutes and then lysed. The cell lysates were separated by SDS-PAGE and immunoblotted as in (B). In (D), the results in the left panel were obtained from cells treated with 1 μM TPA for the indicated periods of time after which whole-cell lysates were analyzed by immunoblotting for PKC isoforms α, δ and ε. The right panel of (D) show results from cells pre-incubated with TPA, as in the left panel, but then stimulated or not with 80 nM LTD_4 for 3 minutes. After these 3 minutes, whole-cell lysates were prepared and analyzed by immunoblotting with an anti-phospho-Erk-1/2 antibody and then reprobed with an anti-total-Erk-1/2 antibody. (E) shows the results from cells transfected with either an empty vector (lanes 1-2), a RD-PKCε-expressing or a RD-PKCδ-expressing vector that were stimulated or not with 80 nM LTD_4 for 3 minutes. After 3 minutes, whole-cell lysates were prepared and analyzed by immunoblotting with an anti-phospho-Erk-1/2 antibody and then reprobed with an anti-total-Erk-1/2 antibody and finally an anti-GFP antibody. **(**F) shows results from cells transfected with an empty vector (lanes 1-2) or a vector expressing K^-PKCε that were stimulated or not with 80 nM LTD_4 for 3 minutes. After 3 minutes, whole-cell lysates were prepared and analyzed by immunoblotting for phospho-Erk-1/2, total Erk-1/2 and finally K^-PKCε. All blots in this figure are representative of at least three separate experiments.

EGF-receptor inhibitor PD153035 (Fig. 3C). Nor did pretreatment with the FGF-1-receptor inhibitor SU5402 or the more general receptor-tyrosine kinase inhibitor SU4984 (blocking FGF-, PDGF- and insulin-receptors**)** affect the LTD_4-induced activation of Erk-1/2 (data not shown). These results suggest that EGF-induced activation of Erk-1/2 is not mediated through the $CysLT_1$ receptor and that LTD_4 is not involved in stimulation of the EGF receptor.

Identification of a specific PKC isoform(s) involved in the LTD_4-induced activation of Erk-1/2

We have previously shown that LTD_4 induces translocations (i.e. activation) of α, δ and ε PKC isoforms but not of PKCβII, PKCμ or PKCζ (no other novel PKC isoforms is expressed) in intestinal epithelial cells (Thodeti et al., 2001). Furthermore when these cells were subjected to prolonged (24 hours) treatment with TPA they exhibited total downregulation of PKCα and PKCε but only partial downregulation of PKCδ (Thodeti et al., 2001). In the leukemia cell line THP-1, LTD_4 has been suggested to activate Erk-1/2 through a PKCα-dependent pathway (Hoshino et al., 1998). However, LTD_4-induced activation of Erk-1/2 in intestinal epithelial cells was only abolished by a high concentration (30 μM) of the PKC inhibitor GF109203X (Fig. 2C). A lower concentration of GF109203X (2-10 μM) impaired the TPA- but not the LTD_4-

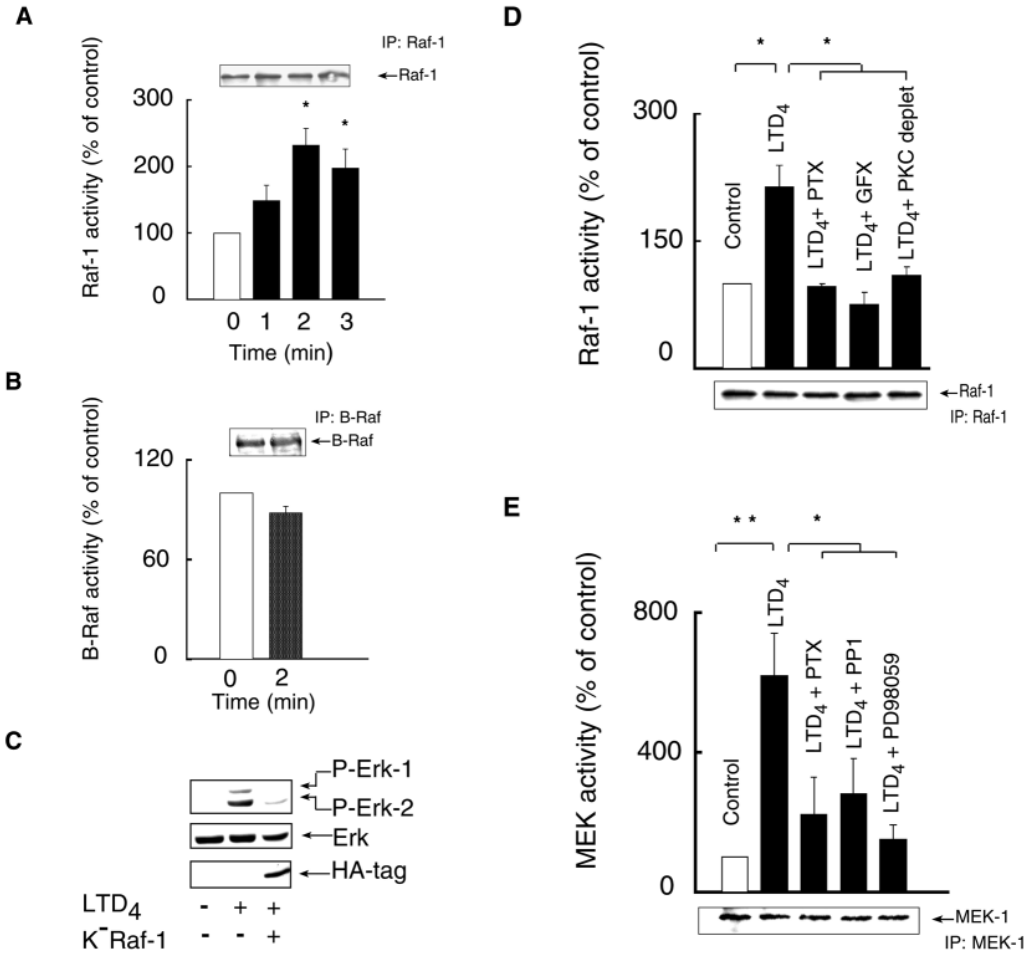

Fig. 5. Effects of LTD_4 on the activities of Raf-1, B-Raf and MEK in Int 407 cells. In (A) and (B), the cells were stimulated with 80 nM LTD_4 for the indicated periods of time, after which kinase assays were performed with anti-Raf-1 (A) and anti-B-Raf (B) immunoprecipitates (as described in the Materials and Methods). In (C), cells were transfected with an empty vector or a HA-tagged K^-Raf-1 expressing vector and then stimulated or not with 80 nM LTD_4 for 3 minutes. After 3 minutes, whole cell lysates were prepared and analyzed by immunoblotting with an anti-phospho-Erk-1/2, an anti-total-Erk-1/2 and finally an anti-HA antibody. In (D), cells were pre-incubated in the absence or presence of PTX (500 ng/ml for 2 hours), GF109203X (GFX; 30 μM for 30 minutes), TPA (1 μM for 24 hours; PKC deplet) and thereafter stimulated or not with 80 nM LTD_4 for 3 minutes. After 3 minutes, the Raf-1 kinase activities were performed as in (A). In (E), cells were pre-incubated in the absence or presence of PTX (500 ng/ml for 2 hours), PP1 (10 μM for 15 minutes) or PD98059 (50 μM for 30 minutes) and thereafter stimulated or not with 80 nM LTD_4 for 3 minutes. After 3 minutes, the MEK kinase activities were performed as in (B). Representative control blots of the different immunoprecipitates are shown in panels (A,B,D,E). The kinase activity values are expressed as a percentage of untreated control cells and given as mean±s.e.m. of four separate experiments. Statistically significant effects (compared with untreated cells) were evaluated using an unpaired Student's *t*-test, $*P < 0.05$ and $**P < 0.01$.

induced activation of Erk-1/2 (data not shown), suggesting that a novel PKC isoform(s) is involved in producing the effects of LTD_4 on Erk-1/2. In order to identify the PKC isoform involved in Erk-1/2 activation in intestinal cells, we first investigated the effect of PTX, which blocks the LTD_4-mediated activation of Erk-1/2, on the LTD_4-induced translocation of PKCα, δ and ε. LTD_4 induced a rapid PTX-dependent translocation of PKC α, δ and ε to a membrane fraction and a subsequent reduction of these isoforms in a cytosolic fraction from these cells (Fig. 4A). We then investigated a possible involvement of calcium-dependent PKCs. Cells were preincubated with the calcium chelator MAPTAM (10 μM for 1 hour) before stimulation with LTD_4. Such a chelation of cytosolic free calcium did not reduce the LTD_4-induced activation Erk-1/2 (Fig. 4B). Furthermore, addition of the calcium ionophore ionomycin (1 μM for 5 minutes) did not stimulate Erk-1/2 nor did it affect the LTD_4-induced activation of ERK-1/2 (Fig. 4B). These data make participation of the calcium-dependent PKCα isoform unlikely. We also noted that the PKCδ inhibitor rottlerin (10 or 30 μM was added for 30 minutes) had no effect on the LTD_4-induced activation of Erk-1/2 (Fig. 4C). These data argue against an involvement of PKCδ in the LTD_4-induced activation of Erk-1/2. To obtain more direct evidence for a role of PKCε in the LTD_4-induced Erk-1/2 activation, we performed the following three experiments. Firstly, we studied the time course of TPA-induced downregulation of the different PKC isoforms, and this revealed that PKCε was downregulated much earlier (4 hours) than PKCα and PKCδ (Fig. 4D). Parallel experiments showed that such a pretreatment with TPA for only 4 hours abolished the LTD_4-induced activation of Erk-1/2 (Fig. 4D), suggesting that PKCε is involved in such activation. Secondly, we examined the effect of LTD_4 on Erk-1/2 activation in cells transfected with either the regulatory domain of PKCε (RD-PKCε) or the regulatory domain of PKCδ (RD-PKCδ). The isolated regulatory domains have been suggested to work as isoform-specific dominant-negative inhibitors of PKC (Jaken, 1996), and inhibition of specific isoforms with these domains has been successfully utilized in several studies (Cai et al., 1997; Kiley et al., 1999; Massoumi and Sjölander, 2001). We noted that expression of RD-PKCε blocked the LTD_4-induced activation of Erk-1/2, whereas expression of RD-PKCδ did not (Fig. 4E). These results were obtained even though the expression level

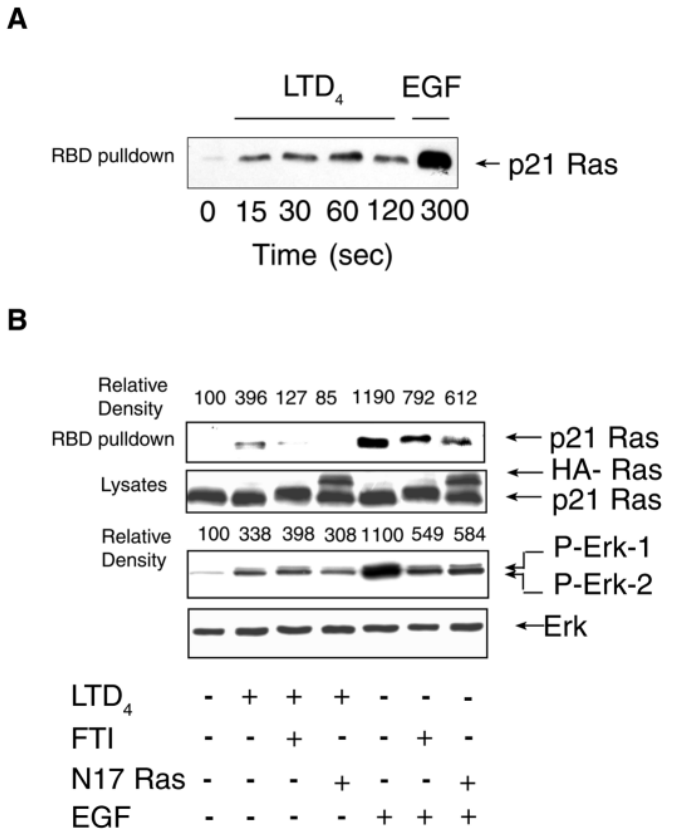

Fig. 6. LTD_4-induced activation of Ras and its role in Erk-1/2 activation. In (A), cells were stimulated with 80 nM LTD_4 for the indicated periods of time or with 100 ng/ml EGF for 5 minutes, after which the cells were lysed. The active form of Ras was then precipitated with the minimal RBD fragment of Raf-1 fused with GST and then separated by SDS-PAGE and immunoblotted with an anti-Ras antibody. A similar analysis is shown in the top panel of (B), but here the cells were pre-incubated in the absence or presence of the Ras inhibitor FTI-277 (20 μM for 48 hours) or transfected with HA tagged N17 Ras as indicated and then stimulated with LTD_4 (80 nM) for 1 minute or EGF (100 ng/ml) for 5 minutes. In the second panel of (B), whole lysates from the samples used in the top panel were separated by SDS-PAGE and immunoblotted with an anti-Ras antibody. The blot show a gelshift of Ras derived from FTI-277 treated cells and the two bands of Ras from cells transfected with HA-tagged N17 Ras (the lower endogenous Ras band and the upper HA-tagged Ras band). In the third and fourth panels, the blot was probed with an anti-phospho-Erk-1/2 antibody and then reprobed with an anti-total-Erk-1/2 antibody. The relative densities given in the figure refer to densitometric analysis of LTD_4 and EGF-induced activation of Ras and Erk-1/2. The blots shown are representative of four separate experiments.

of the GFP-tagged RD-PKCε is less than that of the GFP-tagged RD-PKCδ, which was revealed by reprobing the western blot with an anti-GFP antibody (Fig. 4E). Thirdly, we transfected cells with kinase-dead PKCε (K^-PKCε) or the corresponding empty vector and examined their effects on LTD_4-induced Erk-1/2 activation. We noted that expression of K^-PKCε totally inhibited the LTD_4-induced activation of Erk-1/2, whereas transfection of the empty vector had no effect (Fig. 4F). These results clearly show that PKCε is the isoform involved in the LTD_4-induced activation of Erk-1/2 in intestinal cells.

LTD_4 induces activation of Raf-1 and MEK via a PKC-dependent signaling pathway

Employing in vitro kinase assays, we found that LTD_4 induced rapid activation of Raf-1 (Fig. 5A), but not B-Raf (Fig. 5B), in intestinal epithelial cells. The stimulation of Raf-1 peaked approximately 2 minutes after addition of the leukotriene (Fig. 5A). G-protein- and PKC-dependent activation of Erk has also been found to be mediated by MEKK1 rather than Raf-1 (Vuong et al., 2000). To investigate whether the LTD_4-induced activation of Erk-1/2 is mediated by Raf-1, we transfected cells with either a HA-tagged kinase-dead Raf-1 expressing vector [K^-Raf-1 (Sutor et al., 1999)] or an empty vector and examined their effect on LTD_4-induced Erk-1/2 activation in these cells. The results clearly show that expression of K^-Raf-1 inhibited the LTD_4-induced activation of Erk-1/2, whereas the empty vector had no such effect (Fig. 5C). In subsequent experiments, LTD_4-mediated activation of Raf-1 was demonstrated to be abolished by pre-incubation with PTX (500 ng/ml for 2 hours) or GF109203X (30 μM for 30 minutes) or by TPA-induced downregulation of PKC (Fig. 5D). These data indicate that Raf-1 is located downstream of PKC in LTD_4-induced activation of Erk-1/2 (Fig. 5D). On the basis of these results, we performed immunoprecipitations to examine the possibility of an LTD_4-mediated association between PKCε and Raf-1, but we found no such association (data not shown). It is quite likely that Raf-1 is activated by PKCε, even if there is no physical connection between the two proteins; alternatively PKCε could stimulate Raf-1 indirectly via activation of Ras. It has previously been demonstrated that PKC can play a role in the activation of Ras in lymphocytes (Downward et al., 1990). To test the ability of PD98059 to inhibit the LTD_4-induced activation of MEK, we employed an in vitro kinase assay (Fig. 5E). We found that pre-incubation with PD98059 (50 μM for 30 minutes), PTX (500 ng/ml for 2 hours) or PP1 (10 μM for 15 minutes) inhibited the LTD_4-induced activation of MEK.

LTD_4 activates Erk-1/2 via a Ras-independent mechanism

In the present study we clearly show that LTD_4 causes a rapid and transient activation of Ras (Fig. 6A). To explore a possible role for active Ras in the LTD_4-induced activation of Erk-1/2, we either incubated the cells with the Ras farnesyltransferase inhibitor FTI-277 (20 μM for 48 hours) or transfected them with HA-tagged N17 Ras. The latter is an Asn-17 mutant of Ha-Ras, which blocks multiple downstream signals such as activation of Raf-1 and phosphorylation of MAPK (Odajima et al., 2000). In cells pre-incubated with FTI-277, we detected almost no increase in GTP-Ras in the pull-down assay of cells stimulated with LTD_4 for 1 minute (Fig. 6B); however the LTD_4-induced activation of Erk-1/2 was not affected at all. Under identical conditions we noted reductions of EGF-induced Ras activation and simultaneous and similar reductions in EGF-induced Erk-1/2 activation. To gain further support for LTD_4-induced Ras-independent activation of Erk-1/2, we cotransfected cells with N17 Ras and empty EGFP vector, and following stimulation with LTD_4 or EGF the cells were immunostained with a phospho-Erk antibody (Fig. 7). In unstimulated cells, the staining with the phospho-Erk antibody was weak in both N17-Ras-transfected and non-transfected cells. In contrast, cells stimulated with LTD_4 stained brightly

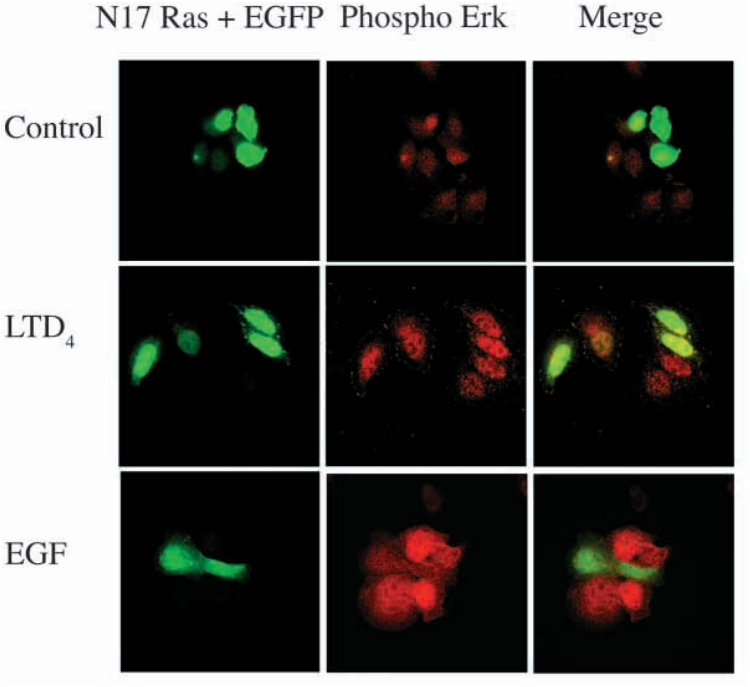

Fig. 7. Effect of N17 Ras expression on LTD_4- and EGF-induced Erk-1/2 activation. The cells were cotransfected with a vector expressing dominant-negative N17 Ras along with an empty EGFP vector and then stimulated with LTD_4 (80 nM for 1 minute), EGF (100 ng/ml for 5 minutes) or not at all. The cells were then fixed, permeabilised and stained for phospho-Erk-1/2 as described in the Materials and Methods. The top three panels show from the left to the right: EGFP expression, phospho-Erk-1/2 staining and an overlay image of non-stimulated control cells. The middle three panels and the bottom three panels show the same analysis from cells stimulated with LTD_4 and EGF, respectively. The results illustrated are representative of three separate experiments.

with the phospho-Erk antibody regardless, of whether the cells were transfected with N17 Ras or not (Fig. 7), thus indicating that LTD_4-induced Erk-1/2 activation is Ras-independent. As a positive control, cells transfected with or without N17 Ras were stimulated with EGF (Fig. 7). Cells stimulated with EGF stained brightly with the phospho-Erk antibody, provided that they were not transfected with N17 Ras (Fig. 7). These latter control data are in agreement with the previously reported Ras-dependency of EGF-induced Erk-1/2 activation (Daub et al., 1996). Furthermore, parallel experiments revealed that neither preincubation with PTX or GF109203X nor TPA downregulation of PKC impaired the LTD_4-induced activation of Ras (data not shown). These findings clearly indicate that LTD_4-mediated activation of Ras is separate from the Erk-1/2 signaling pathway.

Discussion

We found that in intestinal epithelial cells the LTD_4-induced proliferative response is regulated by dual intracellular signaling pathways: one that is initiated by a pertussis toxin-sensitive G-protein and requires activation of Erk-1/2 and one that is pertussis toxin-insensitive but dependent on Ras activation (Fig. 8). The initiation and existence of two parallel pathways is in good agreement with previous reports regarding the Ca^{2+} signaling properties of the LTD_4 receptor (Sjölander et al., 1990; Adolfsson et al., 1996; Grönroos et al., 1996; Thodeti et al., 2000). Our initial observation that Erk-1/2 was involved in the LTD_4-induced proliferative response suggests that a G-protein-Erk-1/2 signaling pathway is activated in these epithelial cells. This pathway may involve receptor crosstalk, because it is well known that Erk-1/2 and the other MAPKs integrate signals from different receptors and second messengers (Garrington and Johnson, 1999; Robinson and Cobb, 1997), and communication between the EGF and the LTD_4 receptor has been reported (Peppelenbosch et al., 1995). However, in our study, EGF-induced Erk-1/2 activation was not affected by PTX, PP1, GFX or PKC depletion, and LTD_4 did not trigger activation of the EGF receptor, findings that argue against a possible transactivation between these two receptors. Furthermore, pre-incubation with the EGF-receptor inhibitor PD153035 did not affect the LTD_4-induced activation of Erk-1/2. Consequently, our results suggest that a distinct LTD_4–G-protein–Erk-1/2 signaling pathway mediates proliferation of intestinal epithelial cells. In agreement with this, other investigators (Le Gall et al., 2000) have proposed that the MAP kinase pathway plays a role in protecting cells against apoptosis, possibly by counteracting cell death induced by loss of matrix contact, cytoskeletal integrity or extracellular mitogenic factors. Therefore, we extended our characterization of the signaling elements that are involved in LTD_4-mediated regulation of Erk-1/2 activity and thereby also participate in the proliferative response of intestinal epithelial cells.

Our finding that LTD_4 causes a transient, time- and concentration-dependent activation of Erk-1/2 in intestinal epithelial cells is compatible with the effect of PD98059, a

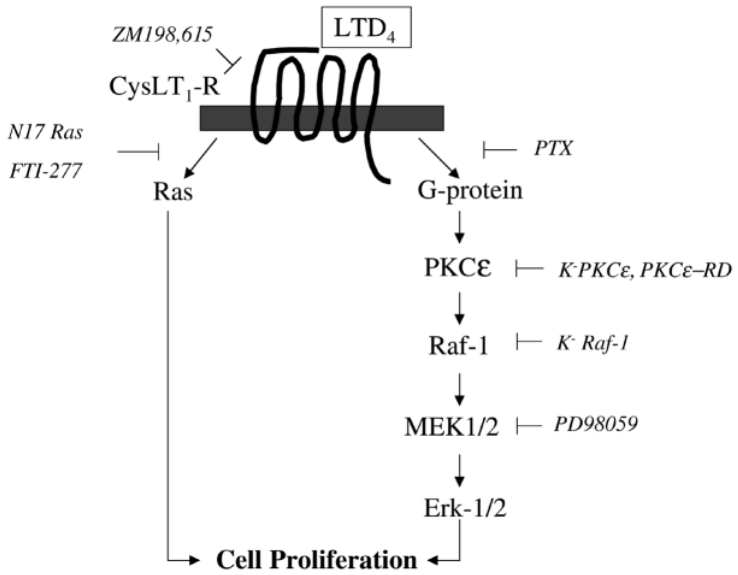

Fig. 8. A schematic model of the LTD_4-induced signalling pathways involved in the regulation of proliferation in human intestinal epithelial cells.

specific MEK inhibitor, on the LTD_4-induced proliferative response in these cells. Also in accordance with effects on LTD_4-mediated cell proliferation, we found that activation of Erk-1/2 by LTD_4 involves stimulation of a PTX-sensitive G-protein, a Src-like protein and MEK. Other types of G_i-protein-coupled receptors in other kinds of cells have been shown to initiate the MAPK signaling cascade through release of Gβγ subunits and activation of a Src-like kinase(s) (van Biesen et al., 1996). We found both similarities and discrepancies between the signaling pathways leading to activation of Erk-1/2. These observations agree with the conclusion drawn by Luttrell and coworkers that the mechanisms involved in activation of MAPK are heterogeneous and appear to depend not only on the nature of the G-protein-coupled receptor but also on the cell type (Luttrell et al., 1996). In addition to the signals discussed above, our findings that PKC inhibitors and downregulation of PKC isoforms impair the LTD_4-induced activation of Erk-1/2 in intestinal epithelial cells suggest that PKC plays an important role in this signaling cascade.

Hoshino and colleagues (Hoshino et al., 1998) have demonstrated that LTD_4 activates Erk-1/2 via a PTX-insensitive but PKCα- and Raf-1-dependent pathway in the monocytic leukemia cell line THP-1. However, we found that LTD_4 activates Erk-1/2 via a PTX-sensitive G-protein/PKC-dependent pathway in Int 407 intestinal epithelial cells. We also obtained evidence that it is the ε isoform of PKC that is involved in LTD_4-mediated stimulation of Erk-1/2. First, a high concentration of GF109203X was required to impair LTD_4-induced activation of Erk-1/2, and this compound is known to be a more potent inhibitor of classical PKC than of novel PKC isoforms (Chen et al., 1999), implying involvement of a novel isoform, such as PKCε. Second, we have earlier shown that LTD_4 not only resulted in translocation/activation of PKC α but also of the δ and ε isoforms (Thodeti et al., 2001). Third, exposing the cells to TPA for 4 hours, a sufficient amount of time for such a TPA treatment to abolish the LTD_4-induced activation of Erk-1/2, caused a significant downregulation only of PKCε. Fourth, by transfecting and using the regulatory domains of PKCε and PKCδ as isoform-specific dominant-negative inhibitors of PKC (Jaken, 1996), we conclude that PKCε, but not PKCδ, is involved in the LTD_4-induced activation of Erk-1/2. Finally, the LTD_4-induced activation of Erk-1/2 was totally inhibited in cells transfected with K^-PKCε.

The exact location at which PKC takes part in the activation of Erk-1/2 most probably depends on the stimulus and the cell type examined. It has been shown that the signaling pathway triggered by activation of the T-cell receptor on T-lymphocytes involves a PKC upstream of Ras (Downward et al., 1990). Nonetheless, we found that LTD_4-induced activation of Ras was insensitive to both PKC inhibitors and downregulation of PKC. In addition, despite our demonstration that LTD_4 can activate Ras, this activation does not seem to be involved in the LTD_4-effected stimulation of Erk-1/2. This conclusion was formed on the basis of the observation that inhibition of Ras, by either the Ras inhibitor FTI-277 or transfection with N17 Ras, had no effect on the LTD_4-induced activation of Erk-1/2. Our results instead agree with data showing that activation of Erk-1/2 by the M1 receptor involves PKC at a point that is upstream of Raf-1 activation (Marais et al., 1998).

We performed in vitro assays for Raf activities and observed that LTD_4 caused activation of Raf-1 with a peak around 2 minutes, which is in line with the LTD_4-induced activation of Erk-1/2. LTD_4-mediated activation of Raf-1 is sensitive to PTX, PKC inhibitors and downregulation of PKC by TPA. In accordance with our data indicating involvement of PKCε in LTD_4-induced activation of Erk-1/2, several investigators have shown that, once activated, PKCε can stimulate Raf-1 via phosphorylation of serine (Kolch et al., 1993; Khalil and Morgan, 1993; Cai et al., 1997). In support of our results, Velarde (Velarde et al., 1999) studied vascular smooth muscle cells and demonstrated that activation of PKCε is required for bradykinin-induced stimulation of Erk-1/2.

In conclusion, our results demonstrate that LTD_4 can promote proliferation of intestinal epithelial cells by a traditional Ras-dependent pathway but, more interestingly, even in the absence of Ras activity a G-protein/PKCε/Raf-1/MEK signaling pathway can induce proliferation via activation of Erk-1/2 in these cells. Activation of such signaling pathways and the subsequent increase in proliferation indicate that this inflammatory mediator can contribute to growth of intestinal cells during pathological inflammatory conditions.

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References

Adolfsson, J. L. P., Öhd, J. F. and Sjölander, A. (1996). Leukotriene D_4-induced activation and translocation of the G-protein α_{i3}-subunit in human epithelial cells. *Biochem. Biophys. Res. Commun.* **226**, 413-419.

Cai, H., Smola, U., Wixler, V., Eisenman-Tappe, I., Diaz-Meco, M. T., Moscat, J., Rapp, U. and Cooper, G. M. (1997). Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. *Mol. Cell. Biol.* **17**, 732-741.

Chen, N., Ma, W. Y., Huang, C. and Dong, Z. (1999). Translocation of protein kinase C epsilon and protein kinase C delta to membrane is required for ultraviolet B-induced activation of mitogen-activated protein kinase and apoptosis. *J. Biol. Chem.* **274**, 15389-15394.

Cheng, J.-J., Wung, B.-S., Chao, Y.-J. and Wang, D. L. (2001). Sequential activation of protein kinase C (PKC)-α and PKC-ε contributes to sustained Raf/Erk1/2 activation in endothelial cells under mechanical strain. *J. Biol. Chem.* **276**, 31368-31375.

Cory, A. H., Owen, T. C., Barltrop, J. A. and Cory, J. G. (1991). Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* **3**, 207-212.

Daub, H., Weiss, F. U., Wallasch, C. and Ullrich, A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**, 557-560.

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997). Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* **16**, 7032-7044.

Daulhac, L., Kowalski-Chauvel, A., Pradayrol, L., Vaysse, N. and Seva, C. (1999). Src-family tyrosine kinases in activation of ERK-1 and p85/p110-phosphatidylinositol 3-kinase by G/CCKB receptors. *J. Biol. Chem.* **274**, 20657-20663.

Downward, J., Graves, J. D., Warne, P. H., Rayter, S. and Cantrell, D. A. (1990). Stimulation of $p21^{ras}$ upon T-cell activation. *Nature* **346**, 719-723.

Ekbom, A., Helmick, C., Zack, M. and Adami, H.-O. (1990). Ulcerative colitis and colorectal cancer. A population-based study. *N. Engl. J. Med.* **323**, 1228-1233.

Garrington, T. P. and Johnson, G. L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* **11**, 211-218.

Grönroos, E., Andersson, T., Schippert, Å., Zheng, L. and Sjölander, A. (1996). Leukotriene D_4-induced mobilization of intracellular Ca^{2+} in epithelial cells is critically dependent on activation of the small GTP-binding protein Rho. *Biochem. J.* **316**, 239-245.

Hallberg, B., Rayter, S. I. and Downward, J. (1994). Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. *J. Biol. Chem.* **269**, 3913-3916.

Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M. and Lefkowitz, R. J. (1995). Distinct pathways of G_i- and G_q-mediated mitogen-activated protein kinase activation. *J. Biol. Chem.* **270**, 17148-17153.

Henle, G. and Dienhardt, F. (1957). The establishment of strains of human cells in tissue culture. *J. Immunol.* **79**, 54-59.

Hoshino, M., Izumi, T. and Shimizu, T. (1998). Leukotriene D_4 activates mitogen-activated protein kinase through a protein kinase Cα-Raf-1-dependent pathway in human monocytic leukemia THP-1 cells. *J. Biol. Chem.* **273**, 4878-4882.

Horwitz, R. J., McGill, K. A. and Busse, W. W. (1998). The role of leukotriene modifiers in the treatment of asthma. *Am. J. Respir. Crit. Care Med.* **157**, 1363-1371.

Jaken, S. (1996). Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8**, 168-173.

Khalil, R. A. and Morgan, K. G. (1993). PKC-mediated redistribution of mitogen-activated protein kinase during smooth muscle cell activation. *Am. J. Physiol.* **265**, C406-C411.

Kiley, S. C., Clark, K. J., Goddnough, M., Welch, D. R. and Jaken, S. (1999). Protein kinase C delta involvement in mammary tumor cell metastasis. *Cancer Res.* **59**, 3230-3238.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993). Protein kinase C alpha activates Raf-1 by direct phosphorylation. *Nature* **364**, 249-252.

Le Gall, M., Chambard, J.-C., Breittmayer, J.-P., Grall, D., Pouysségur, J. and van Obberghen-Schilling, E. (2000). The p42/p44 MAP kinase pathway prevents apoptosis induced by anchorage and serum removal. *Mol. Biol. Cell* **11**, 1103-1112.

Lerner, E. C., Qian, Y., Blaskovich, M. A., Fossum, R. D., Vogt, A., Sun, J., Cox, A. D., Der, C. J., Hamilton, A. D. and Sebti, S. M. (1995). Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. *J. Biol. Chem.* **270**, 26802-26806.

Li, X., Lee, J. W., Graves, L. M. and Earp, H. S. (1998). Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein couple receptor-EGF receptor transactivation pathway. *EMBO J.* **17**, 2574-2583.

Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J. and Lefkowitz, R. J. (1996). Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gβγ subunit-mediated activation of mitogen-activated protein kinases. *J. Biol. Chem.* **271**, 19443-19450.

Lynch, K. R., O'Neill, G. P., Liu, Q., Im, D.-S., Sawyer, N., Metters, K. M., Coulombe, N., Abramovitz, M., Figueroa, D. J., Zeng, Z. et al. (1999). Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor. *Nature* **399**, 789-793.

Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J. and Marais, R. (1999). Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J.* **18**, 2137-2148.

Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F. and Marshall, C. J. (1998). Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science* **280**, 109-112.

Massoumi, R. and Sjölander, A. (2001). Leukotriene D_4 affects localisation of vinculin in intestinal epithelial cells via distinct tyrosine kinase and protein kinase C controlled events. *J. Cell Sci.* **114**, 1925-1934.

Miranti, C. K., Ohno, S. and Brugge, J. S. (1999). Protei n kinase C regulates integrin-induced activation of the extracellular regulated kinase pathway upstream of SHC. *J. Biol. Chem.* **274**, 10571-10581.

Odajima, J., Matsumura, I., Sonoyama, J., Daino, H., Kawasaki, A., Tanaka, H., Inohara, N., Kitamura, T., Downward, J., Nakajima, K. et al. (2000). Full oncogenic activities of v-Src are mediated by multiple signaling pathways. *J. Biol. Chem.* **275**, 24096-24105.

Öhd, J. F., Wikström, K. and Sjölander, A. (2000). Leukotrienes induce cell-survival signaling in intestinal epithelial cells. *Gastroenterology* **119**, 1007-1018.

Peppelenbosch, M. P., Qiu, R.-G., de Vries-Smits, A. M. M., Tertoolen, L. G. J., de Laat, S. W., McCormick, F., Hall, A., Symons, M. H. and Bos, J. L. (1995). Rac mediates growth factor-induces arachidonic acid release. *Cell* **81**, 849-856.

Rao, G. N., Delafontaine, P. and Runge, M. S. (1995). Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor substrate-1, and phospholipase C-γ1 in rat aortic smooth muscle cells. *J. Biol. Chem.* **270**, 27871-27875.

Robinson, M. J. and Cobb, M. H. (1997). Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* **9**, 180-186.

Samuelsson, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**, 568-575.

Samuelsson, B. (2000). The discovery of leukotrienes. *Am. J. Respir. Crit. Care. Med.* **161**, S2-S6.

Sarau, H. M., Ames, R. S., Chambers, J., Ellis, C., Elshourbagy, N., Foley, J. J., Schmidt, D. B., Muccitelli, R. M., Jenkins, O., Murdock, P. R. et al. (1999). Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. *Mol. Pharmacol.* **56**, 657-663.

Sheng, H., Shao, J., Kirkland, S. C., Isakson, P., Coffey, R. J., Morrow, J., Beauchamp, R. D. and DuBois, R. N. (1997). Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J. Clin. Invest.* **99**, 2254-2259.

Sjölander, A., Grönroos, E., Hammarström, S. and Andersson, T. (1990). Leukotriene D_4 and E_4 induce transmembrane signaling in human epithelial cells. Single cell analysis reveals diverse parhways at the G-protein level for the influx and the intracellular mobilization of Ca^{2+}. *J. Biol. Chem.* **265**, 20976-20981.

Smalley, W. E. and DuBois, R. N. (1997). Colorectal cancer and nonsteroidal anti-inflammatory drugs. *Adv. Pharmacol.* **39**, 1-20.

Sutor, S. L., Vroman, B. T., Armstrong, E. A., Abraham, R. T. and Karnitz, L. M. (1999). A phosphatidylinositol 3-kinase-dependent pathway that differently regulates c-Raf and A-Raf. *J. Biol. Chem.* **274**, 7002-7010.

Thodeti, C. K., Adolfsson, J., Juhas, M. and Sjölander, A. (2000). Leukotriene D_4 triggers an association between Gβγ subunits and phospholipase C-γ1 in intestinal epithelial cells. *J. Biol. Chem.* **275**, 9849-9853.

Thodeti, C. K., Kamp Nielsen, C., Paruchuri, S., Larsson, C. and Sjölander, A. (2001). The epsilon isoform of protein kinase C is involved in regulation of the LTD_4-induced calcium signal in human intestinal epithelial cells. *Exp. Cell. Res.* **262**, 95-103.

van Biesen, T., Luttrell, L. M., Hawes, B. E. and Lefkowitz, R. J. (1996). Mitogenic signaling via G protein-coupled receptors. *Endocr. Rev.* **17**, 698-714.

Velarde, V., Ullian, M. E., Morinelli, T. A., Mayfield, R. K. and Jaffa, A. A. (1999). Mechanisms of MAPK activation by bradykinin in vascular smooth muscle cells. *Am. J. Physiol.* **277**, C253-C261.

Vuong, H., Patterson, T., Shapiro, P., Kalvakolanu, D. V., Wu, R., Ma, W. Y., Dong, Z., Kleeberger, S. R. and Reddy, S. P. (2000). Phorbol ester-induced expression of airway squamous cell differentiation marker, SPRR1B, is regulated by protein kinase Cdelta/Ras/MEKK1/MKK1-dependent/AP-1 signal transduction pathway. *J. Biol. Chem.* **275**, 32250-32259.

Watanabe, T., Shimizu, T., Miki, I., Sakanaka, C., Honda, Z., Seyama, Y., Teramoto, T., Shimizu, T., Ui, M. and Kurokawa, K. (1990). Characterization of the guinea pig lung membrane leukotriene D_4 receptor solubilized in an active form. *J. Biol. Chem.* **265**, 21237-21241.

Zeidman, R., Löfgren, B., Påhlman, S. and Larsson, C. (1999). PKCε, via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells. *J. Cell Biol.* **145**, 713-726.