The Pro-inflammatory Mediator Leukotriene D4 Induces Phosphatidylinositol 3-Kinase and Rac-dependent Migration of Intestinal Epithelial Cells*

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Inflammatory bowel diseases are associated with increased risk of developing colon cancer. A possible role of the pro-inflammatory leukotriene D4 (LTD4) in this process has been implicated by the findings that LTD4 can signal increased proliferation and survival, both hallmarks of a cancer cell, in non-transformed intestinal epithelial cells. Here we make the novel finding that LTD4 can also signal increased motility in these cells. In parallel, we found that LTD4 induced a simultaneous transient 10-fold increase in Rac but not Cdc42 activity. These data were also supported by the ability of LTD4 to activate the Rac GDP/GTP exchange factor Vav2. Further, LTD4 triggered a 3-fold transient increase in phosphatidylinositol 3-kinase (PI3K) phosphorylation, a possible upstream activator of the Vav2/Rac signaling pathway. The activation of Rac was blocked by the PI3K inhibitors LY294002 and wortmannin and by transfection of a kinase-negative mutant of PI3K or a dominant-negative form of Vav2. Furthermore, Rac was found to co-localize with actin in LTD4-generated membrane ruffles that were formed by a PI3K-dependent mechanism. In accordance, the inhibition of the PI3K and Rac signaling pathway also blocked the LTD4-induced migration of the intestinal cells. The present data reveal that an inflammatory mediator such as LTD4 cannot only increase proliferation and survival of non-transformed intestinal epithelial cells but also, via a PI3K/Rac signaling pathway, trigger a motile response in such cells. These data demonstrate the capacity of inflammatory mediators to participate in the process by which inflammatory bowel conditions increase the risk for colon cancer development.

Migration of epithelial cells is essential during the development of the gut and during different pathological situations such as wound healing and tumor metastasis (1). The conversion from a sessile to a migratory phenotype requires an extensive remodeling of the actin cytoskeleton (2). In response to different chemotactic substances, cell migration is initiated by the formation of lamellipodia or membrane ruffles at the leading front of a migrating cell (3, 4).

It is well established that epithelial cell migration is stimulated by activation of receptor tyrosine kinases (5, 6). The intracellular signaling pathways activated by such receptors and responsible for the coordinated changes in the actin cytoskeleton seen during cell motility generally include the activation of different members of the Rho family of GTPases (3). The Rho family consists of three main members: Rho; Rac; and Cdc42. In most cell types, these proteins execute specific functions, i.e. Rho promotes the formation of stress fibers and focal adhesion complexes and Rac initiates actin polymerization at the cell membrane and is responsible for the generation of lamellipodia and membrane ruffles, whereas Cdc42 promotes the formation of filopodia and microspikes at the cell periphery (7). These monomeric GTPases cycle between a GDP-bound inactive state and a GTP-bound active state. In their inactive state, Rho GTPases are bound to proteins called guanine nucleotide dissociation inhibitors. Guanine nucleotide exchange factors (GEFs),1 activated by extracellular stimuli, catalyze the exchange of GDP for GTP on Rho GTPases and thereby activate these proteins. In their GTP-bound state, these proteins interact with specific effectors to initiate downstream signals and functions (7). The subsequent hydrolysis of bound GTP to GDP is catalyzed by the family of GTPase-activating proteins (8).

The Dbl family constitutes the main group of GEFs acting on Rho GTPases (9). A hallmark of these exchange factors is that they contain two key conserved domains, a Dbl homology domain that is believed to be responsible for catalyzing GDP/GTP exchange on Rho GTPases and a pleckstrin homology (PH) domain that is important for cellular localization through its interaction with lipids and/or proteins (10). Notably, the PH domain on GEFs are known to bind phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, a substrate and a product of the PI3K, respectively. Such a lipid/protein interaction offers a reasonable explanation for how the PI3K can enable activation of Rac GTPase. It has been suggested that the association between GEF and phosphatidylinositol 3,4,5-trisphosphate may cause dissociation of the GEF from its binding to guanine nucleotide dissociation inhibitor (9). Possibly, such a mechanism could explain how agonist-induced activation of PI3K and generation of phosphatidylinositol 3,4,5-trisphosphate could enable a downstream phosphorylation and acti-

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activation of Vav (11). Src tyrosine kinase, a kinase known to be activated by LTD₄ (12), has been shown to phosphorylate and activate Vav2 and subsequently activate Rac (13). PI3Ks are composed of a catalytic subunit (p110) and a regulatory (p85/p55) subunit (14). Phosphorylation of the p85 subunit is essential for the translocation and activation of the p110/p55 complex from the cytosol to the plasma membrane where it interacts with its effectors (15). Interestingly, the inhibition of PI3K by both molecular and pharmacological approaches blocks membrane ruffling and inhibits cell migration (16).

Leukotrienes belong to an important group of pro-inflammatory mediators derived from arachidonic acid via the 5-lipoxygenase pathway and have been implicated in many inflammatory conditions (17, 18). LTD₄ is the most potent of the cysteinyll leukotrienes (LTC₄, LTD₄, and LTE₄), and it has been implicated in conditions such as asthma and inflammatory bowel diseases (19). LTD₄ mediates its effects by the binding to two different G protein-coupled receptors, CysLT₁ and CysLT₂ (20). The CysLT₁ receptor has a much higher affinity for its ligand than the CysLT₂ receptor and has been shown to couple to both a pertussis toxin (PTX)-sensitive and a PTX-insensitive G protein, indicating that different signal transduction pathways can be initiated and possibly mediate distinct downstream functions (12, 21, 22). We have previously shown that prolonged exposure of non-transformed intestinal epithelial cells to LTD₄ resulted in up-regulation of factors related to colon carcinogenesis such as cyclooxygenase-2, &-catenin, and Bcl-2 (23). More recently, we have shown that LTD₄ induces proli-
The absence or presence of 50 μM LY294002 for indicated periods of time, after which the cells were lysed. A, the cell lysates were immunoprecipitated (IP) with an anti-phosphotyrosine (PTyr) antibody (4 μg/ml) and immunoblotted with an anti-p85 subunit antibody (1:1000). B, the cells were immunoprecipitated with an anti-p85 subunit antibody (5 μg/ml) and immunoblotted with anti-phosphotyrosine antibody (1:5000). These blots were then reprobed with an anti-p85 antibody to confirm equal loading. Representative blots and the accumulated results of a densitometric analysis of the LTD4-induced phosphorylation of the p85 subunit are shown. The p85 tyrosine phosphorylation values were calculated as percentages of those seen in unstimulated cells and are given as the means ± S.E. of three separate experiments. C, the cells were preincubated in the absence or presence of 50 μM LY294002 (LY) for 30 min before LTD4 stimulation. The cells then were lysed, and the proteins were separated by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated Akt (Ser473). Thereafter, the blots were stripped and reprobed with an antibody for total Akt. The accumulated results of densitometric analysis of the LTD4-induced phosphorylation of Akt are shown. The illustrated blots are representative of at least three separate experiments. All of the statistical significances were evaluated using the unpaired Student’s t test. *, p < 0.05; **, p < 0.01.

Experimental Procedures

Materials—Antibodies for Rac and p85 subunit of PI3K were purchased from Upstate Biotechnology. Akt antibodies (phospho-Akt, Ser473, total Akt) were from Cell Signaling technology (Beverly, MA). LTD4 was purchased from Cayman Chemical Company (Ann Arbor, MI). ZM198615 was a gift from (AstraZeneca, R&D Lund). ECL Western blot detection reagents and Hyperfilms were from Amersham Biosciences (Buckinghamshire, United Kingdom). Wortmannin and PTX were obtained from Speywood Biosciences (Buckinghamshire, United Kingdom). Alexa 546 phalloidin is from Molecular Probes Inc. (Eugene, OR). All other chemicals were of analytical grade and obtained from Sigma.

Cell Culture—The Intestine 407 cell line was isolated from the intestine of a human embryo of ~2-month gestation and was successfully maintained in culture without any immortalizing transfections (28). These human intestinal epithelial cells (Int 407, Flow Laboratory), which exhibit typical epithelial morphology and growth (28), were cultured as a monolayer to ~80% confluence for 5 days. Cell cultures were kept at 37 °C in a humidified atmosphere of 5% CO2 and 95% air in basal medium Earle’s supplemented with 15% newborn 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 dominant negative p85 of PI3K or dominant-negative (DN) form of Vav2, which lacks an active dbl domain (L342R/L343S) for 6 h and were allowed to grow in serum containing medium for another 24 h. The DN-p85 construct was generously provided by Dr. Arthur Mercurio (Beth Israel Deaconess Medical Center, Boston, MA), whereas the DN-Vav2 construct was generously provided by Dr. Christopher Carpenter (Beth Israel Deaconess Medical Center). Control cells were transfected with empty pEGFP-N1 vector (Clontech). Transient transfections of the cells were achieved using 3.5 μl of Lipofectamine (Invitrogen) and 1.8 μg of plasmid DNA/ml and were performed in serum-free medium, essentially according to the protocol provided by the supplier. In all of the transfection experiments, it was routinely confirmed that the empty vector had no effect.

GST Pull-down Assays—The cDNA clone encoding the GST fusion protein of the PAK1B binding domain of Rac and Cdc-42 (PAKrib; amino acids 56–267) was cloned into the bacterial expression vector pGEX-2T and was expressed in Escherichia coli and cultured at 30 °C (29). The expression of GST fusion proteins was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and the E. coli were subsequently collected by centrifugation at 5000 × g for 15 min followed by sonication in phosphate-buffered saline. Triton X-100 (final concentration 1%) was added to lysate, and particulate matter was removed by centrifuging at 5000 × g for 15 min. The cleared lysate was incubated with glutathione-Sepharose beads (Sigma) for 1 h at 4 °C, and the beads were subsequently washed three times with ice-cold PBS. Lysates of unstimulated or stimulated cells were prepared in 1.0 ml of the lysis buffer supplemented with 10 mM MgCl2. GST fusion protein pre-bound to Sepharose beads was incubated with 1.0 ml of the lysis buffer supplemented with 0.5 M NaCl and twice with buffer A (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).

FIG. 2. LTD4 induces phosphorylation of the p85 regulatory subunit of PI3K and Akt in Int 407 cells. Cells were incubated in the absence or presence of 80 nM LTD4 for indicated periods of time, after which the cells were lysed. A, the cell lysates were immunoprecipitated (IP) with an anti-phosphotyrosine (PTyr) antibody (4 μg/ml) and immunoblotted with an anti-p85 subunit antibody (1:1000). B, the cells were immunoprecipitated with an anti-p85 subunit antibody (5 μg/ml) and immunoblotted with an anti-phosphotyrosine antibody (1:5000). These blots were then reprobed with an anti-p85 antibody to confirm equal loading. Representative blots and the accumulated results of a densitometric analysis of the LTD4-induced phosphorylation of the p85 subunit are shown. The p85 tyrosine phosphorylation values were calculated as percentages of those seen in unstimulated cells and are given as the means ± S.E. of three separate experiments. C, the cells were preincubated in the absence or presence of 50 μM LY294002 (LY) for 30 min before LTD4 stimulation. The cells then were lysed, and the proteins were separated by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated Akt (Ser473). Thereafter, the blots were stripped and reprobed with an antibody for total Akt. The accumulated results of densitometric analysis of the LTD4-induced phosphorylation of Akt are shown. The illustrated blots are representative of at least three separate experiments. All of the statistical significances were evaluated using the unpaired Student’s t test. *, p < 0.05; **, p < 0.01.
Leukotriene D₄ Induces PI3K and Rac Activation

RESULTS

LTD₄ Induces Cell Migration and Activation of Vav and Rac in the Intestinal Epithelial Cell Line, Int 407—As shown in Fig. 1A, LTD₄ could induce migration of intestinal epithelial cells in a concentration-dependent manner, although less prominent than that seen in a colon cancer cell line, Caco-2 (30). The Rac and Cdc42 GTPase have both been implicated in the regulation of migration of different cell types (7). To examine the ability of LTD₄ to activate Rac and Cdc42, we measured the activity of Rac and Cdc42 in cells using the affinity precipitation assay in which the GST-PAKcrib fusion protein binds the active GTP-
bound form of Rac or Cdc42. We found that the amount of active Rac-GTP increased in a time-dependent manner upon LTD₄ stimulation of Int 407 cells with a maximum after 15 min (Fig. 1B). Densitometric analysis confirmed the transient activation of Rac with a maximum of 942 ± 118%, 15 min after the addition of LTD₄. Under similar conditions, we were unable to detect any activation of Cdc42 (Fig. 1B). Whole cell lysates were used to ensure that the total amounts of Rac and Cdc42 did not change upon LTD₄ stimulation (Fig. 1B). As a positive control for the activation of Rac and Cdc42, the cells were stimulated with the epidermal growth factor (EGF) (Fig. 1B). Rac is known to be activated by GEFs such as Vav and Sos. Therefore, we investigated whether Vav2 or Sos was activated by LTD₄. We could not detect any activation of Sos by LTD₄ in these cells (data not shown); however, Vav2 immunoprecipitated from cells stimulated with LTD₄ for 15 min, the time for maximal activation of Rac, exhibited a distinctly increased tyrosine phosphorylation (Fig. 1C). This tyrosine phosphorylation of Vav2 is indicative of its activation (31). We transfected cells thereafter with a previously described (13) DN form of Vav2 (DN-Vav2) and noted that, in such transfected cells, the LTD₄-induced activation of Rac was abolished (Fig. 1D). These results clearly show that the LTD₄-induced activation of Rac is mediated through Vav2.

To further analyze the LTD₄-induced Rac signaling pathway, we investigated whether LTD₄ could activate PI3K in these cells. The activity of this enzyme was first assessed by investigating the degree of tyrosine phosphorylation of p85 subunit of PI3K (15). The cells were stimulated with LTD₄ for indicated periods of time, and immunoprecipitates of tyrosine phosphorylated proteins were then blotted with an anti-p85 antibody (Fig. 2A) or vice versa (Fig. 2B). As seen in Fig. 2, A and B, LTD₄ caused an increased tyrosine phosphorylation of the p85 regulatory subunit of PI3K. The time kinetics of this activation of PI3K was similar to the LTD₄-induced activation Rac (Fig. 1B). Secondly, we also investigated the ability of LTD₄ to induce activation of the serine/threonine kinase Akt, a well known downstream target of PI3K activity. Cells stimulated with LTD₄ exhibited a clear activation of Akt that was also confirmed to be sensitive to the PI3K inhibitor LY294002 (Fig. 2C). These results clearly argue for the ability of LTD₄ to cause activation of PI3K in intestinal epithelial Int 407 cells.

Involvement of Gₛ and PI3K in the LTD₄-induced Activation of Rac—To explore whether Gₛ, PI3K signals are involved in the LTD₄-induced activation of Rac, we pretreated the cells with the Gₛ protein inhibitor PTX (500 ng/ml for 2 h) and either of
two different PI3K inhibitors, LY294002 (50 μM for 30 min) or wortmannin (100 nM for 30 min) (32). The cells then were stimulated with LTD₄ for 15 min, after which they were lysed. Thereafter, the different lysates were analyzed in the GST-PAKcrib binding assay to assess the activity of Rac. We have reported earlier that LTD₄ can signal via a PTX-sensitive G protein (21, 24). Consistent with those results, preincubation with PTX significantly inhibited the LTD₄-mediated activation of Rac (Fig. 3A). We also observed that preincubation with either of the PI3K inhibitors led to an almost complete block of the LTD₄-mediated activation of Rac, suggesting that a PI3K signals upstream of Rac in these cells (Fig. 3A). This conclusion was confirmed by transfecting the cells with a DN p85 expressing vector (DN-p85). As shown in Fig. 3B, the expression of DN-p85 in these cells also blocked the LTD₄-induced activation of Rac.

**LTD₄ Mediates PI3K- and Rac-dependent Formation of Membrane Ruffles in Intestinal Epithelial Cells**—The Rac GTPase has previously been shown to induce the formation of lamellipodia and membrane ruffles in different cell types (33, 34). Here we show that cells stimulated with LTD₄ formed more membrane ruffles when compared with unstimulated control cells (Fig. 4). The formation of these membrane ruffles was blocked effectively by inhibiting the PI3K/Rac signaling pathway with either wortmannin or LY294002 (Fig. 4). We also examined whether and how LTD₄ influenced the distribution of Rac during the formation of membrane ruffles. Unstimulated control cells immunostained with an anti-Rac antibody exhibited a fairly homogenous staining of Rac throughout the cell (Fig. 5, top left). In contrast, LTD₄ stimulation caused a redistribution of Rac to the actin-containing membrane ruffles (Fig. 5, bottom left and middle). Merge pictures more clearly show the LTD₄-induced co-localization of Rac and actin in the membrane ruffles (Fig. 5, the two panels to the right). To ascertain that the LTD₄-induced membrane ruffles are indeed mediated via the CysLT₁ receptor, we also preincubated cells with the CysLT₁ receptor antagonist ZM198615. Alone this antagonist did not affect the number of ruffles of unstimulated cells, 17 ± 2 ruffles/cell in unexposed control cells and 20 ± 2 ruffles/cell in cells incubated with ZM198615 alone. The obtained results clearly show that, in the presence of ZM198615, all of the LTD₄-induced membrane ruffles were abolished (Fig. 5).

**The LTD₄-induced Migration Is Dependent on the PI3K/Rac Signaling Pathway**—Based on the finding that LTD₄ generates the formation of membrane ruffles via a PI3K/Rac signaling pathway, we investigated whether the same pathway was also involved in the LTD₄-induced migration of intestinal epithelial cells. As shown in Fig. 6A, the intestinal epithelial cell motility initiated by LTD₄ was effectively impaired by PTX and both of the PI3K inhibitors, LY294002 and wortmannin. Finally, a simplified schematic model of the signaling pathway participating in the regulation of LTD₄-induced motility in intestinal epithelial cell is outlined in Fig. 6B.

**DISCUSSION**

The CysLT₁-receptor has been shown previously to activate both a PTX-sensitive and a PTX-insensitive heterotrimeric G protein signaling pathway (12, 21, 22). This receptor has also previously been shown to mediate the activation of the small RhoA GTPase via a PTX-insensitive signaling pathway (12). In contrast, here we show that LTD₄-triggered activation of the small GTPase Rac was inhibited by PTX similar to the LTD₄-induced increase in survival and proliferation of intestinal epithelial cells (21, 24). Investigation of leukotriene-mediated activation of Rac in other cell types supports the present observation. In HL-60 cells, LTB₄-induced Rac activation and chemotaxis were blocked by pretreatment with PTX (35), as was LTD₄-mediated Rac activation in THP-1 cells (22). The notion that two different signaling pathways regulate the activity of RhoA and Rac in intestinal epithelial cell probably provides the CysLT₁-receptor with a more sophisticated control of epithelial cell motility. Subsequent to these findings, we then sought to identify the signaling molecules downstream of CysLT₁-receptor activation of a PTX-sensitive heterotrimeric G protein that could be implicated in the activation of Rac. One obvious candidate to look for is the PI3K. Indeed, it has been reported that lipids generated by the PI3K can participate in the regulation of small G proteins as well as cell motility (11, 36). We not only found that LTD₄ induced tyrosine phosphorylation of the p85 subunit of PI3K, which was used here as an index of its activation, but that the time kinetics of LTD₄-induced phosphorylation of the p85 subunit of PI3K was also parallel to the activation of Rac in intestinal epithelial cells. To further investigate the possibility that PI3K has a role in the LTD₄-induced activation of Rac, we blocked the PI3K activity by different means and tested the effects of such treatments on
the Rac activity. We found that pretreatment of Int 407 cells with wortmannin or LY294002, two structurally unrelated PI3K inhibitors, and also transfection of these cells with a DN pd85 construct halted the LTD4-induced activation of Rac. The fact that we also found that LTD4 had the ability to cause activation of Akt, a well known downstream target of PI3K, further supports our conclusion that LTD4 causes the activation of PI3K in intestinal epithelial Int 407 cells. In keeping with these results, a requirement of PI3K activity for the agonist-induced activation of Rac has also been reported in other cell systems (5). It is likely that the PI3K dependence in these experiments is related to the fact that the PI3K-generated phosphatidylinositol 3,4,5-trisphosphate lipid enables the activation of a number of Rac-specific GEFs (14). Vav2 seems to be the GEF involved in the activation of Rac by LTD4, because not only does it contain a PH domain and is therefore a target of the PI3K lipid product, we also found that Vav2 was tyrosine-phosphorylated, indicative of its activation. In addition, the LTD4-induced activation of Rac was totally blocked in intestinal epithelial 407 cells transfected with a dominant-negative form of Vav2.

In this study, we found that stimulation of Int 407 cells with LTD4 led to the formation of circular membrane ruffles rather than the classical Rac-induced lamellipodia (37). This observation was somehow surprising, because circular membrane ruffle formation has mainly been described in response to different growth factors and we have previously shown that stimulation of Int 407 cells with LTD4 does not result in a transactivation of either EGF or platelet-derived growth factor receptors (30). Although we cannot totally rule out the possibility of a cross-talk between the Cyst-LT1-receptor and alternative growth factor receptors, we do believe that our present results reflect a direct effect of the Cys-LT1-receptor on the cytoskeleton. Several additional findings support the view that the activation of PI3K and Akt, a well known downstream target of PI3K, also cause a simultaneous activation of Cdc42 in their target cells (30).

Cell migration is central to several important biological processes including tissue morphogenesis, wound healing, and cancer cell metastasis (41). Our previous results demonstrating that the pro-inflammatory mediator LTD4 can induce significant increases in proliferation and survival (27) can now be extended by the present finding that it also increases the motility of these cells, all hallmarks of tumor cell behavior. Consequently, the present results add further support for an important role of LTD4 as a link between inflammatory bowel diseases and subsequent development of colon cancer.

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