Activation of Phosphatidylinositol 3-Kinase by Interleukin-13

AN INHIBITORY SIGNAL FOR INDUCIBLE NITRIC-OXIDE SYNTHASE EXPRESSION IN EPITHELIAL CELL LINE HT-29

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The human colonic epithelial cell line HT-29 can be induced by a combination of the cytokines interleukin (IL)-1α, tumor necrosis factor α, and interferon-γ to express the inducible form of nitric-oxide synthase (iNOS; Kolios, G., Brown, Z., Robson, R., Robertson, D. A. F., & Westwick, J. (1995) Br. J. Pharmacol. 116, 2866–2872). IL-13 is a potent inhibitor of cytokine-induced iNOS mRNA expression and nitric oxide generation in HT-29 cells via an unknown mechanism. We report here that in HT-29 cells, IL-13 induces a concentration and time-dependent increase in the formation of the lipid products of phosphatidylinositol (PtdIns) 3-kinase, namely phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate. IL-13 also induces a parallel concentration and time-dependent increase in the in vitro lipid kinase activity present in immunoprecipitates of the p85 regulatory subunit of PtdIns 3-kinase. In addition, we also demonstrate that IL-13 stimulates the tyrosine phosphorylation of the adaptor molecule insulin receptor substrate 1, which may facilitate receptor coupling to PtdIns 3-kinase. Both the increases in D-3 phosphatidylinositol lipids and the increased in vitro lipid kinase activity of p85 immunoprecipitates were inhibited by wortmannin and LY294002. Inhibition of the PtdIns 3-kinase activity was paralleled by a reversal of the ability of IL-13 to inhibit iNOS mRNA expression and nitrite generation in HT-29 cells. These data demonstrate that the activation of PtdIns 3-kinase by IL-13 is a key signal that is responsible for the inhibition of iNOS transcription in activated epithelial cells.

Interleukin-13 (IL-13) is a pleiotropic cytokine secreted by activated Th-2 T lymphocytes that regulates a variety of immune target cells (1, 2). In B lymphocytes, IL-13 induces proliferation and differentiation and promotes CD23 expression and production of certain immunoglobulins such as IgG4 and IgE (3–5). In monocytes, IL-13 induces morphological changes (2), up-regulates expression of members of the integrin superfamily and major histocompatibility complex class II antigen expression, and down-regulates expression of CD14 and FcγR receptors (6). In lipopolysaccharide-stimulated monocytes, IL-13 also acts as a suppressor of proinflammatory cytokines (e.g. tumor necrosis factor (TNF) type α, IL-1, and IL-6), chemokines (e.g. IL-8, macrophage inflammatory protein 1α), and hematopoietic growth factors (e.g. granulocyte/macrophage-colony stimulating factor, granulocyte-colony stimulating factor) expression by activated monocytes/macrophages or endothelial cells (1, 6). Another target of IL-13 is epithelial cells, and we have recently demonstrated that IL-13 can modulate IL-8 generation from the human colonic epithelial cell line HT-29 (7).

Little is currently known about IL-13 signal transduction, although evidence points to similarities with IL-4 signal transduction. For instance, IL-13 can cross-compete with IL-4 for binding, leading to the suggestion that their receptors (IL-4R and IL-13R) share a common component (8). Both the IL-4Ra chain and the common γ chain subunit have been proposed as likely shared components (9–11). More recently, it has been proposed that the IL-13R may comprise the recently cloned IL-13Rα chain (12), an IL-13-binding protein related to the IL-5 receptor α chain (13) and the IL-4Ra chain (12). A common receptor may explain the observations that IL-4 can mimic every cellular response mediated by IL-13. In contrast, human T cells and mouse T and B cells respond to IL-4 but not to IL-13 (14). Unlike other cytokines, IL-4 and IL-13 do not induce tyrosine phosphorylation of the adaptor protein Shc or its association with Grb-2, and they do not activate the mitogen-activated family of protein kinases such as extracellular signal regulated kinases 1 and 2 (11). However, Janus family kinases and STAT proteins have been shown to be significant signal transduction components of cytokine receptors (15). Indeed, the IL-4Ra chain associates with JAK1, whereas the common γ chain associates with JAK3, and both are required for IL-4 activation of STAT-6 (15–17). JAK1, TYK2, and STAT-6 are activated family of protein kinases such as extracellular signal regulated kinases 1 and 2 (11).

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IL-4 and IL-13 share the ability to induce phosphorylation of several cellular proteins including the IL-4Ra chain itself (11, 18) and in particular, a p170 protein (11, 19) recently identified as IRS-2 (20). IRS-2, like its homolog, IRS-1, contains multiple specific YXXM motifs, which after tyrosine phosphorylation may bind the SH2 domains of the p85 regulatory subunit of phosphatidylinositol (PtdIns) 3-kinase, which associates through SH2 domains in the p85 subunit with proteins that are tyrosine-phosphorylated by protein-tyrosine kinases (20, 21). Indeed, several groups have shown that phosphorylation of the 170-kDa protein results in its tight association with PtdIns 3-kinase after IL-4 and IL-13 treatment (11, 19, 22). PtdIns 3-kinase belongs to a growing family of lipid kinases (23) and can potentially generate three products: phosphatidylinositol (3)-monophosphate (PtdIns(3)P), phosphatidylinositol (3,4)-

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biophosphate (PtdIns(3,4)P₂), and phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P₃), which are referred to collectively as D-3 phosphatidylinositol lipids (reviewed in Ref. 24). PtdIns 3-kinase may in fact be a multifunctional molecule because the different lipid products formed by the p110 catalytic subunit (24) may mediate different functions such as trafficking of cell-surface receptors (e.g. PtdIns(3)P) and cell signaling (e.g. PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃) (24). Moreover, the p85 subunit may act as an adaptor molecule that facilitates protein-protein interactions via its SH2 and SH3 domains and/or its proline-rich regions (24, 25). The significance of the association of PtdIns 3-kinase with IRS-2 after IL-4 and IL-13 treatment has yet to be established, but IL-4 treatment of mast cells, myeloid cells, and T cells results in the accumulation of D-3 phosphatidylinositol lipids (26). This implies that the function of PtdIns 3-kinase is probably not restricted to that of a p85 subunit-mediated adaptor function and that the putative signaling cascade regulated by PtdIns 3-kinase has been implicated in the regulation of a variety of receptors in numerous cell systems (reviewed in Ref. 25), including cellular responses and biochemical events initiated by a variety of stimuli (25, 27). Because wortmannin inhibits several pathways (25, 27). Because wortmannin irreversibly inhibits, at concentrations up to 100 nM (K, 1–10 nM), both the lipid kinase and serine kinase activity of PtdIns 3-kinase through covalent interaction with the p110 catalytic subunit (29). With the possible exception of a soluble PtdIns 4-kinase (30) and phospholipase A₂ (31), PtdIns 3-kinase is the only high-affinity target for wortmannin in mammalian cells, but higher concentrations (>1 μM) have been shown to inhibit other enzymes and signaling pathways (25, 27). Because wortmannin inhibits several cellular responses and biochemical events initiated by a variety of receptors in numerous cell systems (reviewed in Ref. 25), PtdIns 3-kinase has been implicated in the regulation of a growing number of diverse physiological events, in particular cell growth and proliferation (25).

We have recently demonstrated that epithelial cells of the colonic mucosa derived from patients with ulcerative colitis are a rich source of the nitric oxide-generating enzyme iNOS (25). A cell line HT-29 can be used to express iNOS mRNA, protein, and nitrite in response to a mixture of the proinflammatory cytokines IL-1α, TNF-α, and interferon-γ (IFN-γ) (34). Furthermore, iNOS expression can be inhibited by pretreatment of the HT-29 cells with IL-13 or IL-4, but not by IL-10 (25). Thus, we have used the colonic epithelial cell line HT-29 to explore the signaling mechanism of IL-13-induced suppression of iNOS induction. In this report, we describe the activation of the PtdIns 3-kinase signaling pathway after IL-13 treatment in HT-29 cells and that inhibition of this signaling pathway reverses the suppressive effect of IL-13 on iNOS expression and activity that is induced in response to a combination of IL-1α, TNF-α, and IFN-γ. Thus, PtdIns 3-kinase may be a pivotal pathway in determining the anti-inflammatory actions of IL-13.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IL-13 was kindly provided by Dr A. Minty (Sanofi Aventis, Bures-sur-Yvette, France). Human recombinant IL-1α (specific activity, 5 x 10⁷ units/mg) and TNF-α (specific activity, 6 x 10⁷ units/mg) were generous gifts from Glaxo (Greenford, United Kingdom) and Bayer (Slough, United Kingdom), respectively. Human recombinant IFN-γ (specificity, >2 x 10⁷ units/mg) was purchased from Boehringer Mannheim. Mouse p85α monoclonal antibody (6A) was a gift from Doreen Cantrell (Imperial Cancer Research Fund, London, United Kingdom). Wortmannin and the structurally unrelated synthetic quercetin derivative LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was purchased from Affiniti (Exeter, United Kingdom).

**Cell Culture**—The human colon epithelial carcinoma cell line HT-29 was obtained from the European Collection of Animal Cell Cultures. Cells were cultured in humidified incubators at 37 °C, 5% CO₂ in McCoy’s 5A medium supplemented with 10% (v/v) fetal bovine serum, 10 units/ml penicillin, 10 μg/ml streptomycin, and 0.5 μg/ml fungizone. For experiments, HT-29 cells were seeded at 2–3 x 10⁶ cells/ml until confluent. Confluent cells were cultured and fresh medium without fetal bovine serum 24 h before stimulation. Growth-arrested cells were treated with the appropriate concentration of stimuli in medium without serum as described above. Cell count and viability were checked by trypan blue exclusion at the beginning and the end of each experiment using representative wells. Cell viability was always greater than 95%.

**Fluorometric Assay for Nitric Oxide**—Nitric oxide production by HT-29 cells was determined by measuring the stable end-product nitrite in the cell culture supernatants by a fluorometric assay that is based upon the reaction of nitrite with 2,3-diaminonaphthalene to form the fluorescent product 1-(H)-naphthotriazole, as described previously (35). 200 μl of freshly prepared 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) were added in 2 ml of sample and mixed immediately. After 10 min of incubation at room temperature in the dark, the reaction was terminated with 100 μl of 2.8 N NaOH. The samples were measured using a Photon Technology International Inc. dual-wavelength spectrophotometer (excitation at 365 nm and emission at 405 nm) and compared with known concentrations of sodium nitrite. The sensitivity of the assay is 10 μM.

**Northern Blot Analysis**—Total cellular RNA from HT-29 cells was isolated as described previously (34, 36). Briefly, HT-29 monolayers were washed in a solution containing 25 mM Tris (pH 8.0), 4 M guanidine isothiocyanate, 0.5% (v/v) Sarcoyl, and 0.1 M 2-mercaptoethanol. After homogenization, the above-described suspension was added to an equal volume of 100 mM Tris (pH 8.0), 10 mM EDTA, and 1% (w/v) SDS. The RNA was then extracted with chloroform-phenol (1:1, v/v) and chloroform-isomyl (24:1, v/v). The total RNA was alcohol-precipitated, and the pellet was dissolved in diethyl pyrocarbonate-treated water. The concentration of RNA was measured by obtaining the absorbance at 260 nm and 280 nm, and 10 μg of RNA was loaded into each well of the agarose gel. Total RNA was separated by electrophoresis using formaldehyde and 1% (w/v) agarose gels and transferred overnight to nylon membrane (Boehringer Mannheim) by capillary blotting, and iNOS mRNA was detected using a digoxigenin-labeled probe according to the manufacturer’s instructions. Bound probes were detected using anti-digoxigenin Fab fragments conjugated to alkaline phosphatase with lumigen PPD (Boehringer Mannheim) as the chemiluminescent substrate. Blots were exposed after hybridization to x-ray film. Equivalent amounts of total RNA loaded/lane were assessed by monitoring 18 and 28 S RNA (34).

**Cell Lysis and In Vitro Lipid Kinase Assays**—3.5 x 10⁶ cells/ml were stimulated and incubated at 37 °C in RPMI 1640 medium as indicated. Reactions were terminated by the addition of 1 ml of ice-cold lysis buffer (1% (v/v) Nonidet P-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM iodoacetamide, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml chymostatin, 1 mM/ml pepstatin A, and 1 mM sodium orthovanadate). lysates were incubated at 4 °C for 15 min, followed by centrifugation at 14,000 rpm. The supernatant was preclarified with protein G-Sepharose beads for 1 h at 4 °C. Immunoprecipitates were performed for 2 h at 4 °C as described (37) using p85α mAb (1 μg/ml) precoupled to protein G-Sepharose beads (Pharmacia Biotech Inc.). Immunoprecipitates were washed and subjected to in vitro lipid kinase assays as described (37) using a lipid...
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mixture of 100 μl of 0.1 mg/ml PtdIns and 0.1 mg/ml phosphatidylserine dispersed by sonication in 20 mM HEPES, pH 7.0, and 1 mM EDTA. The reaction was initiated by the addition of 20 μCi of [γ-32P]ATP (3000 Ci/mmol; DuPont NEN) and 100 μM ATP to the immunoprecipitates suspended in 80 μl of kinase buffer. The reaction was terminated after 15 min, and phospholipids were then separated by TLC (37).

Immunoblotting with 4G10 mAb—When appropriate, cells were lysed after stimulation by IL-13 as already described. Immunoprecipitates were generated using either anti-p85 (1 μg/ml), anti-IRS-1 (1 μg/ml), or anti-IRS-2 (1 μg/ml) antibodies, and the immunoprecipitated samples for immunoblotting were electrophoresed through 10% acrylamide gels by SDS-polyacrylamide gel electrophoresis and transferred by electroblotting onto nitrocellulose (Schleicher & Schuell) as described previously (11, 38). Blots were probed with the anti-phosphotyrosine mAb 4G10 (0.5 μg/ml), and proteins were visualized by a chemiluminescence detection system (ECL; Amersham) with a goat anti-mouse Ig (0.1 μg/ml) conjugated with horseradish peroxidase as a secondary antibody (Dako). When appropriate, blots were completely stripped of antibodies by incubation at 55 °C for 60 min with stripping solution (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, and 100 mM 2-mercaptoethanol). After extensive washing, blots were reblocked before reprobing.

D-3 Phosphatidylinositol Lipid Labeling, Extraction, and HPLC Separation—4 × 10^6 HT-29 cells were labeled with 1 μCi of [32P]orthophosphate (8500–9120 Ci/mmol; DuPont NEN) as described (39). [32P]-labeled HT-29 cells (2 × 10^6 cells/120 μl) and stimulated as described in the figure legends, and phospholipids were extracted with 750 μl of chloroform:methanol:H₂O (32.6:65.3:2.1%, v/v/v, respectively) as described (39). The samples were deacylated and analyzed by anion exchange HPLC analysis using a Partisphere SAX column (Whatman). The eluate was fed into a Canberra Packard A-500 Flo-One on-line radio detector, and the results were analyzed by the Flo-One data program. Radiometric eluted peaks were compared with retention times for standards prepared from [H]-labeled phosphatidylinositol lipids (Amersham) and [32P]-labeled D-3 phosphatidylinositols as described elsewhere (39).

RESULTS

IL-13 Induces the Accumulation of D-3 Phosphatidylinositol Lipids—To investigate the signaling mechanisms activated by IL-13 treatment, [32P]-labeled HT-29 cells were stimulated with IL-13, and the outcome on PtdIns 3-kinase activation was determined by measuring the accumulation of D-3 phosphatidylinositol lipids. Treatment of HT-29 cells with IL-13 (0.3–30 ng/ml) resulted in the concentration-dependent accumulation of PtdIns(3,4,5)P₃ (Fig. 1A). Furthermore, the accumulation of PtdIns(3,4,5)P₃ was transient, with maximum accumulation occurring at 30 s post-IL-13 treatment (Fig. 1B). The levels of PtdIns(3,4,5)P₃ had declined back to basal levels within 10 min of IL-13 treatment. In addition, IL-13 also induced the accumulation of another D-3 phosphatidylinositol, namely PtdIns(3,4)P₂. However, the accumulation of PtdIns(3,4)P₂ occurred with slower kinetics, such that maximum accumulation of PtdIns(3,4)P₂ occurred at 1 min post-IL-13 treatment (Fig. 1B), although the levels of PtdIns(3,4)P₂ also declined back to basal levels within 10 min. The apparent lag time for the accumulation of PtdIns(3,4)P₂ compared with the accumulation of PtdIns(3,4,5)P₃ is consistent with the proposal that PtdIns(3,4)P₂ is the metabolic breakdown product of PtdIns(3,4,5)P₃ (24).

IL-13 Enhances the in Vitro Lipid Kinase Activity of p85 Immunoprecipitates—PtdIns 3-kinase activation in HT-29 cells was also determined by assaying the in vitro lipid kinase activity present in immunoprecipitates of the p85 regulatory subunit of IL-13-activated HT-29 cells with IL-13 (0.3–30 ng/ml) resulting in concentration-dependent increase in the in vitro lipid kinase activity present in p85 immunoprecipitates (Fig. 2A). As was observed for the IL-13-induced accumulation of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂, the IL-13-induced increase in in vitro lipid kinase activity was transient, with maximum lipid kinase activity present in p85 immunoprecipitates derived from HT-29 cells stimulated for 30 s with IL-13 (Fig. 2B). The p85-associated lipid kinase activity declined back to basal levels in p85 immunoprecipitates derived from cells treated for 10 min with IL-13 (Fig. 2B).

IL-13 Induces Tyrosine Phosphorylation of IRS-1 and Association with p85—To define more clearly the mechanism of interaction of the IL-13R with PtdIns 3-kinase, we performed experiments to investigate whether or not the coupling of the IL-13R to PtdIns 3-kinase involved the adaptor molecules IRS-1 and/or IRS-2. Both IRS-1 and IRS-2 have previously been implicated in IL-4R- and IL-13R-mediated signal transduction events (11, 19, 40, 41), and both can associate with PtdIns 3-kinase via specific phosphotyrosine-containing sequences (11, 19, 40, 41). To determine whether or not the coupling of the IL-13R to PtdIns 3-kinase involved the adaptor molecules IRS-1 and/or IRS-2, both IRS-1 and IRS-2 were immunoprecipitated from IL-13-stimulated HT-29 cells using antibodies directed against either the p85 subunit of PtdIns 3-kinase, IRS-1, or IRS-2 (Fig. 3). The resulting precipitates were immunoblotted with the 4G10 anti-phosphotyrosine mAb. Two co-immunoprecipitating tyrosine-phosphorylated proteins migrating at Mr 165,000–170,000, consistent with the molecular weights of IRS-1 and IRS-2, respectively, were precipitated by the p85 mAb (Fig. 3A). Immunoblotting of the p85 immunoprecipitates with the polyclonal anti-IRS-1 at 1:2000 revealed a time-dependent increase

![Fig. 1. IL-13-mediated accumulation of D-3 phosphatidylinositol lipids.](http://www.jbc.org/)}
in the association of IRS-1 with p85 that correlated with the kinetics of tyrosine phosphorylation of the proteins migrating at 165–170 kDa (data not shown). Furthermore, immunoblotting of the IRS-1 immunoprecipitates from IL-13-stimulated HT-29 cells with the 4G10 anti-phosphotyrosine mAb confirmed that IL-13 stimulation induced the strong tyrosine phosphorylation of IRS-1 (Fig. 3B). However, similar experiments using IRS-2 immunoprecipitates (Fig. 3C) revealed no detectable increase in IRS-2 tyrosine phosphorylation after IL-13 stimulation. Each blot was stripped and reprobed with 0.5 μg/ml anti-p85 (Fig. 3A) polyclonal anti-IRS-1 at 1:2000 (Fig. 3B) and polyclonal anti-IRS-2 at 1:4000 (Fig. 3C) to confirm efficiency of immunoprecipitation in each case (data not shown).

Inhibition of IL-13-induced Activation of PtdIns 3-Kinase by Wortmannin and LY294002—The accumulation of PtdIns(3,4,5)P3 (Fig. 4A and B) and PtdIns(3,4)P2 (data not shown) after IL-13 treatment was inhibited by pretreatment with the PtdIns 3-kinase inhibitors wortmannin (100 nM) (27) and the structurally unrelated LY294002 (1–30 μM) (28) 10 min before the addition of IL-13. Moreover, the IL-13-induced increase in lipid kinase activity present in p85 immunoprecipitates was also inhibited by 10 min pretreatment with wortmannin (Fig. 4C) and LY294002 (data not shown).

IL-13 Suppression of Proinflammatory Cytokine-induced iNOS Expression and Nitrite Production Is Prevented by PtdIns 3-Kinase Inhibitors—As shown previously (34), growth-arrested HT-29 monolayers stimulated with the proinflammatory cytokines IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 units/ml) result in the optimal generation of nitrite and expression of iNOS mRNA (Fig. 5). Both the expression of iNOS mRNA (Fig. 5A) and the generation of nitrite (Fig. 5B) induced by the proinflammatory cytokine mixture can be prevented by pretreatment of HT-29 cells with IL-13 (0.3–30 ng/ml) for 1 h before the addition of the cytokine mixture. However, addition of wortmannin (Fig. 5) or LY294002 (data not shown) to HT-29 cells 10 min before IL-13 treatment prevents the inhibitory effects of IL-13 on iNOS mRNA expression (Fig. 5A) and nitrite production (Fig. 5B).

DISCUSSION

In this report we demonstrate that IL-13 activates PtdIns 3-kinase in a human colon cancer cell line as determined by the accumulation of D-3 phosphatidylinositol lipids in vitro and increased lipid kinase activity present in immunoprecipitates of the p85 regulatory subunit of PtdIns 3-kinase. In addition, we also demonstrate that IL-13 stimulates the tyrosine phosphorylation of the adaptor molecule IRS-1, which has previously been reported to facilitate receptor coupling to PtdIns 3-kinase (42). This is the first evidence that IL-13 can stimulate the increased lipid kinase activity of PtdIns 3-kinase inhibitors.
FIG. 4. Wortmannin and LY294002 inhibit IL-13-induced activation of PtdIns 3-kinase. [32P]orthophosphate-labeled HT-29 cells (2 × 10^7 cells/120 μl) were incubated with (A) 100 nM wortmannin and (B) 1–30 μM LY294002 for 10 min at 37 °C. Cells were stimulated with 30 ng/ml IL-13
and is an important observation given that D-3 phosphatidylinositol lipids are increasingly thought to act as important regulatory molecules utilized by a plethora of receptors involved in diverse outcomes (reviewed in Ref. 24).

Accordingly, we have used the two structurally unrelated PtdIns 3-kinase inhibitors wortmannin and LY294002 to demonstrate that the IL-13-mediated activation of PtdIns 3-kinase and the resulting accumulation of D-3 phosphatidylinositol lipids is necessary for the IL-13-induced suppression of iNOS mRNA expression and nitrite production induced by the mixture of proinflammatory cytokines IL-1α, TNF-α, and IFN-γ. The ability of IL-13 to inhibit nitric oxide production and iNOS activity in macrophages (43, 44), mesangial cells (45), and HT-29 carcinoma cells2 induced by proinflammatory cytokines seems to be an important function of IL-13. Until now, however, the signaling cascades that mediate the effects of IL-13 on iNOS have not previously been characterized. Our demonstration that PtdIns 3-kinase inhibitors prevent the effects of IL-13 on iNOS is similar to a previous report that demonstrated that insulin-induced inhibition of phosphoenolpyruvate carboxykinate is dependent on PtdIns 3-kinase activation (46) and further demonstrates an important role for PtdIns 3-kinase in the negative regulation of the induction of specific mRNA.

To date, at least three forms of mammalian PtdIns 3-kinase have been identified, and these include various isoforms of the p85/p110 heterodimer (47), the G protein-coupled PtdIns 3-kinase γ (48), and the PtdIns-specific 3-kinase (49). The p85/p110 heterodimer is the best studied of these lipid kinases, and reagents to either p85 or p110 are readily available and reliable. In contrast, studies relating to PtdIns 3-kinase γ and PtdIns 3-kinase δ are hampered by a lack of reliable and commercially available antibodies. Immunoblotting experiments revealed that HT-29 cells predominantly expressed the p85a subunit of PtdIns 3-kinase δ, and we were unable to detect p85b in these cells.3 Furthermore, in vitro assays for associated lipid kinase activity revealed that IL-13 treatment increased the amount of lipid kinase activity present in p85a immunoprecipitates. These data strongly indicate that the p85/p110 heterodimeric PtdIns 3-kinase is coupled to and activated by the IL-13R. However, coupling of the IL-13R to other PtdIns 3-kinase family members cannot be entirely discounted, and these lipid kinases may potentially contribute to the production of total PtdIns(3,4,5)P3 extracted from IL-13-stimulated [32P]P-labeled HT-29 cells. Although the precise nature of the IL-13R is unclear, the known p85 binding motifs have not so far been identified in the intracellular domain of the related IL-4R. Nevertheless, the IL-4R has been demonstrated to associate with and/or activate PtdIns 3-kinase (22, 26, 50), and this coupling is thought to occur via an intermediate adaptor such as IRS-2, which does contain several YXXM motifs (20). IRS-2 is tyrosine-phosphorylated after IL-4 treatment, and this phosphorylation may be required for association of IRS-2 with PtdIns 3-kinase (20). IRS-2 has also been demonstrated to be tyrosine-phosphorylated and to associate with PtdIns 3-kinase after IL-13 treatment (11, 19), suggesting that IRS-2 may also facilitate the coupling of the IL-13R to the putative signaling cascades regulated by PtdIns 3-kinase. Interestingly, this study revealed that IL-13 was not able to induce detectable tyrosine phosphorylation of IRS-2 in HT-29 cells, although it did stimulate a rapid and strong tyrosine phosphorylation of IRS-1 within 1 min of poststimulation. Generally, the kinetics of IL-13-stimulated IRS-1 tyrosine phosphorylation correlate with the kinetics of IL-13-stimulated PtdIns 3-kinase activation. Data indicate that IRS-1 may potentially have an important role in coupling the IL-13R to PI3-kinase in HT-29 cells. However, a role for IRS-2 in the coupling of IL-13R to PtdIns 3-kinase in HT-29 cells cannot be entirely ruled out because low stoichiometry tyrosine phosphorylation of IRS-2 after IL-13 stimulation may be sufficient to allow IRS-2 to recruit PtdIns 3-kinase to the IL-13R.

Our demonstration that IL-13 differentially stimulates the tyrosine phosphorylation of IRS-1 but not IRS-2 correlates with previous observations that IRS-1 is a major phosphoprotein of nonhematopoietic cells, whereas IRS-2 phosphorylation is induced principally in murine hematopoietic cell types by various growth factors and cytokines (40, 51–55). Other evidence exists to indicate considerable heterogeneity in both receptor structure and signal transduction in different cell types for both IL-4 and IL-13. For instance, in immune cells IL-4 is a growth and differentiation factor, and the common γ chain is associated with the IL-4R (14). However, the common γ chain is not expressed in colon carcinoma cells in which IL-4 mediates a growth inhibitory effect (56). Moreover, both IL-4 and IL-13 induce the tyrosine phosphorylation of JAK2 tyrosine kinase in colon carcinoma cells (56, 57) but are unable to tyrosine-phosphorylate JAK2 in immune cells (11, 56, 58–60). Similarly, IL-4 is unable to phosphorylate JAK3 in colon carcinoma cells (56) but is able to phosphorylate JAK3 in immune cells (11, 58, 60). In addition to phosphorylation of cellular proteins, IL-4 has also been shown to trigger a unique second messenger pathway in human but not mouse B cells. This is characterized by a rapid, transient production of inositol (1,4,5)-trisphosphate and mobilization of Ca2+2, followed after a brief lag period by an increase in intracellular cAMP (61). This pathway is also activated by IL-13 in human monocytes and is required for the IL-13-mediated inhibition of protein kinase C-triggered respiratory burst (62). It is interesting to note, however, that we were unable to detect any changes in intracellular Ca2+2 concentration in the HT-29 cells after IL-13 stimulation.3 The diversity and heterogeneity of both receptors and signaling pathways activated by IL-4 and IL-13 may therefore facilitate the different functional effects of these related cytokines. Given the possible heterogeneity of signal transduction pathways coupled to the IL-13R, it will be necessary to determine whether IL-13 is able to activate PtdIns 3-kinase in other cell types such as immune cells and to investigate the relevance of PtdIns 3-kinase to other IL-13 functional events. This will undoubtedly help in evaluating the potential of the PtdIns 3-kinase pathway as a new and specific therapeutic target for intestinal inflammation.

We have therefore provided the first evidence to demonstrate that PtdIns 3-kinase activation is an important signal in determining the IL-13R-mediated inhibition of iNOS mRNA expression in response to inflammatory cytokines in the epithelial cell line HT-29. Additional experiments are necessary to

3 K. Wright, S. Ward, G. Kolios, and J. Westwick, unpublished observations.
determine the precise downstream events that occur after PtdIns 3-kinase activation in this system. For instance, it will be necessary to determine the effect of IL-13 on the activity of known downstream effectors of PtdIns 3-kinase such as protein kinase B and p70 S6 kinase (25) and to determine whether constitutively active mutants of PtdIns 3-kinase and known downstream effectors can mimic the effects of IL-13 on iNOS expression.

**Fig. 5.** PtdIns 3-kinase inhibitors prevent IL-13 suppression of iNOS mRNA expression and nitrite production. Growth-arrested monolayers of HT-29 cells were treated for 10 min with vehicle or with wortmannin (10–300 nM) as indicated at 37 °C. Cells were then either left untreated or treated with 30 ng/ml IL-13 for 1 h. Where indicated, the cells were then treated with IL-1α (10 ng/ml) TNF-α (100 ng/ml), and IFN-γ (300 units/ml) or left untreated. A, total cellular RNA was isolated after 12 h at 37 °C, and iNOS mRNA was detected as described under “Experimental Procedures.” The upper panel is the Northern blot analysis of iNOS mRNA expression, the middle panel is the densitometric analysis of the Northern blot, and the lower panel is the ethidium bromide-stained 18 and 28 S bands indicating equal loading of the lanes. Data is from a single experiment representative of three other experiments. B, nitrite production after the indicated treatments was determined in supernatants after 24 h at 37 °C as described under “Experimental Procedures.” The data are the mean ± S.E. of three separate experiments.
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