Phospholipase D Is Required in the Signaling Pathway Leading to p38 MAPK Activation in Neutrophil-like HL-60 Cells, Stimulated by N-Formyl-methionyl-leucyl-phenylalanine*

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Human acute myelogenous leukemia cells (HL-60 cells) can be induced to differentiate to neutrophils by exposure to dibutyryl-cyclic AMP. The differentiation of HL-60 cells allowed the mitogen-activated protein kinases p38 and p44/p42 to be rapidly and transiently activated upon stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP). Western blot analysis using phosphospecific p38 and p44/p42 mitogen-activated protein kinase antibodies showed that increasing concentrations of ethanol or 1-butanol but not 2-butanol (0.05–0.5%) inhibited fMLP-induced p38 activation but did not inhibit p44/p42 activation. These data indicated that activation of phospholipase D (PLD) was required for activation of p38 but not p44/p42. We compared the effect of fMLP with those of tumor necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF). We found that ethanol did not inhibit p38 phosphorylation upon stimulation with either GM-CSF or TNFα. These results suggested that in cells stimulated with fMLP, PLD was upstream of p38. To further test the involvement of PLD, we used antisense inhibition of human PLD1 expression. Treatment with antisense oligonucleotides inhibited p38 but not p44/p42 phosphorylation. These data supported a role for human PLD1 in fMLP-induced p38 activation in neutrophil-like HL-60 cells. In addition, the results obtained with TNFα and GM-CSF demonstrated that p38 activation occurred independently of PLD activation.

Human neutrophils constitute the first line of defense against invading microorganisms and are a major cellular component of acute inflammatory reactions (1). Neutrophils become rapidly activated (2, 3) through plasma membrane receptors that trigger responses to various compounds including proinflammatory cytokines granulocyte-macrophage colony stimulating factor (GM-CSF),† tumor necrosis factor α (TNFα), and the chemotactic factor N-formyl-methionyl-leucyl-phenylalanine (fMLP). The fMLP receptor is a seven-transmembrane-spanning receptor that is linked to heterotrimeric GTP-binding proteins (4). GM-CSF and TNFα are potent proinflammatory cytokines secreted by a variety of cells in response to infection and exert profound effects on the host immune system (5–7). The exposure of neutrophils to GM-CSF and TNFα, whose effects are mediated through non-G-protein-coupled receptors, primes the cells for enhanced release of microbicidal metabolites in response to other signals (6, 8, 9).

One of the early intracellular events to occur during neutrophil activation is the rapid induction of protein phosphorylation, which plays an essential role in the regulation of many neutrophil functions (8–11). Mitogen-activated protein kinases (MAPKs) have been demonstrated to play a central role in mediating intracellular signal transduction and regulating cellular functions in response to extracellular stimuli. The phosphorylation and activation of MAP kinases can be effected by both G-protein-coupled receptors and non-G-protein-coupled receptors (12).

The MAPKs are activated by diverse stimuli in the transduction of signals from the cell membrane to the nucleus. Three distinct MAP kinases have been identified to date in mammalian cells: p44/p42 ERKs are activated by growth factors (13); JNK/SAPK is potently activated by irradiation and other environmental stresses such as hyperosmolarity (14, 15); and p38 MAP kinase is activated by proinflammatory cytokines, osmotic stress, and UV irradiation (15). The p38 MAP kinase is associated with immune cell activation, because it is activated by a variety of inflammatory mediators (16, 17). It is well established that p38 plays an important role in gene expression in monocytes and macrophages (18–20). A specific function for p38 in the neutrophil remains unclear, but it has been suggested to play an important role in the respiratory burst, interleukin-8 production, and apoptosis (21–24). Activation of p38 follows phosphorylation of a distinctive TGY motif by the upstream kinase MEK3 or MEK6 (25–27). Activation of p38 leads to phosphorylation of specific transcription factors in vitro and in intact cells and thus may regulate gene expression (12, 25, 28). The activity of p38 MAP kinase is rapidly increased in neutrophils in response to extracellular stimuli, suggesting that this kinase cascade plays a pivotal role in regulating neutrophil function. Activation of p38 is involved in the signaling elicited by IMLP, TNFα, and GM-CSF (29).

Phospholipase D (PLD) is an important signal-transducing

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myelogenous leukemia cells; MAP, mitogen-activated protein; MAPK, MAP kinase; P38, phosphatidylethanol; PLD, phospholipase D; hPLD, human PLD; TNFα, tumor necrosis factor α; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase.
enzyme in a wide variety of cells and catalyzes the hydrolysis of phosphatidylcholine to produce the potential second messenger phosphatidic acid (reviewed in Refs. 30–33). In the presence of primary alcohols, PLD catalyzes a transphosphatidylation reaction producing phosphatidylalcohols at the expense of phosphatidic acid; this feature provides a tool to implicate PLD in cellular responses. The activation of human neutrophils by fMLP stimulates PLD (34). Upon the addition of N\textsuperscript{6},N\textsuperscript{2} -O-dibutryl-adenosine 3\textprime;:5\textprime; -cyclic monophosphate (dbcAMP), human acute myelogenous leukemia cells (HL-60 cells) differentiate into cells that display functions similar to neutrophils and have been used to study the regulatory mechanisms of differentiation and functions of neutrophils (35).

HL-60 cells express PLD1, but little (36) or no PLD2 (37). The regulation of expression of PLD has also been examined in HL-60 cells, which is used as a model for studies of PLD1 regulation (38). Moreover, Ohguchi et al. (39) found that the hPLD1 mRNA level is up-regulated during HL-60 cell differentiation. Therefore, we used the HL-60 cell model to determine the potential role of PLD1 in the activation of the MAP kinases p38 and p44/42.

So far, studies have not demonstrated a relationship between PLD and p38 activation in neutrophils stimulated with chemotactic factors. Using differentiated HL-60 cells as a model for studying regulatory mechanisms and functions of neutrophils, we determined the effect of fMLP on the activation of p38 and p44/42 as well as the involvement of phospholipase D in the signaling pathway utilized by fMLP. We compared the fMLP effect, which is mediated through a seven-transmembrane G-coupled-receptor, with TNF\textalpha{} and GM-CSF, which, unlike fMLP, act through non-G-coupled receptors (6, 9, 10).

One method to investigate PLD involvement in the signaling pathway leading to p38 activation is through the use of antisense oligonucleotides designed to hybridize to complementary mRNA sequences and block production of proteins encoded by the targeted mRNA transcripts. In general, most studies target antisense oligonucleotides to the AUG translation initiation codon (40–43), and this strategy has proven useful in designing the oligonucleotides targeted to PLD1 inhibition. These studies showed through the use of primary alcohols and antisense oligonucleotides that PLD was upstream of p38 in differentiated HL-60 cells stimulated with fMLP. However, the signaling pathways leading to the activation of p38 by the cytokines GM-CSF and TNF\textalpha{} did not require PLD. The results demonstrated that in HL-60 neutrophil-like cells, diverse agonists could activate p38 and that this response was mediated by PLD-dependent or PLD-independent mechanisms.

**EXPERIMENTAL PROCEDURES**

**Materials**—HL-60 cells were purchased from the American Type Culture Collection (Manassas, VA). Fetal bovine serum, RPMI 1640 medium, and insulin-transferrin-selenium-X (100 \times) were obtained from Life Technologies, Inc. dbcAMP, formyl-Met-Leu-Phe, leupeptin, and pepstatin were from Sigma. TNF\textalpha{} was from PeproTech EC Ltd. (London, United Kingdom), and GM-CSF was from R&D Systems (Minneapolis, MN). Ethanol, 1-butanol, and 2-butanol were from Fisher. [\textsuperscript{5,6,8,9,11,12,14,15}\textsuperscript{3}H]Arachidonic acid was from Amersham Pharmacia Biotech. Rabbit polyclonal antiphospho-p38 MAP kinase (Thr\textsuperscript{180}/ Tyr\textsuperscript{182}) and the horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from New England Biolabs, Inc. (Beverly, MA).

Phosphorylated ERK (p-ERK) (E-4) mouse monoclonal IgG\textalpha{} antibody against a peptide corresponding to amino acids 196–209 of ERK1 of human origin phosphorylated on Tyr\textsuperscript{202} (identical to corresponding to ERK2 sequence) and the horseradish peroxidase-conjugated goat anti-mouse antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Gel electrophoresis Kaleidoscope prestained standards were purchased from Bio-Rad. Enhanced chemiluminescence detection reagents were from Amersham Pharmacia Biotech. Nitrocellulose membranes were obtained from Schleicher and Schuell. BCA protein assay reagents were obtained from Pierce. The human PLD1 antisense was initially synthesized and tested at the Institut de Recherche-Jouvenel/Parvis-Davis (Fresnes, France). The PLD antisense cDNA was synthesized based on oligonucleotide primers designed in the Wake Forest University School of Medicine DNA synthesis core laboratory as phosphorothioate derivatives the first two and last two deoxynucleotides and purified by high performance liquid chromatography. The sequences used were as follows: hPLD1 antisense (CCGTGGCTCGTTTT-TCACTGACAT) and hPLD1 5′Rd (CTTCTCGTAGGCTGTGTCATAG). The sequences used were screened for their uniqueness using Blast 2.1.2 (NCBI). Gel silica 60 thin layer chromatography plates and Kodak X-Omat AR film were purchased from VWR (Suwannee, GA). Phosphatidylethanolamine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Preparation of Synthetic Oligonucleotides**—Synthesis of phosphorothioate derivatives the first two and last two deoxynucleotides primers were synthesized in the Wake Forest University School of Medicine DNA synthesis core laboratory as phosphorothioate derivatives the first two and last two deoxynucleotides and purified by high performance liquid chromatography. The sequences used were as follows: hPLD1 antisense (CCGTGGCTCGTTTT-TCACTGACAT) and hPLD1 5′Rd (CTTCTCGTAGGCTGTGTCATAG). The sequences used were screened for their uniqueness using Blast 2.1.2 (NCBI). Gel silica 60 thin layer chromatography plates and Kodak X-Omat AR film were purchased from VWR (Suwannee, GA). Phosphatidylethanolamine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Cell Cultivation and Cell Lysis**—Differentiated HL-60 cells were harvested, washed, and resuspended in RPMI 1640 insulin-transferrin-selenium at a density of 2.5 \times 10\textsuperscript{5} cells/ml. Cells were then incubated at 37 °C under gentle agitation for 1 h before adding the agonist. fMLP was diluted in RPMI 1640 prior to stimulation and was added to medium to a final concentration of 100 nM. TNF\textalpha{} was prepared in Tris, pH 8.0, was added to medium to a final concentration of 25 ng/ml, and GM-CSF prepared in PBS was added to a final concentration of 200 pg/ml. In the experiments studying the effect of ethanol, 1-butanol, and 2-butanol on the phosphorylation of the MAP kinases in response to stimulation with fMLP (100 nM; 2 min), GM-CSF (200 pg; 5 min), and TNF\textalpha{} (25 ng/ml; 5 min), varying concentrations of ethanol, 1-butanol, or 2-butanol (0.025–0.5%) were added just prior to the addition of the agonist. The reaction was stopped on ice, and the cell suspensions were centrifuged. The supernatants were removed, and the pellets were resuspended in 100 \mu l of lysis buffer consisting of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% Triton X-100, 20 mM \beta-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 \mu g/ml leupeptin, 1 \mu g/ml antipain, 1 \mu g/ml Na\textsubscript{2}VO\textsubscript{4}, and 50 mM Na\textsubscript{2}F. Cell lysates were then sonicated with 12 × 1-s bursts with a Branson probe sonicator (Branson Sonic Power Co., Danbury, CT) set at 20% of maximum energy. Insoluble material was removed by centrifugation at 16,000 \times g for 10 min at 4 °C. Protein content was determined with the BCA protein assay (Pierce) using bovine serum albumin as a standard.

**Western Blots**—The proteins were combined with equal volumes of 2× Laemmli sample buffer and boiled for 4 min. For both p44/42 and p38, 50 \mu g of proteins were resolved by SDS-polyacrylamide electrophoresis (4.5% stacking gel and 12% running gel) and electrotransferred to nitrocellulose membranes for 75 min. Prestained protein standards (Bio-Rad) were run in each gel. The blots were blocked overnight at 4 °C in Tris-buffered saline/Tween 20 (TBS-T containing 10 mM Tris-base, pH 7.4, 154 mM NaCl, and 0.1% Tween 20) supplemented with 3% Carnation nonfat milk. The membranes were hybridized with phosho-specific antibodies (phospho-p44/42 and phospho-p38) or nonphospho-specific anti-p44/42 or anti-p38. After three washes of 5 min each with TBS-T (1×), the blots were incubated with horseradish peroxidase-linked anti-IgG antibodies, washed again in the same conditions, and visualized with enhanced chemiluminescence reagents. The phosphorylated or nonphosphorylated MAP kinases were detected by autoradiography for variable lengths of time with Kodak X-Omat film. To confirm that the same amount of cellular proteins was loaded on each lane, the primary antibody-secondary antibody complex was removed by incubating the blot in stripping buffer (100 mM \beta-mercaptoethanol, 2% SDS, 300 mM Tris-HCl, pH 6.7, 50 mM \beta-mercaptoethanol, 2% SDS, 300 mM Tris-HCl, pH 6.7, 50 mM \beta-mercaptoethanol, 2% SDS, 300 mM Tris-HCl, pH 6.7, 50 mM \beta-mercaptoethanol, 2% SDS, 300 mM Tris-HCl, pH 6.7, 50 °C). After this procedure, the blots were washed with TBS-T (1×), blocked with buffer containing 3% Carnation nonfat milk, and reprobed with rabbit antibodies against p38 MAP kinase, followed by incubation with horseradish peroxidase-conjugated antibodies, as described above. Proteins were detected by the enhanced chemiluminescence reagents method as described by the manufacturer.

**Oligonucleotide Treatment—**HL-60 cells were treated with hPLD1

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2 A. J. Melendez, personal communication.
antisense oligonucleotides or hPLD1 randomized oligonucleotides in the presence of LipofectAMINE reagent (2 mg/ml). LipofectAMINE reagent was mixed with oligonucleotides (both prepared in Opti-MEM), and complexes were allowed to form at room temperature for 1 h. Meanwhile HL-60 cells in suspension in serum-free medium (RPMI and complexes were allowed to form at room temperature for 1 h. The reaction was stopped on ice, and the cell suspensions were centrifuged, and samples were analyzed by Western blot. Equal amounts of proteins were loaded in duplicate, and p44 and p38 were detected with specific antibodies. The blots are representative of three different experiments. The data were analyzed by Student’s t test and indicated a significant difference between undifferentiated and differentiated cells (p < 0.05).

**Effect of Differentiation of HL-60 Cells on p38 and p44/p42 Levels and Activation by fMLP**—The total amount of the MAP

**Assay of PLD Activity in Differentiated HL-60 Cells**—Treated HL-60 cells were differentiated to a neutrophil-like phenotype with dbcAMP. After the addition of dbcAMP for 48 h, the cells were radiolabeled for 24 h. Following incubation, 4 ml of the complete growth medium (with serum) was added, and the cells were incubated for 24 h. HL-60 cells were then induced to differentiate, treated with fMLP, and lysed as described above.

**Immunoblot Analysis**—The analysis was performed using antibodies specific for doubly phosphorylated p38 (Thr180/Tyr182) or antibodies recognizing the tyrosine 204-phosphorylated sequence 196–209 of either p42 or p44 and were quantified by laser-scanning densitometry.

**RESULTS**

**Effect of Differentiation of HL-60 Cells on p38 and p44/p42 Levels and Activation by fMLP**—The total amount of the MAP

**Characterization of p38 and p44/p42 Activation by fMLP in Neutrophil-like HL-60 Cells**—We studied the fMLP-induced activation of p38 and p44/p42 in differentiated HL-60 cells using phosphospecific antibodies that bind only the activated form of p38 or p44/p42. Immunoblot analysis demonstrated that fMLP, throughout the dose range of 10^{-10} to 10^{-8} M, activated the MAP kinases p38 (Fig. 3A) and p44/p42 (Fig. 4A) in a dose-dependent manner. The maximal level of stimulation was reached at 10^{-8} M of fMLP for p38 and 10^{-8} M for p44/p42. As shown in Fig. 3B, fMLP induced a time-dependent, transient phosphorylation of p38. Phosphorylation of p38 was ap-
PLD Is Upstream of p38 in Differentiated HL-60 Cells

Fig. 3. Concentration response and time course of p38 activation by fMLP. Differentiated HL-60 cells (2.5 × 10^6 cells/ml) were stimulated with the indicated concentrations of fMLP for 2 min (A) or stimulated for the indicated times with 100 nM fMLP at 37 °C (B). Cells were sonicated, and samples were analyzed by immunoblot. Equal amounts of proteins were loaded, and p38 phosphorylation was detected by immunoblot with phosphospecific antibodies. Data are representative of three experiments.

Fig. 4. Concentration response and time course of ERK activation by fMLP. Differentiated HL-60 cells (2.5 × 10^6 cells/ml) were stimulated with the indicated concentrations of fMLP for 2 min (A) or stimulated for the indicated times with 100 nM fMLP at 37 °C (B). Cells were sonicated, and samples were analyzed by immunoblot. Equal amounts of proteins were loaded, and p44/p42 phosphorylation was detected by immunoblot with phosphospecific antibodies. Data are representative of three experiments.

Inhibition of fMLP-induced p38 MAP Kinase Phosphorylation by Ethanol—We next investigated the involvement of PLD in the activation of the MAP kinases p38 and p44/p42 upon stimulation with fMLP. To determine if PLD-derived products were involved in activation of MAP kinases, we stimulated cells in the presence of ethanol. As shown in Fig. 5D, increasing concentrations of ethanol (0.025–0.5%) inhibited fMLP-induced activation of p38 MAPK. Inhibition of fMLP-induced p38 MAP kinase phosphorylation occurred with low concentrations of ethanol (0.125%). Significant inhibition (p < 0.05, Student’s t test, n = 4) of p38 phosphorylation was observed with 0.25 and 0.5% of ethanol (Fig. 5D). The inhibition was calculated by comparing stimulation in the presence of ethanol versus stimulation in the absence of ethanol (control). We found 59 and 71% inhibition of p38 phosphorylation with 0.25 and 0.5% of ethanol, respectively. In contrast, ethanol did not inhibit fMLP-induced activation of p44/p42 (Fig. 5, inset blot C). To rule out a less specific effect of ethanol on the system, we used a longer chain primary alcohol (1-butanol) and included a secondary alcohol (2-butanol), which is not a PLD substrate. We found that 1-butanol, like ethanol, inhibited p38 phosphorylation (Fig. 6A). However, 2-butanol, as expected, did not inhibit p38 phosphorylation (Fig. 6B). The inhibition of p38 phosphorylation by 1-butanol was more than that with ethanol (86, 80, and 87% inhibition, respectively, with 0.125–0.25 and 0.5% of 1-butanol versus 31, 59, and 71% inhibition with 0.125–0.25 and 0.5% ethanol, respectively).

Ethanol Does Not Affect TNFα-induced and GM-CSF-induced Activation of MAP Kinases p38 and p44/p42—To determine whether the observed inhibition by ethanol and 1-butanol of fMLP-induced phosphorylation of p38 was specific to fMLP, we examined the actions of other known agonists on the phosphorylation of p38 MAP kinase and the activation of this cascade.

TNFα- and GM-CSF-stimulated PLD activities were measured. PLD activity was assessed by measuring [3H]PEt formation (see “Experimental Procedures”) in differentiated HL-60 cells treated with either TNFα or GM-CSF. We found that neither TNFα nor GM-CSF increased PLD activity in differentiated HL-60 cells (no significant differences between control: +TNFα or GM-CSF/no ethanol versus +TNFα or GM-CSF/with ethanol; data not shown). These results are in agreement with other reports showing that TNFα or GM-CSF alone do not activate PLD. However, it has been shown that those two compounds do enhance PLD activity in neutrophils when subsequently stimulated with another agonist (47–49).

Next, we exposed differentiated HL-60 cells to TNFα and GM-CSF in the presence of increasing concentrations of ethanol (0.025–0.5%). Following stimulation, the activation of cellular MAP kinases was evaluated by using Western blots to detect the induced protein phosphorylation as described under “Experimental Procedures.” As shown in Figs. 7 and 8, p38 MAP kinase became phosphorylated in differentiated HL-60 cells in response to stimulation of TNFα (Fig. 7A, lane 2) and GM-CSF (Fig. 8A, lane 2). Unlike the previous data showing an inhibitory effect of ethanol on fMLP-induced phosphorylation of p38, ethanol did not reduce the TNFα-induced (Fig. 7A) or the GM-CSF-induced (Fig. 8A) phosphorylation of p38. The p44/p42 was also activated by exposure of the differentiated cells to GM-CSF (Fig. 8B) but not to TNFα (Fig. 7B). These findings are in agreement with previous studies showing that in neutrophils, TNFα activates only p38 (50). Ethanol was unable to block GM-CSF-induced phosphorylation of p44/p42 (Fig. 8B). Taken together, those data suggested that PLD was not involved in the activation of the MAP kinases when the cells are stimulated with either TNFα or GM-CSF.

Treatment of HL-60 Cells with Oligonucleotides—The phosphorylation of p38 by fMLP in differentiated HL-60 cells was blocked by primary alcohols. This observation suggested that PLD was involved in the signaling pathway leading to the activation of p38. Therefore, we examined the effect of hPLD1-specific antisense oligonucleotides and hPLD1 randomized oligonucleotides on the phosphorylation of p38 from activated HL-60 neutrophil-like cells. The antisense oligonucleotide was complementary to nucleotides 96–119 of the hPLD1. Chiang et al. (51) have demonstrated that oligonucleotides complexed with Lipofectin were more rapidly taken up by cells than were...
free oligonucleotides. We used this strategy to introduce the oligonucleotides into HL-60 cells.

HL-60 cells were treated for 24 h with either LipofectAMINE plus oligonucleotides (0.5–50 μM) or LipofectAMINE alone, incubated in the presence of serum for 24 h, and differentiated for 3 days with dbcAMP (0.5 mM). After differentiation, the cells were activated or not with fMLP (100 nM, 2 min), and whole cell homogenates were prepared for Western blot analysis.

Fig. 9A showed that hPLD1 antisense oligonucleotides inhibited fMLP-induced p38 phosphorylation but did not inhibit fMLP-induced p44/p42 phosphorylation (Fig. 9B). These data demonstrated that PLD was required in the activation of p38 upon stimulation with fMLP. The incubation of HL-60 cells with increasing concentrations of hPLD1 randomized oligonucleotides did not affect the phosphorylation of p38 upon stimulation with fMLP (Fig. 9C).

hPLD1 Antisense Oligonucleotides Inhibited [3H]PEt Formation in Differentiated HL-60 Cells Stimulated with fMLP—To confirm that the inhibition of p38 phosphorylation by the antisense oligonucleotides correlated with inhibition of PLD ac-
Differentiated HL-60 cells (2.5 × 10^6 cells/ml) were stimulated with TNFα (25 ng/ml; 5 min) in the presence of varying concentrations of ethanol (0.025–0.5%) at 37 °C (A). The alcohol was added just prior to the addition of TNFα. Cells were sonicated, and samples were analyzed by Western blotting. Equal amounts of proteins were loaded, and either p38 (A) or p44/42 (B) phosphorylation was detected by immunoblot with phosphospecific antibodies. The blots are representative of three experiments.

Fig. 7. Ethanol did not inhibit TNFα-induced p38 phosphorylation. Differentiated HL-60 cells (2.5 × 10^6 cells/ml) were stimulated with TNFα (25 ng/ml; 5 min) in the presence of varying concentrations of ethanol (0.025–0.5%) at 37 °C (A). The alcohol was added just prior to the addition of TNFα. Cells were sonicated, and samples were analyzed by Western blotting. Equal amounts of proteins were loaded, and either p38 (A) or p44/42 (B) phosphorylation was detected by immunoblot with phosphospecific antibodies. The blots are representative of three experiments.

Experimental Procedures

Activity was assessed by measuring [3H]PEt formation (see “Experimental Procedures”) in differentiated HL-60 cells treated with antisense oligonucleotides upon stimulation with fMLP (100 nM; 2 min). We used oligonucleotide concentrations (25 μM and 50 μM) that inhibited p38 activation. As shown in Fig. 10A, both concentrations of the antisense oligonucleotides decreased the production of [3H]PEt by 2.3-fold (control + fMLP/no antisense) [3H]PEt = 1.4% of total lipids; versus 0.60 and 0.61%, respectively, for stimulated cells in the presence of either 25 or 50 μM concentration of the antisense oligonucleotides). We used hPLD1 randomized oligonucleotides as a control, and we did not observe an inhibitory effect of the randomized oligonucleotides on the [3H]PEt formation (Fig. 10C). These data suggested that the oligonucleotides inhibited the expression of the targeted protein, hPLD1.

The quantitation of p38 (Fig. 10B) showed a 72 and 62% inhibition with a 25 and 50 μM concentration, respectively, of antisense hPLD1 ODNs. The inhibition was calculated by comparing phosphorylation of p38 in HL-60 neutrophil-like cells stimulated with fMLP in the presence of antisense hPLD1 ODNs versus phosphorylation of p38 in HL-60 neutrophil-like cells stimulated with fMLP in the absence of antisense hPLD1 ODNs.

PLD Is Upstream of p38 in Differentiated HL-60 Cells

It has been reported that stimulation of neutrophils with fMLP activates cellular p44/p42 MAP kinase via a Ras- and Raf-mediated pathway (52). In addition, investigation of human neutrophils has revealed that in response to fMLP, p38 is activated (29, 53) as well as PLD (34, 54, 55). However, the mechanism by which chemotactic factors such as fMLP stimulate p38 has not yet been elucidated. Krump et al. (53) have shown that the activation of p38 by chemotactic peptides in human PMN involved phosphatidylinositol 3-kinase, protein kinase C, and calcium. However, none of those studies found a link between PLD and p38.

To elucidate the role of PLD in the activation of p38, we used the property that PLD has to catalyze a transphosphatidylation reaction. The most commonly used alcohol has been ethanol, which leads to the formation of phosphatidylethanol. We also used 1-butanol and 2-butanol to rule out a less specific...
Effect of ethanol. We found that ethanol and 1-butanol inhibited fMLP-induced phosphorylation of p38, whereas 2-butanol, as expected, did not. Furthermore, ethanol did not inhibit fMLP-induced phosphorylation of p44/p42. Our results provide evidence that one of the signal transduction pathways initiated by fMLP leading to activation of p38 involves PLD. However, there are reports showing that PLD is downstream of ERK MAP kinases. Indeed, Muthalif et al. (56) have shown that norepinephrin-induced PLD activity in vascular smooth muscle cells was independent of PKC activity, required the Ras/MAP kinase pathway, and was phosphorylated by ERK2 MAP kinase (56).

To determine whether the observed inhibition was only seen when the agonist was fMLP, we compared the effects of GM-CSF and TNFα, which mediate their actions through non-G-protein-coupled receptors, on p38 and p44/p42 phosphorylation in the presence or absence of ethanol. As previously described by other groups, GM-CSF induced the activation of p38 and p44/p42 MAP kinases (53), but stimulation with inflammatory cytokine TNFα triggered the activation of p38 MAP kinase only (53). Unlike its effects with fMLP, ethanol did not inhibit GM-CSF or TNFα-induced p38 phosphorylation and did not inhibit GM-CSF-induced p44/p42 phosphorylation. Although GM-CSF, TNFα, and fMLP behave similarly in priming some functions of mature neutrophils, such as adherence, chemotaxis, phagocytosis, and superoxide anion production, these results showed that different signaling pathways can be utilized by these agents.

Unlike fMLP, neither GM-CSF nor TNFα increased PLD activity in differentiated HL-60 cells. The addition of GM-CSF or TNFα to human peripheral blood neutrophils primes PLD to subsequent stimulation by fMLP or phorbol myristate acetate (47, 57–59). However, PLD activity was observed to be present in GM-CSF-stimulated adherent neutrophils, but not in neutrophils maintained in suspension (60). According to the data presented in this study, PLD did not seem to be involved in the signaling pathway leading to p38 activation by GM-CSF and TNFα. We have demonstrated that antisense oligonucleotides that target human PLD1 inhibit the phosphorylation of p38 in differentiated HL-60 cells stimulated with fMLP. The data support the hypothesis that PLD is upstream of p38. These results indicated that multiple signaling pathways were involved in stimulating p38 and that p38 MAP kinase played different roles in regulating neutrophil function in response to distinct stimulation. Therefore, different agonists may be coupled to one MAP kinase by different signal transduction pathways. Indeed, previous reports (58) found that fMLP-induced phosphorylation of p38 in neutrophils is dependent on phos-
phatidylinositol 3-kinase and PLC. Finally, our data indicated that PLD was involved with neither the fMLP nor the GM-CSF signaling pathway leading to p44/p42 activation. In conclusion, depending on the nature of the agonist used (chemotactic peptide or chemokines), distinct signaling pathways may lead to the activation of p38. In addition, the activation of p38 could occur dependently or independently of PLD activation.

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