The activation of human polymorphonuclear neutrophil leukocytes (neutrophils) is associated with an increased synthesis of the highly phosphorylated phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3). The aims of the present investigation were to determine whether the newly described, G protein-dependent phosphatidylinositol 3-kinase (PI3K), p110γ, was involved in the responses to chemotactic factors interacting with G protein-coupled receptors. The presence of p110γ in neutrophils was first established both at the protein and the mRNA level. Stimulation of the cells with fMet-Leu-Phe or interleukin-8 increased the PI3K activity in p110γ, but not p85, immunoprecipitates. The time course of this effect (threshold within less than 5 s, maximal activation at 10–15 s) was consistent with that of the generation of PtdIns(3,4,5)P3. Wortmannin, a PI3K inhibitor, abrogated the effects of fMet-Leu-Phe, which were also significantly inhibited by pertussis toxin. Finally, fMet-Leu-Phe also induced a significant translocation of p110γ to a particulate fraction derived from these cells. These data indicate that p110γ represent the major PI3K activated by fMet-Leu-Phe and interleukin-8 at very early time points following the stimulation of human neutrophils.

The activation of human polymorphonuclear neutrophil leukocytes (neutrophils) by chemotactic factors is associated with the generation of polyphosphorylated phosphoinositides through the stimulation of the activity of phosphatidylinositol 3-kinase(s) (PI3Ks)1 (1–8). Although the characteristics of the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) (2, 3) and the functional effects of inhibition of PI3Ks by compounds such as wortmannin and LY294002 (6, 7, *31), was inhibited by tyrosine kinase inhibitors (31) and one of the catalytic p110 subunit (a, α, β). At present, class IA includes heterodimers consisting of a regulatory p85 subunit (a or β) and one of the catalytic p110 subunit (α, β, or δ). Class IA consists of at least two isoforms, p110α and p110β, which are encoded by separate genes.

The PI3K family comprises three classes depending on substrate specificity and protein structure (20–22). Of particular interest to neutrophil physiology are class I PI3Ks, which are further divided into class IA and class IB, and which are involved in receptor-induced cellular responses. Class IA includes heterodimers consisting of a regulatory p85 subunit (α or β) and one of the catalytic p110 subunit (α, β, or δ). At present, class IA includes at least one member, p110γ, which, possibly in association with a regulatory p101 subunit (23, 24), is regulated preferentially by interaction with heterotrimeric G protein subunits (and more particularly the βγ subunit) (23, 25).

A critical role for p110γ in neutrophil migration has recently been shown in p110γ knockout mice (26–28). Phagocyte chemotaxis in response to fMet-Leu-Phe and C5a was reduced, as was their accumulation in the peritoneal cavity in vivo in response to inflammatory stimuli. In addition, T lymphocyte development and activation were impaired in p110γ−/− mice. Although these data show that neutrophil recruitment in response to fMet-Leu-Phe and C5a is severely diminished in mice genetically deficient in p110γ (26–28), direct evidence that human neutrophils express p110γ has not been provided as yet.

Partial and conflicting data are available related to the specific species of PI3K activated upon stimulation of human neutrophils by chemotactic factors, which has not been directly examined as yet. Two studies (29, 30) have reported data suggesting that p85/p110 is not, or is only minimally, involved in the responses of human neutrophils. A similar conclusion was drawn very recently in murine neutrophils, based on the generation of p110γ−/− knockout mice (26–28). These data, however, are difficult to reconcile with those of other studies indicating that the formation of PtdIns(3,4,5)P3 stimulated by fMet-Leu-Phe was inhibited by tyrosine kinase inhibitors (31) or that enhanced PI3K activity was recovered in phosphotyrosine immunoprecipitates from fMet-Leu-Phe-stimulated cells (7). On the other hand, it is well established that the formation of PtdIns(3,4,5)P3 stimulated by fMet-Leu-Phe is inhibited by pertussis toxin (1, 2). Furthermore, it has also been shown that the introduction of GTP into permeabilized neutrophils led to the accumulation of PtdIns(3,4,5)P3 (32). These two sets of data suggest that heterotrimeric GTP-binding proteins regulate the formation of the highly phosphorylated phosphoinositides. It is presently unclear, however, whether the implication of heterotrimeric GTP binding proteins in this response is direct or indirect.

The present study was initiated to examine the potential involvement of the class IA PI3K p110γ in the responses of native human neutrophils isolated from the peripheral blood to chemotactic factors. To test this possibility as directly as possible, the effects of fMet-Leu-Phe and interleukin-8 (IL-8) on the activity and subcellular distribution of immunoprecipitated

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Stimulation of Human Neutrophils by Chemotactic Factors Is Associated with the Activation of Phosphatidylinositol 3-Kinase γ*

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‡ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-monophosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; DFP, diisopropylfluorophosphate; Me3SO, dimethyl sulfoxide; IL-8, interleukin-8; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Lipids (Alabaster, AL). Adenosine-5′-serum albumin, 2 mM orthovanadate, 10 μM for 30 min at 4 °C for 30 min in the presence of 1% Nonidet P-40, 0.05% bovine agents. The filtered lysates were precleared with protein A-Sepharose fluoride, dimethyl sulfoxide (Me2SO), fMet-Leu-Phe, wortmannin, and mobilon polyvinylidene difluoride membranes from Millipore Corp. P-40 were purchased from Calbiochem-Novabiochem. IL-8 was a gen-
gogy except with pig PI3K

37 °C and then stimulated with 100 nM fMet-Leu-Phe or IL-8 in the

glycerol, 2.5 mM orthovanadate, 10 μM for 5 min or pertussis toxin, 1 μg/ml for 2 h. The stimulation was quickly stopped by the addition of ice-cold buffer I (phosphate-buffered saline containing 1 mM CaCl2, 1 mM MgCl2, 100 mM Na2VO3). The samples were centrifuged and then washed twice in ice-cold buffer II (50 mM HEPES, pH 7.4, 157 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 100 μM Na2VO3). The pellets were then dissolved quickly in 1 ml of buffer for 1% (v/v) Nonidet P-40, HEPES 50 mM, pH 7.4, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 2 mM Na2VO3, 10% (v/v) glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 100 mM NaF, 10 mM Na3PO4, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and left on ice for 15 min before being centrifuged for 10 min at 13,000 × g at 4 °C. The supernatants were incubated for 1 h at 4 °C on a rotating wheel with the specific antibodies (pig-pi30 or rabbit-pi30). Upstate Biotechnology (Madison, Lawrence, WI) was purchased for 30 min at 30 °C with constant shaking. Twenty μl of 1N HCl was added to stop the reaction, and PtdIns(3,4,5)P3 was extracted by the addition of 200 μl chloroform/methanol (1:1, v/v). The samples were dried and then solubilized in diethyl pyrocarbonate-treated H2O. Ten μg of total RNA diluted in 45 μl of a solution of 1 % diethyl pyrocarbonate in ddH2O were heated at 65 °C for 10 min. The reverse transcrip-
tion reaction was then performed for 90 min at 37 °C in 80 μl of a solution of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 15 mM MgCl2, 0.01 μM dNTPs, 0.66 μM random hexamer primers, (dT)20, 20 primers, 1 mM dextronucleotides, 0.35 units/μl RNasin, and 1 unit/μl Moloney murine leukemia virus reverse transcriptase.

The reaction was stopped by heating the mixture at 95 °C for 5 min. PCR assays were performed as described previously (36). Briefly, 5 μl of reverse-transcribed RNA was added to a solution to obtain a final concentration of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 50 μM dNTPs, 0.5 μM MgCl2, 0.1 μM dextronucleotides, 0.5 μM random hexamer primers, Taq polymerase, and 1 pmol/μl each sense and antisense specific primers. The primer sequences are as follows: pup110y P32Kf GTGTTGCT-GAGAGAGGACAA, pup110y P32Kr CTATACAGCGAGGCTTCACA (1.38-kilobase fragment); pup110y P32Kr ACAGATTCCAGATTAGGCAGTTAGTACG, pup110y P32Kr GATCGTTGCTGCGCCAGTGTACTCG, pup110y P32Kr GATCGTTGCTGCGCCAGTGTACTCG (0.58-kilobase fragment), GAPDH TGATGACATAC-AAGTTTGGAAG. The sequence of PCR amplification was one cycle of denaturation at 95 °C for 2 min followed by annealing at 56 °C for 30 s and extension at 72 °C for 1 min. This cycle was followed by 30 s at 95 °C, 30 s at 56 °C, and 1 min at 72 °C, repeated 38 times. The PCR reaction was sampled every 5 cycles from cycle 25 to 40, inclusively. The samples were migrated on a 2% agarose gel, stained with ethidium bromide, and compared for intensity. Membrane Preparation and Translocation Assay—Neuropilins (4 × 107 cells/ml) were treated with 1 mM DFP for 30 min at room temperature. The cell suspensions were centrifuged and resuspended in Hanks' balanced salt solution at the same cell concentration. The cells were pre-heated for 5 min at 37 °C and stimulated with Met-Leu-Phe (1 μM) or an equal volume of the diluent (Methanol or PBS, 10, or 15 μM). The incubation was stopped for 1 min. One hundred μM KCl—HEPES relaxation buffer (100 mM KCl, 50 mM HEPES, 5 mM NaCl, 1 mM MgCl2, 0.5 mM EGTA, 2.5 μM aprotinin, 2.5 μg/ml leupeptin, 2.5 mM phenylmethylsulfonyl fluoride, 1 mM DFP, pH 7.2) was added. The cell suspensions were centrifuged for 7 min at 700 × g. Unbroken cells and nuclei were discarded and the supernatants ultracentrifuged at 180,000 × g for 45 min in a Beckman TL-100 ultracentrifuge using a 100 μl of protein A-Sepharose (Amersham Pharmacia Bio-
tech) for an additional hour. The beads were then washed 3 times with buffer IV (phosphate-buffered saline, 1% (v/v) Nonidet P-40, 100 μM Na2VO3), 3 more times with buffer V (100 μM Tris-HCl, pH 7.5, 500 μM LiCl, 100 μM Na2VO3) and finally twice with buffer VI (10 μM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 100 mM Na2VO3). 10 μl of PtdIns (2 mg/ml phosphatidylinositol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was sonicated for 10 min, added to 50 μl of buffer VI and 10 μl of 100 μM MgCl2, and mixed with the beads for 10 min on ice. The reaction was initiated by adding 10 μl of 440 μM ATP containing 30 μCi of [γ-32P]ATP (NEW Life Science Products, 3000 Ci/mmol) and incubated for 15 min at 30 °C with constant shaking. Twenty μl of 1 N HCl was added to stop the reaction, and PtdIns(3,4,5)P3 was extracted by the addition of 200 μl chloroform/methanol (1:1, v/v). The samples were dried and then solubilized in diethyl pyrocarbonate-treated H2O. Ten μg of total RNA diluted in 45 μl of a solution of 1% diethyl pyrocarbonate in ddH2O were heated at 65 °C for 10 min. The reverse transcrip-
tion reaction was then performed for 90 min at 37 °C in 80 μl of a solution of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 15 mM MgCl2, 0.01 μM dNTPs, 0.66 μM random hexamer primers, (dT)20, 20 primers, 1 mM dextronucleotides, 0.35 units/μl RNasin, and 1 unit/μl Moloney murine leukemia virus reverse transcriptase.

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Results

Presence of p110γ in Human Neutrophils—Although the presence of p85/p110 PI3K in human neutrophils has been firmly and repeatedly demonstrated, that of p110γ is much less well established (37). A polyclonal antibody to a peptide sequence derived from amino acids 745–756 of p110γ, as reported by Stoyanov et al. (25), was raised and used in immunoblotting and immunoprecipitation protocols to examine the expression of p110γ in human neutrophils directly. As shown in Fig. 1A, this antibody detected closely spaced protein bands at an approximate molecular mass of 110 kDa. This detection was specific in that the staining was displaced by an excess of the immunizing peptide and was not present if the p110γ antiserum was omitted from the immunoprecipitation step (data not shown). The polyclonal antibody was also able to immunoprecipitate p110γ under reducing and denaturing conditions (Fig. 1B). The efficiency of the immunoprecipitation was not altered in cell lysates derived from cells stimulated by fMet-Leu-Phe. On the other hand, the immunoprecipitation was abrogated if the antiserum was first neutralized with the immunizing peptide. It should be noted, however, that the antiserum was significantly less efficient at immunoprecipitating p110γ under native, nondenaturing conditions (data not shown). Finally, PCR amplification revealed the presence of fragments of the expected sizes of 1377 and 585 base pairs using primers derived from the sequence of human p110γ (Fig. 1C).

Activation of p110γ by Chemoattractants—Neutrophils were stimulated by two separate chemoattractants that interact with distinct G protein-coupled receptors, namely fMet-Leu-Phe and IL-8, for various times ranging from 5 s to 1 min. These times correspond to those during which the levels of PtdIns(3,4,5)P3 have previously been reported to rise following the stimulation of human neutrophils by fMet-Leu-Phe (maximal levels attained after about 10–15 s) (1, 2, 38). The cells were then lysed and immunoprecipitated, using the anti-p110γ antiserum or an anti-p85 antibody, and the PI3K activities present in the immunoprecipitates were assayed. The results of representative experiments using fMet-Leu-Phe are illustrated in Fig. 2. These data show that fMet-Leu-Phe (10^{-7} M) very rapidly induced an increase in the formation of PtdIns(3)P (using PtdIns as substrate). This increase was detectable within 5 s of stimulation with the chemoattractant factor, reached a maximum at 5–10 s, and progressively decreased thereafter. The data from several experiments are pooled in Fig. 2, lower panel. These demonstrate that the increase of the activity of immunoprecipitated p110γ was statistically significant as rapidly as 5 s after the addition of the chemoattractant factor. Control experiments indicated that no PI3K activity was recovered if the antiserum was omitted from the immunoprecipitation step or if the anti-p110γ antiserum was neutralized with the immunizing peptide (data not shown). Similar results were observed if the cells were stimulated with IL-8 (Fig. 3). Again, detectable increases in the formation of PtdIns(3)P were detected within 5 s of the addition of the agonist, and maximal levels of activity were reached at 10–15 s post-stimulation. The formation of PtdIns(3)P induced by incubation of PtIns with the immunoprecipitates was abrogated if the cells were pre-incubated in the presence of 50 nM wortmannin before being stimulated with fMet-Leu-Phe (Fig. 4), thereby providing additional evidence for the involvement of a PI3K activity in the monitored reaction (39, 40). In additional experiments, we also tested the potential effects of granulocyte/macrophage colony-stimulating factor...
Activation of p110γ by Chemoattractants in Human Neutrophils

**Fig. 3.** Stimulation of the activity of p110γ by IL-8. p110γ immunoprecipitates were prepared from cell lysates derived from cells stimulated with IL-8 (10^{-7} M) for the indicated times. The upper panel depicts the result of a representative TLC plate. Average densitometric values derived from experiments (n > 4) such as that shown in the upper panel are shown in the lower panel. The asterisks indicate statistical significance (p < 0.01) (difference from unstimulated controls) using the Wilcoxon rank sum test.

**Fig. 4.** Effect of wortmannin on the stimulation of the activity of p110γ by fMet-Leu-Phe. p110γ activity in immunoprecipitates derived from cells left untreated or incubated with 200 nM wortmannin for 5 min at 37°C was monitored as described under “Experimental Procedures.” The amounts of p110γ in the individual fractions were assayed by immunoblotting. A representative blot is shown in the upper panel. The intensities of the bands corresponding to p110γ were quantitated by densitometry and normalized in each experiment to the value of the particulate fraction derived from control unstimulated cells. The results (Density) represent the mean ± S.E. of four independent determinations.

**DISCUSSION**

Although the G protein-activated isoform of PI3K, p110γ, has been cloned and sequenced (23, 25), its biochemical and domain characteristics described in detail (see reviews inRefs. 21, 22, 42), and its tissue distribution examined (e.g.,Refs. 26 and 43), its role and function in native or unengineered cells remains only very partially investigated and in only a very few cases, e.g., NK cells (44) and Jurkat cells (45) and very recently in knock-out mice (26–28). In the present study, the presence of p110γ in isolated human peripheral blood neutrophils was demonstrated as was its activation and translocation to a particulate fraction upon stimulation by pathophysiologically relevant agonists interacting with G protein-coupled receptors.

The presence of p110γ in human neutrophils was examined first by immunoblotting and by immunoprecipitation. The rabbit antiserum against amino acids 742–757 of the sequence of human p110γ (25), which was raised and utilized in this study,
consistently detected a doublet of approximately 110 kDa (Figs. 1 and 5). The specificity of the detection was established by displacement experiments with the immunizing peptide. Additional evidence for the presence of p110γ was obtained from the ability to amplify by reverse transcription-PCR sequences of the expected base pair lengths. The nature of the p110γ doublet detected in neutrophils is unclear at present, although it may correspond to the two isoforms of G protein-activated PI3Ks isolated by Stephens et al. (46). Alternatively, the doublet may represent presently unidentified post-translational modifications of p110γ. A similar doublet was seen in the undifferentiated or Mε3SO-differentiated human promyelocytic cell line, PLB-985 (data not shown).

Rapid increases in the activity of immunoprecipitated p110γ were seen following the addition of two unrelated chemotactic factors, namely fMet-Leu-Phe and IL-8. Both of these neutrophil agonists interact with G protein-coupled receptors (47–50). The time course of the stimulation of the activity of p110γ corresponded closely to that of the accumulation of PtdIns(3,4,5)P3 in intact cells (1, 2, 38), with transient responses detectable within the first 5 s and peaking at 10 to 15 s after the addition of either chemotactic factor. This was followed by a return to basal levels of activity within 60 s of stimulation. Thus, the activation of p110γ occurred with a time course that makes it compatible with a role in the synthesis of PtdIns(3,4,5)P3 and the very early signaling events in these cells. The inhibition of the stimulation of the activity of p110γ by pertussis toxin provides evidence that this effect is mediated by members of the G family. It should be noted that the magnitude of the increases in the activity of p110γ observed in the present study are likely to represent underestimates of the actual effects, as the efficacy of the antiserum to precipitate p110γ under native conditions was relatively low. Whether this low level was attributable to the low affinity of the antiserum, to the poor accessibility of the immunizing peptide sequence under native conditions, or to masking effects because of protein interactions remains to be examined and will require the development and characterization of new antibodies against other epitopes of p110γ.

Under the conditions used in the present study, no stimulation of the activity of p85-associated PI3K activity could be detected within the first minute of stimulation with fMet-Leu-Phe. This result is in accord with those of Vlahos and Matter (30), who found no increased PI3K activity in antiphosphotyrosine immunoprecipitates from fMet-Leu-Phe-stimulated human neutrophils, with those of Stephens et al. (29), who concluded that the p85/p110-dependent pathway of synthesis of PtdIns(3,4,5)P3 played only a minor role in the increases in this polyphosphoinositide following stimulation by G protein-coupled agonists in myeloid cells, and with the complete inhibition of the chemotactic factor-stimulated generation of PtdIns(3,4,5)P3 in murine p110γ−/− neutrophils (26–28). It should be noted that this conclusion does not exclude an indirect and secondary activation of p85/p110 by fMet-Leu-Phe, as the latter may occur as a result of the stimulation of the activity of various Src family tyrosine kinases including Lyn (31, 51), which is also induced by chemotactic factors (52). These secondary effects on src kinases may underlie the apparent discrepancy between the above results and those of Ptasznik et al. (31), who attenuated the formation of PtdIns(3,4,5)P3 induced by fMet-Leu-Phe using tyrosine kinase inhibitors.

Finally, evidence also was obtained that the subcellular distribution of p110γ was altered upon stimulation with a statistically significant proportion of the enzyme translocating to a particular fraction. The latter presumptively contains various cellular membranes, including the plasma membrane, in which the physiological substrate of p110γ, namely PtdIns(4,5)P2, is present. It is worthwhile to note that the kinetics of the translocation of p110γ correlates closely with that of the formation of PtdIns(3,4,5)P3 (1, 2, 38) and of the stimulation of its activity (Fig. 2), thereby supporting its causal significance. A similar translocation of p110γ to a membrane-containing fraction has previously been described in chemokine-stimulated NK cells, although with significantly slower kinetics (44).

In conclusion, the results of the current study present evidence that the activity of p110γ is rapidly stimulated upon activation of G protein-coupled receptors in human peripheral blood neutrophils. The characteristics of this effect indicate that the stimulation of p110γ underlies, at least in part, the previously described rapid increases in the levels of PtdIns(3,4,5)P3 induced by chemotactic factors and possibly also the effects of PI3K inhibitors on various neutrophil functions (chemotaxis, phagocytosis, oxidative burst). The causal positioning of the stimulation of p110γ in the various signaling pathways summoned upon neutrophil activation remains to be directly examined. The possibility that it may play a role in the mediation of the recruitment of tyrosine kinases of the Tec family, which contain phosphoinositide-interacting pH domains (53), is particularly intriguing in view of the sensitivity of the stimulation of tyrosine phosphorylation in human neutrophils to PI3K inhibitors such as wortmannin and LY294002 (54).

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