Van Keymeulen et al., http://www.jcb.org/cgi/content/full/jcb.200604113/DC1

Results

Selectivity of PI3K inhibitors

Our experiments used five PI3K inhibitors chosen from the much larger number of compounds characterized by Knight et al. (2004, 2006). Our initial goal was to determine which of the four known class I PI3K isoforms are responsible for ligand-stimulated PIP3 accumulation in dHL60 cells. Table S1 shows the in vitro IC50 values of these compounds (Knight et al., 2006) against the four PI3K isoforms (for their effect on other kinases, see Knight et al., 2006).

As a readout for fMLP- and insulin-stimulated PI3K activities in dHL60 cells, we assessed the phosphorylation of Akt, a kinase downstream of PIP3, by immunoblotting extracts of ligand-treated cells with antibody directed against pAkt (phosphorylated at Thr308; Fig. S1). All five compounds tested were able to penetrate dHL60 cells, as shown by their ability to inhibit the pAkt response to insulin. Patterns of relative inhibitory potencies in vitro (Table S1) versus intact dHL60 cells (Fig. S1, A and B; left) suggest that PI3Kα and PI3Kδ mediate most of the insulin response. Compounds that inhibit both isoforms completely prevent the insulin response, whereas IC87114, a relatively "pure" δ inhibitor, partially reduces the response at 10 μM, suggesting a possible role for PI3Kδ. TGX-115, which inhibits both PI3Kδ and PI3Kβ in vitro, does not block the insulin response substantially better than IC87114, making it less likely that PI3Kβ plays a key role.

Relative potencies of these compounds for inhibiting the cellular pAkt response to fMLP (Fig. S1 A and B, right; and Table S2) suggest that PI3Kγ plays a dominant role in mediating the PIP3 response to this ligand, although a possible contribution from PI3Kδ cannot be ruled out. Specifically, high concentrations (≥10 μM) of IC87114, a PI3Kδ inhibitor, and TGX-115, a β/δ inhibitor, reduce the pAkt response to fMLP only by 25–40%, making it impossible to calculate their IC50 values (Table S2). In contrast, PIK-90 and -93 potently inhibit PI3Kγ (and PI3Kδ) in vitro and abrogate the fMLP response in intact cells at 1 μM (IC50 values of 0.06 and 0.12 μM; Table S2). The inference that PI3Kγ mediates most of the pAkt response to fMLP is underlined by the intermediate IC50 (2 μM) of PI-103, which inhibits the γ isoform in vitro 10-fold less potently than PIK-90 and -93 but ~100-fold more potently than IC87114 or TGX-115. PI3Kα is probably not a primary regulator of the fMLP response because PI-103 inhibits the fMLP response less potently than do PIK-90 and -93 but is at least equipotent with these two compounds as an inhibitor of PI3Kα. Results of additional experiments with additional selective inhibitors (Knight et al., 2004, 2006) were in keeping with these inferences (unpublished data).

The inference that PIP3 generated by PI3Kγ plays a dominant role in polarity and chemotaxis of dHL60 cells is consistent with inferences drawn from functional studies of neutrophils from PI3Kγ$^{-/-}$ mice (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). However, other studies (Sadhu et al., 2003; Puri et al., 2004; Condliffe et al., 2005) have suggested that PI3Kδ may also play a role. Our data do not rule out a possible adjunctive role of PI3Kδ in dHL60 cells because TGX-115 and IC87114 at high concentrations do partially reduce the maximal pAkt response to fMLP (Fig. S1) even though they inhibit PI3Kγ >100-fold less potently than PI3Kδ in vitro (Table S1). Indeed, IC87114, the PI3Kδ-selective inhibitor, is reported (Sadhu et al., 2003; Puri et al., 2004) to alter cell morphology and inhibit chemotaxis of

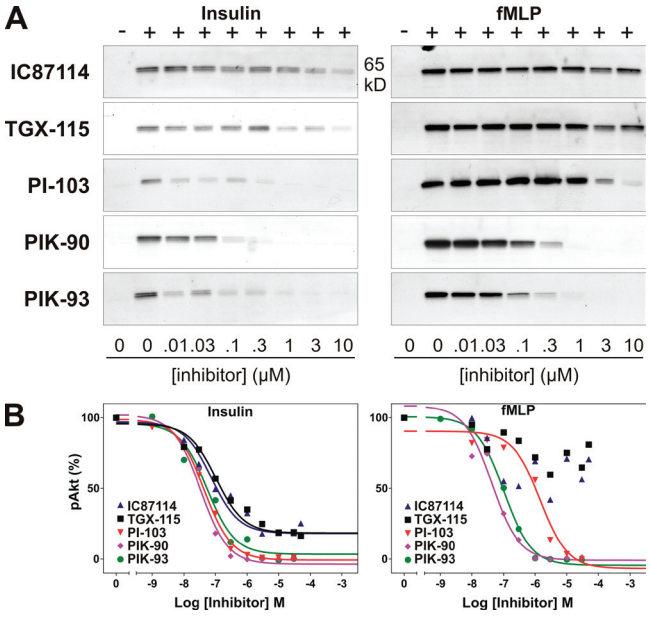

Figure S1. **Effects of five PI3K inhibitors on the phosphorylation of Akt in dHL60 cells.** PIK-90 and -93, which inhibited PI3Kγ selectively in vitro (Table S1), also selectively inhibited Akt phosphorylation stimulated by fMLP. (A) Immunoblots probed with antibody to Akt phosphorylated at threonine 308 (pAkt). dHL60 cells were treated with the indicated concentrations of PI3K inhibitors for 40 min before the addition of 10 μg/ml insulin (left) or 100 nM fMLP (right); 1 min later, the cells were lysed, and extracts were processed for immunoblotting. Immunoblots shown are results of representative experiments. Note that the pAkt response to fMLP alone was quantitatively ~10-fold greater than that of insulin alone; this difference is obscured in the immunoblot images because the fMLP and insulin blots were exposed for different times in order to optimize the signal. (B) Quantification of pAkt immunoblots. Band intensities were assessed using the Image program (Scion). Background signal was subtracted, and all values were normalized to the signal detected in the absence of inhibitor. Each point in the graph represents the mean of at least three independent experiments. Loading controls (Akt blots) showed that similar amounts of protein were assessed in each lane of each experiment. Curves were fitted to a nonlinear sigmoidal concentration–response curve with Prism software (GraphPad), and IC50 values were calculated (Table S2). Weak inhibitions of the fMLP response made it impossible to fit curves for IC87114 and TGX-115.

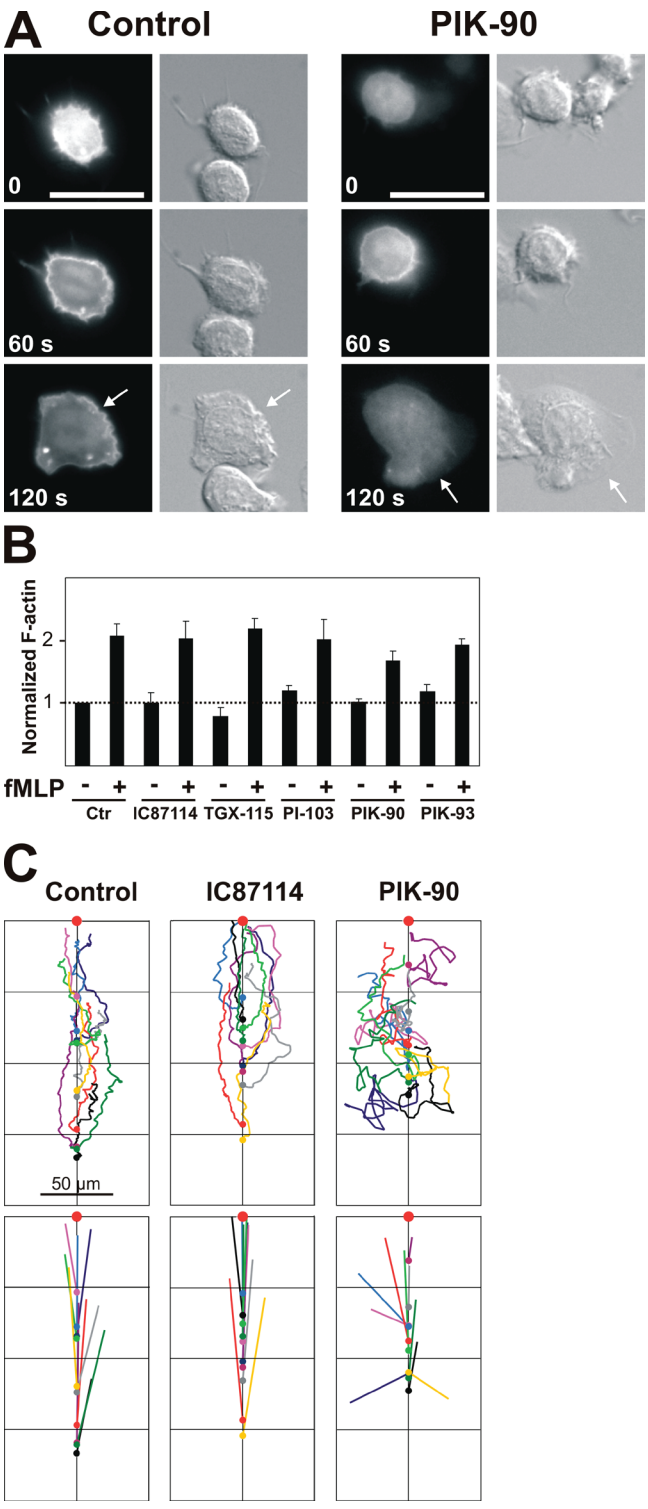

Figure S2. **Effects of PIK-90 on PIP3 localization, accumulation of F-actin, and chemotaxis.** (A) fMLP-stimulated membrane translocation of PH-Akt-YFP. Cells expressing PH-Akt-YFP were pretreated in suspension with or without 1 μM PIK-90 for 40 min, allowed to stick to a coverslip for 15 min, and exposed to a uniform concentration of 100 nM fMLP. Images show the time course of response of a single control or PIK-90–treated cell at the times indicated after the addition of fMLP. Arrows point to pseudopods. Bars, 10 μm. (B) Quantification of F-actin accumulation. Cells were exposed for 40 min to no drugs, to 10 μM IC87114 and TGX-115, or to 1 μM of the compounds PI-103, PIK-90, and PIK-93 and were treated with 100 nM fMLP for 3 min before extraction and quantitation of F-actin in Triton X-100 insoluble fractions, as described in supplemental Materials and methods. Results are normalized to the density of F-actin in unstimulated control cells. Bars show the mean + one SEM (error bars) for four independent experiments. None of the PI3K-inhibiting compounds significantly affected fMLP-stimulated F-actin accumulation in comparison to control cells. In particular, P values for cells treated with PIK-90 and -93 were 0.2 and 0.6, respectively (paired test relative to controls). (C) Chemotaxis is markedly inhibited by PIK-90, which selectively inhibits PI3K-γ in vitro, but not by IC87114, TGX-115, or PI-103, which inhibit PI3K-γ weakly or not at all. (top) Migration trajectories (10 cells shown) of control cells (left) or cells treated with IC87114 (middle) and PIK-90 (right) moving toward a point source of fMLP for 25 min. Trajectories were tracked with SoftWorx software and realigned to the same relative vertical axis. Red circles at the top indicate positions of the fMLP source in each panel, and the colored circles below it on the vertical axis indicate the starting positions for trajectories of the corresponding individual cells. (bottom) Migratory displacements of the cells shown in the top panels. Straight lines in the plot connect positions of individual cells at time 0 and at 25 min, realigned to the same relative vertical axis as in the top panels. PI3K-γ–inhibited cells clearly detected and interpreting the fMLP gradient; mean displacement of PIK-90–treated cells toward the micropipette differed from zero by more than four SEM (not depicted).

human and mouse neutrophils. In our experiments, IC87114 had no effect whatsoever on fMLP-dependent polarity or chemotaxis (Fig. 1 and Fig. S2 C). This discrepancy could reflect a difference between dHL60 cells in culture and neutrophils obtained from blood.

None of the conclusions of this study depends on identifying a specific PI3K isoform that is responsible for PIP3 accumulation in fMLP-treated dHL60 cells. Instead, for our purposes, the principal value of PIK-90 and -93 is that they have unambiguous effects on polarity and chemotaxis at concentrations that almost completely abrogate PIP3 accumulation (as reflected in pAkt assays). The other PI3K inhibitors we tested had none of these effects. Just as important, the classic broad-spectrum PI3K inhibitors previously used in our (Wang et al., 2002; Xu et al., 2003) and another laboratories (Benard et al., 1999) to study neutrophil responses are much less potent and selective than PIK-90 and -93, not only in vitro (Knight et al., 2004, 2006) but also in intact dHL60 cells. For example, we reported (Wang et al., 2002) that wortmannin and LY294002 substantially reduce pAkt responses to fMLP in these cells only at concentrations much higher than required to reduce PIP3-dependent responses in fibroblasts and other cells. At such high concentrations, wortmannin and LY294002 almost completely prevented polarity and migration in fMLP gradients (Wang et al., 2002). This is in sharp contrast to PIK-90 and -93, which have less severe and more informative functional effects at concentrations that similarly reduce pAkt responses. High concentrations of the two broad-spectrum inhibitors are also likely to inhibit multiple lipid and protein kinases in addition to PI3Ks (Knight et al., 2006).

Materials and methods

F-actin

Cellular content of F-actin was measured as described previously (Steimle et al., 2001). dHL60 cells suspended in RPMI/Hepes medium were preincubated with the indicated agents for 40 min, centrifuged in a J6-B centrifuge (Beckman Coulter) for 5 min at 2,000 rpm at room temperature,

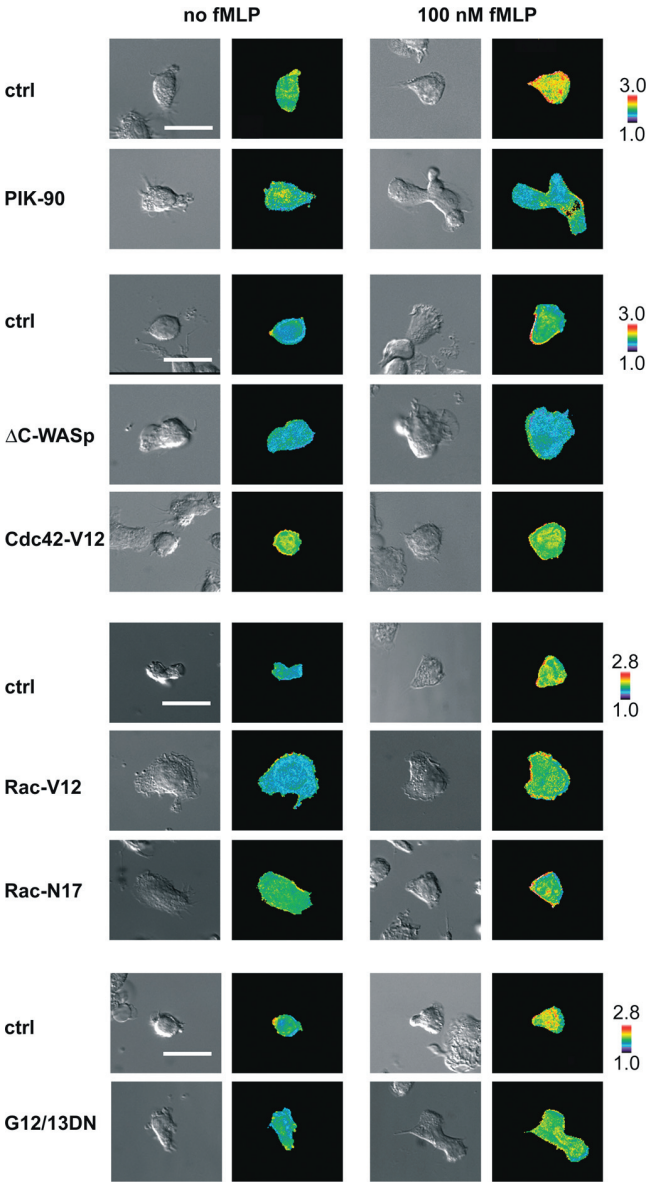

Figure S3. **Subcellular distribution of the FRET signal.** DIC and FRET/CFP ratio images of representative cells stimulated with (right) or without (left) fMLP. Cells were treated with PIK-90 or transiently transfected with Cdc42-V12, ΔC-WASp, Rac-V12, Rac-N17, or G12/13DN as indicated. Pseudocolored images within each set of data were scaled so that the intensities were comparable within each group. Bars, 10 μM.

suspended in modified HBSS (10^7 cells in 0.5 ml for each condition), and stimulated for 3 min with 100 nM fMLP. Stimulation was stopped by the addition of 0.5 ml of 2× stopping buffer at room temperature (1× stopping buffer: 100 mM MES, pH 6.8, 5 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, and protease inhibitor cocktail). Samples were incubated at room temperature for 15 min and centrifuged at 14,000 rpm for 5 min at room temperature in a benchtop centrifuge. Pellets were resuspended in laemmli sample buffer, boiled for 10 min, and subjected to PAGE. Intensities of the Coomassie-stained actin bands (42 kD) were measured using the Image program (Scion).

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