Polarization of Chemoattractant Receptor Signaling During Neutrophil Chemotaxis

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Supplementary Material

To view these movies, download a QuickTime viewer.

- Movie 1
- Movie 2
- Movie 3
- Movie 4
- Movie 5
- Movie 6

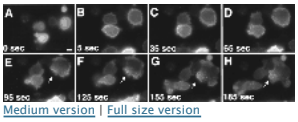
Further details on experimental methods:

Electroporation and establishment of stable cell lines. Cells grown to log phase (0.6×10^6 to 1.0×10^6 cells/ml) were washed twice with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} and resuspended at a density of 2×10^7 cells/ml in ice-cold RPMI 1640 supplemented with 25 mM Hepes. A 400-ml cell suspension (8×10^6 cells) was transferred to an electroporation cuvette (Gene Pulser Cuvette, 0.4-cm electrode, Bio-Rad, Hercules, CA) and mixed with 30 mg linear vectors in 30 ml sterile water. Cells were put back on ice for an additional 10 min and then electroporated using a Gene Pulser II electroporator (Bio-Rad). The settings were 350 mV and 975 mF, resulting in a time constant of ~60 ms. Electroporated cells were put back on ice for 30 min and then transferred to 20 ml fresh culture medium. At 48 hours after electroporation, cells were harvested, spun down, and resuspended in 20 ml conditioned medium supplemented with 1 mg/ml active G-418. Conditioned medium was collected from HL-60 cells plated at 2×10^5 cells/ml and grown for 2 days until they reached a density of 6×10^5 cells/ml. Cells were spun down, and the medium was harvested and filtered through a 0.22-mm filter. During selection, the cell medium was changed every other week with fresh conditioned medium. Cells were selected for 4 to 6 weeks until the G-418-resistant cell population reached a density of ~1.0×10^6 cells/ml. GFP expression was verified by fluorescence-activated cell sorting (FACS).

***C. difficile* toxin-B treatment.** Under the conditions used, 35 ± 15% (mean ± SD of 21 determinations) of cells were dead or moribund, as indicated by the presence of highly fluorescent granules, abnormal cytoplasmic exclusion of the GFP signal, and/or abnormal morphology; the dying cells did not translocate PHAKT-GFP in response to *f*MLP or insulin. Because live *C. difficile* toxin-B-treated cells showed robust translocation of PHAKT-GFP in response to insulin (Fig. 4), we also used insulin-induced translocation as a marker of cell viability. Using this protocol, we measured a similar proportion (41 ± 14%; mean ± SD of 21 determinations) of dead cells. The cells that did not translocate PHAKT-GFP in response to insulin also exhibited highly fluorescent granules, abnormal cytoplasmic exclusion of the GFP signal, or abnormal morphology. In Web figure 4D, the *f*MLP-induced PHAKT-GFP translocation for *C. difficile* toxin-treated cells is expressed as (the number of cells showing translocation in response to *f*MLP)/(the number of cells showing translocation in response to *f*MLP + insulin), a value which reflects the total number of live cells.

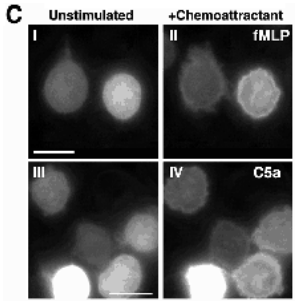
LY 294002 treatment. It was necessary to assure ourselves that assessments of PHAKT-GFP translocation were reproducible, because inhibition of PHAKT-GFP translocation depended on LY 294002 concentration and because the translocation is difficult to quantitate by eye. Accordingly, we asked a second "blind" observer to assess the proportion of cells showing PHAKT-GFP translocation in a series of images presented in random order and without clues as to the previous treatment. This observer's assessments did not differ by more than 10 to 15% from those of the observer whose observations are recorded here and in Web figure 4D (see legend below).

Supplemental Figure 1. Time course of PHAKT-GFP translocation in response to a uniform stimulation with chemoattractant. Differentiated cells (*7*), plated on glass cover slips as described (*4*), were stimulated with 100 nM *f*MLP at time 0 seconds (A) and images were recorded in the fluorescein isothiocyanate (FITC) channel every 5 s as described (*4*). The arrows in panels (E) through (H) point to a portion of a neutrophil's surface that show persisting translocation of PHAKT-GFP. This experiment is representative of at least 10 similar sessions. Bar, 10 mm.



Medium version | Full size version

Web figure 4C. Effect of 100 mM LY 294002 on chemoattractant-induced plasma membrane translocation of PHAKT-GFP in neutrophil-differentiated HL-60 cells. Panels I and III correspond to unstimulated LY 294002-treated cells (zero time). Panels II and IV show responses of the same cells to *f*MLP and C5a, respectively, in the presence of LY 294002 (100 mM). Stimulation times with agonists were as follows: panel II, 65 s; panel IV, 38 s.



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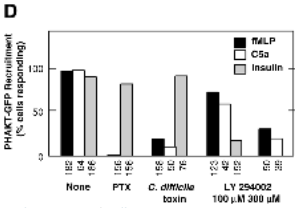
Web figure 4D. Cumulative histogram of PHAKT-GFP responses to chemoattractants and insulin the presence of inhibitors. For responses in the presence of PTX or LY 294002, results are expressed as the percent of cells showing translocation of PHAKT-GFP, compared to the total number of cells analyzed; this number is shown below each of the appropriate columns. For *C. difficile* toxin-B-treated cells, the results for both *f*MLP-induced and insulin-induced recruitment are also expressed as the percent of responding cells, but in this case, the denominator (100%) is restricted to live cells (*14*); this number is shown below each of the appropriate columns.



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