Neutrophil polarization: Spatiotemporal dynamics of RhoA activity support a self-organizing mechanism

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Chemoattractants like fMet-Leu-Phe (fMLP) induce neutrophils to polarize with phosphatidylinositol 3,4,5-trisphosphate (PIP3) and protrusive F-actin at the front and actomyosin contraction at the sides and back. RhoA and its downstream effector, myosin II, mediate the “backness” response, which locally inhibits the “frontness” response and constrains its location to one part of the cell. In living HL-60 cells, we used a fluorescent PIP3 probe or a single-chain FRET biosensor for RhoA-GTP to assess spatial distribution of frontness or backness responses, respectively, during the first 3 min after exposure to a uniform concentration of fMLP. Increased PIP3 signal or RhoA activity initially localized randomly about the cell’s periphery but progressively redistributed to the front or to the back and sides, respectively. Cells rendered unable to mount the frontness response (by inhibiting actin polymerization or Gαi, a trimeric G protein) responded to a micropipette source of attractant by localizing RhoA activity at the up-gradient edge. We infer that protrusive F-actin, induced by the frontness response, constrains the spatial distribution of backness by locally reducing activation of RhoA, thereby reducing its active form at the front. Mutual incompatibility of frontness and backness is responsible for self-organization of neutrophil polarity.

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Abbreviations: fMLP, fMet-Leu-Phe; dHL-60, differentiated HL-60; PIP3, phosphatidylinositol 3,4,5-trisphosphate; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; PTX, pertussis toxin; DIC, differential interference contrast.

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and the RhoA-binding sequence of its effector, rhotekin, as well as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), which serve respectively as the FRET donor and acceptor. This biosensor design has the advantage that the C terminus of the RhoA sequence remains intact, thereby preserving normal regulation by guanine dissociation inhibitor and reversible membrane localization. FRET was assessed as the ratio of the FRET signal to the CFP signal in each pixel of the images; details of FRET measurements are described in Supporting Text, which is published as supporting information on the PNAS web site.

To confirm that the FRET/CFP ratio detected transfer of fluorescence resonance energy, we measured FRET and then photobleached the acceptor YFP. As expected, photobleaching the acceptor by >75% increased CFP emission from individual cells, indicating an overall FRET efficiency of ~20% (see Fig. 5A, which is published as supporting information on the PNAS web site, and Supporting Text). In addition, the spatial distribution of the FRET/CFP signal in individual cells was virtually identical to the relative increase in CFP emission detected after photobleaching (45% YFP bleached; ∆CFP; see Fig. 5B), validating use of the biosensor as a reliable indicator of subcellular location of Rho activation (FRET/CFP; see Fig. 5B).

The response of the biosensor to GTP loading was also validated by coexpressing the probe with a GTPase-activating protein, p50RhoGAP, which inactivates Rho by catalyzing its GTPase activity (31). Coexpression of p50RhoGAP reduced the average FRET/CFP signal in fMLP-treated cells by 20% (see Fig. 5C), indicating that the biosensor was regulated by GTP loading. A large fraction of RhoA was not affected by the GTPase-activating protein because it was already in the GDP state, consistent with observations in other cell types (32, 33), indicating that relatively small proportions of the total pools of Rho GTPases are activated and translocated to the membrane to produce biological effects. Extensive characterization of the RhoA biosensor was reported in fibroblasts (O.P., L. Hodgson, R. Klemke, and K.H., unpublished work). Results in both cell types show that the RhoA biosensor can be regulated and accurately reflects cellular RhoA activity.

As we expected, the transiently expressed biosensor showed higher RhoA activity at the back of polarized dHL-60 cells; this increase was seen in live (Fig. 1A) as well as fixed cells (not shown). More specifically, the highest FRET/CFP signals in polarized cells were localized predominantly to the cell periphery at the sides and back (Fig. 1A), whereas the biosensor itself, like endogenous RhoA (16), was distributed through the cytoplasm behind the nucleus (CFP image; Fig. 1A); this localization was quantified in 10 cells (Fig. 1B; see Fig. 6B, which is published as supporting information on the PNAS web site, for plots of individual cells). Such an asymmetric distribution of the FRET/CFP signal was detected in the majority of polarized cells examined (73% in the back, 7% in the front; n = 69), whereas only 25% of unstimulated cells (n = 72) showed asymmetrical FRET/CFP signals (Fig. 1C).

The front of the pseudopod in most polarized cells contained no detectable amount of probe and, consequently, could not be analyzed for RhoA activation. This very low quantity of Rho in the front of the cell, even if fully activated, would contribute far less activity than that in the back, where Rho is much more abundant. Some polarized cells (20%) exhibited increased Rho activation all around the cell periphery (Fig. 1C), and a few cells showed increased activation behind the nucleus (data not shown). In those cells that did show detectable RhoA activation at the leading edge, activation was low compared to activation at the back (Figs. 1A and 2C; see Fig. 7, which is published as supporting information on the PNAS web site).

It is important to stress that spatial distribution of the FRET/CFP signal always differed from that of the probe itself in fMLP-treated polarized cells (Fig. 1A; see also below), indicating that measured increases in FRET/CFP ratios did not mirror local concentrations of the probe itself but instead authentically reflected activation of RhoA. This finding was to be expected because ratiometric measurements of the unimolecular RhoA biosensor cancel out differences in probe distribution.

For collecting data from larger numbers of cells (n ≥ 25) in a single experiment, we used lentiviral-mediated gene transfer to generate a quasistable HL-60 cell line expressing the RhoA biosensor (RhoA biosensor cells). Polarized dHL-60 cells transiently or quasiternly expressing the RhoA biosensor showed similar FRET/CFP distributions (compare Fig. 2C vs. controls in Fig. 5A, and see Fig. 6B Lower).

Spatial Distributions of PH-Akt-YFP and RhoA Activity During Polarization. Like human neutrophils (27), dHL-60 cells respond to application of a uniform concentration of fMLP by forming ruffles all around the cell periphery at 1 min and become morphologically polarized with a protruding front and a contracting back 2–3 min after stimulation. Similarly, as shown in Fig. 2A, a marker for the front, PH-Akt-YFP, translocated from cytoplasm to the cell periphery by 1 min after exposure to fMLP; during the next 1–2 min, PH-Akt-YFP fluorescence aggregated to become concentrated at the leading edge. In retrospect, we overlooked the potential importance of our earlier observations of this phenomenon (1, 29).

To assess variations in average cellular RhoA activity during the same period, we determined the FRET/CFP ratio of individuals.
Actin Polymerization Suppresses and Localizes RhoA Activity. Latrunculin B, a toxin that prevents actin polymerization by sequestering monomeric actin (34), increased basal cellular RhoA-GTP in dHL-60 cells as assessed in pull-down assays (16); this finding demonstrated that actin polymerization was essential for suppressing RhoA activity. Experiments with the RhoA biosensor confirmed this inference: latrunculin B substantially increased the average FRET/CFP signal in unstimulated cells, but allowed fMLP to do so (data not shown), in keeping with Rho pull-down results (16). Despite its ability to increase RhoA FRET/CFP, a uniform concentration of fMLP did not induce morphologic polarity in PTX-treated cells, and the increased RhoA FRET/CFP was not polarized but instead was randomly distributed (Fig. 3A). PTX did substantially inhibit Gi-dependent pseudopod formation as assessed by its prevention of fMLP-stimulated accumulation of F-actin: 96% of 53 control cells formed distinct F-actin-rich pseudopods, but this staining pattern was observed in only 12% of 86 cells treated with PTX; representative cells are shown in Fig. 3B.

PTX-treated dHL-60 cells respond to a point source of fMLP by forming uropod-like structures at their up-gradient edges (16). In accord with this observation, exposure to a micropipette containing fMLP caused PTX-treated cells to localize the increase in RhoA activation to their up-gradient edges (Fig. 3 C and D). This behavior contrasts with that of control cells, which show elevated RhoA activity distributed to regions of the periphery away from the micropipette (Fig. 3C and D). We infer that fMLP-stimulated frontness restricts the localization of the RhoA backness response.

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either in the absence of fMLP or after addition of a uniform fMLP concentration (Figs. 3A and 4B). Exposure of latrunculin B-treated cells to a point source of fMLP did not induce the RhoA activation signal to distribute predominantly at the cells’ down-gradient edges as seen in normal cells; instead, activated RhoA was greater at the up-gradient edges in most cells (10 of 13, 77%; Fig. 4D). A plot of FRET/CFP in pixels at the cell periphery shows this up-gradient distribution in a representative cell (relative to the micropipette, located at position 0) (Fig. 4C); this distribution contrasted sharply with the down-gradient localization of RhoA activation in normal cells (9 of 10 cells, 90%; Fig. 4D).

Discussion

The present evidence, in combination with previous observations (16), lead us to propose a model for the mechanism of self-organizing polarity in dHL-60 cells. We hypothesize that a uniform concentration of fMLP induces a break in symmetry by stimulating formation of two different actin assemblies, prorutive F-actin and contractile actomyosin complexes, and that each assembly locally inhibits signals necessary for promoting the other. The two assemblies initially compete for the entire cell periphery but soon segregate into two clearly demarcated domains, controlled by different signals. Acting downstream of Gαi, a trimeric G protein, PI3K and Rac promote formation of pseudopods rich in F-actin (i.e., frontness). Myosin II-dependent contraction (i.e., backness) is activated by a Rho/ROCK signaling pathway downstream of different trimeric G proteins, G12 and G13.

This symmetry-breaking mechanism is based on a unifying principle: actin assemblies at the front and back serve not only as downstream “readouts” of signaling pathways but also as regulators of upstream signals. Polarity is initiated and stabilized by the ability of each actin assembly to inhibit locally the signals responsible for the other. In this way, frontness confines backness signals to regions of the cell periphery outside the pseudopod, whereas backness confines frontness signals to the pseudopod. The same principle underlies the positive feedback circuits responsible for robust pseudopods at the leading edge of neutrophils and dHL-60 cells: in this case, protruding actin polymers enhance upstream signals, including PI3K and Rac, that are responsible for their formation (22, 23, 28, 35).

Evidence for the Proposed Symmetry-Breaking Mechanism. As we reported in ref. 16, Rho-dependent backness strongly inhibits frontness and constrains it to a single region at the front of the cell: global activation of backness by expression of constitutively active mutant G12, G13, RhoA, or myosin light chains prevented accumulation of F-actin and PH-Akt-GFP at the cell membrane and blocked pseudopod formation (16). In the opposite direction, cells treated with backness inhibitors (dominant negative mutants or pharmacologic inhibitors of p160-ROCK or myosin activities) responded to fMLP with enhanced increases in Rac activity and association of PH-Akt-GFP with particulate cell fractions and by forming multiple pseudopods unconstrained by competition from backness (16, 36). In both directions, perturbing myosin activity produced effects identical to those caused by perturbing signals upstream of myosin, indicating that the final cytoskeletal readout of the backness response suffices to negatively regulate and confine the location of frontness signals and the pseudopods they promote.

Experiments presented in this article used an intramolecular FRET biosensor for RhoA activation to test the reciprocal element of the proposed symmetry-breaking mechanism, negative regulation of backness signals by frontness (F-actin). The biosensor revealed that frontness normally confines a key upstream backness signal to the portion of the cell periphery not occupied by the pseudodop. In cells exposed to a point source of fMLP, loss of frontness, produced either by inhibiting upstream signals with a Gαi inhibitor PTX (Fig. 3) or by inhibiting actin polymerization with latrunculin B (Fig. 4), produced cells in which RhoA was activated predominantly at the up-gradient edge.

The precise biochemical mechanism(s) by which frontness locally inhibits RhoA activation are unknown. We suspect, however, that this negative regulation is mediated primarily by intrusive actin polymers or, perhaps more likely, by as yet unidentified signals or RhoGAPs and other proteins that may bind to such polymers and depend on them for their activation. In keeping with this idea, latrunculin B, a frontness inhibitor that prevents morphologic polarity by sequestering monomeric actin and inhibiting actin polymerization, markedly elevates RhoA activity even in the absence of fMLP, as assessed either by the RhoA biosensor (Fig. 4) or by a RhoA-GTP pull-down assay (16). This finding suggests that some process dependent on actin polymers suppresses basal RhoA activity even in unstimulated dHL-60 cells. Relative to the elevated baseline of latrunculin-treated cells, uniformly applied fMLP does not further increase RhoA activity (Fig. 4), although an fMLP gradient does cause RhoA activity to localize predominantly at the up-gradient edge. Gi-dependent signals upstream of actin polymerization are less likely to play essential roles in locally inhibiting RhoA activity because such signals are at least partly intact in latrunculin-treated cells. Thus, latrunculin treatment allows fMLP-induced...
accretion of PH-Akt-GFP at the up-gradient edge (1, 28), as well as substantial fMLP-dependent activation of Cdc42, PAK1, Rac, and phosphorylated Akt (A. Van Keymeulen, K.W., Z. Knight, C. Govaerts, K.H., K. Shokat, and H.B., unpublished data).

A critical prediction of the proposed symmetry-breaking mechanism is that the cell’s first response to uniform application of fMLP is to activate backness and frontness signals transiently at multiple random locations on the cell periphery; later, however, prosurposing F-actin and myosin-dependent contraction cause these signals to segregate into freely demarcated domains as each actin assembly evolves without locally inhibiting upstream signals responsible for the other. In keeping with this prediction, at early times after fMLP treatment, both PH-Akt-YFP and the RhoA FRET/CFP appear at multiple regions of the cell periphery (Fig. 2 A and C); RhoA activity is often observed at regions that simultaneously exhibit ruffles (Fig. 2C). Over the course of the next 3 min, however, PH-Akt-YFP or RhoA FRET/CFP become confined to the front or the back and sides, respectively, of the polarized cell (Fig. 2 A and C). The time course of these responses and of their segregation into separate domains of the plasma membrane are in keeping with a self-organizing polarization process that depends on mutual inhibition of frontness by backness and vice versa.

**Perspective.** Our symmetry-breaking hypothesis states that mutual local inhibition and competition between frontness and backness responses creates asymmetry. In the form presented here, however, the simple competition hypothesis may not fully account for the stable asymmetry of neutrophils and dHL-60 cells, which remain completely polarized, with a single pseudopod as long as fMLP is present. The competition might result in triumph of one response over the other unless the cell can preserve a precise, potentially delicate balance between frontness and backness signals. Observations reported in a separate paper (A. Van Keymeulen, K.W., Z. Knight, C. Govaerts, K.H., K. Shokat, and H.B., unpublished data) identify a mechanism for preserving stable polarity that depends on the ability of some frontness signals to reinforce RhoA activity and actomyosin contraction at the trailing edge.

The spatiotemporal pattern of RhoA activation in randomly migrating fibroblasts, assessed with the same RhoA biosensor (O.P., L. Hodgson, R. Klemke, and K.H., unpublished work), differs strikingly from that of polarized dHL-60 cells. Unlike polarized dHL-60 cells, the fibroblasts show only transient activation of RhoA at the back during tail retraction but persistently high RhoA activity at their leading edges. This difference presumably reflects marked differences in migration speeds and roles of the leading and trailing edges between the two cell types. Neutrophils migrate dramatically faster than fibroblasts, continuously contracting their backs to restrict location of the pseudopod and to allow rapid forward movement of the trailing edge. The pseudopod of the neutrophil moves more or less continuously forward, adhering loosely to the substratum, in contrast to the fibroblast’s leading edge, where slower forward motion is accomplished by tight adhesion to the substratum, whereas many peripheral ruffles extend and retract without sticking. We suspect that the persisting RhoA activation at the fibroblast leading edge plays a key role in forming adhesive structures regulated by F-actin (37) and that these structures are absent or much less important in neutrophils. Thus, persistent RhoA activation at the fibroblast’s front allows it to probe and enhance structural integrity of connective tissue, whereas predominant RhoA activation at the rear makes the neutrophil stick less tightly to extracellular matrix so that it can more rapidly penetrate and explore damaged or inflamed tissues.

**Materials and Methods.** Materials and methods used in cell culture, transient expression of exogenous cDNA, micropipette assays, and fixation and staining for actin, drugs, and toxins have been described in detail (1, 16, 28, 29). All methods are described in further detail in Supporting Text.

**DNA Constructs and Lentiviruses Expressing the RhoA Biosensor and PH-Akt-YFP.** To generate lentiviruses, pSVVG.P, pCMVD8.9 (gifts from Todd Brennan, University of California, San Francisco), and the FuPw vector containing either the RhoA biosensor or PH-Akt-YFP were cotransfected into human embryonic kidney (HEK) cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. The concentrated solution of lentiviral particles was added to undifferentiated HL-60 cells.

**Image Acquisition and Data Processing.** For time-lapse and fixed cell imaging, FRET, CFP, and YFP images were captured using filter sets S436/10 and S535/30, S436/10 and S470/30, and S500/20 and S553/35, respectively (Chroma Technology, Rockingham, VT). DIC images were acquired for each cell and binning was set at 2 × 2.

For FRET/CFP ratiometric processing, CFP and FRET images...
were background-subtracted. (Note that the initial analysis showed that applying a shading correction was not necessary; that is, higher activity on one side of the cell was consistent regardless of cell orientation and not attributable to uneven illumination across the image field.) The resulting background-corrected FRET image was divided by that of the CFP image to obtain a pixel-to-pixel FRET/CFP ratio image. Cells were thresholded to discard any portions of the image with insufficient intensity to provide reasonable signal/noise. Unless stated otherwise, the final FRET images were displayed in pseudocolors scaled linearly from the lowest to the highest signal within each cell.

Assessing Polarity and Peripheral Distribution of RhoA Activity. Cells were defined as polarized on the basis of morphology under DIC microscopy. Polarized cells showed a broad front containing a pseudopod on one side and a round, narrow back at the other side. Cells normally became fully polarized after a 2- to 3-min treatment with 100 nM fMLP. Symmetry or asymmetry of FRET distribution in a cell was determined by examining the profile of relative FRET intensity along the cell’s periphery, in pseudocolor images. Similarly, cells exposed to an IMLP gradient with the highest FRET facing toward or away from the micropipette were categorized as “up-gradient” or “down-gradient,” respectively. Cells that showed a relatively even distribution around the periphery were assigned as “other.”

Statistical Analysis. To compare FRET/CFP intensities of cells treated under different conditions, the average FRET/CFP ratio of each cell was determined by dividing the cell’s total FRET/CFP ratio by its entire cell area. Results were normalized against the control unstimulated sample (set to 1.0). Unless stated otherwise, values from at least 25 cells were used to assess mean FRET/CFP ratios for each condition, and the effect of each condition was compared with the unstimulated control by using Student’s t test. Each set of experiments was repeated at least three times.

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