Expression and translocation of fluorescent-tagged p21-activated kinase-binding domain and PH domain of protein kinase B during murine neutrophil chemotaxis

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Abstract: Neutrophils are key cells of the innate immune system; they are terminally differentiated and therefore difficult to genetically manipulate and study in vitro. In the present study, we describe a protocol to transiently express two fluorescent markers, the PH domain of protein kinase B fused to red fluorescent protein and the p21-activated kinase-binding domain fused to a yellow fluorescent protein, in primary neutrophils. Using this approach, we are able to achieve a transfection efficiency of ~30%. The expression of the transfected probes occurred within 2 h and allowed for real-time monitoring of intermediates in key neutrophil activation pathways at the leading edge of migrating cells. We describe here a transfection protocol for primary neutrophils, which preserves fMLP-mediated cell polarization and cytoskeleton reorganization with simultaneous accumulation of PI-3K products and active Rac at the leading edge. The visualization and analysis of transfected fluorescent markers in primary neutrophils are a powerful technique to monitor chemotaxis signaling pathways in real time. J. Leukoc. Biol. 82: 559–566; 2007.

Key Words: transfection · PI-3K · Rac activation

INTRODUCTION

Neutrophils are key cells of the innate immune system [1], carrying out a wide range of functions during the inflammatory response. Neutrophil-mediated bacterial killing is a multistep process, which involves a series of complex signaling networks [2, 3], resulting in neutrophil recruitment to sites of infection, phagocytosis of bacteria, and generation of reactive oxygen species [4–6]. Although neutrophils are key innate immune cells, it is difficult to use standard transfection and microinjection techniques to study them in vitro, as they are small, short-lived, and terminally differentiated cells [7, 8].

Significant efforts are being made to develop alternative models to study neutrophil signaling pathways. Leukemic cell lines such as human NB4, HL-60, and the murine 32Dcl3 and MPRO cells are used currently as models to study neutrophil signaling pathways using mammalian expression vectors. Despite their recognized use as transfection models, yielding transfection efficiencies, varying from 10% to 20% [9–11], these available cell lines have some limitations when used to study neutrophil functions. These include impaired responses to fMLP and IL-8 chemoattractants [12–14], failure to express secondary granule proteins causing mislocalization of cytochrome b558 [7, 15, 16], and impaired respiratory burst and bacterial killing [7, 17–21]. These limitations inherent in the use of cell lines and the development of new nucleoporation techniques have shifted the focus of recent investigations and have allowed the transfection of previously resistant primary cells, including primary neutrophils [3, 22].

The ability to visualize the distribution of activated or recruited molecules in neutrophil signaling pathways in real-time represents an important step in the study of the underlying mechanisms of neutrophil directional migration [23]. In the present study, we describe for the first time a protocol using primary neutrophils, which allows for the real-time study of the initial steps of cell polarization and migration using fluorescent probes to determine the spatial distribution and translocation of PI-3K products, such as phosphatidylinositol (3,4,5,)-3 trisphosphate (PIP3), and active Rac during fMLP-mediated chemotaxis.

MATERIALS AND METHODS

Animals

C57BL/6j wild-type (WT) mice of 6–13 weeks old were used in accordance with the “Guide for the Human Use and Care of Laboratory Animals” and the approval of the University of Toronto Animal Care Committee (Ontario, Canada).

Neutrophil preparations

WT littermates were killed by CO₂ inhalation. Femurs and tibias were removed, and bone marrow (BM) was isolated. Recovered BM cells were layered...
onto discontinuous Percoll (Sigma-Aldrich, Ontario, Canada) gradients of 82%/65%/55% [18]. Mature neutrophils (WT) were recovered at the 82%/65% interface and were found to be positive for Gr-1 and membrane-activated complex-1 by flow cytometry. More than 85% of cells isolated were neutrophils as assessed by Wright-Giemsa staining. Viability determined by trypan blue exclusion was >90%.

Neutrophil immunostaining for Rac and PIP3

BM-isolated neutrophils were reuspended on a 1% gelatin HBSS and allowed to attach to BSA-coated, eight-well tissue-culture slides (Falcon, Becton Dickinson, San Jose, CA, USA) for 10 min at 37°C. Adherent cells were stimulated with a uniform concentration of 1 μM fMLP for 120 s followed by fixation in 4% paraformaldehyde for 10 min at room temperature. Fixed neutrophils were washed three times in PBS solution without calcium and magnesium and permeabilized by 0.5% Triton X-100 at room temperature for 15 min. After fixation and permeabilization, neutrophils were incubated in 5% BSA/PBS for 30 min at 37°C and immunostained for PIP3 (PIp3-FITC, Cytoskeleton Inc., Denver, CO, USA) and Rac (Anti-Rac, Upstate Biotechnology, Lake Placid, NY, USA). For the Rac immunostaining, primary antibody was used at a dilution of 1:100 for 1 h at room temperature followed by incubation with goat anti-mouse Alexa antibody 555 (1:400). The PIp3-FITC antibody was added for 1 h at a dilution of 1:100.

DNA constructs

Plasmid constructs encoding the p21-binding domain of p21-activated kinase fused to yellow fluorescent protein (PAK-PBD-YFP) and the pleckstrin homology domain of Akt fused to a red fluorescent protein (PH-AKT-RFP) were a kind gift from Dr. Siegfried Grinstein (Hospital for Sick Children, Toronto, Ontario, Canada). The PAK-PBD-YFP [24] construct was encoded in a pDNA3.1 vector (Invitrogen, Ontario, Canada) and comprised residues 65–150 of human PAK1 with enhanced YFP linked to its COOH terminus. The PH-Akt-monomeric (m)RFP [25, 26] was made by digestion of GFP-PH-Akt at XhoI of human PAK1 with enhanced YFP linked to its COOH terminus. The PAK-PBD-YFP [24] construct was encoded in a DNA construct containing 1 μM fMLP for 120 s followed by fixation in 4% paraformaldehyde for 10 min at room temperature. Fixed neutrophils were washed three times in PBS solution without calcium and magnesium and permeabilized by 0.5% Triton X-100 at room temperature for 15 min. After fixation and permeabilization, neutrophils were incubated in 5% BSA/PBS for 30 min at 37°C and immunostained for PIP3 (PIp3-FITC, Cytoskeleton Inc., Denver, CO, USA) and Rac (Anti-Rac, Upstate Biotechnology, Lake Placid, NY, USA). For the Rac immunostaining, primary antibody was used at a dilution of 1:100 for 1 h at room temperature followed by incubation with goat anti-mouse Alexa antibody 555 (1:400). The PIp3-FITC antibody was added for 1 h at a dilution of 1:100.

RESULTS

Expression of PH-AKT-RFP and PAK-PBD-YFP in primary neutrophils

Primary neutrophils were transfected efficiently with PH-AKT-RFP and PAK-PBD-YFP. The PH-AKT-RFP probe was used to analyze the cellular localization of PI-3K products, including PIp3 (Fig. 1, B and E), and the PAK-PBD-YFP probe was used to analyze the cellular localization of activated Rac [24] (Fig. 1, A and D). The pmxGFP vector was used as a control for the transfection experiments (Fig. 1, C and F) and showed no evidence of polarized accumulation in the transfected neutrophils. To assess the transfection efficiency of the DNA constructs (see Materials and Methods), cells were analyzed by flow cytometry and fluorescence microscopy (Fig. 2, A–C). The transfection efficiency (based on microscopic analyses) achieved using this protocol was 29.49 ± 1.03%, and ~70% of the cells expressed PAK-PBD-YFP and PH-AKT-RFP vectors. Viability using trypan blue exclusion was 71.86% ± 3.68%. Primary murine neutrophils immunostained for Rac and PIp3 showed a similar distribution of the protein targets used in the transfection experiments (Fig. 3, A–H).
Translocation of PH-AKT-RFP in primary neutrophils after fMLP stimulation

During neutrophil chemotaxis, highly regulated signaling mechanisms determine the cell response and actin cytoskeleton reorganization necessary for leukocyte function [11, 24, 28]. During this process, the activation of G protein-coupled receptors, including the fMLP receptor, induces the activation of PI-3K and the accumulation of PIP3 and other phosphorylated lipid products [3, 29]. The Gβγ subunit and PIP3 formation activate important signaling cascades, leading to activation of the small GTPases cycle, including Rap1, Rac, and Cdc42, which will ultimately regulate PI-3K and actin polymerization [30–32]. Neutrophils expressing the tested fluorescent vectors were mounted in a live cell imaging chamber and exposed to uniform stimulation with 1 μM fMLP. The recovered confocal image sequences showed that PH-AKT-RFP was recruited to the plasma membrane of activated, primary neutrophils (Fig. 4, A–C). In addition, a significant increase (1.24 ± 0.04) in the fluorescence intensity was observed at the leading-edge membrane compared with the average cytoplasmic levels, although the PH-AKT-RFP increase was not significantly different from the membrane increase revealed by the Alexa-CTB marker at the leading edge and trailing edge of the cell (Fig. 4C). The linear fluorescence intensity showed that

![Image of confocal images](fig1.png)

**Fig. 1.** Confocal images of transfected murine neutrophils, which were isolated from murine BM and transfected with PH-AKT-RFP and PAK-PBD-YFP or the control pmaxGFP following the protocol described (see Materials and Methods). After the recovery period, cells were transferred to a live cell chamber and stimulated with a uniform concentration of fMLP (1 μM). Image sequences were recovered using a spinning disk confocal microscope. (A) Neutrophil expressing PAK-PBD-YFP at the resting state. (B) Neutrophil expressing PH-AKT-RFP at the resting state. (C) Neutrophil transfected with the control pmaxGFP fluorophore at the resting state, where probes were distributed equally throughout the cell area. (D) Translocation and accumulation of PAK-PBD-YFP at the leading edge after fMLP stimulation. (E) Cellular localization of PH-AKT-RFP after fMLP stimulation. (F) Stimulated pmaxGFP control neutrophil showing no significant accumulation of the GFP marker. Images represent individual cells. A total of 70 cells was analyzed in five independent experiments.

![Image of flow cytometry results](fig2.png)

**Fig. 2.** Flow cytometry results and microscopic images from transfected murine neutrophils, which were isolated and transfected as described in Materials and Methods. After the recovery period, cells were resuspended in 500 μL HBSS and analyzed for the expression of the fluorescent marker by flow cytometry. (A) Fluorescence log (PMT3) of control nontransfected neutrophils. (B) Fluorescence log of PAK-PBDYFP-transfected neutrophils showing 32.2% of the cells expressing the fluorescent probe. (C) An example of paired epifluorescence and differential interference contrast (DIC) images of a 20× microscopic field, which illustrates the percentage of transfected neutrophils. Approximately 250 cells were counted in each of five independent experiments. Flow cytometry data shown are representative of a single experiment. A total of six independent flow cytometry analyses was performed.
the PH-AKT-RFP distribution profile correlated with the membrane dye levels within the cell (Fig. 5, B–D). In contrast, the pmmaxGFP control was distributed equally throughout the cell (Fig. 5A). These results confirm previous immunostaining experiments, which showed a similar increase of PI-3K products at the leading edge of activated neutrophils [33] (Fig. 3, G and H) and previous experiments using HL-60 cells [29].

Translocation of PAK-PBD-YFP in primary neutrophils after fMLP stimulation

The expressed PAK-PBD-YFP may bind to the active forms of Rac or Cdc42, although previous in vivo studies have shown that this YFP-tagged PAK domain binds preferentially to active forms of Rac [24]. Substantial data affirm that Rac is recruited to the leading edge of fMLP-stimulated neutrophils and neutrophilic cell lines. Confocal microscopy images were recovered from polarized cells with a distinguishable leading edge and trailing edge after fMLP stimulation (Fig. 4A). Primary neutrophils showed a remarkable accumulation of the PAK-PBD-YFP construct at the leading edge, colocalizing with the PH-AKT and membrane dye at maximum intensity levels; yet, there was no increase in the back of the cell compared with the mean cytoplasmic fluorescence intensity (Figs. 4C and 5C). The recruitment of the fluorescent probe corroborates the im-

Fig. 4. Murine neutrophils expressing the transfected vectors were mounted on a live cell chamber and exposed to 1 μM uniform fMLP stimulation. Cells polarizing with a clear lamellipodium were divided in three separate compartments, encompassing the leading edge (L), cytoplasm (C), and trailing edge (T) and measured for their mean pixel intensity (MPI). Results were normalized to the average cytoplasmatic intensity in each fluorescent channel for each cell. (A) Representation of the measured areas, (B) Average cell area measured during the experiments. The new lamella formation and spreading encompassed a slightly greater measured leading-edge area. (C) Translocation of PAK-PBD-YFP and PH-AKT-RFP probes in primary neutrophils after fMLP stimulation. Polarized cells showed a significant increase of PAK-PBD-YFP and PH-AKT-RFP at the leading-edge area of the cell. As expected, the membrane fluorescence intensity levels at the back and front of the cell were similar and significantly higher than the mean cytoplasmic level. In contrast to results for the PAK-PBD-YFP probe, the PH-AKT-RFP increase in fluorescence intensity at the leading edge was not significantly different from the membrane levels in the same area. Results are based on the analysis of 18 cells (*, P<0.0001; **, P<0.003).
munostaining experiments shown in Figure 3, C and D, and previous results from our group [33]. The increase of PAK-PBD-YFP was also found to be significant when normalized to the increased fluorescence intensity of Alexa-CTB at the leading-edge membrane.

**Distribution of the fluorescent markers on the z-axis**

Transfected neutrophils were analyzed for the variance of the fluorescence intensity along the z-axis. In Figure 6A, each channel was normalized to the MPI of the measured area, allowing us to directly compare all the fluorophores simultaneously. Cell images were analyzed for the distribution of probes from the ventral interface to the dorsum of the cell in the z,x- and z,y-axis. Analysis of the optical sections of the cells showed that the PAK-PBD-YFP construct preferentially localized at the leading-edge membrane (Fig. 6B) and along the ventral half of the cells. As the optical sections were generated from the top to the bottom of the cell, and the peak membrane dye levels were similar among the optical sections, the difference observed was probably not a result of photobleaching but may be attributed to the physiologic localization of the probes (Fig. 6, A, C, G, H). The PH-AKT-RFP probe distribution was not significantly different from the membrane dye distribution (Fig. 6, A, B, E, F, I, J).

**Chemotaxis of transfected neutrophils**

Murine neutrophils transfected with PAK-PBD-YFP and PH-AKT-RFP were mounted on a Zigmond chamber and exposed to a 1 μM fMLP gradient. Recovered time sequences showed that transfected primary neutrophils responded to the chemoattractant and were able to migrate toward the source of the chemoattractant (Fig. 7). Although the cells were able to detect the chemoattractant gradients, the mean migration speed (4.03±0.63 μm/min) was significantly lower than that of nontransfected neutrophils (5.20±0.40 μm/min; P<0.05).

**DISCUSSION**

Neutrophils are essential for a protective immune response, representing the initial cellular defense against invading organisms. These unique cells migrate toward sites of tissue injury and inflammation, performing microbicidal reactions and helping tissue healing [22, 34]. Although neutrophil activation may lead to effective bacterial killing and wound healing, these cells may be responsible for major tissue damage in periodontal diseases, sepsis, transplant rejection, and granulomatous disease when regulatory mechanisms fail [5].

Recent publications have reported transfection of neutrophils using fluorescent probes with efficiencies varying from 0.4% to 1% [23], overexpression of CD11b, responsive NADPH activity, and phagocytosis, but no data about cell migration were reported. In the present investigation, primary neutrophils were transfected with ~30% efficiency after 2 h. Although the transfection efficiency reported for HL-60 cells using this same method yielded higher efficiencies, the recovery time for these cells varied from 12 h to 24 h. As a result of
Fig. 6. Three-dimensional localization of PH-AKT-RFP, PAK-PBD-YFP, and Alexa-CTB 647 in murine neutrophils. (A) Two independent, linear measurements were collected from the $z_x$- and $z_y$-axis of 10 neutrophils, starting from the ventral to the dorsal sum of the leading edge of the cell. Scores were compared with the MPI of the specific probe in the measured area. The deviation of the scores from the MPI was plotted and analyzed statistically. All observations were statistically significant ($P<0.0001$). As expected, the Alexa-CTB 647 membrane dye had peak levels at the bottom and the top of the cell, showing no significant difference between the scores. The PAK-PBD-YFP probe was localized preferentially at the ventral surface of the cell ($P<0.005$) and colocalized with the membrane dye increase at the area. PH-AKT-RFP fluorescence showed the same variance as that for the distribution of the membrane dye ($P=0.9$). The ratio of dorsal:ventral PH-AKT-RFP fluorescence intensities appeared to differ clearly in the images, but their mean difference in intensity fell below statistical significance ($P<0.07$). (B) Distribution of PAK-PBD-YFP and PH-AKT-RFP at the leading-edge membrane area on the $z$-axis; both probes localized preferentially in the ventral half of the cell ($P<0.001$). (C) Three-dimensional reconstruction of a neutrophil transfected with PH-AKT-RFP and PAK-PBD-YFP and stained with Alexa-CTB. (D) Three-dimensional reconstruction of a neutrophil transfected with the pmaxGFP control. (E and F) Top ($z_x$) and frontal ($y,z$) view of the Alexa-CTB 647 membrane dye distribution. (G and H) Top ($z_x$) and frontal ($y,z$) view of the PAK-PBD-YFP probe three-dimensional distribution. (I and J) Top ($z_x$) and frontal ($y,z$) view of the PH-AKT-RFP probe three-dimensional distribution. Images E–J represent a single neutrophil. A total of 10 cells was analyzed.
the unquestionable importance of fast-moving neutrophils for studies of chemotactic pathways, we tested and validated a transient transfection protocol for primary murine BM neutrophils to visualize, in real time, the translocation of two widely used fluorescent markers for cytoskeletal reorganization. The proposed technique achieved much higher transfection efficiencies with similar cell viability achieved by previous studies [23].

The translocation of PAK-PBD-YFP in transfected primary neutrophils demonstrated the maintenance of Rac small GTPase activation, a well-known molecular switch responsible for leading-edge actin cytoskeletal remodeling [11, 22, 24]. Spatial analysis of the YFP-tagged PAK probe translocation showed recruitment to the membrane followed by fluctuations of signal leading to the formation of a distinguishable leading edge. At this point, the PAK probe was associated mostly with the leading-edge membrane. This observation also corroborates the immunostaining experiments shown in Figure 3, C and D, where nontransfected neutrophils have an important increase of Rac at the leading edge after fMLP stimulation. Time-series analysis of the activation sequence showed that recruitment occurred in the early seconds of neutrophil activation (data not shown). In addition, analysis of the pmaxGFP control validated the functional applicability of the method, as there was a uniform distribution of this marker throughout the cell. In our model, the PH-AKT-RFP probe was initially recruited to the plasma membrane with a significant increase at the leading edge when compared with its cytoplasmic levels. The PH-AKT-RFP was found to be associated with the plasma membrane in all axes analyzed, and the fluctuations of the PH probe and membrane dye signals were not significantly different. As the targets of this probe are essentially located near the plasma membrane, this observation shows that the probe we used was functional, and it suggests that the membrane conformation may be responsible, in part, for the PH-AKT-RFP distribution. In addition, preliminary results in our lab using immunostaining and PH-AKT-RFP transfections have shown that the regulation of PI-3K is compromised severely in Rac1 null neutrophils (data not shown). The positive-feedback loop between Rho small GTPases and the phosphorylated products of PI-3K has been studied extensively. It is a complex signaling network, which evidently drives the amplification of shallow, extracellular chemotactic signals and regulates the directional recruitment of activated intermediates, which catalyze polarized actin filament assembly. The expression of both constructs at the same time allowed us to compare the localization of PI-3K-phosphorylated products, including PIP3, and active Rac in the primary neutrophils in real time. Confirming previous observations, there was a strong association in the temporal and spatial dynamics of these signaling molecules [35]. Previous studies have shown that the concentration of extracellular matrix components in the substratum affects Rac and Cdc42 activity of the cells [36–38]. Thus, the localization of the probes toward the leading-edge ventral compartment of the transfected neutrophils in our study may indicate cross-regulation of integrin binding and Rac/PI-3K activation. PH-AKT-RFP was constrained to a small area in and around the plasma membrane, and the PAK-PBD-YFP probe was distributed throughout the leading-edge area, preferentially at the ventral portion of the cell. As suggested here by the temporal and spatial correlation in the distribution of these probes, a relatively small increase in the PI-3K products in a specific cell location may translate into the augmented accumulation of active Rac and the formation of a lamellipodium [39].

Similar to previous results using HL-60 cells [24], transfection with the two fluorescent probes yielded a reduction in the primary neutrophil migration speed, although the mechanisms involved in this process still need to be studied. Conceivably, the interference of the fluorescent markers with endogenous protein availability may play a role in this process [40]. Despite their ~20% slower speed, the transfected cells were able to migrate toward a fMLP gradient, confirming the integrity of the complex directionally oriented signaling cascade, which leads to cytoskeleton reorganization. Our verification and improvement of transfection protocols for the fluorescent probes raise new possibilities to apply genetic expression strategies in primary neutrophils to study cell migration.

The data generated in this study along with recent investigations show that primary neutrophils can be transfected efficiently with mammalian expression vectors, allowing for real-time monitoring of the distribution of key signaling intermediates in these cells. The application of this protocol should encourage and help investigators to use primary neutrophils for a wide range of signal transduction investigations.