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Platelet-Activating Factor-Mediated Endosome Formation Causes Membrane Translocation of p67phox and p40phox That Requires Recruitment and Activation of p38 MAPK, Rab5a, and Phosphatidylinositol 3-Kinase in Human Neutrophils

Nathan J. D. McLaughlin,*† Anirban Banerjee,‡ Samina Y. Khan,*† Janet L. Lieber,§ Marguerite R. Kelher,*,‡ Fabia Gamboni-Robertson,‡ Forest R. Sheppard,‡ Ernest E. Moore,‡ Gary W. Mierau,§ David J. Elzi,*‡ and Christopher C. Silliman2*†‡

Neutrophils (polymorphonuclear leukocytes, PMNs) are vital to innate immunity and receive proinflammatory signals that activate G protein-coupled receptors (GPCRs). Because GPCRs transduce signals through clathrin-mediated endocytosis (CME), we hypothesized that platelet-activating factor (PAF), an effective chemoattractant that primes the PMN oxidase, would signal through CME, specifically via dynamin-2 activation and endosomal formation resulting in membrane translocation of cytosolic phagocyte oxidase (phox) proteins. PMNs were incubated with buffer or 2 μM PAF for 1–3 min, and in some cases activated with PMA, and O2


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pathogens (1, 4, 15, 16). This migration to the tissues causes PMN priming resulting in physiologic changes in the PMN, including: changing the phenotype from nonadhesive to adhesive, alteration in the actin cytoskeleton manifested as changes in PMN morphol-
geny, and augmentation of the oxidative response to a given stim-
ulus through translocation of specific cytosolic oxidase compo-
dnents to the plasma membrane (18–23). Platelet activating factor (PAF) is a prototypal lipid chemotractant that binds to a spe-
cific seven-transmembrane domain receptor (PAFR) (24). PAF oc-
cupation of this receptor results in release of the Gq, and Goq 
hetromeric subunits, which mediate chemotactic, cytotoxic, and
cross-regulatory signals as well as initiating inhibitory signals,
β-arrestins and G protein receptor kinases, to desensitize these
activated receptors to repeated stimulation (25, 26). Ligand occu-
dation of PAFR, all before activation of dynamin-2 at the PMN mem-
brane (4, 28). We hypothesize that PAF ligation of the PAFR in-
duces dynamin-2 scission of the endosome from the plasma mem-
brane and recruitment of PI3K, Rab5a, EEA-1, and the p38 MAPK
signalosome to the nascent endosome, triggering phosphorylation
and translocation of the cytosolic 67-kDa phagocyte oxidase pro-
tein (p67phox) with the 40-kDa phagocyte oxidase protein (p40phox)
to the plasma membrane.

Materials and Methods

Materials

Unless otherwise indicated, all chemicals were purchased from Sigma-
Aldrich. All reagents were endotoxin-free, and all solutions were made
from sterile water (United States Pharmacopeia). Acrylamide, N,N'-
methylene-bis-acrylamide, and N,N,N',N'-tetramethylethylenediamine
(TEMED) were obtained from Bio-Rad. ECL reagents were obtained
from Amersham Biosciences. Nitrocellulose paper, x-ray film, and Nunc 96-well
plates were purchased from Life Science Products. Abs to the PAFR, dy-
amin-2, ASK1, MAPK kinase-3 (MKK3), Rab5a, Rab GDP dissociation
inhibitor (RabGDI), EEA-1, and the p38 MAPK signalosome from the nascent endosome, triggering phosphorylation
and translocation of the cytosolic 67-kDa phagocyte oxidase protein
(p67phox) with the 40-kDa phagocyte oxidase protein (p40phox)
to the plasma membrane.

PMNs (5.0 × 10^5 cells) were warmed to 37°C and then incubated
with either buffer or 2 μM PAF for 1 min and prepared as previously described
(4). Images were acquired with a Zeiss Axiosvert fitted with a Cooke CCD
SensiCam using a Chroma Sedit Multiple Bandpass filter wheel and Sutter
filter control. Images were acquired using Intelligent Imaging Innovations
SlideBook software. All images compared within a single figure were ac-
quired as Z-stacks in 0.2-μm intervals and were deconvoluted by applying
constrained iterative deconvolution and Gaussian noise smoothing from
system-specific point spread functions (32–36). Following deconvolution,
images were cropped to represent the middle-most planes (center ± 10
planes), and the proteins in question were masked to represent zero
fluorescence in IgG-negative controls. Cell polarization was assessed
using Nomarski differential interference contrast microscopy (Nomarski
images) (37).

Fluorescent resonance energy transfer (FRET) microscopy

FRET determinations were obtained using the 3-cube method to account
for possible bleed-through of light (38). Images are acquired sequentially
through three filter settings: donor filter, acceptor filter, and the FRET
transfer filter, which is excitation through the donor filter and emitted light
collected through the acceptor filter. To account for bleed-through from
the donor to the acceptor or vice versa, images of samples with a single Ab
(either donor or acceptor) are acquired under identical conditions as the
experimental group, with correction coefficients being calculated using the
“fit data” operation within SlideBook imaging software. In all microscopy
experiments, the fluorophore-antibody combination was varied, and similar
results were found. These data would suggest that even if the donor flu-
ophore vastly outnumbered the acceptor, giving a false-positive, or the
acceptor outnumbered the donor, giving a false-negative, the proximity of
the Abs against proteins of interest was sufficient and necessary to allow
for real energy transfer. FRET indices may then be calculated using: FRET
channel (FRET^\text{channel}) = F_{\text{E}} - D(F_{\text{D}}/D_{\text{A}}, A_{\text{E}}/A_{\text{A}}), where F_{\text{E}}, D_{\text{A}}, and A_{\text{A}}
represent the transfer, donor, and acceptor channels in the presence of the
Ab, respectively. FRET is the energy transfer from donor to
acceptor, which is then corrected for bleed-through as follows. Be-
cause the method of data processing is paramount to its interpretation,
each image is acquired as a Z-stack of 25 planes. Bleed-through is corrected
by a constraint deconvolution iterative in which the imaging properties of
the optimal system are used as a measured point spread function such that
one mathematically “puts light back where it came from” (39). This point
spread function can be used for a calculation of a likely model of the object
from the recorded data set in an iterative process (39). Deconvolution elim-
inates effective blur caused by distortion, and by assuming a Poisson dis-
tribution of stray light it suppresses background light to very low levels
(39). Not only is bleed-through eliminated but resolution is increased,
and is paramount importance in cases involving proliferation of objects
near resolution size (39). FRET efficiencies (E) were calculated using pre-
viously published techniques, and images are displayed in pseudocolor,
using arbitrary linear units of fluorescent intensity (a.u.f.i.), where blue is

Digital fluorescent microscopy

PMNs (5.0 × 10^5 cells) were warmed to 37°C and then incubated
with either buffer or 2 μM PAF for 1 min and prepared as previously described
(4). Images were acquired with a Zeiss Axiosvert fitted with a Cooke CCD
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images) (37).
“cold” (no FRET) and red is “hot” (most FRET). Such a visual representation was used rather than “white light” in which brightness is the representation of high FRET (4). Additionally, for all the FRET images there are a number of FRET-positive voxels even in the control (2–5) if one looks closely. A positive result was only considered if the number of FRET-positive voxels increased by at least 2 logs over the control, and in each of the FRETs in the present paper the numbers of positive FRET voxels were between 2 and 3 logs greater than the paired controls. Furthermore, at least two AbS were used for each and every protein of interest and these Abs are suitable for immunoprecipitation, for most figures have such immunoprecipitations done in concert with the FRETs. Therefore, the immunoreactivity is for the native protein and not an epitope that may be “unmasked” by separation using reducing conditions and immunoblotting. Lastly, in a number of figures we overlay the FRET images, termed “FRET overlay”, or the cellular immunoreactivity of a single protein (p38 MAPK) with a FRET image, termed “overlay”, and examine whether the maximal FRET-positive physical interactions between proteins or the cellular immunoreactivity of a specific kinase and a possible substrate occurs in the identical cellular locales. These overlays visually demonstrate the regions that contain maximal overlay of FRET-positive voxels (e.g., proteins A and B and proteins A and C; all proteins must be on different fluorescent channels) to determine whether these interactions are occurring in the same cellular area. The maximal overlay of voxels is red with areas in high colocalization or blue in areas with little colocalization as visually expressed in a.u.f.i.

**Intracellular neutralization of specific proteins**

BioPORTER was reconstituted according to the manufacturer’s directions. Briefly, individual tubes were reconstituted to a total volume of 40 μl with Krebs-Ringer phosphate buffer with 2% dextrose ± 4 μg of the Ab specified in the text for 5 min at room temperature. PMNs (5 × 10⁶ cells) were incubated with buffer, vehicle only, or vehicle with the Ab for 2 h at 37°C. Following incubation, PMNs were centrifuged for 3 min at 400 g at 4°C and resuspended to 5 × 10⁶ cells/ml. To control for IgG introduction, a FITC-IgG was introduced, and Z-stack images were acquired as described above. β-actin-1 and dynamin-2 are sufficient for native protein as demonstrated by use in immunoprecipitation. Furthermore, for intracellular neutralization two different Abs to different epitopes were used.

**Electron microscopy**

Isolated PMNs were primed with 2 μM PAF or buffer control for 1 and 3 min and immediately fixed with 4% phosphate-buffered paraformaldehyde (pH 7.35). PMNs (7.5 × 10⁶) were added to the 8-well Permanex chambered slides (Nunc) that were shaken to allow for equal distribution of PMNs. The slides were dried, washed with PBS (pH 7.0), and the PMNs permeabilized with 0.1% Triton-X. The permeabilized PMNs were blocked with 200 μl 2% BSA-5% NDS (BSA-C) for 60 min at room temperature. The PMNs were incubated with the primary Abs (1/40 dilution in BSA-C) overnight and then washed with PBS. The secondary Abs, bound to immunogold (5- or 25-nm particles) by standard techniques, were then added at a dilution of 1/100 in BSA-C and incubated for 15 min at room temperature (40, 41). The cells were washed with PBS and fixed with 2.5% cacodylate-buffered glutaraldehyde for 60 min. The PMNs were silver enhanced for 60 min at room temperature and then washed with deionized cacodylate-buffered glutaraldehyde for 60 min. The slides were dried, washed with PBS (pH 7.0), and the PMNs permeabilized with 0.1% Triton-X. The permeabilized PMNs were blocked with 200 μl 2% BSA-5% NDS (BSA-C) for 60 min at room temperature. The PMNs were incubated with the primary Abs (1/40 dilution in BSA-C) overnight and then washed with PBS. The secondary Abs, bound to immunogold (5- or 25-nm particles) by standard techniques, were then added at a dilution of 1/100 in BSA-C and incubated for 15 min at room temperature (40, 41). The cells were washed with PBS and fixed with 2.5% cacodylate-buffered glutaraldehyde for 60 min and then dehydrated in a graded series of ethanol, embedded in Embed 812, and sectioned at a thickness of 80 nm. Sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM-10CA transmission electron microscope at 60 kV accelerating voltage (43).

**Statistical analysis**

The data are expressed as the means ± the SEM. For FRETs the data are expressed as the mean fluorescent intensity of the FRET, and for better visualization the FRETs are represented in color (a.u.f.i.) as described previously. Statistical significance among groups was calculated by paired ANOVA with a Bonferroni-Dunn post hoc analysis using p ≤ 0.05 or p ≤ 0.01 as the level of statistical significance.

**Results**

**Dynamin activation and the formation of the early endosome**

Dynamin-2 is responsible for pinching off the endosome from the plasma membrane and propelling it into the cytosol (1, 7–10). PAF caused activation of dynamin-2 as demonstrated by tyrosine Y-phosphorylation of dynamin (Fig. 1) and colocalization with Grb2, a known signaling protein associated with activated dynamin-2, which was present at 1 and 3 min (Fig. 1) (44, 45). Importantly, intracellular neutralization of dynamin using two distinct neutralizing Abs inhibited internalization of the PAF receptor by 97 ± 4% and 99 ± 8%, respectively (data not shown).

**PI3K** has been implicated in the formation of the nascent endosome; therefore, it was important to discern whether it was required for colocalization of the Rab5a-GTPase with the sorting protein EEA-1 (14, 46, 47). PAF-induced dynamin-2 activation caused colocalization of PI3K with the Rab5a-GTPase in the cytosol at 3 min, as demonstrated by a positive FRET between Rab5a and PI3K, which is inhibited by the intracellular neutralization of dynamin-2 but unaffected by the isotopic control IgG (Fig. 24). PI3K also has a FRET-positive colocalization with EEA-1, which was corroborated by coprecipitation of PI3K with EEA-1 in PMNs primed by PAF as compared with buffer-treated controls of EEA-1 (Fig. 2, B and C). The FRET efficiencies for Fig. 2, A and C, were 34 ± 8% and 32 ± 4%, which correlate with a distance of <8 nm between these proteins. Furthermore, PAF caused a FRET-positive colocalization of PI3K with both EEA-1 and Rab5a in the identical PMNs and localized these protein interactions to the cytosol as demonstrated by light microscopy (Nomarski image, Fig. 3A). The microscopy was reconfirmed by immunoprecipitating Rab5a and demonstrating colocalization of both EEA-1 and PI3K at 1 min that increased at 3 min (Fig. 3B) and by immunoprecipitating EEA-1 and finding coprecipitation of Rab5a (Fig. 3C).

Because Rab5a requires dissociation from RabGDI for activity, it was imperative to demonstrate that PAF decreased Rab5a activation (48, 49). In buffer-treated control PMNs, Rab5a and RabGDI evidenced a FRET-positive interaction (Fig. 4A). PAF caused dissociation of this interaction as demonstrated by the loss of the FRET-positive interaction (Fig. 4A). Importantly, these experiments were also done with the introduction of an IgG isotopic control because in the third group of PMNs we neutralized dynamin-2 by intracellular introduction of an Ab directed against dynamin-2. Intracellular delivery of specific Abs to dynamin-2 abrogated the dissociation of RabGDI from Rab5a, as demonstrated by the FRET-positive interaction between Rab5a and RabGDI (Fig. 4A). This microscopy was reinforced by immunoprecipitations of Rab5a and probing for EEA-1 and RabGDI, which demonstrated that PAF caused a decrease colocalization of Rab5a with RabGDI and an increased coprecipitation with EEA-1, as compared with buffer-treated controls (Fig. 4B).

Previous studies have demonstrated that a potential role for MAPks is the activation of Rab5a via phosphorylation of RabGDI, which facilitates the release of Rab5a (49–53). To determine whether this was true of PMNs in response to PAF, the relationship between Rab5a and the MKK3/p38 MAPK signaling cassette was
first investigated. The role of the p38 MAPK signaling cassette in the activation of Rab5a was then investigated. In lysates immunoprecipitated for p38 MAPK from buffer-treated PMNs, there was coprecipitation of ASK1, a MAP kinase kinase kinase (MAP3K), which did not increase upon PAF stimulation (Fig. 5A). Upon PAF stimulation (3 min), MKK3 coprecipitated with p38 MAPK and Rab5a (Fig. 5B), implying that assembly of the p38 MAPK signalosome occurs on the endosome in the proximity of Rab5a and becomes activated by the recruitment of MKK3. Moreover, pretreatment of PMNs with 1–10 μM PD98059, a selective p42/44 MAPK inhibitor, did not affect PAF priming of the PMA respiratory burst (4.0 ± 0.7 to 8.2 ± 4.1% inhibition), whereas using a selective p38 MAPK inhibitor SB203580 (1 μM) significantly inhibited PAF priming of the PMA-activated respiratory burst by 74 ± 6% as compared with DMSO-treated control PMNs activated with 200 ng/ml PMA (p < 0.05, n = 7). Furthermore, p42/44 MAPK did not localize with RabGDI (data not shown).

PAF also elicited the following events: 1) a FRET-positive association of RabGDI with phosphorylated (diphosphorylated: Y138, T142) p38 MAPK; 2) a FRET-positive interaction of RabGDI and PS; and 3) whether one overlays the cellular immunoreactivity of phosphorylated RabGDI and activated p38 MAPK, there are obvious areas of strong (red pseudocolor) colocalization between the phosphorylated RabGDI and activated p38 MAPK (Fig. 6A). These data directly implicate p38 MAPK in the S-phosphorylation of RabGDI by a FRET-positive interaction of active p38 MAPK and RabGDI leading to activation of Rab5a. Lastly, because a number of reports have documented that EEA-1 is phosphorylated by MAPKs, the phosphorylation of EEA-1 by p38 MAPK was also investigated.
investigated. Fig. 6B shows that PAF caused a FRET-positive interaction between active p38 MAPK and EEA-1 compared with buffer-treated controls. In these same PMNs there is an increase in PS, compared with control PMNs, which demonstrates a FRET-positive interaction with EEA-1 (Fig. 6B). If one then overlays these two FRETs (activated p38 MAPK/EEA-1 and EEA-1/PS) and displays the cellular colocalization in pseudocolor, it appears that these events are occurring in the same cellular location (Fig. 6B). However, one may not expect an interaction of p38 MAPK with PS because the kinase has already phosphorylated the substrate (EEA-1) and has dissociated from it. Thus, these data implicate p38 MAPK in the phosphorylation of EEA-1 based on a FRET-positive interaction of activated p38 MAPK and EEA-1, which demonstrates that these proteins were within 5–8 nm. This physical association of an active S/T kinase with its substrate provides provocative evidence that p38 MAPK is responsible for the observed S-phosphorylation of EEA-1.
Because p38 MAPK has a prominent role in the formation of actin bundles in the plasma membrane at sites for endosomal scission, it was necessary to ensure the spatial locality of activated p38 MAPK, which colocalizes with β-arrestin-1 at 1 min, and the activated p38 MAPK, which colocalizes with EEA-1 in the endosome at 3 min (4). Small immunogold particles (5 nm) were bound to Abs specific for active, diphosphorylated p38 MAPK, and large immunogold particles (25 nm) were linked to either Abs specific for active, diphosphorylated p38 MAPK, which colocalizes with EEA-1 in the endosome (54–57). As demonstrated colocalization of p40phox and Rab5a at 3 min. In resting PMNs, the p40phox–p67phox complex is present but does not colocalize (Fig. 8B). In buffer-treated PMNs, minimally activated p38 MAPK was visualized with scattered immunoreactivity for either β-arrestin-1 or Rab5a (Fig. 7A and C). In contrast, at 1 min, activated p38 MAPK (small immunogold particle, 5 nm) was present near the membrane and colocalized with β-arrestin-1 (large immunogold particle, 25 nm), and in this case two active p38 MAPKs are seen with two β-arrestin-1 molecules (Fig. 7B). At 3 min, activated p38 MAPK (small particle) in the cytosol colocalized with Rab5a (large particle) in an endosome, and, as illustrated, there are two large immunogold particles with one small particle (Fig. 7D). These data provide qualitative evidence that PAF induces sequential activation of p38 MAPK, which occurs on the β-arrestin-1 scaffold at or near the membrane at 1 min and in the cytosol localized with Rab5a at 3 min.

**Recruitment of the p40phox–p67phox complex to the early endosome**

The cytosolic oxidase components p40phox and p67phox are present as a complex in the cytosol (54–57). As demonstrated in Fig. 8A, the FRET-positive interaction between p40phox and p67phox remained unaltered with PAF priming; however, PAF priming does appear to modestly change the cellular location of this complex as compared with the buffer-treated controls. Importantly, p67phox does not contain a lipid-binding domain, and because p40phox does contain this lipid-binding domain, it has the capacity to serve as a chaperone for p67phox to allow this p40phox–p67phox complex to bind to phosphatidylinositol 3,4,5-triphosphate (PIP3) domains in the membrane, or like structures, for translocation and binding to the plasma membrane (58–61). In comparison to buffer-treated controls, PAF caused increased colocalization of p40phox and p67phox with EEA-1 (Fig. 8B) as determined by immunoprecipitation of EEA-1 and probing for p40phox and p67phox (Fig. 8B). Additionally, PAF (3 min) also elicited a FRET-positive colocalization of p67phox with Rab5a, which was blocked with intracellular neutralization of dynamin-2 (FRET efficiency 39%, the fluorophores are ~7 nm apart, and the IgG isotype had no effect on this PAF-induced FRET-positive colocalization). D, To confirm these results, Rab5a was immunoprecipitated and PAF (3 min) caused coprecipitation of Rab5a with p67phox. These data are representative of three to four independent experiments that yielded identical results.
epitope would be “hidden” and there would be no immunoreactivity. As demonstrated previously, control PMNs demonstrated a FRET-positive interaction between the p40\textsuperscript{phox}-p67\textsuperscript{phox} complex, which does not colocalize with EEA-1 (Fig. 9A). At 3 min of PAF stimulation, three important protein interactions occur: 1) the p40\textsuperscript{phox}-p67\textsuperscript{phox} FRET disappears, 2) the total cellular immunoreactivity of p40\textsuperscript{phox} decreases dramatically, and 3) p67\textsuperscript{phox} displays colocalization with EEA-1 (Fig. 9B). Such findings may be explained by whether most p40\textsuperscript{phox} becomes lipid bound to the endosome via PIP3 residues, which could be available on the FYVE domain of EEA-1, or to other PIP3 sites (14, 46). If this supposition is true, then PI3K inhibition would 1) return the FRET-positive interaction of the p40\textsuperscript{phox}-p67\textsuperscript{phox} seen in control PMNs, 2) increase total cellular p40\textsuperscript{phox} immunoreactivity because the PX domain is now free to bind the Ab, and 3) decrease the colocalization of EEA-1 and p67\textsuperscript{phox}. As shown in Fig. 9C, these described events all occurred with PI3K inhibition. Importantly, no other oxidase components were associated with either EEA-1 or Rab5a including p47\textsuperscript{phox}, Rac2, or gp91\textsuperscript{phox} (data not shown), confirming that PAF caused recruitment of substrates to the endosome, namely the p40\textsuperscript{phox}-p67\textsuperscript{phox} complex, and not oxidase assembly.

Because phosphorylation of oxidase components has been reported as a necessary prerequisite for translocation and also appears to be important in priming of the NADPH oxidase, PAF-induced phosphorylation of p40\textsuperscript{phox} and p67\textsuperscript{phox} was investigated (19, 23, 62–64). At 3 min, PAF markedly enhanced S-phosphorylation of p40\textsuperscript{phox}, as one would expect if PI3K were involved with this S-phosphorylation. Because PI3K phosphorylates lipids, the role of Akt1 was investigated. C and D, PAF (3 min) caused the coprecipitation of Akt1 with both p40\textsuperscript{phox} (C) and p67\textsuperscript{phox} (D) as compared with buffer-treated controls.
also showed coprecipitation with Akt1 (Fig. 10, C and D). Additionally, immunoprecipitation of Akt1 also demonstrated coprecipitation of both p40\textsuperscript{phox} but not p47\textsuperscript{phox}. Thus, PI3K appears to recruit Akt1 for phosphorylation of the p40\textsuperscript{phox} and p67\textsuperscript{phox} and consequently the FRET-positive interaction of PI3K with the nonphosphorylated p40\textsuperscript{phox} and p67\textsuperscript{phox} and a decrease in the FRET-positive interaction with these phox proteins after S-phosphorylation (Fig. 10B). The latter decrease in the FRET-positive interaction between the cytosolic oxidase components and PI3K is to be expected because if PI3K is necessary for the phosphorylation of p40\textsuperscript{phox}, then phosphorylation of the physical relationship between p40\textsuperscript{phox} and PI3K should diminish as demonstrated (Fig. 10B).

Translocation of p40\textsuperscript{phox}-p67\textsuperscript{phox} and PI3K to the membrane

Previous data have demonstrated that PAF causes translocation of p67\textsuperscript{phox} to the plasma membrane without concomitant translocation of p47\textsuperscript{phox} (14, 54, 65, 68). The amount of p67\textsuperscript{phox} is sufficient to run the NADPH oxidase in an SDS cell-free system using both membranes for PAF-primed PMNs with cytosol that was p67\textsuperscript{phox} depleted (14, 54, 65, 68). Such p67\textsuperscript{phox} translocation alone appears counterintuitive due to the inability of p67\textsuperscript{phox} to bind lipids and to a relative dearth of data with regard to p67\textsuperscript{phox} membrane translocation without p47\textsuperscript{phox} (14, 54, 65, 68). Therefore, PAF-mediated translocation of the p40\textsuperscript{phox}-p67\textsuperscript{phox} complex was investigated. At 3 min, PAF caused the translocation of this complex to the membrane, compared with buffer-treated controls, and this complex colocalized with the gp91\textsuperscript{phox} membrane oxidase component, which also exhibited a FRET-positive relationship with PI3K at the plasma membrane (data not shown) (68). Additionally, because the small GTPase Rac2 has been associated with the translocation of p47\textsuperscript{phox} to the membrane with priming and activation of the oxidase, PAF-mediated translocation of Rac2 was also investigated. PAF did not cause membrane translocation of Rac2 with the p47\textsuperscript{phox}-p67\textsuperscript{phox} complex (results not shown, n = 5) (32, 60, 69). Lastly, as stated above, p67\textsuperscript{phox} translocation is dependent on p47\textsuperscript{phox} translocation, as demonstrated in the PMNs from patients with chronic granulomatous disease who lack p47\textsuperscript{phox} (70). The roles of p40\textsuperscript{phox} and p47\textsuperscript{phox} were investigated in PAF-mediated translocation of p67\textsuperscript{phox} in intact PMNs using intracellular immunodepletion of p47\textsuperscript{phox} and p47\textsuperscript{phox} with two disparate neutralizing Abs for each phox protein and comparing these PMNs to controls loaded with isotypic IgG. PAF (3 min) caused the translocation of p67\textsuperscript{phox}, which was inhibited by intracellular neutralization of p47\textsuperscript{phox} but was not affected by identical intracellular neutralization of p40\textsuperscript{phox}.

Discussion

The data presented demonstrated that PAF ligation of its receptor on PMNs resulted in dynamin-2 activation, as shown by tyrosine phosphorylation, colocalization with Grb2, and endosome formation (Fig. 12). Assembly of the endosome consisted of PI3K, Rab5a, and EEA-1, and activation of Rab5a occurred through priming and activation of the RabGDI, resulting in dissociation from Rab5a. The p38 MAPK phosphatase-anchored scaffold (4) (Fig. 12). The tethering protein EEA-1 served as a scaffold, which first required PI3K phosphorylation to change the phosphatidylinositol 4,5-bisphosphate (PIP2) sites to PIP3, required for binding of the p40\textsuperscript{phox}-p67\textsuperscript{phox} complex through the PX domain on p40\textsuperscript{phox} (Fig. 12). Following this tethering to EEA-1, these cytosolic phox proteins were most likely phosphorylated by Akt1, as demonstrated by coprecipitation of the p40\textsuperscript{phox} and p67\textsuperscript{phox} with Akt1, which is a common finding among priming agents (18, 19, 23). The p40\textsuperscript{phox}-p67\textsuperscript{phox} complex subsequently translocated to the plasma membrane and localized to gp91\textsuperscript{phox} along with PI3K and did not require p47\textsuperscript{phox} or Rac2 translocation.

Dynamin activation as a function of Y-phosphorylation and its colocalization with Grb2 is congruent with previous reports (1, 44, 45, 71). Furthermore, inhibition of dynamin disrupted endosome formation as demonstrated by the loss of Rab5a activity, the
FRET-positive colocalization of PI3K with Rab5a, and the FRET-positive interaction between Rab5a and p67phox (1, 44, 45, 71). Additionally, the colocalization of Rab5a and EEA-1 in the endosome and PI3K-dependent activation of Akt1 also confirm prior reports in transformed cells or cells that have been manipulated to specifically study the interactions of these specific signaling proteins that are integral to CME (46, 47, 72–76). The Rab GTPases, especially Rab5a, are mediators of intracellular trafficking, control the fusion of vesicles and/or lysosomes with intracellular organelles, and play important roles in CME, and their mutation results in human disease (17, 47, 72–80). Moreover, Rab5a is required for CME of activated receptors and is regulated by binding of RabGDI in the cytosol (17, 47, 49, 53, 72, 75, 76, 78–80). Previous data have demonstrated that activation of Rab5a is controlled by the p42/44 MAPKs that phosphorylate the RabGDI and cause its dissociation (49–53). Contrary to these data, PAF caused a likely p38 MAPK-dependent activation of Rab5a by S-phosphorylation of RabGDI and its subsequent dissociation from Rab5a. This conclusion is drawn from an overlay of the cellular immunoreactivity of active p38 MAPK and the FRET-positive physical interaction of RabGDI with phosphoserine demonstrating a high degree of identical cellular locale. However PAF-induced S-phosphorylation of RabGDI is based on: 1) the physical interactions between proteins, RabGDI/active p38 MAPK, and RabGDI/PS; and 2) the locality of active p38 MAPK with the FRET between RabGDI and PS. Importantly, RabGDI has been reported to be a MAPK substrate, congruent with these observations (49–53). This p38 MAPK phosphorylation is novel in GPCR signaling in primary cells and confirms the in vivo data from peroxide or UV-stimulated PMNs that suggested a role for p38 MAPK in Rab5a activation (50). The assembly of the ASK1/MKK3/p38 MAPK signalosome on the endosome, associated with Rab5a, is also novel in CME, as is the PAF-induced p38 MAPK activation in two cellular locales at disparate times: 1) at the plasma membrane on the β-arrestin-1 scaffold at 1 min (4), and 2) in the cytosol on the endosome at 3 min as delineated by electron microscopy. Activation of p38 MAPK on both the β-arrestin-1 scaffold and on the endosome associated with Rab5a occurred through the recruitment of MKK3 into the signalosome, as in resting PMNs, both ASK1 and p38 MAPK colocalized with β-arrestin-1 and Rab5a, respectively (4). In our initial experiments, PAK1 was suspected to be the MAP3K in the p38 MAPK signalosome, but we were unable to colocalize PAK1 to either the β-arrestin-1 scaffold or to Rab5a (4). For PAF priming of the PMN NADPH oxidase, inhibition of p42/44 MAPK with PD98059 had little effect on the PAF-induced changes in PMN physiology, as others have demonstrated (81, 82). Conversely, p38 MAPK inhibitors did inhibit the PAF priming of the PMA-activated respiratory burst; however, one must interpret these data with caution, as p38 MAPK is required for actin bundle formation at the site of PAFR CME, and thus one may not be inhibiting the activation of Rab5a, which is downstream of these initial signaling events (4). PAF elicited the tethering of the p40phox-p67phox complex to the endosome, as shown by its colocalization with both EEA-1 and Rab5a. This colocalization occurs through binding of the p40phox PX domain, because it required PI3K activity and the lipid-binding
PK domain of the p40phox as based on Ab inhibition of this PK domain and P13K and the lack of a lipid-binding PK domain on p67phox. These data implicate an interaction between the PK domain of p40phox and PIP3 residues in the endosome, which are rich in PIP2 domains (83, 84). Upon specific receptor activation, these PIP2 domains may be phosphorylated by P13K and then may play a role in the regulation of endocytic membrane trafficking, signal transduction, and cytoskeletal reorganization by interacting with the PK domains of the phox proteins and other effectors, including EE1-A, through the phox homology region in its FYVE domain, and the sorting nexins (84–88). Moreover, the recruitment of the p40phox-p67phox complex to EE1-A allowed for their S-phosphorylation by Akt1 in a P13K-dependent manner. These data also extend the relevance of Akt1 from phosphorylation of p47phox to its ability to phosphorylate the p40phox-p67phox complex, which then resulted in the membrane translocation during PAF priming of the NADPH oxidase, both of which were abrogated by intracellular neutralization of the catalytic domain of P13K (89, 90). Additionally, no other oxidase components were recruited to the endosome; therefore, PAF-mediated enlistment of the p40phox-p67phox complex to the EE1-A sort protein is indicative of substrate recruitment rather than assembly or partial assembly of the NADPH oxidase.

Several priming agents, including endotoxin and IL-1β, cause the translocation of p47phox to the plasma membrane (18, 23). In contrast, PAF elicited translocation of the p40phox-p67phox complex to the PMN membrane in the region of gp91phox. Additionally, P13K-dependent formation of PIP3 domains on EE1-A was required for phosphorylation of the p40phox-p67phox complex, a prerequisite for translocation, with binding of the PK domain of p40phox to the FYVE domain of EE1-A. One may expect a similar regulatory role for p40phox with membrane translocation because p67phox does not contain the PK domain; however, more work is required to test this hypothesis (56, 58, 91). Furthermore, the amount of the p40phox-p67phox complex translocated to the plasma membrane was not trivial based upon the ability of PAF-primed membranes to provoke oxidase activity when combined with membrane was not trivial based upon the ability of PAF-primed membranes to provoke oxidase activity when combined with

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