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Platelet-Activating Factor-Mediated Endosome Formation Causes Membrane Translocation of p67\textsuperscript{phox} and p40\textsuperscript{phox} That Requires Recruitment and Activation of p38 MAPK, Rab5a, and Phosphatidylinositol 3-Kinase in Human Neutrophils\textsuperscript{1}

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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 O$_2^-$ was measured, whole-cell lysates and subcellular fractions were prepared, or the PMNs were fixed onto slides for digital or electron microscopy. PAF caused activation of dynamin-2, resulting in endosomal formation that required PI3K and contained early endosomal Ag-1 (EEA-1) and Rab5a. The apoptosis signal-regulating kinase-1/MAPK kinase-3/p38 MAPK signalosome assembled on Rab5a and phosphorylated EEA-1 and Rab GDP dissociation inhibitor, with the latter causing Rab5a activation. Electron microscopy demonstrated that PAF caused two distinct sites for activation of p38 MAPK. EEA-1 provided a scaffold for recruitment of the p40\textsuperscript{phox}-p67\textsuperscript{phox} complex and PI3K-dependent Akt1 phosphorylation of these two phox proteins. PAF induced membrane translocation of p40\textsuperscript{phox}-p67\textsuperscript{phox} localizing to gp91\textsuperscript{phox}, which was PI3K-, but not p47\textsuperscript{phox}-, dependent. In conclusion, PAF transduces signals through CME, and such GPCR signaling may allow for pharmacological manipulation of these cells to decrease PMN-mediated acute organ injury. \textit{The Journal of Immunology, 2008, 180: 8192–8203.}

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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 morphology, and augmentation of the oxidative response to a given stimulus through translocation of specific cytosolic oxidase components to the plasma membrane (18–23). Platelet activating factor (PAF) is a prototypic lipid chemooattractant that binds to a specific seven-transmembrane domain receptor (PAFR) (24). PAF occupation of this receptor results in release of the Goq and Gs heterotrimeric 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 occupation of many GPCRs initiates signal transduction through recruitment of the cytosolic 67-kDa phagocyte oxidase protein (p67phox) from the activated receptor (1, 27). Previous work has demonstrated that activation of the PAFR induces CME, recruits a β-arrestin-1 scaffold for assembly of the p38 MAPK signalosome, and activation of p38 MAPK causes the formation of actin bundles at the PAFR, all before activation of dynamin-2 at the PMN membrane (4, 28). We hypothesize that PAF ligation of the PAFR induces dynamin-2 scission of the endosome from the plasma membrane 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 protein (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 endothelin-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. Nicktulose paper, x-ray film, and Nunc 96-well plates were purchased from Life Science Products. Abs to the PAFR, dynamin-2, ASK1, MAPK kinase-3 (MKK3), Rab5a, and Rab GDP dissociation inhibitor (RabGDI), EEA-1, and the p38 MAPK signalosome to 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.

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 fluorophore vastly outnumbered the acceptor, 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 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 fluorophore vastly outnumbered the acceptor, 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:
“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-arrestin-1 and dynamin-2 were 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 × 10³) 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 Ab (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% cationic buffered glutaraldehyde for 60 min. The PMNs were silver enhanced for 60 min at room temperature and then washed with deionized water (Nanopure filtered) (42). Finally, the PMNs were again washed with 2% cationic buffered osmium tetroxide 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 FRET², 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 previously. Statistical significance among groups was calculated by paired visualization the FRETs are represented in color (a.u.f.i.) as described.

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 isotypic control IgG (Fig. 2A). 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 reinforced 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 caused 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 isotypic 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
The role of the p38 MAPK signaling cassette in the activation of Rab5a was then investigated. In lysates immuno-precipitated 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, pre-treatment of PMNs with 1–10 μM PD98059, a selective p42/44 MAPK inhibitor, did not affect PAF priming of the PMN 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 activated (diphosphorylated: Y138, T142) p38 MAPK; 2) a FRET-positive interaction of RabGDI and PS; and 3) whether one overlays the cellular immunoreactivity of activated p38 MAPK and the FRET of RabGDI and PS, 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 first 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.

FIGURE 4. PAF causes activation of Rab5a. A, In buffer-treated PMNs the RabGDI and Rab5a are physically attached to one another, as demonstrated by a FRET-positive interaction with an efficiency of 58% (<5 nm between fluorophores), which was not affected by loading the PMNs with isotypic IgG controls (upper panels). PAF caused dissociation of Rab5a and RabGDI as demonstrated by the loss of the FRET-positive interaction between these two proteins (middle panels). Intracellular neutralization of dynamin-2 with specific Abs inhibited the PAF-induced dissociation of RabGDI from Rab5a, indicating that it was downstream of dynamin-2 activation (bottom panels). B, These data were confirmed by immunoprecipitation of Rab5a, which demonstrated cophosphorylation of Rab5a with RabGDI in buffer-treated controls, which was reduced with PAF priming. Moreover, PAF also elicited cophosphorylation with EEA-1, which was not present in the control PMNs.

FIGURE 5. PAF priming induces assembly of the ASK1/MKK3/p38 MAPK signalosome. A, Immunoprecipitations of p38 MAPK from buffer-treated or PAF primed PMNs documented that ASK1, a MAP kinase kinase kinase, coprecipitates with p38 MAPK. B, However, as compared with buffer-treated controls, PAF priming causes the recruitment of MKK3, as denoted by the cophosphorylation of MKK3 with both p38 MAPK and Rab5a. The blots shown are representative of two and three independent experiments with identical results using different blood donors.

FIGURE 6. PAF-induced p38 MAPK activation resulting in phosphorylation of RabGDI and EEA-1. A, PAF caused activation (dual phosphorylation: Y138/T142) of p38 MAPK, which demonstrated a FRET-positive colocalization with RabGDI as compared with buffer-treated controls (FRET efficiency 43%, fluorophores <6 nm apart). Additionally, PAF caused a FRET-positive interaction between Abs to PS and RabGDI (FRET efficiency of 49%, indicating that the fluorophores are <5 nm apart), indicating that RabGDI was phosphorylated on serine residues. As an internal control, the overlay of the cellular immunoreactivity of activated p38 MAPK and PS did not demonstrate much interaction, presumably because once the GDI is phosphorylated it rapidly dissociates. However, if one overlays the FRET between RabGDI and PS with the distribution of activated p38 MAPK, one sees that they do show colocalization, which provides evidence that the kinase involved in S-phosphorylation of RabGDI is p38 MAPK. B, PAF elicited a FRET-positive interaction between activated p38 MAPK and EEA-1 and also caused a FRET-positive colocalization between EEA-1 and PS as compared with buffer-treated PMNs. Overlays of these two FRETs (activated p38 MAPK/EEA-1 and EEA-1/PS) demonstrated that activated p38 MAPK, EEA-1, and PS all colocalized to the same general area and provide evidence that PAF causes S-phosphorylation of EEA-1 through activation of p38 MAPK. This figure is a representative digital image of three independent experiments that yielded identical results.
The cytosolic oxidase components p40phox and p67phox were linked to either Abs specific for active, diphosphorylated p38 MAPK, and large immunogold particles (5 nm) were bound to activated p38 MAPK, which colocalizes with EEA-1 in the endosome (Fig. 8), and to Rab5a. In resting PMNs, the p40phox-p67phox complex increased with PAF priming and reached a relative maximum at 3 min. C, Additionally, compared with buffer-treated controls, PAF elicited a FRET-positive interaction of the 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 the p40phox-p67phox complex; however, the cellular distribution appeared different. B, Immunoprecipitation of EEA-1 demonstrated colocalization of p40phox and p67phox that increased with PAF priming and reached a relative maximum at 3 min. 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.

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 ≥ p47phox (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 the 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.

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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 the 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 (Fig. 8C); moreover, PAF induced a coprecipitation of Rab5a with p67phox, which was not present in buffer-treated controls (Fig. 8D), and this further reinforces the data that implicated PAF-induced recruitment of the p40phox-p67phox complex to both EEA-1 and Rab5a, which are constituents of the endosome. Thus, PAF appears to cause localization of the p40phox-p67phox complex to the endosome; however, one must consider how this complex attaches to the endosome and why. To gain insight into this question we used an Ab against p40phox that spanned the PX (lipid binding) domain; therefore, if the p40phox PX domain is already bound to PIP3 residues, the
epitope would be “hidden” and there would be no immunoreactivity. As demonstrated previously, control PMNs demonstrated a FRET-positive interaction between the p40<sup>phox</sup>-p67<sup>phox</sup> complex, which does not colocalize with EEA-1 (Fig. 9A). At 3 min of PAF stimulation, three important protein interactions occur: 1) the p40<sup>phox</sup>-p67<sup>phox</sup>-FRET disappears, 2) the total cellular immunoreactivity of p40<sup>phox</sup> decreases dramatically, and 3) p67<sup>phox</sup> displays colocalization with EEA-1 (Fig. 9B). Such findings may be explained by whether most p40<sup>phox</sup> 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 between PI3K and p40<sup>phox</sup>, which was diminished with S-phosphorylated p40<sup>phox</sup>, 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<sup>phox</sup> (C) and p67<sup>phox</sup> (D) as compared with buffer-treated controls.

![Figure 9](http://www.jimmunol.org/)

**FIGURE 9.** PAF-mediated colocalization of the p40<sup>phox</sup>-p67<sup>phox</sup> complex required PI3K and occurred through binding of p40<sup>phox</sup> to EEA-1. A, Buffer-treated control demonstrated a FRET-positive interaction between the p40<sup>phox</sup>-p67<sup>phox</sup>, which had minimal colocalization with EEA-1. B, PAF caused the loss of a FRET-positive interaction between the p40<sup>phox</sup>-p67<sup>phox</sup> complex and EEA-1 but obvious colocalization of EEA-1 and p67<sup>phox</sup>. C, These results may be explained because the Ab used for p40<sup>phox</sup> spanned the PX domain, and if this domain (epitope) became bound (hidden) to EEA-1, there would be a concomitant loss of p40<sup>phox</sup> immunoreactivity without affecting the colocalization between EEA-1 and p67<sup>phox</sup>. By inhibiting PI3K through intracellular neutralization, there is an increase in p40<sup>phox</sup> immunoreactivity, which demonstrates a FRET-positive interaction with p67<sup>phox</sup>, similar to the control state, and there is a loss of the colocalization of p67<sup>phox</sup> with EEA-1. These data provide supportive evidence that PAF elicited an interaction between the p40<sup>phox</sup>-p67<sup>phox</sup> complex and EEA-1 through the PX domain of p40<sup>phox</sup>.
also showed coprecipitation with Akt1 (Fig. 10, C and D). Additionally, immunoprecipitation of Akt1 also demonstrated coprecipitation of both p40phox and p67phox (data not shown). Thus, PI3K appears to recruit Akt1 for phosphorylation of the p40phox and p67phox, and consequently the FRET-positive interaction of PI3K with the nonphosphorylated p40phox and p67phox, 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 p40phox, then following phosphorylation the physical relationship between p40phox and PI3K should diminish as demonstrated (Fig. 10B).

Translocation of p40phox-p67phox and PI3K to the membrane

Previous data have demonstrated that PAF causes translocation of p67phox to the plasma membrane without concomitant translocation of p47phox (14, 54, 65, 68). The amount of p67phox is sufficient to run the NADPH oxidase in an SDS cell-free system using both membranes for PAF-primed PMNs with cytosol that was p67phox-depleted (14, 54, 65, 68). Such p67phox translocation alone appears counterintuitive due to the inability of p67phox to bind lipids and to a relative dearth of data with regard to p67phox membrane translocation without p47phox (14, 54, 65, 68). Therefore, PAF-mediated translocation of the p40phox-p67phox 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 gp91phox 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 p47phox 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 p40phox-p67phox complex (results not shown, n = 5) (32, 60, 69). Lastly, as stated above, p67phox translocation is dependent on p47phox translocation, as demonstrated in the PMNs from patients with chronic granulomatous disease who lack p47phox (70). The roles of p40phox and p47phox were investigated in PAF-mediated translocation of p67phox in intact PMNs using intracellular immunodepletion of p47phox and p49phox 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 p67phox, which was inhibited by intracellular neutralization of p40phox but was not affected by identical intracellular neutralization of p47phox, when the colocalization of p67phox and the membrane counterstain wheat germ agglutinin were analyzed (Figs. 11). Additionally, intracellular neutralization of p40phox inhibited PAF priming of the PMA-activated respiratory burst by 74 ± 9% as compared with PAF priming of the PMA-activated respiratory burst in PMNs loaded with isotypic IgG controls (n = 5, p < 0.05). Conversely, intracellular neutralization of the p47phox only inhibited PAF priming by 21 ± 2% (n = 5), which was not different from the IgG-loaded control PMNs (data not shown).

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 EE1, and activation of Rab5a occurred through the p38 MAPK signalosome assembled on the endosome, as documented by its colocalization with Rab5a, RabGDI, resulting in dissociation from Rab5a. The p38 MAPK signalosome assembled in intact PMNs using intracellular immunodepletion of p47phox and p49phox (results not shown, n = 5), then following phosphorylation of the RabGDI, resulting in Rab5a dissociation from Rab5a. The p38 MAPK signalosome consisted of PI3K, Rab5a, and EE1, and activation of Rab5a occurred through the p38 MAPK signalosome assembled on the endosome, as documented by its colocalization with Rab5a, RabGDI, resulting in dissociation from Rab5a. The p38 MAPK signalosome assembled on the endosome, as documented by its colocalization with Rab5a, RabGDI, resulting in dissociation from Rab5a. The p38 MAPK signalosome assembled on the endosome, as documented by its colocalization with Rab5a, RabGDI, resulting in dissociation from Rab5a. The p38 MAPK signalosome assembled on the endosome, as documented by its colocalization with Rab5a, RabGDI, resulting in dissociation from Rab5a. The p38 MAPK signalosome assembled on the endosome, as documented by its colocalization with Rab5a, RabGDI, resulting in dissociation from Rab5a.
FIGURE 12. A representation of PAF-mediated signaling events beginning with the activation of dynamin-2. A, PAF causes dynamin-2 activation as determined by Y-phosphorylation and the recruitment of Grb2. B and C, Dynamin-2 scission (B) of the early endosome (shears) recruits PI3K, Rab5α, RabGDI complex, and EEA-1 (C). D, The ASK1/MKK3/p38 MAPK signalosome then assembles on Rabβ5 and S-phosphorylates both RabGDI, to allow for activation of Rab5α, and EEA-1. E, Rab5α recruits PI3K and the p40^phox-p67^phox complex, and both of these p40^phox proteins are S-phosphorylated by Akt1. F, Following S-phosphorylation, the p40^phox-p67^phox complex translocates to the plasma membrane to the area of the membrane-associated gp91^phox, with concomitant recruitment of PI3K to aid in the membrane binding of p40^phox-p67^phox, most likely through the conversion of membrane PIP2 residues to PIP3 by PI3K gp91^phox. It is important to note that the oxidase complex is not fully assembled and that the partial assembly of the p40^phox-p67^phox complex at the site of the membrane-bound gp91^phox represents the PAF-mediated reorganization of the NADPH oxidase important for PAF priming of the respiratory burst.

FRET-positive colocalization of PI3K with Rab5α, and the FRET-positive interaction between Rab5α and p67^phox (1, 44, 45, 71). Additionally, the colocalization of Rab5α 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 GTases, especially Rab5α, 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, 44, 72, 75–80). Moreover, Rab5α is required for CME of activated receptors and is regulated by binding of RabGDI in the cytosol (17, 44, 49, 53, 72, 75, 76, 78–80). Previous data have demonstrated that activation of Rab5α 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 Rab5α by S-phosphorylation of RabGDI and its subsequent dissociation from Rab5α. 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 Rab5α activation (50). The assembly of the ASK1/MKK3/p38 MAPK signalosome on the endosome, associated with Rab5α, 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 Rab5α occurred through the recruitment of MKK3 into the signalosome, as in resting PMNs, both ASK1 and p38 MAPK colocalized with β-arrestin-1 and Rab5α, 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 Rab5α (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 Rab5α, which is downstream of these initial signaling events (4).

PAF elicited the tethering of the p40^phox-p67^phox complex to the endosome, as shown by its colocalization with both EEA-1 and Rab5α. This colocalization occurs through binding of the p40^phox PX domain, because it required PI3K activity and the lipid-binding
PX domain of the p40phox as based on Ab inhibition of this PX domain and PI3K and the lack of a lipid-binding PX domain on p67phox. These data implicate an interaction between the PX domain of p40phox and PI3P residues in the endosome, which are rich in PI(3)P domains (83, 84). Upon specific receptor activation, these PI(3)P domains may be phosphorylated by PI3K and then may play a role in the regulation of endocytic membrane trafficking, signal transduction, and cytoskeletal reorganization by interacting with the PX domains of the phox proteins and other effectors, including EEA-1, through the phox homology region in its FYVE domain, and the sorting nexins (84–88). Moreover, the recruitment of the EEA-1, through the ability to phosphorylate the p40phox, may not require the small G-protein-coupled receptor activation of the NADPH oxidase. Additionally, no other oxidase components were recruited to the endosome; therefore, PI(3)P-mediated enlistment of the p40phox-p67phox complex to the EEA-1 sortin protein is indicative of substrate recruit 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, PI3K-dependent formation of PI(3)P domains on EEA-1 was required for phosphorylation of 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 PI3K (89, 90). Additionally, no other oxidase components were recruited to the endosome; therefore, PAF-mediated enlistment of the p40phox-p67phox complex to the EEA-1 sortin protein is indicative of substrate recruit rather than assembly or partial assembly of the NADPH oxidase.

References


