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Sequential Binding of Cytosolic Phox Complex to Phagosomes through Regulated Adaptor Proteins: Evaluation Using the Novel Monomeric Kusabira-Green System and Live Imaging of Phagocytosis¹

Takehiko Ueyama,* Tomoko Kusakabe,* Satoshi Karasawa,†‡§ Takumi Kawasaki,* Aya Shimizu,* Jeonghyun Son,* Thomas L. Leto,¶ Atsushi Miyawaki, † and Naoaki Saito²*

We engineered a method for detecting intramolecular and intermolecular phox protein interactions in cells by fluorescence microscopy using fusion proteins of complementary fragments of a coral fluorescent reporter protein (monomeric Kusabira-Green). We confirmed the efficacy of the monomeric Kusabira-Green system by showing that the PX and PBI domains of p40phox interact in intact cells, which we suggest maintains this protein in an inactive closed conformation. Using this system, we also explored intramolecular interactions within p47phox and showed that the PX domain interacts with the autoinhibited tandem Src homology 3 domains maintained in contact with the autoinhibitory region, along with residues 341–360. Furthermore, we demonstrated sequential interactions of p67phox with phagosomes involving adaptor proteins, p40phox and p47phox, during FcγR-mediated phagocytosis. Although p67phox is not targeted to phagosomes by itself, p47phox functions as an adaptor for the ternary complex (p47phox–p67phox–p40phox) in early stages of phagocytosis before phagosome closure, while p40phox functions in later stages after phagosomal closure. Interestingly, a mutated “open” form of p40phox linked p47phox to closed phagosomes and prolonged p47phox and p67phox retention on phagosomes. These results indicate that binding of the ternary complex to phagosomes can be temporally regulated by switching between adaptor proteins that have PX domains with distinct lipid-binding specificities. The Journal of Immunology, 2008, 181: 629–640.

In phagocytic cells, reactive oxygen species (ROS)³ are produced by NADPH oxidase, also known as the Nox2 system. The enzyme is a multiprotein complex assembled from the membrane-spanning flavocytochrome b₅₅₈ (composed of p91phox (Nox2) and p22phox) and four cytosolic components (p47phox, p67phox, p40phox, and Rac) (1, 2). In unstimulated phagocytes, the oxidase is dissociated and inactive: the flavocytochrome b₅₅₈ is stored on the membranes of intracellular granules (3, 4), in particular, secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles (5); Rac is maintained in a GDP-bound cytosolic complex dimerized with Rho-GDI (6); and the other phox proteins associate in a separate ternary cytoplasmic complex (p47phox–p67phox–p40phox) (7) in a dephosphorylated state (8–10). During phagocyte activation, intracellular granules containing flavocytochrome b₅₅₈ fuse to newly forming phagosomes, and the ternary cytoplasmic complex and Rac bind to the membrane by independent mechanisms: p47phox is phosphorylated, thereby inducing conformational changes in p47phox that promote the interaction of the ternary cytoplasmic complex with the flavocytochrome b₅₅₈. We recently showed that p40phox also undergoes conformational changes by disruption of the intramolecular PX-PBI domain interaction to enable the ternary cytoplasmic complex to bind to PI(3)P-enriched membranes (11). Finally, Rac translocates to phagosomes (12, 13) in a GTP-dependent manner (14, 15), resulting in generation of superoxide anion by the transfer of electrons from cytoplasmic NADPH to molecular oxygen.

Chronic granulomatous disease (CGD), characterized by defective microbial killing by phagocytic cells, is caused by defects or deficiencies in any one of four oxidase components: Nox2, p22phox, p47phox, or p67phox. An essential role for Rac in Nox2 activation was also demonstrated in an oxidase-deficient patient who expressed mutated Rac2 (16, 17) and in mice rendered genetically deficient in Rac2 or in Rac1 plus Rac2 (18, 19). However, there have been no reports of p40phox defects or deficiencies resulting in CGD. Rac and p67phox together are minimum essential cytoplasmic components regulating electron flow through the flavocytochrome b₅₅₈ through GTP-dependent interactions (20, 21), hence, p67phox is called an “activator” component. In contrast, p47phox is called an “adaptor” or “organizer” component because it

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²Abbreviations used in this paper: ROS, reactive oxygen species; CGD, chronic granulomatous disease; PA, phosphatidic acid; SH3, Src homology 3; AA, arachidonic acid; mKG, monomeric Kusabira Green; AIR, autoinhibitory region; EEA1, early endosome Ag-1; RT, room temperature; LZ, leucine zipper basic protein; AD, activation domain; IRES, internal ribosomal entry site; PR, proline rich.
binds to membrane lipids (PI(3,4)P₂, phosphatidic acid (PA)) through its PX domain (22), is tethered to the flavocytochrome b₅₆₅ through direct interactions between p22phox and its Src homology 3 (SH3) domain, and linked other cytoplasmic phox proteins to this complex (23, 24). CGD patients who lack p47phox show impaired translocation of p67phox to the particulate or membrane fraction, whereas CGD patients who lack p67phox show normal translocation of p47phox to the particulate fraction (25–27).

Although p40phox was shown to act as an oxidase inhibitory factor in some reconstituted systems (28–31), studies in p40phox-deficient mice (32), in p40phox/58K/65K transgenic mice (33), or in FcγRII-reconstituted cells (34) indicate p40phox functions as an essential positive regulator of the Nox2 system. p40phox also has a PX domain that specifically binds to PI(3)P (35, 36), a phospholipid enriched in phagosomes during phagocytosis (37) and in early endosomes which fuse to phagosomes (38). Thus, p40phox was suggested to serve as an “adaptor” component that recruits p67phox and p47phox to membranes (39). In recent work, we showed that both p47phox and p40phox function as “adaptor” or “carrier” proteins of p67phox using arachidonic acid (AA) as a stimulus in the RAW264.7 model (11).

In this study, we developed a new complementation reporter system that can detect protein-protein interactions at cellular levels under confocal fluorescence microscopy based on fusion proteins of fragments of monomeric coral fluorescent reporter protein (monomeric Kusabira-Green (mKG)). The efficacy and specificity of this system was confirmed when used to detect the interaction of leucine zipper proteins produced as various fusion constructs. We used this system to demonstrate the recently reported PX-PB1 domain intramolecular interactions within p40phox (11) in whole live cells. Furthermore, we examined intramolecular interactions within p47phox with this system, and propose a new model for the autoinhibited state of p47phox in which a structure encompassing the tandem SH3 domains bound to the autoinhibitory region (AIR; Supersh3/AIR), and sequence within residues 341–360 are suggested to interact with its N-terminal PX domain. Finally, we examined the adaptor functions of p47phox and p40phox during FcγR-mediated phagocytosis, in which targeting of p67phox to phagosomes depends on both of these proteins. p47phox functions as an early stage adaptor protein of the ternary cytoplasmic complex, while p40phox functions as a late stage adaptor protein of the complex that links p47phox to closed phagosomes and prolonged retention of p47phox and p67phox on phagosomes.

**Materials and Methods**

**Materials**

Goat polyclonal Ab against human p47phox or p67phox and rabbit polyclonal Ab against human p40phox were described previously (28, 40). Rabbit polyclonal Ab against GST and mouse mAb against His tag were obtained from Santa Cruz Biotechnology and Amersham Biosciences, respectively. Mouse mAb (1E6B5) against the N-terminal fragment of mKG (mKGN) was a gift from Medical & Biological Laboratories. Mouse polyclonal Ab against early endosome Ag-1 (EE1A1) and rabbit polyclonal Ab against EE1A were obtained from BD Biosciences and Affinity BioReagents, respectively.

**Cell culture**

RAW264.7 macrophages (13) were maintained in DMEM (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated FBS (Invitrogen) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO₂. HEK293 cells (American Type Culture Collection) were maintained in EMEM (Wako Pure Chemical Industries) containing 10% heat-inactivated FBS (Invitrogen), 100 μM nonessential amino acids (Invitrogen), and antibiotics at 37°C in 5% CO₂.

**Immunoprecipitation, immunoblotting, immunocytochemistry**

Immunoprecipitation and immunoblotting were performed, as described previously (11). HEK293 cells were transfected using Fugene 6 (Roche). Forty-eight hours after the transfection, cells were lysed in homogenizing buffer. For detection of protein expression, the total cell lysates were subjected to SDS-PAGE and immunoblotting. For detection of protein-protein interaction, the total cell lysates were centrifuged at 800 × g for 5 min at 4°C, the supernatants were incubated with anti-p67phox Ab for 2 h at 4°C, and then with protein G-Sepharose 4B (Amersham Biosciences) for an additional 12 h at 4°C. The precipitates were washed three times and the aliquots of precipitates were subjected to SDS-PAGE followed by immunoblotting using anti-p40phox or p47phox Abs (1/1000, room temperature (RT) for 2 h). For Western blotting, the cytosolic proteins (transfected or untransfected cell lysates or cell supernatants) were fixed and permeabilized, as described (41), and were stained using primary Abs at RT for 2 h. Primary Abs were visualized by confocal microscopy using Alexa 488-conjugated anti-rabbit IgG (1/2000, 0.5 h at RT; Molecular Probes).

**Engineering mKG fluorescent protein and a complementation-based interaction detecting system using mKG**

To get a monomeric form of the Kusabira-Green protein (mKG; GenBank No. AB359198; excitation maximum at 494 nm, emission maximum at 507 nm), we introduced 7 mutations into the bacterial reporter (excitation maximum at 548 nm, emission maximum at 559 nm) (42) using semirandom mutagenesis as described (43). Proteins were expressed in Escherichia coli strain JM109(DE3), purified, and characterized spectroscopically as previously described (44). Fluorescence quantum yields were determined using enhanced GFP as a standard.

To develop a complementation method using separate fragments of mKG fluorescent protein to detect protein-protein interactions under a confocal fluorescence microscope, we chose the position between Gly168 and Gly169 of mKG as a cleavage point for constructing fusion-proteins (mKG(N): 168 aa, mKG(C): 51 aa), because it is predicted to form a flexible loop structure within this region in mKG according to a previous report (42, 45). To systemically use these protein fragments to detect protein-protein interactions, we constructed four constructs (see Fig. 1A) in which the divided mKG protein fragments, mKG(N) and mKG(C), are fused to N-terminal portions of interacting proteins of interest, and c and d, in which the divided mKG protein fragments are fused to C-terminal portions of interacting proteins. The efficacy of this system was examined using the well-characterized heterodimeric complex of leucine zipper acidic protein (LZa): AQLKLEQALEKENAQLEWALEQLAELQKLEQLEK (Kusabira-Orange protein (excitation maximum at 548 nm, emission maximum at 559 nm) (42) using semirandom mutagenesis as previously described (43). Fluorescence quantum yields were determined using enhanced GFP as a standard.

**Detection of intramolecular interaction using mKG system**

All cDNA fragments were cloned into BamHI/EcoRI site of LZA-mKG(N) or LZB-mKG(C), thereby replacing the leucine zipper cDNA sequences, using forward and reverse primers that provide these restriction sites during cDNA PCR amplification. The cDNA of the PX domain of p40phox (1–167 aa) was amplified by PCR and cloned into LZA-mKG(N), designated as p40phox(PX)-mKG(N). Since we (residues 318–328, Ref. 11) and others (residues 339) and p40phox(PX)-mKG(N) (data not shown).

Detection of intramolecular interaction using mKG system

All cDNA fragments were cloned into BamHI/EcoRI site of LZA-mKG(N) or LZB-mKG(C), thereby replacing the leucine zipper cDNA sequences, using forward and reverse primers that provide these restriction sites during cDNA PCR amplification. The cDNA of the PX domain of p40phox (1–167 aa) was amplified by PCR and cloned into LZA-mKG(N), designated as p40phox(PX)-mKG(N), p40phox(PX)-mKG(C), p40phox(PX)-mKG(C) (data not shown).

Cell culture

RAW264.7 macrophages (13) were maintained in DMEM (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated FBS (Invitrogen) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO₂. HEK293 cells (American Type Culture Collection) were maintained in EMEM (Wako Pure Chemical Industries) containing 10% heat-inactivated FBS (Invitrogen), 100 μM nonessential amino acids (Invitrogen), and antibiotics at 37°C in 5% CO₂.
were amplified by PCR and cloned into LZA-mKG(N), designated as p47
phox
(SH3n-SH3c)-mKG(N), p47
phox
(AIR)-mKG(N), p47
phox
(SH3n-AIR)-mKG(N), p47
phox
(SH3n-360)-mKG(N), respectively. p47
phox
(SH3n-360,S303D/S304D/S328D)-mKG(N) and p47
phox
(SH3n-360,W193R)-mKG(N) were made using the QuikChange II XL site-directed
mutagenesis kit, and designated as p47
phox
(SH3n-360,3D)-mKG(N), and p47
phox
(SH3n-360,193R)-mKG(N), respectively. Residues 341–360 of
p47
phox
were cloned into LZA-mKG(N), and designated p47
phox
(341–360)-mKG(N). All modified expression vectors were sequenced to confirm
their identities.

HEK293 cells were transfected by a pair of mKG constructs using Fu-
gene 6. Twenty-four hours after the transfection, fluorescence-positive
cells with nuclei staining by Hoechst 33258 (1:5000, 0.5 h, 37°C; Ref. 47)
were observed and counted under a LSM 510 invert (Carl Zeiss) confocal
laser scanning fluorescence microscope (H11003 40 oil) using 385–470 nm (for
Hoechst 33258) and 505–550 nm (for mKG) narrow band-pass barrier
filters with the same microscopic conditions and settings (lens,
power of excitation laser, pinhole (optical slice), scan time, scan averaging, scan mag-
nification, detection gain, etc.). Transfection efficacies were confirmed us-
using a pair of p40
phox
(PB1)-mKG(N) and p40
phox
(PX)-mKG(C) as a posi-
tive control. Green fluorescence-positive cells/Hoechst 33258-positive
cells were used for statistical analysis. Imaging experiments using the
mKG system were performed at least in duplicate and were repeated in at
least three independent transfection experiments (n = 6).

FIGURE 1. Detection of protein-protein interaction by confocal fluorescence
microscopy using the mKG reporter sys-
tem in transfected cells. A, Plasmids en-
gineered for expression of N-terminally
(a and b) or C-terminally (c and d) mKG
fragment-tagged protein. Shown are the
cDNA insert maps cloned into the
BamHI/XhoI sites of pcDNA3. B, All
combinations of complementary fusion
proteins (left half: a + b, c + d, a + d,
and b + c) are capable of reconstituting
mKG fluorescence in HEK293 cells
when fused to interacting LZA and LZB.
Negative controls of mKG system in the
absence of fused interacting modules
(right half). Bar, 50 μm.
FIGURE 2. Detection of the p40\textsuperscript{phox} PX-PB1 domain interaction in transfected HEK293 cells using the mKG system. A, Interaction between p40\textsuperscript{phox}(PB1)-mKG(N) and p40\textsuperscript{phox}(PX)-mKG(C) (left), but not p40\textsuperscript{phox}(PB1:2A+4A)-mKG(N) and p40\textsuperscript{phox}(PX)-mKG(C) (right). All imaging data are representative of at least three independent transfection experiments. Bar, 50 \textmu m. B, Subcellular localization of p40\textsuperscript{phox}(PB1)-mKG(N) and p40\textsuperscript{phox}(PB1:2A+4A)-mKG(N) stained by anti-p40\textsuperscript{phox} Ab (upper panel). Immunoblotting showing comparable expression of p40\textsuperscript{phox}(PB1)-mKG(N) with p40\textsuperscript{phox}(PX)-mKG(C) and 40\textsuperscript{phox}(PB1:2A+4A)-mKG(N) with p40\textsuperscript{phox}(PX)-mKG(C) (lower panel). Bar, 10 \textmu m. C, GST-based pull-down assays of interactions between purified (His)\textsubscript{6} p40\textsuperscript{phox}(PX) and GST-p40\textsuperscript{phox}(PB1) or GST-p40\textsuperscript{phox}(PB1:2A+4A). Complexes bound to glutathione-Sepharose-4B beads were immunoblotted with anti-(His)\textsubscript{6} Ab (upper panel). Representative of three independent experiments. D, Vesicular (condensed) localization of mDsRed-p40\textsuperscript{phox}(PX)-mKG(N) is observed in cells lacking reconstituted mKG green fluorescence (asterisks), but not in cells with high mKG green fluorescence (marked with circles). Immunoblotting detects expression of mDsRed-p40\textsuperscript{phox}(PX)-mKG(N) and EEA1. Bar, 10 \textmu m. E, Interaction between mKG(C) tagged to the N terminus of full-length p40\textsuperscript{phox} and p40\textsuperscript{phox}(PB1). Bar, 10 \textmu m. F, Interaction between mKG(C) tagged to the N terminus of full-length p40\textsuperscript{phox} and p40\textsuperscript{phox}(PB1). Bar, 10 \textmu m.

GST-based in vitro-binding (pull-down) assay

The purified (His)\textsubscript{6} p40\textsuperscript{phox}(PX, 1–167 aa) and the purified GST-p40\textsuperscript{phox}(PB1:237–339 aa) were described previously (11). p40\textsuperscript{phox}(PB1:259A/269A,318–321(A)) in which residues 259, 269, and 318–321 are replaced by alanine residues, in pGEX-6P-1 (GE Healthcare) was made using the QuikChange II XL site-directed mutagenesis kit, and designated p40\textsuperscript{phox}(259A/269A,318–321(A)). PCR-amplified cDNA of the PX domain of p40\textsuperscript{phox}(1–128) was cloned into BamHI and EcoRI sites of pGEX-6P-1, and designated GST-p47\textsuperscript{phox}(PX). All constructs were sequenced to confirm their identities. The purified GST-p40\textsuperscript{phox}(PB1:2A+4A) and the purified GST-p47\textsuperscript{phox}(PX) were obtained, as described previously (11).

To detect the interaction between p40\textsuperscript{phox}(PX) and each p40\textsuperscript{phox}(PB1) protein, the purified (His)\textsubscript{6} p40\textsuperscript{phox}(PX) (300 nM) was mixed with each purified GST-tagged fragment of p40\textsuperscript{phox} (300 nM) in 500 \textmu l of buffer (11). Glutathione-Sepharose-4B beads were added to the solution, and rotated for 1 h at 4°C. The precipitates were washed three times with the same buffer and the aliquots of precipitates were subjected to SDS-PAGE and followed by immunoblotting using anti-(His)\textsubscript{6} Ab (1/1000, RT for 2 h).

To detect direct interactions between p47\textsuperscript{phox}(PX) and other fragments of p47\textsuperscript{phox}, the purified GST-p47\textsuperscript{phox}(PX) (300 nM) was mixed with the lysates (in the presence of 0.1% Triton X-100 and protease inhibitors) of HEK293 cells that were transfected with various plasmids encoding mKG(N)-tagged fragments of p4\textsuperscript{phox}, and rotated for 2 h at 4°C. Glutathione-Sepharose-4B beads were added to the solution, rotated for 8 h at 4°C, and then washed three times with the same buffer. The material absorbed to beads was eluted with 10 mM glutathione, and the eluants were subjected to SDS-PAGE, followed by immunoblotting using anti-mKG(N) Ab (1/1000, RT for 2 h).

Construction of plasmids for imaging studies of phagocytosis

GFP-p47\textsuperscript{phox}, GFP-p67\textsuperscript{phox}, GFP-p67\textsuperscript{phox}(AD), which is lacking the activation domain (AD: residues 199–212), GFP-p67\textsuperscript{phox}(AD,K355A), GFP-p40\textsuperscript{phox} (D289A), and p40\textsuperscript{phox}(320A) were described previously (11, 48). p40\textsuperscript{phox}(D289A) and p67\textsuperscript{phox}(K355A) were reported as mutations that disrupt interactions between p40\textsuperscript{phox} and p67\textsuperscript{phox} (39); p40\textsuperscript{phox}-internal ribosomal entry site (IRES)-DsRed2, p40\textsuperscript{phox}(D289A)-IRES-DsRed2, and p47\textsuperscript{phox}-IRES-GFP-p67\textsuperscript{phox}(AD) were described previously (11). The fragments encoding p67\textsuperscript{phox}(AD) and GFP-p47\textsuperscript{phox} were amplified by PCR, cloned into BglII/EcoRI sites of MCS of pIRES2-DsRed2 (BD Clontech) and BstXI and XhoI sites of pIRES2-DsRed2 in place of DsRed2, respectively, and designated p67\textsuperscript{phox}(AD)-IRES-GFP-p47\textsuperscript{phox}. The pIRES2-DsRed2 plasmids containing p47\textsuperscript{phox}-IRES-GFP-p67\textsuperscript{phox}(AD), p47\textsuperscript{phox}(AD)-IRES-GFP-p67\textsuperscript{phox}(AD), or p67\textsuperscript{phox}(AD)-IRES-GFP-p47\textsuperscript{phox} were made using the QuikChange II XL site-directed mutagenesis kit. We confirmed that GFP-p4\textsuperscript{phox} and GFP-p6\textsuperscript{phox} behave...
experiments were very reproducible. All imaging data are representative of at least three independent transfection experiments.

B. p47phox(PX)-mKGN interactions are examined using various mKGN(N)-tagged p47phox fragment. Bar, 50 μm. C. Immunoblotting of protein expression of p47phox(PX)-mKGN and comparable expression of mKGN(N)-tagged proteins. D. Percent of mKGN fluorescence-positive cells/Hoechst 33258-positive cells observed in B. Data are obtained from experiments performed in duplicate and repeated in at least three independent set transfection experiments (n ≥ 3), and expressed as means ± SD. E. GST-based pull-down assays between purified GST-p40phox(PX) and HEK293 cell lysates expressing mKGN(N)-tagged fragment of p47phox. Complexes bound to Glutathione-Sepharose-4B beads were immunoblotted with α-mKGN(N) Ab. Representative of three independent experiments. F. Proposed model of intramolecular interactions within p47phox in the resting state, in which the interaction of the PX domain with the SuperSH3/AIR structure along with residues 341–360. In active state, unmasked SuperSH3 and unmasked PX domain can bind to the PR motif of p47phox, and P(i3,4)P2 and PA, respectively. The PR motif of p47phox binds to SH3c of p67phox.

Supplemental video 1 shows the vesicular localization and the accumulation of GFP-p67phox(ΔAD) both on phagosomal cups and phagosomes by coexpression with p40phox(4A) during FcγR-mediated phagocytosis in RAW264.7 cells (total time 295 s). Supplemental video 2 (total time 295 s) shows the vesicular localization and the accumulation of GFP-p67phox(ΔAD) both on phagosomal cups and phagosomes by coexpression with p47phox(318–321:4A) and p47phox(318–321:4A,D289A), respectively. p40phox(318–321:4A) and p40phox(318–321:4A,D289A) in pTRES2-ΔSDRed2 were made using the QuickChange II XL site-directed mutagenesis kit, and designated p40phox(318–321:4A). Supplemental video 3 (total time 430 s) shows vesicular localization and the accumulation of GFP-p47phox both on phagosomal cups and phagosomes by coexpression with p67phox(318–321:4A) and p67phox(318–321:4A,D289A). Supplemental video 4 (total time 295 s) shows cytoplasmic localization and the limited (not prolonged) accumulation of GFP-p47phox on phagosomal cups/phagosomes by coexpression with p67phox(318–321:4A) and p40phox(318–321:4A), in which p67phox can interact with p47phox, but not p40phox.

**Results**

Detection of the intramolecular PX-PB1 interaction in p40phox using the mKGN system

Our previous studies suggested the PX domain of p40phox is hindered from binding to membranes and that mutations or deletions within C-terminal regions of the PB1 domain of p40phox enable direct interactions of p40phox with early endosomes, as seen with the isolated PX domain (11). To confirm and extend these findings...
suggested that the intramolecular contacts within p40^phox prevent its interaction with membranes in resting cells, we used the newly developed mKG fluorescent protein to detect protein-protein interactions between the p40^phox PX domain and the p40^phox PB1 domain in whole cells. Preliminary experiments exploring the efficacy and specificity of the mKG system with interacting LZA and LZB proteins indicated that separate N- and C-terminal portions of the mKG protein fused with LZA and LZB can associate to form fluorescent mKG reporter complexes, regardless of whether the fusions occur at N- or C-terminal ends of the leucine zipper proteins or the mKG fragments (Fig. 1). Cotransfection of p40^phox(PB1)-mKG(N) and p40^phox(PX)-mKG(C) fusion proteins in HEK293 cells reconstituted green cellular fluorescence (Fig. 2A). In contrast, cotransfection of p40^phox(PB1:2A+4A)-mKG(N) and p40^phox(PX)-mKG(C) resulted in only faint green fluorescence (Fig. 2A). Cytoplasmic localization of p40^phox(PB1)-mKG(N) and p40^phox(PX)-mKG(N) was confirmed by indirect immunofluorescence using p40^phox Ab (Fig. 2B, upper panel). Furthermore, comparable expression of p40^phox(PB1)-mKG(N) with p40^phox(PX)-mKG(C) and p40^phox(PB1:2A+4A)-mKG(N) with p40^phox(PX)-mKG(C) was confirmed by Western blotting using p40^phox Ab (Fig. 2B, lower panel).

These results were confirmed by in vitro-binding (pull-down) assays between purified (His)_6-p40^phox(PX) and purified GST-tagged proteins, GST-p40^phox(PB1), or GST-p40^phox(PB1:2A+4A): (His)_6-p40^phox(PX) interacted with GST-p40^phox(PB1), but only weakly with GST-p40^phox(PB1:2A+4A) (Fig. 2C). Comparable amounts of input proteins were confirmed by Ponceau S staining (GST proteins) and by anti-(His)_6 blotting, respectively. Similar binding inhibitory effects were observed using the mKG system and in pull-down assays with mutated p40^phox(PB1:2A+320A), which has substitutions of E259A/D269A (2A) and F320A, sites mapped within the proposed contact interface between the PX and PB1 domains (46) (data not shown). Although some heterogeneity in fluorescence intensities was seen in cells in the field reflecting a range of transfection efficiencies (Fig. 2A), the strength of protein-protein interactions detected by the mKG system correlated with the total intensity of green fluorescence in cells or the number of green fluorescence-positive cells detected. Moreover, using a separate red fluorescent protein reporter (monomeric DsRed), the mKG system detected inhibition of p40^phox(PX) binding to P(3)P when the interacting p40^phox(PB1)-mKG(C) partner was coexpressed; as shown in Fig. 2D, the vesicular localization of mDsRed-p40^phox(PX)-mKG(N) was dramatically reduced in cells exhibiting reconstituted mKG green fluorescence resulting from competitive binding by coexpression of p40^phox(PB1)-mKG(C).

An early endosomal localization of mDsRed-p40^phox(PX)-mKG(N) was confirmed by a staining pattern that closely correlated with anti-EEA1-staining patterns (Fig. 2E). Finally, we examined whether the mKG system could detect direct intramolecular interactions within p40^phox by fusing mKG(C) and mKG(N) to both the N and C terminus of full-length p40^phox. This transfected protein exhibited mKG cellular fluorescence, while mKG(C)-p40^phox(320A)-mKG(N) exhibited faint fluorescence reflecting disruption of the intramolecular p40^phox PX-PB1 domain interaction (Fig. 2F). Together, these results demonstrate that the mKG system is efficient and specific for detecting both intramolecular and intermolecular protein-protein interaction at cellular levels under confocal fluorescence microscopy.
Detection of intramolecular PX domain interactions in p47phox
involving residues 341–360 using the mKG system

It has been reported that p47phox exists in an autoinhibited conformation due to intramolecular interactions in the resting state in which the tandem SH3 domains (SH3n and SH3c) and the PX domain are inaccessible to bind p22phox and membrane lipids, respectively (reviewed in Ref. 50). The first autoinhibited model proposed that two distinct inhibitory interactions occur: between a PXXP motif in the PX domain and the SH3c domain and between the AIR and the SH3n domain (51). Subsequently, the SuperSH3 model was proposed in which the binding surfaces for p22phox formed by both tandem SH3 domains are masked entirely by the AIR in the resting state (52, 53). Although the two interactions (PX domain-SH3c and SuperSH3-AIR) are mutually exclusive in structural terms, the latter interaction is considerably stronger (51, 52); thus, the basis for the proposed masking of the PX domain that prevents membrane lipid interactions remains unclear.

To examine intramolecular interactions within p47phox that mask binding of the PX domain to lipids in a resting state, we used the mKG system. First, we tested the SuperSH3 model by cotransfection of p47phox(AIR)-mKG(N) and p47phox(SH3n-SH3c)-mKG(C), and observed cells with green fluorescence (Fig. 3A). We then examined whether the PX domain could interact with the autoinhibited structure constructed from the tandem SH3 domains and the AIR (SuperSH3/AIR). Cotransfection of p47phox(SH3n-AIR)-mKG(N) and p47phox(PX)-mKG(C) revealed cells with green fluorescence; however, cotransfection of p47phox(PX)-mKG(C) with p47phox(SH3n-SH3c)-mKG(N) or with p47phox(AIR)-mKG(N) revealed significantly fewer fluorescence-positive cells (Fig. 3B). These results indicate that the PX domain interacts with the autoinhibited structure constructed from both the SuperSH3 domain and the AIR (SuperSH3/AIR), but not with the SuperSH3 domain nor the AIR alone. We compared interactions of p47phox(SH3n-AIR)-mKG(N) and p47phox(PX)-mKG(C) with p47phox(SH3n-360)-mKG(N) and p47phox(PX)-mKG(C). The number of green fluorescence-positive cells observed with cotransfection of p47phox(SH3n-360)-mKG(N) and p47phox(PX)-mKG(C) was even greater than that seen with p47phox(SH3n-AIR)-mKG(N) and p47phox(PX)-mKG(C) (Fig. 3B). Furthermore, the number of fluorescence-positive cells observed with cotransfection of p47phox(SH3n-360)-mKG(N) and p47phox(PX)-mKG(C) was dramatically decreased by the substitution with p47phox(SH3n-360,3D)-mKG(N) or p47phox(SH3n-360,193R)-mKG(N) (Fig. 3B). Finally, cotransfection of p47phox(341–360)-mKG(N) and p47phox(PX)-mKG(C) also revealed cells with green fluorescence (Fig. 3B). Expression of p47phox(PX)-mKG(C) and comparable expression of mKG(N)-tagged proteins were confirmed by immunoblotting (Fig. 3C). The number of green fluorescence-positive cells reflecting the strength of protein-protein interactions is statistically analyzed in Fig. 3D. Furthermore, we validated the performance of the mKG system and the accuracy of quantifying detectable interactions by comparison with independent pull-down assays using purified GST-p47phox(PX) and lysates of HEK293 cells transfected with various plasmid encoding mKG(N)-tagged fragment of p47phox. Consistent with previous reports (51, 52), p47phox(SH3n-SH3c)-mKG(N) and p47phox(AIR)-mKG(N) bound weakly to GST-p47phox(PX), while p47phox(SH3n-360)-mKG(N) was strongly bound to GST-p47phox(PX) in comparison to p47phox(SH3n-AIR)-mKG(N) (Fig. 3E). These experiments confirmed the following rank order in affinity for binding to p47phox-PX using both methods: p47phox(SH3n-SH3c)-mKG(N) < p47phox(AIR)-mKG(N) < p47phox(SH3n-360)-mKG(N) < p47phox(SH3n-AIR)-mKG(N). Comparable amounts of input proteins are confirmed by anti-p47phox (GST proteins) and anti-mKG(N) (HEK293 cells lysates) blotting, respectively (Fig. 3E). These results suggest that the PX domain interacts with the inhibited SuperSH3/AIR structure and with downstream residues

FIGURE 5. Accumulation of p67phox on mature phagosomes in RAW264.7 cells expressing active (open) p40phox. A, Cytoplasmic localization of GFP-40phox. All imaging data are representative of at least three independent transfection experiments. B, Colocalization of GFP-p40phox(4A) and a marker of early endosomes, EEA1. C, Disappearance of the early endosome localization of GFP-p40phox(4A) by R105K mutation. D, Vesicular localization of GFP-p40phox(4A,289A). E, Immuno blotting showing protein expression by plasmids transfected into HEK293 cells. F, Subcellular localization of GFP-p67phox(DAD) when coexpressed with p40phox(4A) (before stimulation), and accumulation of GFP-p67phox(DAD) at phagosomes (arrowhead) after phagosomal closure. Cell exhibiting diffuse DsRed2 fluorescence expresses untagged p40phox(4A). Astarisk, circle, and triangle show the same phagosome, respectively. A movie is available in supplemental video 1. IgG-opsonized glass beads (2 μm) were added at time 0, although phagocytosis of each bead begins at different time points. G, Subcellular localization of GFP-p67phox(DAD) by coexpression of p40phox(4A,289A). Cells exhibiting diffuse DsRed2 fluorescence express no tagged p40phox(4A,289A). H, Subcellular localization of GFP-p67phox(DAD,355A) when coexpressed with p40phox(4A). Cells exhibiting diffuse DsRed2 fluorescence express untagged p40phox(4A). I, Interaction of GFP-p67phox(DAD) both with wild-type p40phox(left panel) and p40phox(4A,289A) (right panel) in HEK293 cells. Representative of at least three independent transfection experiments.
that the p47
however, these changes did not influence the interaction with the S345/S348/S359 were substituted by three aspartates or alanines; (11). During Fc
leads to death of transfected RAW264.7 cells, as previously noted.B

accumulation of GFP-p67\textsubscript{phox}(\Delta AD) at phagosomal cups (arrow) and phagosomes (arrowhead) during phagocytosis of BlgG. Cell exhibiting both GFP-p67\textsubscript{phox}(\Delta AD) and diffuse DsRed2 fluorescence also produces untagged p47\textsubscript{phox} and untagged p40\textsubscript{phox}(4A). A movie is available in supplemental video 2. IgG-opsonized glass beads (2 μm) were added at time 0, although phagocytosis of each bead begins at different time points. All imaging data are representative of at least three independent transfection experiments. B, Subcellular localization of GFP-p67\textsubscript{phox}(\Delta AD) when coexpressed with p47\textsubscript{phox} and p40\textsubscript{phox}(4A,289A). Cell exhibiting both GFP-p67\textsubscript{phox}(\Delta AD) and diffuse DsRed2 fluorescence also produces untagged p47\textsubscript{phox} and untagged p40\textsubscript{phox}(4A,289A). C, No accumulation of GFP-p67\textsubscript{phox}(\Delta AD) on phagosomal cups (arrow) or phagosomes (arrowhead) following disruptions of interactions between GFP-p67\textsubscript{phox}(\Delta AD) and p47\textsubscript{phox} and between GFP-p67\textsubscript{phox}(\Delta AD) and p40\textsubscript{phox}(4A). Cell exhibiting both GFP-p67\textsubscript{phox}(\Delta AD,355K) and diffuse DsRed2 fluorescence also produces untagged p47\textsubscript{phox}(\Delta PR) and untagged p40\textsubscript{phox}(4A). D, Immunoblotting showing protein expression by plasmids (HEK293 cells) used in Fig. 6.

Accumulation of p67\textsubscript{phox} by p47\textsubscript{phox} at phagosomes during FcγR-mediated phagocytosis

In this study, GFP-p67\textsubscript{phox}(\Delta AD) was used instead of GFP-p67\textsubscript{phox}, because overexpression of all three active phox proteins leads to death of transfected RAW264.7 cells, as previously noted (11). During FcγR-mediated phagocytosis, GFP-p47\textsubscript{phox} accumulated at phagosomal cups and on phagosomes with an accumulation time of ∼60 s (detected at T = 155–220 (Fig. 4A) and see Fig. 7C), similar to a previous report (48). In contrast, GFP-p67\textsubscript{phox}(\Delta AD) (Fig. 4B), as well as GFP-p67\textsubscript{phox} (data not shown), showed no accumulation at the phagosomal cup nor on phagosomes in the absence of other transfected cytosolic phox proteins. However, in the case when GFP-p67\textsubscript{phox}(\Delta AD) was coexpressed with p47\textsubscript{phox} using p47\textsubscript{phox}-IRES-GFP-67\textsubscript{phox}(\Delta AD), GFP-p67\textsubscript{phox}(\Delta AD) accumulated at phagosomal cups and on phagosomes with accumulation times ∼60 s (detected at T = 180–245) (Fig. 4C), as seen with GFP-p47\textsubscript{phox}. GFP-67\textsubscript{phox}(\Delta AD) did not accumulate at phagosomal cups or phagosomes when coexpressed with p47\textsubscript{phox} using p47\textsubscript{phox}(\Delta PR)-IRES-GFP-67\textsubscript{phox}(\Delta AD), which does not interact with GFP-67\textsubscript{phox}(\Delta AD) (Fig. 4D). The accumulation of GFP-tagged phox protein was further confirmed based on fluorescence intensity profiles transversing the phagosomal cup/phagosome (Fig. 4, A–D, right panel of each). These results demonstrated that p47\textsubscript{phox} functions as a “carrier” for p67\textsubscript{phox} to phagosomal cups or phagosomes during FcγR-mediated phagocytosis, consistent with earlier studies on p7\textsubscript{phox}-deficient CGD neutrophils (27). The protein expression of plasmid (GFP-p47\textsubscript{phox}, GFP-p67\textsubscript{phox}(\Delta AD), p47\textsubscript{phox}-IRES-GFP-p67\textsubscript{phox}(\Delta AD), and p47\textsubscript{phox}(\Delta PR)-IRES-GFP-p67\textsubscript{phox}(\Delta AD)) was confirmed by immunoblotting (Fig. 4E).

Accumulation of p67\textsubscript{phox} by p40\textsubscript{phox} at phagosomes during FcγR-mediated phagocytosis

To clarify the function of p40\textsubscript{phox} as a “carrier” protein for p67\textsubscript{phox}, we used p40\textsubscript{phox}(4A), which has an “open conformation” resulting from disruption of the intermolecular PX-PB1 interaction (11). GFP-40\textsubscript{phox} was localized in cytoplasm (Fig. 5A) and transient vesicular accumulation of GFP-p40\textsubscript{phox} was observed occasionally, which fuses with newly forming phagosomes during FcγR-mediated phagocytosis (11). In contrast, GFP-p40\textsubscript{phox}(4A) was localized predominately on vesicular structures (Fig. 5B, left), was colocalized with a marker of early endosomes, EEA1 (Fig. 5B), and accumulated on phagosomes during FcγR-mediated phagocytosis (data not shown). The early endosome localization of GFP-p40\textsubscript{phox}(4A) was dramatically decreased with GFP-
p40(4A,105K) (Fig. 5C), in which 105K disrupts P(S)P binding.
p40(4A,289A) (Fig. 5D) was translocated to early endosomes in response to AA (11), while p40(4A,289A) was also localized at vesicular structures (Fig. 5D). Consistent protein expression with these plasmids (p40(4A), p40(4A,105K), and p40(4A,289A)) was confirmed by immunoblotting (Fig. 5E). GFP-p67phox(DAD) was localized at vesicular structures when coexpressed with p40(4A)-IRES-DsRed2, which identifies cotransfected cells expressing no tagged p40(4A) by DsRed2 fluorescence (Fig. 5F). GFP-p67phox(DAD) localized at vesicular structures accumulated at phagosomes after phagosomal closure (at least >80 s (T = 175–255); Fig. 5F and supplemental video 1). The retention time of GFP-p67phox(DAD) on phagosomes was longer than 100 s, as seen with GFP-p40(4A)-IRES-DsRed2 (data not shown). These results suggest that p67phox also functions as a “carrier” for p67phox to phagosomes during FcγR-mediated phagocytosis.

Sequential accumulation of p67phox by p47phox and p40phox at phagosomes during FcγR-mediated phagocytosis

To study the kinetics of p67phox localization during FcγR-mediated phagocytosis where both carrier proteins exist, we expressed p47phox and an active, opened confirmation p40phox with GFP-p67phox(DAD) using p47phox-IRES-p67phox(DAD) and p40phox(4A)-RES-DsRed2. GFP-p67phox(DAD) was localized in vesicular structures and the cytoplasm before stimulation, and accumulated at phagosomal cups and on phagosomes during phagocytosis of B1gG (at least 85 s (T = 160–245); Fig. 6A and supplemental video 2). Substitution of p40phox(4A) by p40phox(4A,289A) abolished the vesicular localization pattern of GFP-p67phox(DAD) (Fig. 6B). These results were confirmed in complementary experiments using p47phox-IRES-GFP-p67phox(DAD,355K) and p40phox(4A)-RES-DsRed2 (data not shown). Furthermore, disruptions of both interactions between p47phox and p67phox, and between p40phox and p67phox using p47phox(4A)-RES-DsRed2 and p67phox(DAD,355K), respectively, resulted in no accumulation of GFP-p67phox(DAD) at phagosomal cups or on phagosomes (Fig. 6C). These results were further confirmed in complementary experiments using p47phox(4A)-RES-GFP-p67phox(DAD) and p40phox(4A,289A)-RES-DsRed2 (data not shown). These results suggest that p67phox accumulates at phagosomal cups and on phagosomes through interactions both with p47phox and with p40phox, in which the early stage accumulation involves p47phox (at phagosomal cups...
and phagosomes (~60 s); see Fig. 4C) and the late-stage accumulation involves p40\textsuperscript{phox} (after phagosomal closure; see Fig. 5F). Expression of all three phox proteins by two plasmids (p47\textsuperscript{phox}-IRESGFP-p67\textsuperscript{phox}(ΔAD) + p40\textsuperscript{phox}(4A)-IRESDsRed2, p47\textsuperscript{phox}-IRESGFP-p67\textsuperscript{phox}(ΔAD) + p40\textsuperscript{phox}(4A,289A)-IREDSsRed2, and p47\textsuperscript{phox}(ΔPR)-IRESGFP-p67\textsuperscript{phox}(ΔAD,355K) + p40\textsuperscript{phox}(4A)-IRESDsRed2) was confirmed by immunoblotting (Fig. 6D).

**Prolonged accumulation of p47\textsuperscript{phox} by p40\textsuperscript{phox} at phagosomes during FcγR-mediated phagocytosis**

Although we demonstrate that both p47\textsuperscript{phox} and p40\textsuperscript{phox} function as “carrier” proteins for p67\textsuperscript{phox}, the carrier function of p40\textsuperscript{phox} appears to be stronger than that of p47\textsuperscript{phox} when mutated opened form of p40\textsuperscript{phox} is expressed. To confirm this observation, GFP-p47\textsuperscript{phox} kinetics during FcγR-mediated phagocytosis were monitored with coexpression of p67\textsuperscript{phox}(ΔAD) and p40\textsuperscript{phox}(4A), using p67\textsuperscript{phox}(ΔAD)-IRESGFP-p47\textsuperscript{phox} and p40\textsuperscript{phox}(4A)-IRESDsRed2. GFP-p40\textsuperscript{phox} was localized at vesicular structures and the cytoplasm before stimulation, and during phagocytosis of BiG GFP-p47\textsuperscript{phox} accumulated at phagosomal cups and phagosomes (at least T = 105 s); see Fig. 7A and supplemental video 3). The vesicular structures also fused to phagosomes (supplemental video 3). Substitution of p67\textsuperscript{phox}(ΔAD) with p67\textsuperscript{phox}(ΔAD,355A) revealed no vesicular localization pattern of GFP-p47\textsuperscript{phox} (Fig. 7B); however, it still showed the accumulation at phagosomal cups (limited (not prolonged) accumulation; supplemental video 4). This result was further confirmed in complementary experiments using p67\textsuperscript{phox}(ΔAD)-IRESGFP-p47\textsuperscript{phox} and p40\textsuperscript{phox}(4A,289A)-IRESDsRed2 (data not shown). These results indicate that p40\textsuperscript{phox} functions as a “carrier” even for p47\textsuperscript{phox} through p67\textsuperscript{phox} interactions. To verify the carrier function of p40\textsuperscript{phox} for p47\textsuperscript{phox}, we examined the retention times of GFP-p47\textsuperscript{phox} at phagosomal cups and on phagosomes during phagocytosis of BiG. The retention of GFP-p47\textsuperscript{phox} (n = 8, 63.8 ± 13.8 s) was markedly prolonged (n = 13, 220.0 ± 40.6 s) by coexpression with p40\textsuperscript{phox}(4A), and this prolonged accumulation time was abolished (n = 9, 62.8 ± 12.0 s) by the substitution of p40\textsuperscript{phox}(4A) with p40\textsuperscript{phox}(4A,289A) (Fig. 7C). Expression of all three phox proteins by two plasmids (p67\textsuperscript{phox}(ΔAD)-IRESGFP-p47\textsuperscript{phox} + p40\textsuperscript{phox}(4A)-IRESDsRed2, p67\textsuperscript{phox}(ΔAD,355K)-IRESGFP-p47\textsuperscript{phox} p40\textsuperscript{phox}(4A)-IRESDsRed2, and p67\textsuperscript{phox}(ΔAD)-IRESGFP-p47\textsuperscript{phox} + p40\textsuperscript{phox}(4A,289A)-IRESDsRed2) was confirmed by immunoblotting (Fig. 7D).

**Discussion**

In this report, we engineered a method using complementary fragments of the monomeric coral fluorescent reporter protein (mKG) to detect protein-protein interactions between fusion proteins at the cellular level by direct visualization under a confocal fluorescence microscope. There are several reports using the complementation-based method to detect protein-protein interaction (54–59). The first report of the complementation method using fluorescent protein was based on GFP in E. coli (60). Subsequently, methods using YFP, BFP, or CFP in mammalian cells were also reported (45, 61). Our newly developed mKG system has two distinct advantages from these conventional methods: 1) the background signal observed related to nonspecific binding is very low and 2) because mKG has sharper emission spectra than BFP/CFP (data not shown), the mKG system would be better suited for simultaneous visualization of multiple protein interactions within the same cell, if we can engineer different color fluorescent proteins by through mutations in the N-terminal fragment of mKG. The complementation-based method is a very easy way for screening protein-protein (domain-domain) interaction in real time, compared with conventional methods such as immunoprecipitation, in vitro-binding assays with purified proteins, the yeast two-hybrid system, detection of surface plasmon resonance using BIACORE, and fluorescence resonance energy transfer analysis. The mKG system has high efficacy and specificity for detecting real-time protein-protein interactions in intact cells. As shown in Fig. 2, the specificity of this system was confirmed two ways: 1) in vitro pull-down assays using purified proteins and 2) inhibition of the specific binding of p40\textsuperscript{phox}(PX) to early endosomes by intramolecular contacts between the PB1 and PX domains of p40\textsuperscript{phox}. As shown in Fig. 2D, the mKG system is also very reliable, because the system is based on a GFP and the specific protein-protein interaction could be confirmed by direct visualization of red fluorescent protein. However, the mKG system cannot evaluate the strength of binding affinity among different sets of interacting proteins, because the observed fluorescence intensity of complemented mKG protein depends on the varying intrinsic stabilities and levels of the expressed complementary tagged proteins. Thus, we cannot compare fluorescence intensity values as a direct indication of relative binding affinities among the different interacting partners studied in Figs. 1, 2, 3A, and 3B.

Using the mKG system, we also evaluated interdomain interactions within p47\textsuperscript{phox} that affect the accessibility of its membrane-binding domains. Targeting of p47\textsuperscript{phox} to the phagosomal membrane requires two distinct interactions: binding of its PX domain to membrane phospholipids (both PI(3,4)P2 and PA) and interactions between its tandem SH3 domains and a proline-rich (PR) motif on p22\textsuperscript{phox}. We have reported that disruption of either one of these interactions abolishes membrane targeting of p47\textsuperscript{phox} (11, 48). The SuperSH3 model specifying autoinhibitory AIR interactions responsible for masking of the tandem SH3 domains in the resting, unphosphorylated state has been well-recognized (52, 53). However, details on autoinhibitory interactions responsible for masking the PX domain of p47\textsuperscript{phox} remain unclear, and direct binding of the PX domain PXXP motif to SH3c (51) is incompatible with and would preclude the autoinhibitory SuperSH3/AIR interactions, because the two interactions would involve the same binding surface of SH3c. The present study indicates that the “closed” structure encompassing the tandem SH3 domains bound to the AIR (SuperSH3/AIR) interacts directly with the PX domain, and that this interaction is enhanced by residues 341–360. Recently, Durand et al. (62) reported that p47\textsuperscript{phox} in the resting state adopts an elongated (nonglobular) conformation detected by small-angle x-ray scattering, and suggested weak interactions are plausible between the PX domain and the SuperSH3/AIR structure and could also involve a sequence downstream of the AIR (62), consistent with the interdomain contacts we detected by the mKG reporter system. Furthermore, microcalorimetry titration experiments that suggest the PX domain contributes to or enhances the SuperSH3/AIR interaction (52) are consistent with our observations using the mKG system.

We recently showed using the AA-stimulated RAW264.7 cell model that both p40\textsuperscript{phox} and p47\textsuperscript{phox} function as regulated “carrier” proteins for p67\textsuperscript{phox} through distinct membrane-targeting mechanisms (11). Although neutrophils from CGD patients lacking p47\textsuperscript{phox} or p67\textsuperscript{phox} show impaired translocation of p40\textsuperscript{phox} to the membrane fraction by soluble stimulation (PMA or FMLP) (26), it is well-known that p40\textsuperscript{phox} functions in FcγR-mediated phagocytosis (33, 34). We and others reported that p47\textsuperscript{phox} accumulates transiently on phagosomal cups and mature phagosomes during FcγR-mediated phagocytosis and acts as a “carrier” for p67\textsuperscript{phox} (27, 48); however, little is known on how p40\textsuperscript{phox} functions in the
assembly of the Nox2 complex during FcγR-mediated phagocytosis. Here, we demonstrated that both p40<sup>pos</sup> and p47<sup>pos</sup> function as “carrier” proteins for p67<sup>pos</sup> during FcγR-mediated phagocytosis: p47<sup>pos</sup> functions in early stages of phagocytosis during phagosome formation, while p40<sup>pos</sup> functions in late stages of phagocytosis after phagosomal closure (dependent on PIP3 generation). Furthermore, the accumulation or retention times of p47<sup>pos</sup> on phagosomes are prolonged by coexpression of an active form of p40<sup>pos</sup>, p40<sup>pos</sup>(AA), which has an open conformation accessible to PIP3 (63.8 s–220 s; Fig 7C). Consistent with these observations, the specific affinity of the p40<sup>pos</sup>-PX domain for liposomes containing PIP3 is ~3.5 times higher than that of the p47<sup>pos</sup>-PX domain for PIP3 (3,4)P2 (22, 63), although addition of PA to PIP(3,4)P2 increases the affinity of the p47<sup>pos</sup>-PX domain (22). Although addition of high concentrations (20 μM) of PIP(3,4)P2 to the HEK293 cell line alone promotes the plasma membrane translocation of GFP-p47<sup>pos</sup>(PX) (63), we reported that both the SH3 domain (p47<sup>pos</sup>-PX)-PR motif (p22<sup>pos</sup>) interaction and PX domain are required for p47<sup>pos</sup> translocation to the plasma membrane (11). In contrast to p47<sup>pos</sup>, the carrier function of p40<sup>pos</sup> is dependent on PIP3 binding of the PX domain. However, p47<sup>pos</sup> is essentially absol</p>