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p47^{phox} PX Domain of NADPH Oxidase Targets Cell Membrane Via Moesin-Mediated Association With the Actin Cytoskeleton

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Abstract Activation of phagocytic NADPH oxidase requires association of its cytosolic subunits with the membrane-bound flavocytochrome. Extensive phosphorylation of the p47^{phox} subunit of NADPH oxidase marks the initiation of this activation process. The p47^{phox} subunit then translocates to the plasma membrane, bringing the p67^{phox} subunit to cytochrome b558 to form the active NADPH oxidase complex. However, the detailed mechanism for targeting the p47^{phox} subunit to the cell membrane during activation still remains unclear. Here, we show that the p47^{phox} PX domain is responsible for translocating the p47^{phox} subunit to the plasma membrane for subsequent activation of NADPH oxidase. We also demonstrate that translocation of the p47^{phox} PX domain to the plasma membrane is not due to interactions with phospholipids but rather to association with the actin cytoskeleton. This association is mediated by direct interaction between the p47^{phox} PX domain and moesin. J. Cell. Biochem. 92: 795–809, 2004. © 2004 Wiley-Liss, Inc.

Key words: P47^{phox} PX domain; NADPH oxidase; phosphoinositide; moesin; actin cytoskeleton

Phagocyte NADPH oxidase is the enzyme primarily responsible for cellular superoxide generation. It is preferentially expressed in phagocytic cells and plays an important role in immune defense against microbial infections. Defects in NADPH oxidase subunits result in enzyme dysfunction and cause chronic granulomatous disease (CGD), a hereditary disorder characterized by severe, recurrent bacterial and fungal infections [Babior, 1999, 2000, 2002, 2004].

In phagocytes, assembly of p47^{phox}, p67^{phox}, and Rac 1/2 with membrane-associated cytochrome b558 is essential for NADPH oxidase activity. p47^{phox} and p67^{phox} normally reside in cytosol and translocate to the plasma membrane upon cell activation. The p67^{phox} subunit contains an NADPH binding site and/or an activation domain [Han et al., 1998] and is required for catalyzing electron transport and regulating cytochrome b558 function [Diebold and Bokoch, 2001]. The p47^{phox} subunit acts as a signal-receiving protein that initiates assembly of active oxidase; it also functions as an adaptor to facilitate interactions between p67^{phox} and cytochrome b558 [Heyworth et al., 1991; Diebold and Bokoch, 2001].

Although the detailed activation mechanism for NADPH oxidase is unclear, assembly of active enzyme is initiated by activation of the p47^{phox} subunit. p47^{phox} consists of a novel PX domain followed by two tandem SH3 domains and a C-terminal tail. It exists as a cytosolic subunit in resting cells and translocates to membranes in association with cytoskeleton upon stimulation [El Benna et al., 1994, 1999]. p47^{phox} mediates translocation of p67^{phox} to the plasma membrane, since the p67^{phox} subunit fails to translocate to the membrane in the

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absence of p47^{phox} [Heyworth et al., 1991]. In contrast, translocation of p47^{phox} to the plasma membrane does not depend on the p67^{phox} subunit, since p47^{phox} translocates to the plasma membrane in the absence of p67^{phox} [Rotrosen and Leto, 1990; Heyworth et al., 1991; Uhlinger et al., 1993]. The presence of p22^{phox} in the membrane may act as a signal to recruit p47^{phox} to the membrane [Babior, 1999]. However, p47^{phox} still translocates and accumulates in the peri-phagosomal area in individuals with X-linked CGD who lack both subunits of cytochrome b558. This finding suggests that translocation of p47^{phox} to the membrane can also occur independent of the p22^{phox} subunit [Allen et al., 1999]. Molecular mechanisms of p47^{phox} translocation to the plasma membrane, however, remain unclear.

The p47^{phox} PX domain may play a role in targeting the p47^{phox} subunit to the plasma membrane [Ago et al., 2001; Kanai et al., 2001; Karathanassis et al., 2002; Zhan et al., 2002]. The p47^{phox} PX domain has phosphoinositide-binding ability and targets the plasma membrane upon activation of PI-3 kinase [Kanai et al., 2001; Zhan et al., 2002]. Recently, an additional lipid-binding pocket for phosphatidic acid (PA) has been identified from the crystal structure of the p47^{phox} PX domain, which implies that the p47^{phox} subunit might target the plasma membrane via synergistic interactions with both lipid molecules [Karathanassis et al., 2002]. A previous study showed that a p47^{phox} PX domain mutant (p47-PX-R42Q) lacked the phosphoinositide affinity, but could still target the plasma membrane [Zhan et al., 2002]. However, it remains uncertain whether other phospholipids play a role in translocating p47^{phox} to the plasma membrane. Here, we present data demonstrating for the first time that the p47^{phox} PX domain is responsible for targeting the p47^{phox} subunit to cell membrane. We also demonstrate that the targeting function is not due to its affinity for phosphoinositide, but is regulated through moesin-mediated association with actin.

MATERIALS AND METHODS

Materials

IGF-1 and latrunculin B were obtained from Calbiochem Co. (Biosciences, Inc., La Jolla, CA). Anti-moesin polyclonal (Upstate, Biotechnology, Lake Placid, NY), anti-actin polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GFP monoclonal (CLONTECH, Laboratories, Inc., Palo Alto, CA), anti-HA monoclonal antibodies (Santa Cruz), anti-CD20 polyclonal (Santa Cruz) were used. Rhodamine-conjugated goat-anti-rabbit IgG was purchased from BioSource International, Inc. (Camarillo, CA). Alkaline phosphatase (AP)-conjugated goat-anti-rabbit IgG and goat-anti-mouse IgG were from Bio-Rad Laboratories, Inc. (Hercules, CA), and alkaline phosphatase-conjugated donkey-anti-goat IgG was from Santa Cruz Biotechnology, Inc.

Plasmid Constructs

The full-length p47^{phox} had been cloned from human cDNA (CLONTECH). For green fluorescent protein fusion, the DNA fragment encoding EGFP-p47 (amino acid 2–390), and its truncation mutants EGFP-p47ΔC (amino acid 2–284), EGFP-p47ΔPX (amino acid 155–390), EGFP-p47ΔC-SH3 + ΔC (amino acid 2–214) were PCR-amplified from full-length p47^{phox} and cloned into pEGFP-C2 vector (CLONTECH). The R43Q and R70Q mutants of the p47^{phox} PX domain were made using the QuikChange^{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA). The R43Q/R70Q double mutant and R42Q/R70Q/P73A/P76 quadruple mutant of EGFP-p47PX were similarly generated by using the EGFP-p47PX-R43Q and EGFP-p47PX (R42Q/P73A/P76A) DNA as the templates. For GST-fusion protein expression, the DNA fragments encoding p47^{phox} PX domain (amino acids 2–125) and C-terminal p47^{phox} (amino acids 285–390) were similarly generated and cloned into pGEX 4T-1 vector (Amersham Biosciences, Piscataway, NJ). The DNA encoding the p47^{phox} PX domain was also cloned into pCMV5-3× HA vector for expression of HA-tagged p47PX (HA-p47PX). All DNA constructs were sequence-confirmed.

Cell Culture

COS cells and ECV304 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA). K562 cells and JG cells, an Epstein-Barr virus-transformed lymphoblastoid B cell line from a p47^{phox}-deficient CGD patient [Chanock et al., 1996; Inanami et al., 1998] were grown in RPMI-1640 (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum (Hyclone,

Logan, UT) and 1% of a supplemental solution (L-glutamine (200 mM), penicillin (10,000 U/ml), streptomycin (10 mg/ml) in 0.9% NaCl, Sigma, St. Louis, MO).

Immunoprecipitation

COS cells were cultured to 50~80% confluency, then transfected with HA-p47PX construct or the pCMV5-3× HA vector, using Superfect Transfection Kit (Qiagen, Valencia, CA) and following manufacturer's procedure. After 40 h, cells were scraped from culture plates and lysed in a lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP40, 1 mM EDTA, 0.02% sodium azide, 1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Cell lysates were centrifuged at 800*g* for 20 min to remove cell debris. The supernatants were subjected to immunoprecipitation study.

Immunoprecipitation was performed with an antibody complex of the monoclonal anti-HA antibody conjugated onto agarose beads (Santa Cruz). The antibody–agarose complex was incubated with cell lysates both from the HA-p47PX-transfected cells and cells transfected with empty vectors at 4°C overnight. After three times washing with the same buffer, beads were boiled in SDS sample buffer, and samples were analyzed by SDS–PAGE followed by immunoblotting.

Cell Fractionation and Western Blotting

Cells were cultured as above, and transfected with EGFP-p47PX construct or EGFP vector. After 40 h, cells were washed in cold PBS, scraped from culture plates and resuspended in an extraction buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100). Cell lysates were incubated at 4°C for 20 min, and then centrifuged at 13,000*g* for 20 min to separate into Triton-soluble and -insoluble fractions. The fractions were sampled, analyzed by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk for 1 h at room temperature. Then they were incubated with indicated primary antibodies, followed by incubation with AP-conjugated secondary antibodies and detection with enhanced chemiluminescence (ECL) kit.

Pull-Down Assays and Latrunculin B Treatment

GST and GST fusion proteins were bacterially expressed, purified, and immobilized on glutathione-Sepharose beads (Amersham Pharmacia). The bound proteins were adjusted to equal concentration at approximately 2 mg/ml packed beads. COS cell lysates were prepared as above. Hundred micrograms of cell lysates in each reaction were incubated at 4°C over night with 40 μg of each protein on beads. The beads were boiled with SDS sample buffer and samples were analyzed by SDS–PAGE, and followed by immunoblotting for the indicated proteins. For latrunculin B treatment, cell lysates were preincubated with 10 μM latrunculin B for 6 h at 4°C prior to further incubation with immobilized GST-p47PX. The G-actin Affinity Pull-down Assays were performed as previously described [James et al., 2001].

Immunofluorescent Microscopy

Cells were maintained and transfected by the calcium phosphate precipitation method. Serum-starvation, IGF-1 treatment, and phalloidin staining of cells were performed as previously described [Zhan et al., 2002]. To visualize moesin, cells were fixed with 4% formaldehyde and permeabilized with PBS containing 1% FBS and 0.5% Triton X-100, then incubated with anti-moesin antibody for 2 h at room temperature. The cells were washed three times for 10 min each in PBS before rhodamine-conjugated IgG was applied for 30 min. After a brief wash in PBS, the coverslips were mounted in 90% glycerol containing 2.5% diazabicyclo[2.2.2]octane.

For latrunculin B treatment, serum-starved cells were treated with 10 μM latrunculin B for 1 h at 37°C before fixation.

RESULTS

$p47^{phox}$ PX Domain Is Responsible for Translocating the $p47^{phox}$ Subunit to the Plasma Membrane

We previously demonstrated that the $p47^{phox}$ PX domain was localized to membrane ruffles upon activation of PI-3 kinase [Zhan et al., 2002]. To clarify that the membrane-targeting function of the $p47^{phox}$ PX domain is responsible for translocating the $p47^{phox}$ subunit, we generated EGFP-tagged full-length $p47^{phox}$ as well as several truncation mutants, and studied

their subcellular localization and translocation in cells.

In basal COS cells, we found that EGFP-p47PX was mainly localized in the perinuclear region, while little was seen cortically (Fig. 1B(b)) in colocalization with F-actin (Fig. 4B). IGF-1 treatment dramatically increased the amount of EGFP-p47-PX in the cell periphery (Fig. 1B(h)). Based on our study on IGF-1-stimulated translocation of the $p47^{phox}$ PX domain [Zhan et al., 2002], we anticipated a similar effect of IGF-1 on full-length $p47^{phox}$. Full-length $p47^{phox}$ (EGFP-p47), however, was localized exclusively in nuclei; IGF-1 stimulation did not alter its localization (Fig. 1B(a,g)). The C-terminus-truncated $p47^{phox}$ (EGFP-p47ΔC) and a $p47^{phox}$ mutant with both the C-terminal SH3 domain and C-terminal tail truncated (EGFP-p47ΔC-SH3 + ΔC), with or without IGF-1 stimulation, have similar localization to EGFP-p47PX (Fig 1B(c,d,i,j)). In contrast, the PX domain-deleted $p47^{phox}$ mutant (EGFP-p47ΔPX) lost cortical localization completely and was localized predominantly in nuclei, even under IGF-1 stimulation (Fig. 1B(e,k)).

EGFP-tagged full-length $p47^{phox}$ was reported to localize in the plasma membrane in ECV304 cells [Gu et al., 2002]. EGFP-tagged $p47^{phox}$, however, had a non-membrane distribution in COS cells. To clarify that localization of $p47^{phox}$ is regulated differentially in COS cells and ECV304 cells, cellular localization of the full-length $p47^{phox}$ subunit and its truncation mutants in ECV304 cells were examined. We confirmed that the full-length $p47^{phox}$ (EGFP-p47) could indeed localize in the plasma membrane following IGF-1 stimulation (Fig. 1B(m)). Similar localization was seen for EGFP-p47PX, EGFP-p47ΔC, and EGFP-p47ΔC-SH3 + ΔC (Fig 1B(n–p)), whereas EGFP-p47ΔPX had non-membrane localization similar to EGFP itself (Fig. 1B(q–r)).

Since activation of $p47^{phox}$ is regulated differentially according to cell type and NADPH oxidase is expressed preferentially in hematopoietic cells, especially phagocytic cells, we then investigated localization and translocation of $p47^{phox}$ in leukocytes to gain further insight into the membrane-targeting role of the $p47^{phox}$ PX domain. The JG $p47^{phox}$-deficient lymphoblast B cell line [Chanock et al., 1996; Inanami et al., 1998] was transfected with EGFP vector or EGFP-tagged $p47^{phox}$ constructs, and their localization and translocation were examined. The full-length $p47^{phox}$ (EGFP-p47), with or without IGF-1 stimulation, was localized in the membrane region (Fig. 2A,B) along with CD20, a leukocyte surface antigen marker for B cells (Fig. 2A). In addition, EGFP-p47PX, EGFP-p47ΔC, and EGFP-p47ΔC-SH3 + ΔC were localized in the plasma membrane in a manner similar to full-length $p47^{phox}$ (Fig. 2B). In contrast, EGFP-p47ΔPX showed non-membrane localization (Fig. 2B), similar to EGFP and consistent with observations in COS and ECV304 cells.

Various forms of $p47^{phox}$ that contain the PX domain all tend to target membrane while the mutant without the PX domain shows no membrane localization in NADPH oxidase-containing or -deficient cells. This pattern suggests that the PX domain accounts for translocation of the $p47^{phox}$ subunit to the plasma membrane.

Translocation of the $p47^{phox}$ PX Domain to Plasma Membrane Does not Depend on Its Lipid-Binding Ability

Although the $p47^{phox}$ PX domain has affinity for different phosphoinositides, its R42Q mutant, which has no lipid-binding ability, can still target plasma membrane [Zhan et al., 2002]. The crystal structure of the $p47^{phox}$ PX domain, however, has shown two binding pockets: one for phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) and another for PA. This feature suggests that the membrane-targeting function of the $p47^{phox}$ PX domain could be due to binding to PA or to synergistic binding to $PI(3,4)P_2$ and PA within the plasma membrane [Karathanassis et al., 2002]. Because the R42Q, P73A/P76A, and R42Q/P73A/P76A mutants of the $p47^{phox}$ PX domain, which lost lipid-binding affinity and/or the SH3 domain-binding motif, were still localized to the membrane region after IGF-1 stimulation [Zhan et al., 2002], we sought

Fig. 1. Localization and translocation of full-length $p47^{phox}$ and its truncation mutants in COS cells and ECV304 cells. **A**: Schematic outline of the $p47^{phox}$ subunit and its different truncation mutants. **B**: Intracellular localization of the $p47^{phox}$ subunit and its truncation mutants in COS cells and in ECV304 cells. The cells were transiently transfected with corresponding plasmid DNA and grown for 24 h, then either left untreated or treated with 50 ng/ml IGF-1 for 10 min after cells were starved for another 3 h.

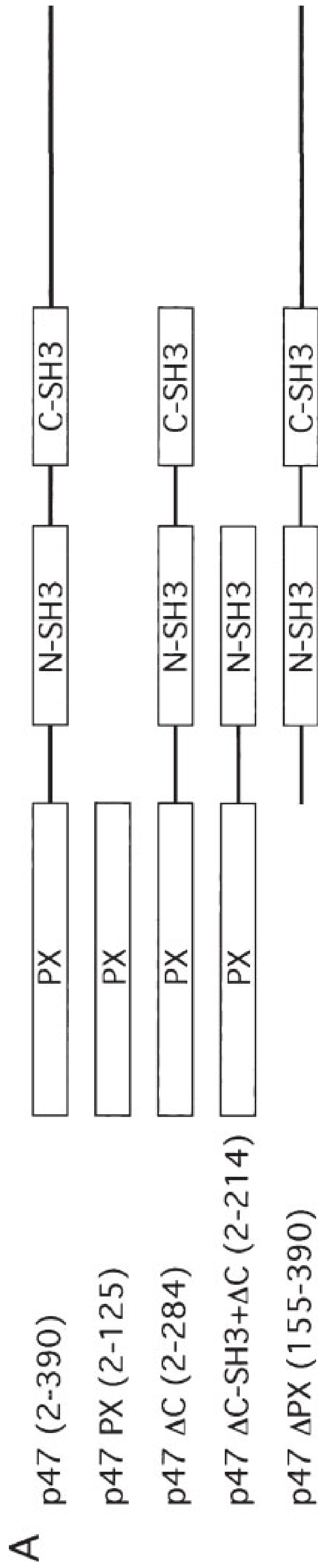

Fig. 1.

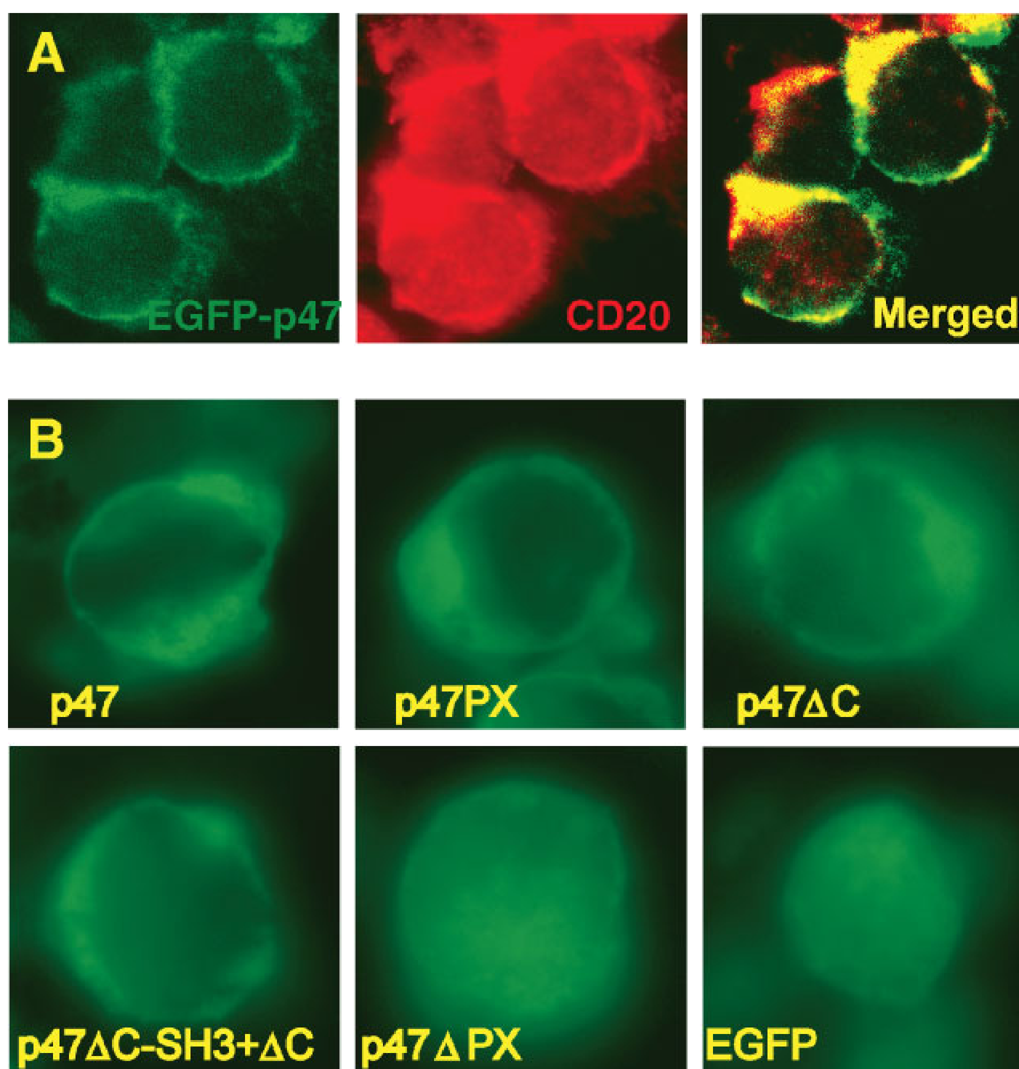

Fig. 2. Localization of full-length p47^{phox} and its truncation mutants in JG p47^{phox}-deficient B cells. **A**: EGFP-p47 is colocalized with CD20, the leukocyte B cell membrane marker. JG-p47^{phox} deficient B cells were transiently transfected with EGFP-p47 and grown for 20 h, and then immunostained with anti-CD20 polyclonal antibody. **B**: The intracellular localization of different p47^{phox} subunit in JG cells on IGF-1 stimulation.

to determine whether the presence of a second lipid-binding pocket for PA could contribute and/or dominate the membrane-targeting ability of the p47^{phox} PX domain. Since Arg43 and Arg70 have been identified as two key lipid-interacting residues in these binding pockets [Karathanassis et al., 2002], we generated mutants of EGFP-p47PX, including R43Q, R70Q, and R43Q/R70Q, and examined their cellular localization. Although these mutants all have decreased lipid affinities to PIP, PA, and PIP/PA [Karathanassis et al., 2002], they were all localized in the plasma membrane in COS cells after IGF-1 stimulation (Fig. 3a–c). In addition, the R42Q/R70Q/P73A/P76A mutant, which has a disrupted PXXP motif and altered binding pockets for PI(3,4)P_2 and PA, was still localized in the plasma membrane after IGF-1 stimulation (Fig. 3d). These data suggest that the lipid-binding ability of the p47^{phox} PX domain is unlikely to contribute to its translocation to the plasma membrane. Other proteins could regulate translocation of the p47^{phox} PX domain to the plasma membrane [Wientjes et al., 2001; Zhan et al., 2002].

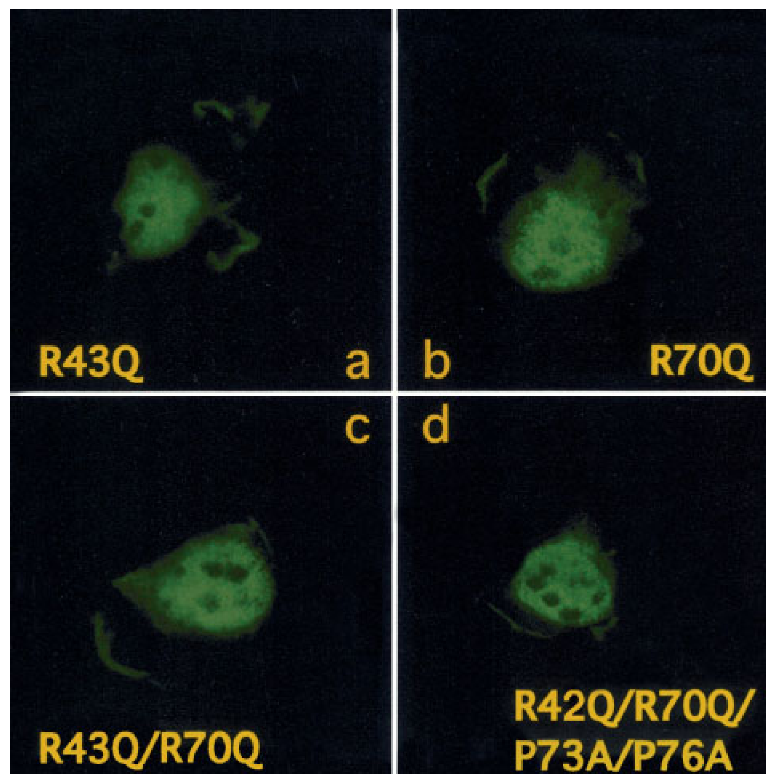

Fig. 3. The p47^{phox} PX domain mutants target the plasma membrane. COS cells were transfected with the EGFP-p47-PX R43Q mutant, R70Q mutant, R43Q/R70Q double mutant or R42Q/R70Q/P73A/P76A quadruple mutant, and stimulated with 50 ng/ml IGF for 10 min.

p47^{phox} PX Domain Is Associated With the Actin Cytoskeleton

Both endogenous p47^{phox} and transfected EGFP-p47^{phox} were reported to associate with cortical actin cytoskeleton, and collect at membrane ruffles upon cell activation [Gu et al., 2002]. Based on our observation that the p47^{phox} PX domain is able to translocate to the plasma membrane in colocalization with F-actin, we hypothesized that the p47^{phox} PX domain associates with actin cytoskeleton. To test this hypothesis, we first examined the cellular distribution and localization of the transfected p47^{phox} PX domain in COS cells. Cells transfected with EGFP-p47PX or EGFP vector were subjected to Triton extraction. Cell lysates were separated into Triton-soluble and -insoluble fractions. Proteins were detected with anti-EGFP antibody. Indeed, EGFP-p47PX was present predominantly in Triton-insoluble fractions, in contrast to EGFP, which was mainly recovered from the Triton-soluble fractions (Fig. 4A). Association of the p47-PX domain with cytoskeleton was also demonstrated from the partial colocalization of the p47^{phox} PX domain with F-actin (Fig. 4B). EGFP-p47PX showed an uneven punctate distribution in COS cells, was enriched in perinuclear regions, and was only partially colocalized with F-actin. EGFP-p47PX was also found colocalized with cortical actin cytoskeleton (Fig. 4B, arrows). When cells were treated with latruculin B, an inhibitor of actin polymerization, cortical actin cytoskeleton was disrupted, but some cytoplasmic actin cytoskeleton remained in the cells (Fig. 4C). In these cells, EGFP-p47PX but not EGFP was observed colocalized with the residual actin cytoskeleton (Fig. 4C). These data suggest that the p47^{phox} PX domain alone can associate with the actin cytoskeleton in COS cells.

p47^{phox} PX Domain Forms a Complex With Moesin and Actin

The association between the p47^{phox} PX domain and actin cytoskeleton could be achieved either through direct interactions or indirect interactions mediated by other proteins. Moesin, a member of the ERM (ezrin-radixin-moesin) family, is responsible for linking F-actin to the plasma membrane [Sato et al., 1992;

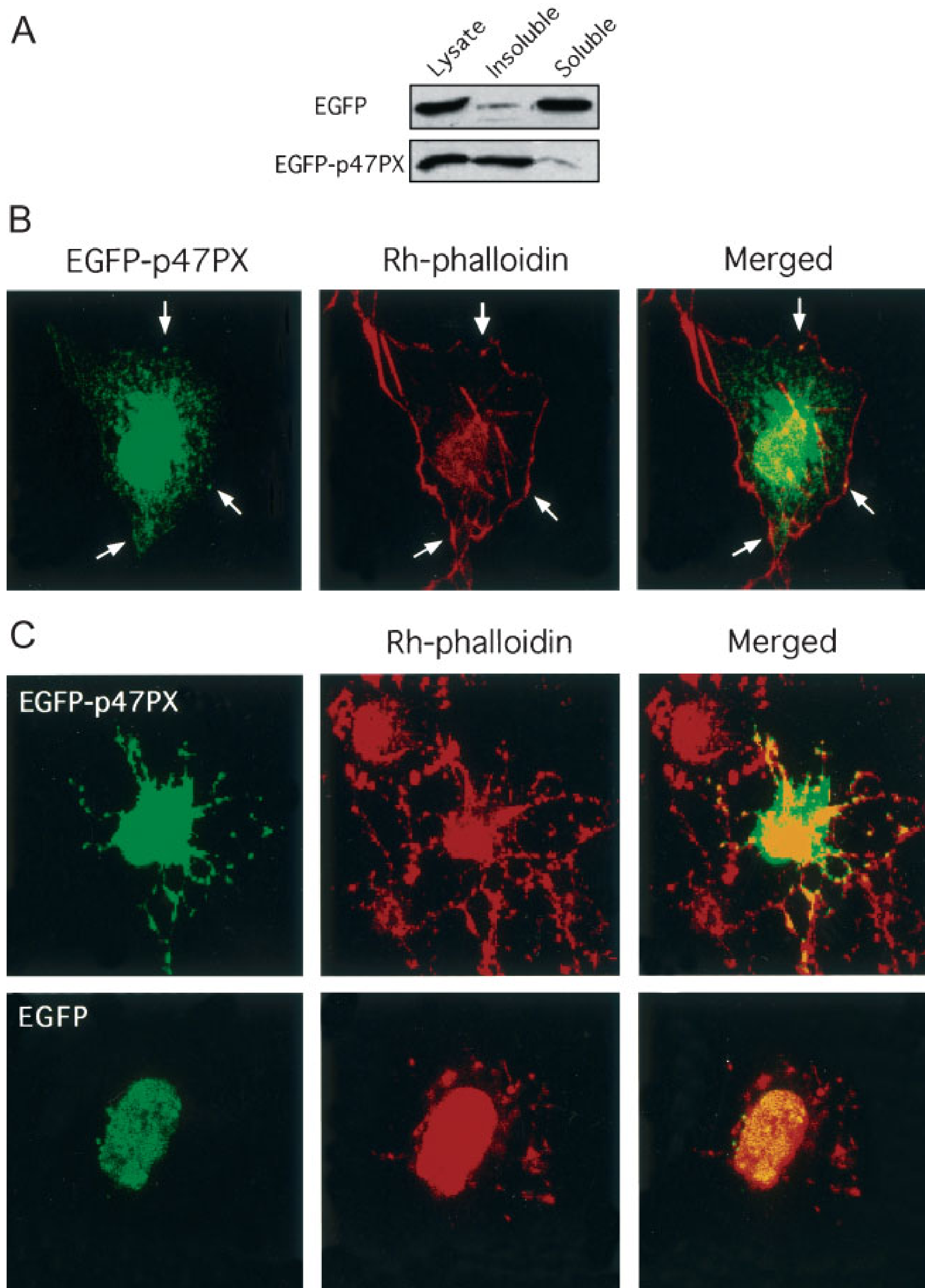

Fig. 4. Cellular distribution and localization of the transfected p47^{phox} PX domain in COS cells. **A**: COS cells were transfected with EGFP or EGFP-p47PX plasmid DNA and lysed after 40 h. Triton-soluble and -insoluble fractions were separated. Samples were analyzed by SDS–PAGE, transferred to membranes, and immunoblotted with anti-GFP antibody. **B**, **C**: COS cells were transfected with EGFP or EGFP-p47PX plasmid DNA and grown for 16 h. Cells were either left untreated (B) or treated (C) with 10 μM latrunculin B for 1 h before fixation. Cells were also stained with rhodamine-conjugated phalloidin for F-actin labeling.

Franck et al., 1993; Gary and Bretscher, 1993; Simons et al., 1998; Pearson et al., 2000]. Moesin has been identified as a binding partner of p47^{phox} with an unclear mechanism [Wientjes et al., 2001]. We hypothesized that moesin serves as a mediator protein to link the p47^{phox} PX domain with actin cytoskeleton. To test the hypothesis, cell lysates of COS cells transfected with HA vector or HA-p47PX plasmid were immunoprecipitated with anti-HA antibody. The anti-HA antibody immunoprecipitated HA-p47PX together with moesin (Fig. 5A). Actin was also detected in the HA-p47PX immunoprecipitation complex. However, neither moesin nor actin was immunoprecipitated with anti-HA antibody in the absence of the p47^{phox} PX domain. The p47^{phox} PX domain alone, therefore, could form a complex with moesin and actin in COS cells.

We then investigated whether the p47^{phox} PX domain interacts directly with actin. The GST-fused p47^{phox} PX domain (GST-p47PX) was bacterially expressed and purified. The C-terminal tail of p47^{phox}, which had been previously shown to interact with actin [Tamura et al., 2000], was also expressed as a GST-fusion protein (GST-p47C). Glutathione-Sepharose beads were conjugated with the purified GST-fusion proteins (Fig. 5B) and then incubated with actin monomers. Western blotting was performed with polyclonal anti-actin antibody to detect bound actin. The C-terminal tail p47^{phox} was shown to interact directly with actin, whereas GST and GST-p47PX did not show binding to actin (Fig. 5C). We also found that GST-p47PX did not bind to polymerized actin in an actin co-sedimentation assay (data not shown).

To clarify further the detailed interactions among these three proteins, we performed in vitro pull-down assays with GST-fusion proteins and cell lysates obtained from COS cells. Consistent with the immunoprecipitation results above, GST-p47PX could pull down both moesin and actin from cell lysates, whereas GST-p47C pulled down actin only and GST had no interaction with either protein (Fig. 5D). Furthermore, when cell lysates were incubated with latrunculin B prior to incubation with GST-p47PX, association of the p47^{phox} PX domain with actin was markedly attenuated, while its binding to moesin was not affected. These data suggest that the p47^{phox} PX domain does not directly interact with actin. The association between the p47^{phox} PX domain and the actin cytoskeleton, moreover, is mediated by moesin.

p47^{phox} PX Domain Colocalizes With Both Moesin and F-Actin in COS Cells and in Leukocytes

In COS cells, EGFP-p47PX partially colocalized with the actin cytoskeleton (Fig. 4B). It was also colocalized with endogenous moesin, however, in the cortical region (Fig. 6, small arrow) and in long filopodia, especially at the tips (Fig. 6, arrow head). When cells were stimulated with IGF-1, both EGFP-p47PX and moesin colocalized in the cortical region (Fig. 6). The accumulation of EGFP-p47PX at membrane ruffles was also accompanied by actin polymerization [Zhan et al., 2002]. Colocalization of cellular moesin and F-actin in the cortical region following growth factor treatment was documented [Franck et al., 1993]. In combination with in vitro binding results, we hypothesized that the p47^{phox} PX domain alone may associate constantly with moesin in COS cells, and associate with F-actin by moesin mediation. To investigate further the link between the p47^{phox} PX domain and the actin cytoskeleton, we treated the EGFP-p47PX-transfected cells with F-actin disruptors, and examined its localization in COS cells. When cells were treated with latrunculin B, both moesin and EGFP-p47PX in the cortical region disappeared. However, the colocalization of EGFP-p47PX and moesin was still observed in the cytoplasm (Fig. 6) in a pattern similar to the colocalization of EGFP-p47PX and F-actin (Fig. 4B). EGFP-p47PX was also observed colocalized with moesin in cytochalasin B-treated cells (data not shown).

The colocalization of EGFP-p47PX with both moesin and F-actin was also confirmed in JG p47^{phox}-deficient B cell (Fig. 7A), and in the K562 cell line, an undifferentiated multi-potent leukemia cell line with endogenous expression of Rac and p22^{phox} (Fig. 7B). Collectively, these data suggest that the p47^{phox} PX domain per se, with or without IGF-1 stimulation, appear as a cytoskeleton-associating domain, and the association between the p47^{phox} PX domain and the actin cytoskeleton is mediated primarily by moesin.

DISCUSSION

In phagocytes, NADPH oxidase functions primarily in conducting microbicidal activity

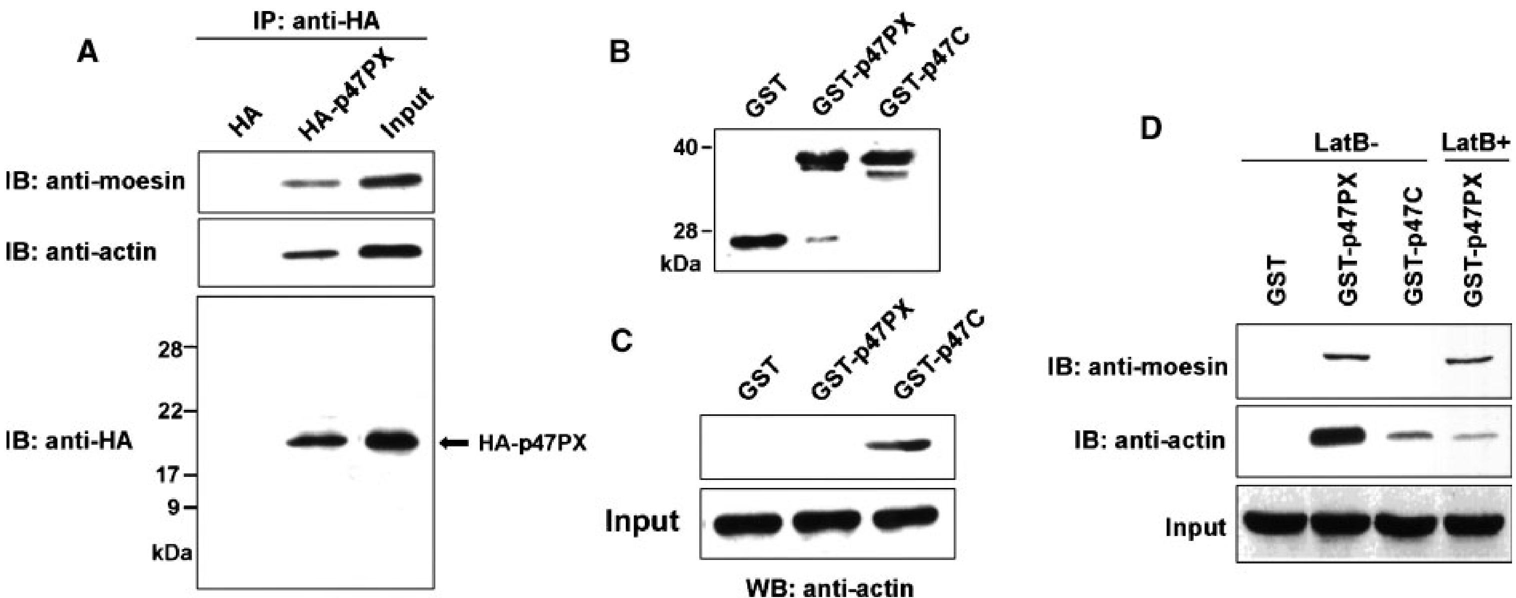

Fig. 5. Characterization of the association of the p47^{phox} PX domain with actin. **A**: p47^{phox} PX domain associates and forms a complex with moesin and actin. COS cells were transfected with pCMV5-3× HA or HA-p47PX DNA, and lysed after 40 h. Co-immunoprecipitation was carried out using COS cell lysates and agarose beads conjugated with anti-HA-antibody. Membranes were immunoblotted with antibodies against moesin, actin, and HA, respectively. Input lane represents 10% of applied cell lysates. **B**: GST and GST-fusion proteins (GST-p47PX and GST-p47C) were bacterially expressed, purified, and immobilized on glutathione-Sepharose beads. Equal amounts of proteins were separated by SDS–PAGE and stained with coomassie blue. **C**: Equal amounts (approximately 30 μg) of GST and the GST-fusion proteins were used to pull down G-actin by incubating with purified actin monomers in an actin-binding buffer. Bound actin was detected with polyclonal anti-actin antibody. Input line shows equal amount of G-actin used for binding study. **D**: The immobilized GST and the GST fusion proteins were incubated with COS cell lysates, with or without latrunculin B treatment (LatB). Bound moesin and actin were detected with immunoblotting. Input line shows equal amount of cell lysates applied and represented by actin expression.

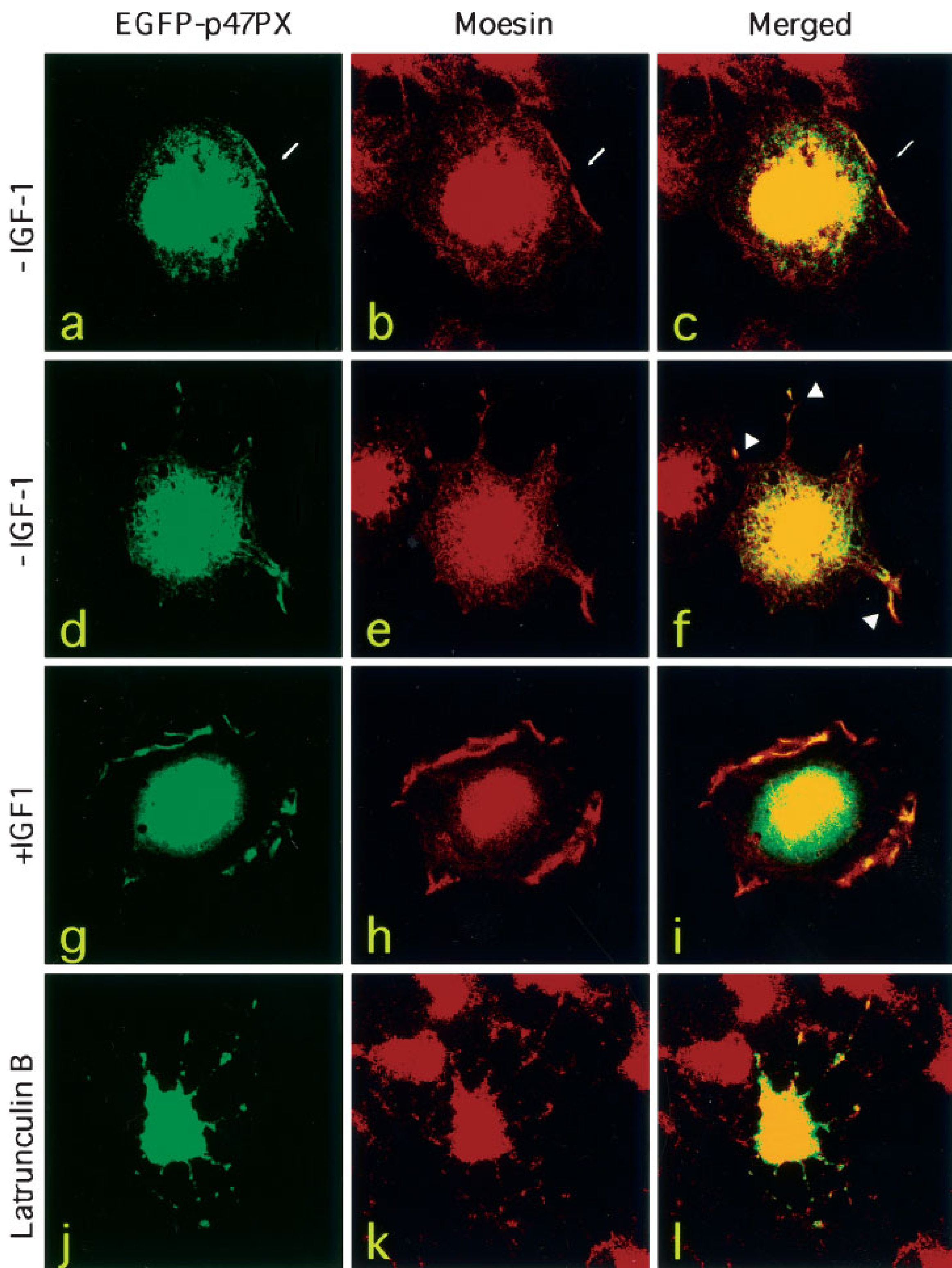

Fig. 6. Colocalization of the p47^{phox} PX domain with moesin in COS cells. COS cells were transfected with EGFP-p47PX. Sixteen hours later, cells were left untreated, treated with 50 ng/ml IGF-1 for 10 min, or treated with 10 μM latrunculin B for 1 h and then stained with anti-moesin antibody followed by rhodamine-conjugated secondary antibody.

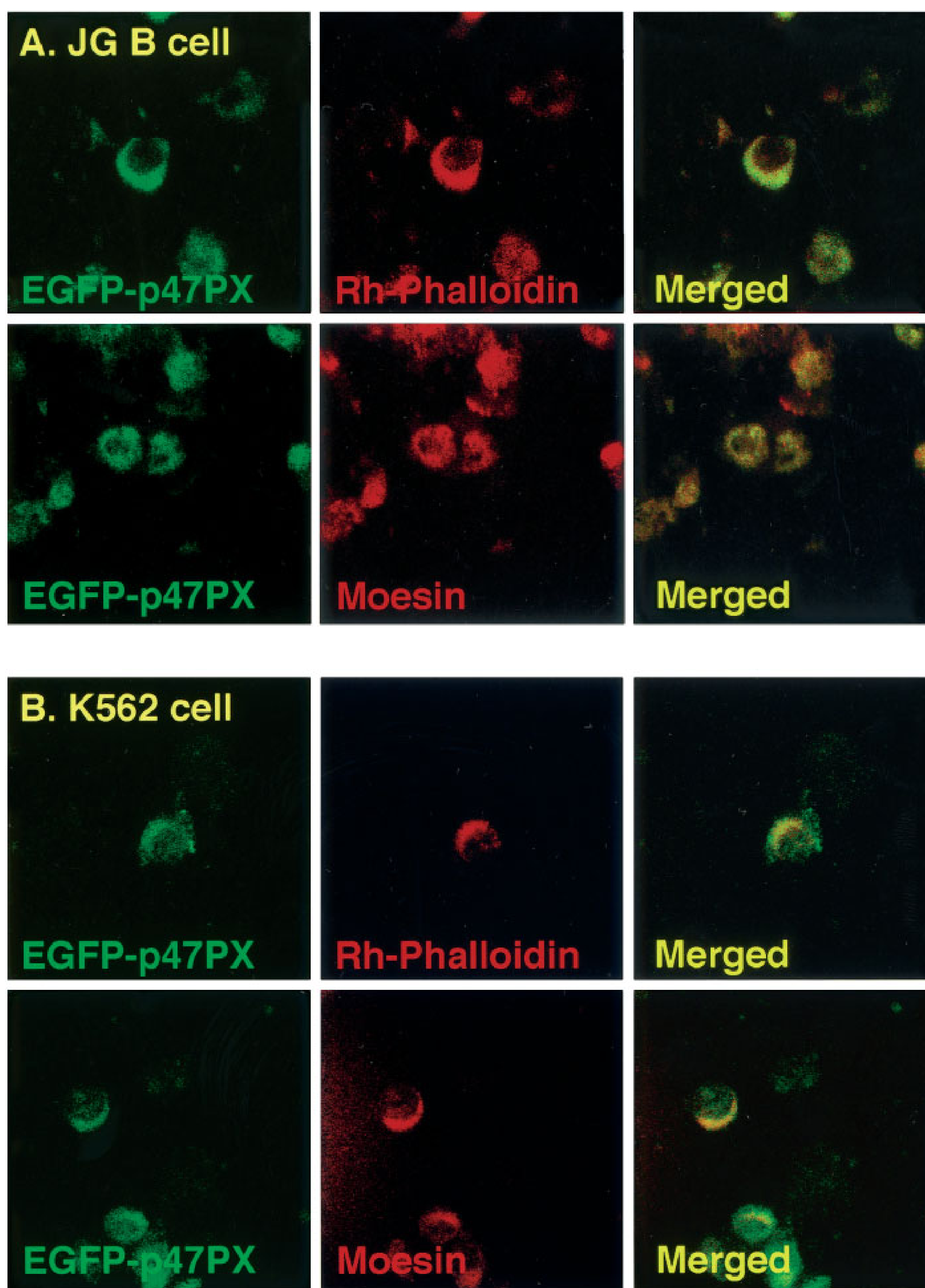

Fig. 7. The p47^{phox} PX domain colocalizes with F-actin and moesin in leukocytes. JG p47^{phox}-deficient B cells (**A**) and K562 cells (**B**) were transfected with EGFP-p47PX for 20 h. After fixation and permeabilization, the cells were labeled with rhodamine-conjugated phalloidin for F-actin, or immunostained with anti-moesin polyclonal antibody.

through production of superoxide. Activation of NADPH oxidase requires translocation of its cytosolic subunits to phagosome membrane for the generation of superoxide. Translocation of the cytosolic subunits is strictly regulated and activated by a variety of mechanisms to prevent harm to host cells. Recently, the PX domains in the p40^{phox} and p47^{phox} subunits of NADPH oxidase have demonstrated lipid-binding affinities, and have been proposed as the membrane-targeting domains of these subunits [Ago et al., 2001, 2003; Kanai et al., 2001; Karathanassis et al., 2002; Zhan et al., 2002]. Moreover, since these two PX domains bind primarily to D-3′ phosphoinositides, the role of phospholipid as well as PI 3-kinase has attracted much attention. The p40^{phox} PX domain binds specifically to phosphatidylinositol 3-phosphate(PI(3)P) and targets endosomes in vivo [Ago et al., 2001; Ellson et al., 2001; Kanai et al., 2001; Zhan et al., 2002], clearly suggesting its important role in activating NADPH oxidase. No data exists, however, to show the critical role of the p47^{phox} PX domain in translocating the subunit to membranes. The present studies provide direct evidence that the p47^{phox} PX domain is responsible for transporting the p47^{phox} subunit to the membrane vicinity. The EGFP-tagged full-length p47^{phox} localizes in the plasma membrane region in COS cells and, more importantly, in leukocytes. In addition, truncation mutants of p47^{phox} in which the PX domain remains possess the ability to localize in the plasma membrane, whereas the PX domain-deleted p47^{phox} mutant completely loses membrane localization, even in the presence of IGF-1 stimulation.

Since PX domains are capable of phosphoinositide-binding, the p47^{phox} PX domain was proposed to translocate to membranes through direct binding with phospholipids. However, unlike the p40^{phox} PX domain, which has distinct PI(3)P affinity, the p47^{phox} PX domain has no specific phosphoinositide ligands, according to several studies [Ago et al., 2001; Kanai et al., 2001; Zhan et al., 2002]. Moreover, our data show that the mutants of the p47^{phox} PX domain with diminished phosphoinositide-binding ability can still translocate to the plasma membrane upon IGF-1 stimulation. This finding suggests that phosphoinositides may not act as signals to attract p47^{phox} to the membrane. Recently, a second lipid-binding pocket, possibly interacting with PA, has been identified from the crystal structure of the p47^{phox} PX domain [Karathanassis et al., 2002]. The membrane-targeting function of the p47^{phox} PX domain could thus be due to the effect of other phospholipids or synergistic binding to PIP and PA in the plasma membrane, which had not been ruled out in our previous study [Zhan et al., 2002]. In the present studies, we found that multiple mutants of the p47^{phox} PX domain, which have decreased affinities to PIP, PA, and PIP/PA, according to previous reports [Karathanassis et al., 2002; Zhan et al., 2002], can still translocate to the plasma membrane area upon stimulation. Our data imply that rather than a recruitment signal, the lipid-binding ability of the p47^{phox} PX domain is more likely to contribute to the activation of p47^{phox} by disrupting the inter-domain interactions and/or maintaining its presence at phagosomal membrane.

It has been long noted that the cytosolic subunits of NADPH oxidase associate with cytoskeleton [Nauseef et al., 1991; El Benna et al., 1994, 1999; Gu et al., 2002]. The association of p47^{phox} with the actin cytoskeleton contributes to the activation of this subunit [El Benna et al., 1994, 1999; Gu et al., 2002]. p47^{phox} is able to translocate to phagosomes in a mechanism dependent on F-actin but not cytochrome b558 [Allen et al., 1999], suggesting a correlation between translocation of cytosolic subunits and actin polymerization. Direct interaction of the C-terminal tail of p47^{phox} with actin and indirect interaction mediated by actin-binding proteins, such as coronnin [Tamura et al., 2000], have both been suggested as mechanisms by which p47^{phox} associates with the actin cytoskeleton. Moreover, the p47^{phox} PX domain has been identified as a binding partner of moesin, suggesting that protein as a cytoskeleton-associating partner [Wientjes et al., 2001]. Thus, we sought more evidence, especially in vivo localization, to determine the relative role of the PX domain or C-terminal tail of p47^{phox} in translocation of this subunit to the membrane.

Consistent with previous observations, we found that the C-terminal tail of p47^{phox} interacts with actin [Faust et al., 1995; Inanami et al., 1998; Ago et al., 2001; Zhan et al., 2002]. However, we did not observe colocalization of the PX domain-deleted p47^{phox} with the actin cytoskeleton, but observed its localization in the

nucleus of COS cells. The C-terminal tail of p47^{phox} mediates the packaging of functional oxidase [Hata et al., 1998; Babior, 1999; Lee and Park, 2000], whereas it normally interacts with the N-terminal SH3 domain of the same subunit in resting phagocytic cells. These data cast further doubt on potential of the C-terminal tail of p47^{phox} in mediating cytoskeleton association in vivo. Although we cannot rule out the possibility that the C-terminal tail of p47^{phox} plays a role in linking the subunit to the actin cytoskeleton in some circumstances, the C-terminal tail of p47^{phox} is unlikely to contribute to the membrane-translocation of the p47^{phox} subunit.

Consistent with the report from Wientjes et al. [2001] that the p47^{phox} PX domain binds to moesin, we have provided evidence that the p47^{phox} subunit associates with the actin cytoskeleton through interaction between its PX domain and moesin. Moesin is also involved in the activation of NADPH oxidase during translocation of p47^{phox} to the plasma membrane. These data further support the hypothesis that the p47^{phox} PX domain is responsible for targeting the p47^{phox} subunit to the plasma membrane. The p47^{phox} PX domain can bind to moesin, thereby directing the subunit to the membrane in parallel with actin polymerization. Normally, the p47^{phox} subunit remains in dormant form with intermolecular interactions between its PXXP motifs and SH3 domains [Leto et al., 1994; Babior, 1999; Hiroaki et al., 2001]. When stimulated, sequential phosphorylation at its C-terminal tail takes place and both its N-terminal PX domain and C-terminal tail are exposed by disruption of intramolecular binding to the SH3 domains [Sumimoto et al., 1994; Ago et al., 1999; Shiose and Sumimoto, 2000], followed by translocation to the plasma membrane via moesin-mediated interaction with actin-cytoskeleton.

In conclusion, we have shown that the p47^{phox} PX domain is responsible for the association of the p47^{phox} subunit with the actin cytoskeleton and for its translocation to the plasma membrane. Accompanied by actin polymerization on phagosomal membranes during phagocytosis, the p47^{phox} PX domain can translocate the p47^{phox} subunit towards the plasma membrane through interaction with moesin. The interaction between the SH3 domain of p47^{phox} and the proline-rich region of p22^{phox} would then occur when p47^{phox} reaches the vicinity of plasma membrane, and help to form a functional NADPH oxidase complex.

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