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The Recruitment of Raf-1 to Membranes Is Mediated by Direct Interaction with Phosphatidic Acid and Is Independent of Association with Ras*

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The serine/threonine kinase Raf-1 is an essential component of the MAPK cascade. Activation of Raf-1 by extracellular signals is initiated by association with intracellular membranes. Recruitment of Raf-1 to membranes has been reported to be mediated by direct association with Ras and by the phospholipase D product phosphatidic acid (PA). Here we report that insulin stimulation of HIRcB fibroblasts leads to accumulation of Ras, Raf-1, phosphorylated MEK, phosphorylated MAPK, and PA on endosomal membranes. Mutations that disrupt Raf-PA interactions prevented recruitment of Raf-1 to membranes, whereas disruption of Ras-Raf interactions did not affect agonist-dependent translocation. Expression of a dominant-negative Ras mutant did not prevent insulin-dependent Raf-1 translocation, but inhibited phosphorylation of MAPK. Finally, the PA-binding region of Raf-1 was sufficient to target green fluorescent protein to membranes, and its overexpression blocked recruitment of Raf-1 to membranes and disrupted insulin-dependent MAPK phosphorylation. These results indicate that agonist-dependent Raf-1 translocation is primarily mediated by a direct interaction with PA and is independent of association with Ras.

The MAPK[1] cascade is a signaling pathway essential for the regulation of mitogenesis by extracellular signals (1). One of the critical regulatory points in this cascade is the activation of the serine/threonine kinase Raf-1. Inactive Raf-1 exists in a large cytoplasmic complex with molecular chaperone proteins (2). Upon stimulation of cell-surface receptors, Raf-1 becomes associated with membranes and undergoes a complex series of activation steps modulated by the small GTPase Ras (3–5), 14-3-3 proteins (6–11), and phosphorylation (12–15). Furthermore, the association of Raf-1 with membranes appears to be essential for its activation. Forced membrane recruitment through attachment of the Ras prenylation moiety to the C terminus of Raf-1 has been shown to induce kinase activation by a mechanism reportedly independent of association with Ras (16, 17). It was also found that overexpression of constitutively activated Ras proteins results in the recruitment of Raf-1 to membranes (16, 17). Although it was clear that Raf-1 associates with activated Ras *in vivo*, *in vitro* activation of Raf-1 by Ras has been difficult to demonstrate (18, 19). Therefore, it was proposed that Ras mediates recruitment of Raf-1 to membranes (20).

The recruitment of Raf-1 to membranes has also been reported to be dependent on its association with phosphatidic acid (21, 24). Disruption of agonist-dependent PLD activity either pharmacologically or by expression of catalytically inactive mutants blocks recruitment of Raf-1 to membranes and Raf-1 activation. Furthermore, addition of exogenous PA reverses the effects of PLD inhibition on Raf-1 translocation and MAPK phosphorylation. Although PA does not activate Raf-1 *in vitro* or *in vivo*, addition of exogenous PA can induce recruitment of Raf-1 to membranes in HIRcB cells or Ras-transformed cells (21). Therefore, it is clear that PA is required for agonist-dependent Raf-1 translocation. However, the relative contribution of Ras and PA to receptor-stimulated Raf-1 translocation is unknown.

Here we provide definitive evidence that PA is essential for the recruitment of Raf-1 to cell membranes. We show that association between PA and Raf-1 is sufficient and necessary for targeting Raf-1 to the correct subcellular locus. Insulin stimulated the accumulation of Ras, Raf-1, phosphorylated MEK, phosphorylated MAPK, and PA in early endosomes. Mutation of a single residue in the PA-binding region of Raf-1 blocked translocation of Raf-1 to endosomes, whereas disruption of Ras-Raf interactions or Raf-1 kinase activity had no effect on translocation. Furthermore, expression of the PA binding-deficient Raf-1 mutant prevented insulin-dependent translocation of endogenous Raf-1 and MAPK activation. We also show that expression of the PA-binding region of Raf-1 can block MAPK activation by preventing Raf-1 translocation to endosomes. These results support a model in which agonist-dependent Raf-1 translocation is primarily mediated by PA, whereas Raf-1 activation is dependent on its interactions with Ras. According to this model, the translocation of Raf-1 to membranes and the activation of Raf-1 are distinct processes. Raf-1 recruitment is a function of PA, whereas the activation of Raf-1 is a consequence of the interaction between Raf-1 and activated Ras.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat-1 fibroblasts that overexpress the human insulin receptor (HIRcB cells) were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 10% fetal bovine serum as described previously (21). Transfections were performed using Superfect transfection reagent (QIAGEN Inc.) for imaging studies or LipofectAMINE (Life Technologies, Inc.) for biochemical assays. Trans-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; PLD, phospholipase D; PA, phosphatidic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GFP, green fluorescent protein; EEA-1, early endosome antigen-1; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; BFA, brefeldin A; PABR, phosphatidic acid-binding region.

fection reagents were used according to the manufacturer's instructions. Transfection efficiencies reached ~50% using Superfect and were ~70–90% using LipofectAMINE. Expression of GFP-tagged constructs was within the same order of magnitude as expression of endogenous Raf-1 as determined by Western blotting of cell lysates.

Materials—The primary antibodies used were as follows: mouse anti-Raf-1 and mouse anti-EEA-1 (Transduction Laboratories), mouse anti-Ras clone Ras10 (Upstate Biotechnology, Inc.), rabbit anti-insulin receptor (Santa Cruz Biotechnology), rabbit anti-phosphorylated MEK and mouse anti-phosphorylated ERK1/2 clone E10 (New England Biolabs Inc.), and mouse anti-GFP (CLONTECH). Donkey secondary antibodies conjugated to fluorophores or horseradish peroxidase were obtained from Jackson ImmunoResearch Laboratories, Inc. All reagents were obtained from Sigma unless noted otherwise.

Isolation of Endosomes—Endosomes were isolated from HIRcB cells essentially as described previously by Rizzo *et al.* (21) using sucrose gradient purification followed by immunoisolation with antibodies raised against the C terminus of the insulin receptor (23). Cells were disrupted using a ball homogenizer as described previously (23). Preparations were normalized to the protein content of the cell homogenates prior to sucrose gradient purification. Western blots of the immunoisolates were used to detect the presence of EEA-1, Raf-1, GFP, Ras, phosphorylated MEK, and phosphorylated MAPK as described below. This fraction was found to contain markers for the early endocytic pathway, including clathrin (21) and EEA-1, indicating that this fraction is composed of membranes containing nascent endocytic vesicles. To determine the level of contamination of these endosomal preparations with plasma membrane vesicles, the surface plasma membrane glycoproteins of insulin-treated cells were biotinylated to label exterior membranes. Cells were incubated with 2 mM biotin-LC-hydrazide (Pierce) in 100 mM sodium acetate for 30 min at 4 °C as described by Deal *et al.* (60). Vesicles isolated from these preparations using the fractionation procedure described above did not contain significant amounts of biotinylated cell-surface proteins as determined by probing of Western blots with horseradish peroxidase-conjugated streptavidin and as resolved by chemiluminescence, thus demonstrating that these preparations were essentially free of contaminant plasma membranes vesicles that might have been derived from the homogenization procedure.

Quantitation of PA Levels in Endosomes—HIRcB cells were prelabeled with 10 μCi/ml [^{3}H]palmitate overnight prior to vesicle isolation. Lipids were extracted from isolated vesicles, and PA levels were quantitated as described previously (21, 22). PA levels are expressed as percentage of the total labeled lipid.

Confocal Microscopy—HIRcB cells were plated on poly-L-lysine-coated glass coverslips and transfected with the indicated constructs as described (21). Following stimulation, the coverslips were fixed in fresh 3% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min at 4 °C and permeabilized in 0.1% Triton X-100/PBS (2 min). After permeabilization, cells were washed extensively with PBS (4 × 5 min) and blocked with 3% bovine serum albumin/PBS. Primary antibodies were applied in a 3% bovine serum albumin/PBS solution for 1 h, followed by washing with PBS (3 × 10 min) and staining with primary antibodies as indicated below. Coverslips were then washed with PBS (3 × 10 min) and stained with Cy5-labeled anti-mouse IgG or tetramethylrhodamine B isothiocyanate-labeled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). Coverslips were washed prior to mounting on glass slides and analysis by confocal microscopy with a Leica TCS confocal system. Merged images were generated using Adobe Photoshop software. By convention, the first panel in each merged figure is colored *green*, the second *red*, and the third *blue*. Colocalization of two colors is indicated by *yellow*, and three-color colocalization is indicated by *white*. To more clearly present areas of colocalization between multiple labels, points of colocalization were filtered out using the bitwise "and" function on Invovision ISEE software. Images generated using this function contain only colocalized pixels.

Synthesis of BODIPY-Insulin—The BODIPY® 581/591 succinimidyl ester (Molecular Probes, Inc.) was conjugated to porcine pancreas insulin (Sigma) at room temperature. The reaction was stopped by addition of 25 mM ethanolamine. BODIPY-insulin was purified by Sephadex G-25 column chromatography. Cells were stimulated with 200 nM BODIPY-insulin for 5 min prior to fixation and immunostaining.

Generation of Fusion Proteins—Residue 398 of Raf-1 was mutated from arginine to alanine by the four-primer polymerase chain method and subcloned into the pEGFP-N1 vector (CLONTECH). Other Raf-1 mutants (R89L and K375M) were also subcloned into pEGFP-N1, whereas Ras constructs were subcloned into the pEGFP-C1 vector. The PA-binding region of Raf (residues 390–426) (24) was subcloned out of

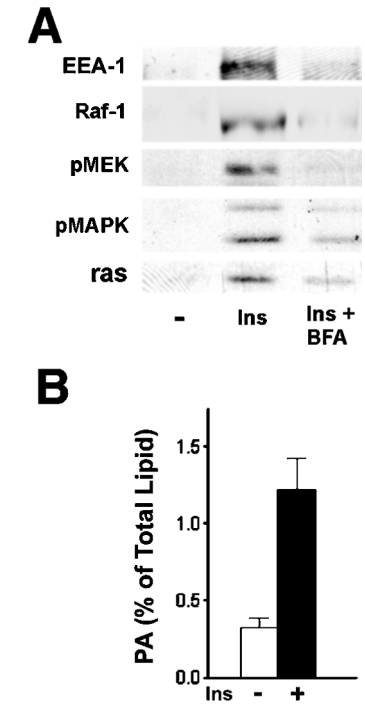

FIG. 1. **Insulin-dependent localization of signaling elements in isolated vesicles.** *A*, pretreatment with 10 μg/ml brefeldin A blocked insulin (*Ins*)-induced localization of EEA-1, Raf-1, phosphorylated MEK (*pMEK*), phosphorylated MAPK (*pMAPK*), and Ras to immunoisolated vesicles as determined by Western blotting. *B*, PA content of immunoisolated vesicles was determined from cells labeled with [^{3}H]palmitate as described under "Experimental Procedures" before and after stimulation with insulin. Cells were treated with brefeldin A 10 min prior to addition of insulin. Insulin stimulation was carried out for 5 min at 37 °C.

Raf-1 into pEGFP-C1 using *Bgl*II and *Eco*RI restriction sites. Single and double arginine-to-alanine mutations that correspond to Raf residues 398 and 401 were also introduced into this construct by site-directed mutagenesis. Successful generation of constructs was verified by sequencing and Western blotting.

Isolation of Recombinant Protein and Analysis of PA Affinity—Mutant and wild-type Raf-GFP fusion proteins were cloned into the pFLAG-MAC vector using *Eco*RI and *Kpn*I restriction sites. Recombinant protein was generated and affinity-purified on an anti-FLAG epitope M2 affinity column (Sigma) as described (25). The purity of recombinant protein was assessed by SDS-polyacrylamide gel electrophoresis; Coomassie Blue staining; and Western blotting using antibodies against the FLAG epitope, Raf-1, and GFP. The purified protein was then tested for its ability to bind PA-coated plates (1 μg of dioleoyl-PA/9 μg of dipalmitoylphosphatidylcholine (Avanti)/well) in an enzyme-linked immunosorbent assay-type assay as described by Ghosh *et al.* (24). Bound protein was detected using mouse anti-GFP antibodies (CLONTECH). Horseradish peroxidase activity was detected using AMPLEX Red reagent (Molecular Probes, Inc.).

Analysis of MAPK Phosphorylation—HIRcB cells were grown in 60-mm dishes and transfected with the indicated constructs using LipofectAMINE transfection reagent. Transfection efficiency was assessed by examination of the cells with a phase-contrast microscope and by determination of the GFP fluorescence. Efficiency of transfection varied between 70 and 90%. Cells were serum-starved for 18–24 h prior to insulin treatment. Cells were then scraped and lysed as described previously (21). Cell lysates were boiled in SDS sample buffer, and equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose membrane. The nitrocellulose membrane was then probed with a phospho-specific anti-MAPK antibody (New England Biolabs Inc.) and peroxidase-conjugated secondary antibodies (21). Immunocomplexes were detected by enhanced chemiluminescence.

RESULTS

Ras, Raf-1, MEK, and MAPK Accumulate in Endosomes in Insulin-treated Cells—Mitogenic signaling cascades are

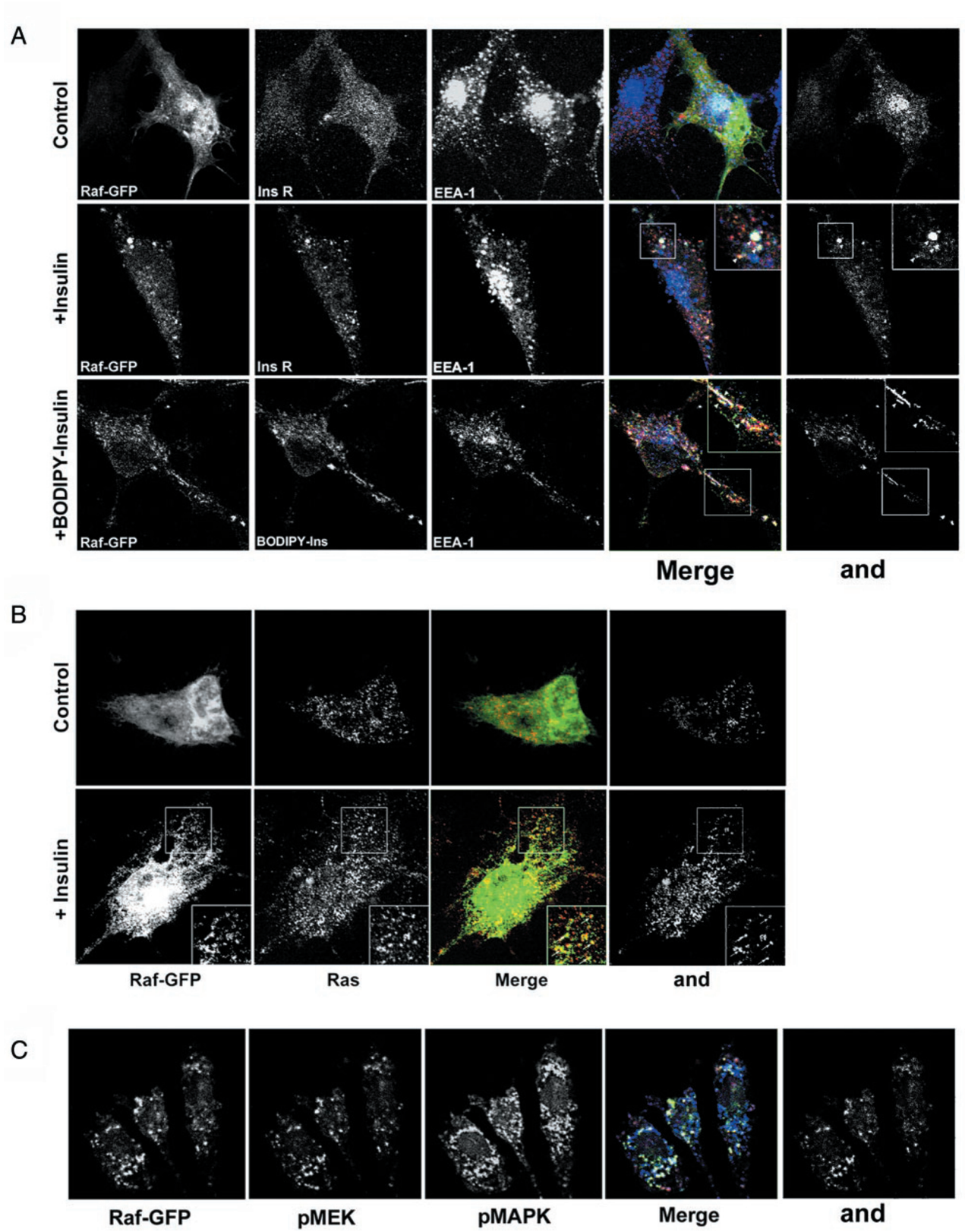

FIG. 2. **Insulin-dependent localization of signaling elements to endosomal structures.** *A*, insulin (200 nM, 5 min) stimulated colocalization of Raf-GFP, the insulin receptor (*Ins R*), and EEA-1 as determined by confocal microscopy of immunolabeled HIRcB cells. BODIPY-labeled insulin (*BIODIPY-Ins*) was also used to track receptor-mediated endocytosis. Merged images were generated as described under "Experimental Procedures." Points of colocalization between the multiple labels were filtered out using a bitwise "and" function as described under "Experimental Procedures." Images containing only colocalized points are labeled *and*. *Arrowheads* indicate colocalization between all markers. *B*, Ras and Raf colocalized in early endosomes in response to insulin as determined by immunostaining of Ras in cells expressing Raf-GFP. *Arrows* indicate colocalization between Ras and Raf. *C*, insulin-stimulated cells (5 min) that express Raf-GFP were labeled with phospho-specific anti-MEK (*pMEK*) and anti-MAPK (*pMAPK*) antibodies.

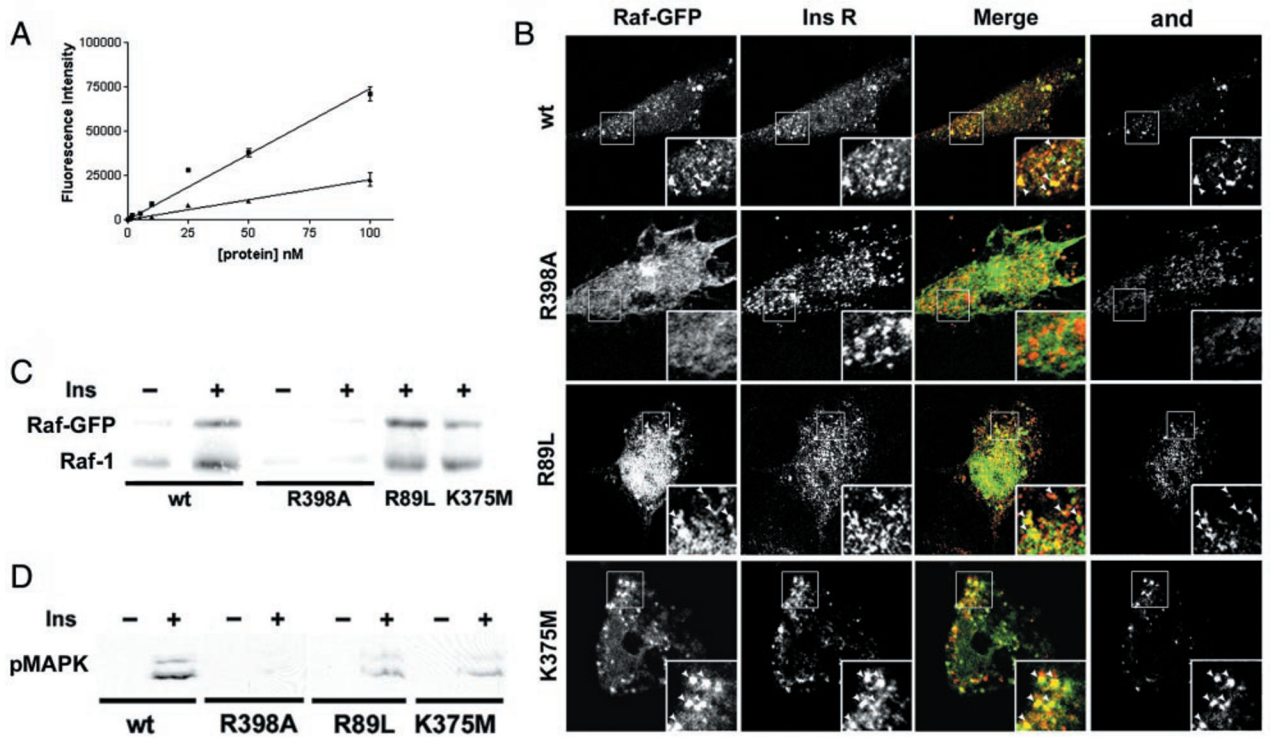

FIG. 3. **Raf-1 translocation requires PA, but is independent of association with Ras.** *A*, the ability of FLAG-tagged recombinant protein (■, FLAG-Raf-GFP; ▲, FLAG-Raf(R398A)-GFP) to bind to PA-coated dishes was examined using an enzyme-linked immunosorbent assay-type assay. The R398A mutation reduced the affinity of Raf-1 for PA. *B*, HIRcB cells expressing wild-type (*wt*) Raf-GFP or Raf-GFP containing mutations in the PA-binding region (R398A), the Ras-binding domain (R89L), or the kinase domain (K375M) were stimulated with insulin (5 min) and immunostained for the insulin receptor (*Ins R*). Points of colocalization between the multiple labels were filtered out using a bitwise "and" function as described under "Experimental Procedures." Images containing only colocalized points are labeled *and*. *Arrowheads* indicate colocalization between Ras and the insulin receptor. *C*, expression of Raf(R398A)-GFP inhibited insulin (*Ins*)-dependent translocation of endogenous Raf-1 to immunoisolated vesicles, whereas disruption of Ras binding or kinase activity had no effect on Raf translocation as shown by Western blotting using anti-Raf-1 antibodies. *D*, expression of Raf(R398A)-GFP inhibited insulin-induced MAPK phosphorylation. MAPK activation was assessed by Western blotting using antibodies specific for phosphorylated MAPK (*pMAPK*).

initiated on the plasma membrane. However, activated receptors are found in endocytic structures shortly after activation, along with many signaling elements, including Raf-1 (26–29). We have found that stimulation of HIRcB cells with insulin results in accumulation of Raf-1 in endocytic structures containing insulin receptors after transient association with plasma membranes (21). The association of Raf-1 with membranes has been linked to both Ras activation (16, 17) and the generation of PA by PLD (21). Therefore, we developed biochemical and imaging assays to examine the relative role of Ras and PA in recruiting Raf-1 to membranes.

Insulin stimulated the accumulation of Ras, Raf-1, phosphorylated MEK, and phosphorylated MAPK in endocytic vesicles containing insulin receptors (Fig. 1*A*). Consistent with our previous findings that Raf-1 recruitment to membranes requires PA, vesicles isolated after insulin treatment were substantially enriched in PA (Fig. 1*B*). We have previously reported that brefeldin A (BFA) blocks signaling through insulin-dependent MAPK phosphorylation and insulin-dependent PA generation (21, 22). Furthermore, we also showed that the effects of BFA on the MAPK cascade are reversed in the presence of PA, indicating that BFA inhibits insulin-dependent MAPK signaling through its effects on PLD activation (21). Therefore, cells were pretreated with BFA to examine the role of PLD activation in the recruitment of signaling molecules to endosomes. As shown, vesicles isolated from BFA-treated cells contained significantly smaller quantities of the early endosome marker EEA-1 (59), Raf-1, phosphorylated MEK, and phosphorylated MAPK compared with vesicles isolated from cells that had not been treated with BFA. The inhibition of the accumulation of insulin receptors, Raf-1, and other signaling molecules in the endosomal compartment is consistent with previous reports showing that the internalization of the insulin receptor is sensitive to BFA treatment (21). These findings are consistent with a role for PA in endocytic trafficking and in recruitment of proteins to endosomes. However, our data do not exclude a role for ADP-ribosylation factor proteins in mediating these effects by mechanisms independent of PLD activation.

The subcellular localization of the components of the MAPK cascade was further examined using confocal microscopy. In the absence of stimulation, Raf-GFP was diffusely localized in the cytosol. Insulin treatment resulted in the accumulation of Raf-GFP in vesicles that contained insulin receptors and EEA-1. To facilitate analysis of multiple labeling studies, points of colocalization between the multiple labels were filtered out using a bitwise "and" function. As shown in Fig. 2*A*, all vesicles containing Raf-GFP also contained the insulin receptor and EEA-1, although many early endosomes contained no Raf-GFP. Furthermore, all Raf-GFP-containing vesicles were labeled with exogenously added BODIPY-conjugated insulin, indicating that the endosomes containing Raf-GFP were derived from the cell surface. Very little Raf-GFP was found on the plasma membrane after 5 min of insulin treatment. This is consistent with our previous studies on the localization of endogenous Raf-1 in HIRcB cells after insulin treatment (21). Ras, phosphorylated MEK, and phosphorylated MAPK also accumulated in endosomes along with insulin receptors and Raf-GFP (Fig. 2, *B* and *C*). The accumulation of activated signaling elements in early endosomes suggests that these

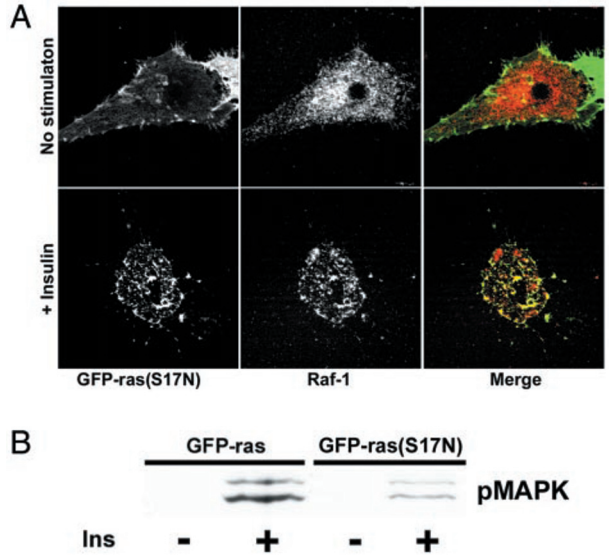

FIG. 4. **Raf-1 translocation does not require Ras activation.** *A*, HIRcB cells expressing Ras(S17N)-GFP were stimulated with insulin (*Ins*; 5 min) and immunostained for endogenous Raf-1 localization. Expression of this dominant-negative Ras did not inhibit insulin-dependent Raf-1 translocation. *B*, Ras(S17N)-GFP blocked insulin-dependent MAPK phosphorylation as shown by Western blotting. *pMAPK*, phosphorylated MAPK.

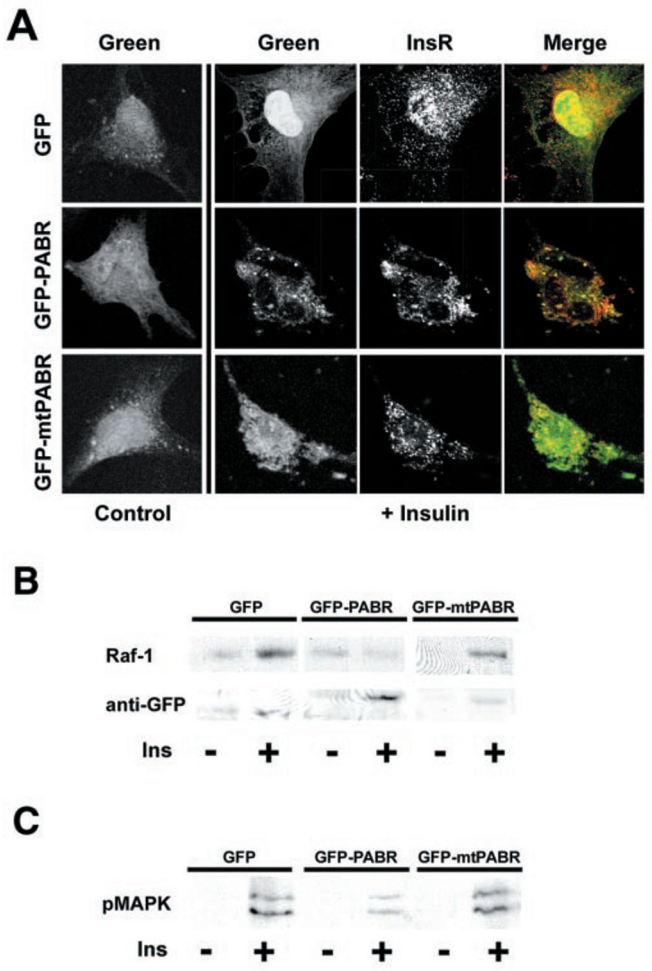

FIG. 5. **The PA-binding region is sufficient to target GFP to the correct subcellular compartment.** HIRcB cells were transfected with GFP, GFP-PABR, or GFP-mtPABR and serum-starved prior to insulin stimulation (200 nM, 5 min) as indicated. *A*, cells that were fixed and stained for the insulin receptor (*InsR*) were analyzed using confocal microscopy. *B*, endosomes containing insulin (*Ins*) receptors were immunopurified from cell lysates. The presence of Raf-1 or GFP-tagged constructs was examined by Western blotting. *C*, cell lysates were examined for phosphorylated MAPK (*pMAPK*) by Western blotting.

endosomes constitute important elements of the MAPK signaling cascade.

Phosphatidic Acid Targets Raf-1 to Endosomes—To assess the relative contributions of Ras and PA to Raf-1 recruitment to membranes, we introduced point mutations in Raf-1 with the purpose of disrupting the interactions of Raf-1 with Ras or PA. The PA-binding region of Raf-1 contains three highly conserved cationic residues (24). Mutation of one of these residues to alanine (R398A) was sufficient to disrupt the ability of recombinant Raf proteins to associate with PA using a microtiter plate binding assay as described by Ghosh *et al.* (24) (Fig. 3*A*). It should be noted that this assay may not be ideal to assess the affinity of Raf-1 for PA in quantitative terms and that the interaction of Raf-1 with other lipids cannot be fully ruled out. This assay only allows the comparison of the relative affinities of different Raf-1 mutants for the adsorbed lipid mixture. As shown, mutation of Arg^{398} to Ala significantly decreased the binding affinity of Raf-1 for the adsorbed lipid.

The PA-binding region is imbedded in the kinase domain of Raf-1. However, mutations in the PA-binding region do not alter the transforming activity of v-Raf (30), indicating that the kinase activity of Raf-1 is unaffected by these mutations. Furthermore, these mutations did not alter the ability of the construct to associate with endogenous Raf-1 and did not disrupt the basal activity of the kinase (data not shown), indicating that this mutation is unlikely to significantly disrupt protein folding. The interaction between Ras and Raf-1 was disrupted by a single mutation in the Ras-binding domain of Raf-1 (R89L). This mutant has been previously described (31). Insulin treatment stimulated the translocation of Raf-GFP, Raf(R89L)-GFP, and catalytically inactive Raf(K375M)-GFP (32) to vesicles containing insulin receptors as determined by confocal microscopy (Fig. 3*B*). However, Raf(R398A)-GFP remained diffuse after insulin treatment and did not significantly associate with vesicles or the plasma membrane. Insulin receptor internalization was unaffected. Disruption of Raf-PA interactions (R398A) also inhibited insulin-dependent recruitment of both the mutant construct and endogenous protein to isolated vesicles, whereas disruption of Ras-Raf interactions or Raf-1 kinase activity (K375M) did not affect Raf-1 translocation (Fig. 3*C*). It is likely that expression of Raf(R398A)-GFP prevents translocation of endogenous Raf-1 via the formation of oligomeric complexes with the native protein (33). In support of this, endogenous Raf-1 co-immunoprecipitated with Raf(R398A)-GFP (data not shown). These data indicate that Raf-1 translocation is independent of its association with Ras, but requires PA binding. The overexpression of each one of these mutations inhibited insulin-dependent MAPK phosphorylation (Fig. 3*D*) to an extent that was consistent with transfection efficiency. This indicates that two different kinds of interactions, namely the association of Ras with Raf-1 and the binding of Raf-1 to PA-containing membranes, are required for the activation of the MAPK cascade.

The role of Ras activation in Raf-1 recruitment was then assessed by expression of a dominant-negative Ras mutant (S17N) that is unable to bind GTP. Expression of this mutant has been shown to suppress Ras activation through sequestration of endogenous guanine nucleotide exchange factors (34, 35). Transfection of this mutant in HIRcB cells did not alter agonist-dependent translocation of endogenous Raf-1 to vesicles (Fig. 4*A*). However, Ras(S17N) significantly inhibited insulin-dependent MAPK phosphorylation to a degree that was con-

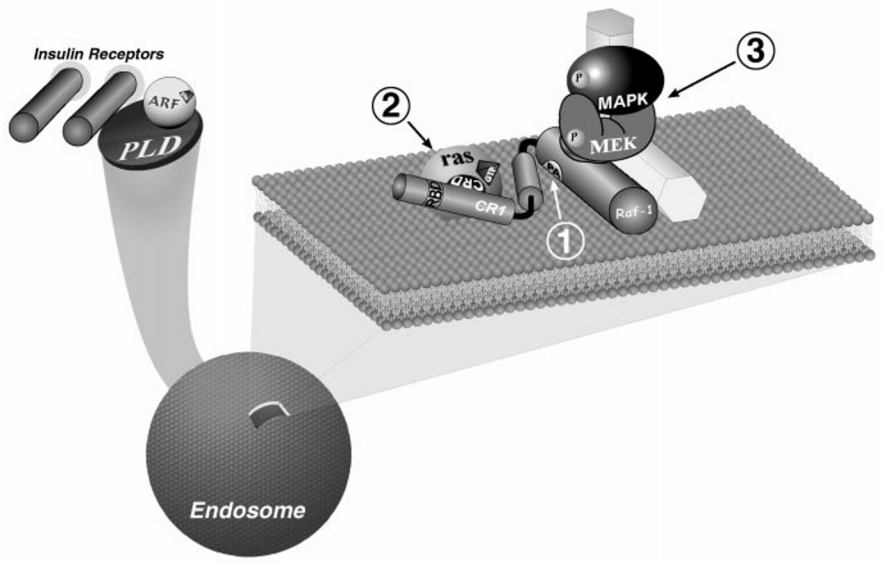

FIG. 6. **Model of agonist-dependent Raf-1 activation.** Activation of the insulin receptor results in stimulation of the PLD pathway through the ADP-ribosylation factor (*ARF*) and activation of Ras through insulin receptor substrate-1 and Grb2/Sos. PA then recruits Raf-1 to the membrane through direct interaction with the PABR (1). Subsequently, interaction between RasGTP and Raf-1 results in activation of Raf-1 (2). Finally, active Raf-1 phosphorylates MEK, which in turn phosphorylates MAPK (3). MEK and MAPK are probably recruited to membranes by a scaffolding protein. Concurrent with activation of this cascade, the components of the MAPK cascade migrate along with internalized insulin receptors and accumulate in early endosomes. *RBD*, Ras-binding domain.

sistent with the observed transfection efficiency. These results demonstrate that, although Raf-1 translocation to membranes does not require Ras activation, Ras signaling has a critical function in the activation of the MAPK cascade.

The PA-binding Region of Raf-1 Targets Proteins to Endosomes—To determine if the association with PA was sufficient to explain the translocation of Raf-1 to membranes, the PA-binding region (PABR) of Raf-1 was fused to GFP and expressed in HIRcB cells. The PABR is a highly conserved 36-amino acid region in the kinase domain of Raf-1 that has been shown to associate with PA *in vitro* (24). Prior to stimulation, GFP-PABR was diffusely localized in the cytosol (Fig. 5*A*). Insulin stimulation resulted in the translocation of GFP-PABR to vesicles containing insulin receptors (Fig. 5*A*). This result demonstrates that association between PA and the PABR is sufficient to explain Raf-1 targeting. Furthermore, expression of GFP-PABR inhibited insulin-dependent Raf-1 translocation to isolated vesicles (Fig. 5*B*) and insulin-dependent MAPK phosphorylation, indicating that association between Raf-1 and PA is absolutely essential for Raf-1 translocation and function. As a control, arginine-to-alanine mutations that would be expected to reduce the affinity of PABR for PA were made in the PABR. The PABR contains a cluster of three basic residues (Arg^{398}, Lys^{399}, and Arg^{401}) of the full-length protein. Mutation of both arginines in GFP-PABR generated a construct (GFP-mtPABR) that failed to significantly associate with endosomes upon stimulation of the cells with insulin (Fig. 5*A*). Some vesicle association (<5%) was observed; however, overexpression of GFP-mtPABR was unable to block the translocation of endogenous Raf-1 to endosomes (Fig. 5*B*) or the phosphorylation of MAPK (Fig. 5*C*). Given that GFP-PABR inhibited both Raf-1 translocation and MAPK phosphorylation and that GFP-mtPABR did not, we conclude that the effects of GFP-PABR are a consequence of the sequestration of PA. It should be noted that a single arginine-to-alanine mutation (position 398 of full-length Raf-1) was insufficient to eliminate the ability of GFP-PABR to block endogenous Raf-1 translocation and MAPK phosphorylation (data not shown). These observations are in contrast with those obtained with full-length Raf-1, where the R398A mutation blocked Raf-1 translocation and inhibited MAPK phosphorylation. The structural basis for these differences is not clear at this time. It is possible that the smaller PABR fragment is sufficiently flexible to bind PA such that the mutation of a single arginine does not prevent the interaction with PA. Full-length Raf-1 may be a significantly more rigid structure such that a single mutation is sufficient to dramatically reduce the affinity of Raf-1 for PA.

DISCUSSION

Endocytosis and mitogenic signaling are parallel functions associated with the activation of extracellular receptors. The two pathways are clearly interrelated and share many common elements. For example, the Ras exchange factor Sos plays an important role in regulating dynamin function (36, 37). Src and phosphatidylinositol 3-kinase are other factors that play key roles in both mitogenesis and endocytosis (38–42). Moreover, the components of the MAPK signaling cascade appear to accumulate in structures associated with membrane traffic, including caveolae (29) and endosomes (26, 27). We show here that insulin stimulation of HIRcB cells results in the accumulation of components of the MAPK signaling cascade in endocytic structures. Agonist-induced translocation of Ras, Raf-1, phosphorylated MEK, and phosphorylated MAPK to plasma membranes was transient at best. Five minutes after insulin stimulation, these components were predominantly localized in a subset of early endosomes. BODIPY-conjugated insulin also labeled these endosomes, indicating that the components of the MAPK cascade traffic with endocytic structures. It is unlikely that these results are due to overexpression of GFP-tagged constructs since the results obtained from experiments examining GFP-tagged constructs or endogenous proteins were similar. Furthermore, expression of GFP-tagged constructs was within the same order of magnitude as expression of endogenous proteins, thus making it unlikely that the results observed were a consequence of massive overexpression of certain cellular components.

Although the physiological significance of the accumulation of these signaling components on endosomal membranes has not been fully elucidated, it is clear that endocytosis is linked to the regulation of the MAPK activity in many receptor systems (43–46). In fact, the compartmentalization of signaling components to endocytic structures is an attractive model for mitogenic signaling. Endosomes are enriched in signaling lipids such as phosphatidylinositol phosphates (47–49) and phosphatidic acid (see Fig. 1*B*). Therefore, endosomes may complement scaffolding proteins via the recruitment of signaling molecules to specialized lipid surfaces. Endosomal localization of specific signaling components also opens the possibility for cross-talk between mitogenic signaling pathways and the endocytic ma-

chinery. For example, recent evidence has shown that ERK1 can phosphorylate Rab5a *in vitro* (50).

The recruitment of proteins to lipid membranes has been shown to be regulated by direct association with resident membrane proteins and with lipid second messengers. For example, recruitment of EEA-1 to membranes requires the direct association of EEA-1 with both activated Rab5 and the lipid second messenger phosphatidylinositol 3-phosphate (51–53). Recruitment of Raf-1 to membranes has also been shown to be regulated by lipid and protein factors. Early reports concluded that Ras determined the recruitment of Raf-1 to membranes. More recently, the PLD product phosphatidic acid was also identified as a second regulator of agonist-dependent Raf-1 translocation. This model is consistent with the ubiquitous nature of PLD activation by mitogenic signals (54). Here we have examined the relative contribution of Ras and PA to Raf-1 translocation, and we conclude that interaction with PA is the primary mechanism that mediates the translocation of Raf-1 to membranes.

The conclusion that the interactions with PA constitute the driving force for the translocation of Raf-1 to membranes is based on several pieces of evidence. First, we have shown that PA promotes translocation of Raf-1 to membranes without promoting its activation (21). Second, Raf-1 mutants that cannot interact with Ras are efficiently translocated to endosomal membranes (Fig. 3). Third, dominant-negative Ras mutants do not block the translocation of Raf-1 to membranes. Fourth, the mutation of a single basic residue in the PA-binding region significantly reduces agonist-dependent Raf-1 translocation and agonist-dependent MAPK phosphorylation in a manner that closely parallels the reduced affinity of this mutant for phosphatidic acid. Fifth, the PA-binding region of Raf-1 is sufficient to target GFP to endosomal structures containing internalized insulin receptors. Finally, overexpression of a GFP-PABR construct blocks recruitment of Raf-1 to endosomes and concurrently inhibits the phosphorylation of MAPK. In contrast, a mutated PABR that contains two arginine-to-alanine mutations in the conserved polybasic cluster does not translocate and does not block insulin-dependent Raf-1 translocation or MAPK phosphorylation. Contrary to most current models (55, 56), the interaction of Ras with Raf-1 was not found to be essential for agonist-dependent Raf-1 translocation. However, Ras activity and Ras-Raf interactions were absolutely required for the downstream effects of Raf-1 such as phosphorylation of MAPK. These data suggest that the currently accepted model of Raf-1 translocation needs to be revised.

The evidence linking Ras to the translocation of Raf-1 to membranes was based in part on the observation that RasGTP could not activate Raf-1 *in vitro* (18–20). Furthermore, the attachment of the C-terminal C*AAX* motif from Ras to Raf-1 resulted in membrane targeting, leading to some Ras-independent kinase (16, 17). It was reasoned that since Ras could not be shown to activate Raf-1 in the test tube and since membrane translocation was sufficient to provide some level of Raf-1 activation, the function of Ras-Raf interactions was mainly the recruitment of Raf-1 to membranes (20). However, subsequent work has demonstrated that Ras does activate Raf-1 by interacting with the cysteine-rich domain of Raf-1 (55). Furthermore, Mineo *et al.* (5) used complementary mutations between Ras and Raf-1 to show that the activation of membrane-localized Raf-1 was in fact sensitive to regulation by Ras, contrary to previous reports. Finally, *in vitro* activation of Raf-1 by Ras was demonstrated by Inouye *et al.* (57). Therefore, the main role of Ras appears to be the activation of Raf-1. Our data support this model. Disruption of Ras activation or the ability of Ras to bind Raf-1 prevented agonist-dependent MAPK phosphorylation, although neither of these treatments prevented Raf-1 translocation. This clearly demonstrates that Raf-1 translocation and Raf-1 activation are independently regulated events.

The data presented here suggest a model for the activation of the MAPK cascade by extracellular signals (Fig. 6). Stimulation of cell-surface receptors results in the concurrent activation of multiple pathways. PA generated by PLD recruits Raf-1 to membranes, where it interacts with Ras on the Ras-binding domain and the cysteine-rich domain, resulting in kinase activation. Concurrently, Ras and Raf-1 are internalized along with the activated receptor. Activated Raf-1 then phosphorylates MEK, which in turn phosphorylates MAPK. Whether or not scaffolding proteins are involved (58) in the recruitment of MEK and MAPK to these endosomes is not known at this time. This is the simplest possible explanation of all the available data.

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