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The Adaptor Protein Grb14 Regulates the Localization of 3-Phosphoinositide-dependent Kinase-1*

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Charles C. King and Alexandra C. Newton‡

From the Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0721

The metabolic actions of insulin are transduced through the phosphatidylinositol 3-kinase pathway. A critical component of this pathway is 3-phosphoinositide-dependent kinase-1 (PDK-1), a PH domain-containing enzyme that catalyzes the activating phosphorylation for many AGC kinases, including Akt and protein kinase C isozymes. We used a directed proteomics-based approach to identify the adaptor protein Grb14, which binds the insulin receptor through an SH2 domain, as a novel PDK-1 binding partner. Interaction of these two proteins is constitutive and mediated by a PDK-1 binding motif on Grb14. Disruption of this motif by point mutation or deletion of the Grb14 SH2 domain prevents the insulin-triggered membrane translocation of PDK-1. The interaction of PDK-1 with Grb14 facilitates Akt function: disruption of the interaction by overexpression of a construct of Grb14 mutated in the PDK-1 binding motif significantly decreases insulin-dependent activation of Akt. Thus, Grb14 serves as an adaptor protein to recruit PDK-1 to activated insulin receptor, thus promoting Akt phosphorylation and transduction of the insulin signal.

Members of the AGC family of protein kinases require phosphorylation at a conserved threonine residue in a segment near the entrance to the enzyme active site, termed the activation loop, for maximal catalytic activity (1). The 3-phosphoinositide-dependent protein kinase-1 (PDK-1)[1] is a ubiquitously expressed, constitutively active enzyme that catalyzes phosphorylation at this key regulatory position. Originally identified as the activator of Akt (2, 3), PDK-1 is now known to phosphorylate many diverse kinases, including $p70^{S6}$ kinase, $p90^{RSK}$, conventional, novel, and atypical protein kinase C (PKC) isozymes, PKC-related kinases, p21-activated kinase, and serum and glucocorticoid kinase (4–6). Each of these kinases controls specific signaling pathways, placing PDK-1 at the apex of complex networks of intracellular signaling. Fidelity and specificity are, in part, maintained by substrate conformation and location, which dictate availability for phosphorylation by PDK-1 (4). However, having one kinase provide the activating step to abundant pathways likely involves other regulatory mechanisms.

PDK-1 plays a central role in transduction of signals from insulin by providing the activating phosphorylation to Akt, thus propagating the signal to downstream targets controlling cell proliferation and survival, as well as glucose and amino acid uptake and storage (6–8). The initial binding of insulin to the insulin receptor activates phosphatidylinositol 3-OH-kinase (PtdIns 3-kinase) (9–11), an event that is required for effective insulin signaling (12–15). Members of the PtdIns 3-kinase family of enzymes phosphorylate inositol phospholipids at the D-3 position of the inositol ring to generate phosphatidylinositol 3,4-bisphosphate (PtdIns-3,4-P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3). These lipids serve as membrane docking sites for many pleckstrin homology (PH) domain-containing proteins, mediating their recruitment to membranes following activation of PtdIns 3-kinase (16–18). Akt is an immediate downstream target of PtdIns 3-kinase activation. It contains a PH domain that specifically binds 3-phosphoinositides, causing the translocation of Akt to membranes. Membrane binding results in phosphorylation by PDK-1 on the exposed activation loop; this latter kinase also contains a PH domain that binds 3-phosphoinositides (2, 3). Thus, Akt translocation triggers its phosphorylation and activation by PDK-1.

PDK-1 contains a carboxyl-terminal PH domain that binds PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 *in vitro* with low nanomolar affinity, however, lipid binding has no significant effect on the intrinsic activity of the enzyme (19). Whether PtdIns 3-kinase controls the spatial distribution of PDK-1 in cells is less clear. It is uniformly observed that PDK-1 is localized to the cytoplasm in quiescent cells, however, there are conflicting studies on whether activation of PtdIns 3-kinase causes membrane redistribution. Anderson *et al.* (20) demonstrated translocation of overexpressed PDK-1 to plasma membranes in porcine aortic endothelial cells stimulated with PDGF, and Kim *et al.* (21) reported translocation of enhanced green fluorescent protein-tagged PDK-1 to plasma membranes in epidermal growth factor-stimulated Rat-2 cells. However, Currie *et al.* (19) reported no translocation of overexpressed PDK-1 in porcine aortic endothelial or 293 cells stimulated with PDGF or insulin-like growth factor-1. The authors noted that the amount of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 in cells is much greater than the amount of PDK-1 within a cell, suggesting that other factors or proteins, which are limiting in cells overexpressing PDK-1, play a role in recruiting PDK-1 to membranes. Studies of endogenous PDK-1 have been limited by the availability of appropriate antibodies.

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of California at San Diego, La Jolla, CA 92093-0721. Tel.: 858-534-4527; Fax: 858-822-5888; E-mail: anewton@ucsd.edu.

[1] The abbreviations used are: PDK-1, 3-phosphoinositide-dependent kinase-1; PDGF, platelet-derived growth factor; PKC, protein kinase C; PtdIns-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; PH, pleckstrin homology domain; HA, hemagglutinin; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; SH2, Src Homology 2; PRR, proline-rich region; PtdIns 3-kinase, phosphatidylinositol 3-kinase)

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Although activation of PtdIns 3-kinase plays an important role in Akt phosphorylation, other mechanisms controlling the phosphorylation of PDK-1 substrates have been identified. Substrates such as conventional protein kinase C isoforms are constitutively phosphorylated by PDK-1 in a reaction that is insensitive to PtdIns 3-kinase activity (22). The derivation of the name for PDK-1 arises from the phosphoinositide dependence of its first discovered substrate, Akt, whose phosphorylation requires PtdIns 3-kinase activation to present itself as a substrate to PDK-1 (23). However, PDK-1 directly docks to substrates such as protein kinase C and $p70^{S6}$ kinase, whose phosphorylation is not dependent on phosphoinositides. This docking is conformationally controlled by the substrate. Other regulatory inputs into PDK-1 activity include tyrosine phosphorylation, which occurs on multiple sites in response to cellular stress and activation (24–26). In addition, the biologically active lipid sphingosine directly activates PDK-1 (27). Tian *et al.* (28) reported that the amino-terminal region of PDK-1 binds and activates the guanine nucleotide exchange factor Ral GDS. Whether scaffold proteins regulate the function of PDK-1 by poising it at specific intracellular sites is largely unexplored.

Grb14 is a multidomain protein that is a member of the Grb7 family of proteins, which also includes Grb7, Grb10, and the *Caenorhabditis elegans* protein Mig 10 (29). Little is known about the function of Grb14; however, it has been reported to localize via an SH2 domain to many different receptor tyrosine kinases, including fibroblast growth factor receptor 1 (30), platelet-derived growth factor (PDGF) receptor (29), epidermal growth factor receptor, and insulin receptor (31). Thus, most of the knowledge about Grb14 comes from studies detailing the receptor binding mediated through the SH2 and BPS domains (29–31). Grb14 has recently been hypothesized to play a role in the termination of insulin signaling (31–33).

In the present study, we used a directed proteomics-based approach to identify the adaptor protein Grb14 as a novel PDK-1 interacting protein. Biochemical studies indicate that the Grb14-PDK-1 interaction is direct and mediated through a conserved motif within Grb14 similar to a previously described PDK-1 consensus binding sequence. Disruption of the PDK-1 binding motif or the SH2 domain in Grb14 blocks PDK-1 membrane translocation in response to insulin, but not PDGF. Phosphorylation of endogenous Akt, but not PKCα, is reduced in the presence of these Grb14 mutants, suggesting only PtdIns 3-kinase-dependent substrates of PDK-1 are subject to regulation by Grb14. These data provide evidence for a novel mechanism to regulate PDK-1 localization.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies to the phosphorylated activation loop of Akt (Thr-308) and PDK-1 (Ser-241) were purchased from Cell Signaling. The polyclonal phosphospecific antibody that recognizes the phosphorylated activation loop of PKC isozymes (P500 based on the nomenclature of the βII isozyme, but referred to as P497 in this report, based on the phosphorylation site of PKCα) was generated as described previously (34). HA-tagged murine Akt α and 9E10 Myc-tagged human PDK-1 with a deleted PH domain (Myc-ΔPH-PDK-1) was a gift of Dr. Alex Toker (Harvard Medical School). Polyclonal antibodies to Grb14 were purchased from Chemicon and from Santa Cruz Biotechnology. PDGF was purchased from Upstate Biotechnology, and insulin was purchased from Sigma. All other materials were of reagent grade.

cDNA Constructs and Plasmids—Human PDK-1 was cloned into the pCMV5M vector containing an amino-terminal 9E10 Myc-epitope tag as described previously (35). The cDNA for Grb14, a kind gift from Pam Maher (Scripps Research Institute), was cloned in pCDNA3 containing an amino-terminal Xpress tag. Grb14 E195A mutations were made by PCR amplification using a QuikChange kit (Stratagene) according to the manufacturer's instructions, followed by sequencing to confirm mutations. The primers for Grb14 E195A were as follows: forward primer, 5′-GCCAAATATgcgTTCTTTAAAAACCC-3′; reverse primer, 5′-GGGTTTTTAAAGAAcgcATATTTGGC-3′. Lowercase base pairs represent the mutated amino acid. Grb14 1–342 truncations were generated by PCR with KpnI/HindIII sites and cloned into pCMV5-HA3 vector (a kind gift from Dr. Frank Zenke, Scripps Research Institute) and amplified. The primers for the truncated Grb14 construct were: forward primer, 5′-GGGGTACCATGACCACTTCCCTGCAAG-3′; reverse primer, 5′-CCCAAGCTTGGGTTACTGCATGCCATACTTAAGC-3′. All constructs were sequenced to confirm mutations and/or truncations.

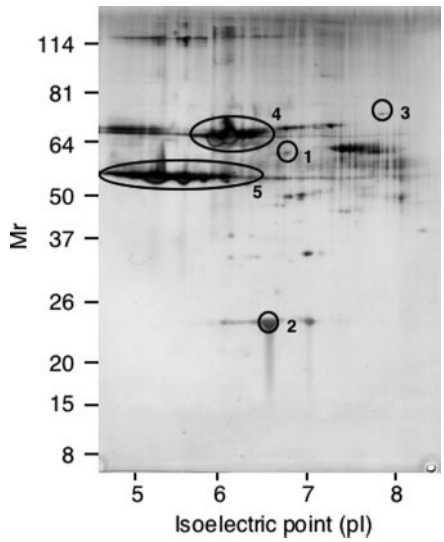

FIG. 1. **Silver-stained two-dimensional gel showing proteins that associate with PDK-1 in HEK 293 cells.** Myc-PDK-1 was immunoprecipitated from the detergent-soluble fraction of HEK 293 cells and bound proteins were separated by two-dimensional electrophoresis, silver stained, and analyzed by MALDI-TOF MS as described under "Experimental Procedures." Five of the identified proteins are indicated: the novel interactors Grb14 (*spot 1*) and heat shock protein 27 (*spot 2*), the known interactor protein kinase Cα (*spot 3*), Myc PDK-1 (*spot 4*), and bovine serum albumin (from coating the Protein A/G beads)
(*spot 5*).

Cell Culture and Transfection—All cell lines used in these studies (HEK 293 and MCF-7) were maintained in Dulbecco's modified Eagle's medium (BioWhittaker) containing 10% heat-inactivated fetal bovine serum (Omega Scientific Inc.). HEK 293 cells (seeded at 0.2×10^6 cells/60-mm dish) were grown to 60–70% confluence and transfected (2 μg of total plasmid DNA and 10 μl of Superfect) according to the manufacturer's protocol (Qiagen). 30 h post-transfection, cells were transferred to serum-free media for 18 h, then stimulated with insulin (100 nM). Cells were subsequently washed with phosphate-buffered saline. Lysis buffer A (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 150 IU/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) containing 1% Triton X-100 was added to each plate, the cell lysates were then centrifuged at 14,000 rpm for 10 min, and the detergent-soluble supernatants were collected.

Cell Fractionation—For experiments involving separation of the cytosol and membrane fractions, HEK 293 cells were resuspended in lysis buffer B (20 mM HEPES, pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM $MgCl_2$, 300 μM phenylmethylsulfonyl fluoride, 1 mM vanadate, 40 μg/ml leupeptin, and 1 μM microcystin) and sonicated (Branson bath sonicator). Whole cell lysates were centrifuged at $100{,}000 \times g$ for 30 min at 4 °C, and the resulting supernatant (cytosol, C) was removed. The pellet was resuspended in lysis buffer B containing 1% Triton X-100 and centrifuged at $100{,}000 \times g$ for 20 min at 4 °C, and the resulting supernatant containing solubilized membrane components (membrane, M) was removed (22).

Immunoprecipitation—An anti-HA antibody and anti-Xpress antibody were used to immunoprecipitate the HA- or Xpress-Grb14, respectively. Cell lysates were immunoprecipitated in the presence of 100 μl of protein A/G-agarose and 1 μl of specific antibodies for 16 h at 4 °C. Agarose beads were washed three times with 300 μl of lysis buffer containing 1% Triton X-100 and twice with 300 μl of 0.5 M NaCl. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. Grb14 (or PDK-1) was detected by Western blot analysis using Chemiluminescence. Chemiluminescence was quantified

TABLE I
Tryptic peptides from a novel PDK-1 interacting protein match the adaptor protein Grb14
Peptides detected by MALDI-TOF from a protein of ~60 kDa co-immuoprecipitating with PDK-1.

Observed mass, MH^+	Predicted mass, MH^+	Difference	Peptide sequence
1052.5935	1052.6104	−0.0169	ALLPLPDGTR
1443.6815	1443.7014	−0.0199	DSPLAAQVCGAAQGR
1669.9393	1670.0005	−0.0612	LIIQQGLVDGVFLVR
2258.9729	2259.1086	−0.1357	HLQFFSEFGNSDIYVSLAGK

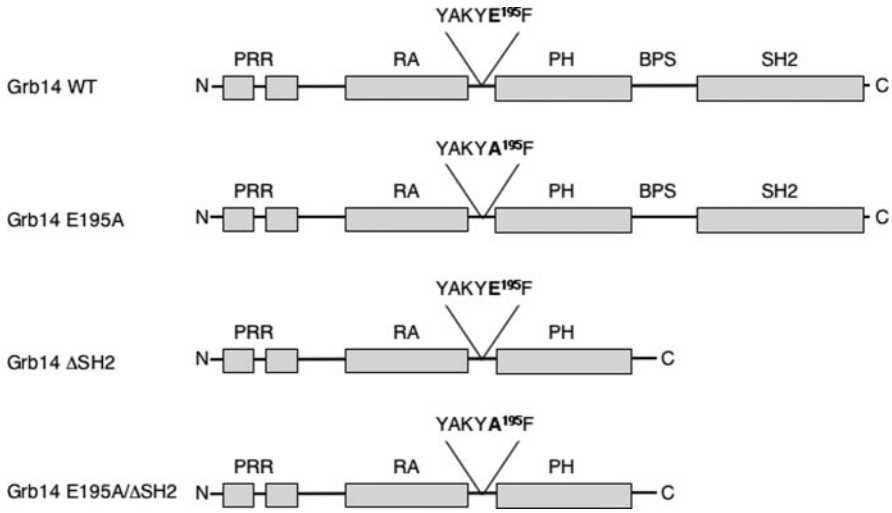

FIG. 2. **Diagram showing domain composition of Grb14 and constructs used in this study.** Grb14 wild type (*Grb 14 WT*) contains two amino-terminal proline-rich regions (*PRR*), a Ras association domain (*RA*), a pleckstrin homology (*PH*) domain, and a carboxyl-terminal SH2 domain. Additionally, He *et al.* (46) identified a region between the PH and SH2 domains (*BPS*) as required for receptor interaction. Also indicated is a consensus PDK-1 binding motif (Φ*XX*ΦEΦ) positioned around Glu-195, identified by the program Scansite (available at scansite.mit.edu/). The following constructs were made: Grb14 E195A in which a key determinant in the PDK-1 binding motif, Glu-195, was mutated to Ala; Grb14 ΔSH2 in which the protein was truncated at amino acid 342 to eliminate the regions required for receptor association; and Grb14 E195A/ΔSH2 in which both the PDK-1 binding site was mutated and the receptor interaction region was deleted.

by with a charge-coupled device camera using a GeneGnome bio-imaging system (Syngene).

Two-dimensional Electrophoresis/MALDI-TOF MS—HEK 293 cells transfected with Myc-PDK-1 were lysed in buffer A containing 1% Triton X-100 and centrifuged at 14,000 rpm, 10 min, to obtain the detergent-soluble fraction. Sample containing ~500 μg of protein was incubated with Myc antibodies and protein A/G beads (Pierce) for 12 h at 4 °C. The beads were washed three times in detergent-containing buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES, pH 7.3, 1% Triton X-100, 1.0 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride). The beads were resuspended in 9 M urea/4% CHAPS containing 15 mg of dithiothreitol and 0.5% ampholytes (pI range 3–10; Bio-Rad) and passively absorbed onto immobilized pH gradient strips (pI range 3–10; Bio-Rad) for 12 h at 20 °C. Isoelectric focusing was performed for 35,000 V-h on a Protean IEF System (Bio-Rad), electrophoresis was carried out using the Criterion gel system (Bio-Rad), and the samples were stained with Coomassie Brilliant Blue or Silver Quest (Invitrogen). Bands containing potentially novel PDK-1-interacting proteins were excised, washed three times in 50% acetonitrile/50% NH_4HCO_3, and lyophilized. Proteins were subjected to in-gel reduction with 10 mM dithiothreitol at 56 °C for 1 h and alkylation with 100 mM iodoacetic acid for 30 min at room temperature, followed by digestion with 0.5 μg of trypsin (Roche Applied Science). Isolated peptides were washed and concentrated in C_{18} ZipTips (Millipore) according to the manufacturer's protocol, mixed with α cyano-4-hydroxycinnamic acid (Sigma), and spotted onto a platform. MALDI-TOF MS was performed on an Applied Biosystems DE STR mass spectrometer. Peptides were analyzed with the online data base at the University of California, San Francisco (available at prospector.ucsf.edu).

RESULTS

To understand the role of scaffold proteins in regulating-PDK-1, we employed a proteomics-based approach to identify novel proteins that interact with the kinase. Myc-tagged PDK-1 was transiently overexpressed in HEK 293 (human embryonic kidney 293) cells and immunoprecipitated using the anti-Myc antibody from cell lysates. The immunoprecipitate was washed in detergent-containing buffer to remove unbound proteins, and bound proteins were separated by one- or two-dimensional electrophoresis as described under "Experimental Procedures." Control experiments included preclearing nonspecific binding proteins by incubation with protein A/G beads alone, mock immunoprecipitations with IgG alone, and immunoprecipitation with Myc antibodies from untransfected cell lysates. Silver staining analysis of two-dimensional gels revealed at least 20 different proteins that appear to associate specifically with PDK-1 (Fig. 1); these included heat shock protein 27 (*spot 2*) and the known PDK-1-binding protein, protein kinase C α (*spot 3*). A spot with an apparent molecular mass of ~60 kDa (*spot 1*) was repeatedly observed in samples immunoprecipitated with anti-Myc antibodies, but not in samples immunoprecipitated with anti-IgG or the irrelevant antibody anti-HA. Four tryptic peptides were consistently observed in analyses of this spot by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) mass spectrometry. A search of the Protein Prospector data base (prospector.ucsf.edu) matched all four peptides to tryptic fragments from Grb14, an adapter protein of ~60 kDa (Table I).

Members of the Grb7 family of proteins, which includes Grb14, have a conserved Ras association domain, a PH domain, and an Src Homology 2 (SH2) domain (Fig. 2). Unique to Grb14 is a conserved amino-terminal proline-rich region (*PRR*, Fig. 2) that may bind SH3 domain-containing proteins. In addition, analysis of the Grb14 sequence (www.scansite.mit.edu) revealed a PDK-1 binding consensus motif (Φ*XX*ΦEΦ, where *X* is a bulky hydrophobic such as Phe) previously referred to as PIF (PDK-1 interacting fragment) (36). To explore the mechanism of interaction of Grb14 with PDK-1, the Grb14 constructs listed in Fig. 2 were generated for bacterial and mammalian cell expression. Specifically, we generated a construct in which the key Glu in the PDK-1 binding motif was mutated to Ala (E195A), a construct in which the SH2 domain was deleted (ΔSH2), and a construct in which the PDK-1 binding motif was mutated and the SH2 domain was deleted (E195A/ΔSH2).

We first asked whether the interaction observed *in vivo* between PDK-1 and Grb14 was direct. GST-PDK-1 and His-Grb14 were expressed in bacteria and affinity-purified. Fig. 3*a*

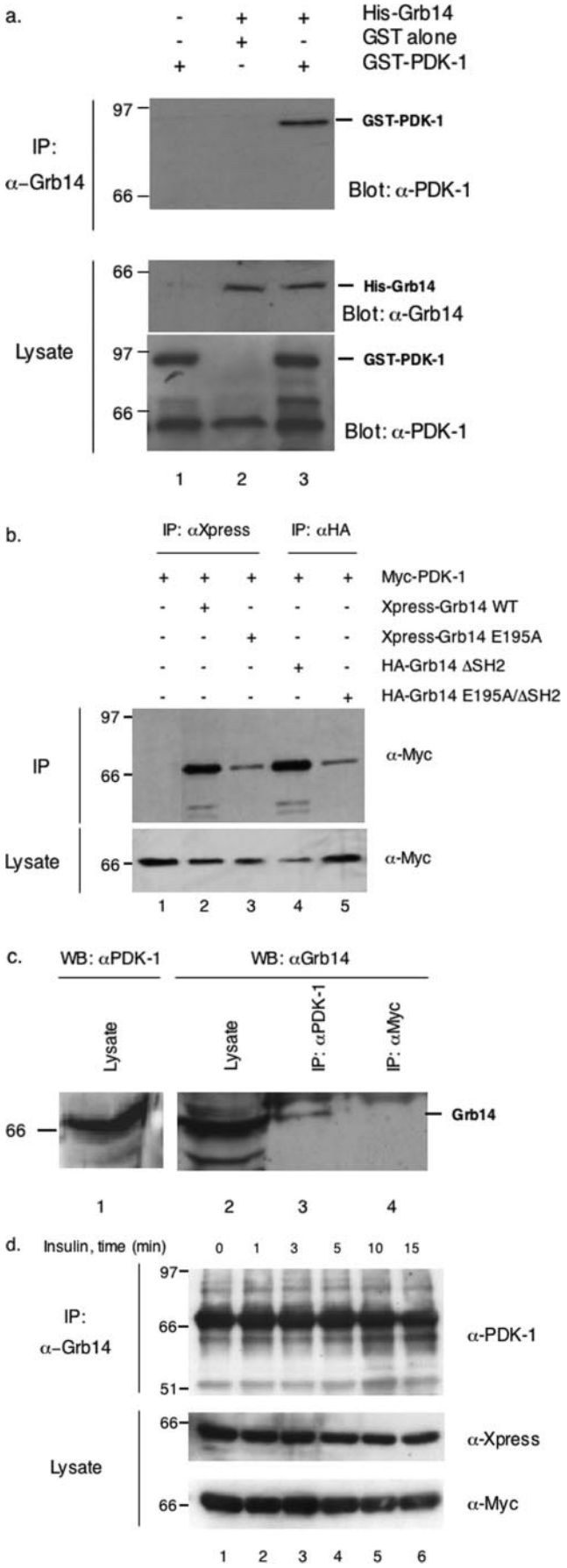

FIG. 3. **Grb14 interacts directly with PDK-1 through a consensus PDK-1 binding sequence.** *a*, Western blot showing PDK-1 associated with Grb14 following incubation of pure bacterially expressed shows GST-PDK-1 associated with His-Grb14 following incubation of the two pure proteins, each present at 2 and 50 μM, respectively (Fig. 3*a*, *lane 3*). Approximately 20% of the PDK-1 was associated with the His-Grb14 suggesting a binding affinity on the order of 100 μM under these conditions. In contrast, control experiments showed that no significant amount of GST-PDK-1 was pulled down by antibody in the absence of His-Grb14 (*lane 1*). Additionally, the GST protein alone did not bind to the His-Grb14 protein (*lane 2*). These data indicate that Grb14 directly interacts with PDK-1 in the absence of secondary protein associations.

We next examined the interaction of PDK-1 and Grb14 in mammalian cells using the Grb14 constructs described in Fig. 2. HEK 293 cells were transiently transfected with Myc-PDK-1 alone or with the following Grb14 constructs: Xpress-tagged wild-type Grb14, Xpress-tagged E195A Grb14, an HA-tagged construct lacking the BPS and SH2 domains (ΔSH2), and the ΔSH2 construct with the E195A mutation. The *lower panel* in Fig. 3*b* shows the amount of Myc-PDK-1 in cell lysates. PDK-1 was effectively immunoprecipitated by wild-type Grb14 (*lane 2*). Significantly less PDK-1 was immunoprecipitated by the E195A construct, both for Xpress-tagged full-length (*lane 3*) and HA-tagged (*lane 5*) Grb14 lacking an SH2 domain. Quantitation of three independent experiments revealed approximately one-third as much PDK-1 bound the E195A constructs compared with wild-type Grb14. Removal of the carboxyl-terminal SH2 domain did not impair the association of PDK-1 with Grb14 (*lane 4*). Control conditions revealed that PDK-1 was absent in immunoprecipitations from cells not transfected with Xpress-Grb14 (*lane 1*). These data indicate that a primary determinant driving the binding of PDK-1 to Grb14 is the PDK-1 binding motif centered near residue Glu-195.

Grb14 was initially identified by a proteomics approach analyzing protein bound to PDK-1 in HEK 293 cells. We selected a different cell line, MCF-7, which has been reported to have relatively high levels of Grb14 (29) and PDK-1, to confirm the interaction of the endogenous proteins. The Western blot in Fig. 3*c* shows that 300 μg of lysate (corresponding to a total on the order of 10^9 cells) was sufficient for robust staining of PDK-1 (*lane 1*) and Grb14 (*lane 2*) with appropriate antibodies. Grb14 was specifically detected in immunoprecipitations using

His-Grb14 (2 μM) and pure bacterially expressed GST-PDK-1 (50 μM). His-Grb14 was pulled down with Ni^{2+}-NTA beads from mixtures containing GST-PDK-1 alone (*lane 1*), His-Grb14 alone (*lane 2*), or GST-PDK-1 and His-Grb14 (*lane 3*) followed by Western blotting with an anti-PDK-1 antibody (Cell Signal; *top* and *lower panels*) or anti-Grb14 (Santa Cruz Biotechnology; *middle panel*). *b*, Western blot showing Myc-PDK-1 associated with the indicated Grb14 constructs following transient transfection of HEK 293 cells with the cDNA for both proteins. Grb14 was immunoprecipitated from detergent-solubilized lysates with anti-Xpress (Santa Cruz Biotechnology; *lanes 1–3*) or anti-HA (Covance; *lanes 4* and *5*) antibodies coupled to protein A/G beads (Pierce). Proteins were separated by SDS-PAGE and co-immunoprecipitated PDK-1 was detected with the anti-Myc antibody (Covance). A Western blot of 10% of the total lysates probed with Myc-PDK-1 is shown in the lower panel. *c*, Western blot showing endogenous PDK-1 and Grb14 interact in the breast cell line MCF-7. Confluent MCF-7 cells (1×10^9 cells) were lysed in detergent-containing buffer and 300 μg of protein separated on 7.5% SDS-polyacrylamide gels. Blots were probed for endogenous PDK-1 (*lane 1*) or endogenous Grb14 (*lane 2*). Either anti-PDK-1 antibody from Upstate (*lane 3*) or the nonspecific antibody anti-Myc (*lane 4*) were used to immunoprecipitate PDK-1 from 2 mg of total protein from MCF-7 cells and associated Grb14 was detected using anti-Grb14 (Santa Cruz Biotechnology). *d*, HEK 293 cells were transiently transfected with Myc-PDK-1 and Xpress-Grb14. Cells were serum-starved for 18 h, then stimulated with 100 nM insulin for the indicated times. Grb14 was immunoprecipitated with anti-Grb14 antibody (Chemicon), and immunoprecipitates were probed for PDK-1 (anti-PDK-1 antibodies, Upstate Biotechnology). *Lower panel*, 10% of total cell lysates were probed for PDK-1 (anti-Myc) or Grb14 (anti-Xpress).

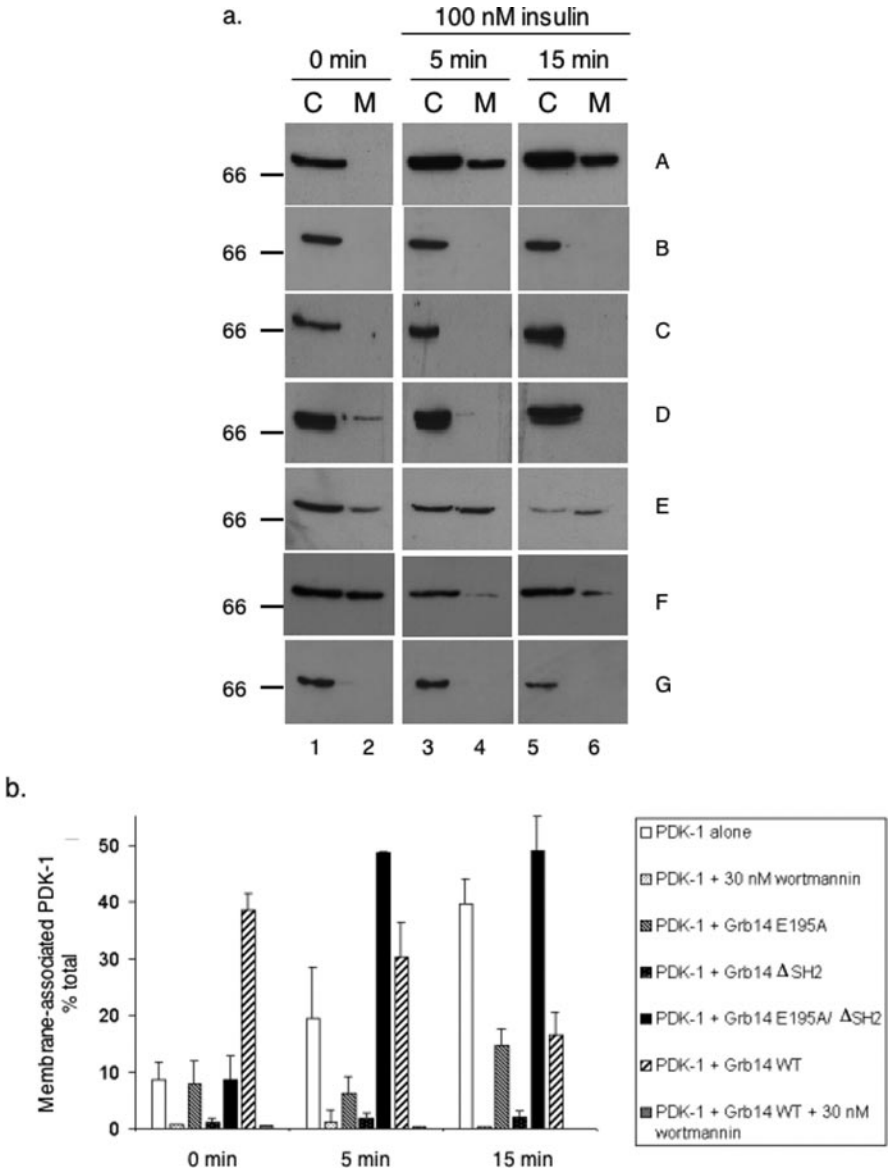

FIG. 4. **PDK-1 distribution in insulin-stimulated HEK 293 cells in the absence or presence of Grb14 constructs.** *a*, Western blots showing subcellular distribution of Myc-PDK-1 in HEK 293 cells transiently transfected with Myc-PDK-1 alone (*lane 1*) or with Grb14 WT, Grb14 E195A, Grb14 ΔSH2, or Grb14 E195A/ΔSH2 E195A. Cells were serum-starved for 18 h, treated with or without wortmannin (30 nM) for 15 min, and then stimulated for 5 or 15 min with 100 nM insulin, lysed, and fractionated as described under "Experimental Procedures" to yield the cytosol (*C*) and detergent-solubilized membrane (*M*) fractions. Samples were analyzed on 10% SDS-polyacrylamide gels and Western blotted using an anti-Myc antibody. Data are representative of the indicated replicates. *Panels* are as follows: *row A*, no addition ($n = 6$); *row B*, +30 nM wortmannin ($n = 3$); *row C*, +Grb14 E195A ($n = 5$); *row D*, +Grb14 ΔSH2 ($n = 6$); *row E*, +Grb14 E195A/ΔSH2 ($n = 3$); *row F*, +wild-type Grb14 ($n = 6$); and *row G*, +wild-type Grb14 plus 30 nM wortmannin ($n = 3$). *b*, graph showing the amount of PDK-1 localized to membrane, obtained from quantitative analysis of Western blots described in *a* ($n = 3–6$) using the GeneGnome software (Syngene).

the PDK-1 antibody (Upstate), but not in immunoprecipitations using the Myc antibody (*lanes 3* and *4*, respectively). These data confirm the initial observation that PDK-1 and Grb14 interact in cells.

We next addressed whether the interaction of PDK-1 with Grb14 changes upon stimulation of cells. HEK 293 cells transfected with the cDNA for wild type Myc-PDK-1 and Xpress-Grb14 were serum-starved for 18 h, then stimulated with insulin for 1–15 min. Fig. 3*d* shows that a constant amount of PDK-1 was present in immunoprecipitates of Grb14 independent of insulin treatment. Similarly, no change in the amount of PDK-1 present in Grb14 immune complexes was observed in HeLa cells treated without or with insulin (data not shown). These data suggest that the interaction of PDK-1 with Grb14 is constitutive and not sensitive to insulin stimulation.

Grb14 translocates to activated tyrosine kinase receptors (20, 29, 37), leading us to us ask whether Grb14 serves as a scaffold to recruit PDK-1 to membranes in response to insulin stimulation. Therefore, we examined the recruitment of PDK-1 and Grb14 to membranes (Figs. 4 and 5, respectively). HEK 293 cells were transfected with the cDNA for Myc-PDK-1 alone, or Myc-PDK-1 and the Grb14 constructs described in Fig. 2. Cells were simulated with insulin for 0, 5, or 15 min and lysed in detergent-free buffer. A fraction of the total cell lysate was removed for Western blot analysis to normalize for expression of the various constructs (data not shown). The lysate was then separated into two fractions: 1) cytosol (high speed supernatant) and 2) membrane (Triton X-100 extraction of pellet) and analyzed for PDK-1 or Grb14 distribution (Figs. 4 and 5). Fig. 4*a* (*row A*), shows that PDK-1 was localized exclusively to the cytosol in unstimulated cells and partially translocated to the membrane fraction following insulin stimulation. This translocation was prevented by prior treatment of cells with the PtdIns 3-kinase inhibitor, wortmannin (*row B*). It was also prevented by co-expression of Grb14 E195A (*row C*) and by co-expression of the Grb14 construct deleted in the BPS and SH2 domains (*row D*). PDK-1 translocation was not significantly affected by the deletion construct that also had a point mutation in the PDK-1 binding motif, Grb14 E195A/ΔSH2 (*row E*). Co-expression of wild-type Grb14 promoted insulin-independent membrane association (*row F*), an association that was prevented by wortmannin (*row G*). Data from at least three independent experiments are quantified in Fig. 4*b*. These data reveal that Grb14 regulates the insulin-sensitive translocation of PDK-1 to membranes. Grb14 constructs unable to bind either PDK-1 (E195A) or the insulin receptor (ΔSH2) serve as dominant negative proteins that prevent translocation of PDK-1. A construct that can bind neither PDK-1 nor the insu-

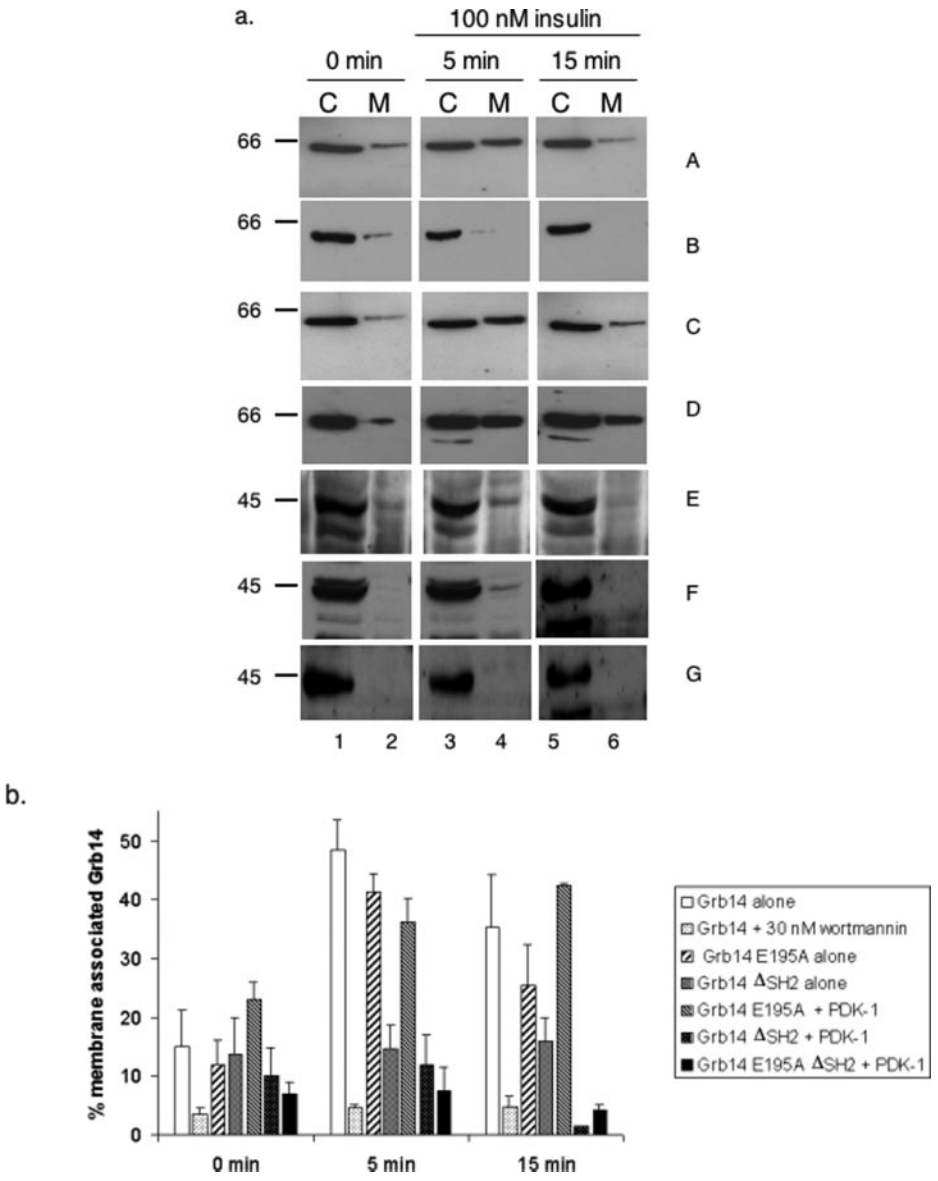

FIG. 5. **Distribution of Grb14 constructs in insulin stimulated HEK 293 cells in the absence or presence of PDK-1.** Western blot showing subcellular distribution of Xpress-Grb14 WT, Xpress-Grb14 E195A, HA-Grb14 ΔSH2, or HA-Grb14 E195A/ΔSH2 in HEK 293 cells transfected with Grb14 constructs alone or co-transfected with Myc-PDK-1. Cells were serum-starved for 18 h then, treated with or without wortmannin (30 nM) for 15 min, and then stimulated for 5 or 15 min with 100 nM insulin, lysed, and fractionated as described under "Experimental Procedures" to yield the cytosol (*C*) and detergent-solubilized membrane (*M*) fractions. Samples were analyzed on 10% SDS-polyacrylamide gels and Western blotted using an anti-Xpress or anti-HA antibodies. A representative Western blot of the indicated replicates is shown. *Panels* are as follows: *row A*, wild-type Grb14 alone ($n = 5$); *row B*, wild-type Grb14 plus 30 nM wortmannin ($n = 3$); *row C*, Grb14 E195A alone ($n = 3$); *row D*, Grb14 E195A plus PDK-1 ($n = 7$); *row E*, Grb14 ΔSH2 alone ($n = 3$); *row F*, Grb14 ΔSH2 plus PDK-1 ($n = 5$); *row G*, Grb14 E195A/ΔSH2 plus PDK-1 ($n = 3$). *b*, graph showing the amount of Grb14 localized to the membrane, obtained from quantitative analysis of Western blots described in *a* ($n = 3$–7) using the GeneGnome software (Syngene).

lin receptor, E195A/ΔSH2, is inert and does not significantly affect insulin-dependent translocation of PDK-1.

Fig. 5 shows the subcellular distribution of wild-type and mutant constructs of Grb14 in HEK 293 cells. Approximately 15% of the total Grb14 was membrane-associated in unstimulated HEK 293 cells (Fig. 5*a*). Stimulation with insulin increased the amount of membrane-associated wild-type Grb14 2.5-fold (Fig. 5*a*, *row A* and Fig. 5*b*). Translocation of wild-type Grb14 was prevented by pre-treatment of cells with the PtdIns 3-kinase inhibitor, wortmannin (*row B*). The E195A construct displayed robust translocation in response to insulin (Fig. 5*a*, *rows C* and *D*), under conditions where translocation of PDK-1 was abolished (compare with Fig. 4*a*, *row B*). Constructs deleted in the BPS and SH2 domains did not translocate to membranes in response to insulin (Fig. 5*a*, *rows E* and *F*). The Grb14 construct in which the SH2 domain was deleted and the PDK-1 binding site was mutated also remained in the cytosol (Fig. 5*a*, *row G*). These data reveal that both the PH domain and the SH2 domain of Grb14 are required for insulin-sensitive translocation to membranes and that this translocation is not affected by mutation of the PDK-1 binding motif.

PDK-1 binds to membranes containing 3′-phosphoinositide lipids through its PH domain. We wanted to determine whether the PH domain of PDK-1 was required for translocation to membranes. cDNA for a PDK-1 construct in which the PH domain was deleted (Myc-ΔPH-PDK-1) was transfected alone or with the Grb14 constructs described in Fig. 2 into HEK 293 cells. The cells were stimulated with insulin for 0, 5, or 15 min, and the distribution of ΔPH-PDK-1 between the cytosol and membrane fractions was analyzed (Fig. 6). Fig. 6 (*panel A*) shows that the ΔPH-PDK-1 was primarily cytosolic in unstimulated cells and mostly translocated to the membrane following insulin stimulation. Translocation of ΔPH-PDK-1 to membranes was not significantly altered by co-expression with wild type Grb14 (*panel B*). Translocation was inhibited when Grb14 E195A (*panel C*) or Grb14 ΔSH2 (*panel D*) were co-expressed. These data indicate that the PH domain of PDK-1 is not required to recruit the enzyme to membranes and that interaction with Grb14 is sufficient for recruitment. Expression of Grb14 constructs that cannot bind PDK-1 (E195A) or the insulin receptor (ΔSH2) prevent PDK-1 translocation.

We next asked whether preventing PDK-1 translocation, by co-expression with the disrupting construct Grb14 E195A, affected Akt translocation. Fig. 7 shows that Akt translocated to membranes in response to insulin and that this translocation was not significantly altered by co-expression with either wild-type Grb14 or the E195A dominant negative construct. Approximately 30% of the total Akt was membrane-associated

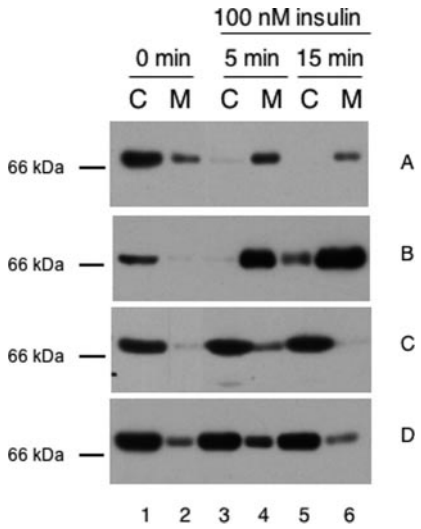

FIG. 6. **The PH domain of PDK-1 is not required for membrane translocation.** Western blots showing subcellular distribution of Myc-ΔPH-PDK-1 in HEK 293 cells transiently transfected with Myc-ΔPH -PDK-1 alone (*panel A*) or with Grb14 WT (*panel B*), Grb14 E195A (*panel C*), or Grb14 ΔSH2 (*panel D*). Cells were serum-starved for 18 h then for 0, 5, or 15 min with 100 nM insulin, lysed, and fractionated as described under "Experimental Procedures" to yield the cytosol (*C*) and detergent-solubilized membrane (*M*) fractions. Samples were analyzed on 10% SDS-polyacrylamide gels and Western blotted using an anti-Myc antibody.

following 5-min insulin treatment in all samples. Co-immunoprecipitation studies revealed that Akt does not interact with Grb14 (data not shown). Thus, Grb14 specifically binds and regulates the location of PDK-1 and does not interact with proteins that translocate in response to insulin but do not bind directly to Grb14 such as Akt.

We next examined the translocation of endogenous PDK-1 to membranes in insulin-stimulated HEK 293 cells in the presence or Grb14 WT or Grb14 E195A (Fig. 8). Commercially available antibodies do not consistently detect endogenous PDK-1, therefore we used a PDK-1 phosphospecific antibody (Cell Signal) that recognizes phospho-serine 241, the constitutively phosphorylated activation loop site on PDK-1 (38). Fig. 8 shows that endogenous PDK-1 partitioned exclusively in the cytosol of unstimulated cells, similar to the overexpressed protein (compare with Fig. 4*a*, *row A*). Insulin caused ~30% of the PDK-1 to redistribute to the membrane in cells expressing wild-type Grb14. This translocation was quantitatively prevented in cells expressing the dominant negative E195A construct of Grb14. Thus, endogenous PDK-1 translocates to membranes following insulin stimulation by a mechanism that involves binding to Grb14.

We next sought to determine whether the Grb14-directed translocation of PDK-1 to membranes modulated the phosphorylation of endogenous PDK-1 substrates. Phosphorylation of the PDK-1 site on endogenous Akt, Thr-308, or endogenous PKCα, Thr-497, was measured in 293 cells transfected with PDK-1 alone or with wild-type Grb14 or the E195A construct. The Western blot in Fig. 9*a* shows that insulin caused an increase in the phosphorylation at Thr-308 on Akt in cells transfected with PDK-1 alone (Fig. 9*a*, *lanes 1–3*). These results were similar to data obtained with untransfected cells (data not shown). In cells co-expressing wild-type Grb14, the rapid increase in Akt phosphorylation was followed by a significant decrease in phosphorylation at 15 min (*lanes 4–6*), consistent with the removal of PDK-1 from membranes (see Fig. 4*a*, *row F*). Phosphorylation at Thr-308 was abolished in cells co-expressing the E195A construct of Grb14 (*lanes 7–9*). Data from five independent experiments are quantified in Fig. 9*b*. In contrast, phosphorylation of the PDK-1 site on PKCα was not significantly altered by insulin, by co-expression of Grb14, or by co-expression of the dominant negative E195A construct of Grb14. These data are consistent with a previous report showing that phosphorylation of conventional isozymes of protein kinase C by PDK-1 is constitutive and not sensitive to regulators of the PtdIns 3-kinase pathway (22). Taken together, these data support the hypothesis that Grb14 recruits PDK-1 to membranes where it accesses and phosphorylates Akt. Disruption of the interaction with Grb14 prevents translocation and thus Akt phosphorylation. Substrates that directly dock PDK-1 such as conventional PKC isozymes are not sensitive to Grb14.

DISCUSSION

This study identifies Grb14 as a scaffold that recruits PDK-1 to membranes in an insulin-dependent manner. Specifically, we show that Grb14 co-recruits PDK-1 to membranes following insulin stimulation of HEK 293 cells through a PDK-1-binding segment. Its ability to recruit PDK-1 to membranes is impaired by either mutating the PDK-1-binding segment or by removal of the SH2 domain to prevent association of Grb14 with activated insulin receptor. The co-recruitment of PDK-1 with Grb14 contributes to Akt activation, suggesting that Grb14 contributes to transduction of the insulin signal by facilitating the localization of PDK-1 near its membrane-bound substrate, Akt.

Grb14 binds the insulin receptor and plays a role in the termination of insulin signaling (31–33). Other recent studies have demonstrated the role of Grb14 in decreasing DNA and glycogen synthesis in response to insulin stimulation. These studies also found that Grb14 overexpression decreases IRS-1 phosphorylation, but not the phosphorylation state of the insulin receptor itself (32). Association of Grb14 with some of receptor tyrosine kinases results in serine/threonine phosphorylation by unknown kinase(s) (29). The finding that Grb14 provides a direct link between the insulin receptor and PDK-1 has important ramifications for understanding the regulation of Akt by insulin.

Regulation of PDK-1—PDK-1 appears to be constitutively active in both resting and stimulated cells (4, 6), leaving major questions as to how the function of this enzyme is regulated. One possibility is that autophosphorylation or phosphorylations by other kinases modulate PDK-1 activity. In this regard, sphingosine promotes PDK-1 autophosphorylation in a serine-rich linker region between the kinase and PH domain, resulting in an 8-fold increase in activity (27). In addition, Src phosphorylation of PDK-1 has been shown to increase PDK-1 activity 2-fold (26). Thus, phosphorylation is one regulatory input for controlling the activity of PDK-1. Protein-protein interactions may also regulate the function of this kinase. Recent work identified Ral-GEF as a PDK-1 interacting protein, indicating that non-substrate binding partners of PDK-1 exist (28). The finding reported here that Grb14 binds PDK-1 and facilitates its insulin-triggered membrane translocation underscores the possibility that protein-protein interactions may emerge as key regulators of this enzyme.

Grb14 Has a PDK-1 Binding Site—A search for a potential PDK-1 binding motif in Grb14 revealed a sequence similar to a PIF (PDK-1 interacting fragment) site centered near glutamic acid 195. This sequence is present on many PDK-1 substrates, including protein kinase C isoforms, Akt, and protein kinase C-related kinase (36, 39, 40). Mutation of glutamic acid 195 to alanine dramatically decreased the interaction between the two proteins, but deletion of the carboxyl terminus of Grb14 containing the BPS/SH2 domain did not. Although glutamic acid at position 195 provides the major interaction site between

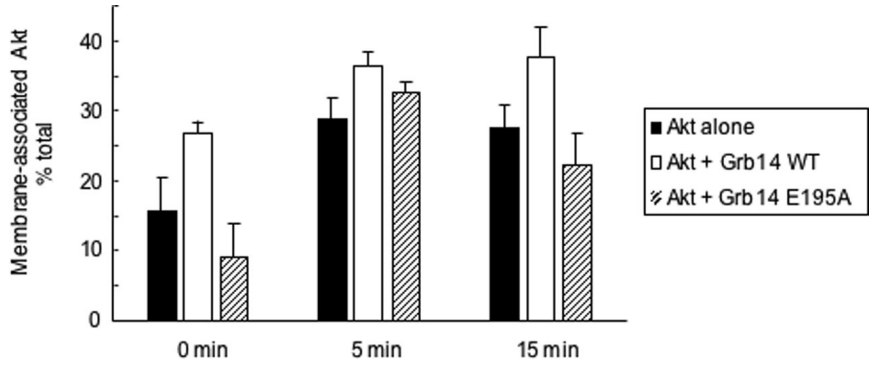

FIG. 7. **Grb14 does not alter the membrane distribution of Akt.** Graph showing the fraction of membrane-associated HA-Akt in HEK 293 cells co-transfected with the cDNA for HA-Akt alone (*black columns*) or co-transfected with the cDNA for HA-Akt and either wild-type Grb14 (*white columns*) or Grb14 E195A (*stippled columns*). Cells were starved for 18 h, then stimulated with 100 nM insulin for 5 or 15 min. Cytosol and membrane fractions were prepared as previously described, and Akt was detected by Western blot analysis with an anti-HA antibody (Covance); data were quantified by using the GeneGnome software (Syngene). Data represent the average ± S.D. of three separate experiments.

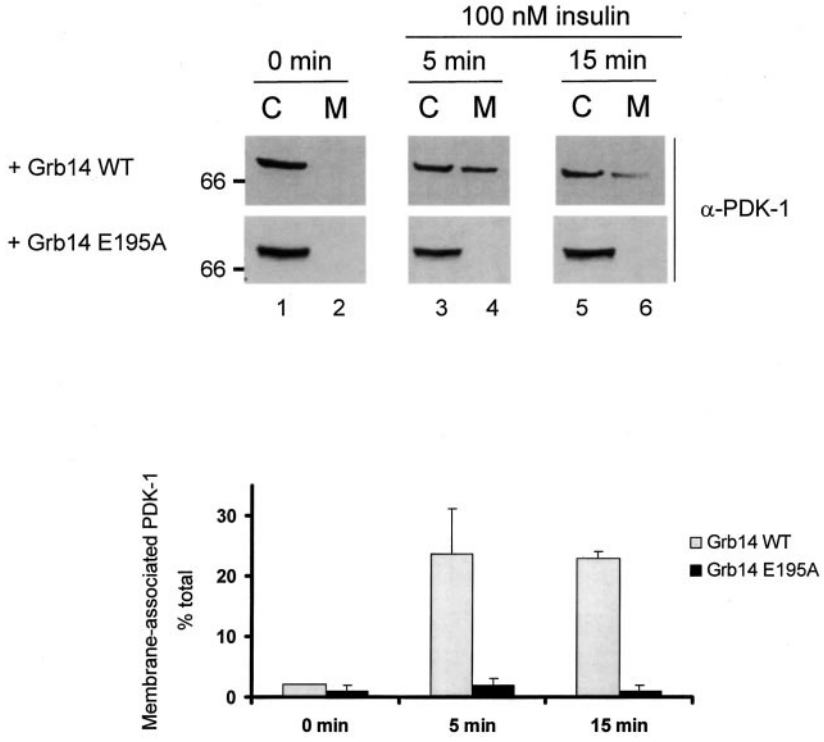

FIG. 8. **Grb14 E195A blocks membrane translocation of endogenous PDK-1 in insulin stimulated cells.** HEK 293 cells were transiently transfected with the cDNA for wild-type Grb14 or Grb14 E195A. Cells were serum starved for 18 h, then stimulated for the indicated times with 100 nM insulin. Cell lysates were fractionated and cytosol and membrane fractions were probed for endogenous PDK-1 using the phospho-Ser 241 antibody. The *upper panel* shows representative Western blot of cytosol (*C*) and membrane (*M*) fractions probed for PDK-1; the *lower panel* shows the result of quantifying the percent membrane-associated PDK-1 from three independent experiments (average ± S.D. shown).

these two proteins, a minimal interaction is seen when the site is mutated, suggesting other amino-terminal determinants on Grb14 exist. Daly (29) reported that Grb14 is phosphorylated by unknown protein kinases. Immunoprecipitated PDK-1 phosphorylates Grb14 *in vitro*, but phosphorylation is substoichiometric under our experimental conditions.[2] Whether serine/threonine phosphorylation of Grb14 by these unknown kinases or PDK-1 phosphorylation by tyrosine kinases alters the affinity of the interaction between these proteins is currently under investigation. Additionally, protein phosphatases may also alter the interaction between Grb14 andPDK-1.

Mechanism of PDK-1 Membrane Recruitment by Grb14—The PH domain of PDK-1 has previously been shown to be required for efficient translocation of this kinase to membranes (20). Our data indicate that the PH domain is not required for translocation of PDK-1 to membranes, because constructs deleted in the PH domain continue to undergo insulin-dependent membrane association. Rather, we show that interaction with Grb14 mediates insulin-sensitive membrane translocation. Thus, the wortmannin sensitivity of PDK-1 translocation noted by us and others (20, 21) may reflect the dependence of Grb14 translocation in phosphatidylinositol 3-kinase. However, we found that pretreatment of HEK 293 cells with wortmannin also prevented PDK-1 translocation. These data, taken together, suggest that Grb14 is necessary to bring PDK-1 in close proximity to PtdIns-3,4-P_2 or PtdIns-3,4,5-P_3 and phosphorylate substrates. An intact Grb14 protein is also required for efficient PDK-1 localization: abolishing the PDK-1 binding site or truncating the receptor binding domain in Grb14 also prevents PDK-1 translocation to membranes. This suggests that Grb14 plays a role in the specific targeting of PDK-1 to membranes that contain active and, therefore, signaling receptor tyrosine kinases.

Grb14 plays a critical role regulating PDK-1 membrane translocation in response to insulin stimulation. Because this adaptor protein associates with many other tyrosine kinase receptors (28–30), we wanted to determine whether this protein-protein interaction represents a general PDK-1 recruitment mechanism. Grb14 E195A did not prevent membrane translocation of PDK-1 in response to PDGF stimulation of NIH3T3 cells,[2] indicating additional mechanisms for PDK-1 membrane recruitment exist. These stimulus-specific requirements for membrane translocation of PDK-1 may account for the differences observed by many groups (19–21) as to the role

[2] C. C. King and A. C. Newton, unpublished results.

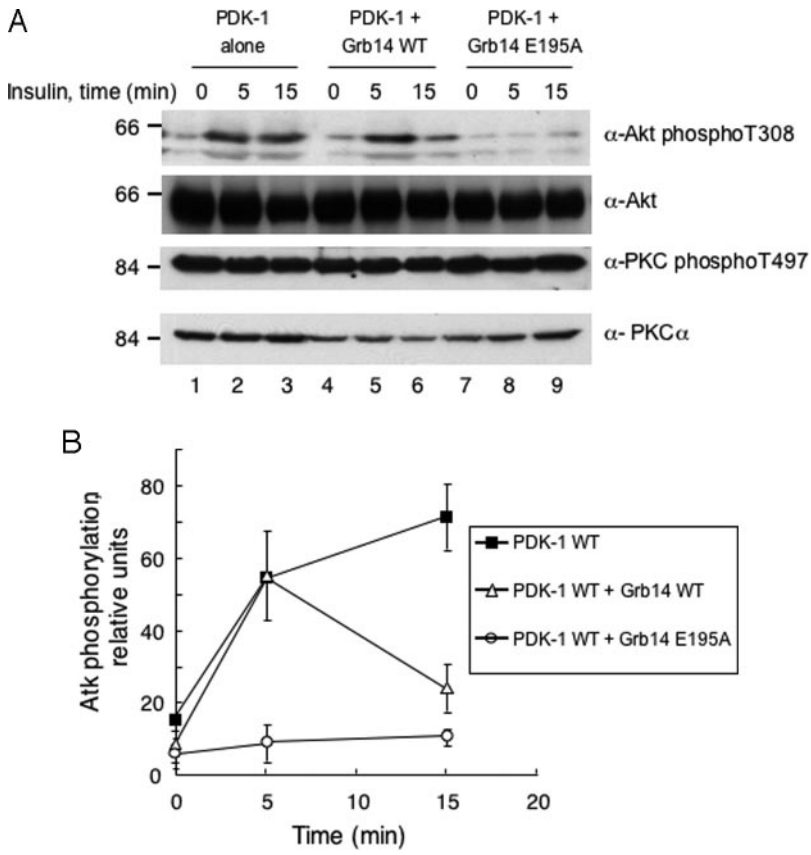

FIG. 9. **Grb14 E195A decreases the insulin-dependent phosphorylation of Akt but does not affect the phosphorylation of PKCα at the PDK-1 site.** *a*, Western blot showing phospho-Akt (α-Akt P308), total Akt (α-Akt), phospho-PKC (α-PKC P497), and total PKC (α-PKCα) in cell lysates of HEK 293 cells transfected with Myc-PDK-1 alone (*lanes 1–3*) or co-transfected with wild type Grb14 (*lanes 4–6*) or Grb14 E195A (*lanes 7–9*) and then stimulated with 100 nM insulin for the indicated times. *b*, graph showing the amount of Akt phosphorylated at threonine 308, obtained by quantitative analysis of Western blots described in *a* ($n = 5$) using Quantity One software (Bio-Rad).

of the PH domain of PDK-1 in membrane recruitment.

A significant amount of PDK-1 is membrane-associated in serum-starved cells co-transfected with wild type Grb14, and stimulation with insulin dramatically decreased the amount of membrane-associated PDK-1 (Fig. 4, *a* (*lane 6*) and *B*). However, endogenous PDK-1 appears to be cytosolic in serum-depleted cells and translocates to membranes in response to insulin. Although it is possible that this is an artifact of transfection or use of phosphospecific antibodies to detect endogenousPDK-1, other possible explanations exist. Recently, a number of papers identified Grb14 as an inhibitor of insulin signaling (32, 33, 41). It is possible that Grb14 plays a role in both recruitment and dissociation of PDK-1 from membranes. Preliminary experiments have found an increase in PDK-1 tyrosine phosphorylation in the presence of wild type Grb14.[2] It is possible that PDK-1 may aid in Grb14-dependent inhibition of insulin receptor signaling. Data supporting this hypothesis come from a recent report by Cariou *et al.* (41) who identified Grb14 as a PKCζ substrate and binding partner. PKCζ requires phosphorylation by PDK-1 for activation (42), and phosphorylation of Grb14 by PKCζ increased upon insulin stimulation. Phosphorylation by PKCζ increased the inhibitory effect of Grb14 on the tyrosine kinase activity of the insulin receptor. PDK-1 interaction with Grb14 and localization to insulin receptors may provide a mechanism by which Grb14 terminates insulin signaling.

Co-recruitment of PDK-1 to Membranes by Grb14 Promotes Akt Activation—To assess the functional significance of the PDK-1-Grb14 interaction, the phosphorylation of known endogenous PDK-1 substrates was examined. Phosphorylation of PKCα, a conventional isoform of this family of protein kinases, was unaltered by the presence of either wild type Grb14 or Grb14 E195A. These data are consistent with previous data identifying PDK-1 phosphorylation of this kinase as a constitutive processing event (22). PDK-1 phosphorylation of Akt has been demonstrated to occur at the membrane following cell stimulation. Phosphorylation of Thr-308 in Akt was observed in cells transfected with PDK-1 alone or Grb14 wild type. Co-transfection with wild type Grb14 resulted in a rapid decrease in Thr-308 phosphorylation after 15 min, consistent with PDK-1 removal from membranes (Fig. 4*a*, *row F*) (43). Co-transfection with Grb14 E195A dramatically decreased phosphorylation of Akt, consistent with the finding that PDK-1 recruitment to membranes is ablated in the presence of this Grb14 construct.

Grb7 Family Members and Insulin Signaling—Akt was recently found to constitutively associate with another Grb7 family member, Grb10 (44). The interaction between these two proteins was reported to be required to bring Akt to membranes in c-kit signaling, although the constitutive nature of the Akt-Grb10 interaction has been challenged recently (45). It is possible that Grb7 family members act as carriers of protein kinases not simply to membranes, but to activated signaling centers. This could help direct and modulate the response of these signaling proteins. PDK-1 contains a PH domain, which binds PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, lipids that are formed at active signaling centers. It is possible that linking protein kinases that signal from activated tyrosine kinase receptors with proteins that bind these receptors allows protein kinases like PDK-1 and Akt to very efficiently search in a limited membrane space for phosphatidylinositol lipids, which bind these proteins to membranes. Such a system would ensure that protein kinases like PDK-1 are efficiently and rapidly brought into close enough proximity to substrates.

Summary—Taken together, our results show that the adaptor protein Grb14 regulates the membrane association of PDK-1 in response to insulin signaling. Identification of Grb14 as a constitutive PDK-1 interacting protein suggests a novel regulatory input into the mechanism by which PDK-1 is recruited to and removed from active signaling centers. Because

PDK-1 is constitutively active in cells, proper localization to substrates is essential, especially during transient signaling events that are mediated by PtdIns 3-kinase activity. Interaction of PDK-1 with an SH2 domain-containing protein that binds activated tyrosine kinase receptors ensures rapid translocation to membranes where it can subsequently bind PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 and phosphorylate substrates in an efficient manner.

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