Identification of P-Rex1 as a Novel Rac1-Guanine Nucleotide Exchange Factor (GEF) That Promotes Actin Remodeling and GLUT4 Protein Trafficking in Adipocytes*

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Background: PREX1 maps to a Type 2 diabetes susceptibility locus; however, its role in insulin-stimulated GLUT4 trafficking is unknown.

Results: P-Rex1 activates Rac1 in adipocytes and thereby actin rearrangement and GLUT4 trafficking, facilitating glucose uptake.

Conclusion: P-Rex1 may contribute to insulin-stimulated glucose homeostasis.

Significance: These studies identify a novel regulator of GLUT4 trafficking in adipocytes.

Phosphoinositide 3-kinase (PI3K) signaling promotes the translocation of the glucose transporter, GLUT4, to the plasma membrane in insulin-sensitive tissues to facilitate glucose uptake. In adipocytes, insulin-stimulated reorganization of the actin cytoskeleton has been proposed to play a role in promoting GLUT4 translocation and glucose uptake, in a PI3K-dependent manner. However, the PI3K effectors that promote GLUT4 translocation via regulation of the actin cytoskeleton in adipocytes remain to be fully elucidated. Here we demonstrate that the PI3K-dependent Rac exchange factor, P-Rex1, enhances membrane ruffling in 3T3-L1 adipocytes and promotes GLUT4 trafficking to the plasma membrane at submaximal insulin concentrations. P-Rex1-mediated GLUT4 translocation to the plasma membrane is a novel development in GLUT4 trafficking. In contrast, expression of other Rho GTPases, such as CDC42 or Rho, did not affect insulin-stimulated P-Rex1-mediated GLUT4 trafficking. P-Rex1 siRNA knockdown or expression of a P-Rex1 dominant negative mutant reduced but did not completely inhibit glucose uptake in response to insulin. Collectively, these studies identify a novel RacGEF in adipocytes as P-Rex1 that, at physiological insulin concentrations, functions as an insulin-dependent regulator of the actin cytoskeleton that contributes to GLUT4 trafficking to the plasma membrane.

Insulin-stimulated glucose uptake in adipocytes and striated muscle cells is primarily mediated by GLUT4, the glucose transporter. GLUT4 resides in storage vesicles in unstimulated cells within specialized intracellular endosomal compartments that include recycling endosomes, the trans-Golgi network and tubulovesicular elements (1). Following insulin stimulation, GLUT4 storage vesicles physically traffic to and fuse with the plasma membrane, facilitating increased cellular glucose uptake. Insulin activates the class I phosphoinositide 3-kinase (PI3K) that transiently phosphorylates phosphatidylinositol 4,5-biphosphate to synthesize PtdIns[3,4,5]P3,2 which recruits and activates cytosolic effectors, including Akt, AS160, PDK1, and atypical protein kinase C (2, 3). In many cell types, including skeletal muscle, activation of PI3K also induces activation of guanine nucleotide exchange factors (GEFs) for small Rho family GTPases, such as Rac1, that promote actin cytoskeletal dynamics and membrane ruffling and contribute to GLUT4 translocation (4).

The cytokine plays a significant role in facilitating GLUT4 exocytosis mediated by both microtubules and actin filaments (5–9). Microtubules direct the transport of GLUT4 vesicles to the cell cortex via the kinesin motor protein KIF5B (10). Insulin causes a rapid and dynamic remodeling of actin into a submembranous cortical network (11, 12) that scaffolds insulin effectors, including GLUT4, vesicle-associated membrane protein 2 (VAMP2), and PtdIns[3,4,5]P3 (11, 12). Several models have been proposed to predict the mechanisms by which actin may contribute to GLUT4 exocytosis. Actin reorganization may physically drive GLUT4 vesicle translocation to the plasma membrane (5, 13). Actin may also contribute to the assembly of the Akt signaling pathway in adipocytes (14). Alternatively, the cortical actin network may regulate the rate of GLUT4 translocation to the plasma membrane (15).
P-Rex1 Promotes GLUT4 Trafficking

GLUT4 vesicle fusion with the plasma membrane in a PI3K/Akt-dependent manner (15).

The insulin-stimulated pathways that control actin dynamics to influence GLUT4 exocytosis may be different in adipocytes and muscle cells. For example, in adipocytes, TC10 (16), N-WASP (neural Wiskott-Aldrich syndrome protein) (17), and Fodrin (18) but not the small GTPase Rac1 contribute to the regulation of actin-mediated GLUT4 translocation. However, in skeletal muscle, Rac1 represents the candidate small GTPase required for insulin-stimulated actin reorganization and GLUT4 translocation (19, 20). Muscle expression of dominant-negative Rac1 blocks insulin-induced actin remodeling and GLUT4 translocation to the cell surface (11). Following intravenous insulin injection, Rac1 is activated in mouse gastrocnemius (21) and human muscle (22). Muscle-specific Rac1 knock-out mice exhibit significantly impaired insulin-stimulated GLUT4 translocation, independent of Akt or AS160 phosphorylation (21), but whether this results in altered glucose uptake is unknown. In contrast, overexpression of constitutively active Rac1 has no significant effect on insulin stimulation of GLUT4 trafficking in 3T3-L1 adipocytes (23) or glucose uptake (24, 25).

P-Rex1 is a member of the P-Rex (P-Rex1, P-Rex2, and P-Rex2B) family of RacGEFs that regulate chemotaxis and reactive oxygen species formation in neutrophils (26). P-Rex1 is a multidomain protein, and at the N terminus, the enzyme contains a catalytic Dbl-homologous domain, which activates Rac1, adjacent to a pleckstrin homology domain, a domain pair present in most small GTPase GEFs (Fig. 2C) (27). Two Dishevelled, Egl-10 and pleckstrin domains are followed by two PDZ domains, which are protein interaction domains. Interestingly, the P-Rex1 DEP domains may also contribute to Rac activation via binding to mTORC2 (28). DEP domains also target proteins to membranes, whereas PDZ domains perform multiple adapter functions (29, 30). The P-Rex1 C-terminal domain exhibits significant homology to the type I and II phosphatidylinositol 3,4-bisphosphate 4-phosphatases (INPP4A and INPP4B) (27, 31), over 300 amino acids, leaving a central region with no known homology (27). Although the 4-phosphatase homology domain of P-Rex1 contains the dual specificity phosphatase consensus CX2R catalytic motif, no phosphoinositide or protein phosphatase activity has been identified (27). P-Rex1 null mice, although small, are apparently healthy. Neutrophils lacking P-Rex1 exhibit decreased Rac2 activation in response to a chemoattractant formylmethionylleucylphenylalanine and attenuated formylmethionylleucylphenylalanine-induced F actin formation and superoxide production (32). In addition, P-Rex1 regulates neurite migration and differentiation (33–34), and it is implicated as an oncogene in a range of malignancies (35–37). Interestingly, a detailed genotypic analysis has mapped PREX1 to a Type 2 diabetes susceptibility locus on chromosome 20q12–q13.1, suggesting its possible association with metabolic disorders; however, the role P-Rex1 plays in insulin signaling remains unexplored (38, 39). P-Rex1 is prominently expressed in cells of the immune system. Preadipocytes and adipocytes share many similarities with cells of the monocyte/macrophage lineage. Preadipocytes can differentiate into either adipocytes or macrophages (40) and display phagocytic properties similar to specialized phagocytes (41–44). Preadipocytes and adipocytes express the MOMA-2 antigen, a marker of monocyte/macrophage lineage (45). In this study, we therefore investigated whether P-Rex1 is expressed in adipocytes and explored its function.

We report in adipocytes P-Rex1 enhances insulin-stimulated membrane ruffling, via its intrinsic RacGEF activity, and promotes GLUT4 translocation to the plasma membrane in a PtdIns(3,4,5)P3- and Rac1-dependent manner. Furthermore, we identify that, consistent with previous results, Rac1 does not promote GLUT4 translocation at saturating doses of insulin (100 nm) (23) in adipocytes, but at physiological insulin concentrations (1–10 nm), Rac1 enhances GLUT4 plasma membrane association. These studies identify Rac1 and its activator P-Rex1 as insulin-dependent regulators of actin cytoskeletal dynamics that promote GLUT4 trafficking in adipocytes.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying and restriction enzymes were from Fermentas, New England Biolabs, or Promega. Texas Red-conjugated phalloidin was from Molecular Probes (Eugene, OR). Monoclonal antibody (mAb) specific for hemagglutinin (HA) tag was from Babco, T7-tag specific mAb was from Novagen, and Myc-tag specific mAb was from Cell Signaling. Rabbit monoclonal phospho-Akt(Ser-473) antibody (catalog no. 4058) and Akt antibody (catalog no. 9252) was from Cell Signaling. Polyclonal antibodies specific for HA tag were from Upstate. Oligonucleotides were obtained either from Micromon (Monash University) or Geneworks (Adelaide, Australia). Oligonucleotides for RNAi experiments involving down-regulation of P-Rex1 were from Dharmacon RNAi Technologies (murine P-Rex1, Product no. D-053658-01-0005; murine negative control, Product no D-001210-01). The 3T3-L1 cell line was from the American Tissue Culture Collection. GFP–GLUT4 and exofacial Myc–GLUT4–GFP were kindly provided by Dr. Jeffrey Pessin and Dr. Alan Saltiel, respectively. pCGT-T7-RhoV14, pCGT-T7-Rac1V12, and pCGT-T7-cdc42V12 plasmids were kind gifts from Linda Van Aelst (Cold Spring Harbor, NY), and pcDNA3-Myc-(DN)Rac1N17 and pcDNA3-Myc-(DN)cdc42N17 plasmids were kind gifts from Alpha Yap (University of Queensland, Australia). Unless otherwise stated, all other reagents were from Sigma.

Cell Culture and Differentiation of 3T3-L1 Preadipocytes—3T3-L1 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) containing 4.5 mg/ml glucose and 10% (v/v) fetal bovine serum (FBS) Commonwealth Serum Laboratories (CSL). Confluent 3T3-L1 preadipocytes were differentiated with DMEM containing 10% (v/v) FBS, 400 μg/ml insulin, 500 μM isobutyl 1-methylxanthine, and 250 nM dexamethasone. Growth medium was replaced with DMEM plus 10% FBS 4 days after the addition of differentiation medium and incubated for a further 4 days.

Production of P-Rex1 Anti-peptide Antibodies—A P-Rex1-specific anti-peptide antibody was generated to a peptide corresponding to a unique sequence ([34S]LYRYNNNGEYNESS) within human P-Rex1 and affinity-purified as described (33).
Transfection of 3T3-L1 Adipocytes and Indirect Immunofluorescence—Adipocytes were transiently transfectected 8 days following differentiation by electroporation as described (46). Electroporation cuvettes containing $1 \times 10^7$ cells and 50 μg of each plasmid DNA were pulsed at 0.17 kV and 975 microfarads. Cells were plated onto 0.01% poly-L-lysine-coated glass coverslips and incubated for 48 h. Cells on coverslips were washed twice with cold PBS supplemented with 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$ and then fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.1% (v/v) Triton X-100 in PBS (5 min), incubated with blocking buffer (5% goat serum in PBS) (30 min), overlaid with primary antibody diluted in blocking buffer (1 h), and washed and incubated with secondary antibody-Alexa Fluor conjugate (Molecular Probes) diluted in blocking buffer (1 h). To detect F-actin or G-actin, cells were labeled with Texas Red-phallolidin (Molecular Probes) or DNase I, as described (33). Coverslips were washed and mounted onto glass slides using Fluorolyte mounting medium (ICN) and imaged by laser-scanning confocal microscopy (Monash Micro Imaging Facility).

siRNA Knockdown of P-Rex1 in 3T3-L1 Adipocytes—Lipofectamine 2000 reagent was used for transfection of siRNA into differentiated 3T3-L1 adipocytes. Annealed RNA oligonucleotides (Dharmacon) specific for murine P-Rex1 (GenBank™ accession NM_177782.3) were labeled using the Silencer siRNA labeling kit (Ambion) according to the manufacturer’s instructions. 250 pmol of siRNA was mixed with 40 μl of Lipofectamine 2000 reagent and 800 μl of Opti-MEM (low serum medium) and allowed to incubate at room temperature for 45 min. The resulting siRNA-liposome complexes were added to 6.4 ml of Opti-MEM, and this mixture was then aliquoted directly onto the cells, which were plated onto 12- or 24-well plates and incubated for 48 h. Cells on coverslips were washed twice with cold PBS supplemented with 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$ supplemented with 1% BSA and 2 mM sodium pyruvate. Adipocytes were equilibrated for 90 min at 37 °C and treated with 100 nM insulin for 30 min or left untreated. Uptake of 50 μM 2-deoxyglucose and 0.5 μCi of 2-deoxy-[U-14C]glucose (PerkinElmer Life Sciences) per well was measured during the final 10 min of insulin stimulation. Cells were harvested and analyzed by scintillation counting. Uptake of 2-deoxyglucose in siRNA-transfected 3T3-L1 adipocytes was assessed using 2-[3H]DOG as described previously (47).

RESULTS

P-Rex1 Is Expressed in Differentiated 3T3-L1 Adipocytes—We first determined whether P-Rex1 was expressed in adipocytes. Affinity-purified P-Rex1 antibodies, generated as we described previously (33), immunoblotted a polypeptide species of ~175 kDa, consistent with the molecular weight of P-Rex1, in the cytosolic fraction of adipocyte subcellular fractions, derived from 3T3-L1 adipocytes (Fig. 1, A and B). Indirect immunofluorescence of 3T3-L1 adipocytes using P-Rex1 antibody revealed endogenous P-Rex1 localized diffusely in the cytosol of both resting and insulin-stimulated (100 nM) 3T3-L1 adipocytes (Fig. 1C), partially colocalizing with the plasma membrane. However, P-Rex1 was not detected in plasma membrane fractions by immunoblotting (Fig. 1B). The localization of GFP-GLUT4 is indistinguishable from endogenous GLUT4 in resting and insulin-treated adipocytes (48, 49). No direct colocalization of P-Rex1 with GFP-GLUT4 was noted either in resting or insulin-stimulated adipocytes (Fig. 1C).

P-Rex1 Promotes Membrane Ruffling in Differentiated 3T3-L1 Adipocytes—Insulin stimulates the rapid polymerization of submembranous actin in 3T3-L1 adipocytes to form membrane ruffles, in a PI3K-dependent manner, which correlates temporally with GLUT4 translocation (50). Formation of actin-rich membrane ruffles can be visualized by phalloidin, which binds F-actin (25). Wild-type P-Rex1 was expressed in 3T3-L1 adipocytes to evaluate its effects on adipocyte cell morphology and F-actin accumulation at membrane ruffles in resting and insulin-treated cells. In vector controls, small patches of punctate actin were detected at the cell base, which represent the adipocyte counterpart of stress fibers (Fig. 2, A and B). Imaging through the center of the cell identified subcortical actin as a thin rim around the cell periphery (Fig. 2A). In vector controls, insulin stimulation dramatically increased the number of cells exhibiting insulin-stimulated membrane ruffling at all insulin concentrations tested (Fig. 2E) but did not induce membrane ruffling in the absence of insulin (Fig. 2, A–C and E). Wild-type HA-P-Rex1
P-Rex1 Promotes GLUT4 Trafficking

A

P-Rex1

1346
LGYRYNNNGEYESS

1360
DH | PH | DEP | DEP | PDZ | PDZ | 4-PTASE

C

P-Rex1

GFP-GLUT4

Merged

+ Insulin

- Insulin

FIGURE 1. P-Rex1 expression and localization in 3T3-L1 adipocytes. A, P-Rex1 domain structure and localization of peptide used for antibody generation. P-Rex1 polyclonal antibodies were raised to the indicated peptide sequence (33). B, 50 μg of cell lysate (LYS), cytosolic (CYT), or plasma membrane (PM) fractions prepared from differentiated 3T3-L1 adipocytes were immunoblotted with affinity-purified P-Rex1 anti-peptide antibodies. C, resting or insulin-stimulated (100 nM) 3T3-L1 adipocytes expressing GFP-GLUT4 (green) were fixed, permeabilized, and stained with P-Rex1 anti-peptide antibodies (red). Yellow regions in merged images indicate areas of colocalization (see arrows). Bar, 2 μm.

localized diffusely in the cytosol and accumulated at membrane ruffles, co-localizing with F-actin (Fig. 2, A and B). However, despite inducing dynamic changes to actin networks at the cell periphery, P-Rex1 did not significantly alter the distribution of punctate F-actin detected at the cell base (Fig. 2B, inset).

To dissect P-Rex1 function in adipocytes, the following series of HA-tagged P-Rex1 mutants was generated: P-Rex1 that lacks the Rac1-activating DH domain (P-Rex1(ΔDH), amino acids 218 – 1659); a mutant construct that deletes the 4-phosphatase homology domain but leaves the DH/PH/DEP/DEP/PDZ/PDZ and central domain intact (P-Rex1(Δ4P), amino acids 36 – 1364); and a mutant containing only the central and 4-phosphatase homology domain (P-Rex1(ΔN-term), amino acids 791 – 1659) (Fig. 2C). A mutant P-Rex1 construct that contains a single point mutation in the putative catalytic cysteine residue located in the CX3R motif within the 4-phosphatase homology domain (P-Rex1(C1583S)) was also generated (Fig. 2C). In dual specificity protein and lipid phosphatases, this renders these enzymes catalytically inactive to their substrates (51, 52). Immunoblot analysis demonstrated intact expression of these recombinant proteins in 3T3-L1 cells, migrating at the predicted molecular mass (Fig. 2D). To examine which domains of P-Rex1 are responsible for insulin-mediated membrane ruffling, the percentage of cells exhibiting membrane ruffling, defined as the presence of extensive bundled F-actin at the cell periphery, was scored for wild-type and mutant P-Rex1 constructs in resting and insulin-stimulated cells (Fig. 2E). Wild type P-Rex1 significantly enhanced the number of cells that exhibited insulin-stimulated membrane ruffling (Fig. 2E). The HA-P-Rex1(ΔDH) mutant, which lacks the catalytic Rac1-activating DH domain, acted as a dominant negative construct, reducing the number of cells that showed insulin-stimulated membrane ruffling. For example, at 10 nM insulin treatment, >40% of cells expressing wild type P-Rex1 exhibited membrane ruffling, which reduced to ~20% following expression of HA-P-Rex1(ΔDH). This is consistent with previous reports that this construct binds and sequesters Rac1 (33, 34). Similarly, deletion of the N-terminal DH/PH/DEP/DEP/PDZ/PDZ domains (HA-P-Rex1(ΔN)) also functioned as a dominant-negative construct, inhibiting insulin-stimulated membrane ruffling at all insulin doses tested. The mechanisms for this construct acting as a dominant negative construct are unclear; however, it lacks the DH domain required for Rac1 activation and two DEP domains that activate Rac via mTORC2 (28). In contrast, expression of HA-P-Rex1(Δ4P), which lacks the 4-phosphatase homology domain or HA-P-Rex1(C1583S), promoted insulin-stimulated membrane ruffling similar to wild type HA-P-Rex1 (Fig. 2E). In control studies, all P-Rex1 mutants localized diffusely in the cytosol and at membrane ruffles in insulin-stimulated cells, similar to wild type HA-P-Rex1 (not shown).

P-Rex1 Enhances Insulin-induced GLUT4 Plasma Membrane Association in 3T3-L1 Adipocytes—We next investigated the role P-Rex1 plays in regulating GLUT4 trafficking. Cells co-transfected with GFP-GLUT4 and vector control or wild-type HA-P-Rex1 were treated with insulin (0–100 nM) and scored for the localization of GFP-GLUT4 at the plasma membrane (Fig. 3A). In unstimulated cells, no increase in GFP-GLUT4 translocation was observed in cells expressing HA-P-Rex1, relative to vector controls. However, HA-P-Rex1 expression significantly increased insulin-stimulated GFP-GLUT4 translocation, with a ~2.0-fold increase at 1 nm treatment and a ~1.5-fold increase at 10 nm insulin treatment relative to vector controls (Fig. 3A). In control experiments, trafficking of the endogenous GLUT1 transporter was not altered with HA-P-Rex1 expression (not shown). To evaluate whether P-Rex1-induced insulin-stimulated GFP-GLUT4
translocation was dependent on PtdIns(3,4,5)P_3-generated via Type 1 PI3K, cells were pretreated with the PI3K inhibitor LY294002 prior to insulin stimulation (Fig. 3B). Notably, LY294002 treatment decreased P-Rex1-induced insulin-stimulated GFP-GLUT4 translocation by 2.0-fold, a degree of inhibition similar to that observed in LY294002-treated vector controls (Fig. 3B). However, insulin-stimulated GFP-GLUT4 translocation induced by P-Rex1 overexpression was not completely abolished upon LY294002 treatment, indicating that activation of PI3K-independent pathways in P-Rex1-expressing cells may play an important role in GLUT4 trafficking by unknown mechanisms.

To evaluate whether HA-P-Rex1-mediated increases in insulin-stimulated GFP-GLUT4 plasma membrane association were dependent on the integrity of the actin cytoskeleton, prior to insulin-stimulation, 3T3-L1 adipocytes were treated with either cytochalasin D, which depolymerizes actin, or latrunculin A, which sequesters actin monomers (5, 6, 12, 53, 54). Treated adipocytes were scored for GFP-GLUT4 plasma membrane fluorescence (Fig. 3C). Latrunculin A treatment significantly inhibited insulin-stimulated GFP-GLUT4 translocation at submaximal insulin (1 nM) concentrations from 61% to 19% in cells expressing HA-P-Rex1, with a similar inhibition following cytochalasin D treatment (from 61% to 29%), indicating dependence on a functional actin cytoskeleton. For vector controls, latrunculin A or cytochalasin D treatment prevented insulin-stimulated GFP-GLUT4 translocation.

Actin cytoskeleton rearrangement may facilitate fusion of GLUT4 vesicles with the plasma membrane via unknown mechanisms (15). Exofacial Myc-GLUT4-GFP, that contains a Myc epitope tag in the large extracellular loop between transmembrane domains 1 and 2 of GLUT4, was used to determine cell surface exposure of GLUT4 in nonpermeabilized cells, using Myc antibody indirect immunofluorescence (47) (Fig. 4, A and B). Wild-type HA-P-Rex1 significantly increased Myc plasma membrane fluorescence relative to vector controls, following 1 nM (2-fold) or 100 nM insulin (1.25 fold) stimulation (Fig. 4, A and B).

P-Rex1-induced GLUT4 Trafficking Requires the N-terminal DH Domain and Is Rac1-specific—In order to dissect which domains of P-Rex1 mediated its stimulatory effect on GLUT4 translocation, P-Rex1 mutants were co-expressed with GFP-GLUT4 in 3T3-L1 adipocytes, and cells were scored for insulin-stimulated plasma membrane GFP-GLUT4 fluorescence. In
In the absence of insulin stimulation, no change in the localization of GFP-GLUT4 was detected following expression of mutant or wild type HA-P-Rex1 (Fig. 5A). However, under insulin-stimulated conditions, expression of dominant negative HA-P-Rex1(ΔDH) or HA-P-Rex1(ΔN) significantly inhibited insulin-stimulated GLUT4 translocation (Fig. 5, A and B). In contrast, substitution of the putative catalytic cysteine residue in the 4-phosphatase homology domain (HA-P-Rex1(C1583S)) or deletion of the entire 4-phosphatase homology domain (HA-P-Rex1(Δ4P)) did not affect insulin-stimulated GLUT4 translocation, relative to wild type P-Rex1 (HA-P-Rex1) (Fig. 5, A and B). Therefore, P-Rex1 dominant negative constructs (HA-P-Rex1(ΔDH) and HA-P-Rex1(ΔN)) inhibit insulin-stimulated membrane ruffling and GLUT4 plasma membrane association.

The effect of various Rho family GTPases on GLUT4 translocation was examined, using a series of constitutively active or dominant negative RhoGTPase constructs. T7-tagged constitutively active Rho, Rac1, or Cdc42 was cotransfected with GFP-GLUT4 in 3T3-L1 adipocytes and scored cells for GFP-GLUT4 plasma membrane fluorescence in response to both submaximal (1 nM) and high insulin (100 nM) concentrations (Fig. 5C). It has previously been demonstrated that expression of constitutively active Rac1 in adipocytes does not alter GLUT4 translocation in response to 100 nM insulin (23–25); however, the effect of Rac1 at lower insulin doses has not been reported. Expression of constitutively active Rac1 (T7-Rac1V12), but not Rho (T7-RhoV14) or Cdc42 (T7-Cdc42V12), significantly increased GFP-GLUT4 plasma membrane association in insulin-stimulated adipocytes to 65% of cells at 1 nM insulin, relative to 40% of mock-transfected cells (Fig. 5C); however, at 100 nM insulin, no increase was noted. In contrast, constitutively active Rho or Cdc42 inhibited insulin-stimulated GFP-GLUT4 plasma membrane association, relative to mock-transfected cells, by 20 and 25% at 1 and 100 nM insulin, respectively, for T7-RhoV14-expressing cells and 25 and 30% at 1 and 100 nM insulin for T7-Cdc42V12-expressing cells, respectively (Fig. 5C). Therefore, Rac1 may enhance GLUT4 plasma membrane association at lower insulin concentrations, and P-Rex1
P-Rex1 Promotes GLUT4 Trafficking

may be a GEF that activates Rac1 in adipocytes. To evaluate this contention, adipocytes were co-transfected with constitutively active Rac1 (T7-Rac1V12) and P-Rex1 lacking the catalytic DH domain (HA-P-Rex1(ΔDH)) to reveal whether GTP-loaded-Rac1 can rescue the dominant negative effect of HA-P-Rex1(ΔDH) on insulin-stimulated GLUT4 translocation. In cells co-expressing HA-P-Rex1(ΔDH) and GFP-GLUT4, co-expression of T7-Rac1V12 induced insulin-stimulated GFP-GLUT4 plasma membrane association to levels exceeding that of vector controls, rescuing the inhibitory effect of HA-P-Rex1(ΔDH) (Fig. 5D). In contrast, in cells co-expressing HA-P-Rex1(ΔDH) and GFP-GLUT4, co-expression of T7-Cdc42V12 did not affect insulin-stimulated GFP-GLUT4 translocation (Fig. 5D). Therefore, the inhibition of insulin-stimulated GFP-GLUT4 plasma membrane association induced by the HA-P-Rex1(ΔDH) mutant can be rescued by overexpression of constitutively active Rac1.

To further dissect the Rho-GTPase target of P-Rex1 in adipocytes, we evaluated whether dominant inhibitory RhoGTPases could prevent HA-P-Rex1-mediated increase in insulin-stimulated GFP-GLUT4 plasma membrane association. Wild-type HA-P-Rex1 was co-expressed with either Myc-tagged dominant negative Rac1 (Myc-Rac1V12N17), or Myc-tagged dominant negative Cdc42 (Myc-Cdc42N17). Co-expression of dominant negative Rac1 (Myc-Rac1V12N17) suppressed HA-P-Rex1-mediated insulin-stimulated GFP-GLUT4 translocation to levels similar to vector controls, whereas expression of dominant negative Cdc42 had no inhibitory effect (Fig. 5E). In control studies, constitutively active and dominant negative Rac1 localized to the plasma membrane and to the cytosol in resting and insulin-stimulated adipocytes, colocalizing with GFP-GLUT4 and partially with HA-P-Rex1 at the plasma membrane. In addition, dominant negative Rac1 co-localized with GFP-GLUT4 and HA-P-Rex1 at intracellular sites in the perinuclear region (Fig. 5E).

P-Rex1 Knockdown and Dominant Negative P-Rex1 Inhibit Insulin-stimulated Glucose Uptake—Because P-Rex1 promotes the plasma membrane engagement of GLUT4, we investigated its role in regulating glucose uptake. The effect of the dominant negative P-Rex1 construct (HA-P-Rex1(ΔN)) on 2-DOG uptake in adipocytes was evaluated (Fig. 6A). For these experiments, which require high transfection efficiencies, we used the HA-P-Rex1(ΔN) construct because we had observed that more than >60% of cells were transfected with this construct, compared with <40% for the HA-P-Rex1(ΔDH) construct, but both constructs exerted similar effects on membrane ruffling and GLUT4 translocation. In unstimulated cells expressing HA-P-Rex1(ΔN), 2-[3H]DOG uptake was similar to vector controls. Following stimulation with 100 nM insulin, glucose uptake was stimulated 7-fold over basal levels in vector-expressing cells, but this was reduced by ~40% in cells expressing HA-P-Rex1(ΔN) (Fig. 6A). This correlates with the inhibition of insulin-stimulated GLUT4 plasma membrane engagement observed in cells expressing HA-P-Rex1(ΔN) (Fig. 5, A and B). To confirm that P-Rex1 positively regulates GLUT4 trafficking, the effects of RNAi-mediated knockdown of P-Rex1 on insulin-mediated GLUT4 trafficking in 3T3-L1 adipocytes were analyzed. siRNA specific for P-Rex1 or a nonspecific scrambled sequence was transfected into 3T3-L1 adipocytes for 72 h. To confirm that the siRNA oligonucleotides successfully targeted P-Rex1 mRNA, leading to reduced protein levels, 3T3-L1 adipocytes were harvested 72 h post-transfection and immunoblotted using C-terminal P-Rex1 antibodies (Fig. 6B). Transient transfection of 250 pmol of siRNA specific for P-Rex1 resulted in significant reduction of endogenous P-Rex1 protein levels in 3T3-L1 adipocytes relative to control siRNA (Fig. 6B). siRNA-transfected 3T3-L1 adipocytes were serum-starved and insulin-stimulated 72 h post-transfection and subjected to 2-[3H]DOG uptake assays (Fig. 6C). P-Rex1 depletion modestly inhibited 2-DOG uptake at both 1 and 10 nM insulin (Fig. 6C).
P-Rex1 Promotes GLUT4 Trafficking

A

% cells with PM GFP-GLUT4

Vector
P-Rex1(ΔDH)
P-Rex1(ΔN)
P-Rex1(ΔC)
P-Rex1(C1S55)
P-Rex1(ΔP)

0 nM
1 nM
100 nM

Insulin

B

Fold increase over basal PM GFP-GLUT4

Vector
P-Rex1(ΔDH)
P-Rex1(ΔN)
P-Rex1(ΔC)
P-Rex1(C1S55)
P-Rex1(ΔP)

1 nM
100 nM

Insulin

C

% cells with PM GFP-GLUT4

Mock
T7-Rac(V12)
T7-Cdc42(V12)
T7-Rac(V12)

0 nM
1 nM
100 nM

Insulin

D

% cells with PM GFP-GLUT4

GFP-GLUT4
P-Rex1(ΔDH)
GFP-GLUT4
+P-Rex1(ΔDH)
GFP-GLUT4
+P-Rex1(ΔC)
GFP-GLUT4
+P-Rex1(C1S55)
GFP-GLUT4
+P-Rex1(ΔP)

0 nM
1 nM
10 nM
100 nM

Insulin

E

% cells with PM GFP-GLUT4

GFP-GLUT4
P-Rex1(ΔDH)
GFP-GLUT4
+P-Rex1(V12)
GFP-GLUT4
+P-Rex1(C1S55)
GFP-GLUT4
+P-Rex1(ΔP)

0 nM
1 nM
10 nM
100 nM

Insulin

+ Insulin (1 nM)

P-Rex1(ΔDH) + Rac(V12) + GFP-GLUT4

P-Rex1(ΔDH) + GFP-GLUT4

P-Rex1(ΔDH) + GFP-GLUT4

P-Rex1 + Rac(V12N17) + GFP-GLUT4

P-Rex1 + GFP-GLUT4

P-Rex1 + GFP-GLUT4

P-Rex1 + GFP-GLUT4

Overlay
DISCUSSION

This study has shown that the RacGEF P-Rex1 significantly enhances insulin-stimulated membrane ruffling, GLUT4 vesicle engagement at the plasma membrane at physiological insulin concentrations in adipocytes. P-Rex1 activity is dependent on a functional actin network and is predominantly mediated by its inherent RacGEF activity in a PI3K-dependent mechanism. Expression of dominant negative P-Rex1 or P-Rex1 siRNA knockdown modestly reduces glucose uptake. Our studies also reveal that at submaximal insulin concentrations, Rac1 contributes to insulin-stimulated GLUT4 plasma membrane association in adipocytes in addition to its known role in skele-

FIGURE 5. P-Rex1-induced insulin-stimulated GLUT4 trafficking is Rac1-specific. A, serum-starved 3T3-L1 adipocytes, co-transfected with the indicated P-Rex1 constructs and GFP-GLUT4, were scored for plasma membrane fluorescence following stimulation with 1 or 100 nM insulin. Results are the mean ± S.E. of three separate experiments in which 50 cells were scored per condition per experiment. B, 250 pmol of siRNA oligonucleotides specific for P-Rex1 or a nonspecific scrambled sequence (control) were transfection using Lipofectamine 2000 reagent into 3T3-L1 adipocytes for 72 h. Cells were immunoblotted for P-Rex1 expression using anti-P-Rex1 antibodies. Duplicate samples were immunoblotted with anti-actin antibodies to confirm equal protein loading. C, 3T3-L1 adipocytes were transfected with 250 pmol of either control or P-Rex1 siRNA oligonucleotides and seeded into 24-well plates. 72 h post-transfection, cells were serum-starved and insulin-stimulated (striped bars, 10 nM insulin; black bars, 100 nM insulin prior to incubation with 100 μM 2-[3H]deoxyglucose (7.5 μCi/ml) in 2-DOG assay buffer for 20 min at 37 °C and assayed in replicates of three per experiment. Data is displayed as a ratio of insulin-stimulated 2-[3H]deoxyglucose uptake over basal levels of 2-deoxyglucose uptake for each siRNA transfection. Over three separate experiments (*, p < 0.05).

FIGURE 6. Dominant negative P-Rex1 or RNAi-mediated depletion of P-Rex1 inhibits insulin-stimulated glucose uptake in 3T3-L1 adipocytes. A, 3T3-L1 adipocytes were transfected with either vector or HA-P-Rex1(ΔN), serum-starved (white bars), and treated with 10 nM insulin (gray bars). Following stimulation, cells were washed three times and then incubated for 20 min in 100 μM 2-[14C]DOG (7.5 μCi/ml) in assay buffer at 37 °C. Cells were washed and assayed in replicates of three per experiment (*, p < 0.05). B, 250 pmol of siRNA oligonucleotides specific for P-Rex1 or a nonspecific scrambled sequence (control) were transfected using Lipofectamine 2000 reagent into 3T3-L1 adipocytes for 72 h. Cells were immunoblotted for P-Rex1 expression using anti-P-Rex1 antibodies. Duplicate samples were immunoblotted with anti-actin antibodies to confirm equal protein loading. C, 3T3-L1 adipocytes were transfected with 250 pmol of either control or P-Rex1 siRNA oligonucleotides and seeded into 24-well plates. 72 h post-transfection, cells were serum-starved and insulin-stimulated (striped bars, 100 nM insulin; gray bars, 10 nM insulin; black bars, 100 nM insulin) prior to incubation with 100 μM 2-[3H]deoxyglucose (7.5 μCi/ml) in 2-DOG assay buffer for 20 min at 37 °C and assayed in replicates of three per experiment. Data is displayed as a ratio of insulin-stimulated 2-[3H]deoxyglucose uptake over basal levels of 2-deoxyglucose uptake for each siRNA transfection. Over three separate experiments (*, p < 0.05).
P-Rex1 Promotes GLUT4 Trafficking

tal muscle. The potential importance of P-Rex1 in insulin-stimulated GLUT4 trafficking is suggested by evidence that the P-Rex1 gene (KIAA1415 or PREX1) maps to a Type 2 diabetes susceptibility locus on chromosome 20 (38, 39).

Insulin induces the release of GLUT4 into the cell surface recycling system in a graded insulin dose-dependent manner, and remodeling of actin is critical for these events. Although the role of Rac1-induced actin remodeling in promoting insulin-stimulated GLUT4 translocation in skeletal muscle is well established (11, 19), this has not been shown in adipocytes. Expression of constitutively active or dominant negative Rac1 in adipocyte cell lines has no effect on GLUT4 trafficking in response to 100 nM insulin (23, 25). Here we establish that at lower submaximal doses of insulin (1–10 nM), which are closer to in vivo physiological concentrations (55, 56), Rac1 exerts a stimulatory effect on GLUT4 trafficking. P-Rex1 mutants that lack the Rac1-activating DH domain do not promote membrane ruffling or GLUT4 trafficking, a phenotype rescued by expression of GTP-loaded Rac1. In contrast, Rho and Cdc42 do not contribute to P-Rex1-mediated GLUT4 plasma membrane translocation. Interestingly, wild type Cdc42 has been previously reported to promote insulin-stimulated glucose transport (57); however, under the conditions described here, constitutively active Cdc42 inhibits insulin-stimulated GLUT4 translocation (Fig. 5C). Cdc42 exhibits 69% amino acid homology and 86% similarity to the related small GTPase TC10 (58). TC10 activation is essential for maximal insulin-stimulated glucose transport; however, constitutively active TC10 paradoxically inhibits insulin-stimulated glucose transport (59), and this may also be the case for constitutively active Cdc42. Remodeling of the cortical actin network by TC10 is required for efficient GLUT4 translocation. Lamellipodia formation occurs in adipocytes in response to insulin, but whether it elicits a stimulatory effect on GLUT4 translocation and fusion remains unclear (5, 60). Insulin-stimulated adipocytes that express P-Rex1 adopt a flatter, less circular morphology with enhanced lamellipodia formation but no changes to cortical actin, as observed with TC10 expression.

We observed that the N-terminal DH, Rac-activating domain of P-Rex1 contributes to both actin cytoskeletal remodeling and GLUT4 translocation in response to insulin. In addition to its Rac1-activating function, the DH domain binds the Gβγ subunit of heterotrimeric G-proteins, significantly enhancing P-Rex1 activity (61). Whether this function of the DH domain also contributes to P-Rex1-induced actin remodeling and GLUT4 trafficking remains to be determined. Deletion of the 4-phosphatase homology domain or mutation of the CXX3R motif had no effect on either membrane ruffling or GLUT4 translocation induced by P-Rex1, and the function of this region and phosphatase motif remains unclear. Notably, deletion of all modular domains at the N terminus, which include the DH, PH, and two PDZ domains, also induces a dominant negative phenotype. The DH and DEP domains contribute to Rac activation via distinct mechanisms, the tandem DH/PH domain via stimulating the guanine nucleotide exchange activity of Rac and the DEP domain by stimulating this activity indirectly via its interaction with mTORC2 (27, 28). GLUT4 trafficking from GLUT4 storage vesicles to the plasma membrane occurs in distinctly regulated steps (vesicle translocation, docking, fusion, and activation), and these events may occur in the absence of glucose uptake (62, 63). Additional signals are required for fused GLUT4 vesicles to become fully functional and facilitate glucose entry, such as GLUT4 C-terminal unmasking (64). We have not shown here at which stage P-Rex1 or Rac1 acts. P-Rex1 promotes insulin-stimulated GLUT4 association with the plasma membrane and may contribute to glucose uptake. We demonstrated that both inhibition of endogenous P-Rex1 activity via expression of dominant negative HA-P-Rex1(ΔN) and reduction of endogenous P-Rex1 protein via its RNAi knockdown exerts a modest inhibitory effect on insulin-stimulated glucose uptake. Although this suggests that P-Rex1 may contribute in part to insulin-stimulated glucose uptake, the distinct stage of GLUT4 exocytosis regulated by P-Rex1 remains to be determined. It is likely that P-Rex1 depletion or inhibition results in impaired GLUT4 translocation, possibly leading to an overall shortage of GLUT4 vesicles at the plasma membrane that are available to facilitate glucose entry into the cell. Significantly, a recent report has indicated that actin remodeling at the cell periphery just beneath the plasma membrane is essential for the efficient fusion of GLUT4 to the plasma membrane (15); therefore, we cannot exclude the possibility that P-Rex1 may be implicated in regulating the distal stages of GLUT4 exocytosis, and this will be investigated in future.

Notably, the P-Rex1 gene (PREX1) has been mapped to a Type 2 diabetes susceptibility locus on chromosome 20q12-q13.1 (39, 65). This region is frequently deleted or amplified in many types of cancer, including malignant myeloid diseases (66), hereditary prostate cancer (67), pancreatic endocrine tumors (68), and ovarian and breast cancers (69). Detailed genotypic analysis of this susceptibility locus on chromosome 20q12-q13.1 identified PREX1 as one of three candidate genes with suggestive evidence of an association with Type 2 diabetes (38). In a recent follow-up study, the influence of PREX1 on Type 2 diabetes and body mass index in two European American case-control populations was investigated by examining common SNPs of these candidate genes (65). The presence of SNPs in the PREX1 coding region associated with Type 2 diabetes and high body mass index was more common in European American diabetic patients compared with healthy controls. SNPs associated with Type 2 diabetes in the PREX1 region were located both within the coding region and 3′ of the gene sequence, which may alter expression levels of the gene in diabetic patients as PREX1 is transcribed in the antisense direction (65). In addition, SNPs in this region 3′ of PREX1 were associated with higher body mass index solely in the diabetic cohort, indicating that genotypic differences near PREX1 may contribute to Type 2 diabetes susceptibility mediated through effects of adiposity (65). It will be of interest to determine in future studies if P-Rex1−/− mice exhibit insulin-resistant phenotypes, given that we have identified P-Rex1 as a novel contributor to insulin-stimulated GLUT4 trafficking.
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