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Overexpression of Rad Inhibits Glucose Uptake in Cultured Muscle and Fat Cells*

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Rad is a Ras-like GTPase that was isolated by subtraction cloning of human muscle and shown to have increased expression in some individuals with Type II diabetes. To ascertain the potential role of Rad in insulin-mediated signaling, we have overexpressed Rad in myocyte and adipocyte cell lines. Expression of Rad resulted in a 50–90% reduction in insulin-stimulated 2-deoxyglucose glucose uptake in C2C12 murine myotubes, L6 rat myotubes, and 3T3-L1 adipocytes and a 25% reduction in 3-*O*-methylglucose uptake in 3T3-L1 adipocytes. This occurred despite unaltered levels of glucose transporter expression, with no detectable change in Glut4 translocation and with no alteration in insulin receptor or substrate phosphorylation or phosphatidylinositol 3-kinase activity. These data indicate that Rad is a negative regulator of glucose uptake and that this effect may be due to a decrease in the intrinsic activity of the transporter molecules, rather than an effect on the translocation of Glut4.

Ras-related guanine nucleotide-binding proteins comprise a large family of molecules that act as molecular switches, binding GTP in their activated state or GDP in the inactive state (1). Ras, itself, becomes activated by a variety of external stimuli, including platelet-derived growth factor, epidermal growth factor, and insulin, which stimulate Ras-GTP formation through signaling cascades involving the Grb2-SOS complex (2–6). Activated Ras then initiates a series of signaling events involving Raf, MEK (MAP[1] kinase kinase), and MAP kinase (7–9). The Ras-related GTPases, Rac, Rho, and cdc42 mediate signals leading to the regulation of membrane ruffling, formation of focal adhesion complexes, and regulate the polymerization of actin to produce stress fibers and lamellipodia (10), whereas small GTP-binding proteins of the Rab subfamily appear to regulate intracellular traffic events (11–13). Rab4 and Rab3D have been suggested to participate in the insulin-stimulated translocation of glucose transporters (14, 15).

Rad is the prototypic member of a new class of Ras-like GTPases, which includes Gem and Kir (16–19). All three of these GTPases encode proteins with several structural features which are distinct from other Ras-like proteins, having extended amino- and carboxyl termini, several divergent residues in the guanine nucleotide binding regions, and unique effector domains. In humans, Rad is most highly expressed in the heart, lung, and skeletal muscle. While the exact role of these proteins is still unclear, Rad was originally identified because its expression is increased in some patients with Type II (non-insulin-dependent) diabetes (16). Recently, our laboratory has also found that Rad associates with the actin-binding protein, β-tropomyosin, suggesting that Rad may participate in regulation of the cytoskeleton (20). Since Type II diabetes is associated with insulin resistance, in the present study we have asked whether Rad might affect insulin-regulated events by overexpressing the protein in two highly insulin-responsive cell types, myocytes and adipocytes.

MATERIALS AND METHODS

Retroviral Transduction of Cell Lines—Full-length human Rad cDNA was introduced into target cells as described previously (20) using the pBABE-Puro retroviral expression vector (35) and the BOSC23 viral packaging cell line (36) (a generous gift from D. Baltimore, MIT). Stable cell lines were established by continued passage in puromycin-containing media (2 μg/ml, Sigma) followed by maintenance in media without puromycin.

Cell Culture and Differentiation—C2C12 murine myocytes and L6 rat myocytes were maintained in the Dulbecco's modified Eagle's medium (DMEM, 4.5 mg/ml glucose) supplemented with 10% fetal bovine serum (FBS) in a 10% CO_2 environment. 3T3-L1 murine fibroblasts were maintained in DMEM supplemented with 10% calf serum in a 10% CO_2 environment. For differentiation of myocytes, confluent cells were fed with DMEM containing 1% calf serum and allowed to differentiate 6–7 days prior to harvesting. Confluent 3T3-L1 cells were fed with DMEM containing 10% FBS, 0.4 μg/ml dexamethasone, 0.5 mM isobutylmethylxanthine, and 5 μg/ml insulin for 48 h followed by media containing 10% FBS and 5 μg/ml insulin for an additional 48 h. Cells were maintained in DMEM containing 10% FBS for an additional 5–7 days. Greater than 95% of cells displayed the fully differentiated adipocyte phenotype.

Immunoblotting and Immunoprecipitation—Quantities of lysates indicated the figure legends were subjected to SDS-PAGE followed by Western immunoblotting using specific antisera and detection with ^{125}I-labeled protein A. Rad polyclonal antiserum was used at a 1:1000 dilution as described (20). Anti-Glut4 (1:400 dilution) and anti-Glut1 (1:2000 dilution) polyclonal antibodies were purchased from East Acres Biologicals; Ras polyclonal antisera (1:500 dilution) and a polyclonal antibody to the p85 subunit of phosphatidylinositol (PI) 3-kinase were purchased from Upstate Biotechnologies, Inc; anti-phosphotyrosine antibody was provided by Dr. M. F. White (Joslin Diabetes Center). Antibodies were raised in rabbits against the COOH termini of the human insulin receptor β-subunit and IRS-1 in our laboratory (37). For MAP kinase immunoprecipitation, 200 μg of cellular extract was subjected to immunoprecipitation with 3 μl of antibody raised in our laboratory against a synthetic peptide corresponding to amino acids 348–367 using protein A-Sepharose (40).

[^{3}H]Thymidine Incorporation, Phosphatidylinositol 3-Kinase, and MAP Kinase Assays—[^{3}H]Thymidine incorporation was assayed in C2C12 and L6 myoblasts as described (38). Radioactivity incorporated into trichloroacetic acid-precipitable material was quantitated by scin-

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[1] The abbreviations used are: MAP, mitogen-activated protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; IRS, insulin receptor substrate; LDM, low density microsomal; PM, plasma membranes; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

tillation counting. All determinations were carried out in triplicate.

PI 3-kinase activity was measured *in vitro* in anti-IRS-1 or anti-phosphotyrosine immunoprecipitates from C2C12 myotubes, which were treated with or without 100 nM insulin for 5 min at 37 °C following overnight serum starvation as described (39). ^{32}P incorporation into PI 3-phosphate was quantitated using a Molecular Dynamics PhosphorImager.

MAP kinase assays were carried out essentially as described by Myers *et al.* in immune complexes from C2C12 myotubes in buffer containing containing 50 mM HEPES, 10 mM $MgCl_2$ and 2 μg myelin basic protein. Reactions were initiated by the addition of 50 μM ATP containing 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and incubated for 10 min at 30 °C. Reaction products were visualized by separation on 15% SDS-PAGE gels and exposure to film.

Glucose Uptake Assays—Cells were assayed for hexose uptake within 10 passages following the establishment of puromycin-resistant populations. For 2-deoxyglucose uptake, differentiated monolayers were treated with the indicated concentrations of insulin for 15 min at 37 °C and exposed to 50 μM 2-deoxy-[^{3}H]D-glucose (0.5 μCi/ml) for the final 4 min of stimulation (41). The incorporated radioactivity was determined by liquid scintillation counting of triplicate points. Nonspecific counts, determined in the presence of 10 μM cytochalasin B, were subtracted from each value. 3-*O*-Methylglucose transport assays were performed essentially as described above for 2-deoxyglucose uptake, except that cells were incubated for 10 min at room temperature prior to the addition of 3-*O*-methly-[^{14}C]glucose (2 μCi/ml) for 5 min, according to the method of Harrison *et al*. (42). Nonspecific influx was was measured in the presence of 250 μM phloretin and subtracted from total influx. Aliquots from each well were used to determine protein concentration using the BCA assay (Pierce).

Subcellular Fractionation—3T3-L1 adipocytes were grown in 150-mm diameter tissue culture dishes and treated with or without 100 nM insulin following overnight serum starvation. Cells were harvested by scraping into ice-cold HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, 1 mM EDTA, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and subcellular fractions were obtained according to the method of Volchuk *et al.* (43). Aliquots (100 μg) from the low-density membranes, plasma membranes, and cytosolic fractions were solubilized in 1% Triton X-100 for 30 min at 4 °C followed by centrifugation at 200,000 $\times$ *g* for 1 h. The pellet was resuspended in 20 mM HEPES, 2 mM EGTA, 1% Triton X-100 with 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. 20 μg of untreated sample and equal volumes of fractionated samples (approximately 40 μg) were analyzed by SDS-PAGE followed by Western immunoblotting with anti-Rad or anti-Ras antibodies.

Sheets Immunofluorescence Assay—Serum-deprived 3T3-L1 adipocytes, grown and differentiated on glass coverslips, were stimulated with or without 100 nM insulin for 15 min at 37 °C, washed, and subjected to sonication for 5 s to expose the inner surface of the plasma membrane, which remained attached to the coverslips (41). The plasma membrane sheets were processed for indirect immunofluorescence using anti-Glut4 polyclonal antibody (1:200, East Acres Biologicals) followed by incubation with Texas Red-conjugated goat anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch Laboratories, Inc.) and visualization using an Olympus BX60 microscope.

RESULTS

Overexpression of Rad Does Not Affect DNA Synthesis, Differentiation, or MAP Kinase Activation—Rad was expressed in C2C12 murine myocytes, L6 rat myocytes, and 3T3-L1 murine fibroblasts using retroviral-mediated transduction. Western blotting of lysates from differentiated cells indicated that Rad was expressed at least 25–30-fold above endogenous levels in C2C12 and 3T3-L1 cells and approximately 10–15-fold above endogenous levels in L6 cells (Fig. 1*A*). Overexpressing cells did not differ from control cells in gross morphology or in their ability to differentiate into mature myotubes or adipocytes. Likewise, overexpression of Rad in C2C12 and L6 myocytes had no effect on [^{3}H]thymidine incorporation in response to increasing concentrations of insulin (Fig. 1*B*) or 10% serum (not shown).

To directly assess MAP kinase activity, immune-complexed MAP kinase from C2C12 myotubes (C2C12), cells infected with vector only (C2C12 Puro) and Rad overexpressors (C2C12 Rad), was assayed for the ability to stimulate incorporation of

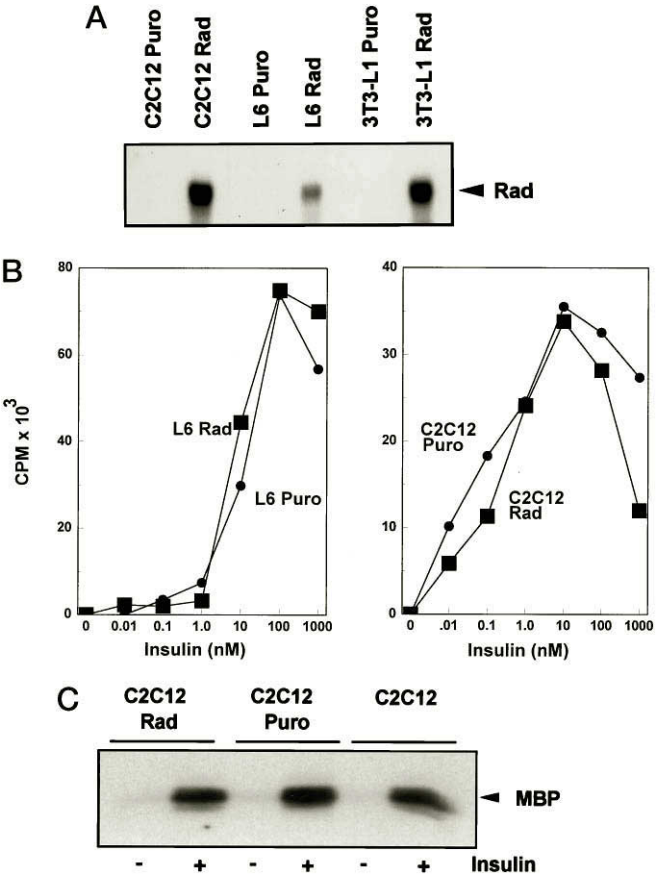

FIG. 1. **[^{3}H]Thymidine uptake and MAP kinase activity in Rad overexpressors.** Expression levels of Rad were assessed by Western immunoblotting lysates (100 μg) from control cells (C2C12 Puro, L6 Puro, and 3T3-L1 Puro) and Rad overexpressors (C2C12 Rad, L6 Rad, and 3T3-L1 Rad) using anti-Rad antibody. Endogenous Rad is visible following long exposure. Control myoblasts (●, C2C12 Puro and L6 Puro) and Rad overexpressors (■, C2C12 Rad and L6 Rad) were stimulated with the indicated concentrations of insulin (in triplicate) for 15 h followed by a 1-h incubation with [^{3}H]thymidine (1 μCi/well). Data show the average counts incorporated ± S.E. in L6 cells (*B, left*) and C2C12 cells (*B, right*). In *C* control myoblasts (C2C12 Puro) or Rad overexpressors (C2C12 Rad) were treated with or without 100 nM insulin for 10 min. Lysates (200 μg) were subjected to immunoprecipitation with anti-MAP kinase antibody, followed by determination of MAP kinase activity, as assessed by the incorporation of [^{32}P]ATP into myelin basic protein. Reaction products were analyzed by SDS-PAGE followed by autoradiography.

[^{32}P]ATP into myelin basic protein. Again, Rad overexpression had no effect on insulin-stimulated MAP kinase activity (Fig. 1*C*). In addition, overexpression of Rad does not induce growth of C2C12 cells in soft agar or affect transformation of C2C12 cells by an oncogenically active (Q61L) form of Ras (not shown). Thus, Rad does not affect the signaling events required for mitogenesis and differentiation induced by insulin (or serum) nor the upstream signals that lead to these events.

Overexpression of Rad Inhibits Insulin-stimulated Glucose Uptake—To determine whether Rad affects signals leading to insulin-stimulated glucose uptake, cells overexpressing Rad were assayed for 2-deoxyglucose uptake in response to increasing concentrations of insulin. In contrast to its lack of effect on mitogenesis, overexpression of Rad almost completely inhibited insulin-stimulated glucose uptake in rat L6 myotubes (Fig. 2*A*), and reduced insulin-stimulated glucose uptake by approximately 50% in C2C12 myotubes (Fig. 2*B*) and 3T3-L1 adipocytes (Fig. 2*C*). Expression of Rad affected glucose transport, since uptake of the nonmetabolizable glucose analog, 3-*O*-methylglucose, was inhibited by Rad (Fig. 2*D*), although to a

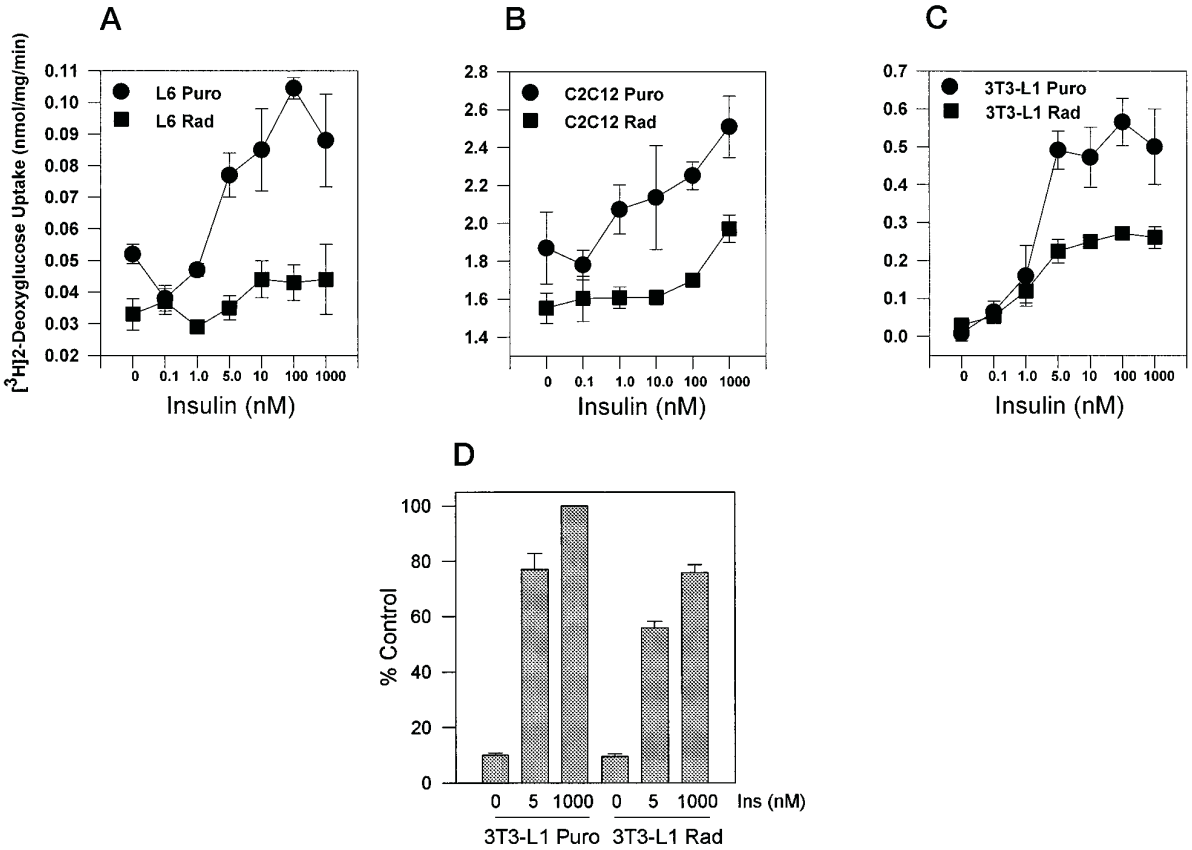

FIG. 2. **Glucose uptake in Rad overexpressors.** Control cells (●, L6 Puro, C2C12 Puro, and 3T3-L1 Puro) and Rad overexpressors (■, L6 Rad, C2C12 Rad, and 3T3-L1 Rad) were assayed for 2-deoxy-[^{3}H]D-glucose (*A–C*) or 3-*O*-methyl-[^{14}C]D-glucose (*D*) uptake in response to various concentrations of insulin as described under "Materials and Methods." *A* and *B* are representative experiments from three and six independent experiments, of L6 and C2C12 cells, respectively, each performed in triplicate. *C* is the average of three independent experiments of 3T3-L1 cells, each performed in triplicate. Data are expressed as nanomoles of glucose taken up/mg of cell protein/min ± S.E. with nonspecific counts, determined by the counts incorporated in the presence of 10 μM cytochalasin B, subtracted from each value. *D* shows the average of two independent experiments expressed as percent of control cells treated with 1000 nM insulin. Nonspecific counts incorporated in the presence of 250 μM phloretin were subtracted to yield specific influx values.

lesser extent than that of 2-deoxyglucose uptake. The effect of Rad on glucose uptake occurred without reduction in expression or phosphorylation of molecules involved in signaling events which are known to affect glucose uptake. Thus, phosphotyrosine immunoblots of C2C12 control cells (C2C12 Puro) and C2C12 Rad overexpressors (C2C12 Rad) revealed similar levels of tyrosine phosphorylation of the insulin receptor and IRS-1 following treatment with 100 nM insulin for 10 min (Fig. 3*A*). Western immunoblotting of lysates from C2C12 control myotubes and Rad overexpressors also indicated no differences in expression of insulin receptors (IR), insulin receptor substrate (IRS), or the p85 subunit of PI 3-kinase (p85) or in the phosphotyrosine content of IRS-1 (Fig. 3*B*). The association of p85 PI 3-kinase with IRS was also unchanged by Rad expression when IRS immunoprecipitates were immunoblotted with anti-PI 3-kinase antibody following insulin stimulation (Fig. 3*B*). Consistent with these findings, Rad overexpressors and control cells exhibited similar IRS-1-associated basal levels of PI 3-kinase activity, and both exhibited an average 7-fold stimulation in response to insulin treatment (Fig. 3*C*). Similar results were obtained in immunoprecipitates using anti-phosphotyrosine antibody (not shown).

Glucose Transporter Translocation and Rad Localization—Glut4 is the major insulin-stimulated glucose transporter and undergoes translocation from internal membrane compartments to the plasma membrane following stimulation, whereas Glut1 tends to be localized to the plasma membrane at all times and accounts for most of the basal glucose uptake (21). Western immunoblotting of differentiated 3T3-L1 adipocytes revealed no detectable differences in Glut1 or Glut4 expression between control cells (3T3-L1 Puro) and Rad overexpressors (3T3-L1 Rad) (Fig. 4*A*).

To investigate the potential effects of Rad on Glut4 translocation, two different methods were used. Glucose transporter translocation in differentiated 3T3-L1 control cells (3T3-L1 Puro) and Rad overexpressors (3T3-L1 Rad) was examined in subcellular fractions of internal membranes and plasma membranes by Western immunoblotting with Glut4 antibody. Insulin stimulation of control cells resulted in a decrease in Glut4 detection in the low density microsomal (LDM) fraction, accompanied by a corresponding increase in Glut4 in plasma membranes (PM) (Fig. 4*B, left*). Similar results were obtained when Rad overexpressors were analyzed (Fig. 4*B, right*).

Differentiated adipocytes from control cells (3T3-L1 Puro) and Rad overexpressors (3T3-L1 Rad) were also subjected to a sheets immunofluorescence assay as described under "Materials and Methods." Fig. 4*C* shows representative regions of coverslips of control cells (*left*) and Rad overexpressors (*right*) from the basal (*top*) or insulin-stimulated (*bottom*) state. Glut4 immunofluorescence was low in the basal state and showed a similar increase following insulin stimulation in both cell lines. Examination of numerous fields in multiple coverslips revealed no overall differences in the intensity of staining between the two cell lines following insulin stimulation. These findings, together with those of the fractionation assay, suggest Rad overexpression does not interfere with glucose transporter

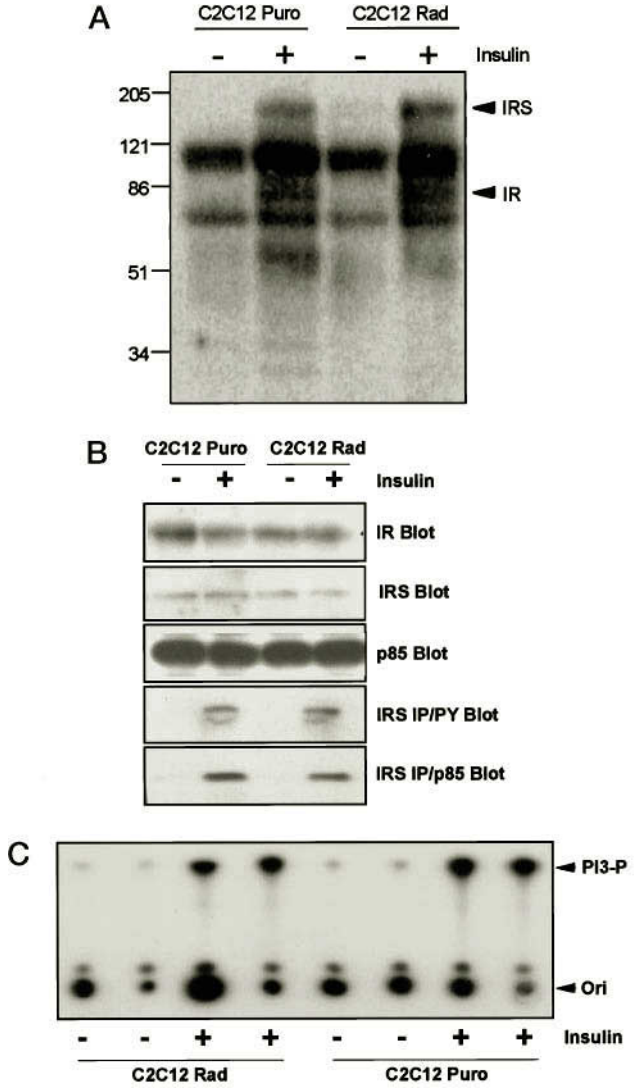

FIG. 3. **Characterization of insulin signaling molecules.** *A*, cellular lysates (100 μg) from control cell (C2C12 Puro) and cells overexpressing Rad (C2C12 Rad), treated with or without 100 nM insulin for 10 min, were subjected to Western immunoblotting using anti-phosphotyrosine antibody. The positions of IRS-1 and the β-subunit of the insulin receptor are indicated. *B*, C2C12 lysates described in *A* were subjected to Western blotting using anti-insulin receptor (IR), anti-IRS-1 (IRS-1), and anti-phosphatidylinositol 3-kinase (p85) antibodies, as described under "Materials and Methods." 500 μg of lysates were subjected to immunoprecipitation with anti-IRS-1 antibody and blotted with anti-phosphotyrosine (*PY*) antibody to detect phosphorylation or anti-PI 3-kinase antibody to detect IRS-associated p85 PI 3-kinase. In *C*, lysates were analyzed for IRS-1-associated PI 3-kinase activity, and the reaction products were visualized by thin layer chromatography and autoradiography.

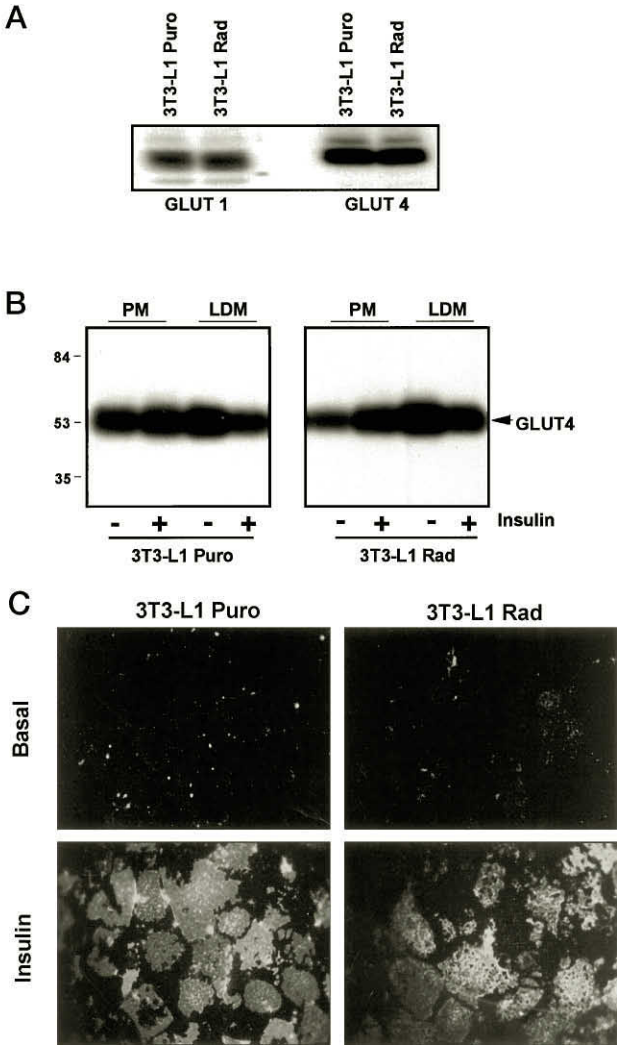

FIG. 4. **Glucose transporter translocation and Rad localization.** *A*, lysates (100 μg) from control adipocytes (3T3-L1 Puro) and cells overexpressing Rad (3T3-L1 Rad) were subjected to Western blotting with anti-Glut1 or anti-Glut4 antibody, as described under "Materials and Methods." In *B*, differentiated control cells (3T3-L1 Puro) and Rad overexpressors (3T3-L1 Rad) were stimulated with or without 100 nM insulin for 15 min prior to subcellular fractionation, as described under "Materials and Methods." 10 μg of the low density microsome (*LDM*) and plasma membrane (*PM*) fractions were then subjected to Western immunoblotting to detect Glut4 protein. In *C*, adipocytes, grown and differentiated on glass coverslips in 6-well dishes, were subjected to sonication to expose the inner surface of the plasma membrane following treatment with or without 100 nM insulin for 15 min. Membrane sheets were assayed by indirect immunofluorescence for detection of Glut4 in the plasma membrane. Plasma membrane sheets from representative regions of the coverslips from control cells (3T3-L1 Puro, *left*) and Rad overexpressors (3T3-L1 Rad, *right*) are shown.

translocation to the plasma membrane in response to insulin.

Western immunoblotting with Rad antibody before and after stimulation with 100 nM insulin for 15 min revealed that Rad was most highly expressed in the cytosol and was significantly enriched in the LDM fraction, in comparison with the PM and high-density microsomal (*HDM*) fractions (Fig. 5*A*). Little or no Rad was detected in the fraction containing nuclear/mitochondrial (NUC/MITO) proteins. Insulin stimulation did not appear to significantly alter the localization of Rad. Interestingly, the Rad protein associated with the LDM fraction could be solubilized in 1% Triton X-100, while that associated with the PM fraction was not (Fig. 5*B*). By contrast, Ras partitioned as expected, being highly expressed in the Triton X-100-soluble PM fraction. These results suggest that Rad stably associates with the plasma membrane, perhaps via the cytoskeletal network, while Rad is more loosely attached to LDM membranes, perhaps by protein-protein interaction.

DISCUSSION

It has previously been shown that Rad is overexpressed in the skeletal muscle of some humans with Type II diabetes, a disease characterized by insulin resistance at the level of muscle and fat (16). To determine if Rad might play a role in insulin resistance, we have examined the effects of Rad overexpression on insulin-stimulated signaling events in C2C12 and L6 skeletal muscle lines and the 3T3-L1 adipocyte line. In all three cell lines, Rad expression inhibits insulin-stimulated 2-deoxyglucose uptake by approximately 50–90% and 3-*O*-methylglucose

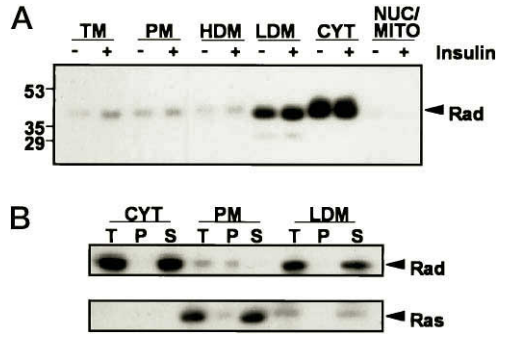

FIG. 5. **Subcellular localization of Rad.** 50 μg of subcellular fractions from control or insulin-stimulated (100 nM, 15 min) 3T3-L1 cells overexpressing Rad were subjected to Western immunoblotting with anti-Rad antibody. *A* shows the relative subcellular distribution of Rad in total membranes (*TM*), high density microsomes (*HDM*), plasma membrane (*PM*), low density microsomes (*LDM*), cytosol (*CYT*), and nuclear and mitochondrial-containing fractions (*NUC/MITO*). In *B*, 100 μg of the CYT, PM, and LDM fractions of unstimulated cells were solubilized in 1% Triton X-100, and total (*T*, before centrifugation), 200,000 $\times$ *g* pellet (*P*) and supernatant (*S*) were analyzed by immunoblotting with anti-Rad or anti-Ras antibodies.

uptake by approximately 25%, while having no effect on insulin-stimulated [^{3}H]thymidine incorporation, MAP kinase activity, PI 3-kinase activity, or receptor and substrate phosphorylation. This occurred without apparent changes in Glut1 or Glut4 expression.

Several studies have suggested that the regulation of glucose transport in cells is a multistep process (22–27). The best studied of these steps is the translocation of Glut4 glucose transporters from an intracellular pool or low density membranes to the plasma membrane. The process of hexose uptake is likely to involve a series of fusion and cycling events, with regulatory steps, similar, but not necessarily identical, to those implicated in the synaptic vesicle cycle, including vesicle budding, translocation, docking, priming, fusion, and exocytosis, followed by internalization, endosome fusion, and neurotransmitter uptake (12). Several of these steps appear to be regulated by Rab-like low molecular weight GTP-binding proteins. Evidence suggests that GTP-binding proteins are also involved in the processes of Glut4 exocytosis and endocytosis (28). Thus some investigators have found that GTPγS and NaF stimulate glucose transport in permeabilized rat epididymal fat cells (29–30), although this has not been uniformly observed (31). Low molecular weight GTP-binding proteins have been found to be associated with Glut4-containing vesicles from the LDM fractions of adipocytes in a manner that is disrupted by treatment with 1% Triton X-100 (30). In addition, some investigators have implicated an "activation" step in glucose uptake, based on the fact that in some systems translocation does not appear to be sufficient to account for the entire insulin effect on glucose uptake and that some agents, which stimulate transport, do so without affecting translocation (27).

Rad diminishes glucose uptake in cells expressing both Glut1 (which does not readily translocate) and Glut4 transporters (which do readily translocate). Rad exerts this effect without apparent alteration of the insulin-stimulated translocation of Glut4 transporters, suggesting that Rad has some effect on the intrinsic activity of Glut1 and Glut4. The mechanism of this effect is unclear. Thus far, we have been unable to demonstrate direct co-precipitation of Rad with Glut4-containing vesicles (not shown), indicating that Rad is either loosely associated with these vesicles or is enriched in other types of low density membranes. It is possible that Rad affects exposure of the transporter to the extracellular mileau or some intermediate step, such as fusion, which is required for full activation of transporter activity. It is also possible that Rad has some effects on other early events which secondarily affect glucose uptake, such as phosphorylation by hexokinase.

The actin cytoskeleton organization has been shown to be important for regulated endocytosis and exocytotic events (32, 33) and has been shown recently to be important in the localization of glucose transporters and for their incorporation into the plasma membrane of L6 cells in response to insulin (34). Insulin causes a reorganization of the actin network involving the aggregation of polymerized actin, and disassembly of the actin network by treatment with cytochalasin D results in an inhibition of glucose uptake associated with a reduction in the translocation of glucose transporters. In this regard it is interesting to note that in skeletal muscle, some Rad associates with β-tropomyosin (20). While we have not detected insulin-induced changes in this association, it is intriguing to speculate that Rad may have some role in actin/cytoskeleton arrangement, which affects glucose uptake. In any case, Rad should provide a valuable tool for understanding the events which regulate glucose uptake and transporter intrinsic activity.

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