Articles

Association of Cytosolic Rab4 with GDI Isoforms in Insulin-Sensitive 3T3-L1 Adipocytes†

Assia Shisheva*,‡ and Michael P. Czech§

Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201, and Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

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ABSTRACT: Translocation of an intracellular pool of GLUT4 glucose transporters to the fat and muscle cell surface is thought to involve small GTP-binding proteins such as the Rab4 protein. The cycling of Rab proteins between cytosol and intracellular membranes necessary for their function appears to be regulated by GDP-dissociation inhibitors (GDI), three of which have been cloned thus far. Previous data suggest that Rab4 binds two of these isoforms of GDI (1 and 2) similarly when purified proteins are employed [Shisheva, A., et al. (1994) *Mol. Cell. Biol. 14*, 3459−3468]. In the present study, we have analyzed the cytosolic Rab4 in complexes with GDI-1 or GDI-2 in intact cells using a coprecipitation technique. We show here that in insulin-sensitive 3T3-L1 adipocytes and other cultured cells, Rab4 simultaneously forms stable cytosolic complexes with both GDI-1 and GDI-2. Acute insulin treatment of the cultured adipocytes significantly increases cytosolic levels of Rab4 which can be quantitatively immunoprecipitated with anti-Rab4 antibodies. Surprisingly, the increased cytosolic Rab4 due to insulin action is predominantly associated with cytosolic GDI-1. The levels of cytosolic Rab4−GDI-2 complexes were virtually unaltered by insulin. Insulin-dependent alterations of Rab4 and GDI-1 phosphorylation were not detected in ^{32}P-labeled 3T3-L1 adipocytes, suggesting another mechanism accounts for the specificity of Rab4 binding to GDI-1. Taken together, these data suggest there is selective formation of Rab4−GDI-1 complexes in response to insulin which plays a role in the action of insulin on membrane trafficking.

Abundant *in vivo* and *in vitro* experimental evidence has indicated that the effect of insulin on hexose transport is in large part mediated by the redistribution of glucose transporters from an intracellular site to the plasma membrane, thereby augmenting the facilitated transport of glucose into the cell [for reviews, see Birnbaum (1989), Bell et al. (1993), James and Piper (1994), Mueckler (1994), and Czech (1995)]. Fat and muscle tissues express two glucose transporter isoforms, the ubiquitous GLUT1 at low levels, and the fat/muscle-specific insulin-sensitive GLUT4. Both isoforms translocate to the adipocyte cell surface in response to insulin. Although numerous immunoelectron microscopy and biochemical studies accumulated for over a decade support this translocation model, originally proposed by Cushman and Wardzala (1990) and Suzuki and Kono (1980), the molecular mechanism(s) underlying these glucose transporter protein movements in basal and insulin-stimulated states is still obscure.

Substantial evidence suggests a role for the *Rab/YPT1/SEC4* gene subfamily of p21^{ras}-like small GTP-binding proteins in the mechanisms regulating membrane trafficking in yeast and mammalian cells [for recent reviews, see Novick and Brennwald (1993), Simons and Zerial (1993), Zerial and Stenmark (1993), Nuoffer and Balch (1994), von Mollard et al. (1994), Pfeffer et al. (1995), Mellman (1995), and De Camililli et al. (1996)]. The mammalian Rab proteins, including over 30 known members, are found associated with distinct compartments of both biosynthetic and endocytic pathways. Although the specific function of each Rab protein in membrane trafficking is currently unknown, these proteins appear to be essential in each step of vesicular transport, including the formation of functional carrier vesicles, vesicle docking, and membrane fusion (Novick & Brennwald, 1993; Simons & Zerial, 1993; Zerial & Stenmark, 1993; Nuoffer & Balch, 1994; von Mollard et al., 1994; Nuoffer et al., 1994; Riederer et al., 1994; Pfeffer et al., 1995). It has been shown that Rab protein is required for vSNARE and tSNARE assembly (Sogaard at al., 1994; Rothman & Wieland, 1996), suggesting that members of the Rab family represent important components of the membrane transport machinery.

Current models suggest Rab proteins undergo a membrane−cytosol localization cycle associated with GTP binding and

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* Correspondence should be addressed to this author at the Department of Physiology, Wayne State University School of Medicine, 540 E. Canfield, 4237 Scott Hall, Detroit, MI 48201. Telephone: (313) 577-5674. Fax: (313) 577-5494. E-mail: ashishev@moose.med.wayne.edu.

‡ Wayne State University School of Medicine.

§ University of Massachusetts Medical Center.

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hydrolysis. This functional cycle is promoted by several accessory proteins, the best well studied among them being GDP-dissociation inhibitor protein-1 [GDI-1;[1] Shisheva et al., 1994a; also known as Rab3AGDI (Ullrich et al., 1993) or GDI d (Nishimura et al., 1994)]. Originally identified by its ability to inhibit GDP-dissociation from Rab3A (Sasaki et al., 1990), it turned out GDI-1 also functions in retrieval and specific delivery of several Rab proteins from and to membranes (Ullrich et al., 1993, 1994; Soldati et al., 1994; Peter et al., 1994; Horiuchi et al., 1995). Three GDI isotypes have been identified and cloned thus far: GDI-1, GDI-2, and GDI β (Matsui et al., 1990; Shisheva et al., 1994a; Nishimura et al., 1994; Yang et al., 1994; Janoueix-Lerosey et al., 1995). They are ubiquitously expressed, highly homologous protein isoforms, tightly conserved among mouse, rat, bovine, and human. Structural studies point a domain at the apex of GDI crucial for Rab protein binding (Schalk et al., 1996). In *in vitro* assays, the three GDIs (at relatively high concentrations) display similar abilities to bind and solubilize membrane-associated Rabs in a GDP/GTP-dependent manner (Shisheva et al., 1994a; Janoueix-Lerosey et al., 1995). However, several lines of biochemical and morphological evidence suggest distinct functional roles for GDI-1 and GDI-2. Thus, they are differentially distributed in several cell types, the relative amounts of membrane-associated GDI-2 being strikingly higher than that of GDI-1 (Shisheva et al., 1994b, 1995). Further, GDI-2 associated with the microsomal fraction significantly decreases upon insulin stimulation of 3T3-L1 adipocytes, as opposed to GDI-1 (Shisheva et al., 1994b). Finally, GDI-2 overexpression in COS cells markedly inhibits transferrin receptor internalization, while the overexpressed GDI-1 has almost no effect (Shisheva et al., 1995). All these findings suggest that GDI isotypes may be selectively involved in Rab function.

In this study, we analyzed the cytosolic complexes of Rab4, a Rab family member thought to play a role in GLUT4 membrane trafficking (Cormont et al., 1993), with GDI-1 and GDI-2 in several cultured cells by a coprecipitation technique. We demonstrate here that Rab4 simultaneously forms stable cytosolic complexes with both GDI isoforms in all cell types examined. Surprisingly, the increased Rab4 released into the cytosol upon insulin stimulation of 3T3-L1 adipocytes is preferentially taken up by the cytosolic GDI-1. These data show further functional differences between GDI-1 and GDI-2, and suggest a selective role of cytosolic Rab4−GDI-1 complexes in the action of insulin on membrane movements.

MATERIALS AND METHODS

Reagents and Antibodies. Human insulin was a gift from Eli Lilly; [^{32}P]orthophosphate and Renaissance chemiluminescence detection kit were purchased from DuPont, New England Nuclear; protein A−Sepharose 4 Fast Flow was from Pharmacia Biotech; dimethyl pimelimidate was from Sigma; Immobilon-P transfer membranes (0.45 μm pore size) were from Millipore Corp. (Bedford, MA); bicinchoninic acid protein assay reagent was from Pierce (Rockford, IL); horseradish peroxidase-coupled donkey anti-rabbit IgG was from Amersham Life Sciences (Arlington Heights, IL). Rabbit polyclonal anti-GDI-1 (R3357) and anti-GDI-2 (R3361) antibodies were directed against a mGDI-1 peptide (amino acids 431−447) or a mGDI-2 peptide (amino acids 387−404), respectively (East Acres, Southbridge, MA), and were characterized as described elsewhere (Shisheva et al., 1994a,b). Rabbit polyclonal anti-human Rab4 antibodies (R10502), a generous gift from Dr. Ira Mellman, were directed against Rab4 fusion protein. Their specificity toward Rab4 was described elsewhere (van der Sluijs et al., 1991) and was confirmed in this study with respect to Rab5.

Cell Culture. 3T3-L1 mouse fibroblasts were differentiated into adipocytes as previously described (Shisheva et al., 1994b, 1995). Briefly, fibroblasts were grown to confluence (100 or 150 mm plate) in DMEM, containing 10% calf serum, 50 units/mL penicillin, and 50 μg/mL streptomycin sulfate. Differentiation was induced with DMEM, supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin sulfate, 5 μg/mL insulin, 0.25 μM dexamethasone, and 0.35 mM 3-isobutyl-1-methylxanthine. On the second day, the medium was replaced with DMEM, containing all but the last two of the above components. Thereafter, the cells were maintained in DMEM, containing 10% fetal bovine serum and the antibiotics with medium changes every 3−4 days. Adipocytes were used between 10 and 17 days after the onset of differentiation. COS-1 and CHO-T cells were grown to confluence (150 mm plate) in DMEM or Ham's F-12 medium, respectively, containing 10% FBS and the above antibiotics.

^{32}P-Labeling. Differentiated 3T3-L1 adipocytes were washed 3 times with phosphate-free DMEM and labeled for 16 h with [^{32}P]orthophosphate (1 mCi/mL) in phosphate-free DMEM containing 0.5% BSA, 25 mM HEPES, pH 7.3, and 2 mM pyruvate. The cells were then stimulated or not with insulin (100 nM) for 10 min, placed on ice, and washed 2 times with DMEM, 2 times with PBS, containing 1 mM PMSF, and 1 time with "homogenization buffer" [20 mM HEPES, pH 7.4, containing 255 mM sucrose, 1 mM EDTA, 25 mM β-glycerophosphate, 100 mM NaF, 2 mM sodium orthovanadate, and 1 × protease inhibitor mixture (1 mM PMSF, 0.5 mg/mL benzamidine, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 1 μg/mL pepstatin)]. The cells were then scraped with the above "homogenization buffer" supplemented with 800 nM okadaic acid, homogenized in a motor-driven Teflon/glass homogenizer at 4 °C, and then fractionated in a Beckman TLA 100.3 rotor as we previously described (Shisheva et al., 1994b). The cytosolic fractions (normalized for equal amounts of radioactivity; 170 × 10^6 cpm; ∼400 μg of cytosolic protein) were immunoprecipitated with anti-GDI-1, anti-Rab4, or preimmune IgG cross-linked to protein A−Sepharose Fast Flow.

Cell Treatment and Fractionation. 3T3-L1 adipocytes, serum-starved for 4 h prior to the experiment, were incubated in the absence or presence of insulin (100 nM) for 20 min. After washes, the cells were scraped at 4 °C in the "homogenization buffer" (same as above containing in addition 5 mM $MgCl_2$), homogenized, and centrifuged to obtain the cytosolic fractions as described above. The cytosols from COS-1 and CHO-T cells were obtained by centrifuging postnuclear supernatants for 24 min at 200000*g* in a Beckman TLA 100.3 rotor. Equal amounts of protein (typically between 1 and 1.5 mg; determined by bicinchoninic

[1] Abbreviations: GDI, GDP-dissociation inhibitor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride.

acid protein assay) were immunoprecipitated with anti-Rab4, GDI-1, or preimmune IgG cross-linked to protein A−Sepharose Fast Flow.

Antibody Cross-Linking, Immunoprecipitation, and Western Blotting. Coupling of anti-GDI-1, anti-Rab4, or preimmune IgG to protein A−Sepharose Fast Flow was performed essentially as described in Sisson and Castor (1990) with modifications. Briefly, anti-GDI-1, anti-Rab4, or preimmune antisera were incubated with protein A−Sepharose Fast Flow beads (5 mg of IgG/mL of packed beads) in 50 mM HEPES, pH 7.6, containing 100 mM NaCl for 16 h at 4 °C. The beads were washed 4 times with 10 volumes of 0.1 M Tris-HCl, pH 8.5, and twice with 10 volumes of 0.2 M borate buffer, pH 9.0, and then resuspended in 10 volumes of 0.2 M borate buffer, pH 9.0, containing 35 mM dimethyl pimelimidate. Following 40 min of gentle mixing, fresh dimethyl pimelimidate solution was added. After 30 min, the reaction was stopped by washing with 10 volumes of 0.2 M ethanolamine, pH 8.0, and incubated for an additional 2 h at room temperature. The beads were finally washed and resuspended in PBS, containing 0.05% azide, and stored at 4 °C.

For the immunoprecipitation experiments, coupled IgG fractions equivalent to 14 μL of packed beads (∼50 μg of IgG) were incubated with fractionated cytosols in the presence of 1 mM GDP for 40 min at 4 °C. The beads were then quickly washed twice with 1.4 mL portions of "cytosol washing buffer", consisting of 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 2 mM EDTA, 25 mM NaCl, 5 mM $MgCl_2$, 0.8 mM GDP, 25 mM β-glycerophosphate, 25 mM NaF, 2 mM sodium orthovanadate, and 1 × protease inhibitor mixture by spinning down for 30 s at 10 000 rpm at 4 °C each time. The beads were then eluted stepwise. First, Rab4-associated proteins were eluted for 20 min at room temperature with 50 μL of RIPA buffer (20 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 132 mM NaCl, 5 mM $MgCl_2$, and the above phosphatase and protease inhibitors). Following centrifugation, 40 μL aliquots were decanted, reduced with Laemmli sample buffer (Laemmli, 1970), and analyzed by SDS−PAGE, using 10.5% acrylamide. After transfer onto Immobilon-P membranes, the blots were saturated with blocking buffer under previously specified conditions (Shisheva & Shechter, 1993) and blotted (16 h at 4 °C) with anti-GDI-1 and anti-GDI-2 antibodies as indicated in the figure legends. After washings, bound antibodies were detected with horseradish peroxidase-bound anti-rabbit immunoglobulins and chemiluminescence. Rab4 was dissociated from the immunoaffinity columns by 3 min boiling in Laemmli sample buffer, and following separation by SDS−PAGE (10.5% acrylamide) was analyzed by Western blotting as described above using anti-Rab4 antiserum.

Protein levels on the immunoblots were quantitated on a laser scanning densitometer (Molecular Dynamics) by area integration. Two exposures of each blot were quantified to ensure that the chemiluminiscence exposures were within the linear range of the film.

RESULTS

Cytosolic Rab4 Is Simultaneously Associated with both GDI-1 and GDI-2 in Intact Cells. We first analyzed whether in intact cells Rab4 can form stable cytosolic complexes with both GDI-1 and GDI-2 protein isoforms as detected by immunoprecipitation of the cytosolic Rab4 and probing for GDIs by immunoblotting. Immunodetections of both GDI-2 and Rab4 are hampered by the fact that these proteins comigrate with IgG heavy and light chains, respectively, during their electrophoretic resolutions. We therefore used cross-linked immunoaffinity columns of anti-Rab4 (or preimmune IgG) and protein A−Sepharose Fast Flow beads. This cross-linking step, combined with subsequent elution of the proteins associated with Rab4 immunoaffinity columns using RIPA buffer, practically eliminated the undesirable appearance of the IgG protein bands and allowed us to unequivocally evaluate GDI-2−Rab4 complexes (Figure 1, compare lanes 1−3 vs lanes 4−6). As demonstrated in Figure 1, SDS−PAGE and Western blotting analysis with anti-GDI-1 and anti-GDI-2 antibodies revealed that both GDI-1 and GDI-2 coprecipitated with cytosolic Rab4 in three cell types examined. These data demonstrate that cytosolic Rab4 is able to simultaneously form complexes with both GDI-1 and GDI-2 in intact cells. Under the conditions used, more than 50% of the cytosolic Rab4 was immunoprecipitated, as judged by quantitative Rab4 depletion from the cytosols of indicated cells (not shown). Levels of GDI-1 and GDI-2 associated with Rab4 accounted for between 1/60 and 1/90 of total cytosolic GDIs. These findings are

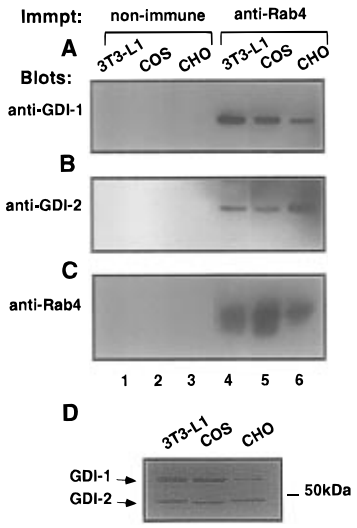

FIGURE 1: Rab4 forms stable cytosolic complexes with both GDI-1 and GDI-2 in different cultured cells. Indicated cultured cells (150 mm dish) were homogenized and then fractionated into total membranes and cytosol. Cytosolic proteins (1.2 mg) were immunoprecipitated (45 min at 4 °C) in the presence of 1 mM GDP with nonimmune IgG or anti-Rab4 IgG cross-linked to protein A−Sepharose Fast Flow. Immunoprecipitates were then quickly washed twice with "cytosol washing buffer". Rab4-associated proteins were eluted with RIPA buffer, reduced with Laemmli sample buffer, and analyzed by SDS−PAGE and Western blotting with anti-GDI-1 antiserum (1:20 000 dilution; A) or anti-GDI-2 IgG (0.24 μg/mL; B) with a stripping step in between. Immunodetection of Rab4 was achieved following its dissociation from the immunoaffinity column with Laemmli sample buffer, resolution by SDS−PAGE, and Western blotting with anti-Rab4 antiserum (1:1000; C). The relative amounts and electrophoretic mobility relative to the 50 kDa protein marker of both GDIs are shown in the initial cytosolic fractions (20 μg), which were resolved by SDS−PAGE, transferred to Immobilon-P membranes, and immunoblotted with a mixture of anti-GDI-1 and anti-GDI-2 antibodies (D). The exposure time in panel B is longer compared to the other blots. Shown are chemiluminescence detections of a representative experiment from two to four independent fractionations of each cell line.

consistent with current models suggesting a limited number of GDI isotypes associate with many members of the Rab protein family.

Acute Insulin Action Increases Cytosolic Rab4 Levels in 3T3-L1 Adipocytes. The 3T3-L1 cell line has been proven as a useful model for the study of insulin action. Under appropriate conditions, these cultured cells differentiate from fibroblast to adipocyte-like cells acquiring many characteristics of insulin-sensitive fat cells. GLUT4 recruitment to the 3T3-L1 adipocyte plasma membrane and augmentation of glucose uptake in response to acute insulin action have been reported by several laboratories including our own. We tested the hypothesis that insulin action in this cell type increases cytosolic Rab4 levels similar to its action in primary fat cells (Cormont et al., 1993). Figure 2A shows results of experiments in which we analyzed the cytosolic levels of immunoreactive Rab4 in 3T3-L1 adipocytes following acute stimulation with the hormone. An increase of cytosolic Rab4 by 70 ± 5% ($x \pm$ SE, $N = 4$) after 20 min insulin stimulation was documented by immunoblotting SDS−PAGE-resolved cytosolic proteins with anti-Rab4 antibodies. The cytosolic levels of both GDI-1 and GDI-2 were not significantly altered under similar treatment with insulin as judged by quantitative analysis of densitometric scans of the Western blots probed with anti-GDI-1 or anti-GDI-2 antibodies (Figure 2C,D). Importantly, these increased Rab4 cytosolic levels were also clearly demonstrated following Rab4 immunoprecipitation and detection by Western blotting. As shown in Figure 2B, Rab4 immunoprecipitates derived from insulin-stimulated intact 3T3-L1 adipocytes display a 44 ± 4% ($x \pm$ SE, $N =$ 4) increase of cytosolic Rab4 compared to untreated cells. These results demonstrate a selective increase of Rab4 cytosolic levels concomitant with the GLUT4 recruitment to the plasma membrane [Figure 4 in Shisheva et al. (1994b), and data not shown] upon insulin stimulation of 3T3-L1 adipocytes.

Acute Insulin Action Selectively Increases the Formation of Cytosolic Rab4−GDI-1 Complexes in 3T3-L1 Adipocytes. The apparent ability of Rab4 to simultaneously form stable cytosolic complexes with both GDI isoforms in intact 3T3-L1 adipocytes, documented in Figure 1, raises the question whether insulin action alters the relative proportions of Rab4−GDI-1 and/or Rab4−GDI-2 complexes. We tested this hypothesis by immunoprecipitating Rab4 from the cytosols of insulin-stimulated or control 3T3-L1 adipocytes and probing for coprecipitation of GDI-1 and GDI-2. Figure 3B shows significantly higher levels (~60−70%) of GDI-1 coprecipitated with Rab4 protein following insulin stimulation. The level of GDI-2 in Rab4 immunoprecipitates was virtually unaltered upon insulin treatment of these cells (Figure 3C). Densitometric scans of control Western blots demonstrated a linear relationship between the amount of protein and the signal detected by chemiluminiscence of immunoblots for each one of the antigens analyzed here at relatively short exposures (Figure 3A′,B′,C′). Taken together, these data demonstrate that the insulin-mediated increase of cytosolic Rab4 becomes selectively associated with GDI-1 and suggest an important role of Rab4−GDI-1 complexes in insulin action on membrane trafficking.

We next attempted to gain initial insight into the mechanism by which insulin causes an increase of the cytosolic Rab4 and its selective binding to GDI-1. Substantial evidence suggests that a phosphorylation step may regulate

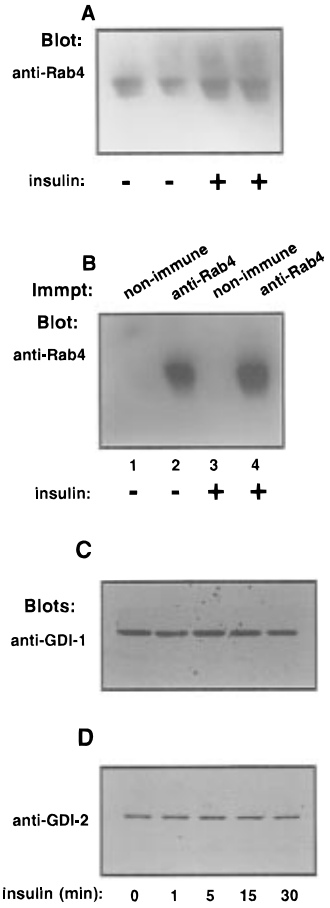

FIGURE 2: Insulin treatment increases the immunoreactive Rab4 in the cytosol of 3T3-L1 adipocytes. 3T3-L1 adipocytes (150 mm dish) were stimulated or not with insulin (10^{-7} M) for 20 min (A and B) or for the indicated time periods (C and D). The cells were homogenized and then fractionated into intracellular membranes, plasma membranes, and cytosol. (A) Cytosolic proteins (70 μg) were resolved by SDS−PAGE, transferred to Immobilon-P membranes, and immunoblotted with anti-Rab4 antiserum (1:1000). (B) Cytosolic proteins (1.5 mg) were immunoprecipitated (45 min at 4 °C) with nonimmune IgG or anti-Rab4 IgG cross-linked to protein A−Sepharose Fast Flow. Immunoprecipitates were washed with RIPA buffer, then solubilized in Laemmli sample buffer, and analyzed by SDS−PAGE and Western blotting with anti-Rab4 antiserum. (C and D) Cytosolic proteins (20 μg) were resolved by SDS−PAGE, transferred to Immobilon-P membranes, and immunoblotted with anti-GDI-1 (C) or anti-GDI-2 antiserum (D) (1:20 000). Shown are chemiluminescence detections of a representative experiment out of four (A and B) or six (C and D) with identical results. The increase of cytosolic Rab4 after 20 min of insulin stimulation was 70 ± 5% and 44 ± 4% ($x \pm$ SE, $N = 4$ independent cytosolic preparations) determined directly by Western blotting or following Rab4 immunoprecipitation, respectively.

Rab4 and/or GDI-1 functional cycles. First, Rab4 is phosphorylated in a cell cycle-dependent manner and is mainly found in the cytosol of mitotic cells (Baily et al., 1991; van der Sluijs et al., 1992b). Further, okadaic acid, a potent Ser/Thr phosphatase inhibitor, increases Rab4 cytosolic localizations (Cormont et al., 1993). Finally, GDI-1 itself is substantially phosphorylated when complexed with several Rab species as demonstrated by the studies in baby hamster kidney cells (Mortimer et al., 1993). We tested the hypothesis that a phosphorylation event is involved in the apparent increase of Rab4−GDI-1 complexes in response to insulin by immunoprecipitating GDI-1 or Rab4 from cytosol of ^{32}P-labeled 3T3-L1 adipocytes, followed by autoradiography of the SDS−PAGE-resolved proteins. As demonstrated in

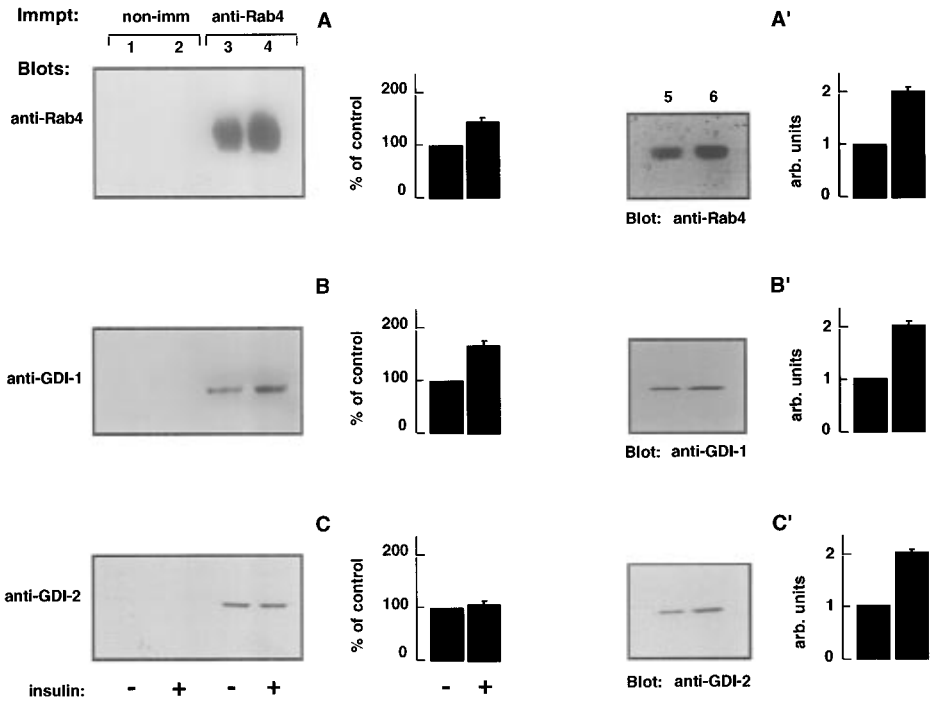

FIGURE 3: Insulin treatment increases Rab4 association with GDI-1 in the cytosol of 3T3-L1 adipocytes. (A−C) 3T3-L1 adipocytes (150 mm dish) were stimulated or not with insulin (10^{-7} M) for 20 min as indicated. Adipocytes were then fractionated into intracellular membranes, plasma membranes, and cytosol. Cytosolic proteins (1.7 mg) were immunoprecipitated (45 min at 4 °C) in the presence of 1 mM GDP with nonimmune IgG (vertical lanes 1 and 2) or anti-Rab4 IgG (vertical lanes 3 and 4) cross-linked to protein A−Sepharose Fast Flow. Immunoprecipitates were then quickly washed twice with "cytosol washing buffer". The proteins associated with Rab4 were eluted with RIPA buffer, reduced with Laemmli sample buffer, and analyzed by SDS−PAGE and Western blotting with anti-GDI-1 antiserum (1:20 000 dilution; B) or anti-GDI-2 IgG (0.24 μg/mL; C). Immunodetection of Rab4 was achieved following its dissociation from the immunoaffinity column with Laemmli sample buffer, resolution by SDS−PAGE, and Western blotting with anti-Rab4 antiserum (1:1000; A). (A′−C′) 20 μL (vertical lanes 5) and 40 μL (vertical lanes 6) of cytosolic protein derived from resting 3T3-L1 adipocytes were analyzed by SDS−PAGE and Western blotting using anti-GDI-1 (1:20 000), anti-GDI-2 (1:20 000), or Rab4 antisera (1:1000) as indicated. A linear relationship between the amount of protein and the signal detected by chemiluminiscence of the immunoblots was typically observed at a relatively low level of intensity. Shown are chemiluminescence detections of a representative experiment and densitometric scans of three independent cytosolic preparations ($x \pm$ SE).

Figure 4A,B, GDI-1 quantitatively immunoprecipitated from cytosols of ^{32}P-labeled cells is slightly phosphorylated in the basal state. No insulin-dependent changes on GDI-1 phosphorylation levels were reproducibly demonstrated in three independent experiments (Figure 4A). Phosphoamino acid analysis after acid hydrolysis of the band excised from the Immobilon-P membrane corresponding to GDI-1 revealed no phosphotyrosine and equal levels of phosphoserine and phosphothreonine in insulin-treated and control cells (data not shown). Phosphorylation corresponding to the Rab4 band was barely detectable in the basal state (Figure 4C,D). No insulin-mediated change in the phosphorylation state of the cytosolic Rab4 was detected, despite the significant increase of the immunoreactive Rab4 in Rab4 immunoprecipitates (Figure 4C,D). Taken together, these data are consistent with the conclusion that alterations in Rab4 and GDI-1 phosphorylation do not contribute to the observed insulin-dependent increase of cytosolic Rab4 and Rab4−GDI-1 complexes in 3T3-L1 adipocytes.

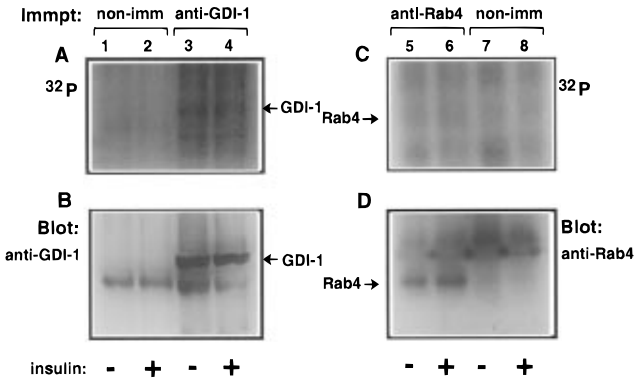

FIGURE 4: Lack of insulin-dependent phosphorylation on the cytosolic populations of Rab4 and GDI-2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes (100 mm dish) were labeled for 16 h with [^{32}P]-orthophosphate (1 mCi/mL) in phosphate-free DMEM. The cells were then stimulated (10 min, 37 °C) or not with insulin (10^{-7} M) as indicated, homogenized, and fractionated as above. Cytosolic proteins (0.5 mg) were immunoprecipitated (4 h, 4 °C) with anti-GDI-1 (A and B) or anti-Rab4 IgG (C and D) cross-linked to protein A−Sepharose Fast Flow. Immunoprecipitates were washed 6 times with RIPA buffer, solubilized in Laemmli sample buffer, and then analyzed by SDS−PAGE. The proteins were then transferred onto Immobilon-P membranes and consequently analyzed by autoradiography (A and C, X-OMAT-AR, Kodak) and by Western blotting with anti-GDI-1 (B) or anti-Rab4 antisera (D). Shown are autoradiograms and chemiluminescence detections of representative experiments out of four independent cell labelings with identical results. The increase of cytosolic Rab4 detected in Rab4 immunoprecipitates after 10 min of insulin stimulation was 30%.

DISCUSSION

Although insulin action on glucose transporter membrane trafficking has been recognized for more than a decade, the molecular mechanism underlying GLUT4 kinesis in insulin-sensitive cells is still elusive. Several recent studies suggest a role of certain Rab protein family members and their binding protein, GDI-2, in insulin-regulated secretory pathways in primary fat cells and cultured adipocytes (Baldini

et al., 1992, 1995; Cormont et al., 1993; Shisheva et al., 1994a,b). Rab4, in particular, attracts considerable attention as it is associated with the membranes of the recycling endosomal system (van der Sluijs, 1991, 1992a) with which GLUT4 recycles [reviewed in Czech (1995)]. The main focus of the present study was to analyze the soluble Rab4 complexes with the two GDI isotypes, GDI-1 and GDI-2, in intact cells including insulin-sensitive adipocytes in culture. We report here that the cytosolic Rab4 is simultaneously associated with both GDI-1 and GDI-2 under basal conditions. The key finding we documented further here is that acute insulin action of 3T3-L1 adipocytes increases cytosolic Rab4 and Rab4−GDI-1 soluble complexes. This result suggests a predominant role of GDI-1 vs GDI-2 in insulin-dependent Rab4 cycling off membranes.

Targeting of Rab proteins to their specific membrane compartments is determined by both proteins of the heterodimer Rab−GDI complex (Pfeffer et al., 1995). GDI protein is also required for selectively stimulating GTP/GDP exchange which follows the attachment of Rab5 to its target membranes (Horiuchi et al., 1995). Thus, whether a particular Rab will form a heterocomplex with GDI-1, GDI-2, or GDI β or with other yet to be discovered GDIs in the intact cell may be important as it might predict Rab protein selective targeting and specific delivery to the appropriate membrane. Selective regulations at the level of Rab association with a specific GDI isotype can be anticipated. The ability of insulin to selectively augment the cytosolic Rab4−GDI-1 complexes in 3T3-L1 adipocytes demonstrated in this study (Figure 3) is consistent with this hypothesis. It should be emphasized at this point that the mass action could not solely account for the observed almost exclusive association of elevated Rab4 with the GDI-1 isotype. The cytosolic concentration of GDI-1 is only slightly higher than that of GDI-2 at basal conditions (0.5 vs 0.35 μg/100 μL of cytosolic protein documented by quantitative Western blotting; Shisheva et al., 1994b), and insulin treatment of 3T3-L1 adipocyte does not change these levels (Figure 2C,D). Given the almost equal potency of recombinant GDI-1 and GDI-2 to bind and solubilize membrane-associated Rab4 in cell-free systems (Shisheva et al., 1994a), and to stably associate with cytosolic Rab4 in intact cells (Figure 1), subtle differences in the regulation of their interactions with Rab4 are probably physiologically significant. The exact molecular mechanism for the observed selective increase of Rab4−GDI-1 complexes in insulin-stimulated 3T3-L1 adipocytes remains to be elucidated. However, the lack of insulin-dependent alterations in Rab4 and GDI-1 phosphorylation states, clearly documented in the present study (Figure 4), suggests a phosphorylation-independent mechanism. These results further indicate that the phosphorylation of Rab4 by insulin-activated extracellular signal-regulated kinase (Cormont et al., 1994) observed in a cell-free system does not take place in intact adipocytes following insulin stimulation.

Biochemical and morphological studies demonstrate cytosolic GDI-1 localizations in 3T3-L1 adipocytes (Shisheva et al., 1994b, 1995). The small amounts of microsome-associated GDI-1 populations are not redistributed upon insulin stimulation of this cell type (Shisheva et al., 1994b, and this study; data not shown). These findings suggest that the portion of GDI-1 which takes up and solubilizes Rab4 in response to insulin is already available in the cytosol. Interestingly, insulin action causes a redistribution of Rab5, a protein associated with the early endosomes, from plasma membranes to low-density microsomes within the time course of its effect on GLUT4 translocation to the plasma membrane of 3T3-L1 adipocytes (Shisheva and Czech, unpublished data). Also, relocation of the microsome-associated GDI-2 coincides with the time course of GLUT4 translocation (Shisheva et al., 1994b). These results are consistent with the idea that GDI-1 may be liberated from other cytosolic interactions as a result of insulin effects on the overall membrane trafficking and protein kinesis, a hypothesis which requires direct experimentation and rigorous evaluation. In any case, the present results demonstrate for the first time selectivity of Rab−GDI complex formation in the context of the intact cell, and suggest a possible role of Rab4−GDI-1 heterocomplexes in the action of insulin on membrane movements.

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