Communication

Differential Intracellular Localizations of GDP Dissociation Inhibitor Isoforms

INSULIN-DEPENDENT REDISTRIBUTION OF GDP DISSOCIATION INHIBITOR-2 IN 3T3-L1 ADIPOCYTES*

(Received for publication, July 27, 1994)

Assia Shishева, Joanne Buxton, and
Michael P. Czech

From the Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Insulin action on fat cell/skeletal muscle glucose transporter (GLUT4) redistribution to cell surface membranes appears to involve small GTP-binding proteins. It has been recently recognized that at least two GDP dissociation inhibitor (GDI) isoforms, GDI-1 and GDI-2, can bind and release GDP-bound Rab proteins from membranes (Shishева, A., Südhof, T. C., and Czech, M. P. (1994) Mol. Cell. Biol. 14, 3459-3468). The present studies show that a strikingly higher level of GDI-2 fractionates with total membranes of COS-1 cells, Chinese hamster ovary cells, and 3T3-L1 adipocytes compared to GDI-1, which is virtually totally cytosolic. In 3T3-L1 adipocytes, most of the membrane-bound GDI-2 was present in a low density, intracellular membrane fraction. Immunodepletion of GLUT4-enriched vesicles from this membrane fraction also depleted significant amounts of GDI-2 proteins. Localization of both GDI-2 and GLUT4 in the same perinuclear regions of these cells was established by immunofluorescence microscopy, whereas GDI-1 displayed a diffuse, cytoplasmic distribution. Insulin acutely decreased both GLUT4 and GDI-2 protein levels in the low density microsomes by about 50%. Concomitantly, GLUT4 but not GDI-2 protein content of plasma membranes increased, suggesting release of GDI-2 into the cytoplasm in response to insulin. Taken together, these data suggest functional differences for the GDI-1 and GDI-2 protein isoforms, as well as a potential role of GDI-2 in the action of insulin on membrane movements.

An important metabolic effect of insulin is the stimulation of hexose uptake into muscle and fat cells. A family of glucose transport proteins mediates the facilitated movement of glucose across the plasma membranes of mammalian cells (reviewed in Ref. 1). The predominant mechanism by which insulin regulates hexose flux involves a redistribution of glucose transport proteins from an intracellular pool to the plasma membrane (reviewed in Ref. 2). Fat cells express two transporter isoforms, the ubiquitous glucose transporter GLUT1 at low levels, and the fat/muscle-specific insulin-sensitive glucose transporter GLUT4 (3-8). Whereas both GLUT1 and GLUT4 translocate to the adipocyte cell surface in response to insulin, the bulk of insulin-stimulated hexose flux is mediated by GLUT4 (9-11). Recent studies indicate that insulin modulates both the exocytosis and endocytosis pathway of GLUT4 trafficking in fat cells (12-14). The molecular mechanism(s) underlying GLUT1 and GLUT4 protein movements in response to insulin is still obscure.

Rab proteins, a family of at least 30 Ras-related small GTP-binding proteins, function as specific regulators of cell membrane trafficking and fusion (reviewed in Refs. 15-18). By alternating between two distinct conformations in a GDP/GTP-dependent fashion, these proteins are hypothesized to function as molecular switches, ensuring fidelity in the process of vesicle targeting to their correct acceptor compartment. Rab protein shuttling between cytosolic and membrane locations appears to be regulated by GDP dissociation inhibitor (GDI) proteins (GDI-1 and GDI-2) (15-21). The two GDI isoforms display a high degree of deduced amino acid sequence identity (86%) and similar abilities to bind and solubilize membrane-associated forms of Rab4 and Rab5 proteins in a GDP/GTP-dependent manner (21). Both GDI isoforms are ubiquitously expressed in various rat and human tissues and cultured cells, including insulin-sensitive cells and tissues (21-23). No functional differences between GDI-1 and GDI-2 have yet been identified (21).

Several considerations suggest that Rab and GDI proteins may also be involved in the membrane movements of GLUT4 transporter proteins. Thus, the nonhydrolyzable GTP analog, GTPγS, stimulates GLUT4 protein translocation in permeabilized fat cells and 3T3-L1 adipocytes (24, 25). Furthermore, increased expression of a Rab3 isotype (Rab3D), as well as the GDI-1 and GDI-2 proteins, appears to be associated with differentiation of 3T3-L1 fibroblasts to insulin-responsive cells (21, 26). Finally, the acute redistribution of Rab4 protein from fat cell low density microsomes to cytosol in response to insulin has recently been observed (27). The aim of the present studies was to characterize the cellular localizations of GDI isoforms in insulin-sensitive 3T3-L1 adipocytes and to determine whether insulin may modulate the distribution of these proteins as well. The results demonstrate a strikingly higher GDI-2 content associated with intracellular membrane compartments compared to that of GDI-1, and an acute effect of insulin to decrease GDI-2 levels in low density, intracellular membrane fractions that contain GLUT4.

EXPERIMENTAL PROCEDURES

Cell Cultures, Antibodies, and Fusion Proteins—3T3-L1 mouse fibroblasts, grown to confluence (100-mm plates or 18-mm round glass coverslips for immunofluorescence studies) in DMEM, containing 10% calf serum, 50 units/ml penicillin, and 50 µg/ml streptomycin sulfate, were differentiated into adipocytes as described previously (28). COS-1 and CHO-K1 cells were grown to confluence (100-mm plate) in DMEM or Ham’s F-12 medium, respectively, containing 10% FBS and the above antibiotics.

* This project was supported by National Institutes of Health Grant DK30898. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Rabbit polyclonal anti-GDI-1 (R3257) and anti-GDI-2 (R3361 and R3362) antibodies were directed against a synthetic mouse GDI-1 peptide (amino acids 431-447) and a synthetic mouse GDI-2 peptide (amino acids 386-404) (21). Antisera (dilution 1:20,000) or protein A-purified immunoglobulins were used for immunoblotting and immunoprecipitation, respectively. Affinity-purified anti-GDI-1 and anti-GDI-2 antibodies (Clonabodies, GammaBind Genex) were used for immunoblotting and immunoprecipitation, respectively. Mouse monoclonal anti-GLUT4 antibody (IPF), a generous gift from Dr. P. Pich, was applied in immunofluorescence microscopy studies. Mouse monoclonal antibody against β-COP (MA5) was generously provided by Dr. S. Doxsey.

GST-GDI-1 and GST-GDI-2 fusion proteins were produced as described previously (21). Their purity and concentrations were estimated electrophoretically by Coomassie Blue staining and, after electrophoresis, on nitrocellulose strips essentially as described (29). Rabbit polyclonal anti-GLUT4 (R1288) antibodies were directed against a synthetic rat GLUT4 peptide (amino acids 496-500) (30). Antisera (dilution 1:1,000) or purified anti-GLUT4 immunoglobulins (Clonabodies, GammaBind Genex) were used for immunoblotting and immunoprecipitation, respectively. Mouse monoclonal anti-GLUT4 antibody (IPF) was used as a reference for the calculation of GDI-1 and GDI-2 cytosolic protein contents by Western blotting (triplicate gels).

**Cell Treatment and Subcellular Fractionation—** 3T3-L1 adipocytes, serum-starved for 4 h prior to the experiment, were incubated in the absence or presence of insulin (10⁻⁷ m) for different time periods, as specified in the figure legends. After washes, the cells were scraped in the homogenization buffer (20 mM Tris/HCl, pH 7.4, 255 mM sucrose, 1 mM EDTA, and 1× protease inhibitor mixture (1× phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM benzamidine)) at 4°C, and fractionated according to previously characterized procedures (6, 28, 30). Briefly, adipocytes were homogenized in a motor-driven Teflon/glass homogenizer and centrifuged at 16,000 × g for 20 min in a Beckman JA-17 rotor. The subcellular fractions, high density microsomes (HDM), low density microsomes (LDM), plasma membranes (PM), and cytosol were then obtained either following the conventional procedure or by a modified protocol adapted in our laboratory for more rapid fractionation of smaller amounts of cells. The latter procedure is based on centrifugation of 16,000 × g supernatant for 5 min at 36,000 × g (HDM) and then for 24 min at 200,000 × g (LDM) in a Beckman TL-100.3 rotor. The PM fraction was collected from the interface of a 1.12 m sucrose cushion following centrifugation of the 16,000 × g pellet at 70,000 × g for 10 min. PM were then resuspended in homogenization buffer and pelleted at 200,000 × g for 4 min. All particulate fractions were resuspended in 10 mM Tris/1% Triton X-100 buffer (pH 7.5), containing 1× EDTA and 1× protease inhibitor mixture to 1× mg/ml protein (bicinchoninic protein assay kit, Pierce). The average yield of HDM, LDM, PM, and cytosol from a 100-mm dish (6.7 × 10⁶ 3T3-L1 adipocytes) was 50 μg, 160 μg, 450 μg, and 1.6 mg, respectively. Aliquots of the fractions were analyzed for GDI-1, GDI-2, or GLUT4 contents by Western blotting (tripplicate gels).

**Immunopurification of GDI-1 and GDI-2—** When equivalent amounts of total membrane and cytosolic protein (-5 μg) were immunopurified from several cultured cell types from various species. GDI-1 appears to reside virtually exclusively in the soluble fractions derived from COS-1 cells, CHO-K1 cells, and 3T3-L1 adipocytes. Only 0.05-0.1% of the total cellular GDI-1 appears to be associated with total membranes. Surprisingly, GDI-2 content in total membranes of these cell types greatly exceeds that of GDI-1 and accounts for 5-8% of the total cellular GDI-2 (Fig. 1).

When equivalent amounts of total membrane and cytosolic protein from the different cell types were compared, the ratios of GDI in membranes:GDI in cytosol were calculated to be between 1:19 and 1:30 for GDI-1 and between 1:2 and 1:3 for GDI-2. Using quantitative immunoblotting with recombinant GST-GDI-1 and GST-GDI-2 fusion proteins as standards (not shown), we also estimated the GDI protein concentrations in 3T3-L1 adipocyte cytosol to be 0.5 and 0.35 μg/100 μg of cytosolic protein (~5× 10⁶ 3T3-L1 adipocytes) for GDI-1 and GDI-2, respectively.

Fractionation of insulin-sensitive 3T3-L1 adipocytes was performed to further characterize the association of GDI-2 with cellular membranes. As shown in Fig. 2, the LDM fraction,
composed of intracellular membranes containing most of the cellular GLUT4 (28, 30), contained relatively high levels of GDI-2 protein. Significant levels of GDI-2 could also be detected in PM (plasma membrane-enriched) and HDM fractions (Golgi- and endoplasmic reticulum-enriched). When equivalent amounts of protein from the four cellular fractions were analyzed, 25–30% of the total immunoreactive GDI-2 detected in the samples was found in the LDM. In contrast, GDI-1 was barely detectable in the LDM and HDM fractions and was not detectable in PM (Fig. 2). The LDM-bound GDI-2 could be released from the membranes by high salt treatment (0.8 M NaCl or 0.6 M KCl), consistent with the concept that it is a peripheral membrane protein (not shown).

The apparent differential subcellular localizations of GDI-1 versus GDI-2 observed in Figs. 1 and 2 were confirmed by immunofluorescence microscopy of 3T3-L1 adipocytes (Fig. 3). Immunoreactive GDI-1 displayed a diffuse pattern of staining (Fig. 3A) throughout the cytoplasm of 3T3-L1 adipocytes, similar to that observed by Ullrich et al. (20) in Madin-Darby canine kidney cells. In contrast, GDI-2 exhibited a vesicular pattern that was concentrated in the perinuclear region of these cells in addition to a cytoplasmic distribution (Fig. 3, C and E). This perinuclear GDI-2 distribution coincided with that observed for β-COP (Fig. 3, B and D), a coat protein associated with the Golgi apparatus (34). Furthermore, the perinuclear localization of GDI-2 also coincided with the fluorescence associated with immunoreactive GLUT4 in 3T3-L1 adipocytes (Fig. 3, E and F). These data are consistent with the concept that significantly higher levels of GDI-2 are associated with perinuclear membrane compartments compared to GDI-1.

The apparent colocalization of GDI-2 and GLUT4 in the perinuclear region of 3T3-L1 adipocytes observed in Fig. 3 suggests the possibility that these proteins might reside in the same membrane structures. We tested this hypothesis by immunoadsorption of isolated LDM preparations with either anti-GDI-2 or anti-GLUT4 antibodies, and we probed for coimmunoadsorption of the GLUT4 or GDI-2 proteins, respectively. Fig. 4A shows that vesicles from the LDM fraction immunoadsorbed with anti-GDI-2 immunoglobulins contained significantly higher immunoreactive GLUT4 levels than vesicles immunoadsorbed with preimmune immunoglobulins. Nonetheless, most of the GLUT4 (~98%) remained in the vesicles that were not immunoadsorbed with anti-GDI-2 (not shown). Conversely, when anti-GLUT4 immunoglobulins were used to immunoadsorb GLUT4-containing vesicles, a significant depletion (22 ± 8% ± S.E., three experiments) of GDI-2 in the remaining vesicles was observed (Fig. 4B) concomitant with depletion of GLUT4-containing vesicles (Fig. 4C). Immunoreactive GDI-2 could not be analyzed in the vesicles isolated with anti-GLUT4 antibodies due to the interference from immunoglobulin heavy chains that migrate in the same region of the electrophoresis gel. Taken together, these data indicate that a significant portion of the LDM-associated GDI-2 resides in GLUT4-enriched membranes from insulin-sensitive 3T3-L1 adipocytes.

We next tested whether insulin action might modulate the cellular localization of GDI proteins in 3T3-L1 adipocytes. The
PM, LDM, and soluble fractions derived from cells incubated with or without insulin for up to 30 min, were subjected to SDS-PAGE and immunoblotting with anti-GDI or anti-GLUT4 antisera. As expected, immunoreactive GLUT4 increased in the LDM fraction of fat cells by insulin treatment (Fig. 5B). Immunoreactive GDI-2 in the LDM fraction also rapidly decreased with insulin treatment of intact cells. Recent reports in vitro suggest that Rab proteins may present Rab proteins to membranes. Whether this hypothesis is correct requires further testing, as does the possibility that insulin action might regulate this or other function of GDI-2. In any case, the present results demonstrate a striking difference between GDI-1 and GDI-2 cellular localizations and a specific regulation of the GDI-2 protein by insulin.

Acknowledgments—We thank Judy Kula for excellent assistance in the preparation of the manuscript and Douglas Bowman from the Biomedical Imaging Group of the Massachusetts Medical School and Silvia Corvera for their help in immunofluorescence microscopy.

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