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Role of RalA downstream of Rac1 in insulin-dependent glucose uptake in muscle cells

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ABSTRACT

The small GTPase RalA has been implicated in glucose uptake in insulin-stimulated adipocytes, although it remains unclear whether RalA has a similar role in insulin signaling in other types of cells. Recently, we have demonstrated that the Rho family GTPase Rac1 has a critical role in insulin-dependent glucose uptake in myoblast culture and mouse skeletal muscle. However, the mechanisms underlying Rac1-dependent glucose uptake, mostly mediated by the plasma membrane translocation of the glucose transporter GLUT4, remain largely unknown. The purpose of this study is to examine the involvement of RalA in Rac1 regulation of the translocation of GLUT4 to the plasma membrane in muscle cells. Ectopic expression of a constitutively activated RalA mutant indeed stimulated GLUT4 translocation, suggesting an important role of RalA also in muscle cells. GLUT4 translocation induced by constitutively activated mutation of Rac1 or more physiologically by upstream Rac1 regulators, such as phosphoinositide 3 kinase and the guanine nucleotide exchange factor FLJ00068, was abrogated when the expression of RalA was downregulated by RNA interference. The expression of constitutively activated Rac1, on the other hand, caused GTP loading and subcellular redistribution of RalA. Collectively, we propose a novel mechanism involving RalA for Rac1-mediated GLUT4 translocation in skeletal muscle cells.

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1. Introduction

Insulin-dependent glucose uptake in fat and muscle cells is largely mediated by translocation of the glucose transporter GLUT4 from intracellular storage sites to the plasma membrane. Insulin enhances exocytosis of GLUT4 vesicles and suppresses their endocytosis, leading to the net accumulation of GLUT4 in the plasma membrane. Accumulating evidence reveals that diverse signaling molecules, including phosphoinositide 3 kinase (PI3K) and the serine/threonine kinase Akt2, exert important roles in insulin signaling. However, the mechanisms underlying insulin-dependent GLUT4 trafficking remain incompletely understood [1–4].

Rho family small GTPases regulate a wide variety of cellular responses such as cytoskeletal rearrangements [5,6]. We and others recently reported that the Rho family member Rac1 is involved in the regulation of GLUT4 translocation in skeletal muscle cells [7–9]. One critical role of Rac1 in GLUT4 trafficking is thought to be the regulation of the actin cytoskeleton. Insulin induces rapid and marked reorganization of the actin cytoskeleton in myocytes, including the formation of membrane ruffles, in which diverse signaling molecules, such as PI3K and Akt, become accumulated [10,11]. This signaling is considered to be crucial for the induction of GLUT4 translocation, and is mediated by Rac1 [10,12]. A subsequent study further showed that Rac1-dependent actin cytoskeletal remodeling and submembrane tethering of GLUT4 vesicles are important for cell surface expression of GLUT4 [13]. However, the regulation of actin cytoskeletal remodeling may not be a sole function of Rac1. It is likely that Rac1 is also involved in a series of insulin-dependent events, such as GLUT4 vesicle trafficking and its docking/fusion to the cell membrane, taking into consideration that constitutively activated Rac1 alone is able to induce GLUT4 translocation in the absence of insulin without enhancing the activity of the serine/threonine kinase Akt [7,8].

The small GTPase RalA has been implicated in intracellular membrane trafficking, including polarized membrane delivery, neurosecretion, and endocytosis [14]. In adipocytes, RalA resides in GLUT4 vesicles, and interacts with the exocyst subunit Sec5 and the motor protein Myo1c, thereby regulating GLUT4 targeting to the plasma membrane in response to insulin [15]. However, it remains uncertain whether RalA is also involved in GLUT4 translocation in other types of cells, such as muscle cells. In particular, the role of RalA downstream of Rac1, a skeletal muscle-specific regulator of insulin signaling, is totally unknown.

In this paper, we show that RalA in fact plays a pivotal role also in Rac1-mediated GLUT4 translocation in muscle cells. These results emphasize a RalA-dependent common mechanism underlying GLUT4

Abbreviations: GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; PI3K, phosphoinositide 3 kinase; siRNA, small interfering RNA; TRITC, tetramethylrhodamine isothiocyanate.

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trafficking in contrast to divergence in upstream signaling pathways in different cell types.

2. Materials and methods

2.1. Materials

Antibodies against Myc (mouse monoclonal (sc-40)) and hemagglutinin (HA) (rat monoclonal (11 867 423 001)) epitope tags were purchased from Santa Cruz Biotechnology and Roche Applied Science, respectively. Antibodies against V5 (rabbit polyclonal (V8137)) and FLAG (rabbit polyclonal (F7425)) epitope tags and tubulin (mouse monoclonal (T4026)) were purchased from Sigma-Aldrich. Anti-RalA (mouse monoclonal (610221)) and anti-Rac1 (mouse monoclonal (610650)) antibodies were purchased from BD Biosciences. An anti-phospho-(Ser/Thr) Akt substrate (rabbit polyclonal (9611)) antibody was purchased from Cell Signaling Technology. Anti-mouse IgG, anti-rabbit IgG, and anti-rat IgG antibodies conjugated with Alexa Fluor® 488/546/647 were purchased from Life Technologies. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (P1951) was purchased from Sigma-Aldrich. Anti-mouse IgG (NA9310) and anti-rabbit IgG (NA9340) antibodies conjugated with horseradish peroxidase were purchased from GE Healthcare.

2.2. Cell culture and RNA interference

L6 and L6-GLUT4 rat myoblasts [7] were cultivated in minimum essential medium Eagle (M4655; Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Cell Culture Bioscience), 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. A mixture of three small interfering RNA (siRNA) duplexes designed from the rat RalA cDNA sequence (RalA siRNA) (duplex-1, 5′-AAGAUGUCGAUCU GCACUUCCUCCC-3′; duplex-2, 5′-AAAUCUGUUCCCUGAAGUCCGCU GU-3′; duplex-3, 5′-CAUAGUUAACGUUCCACUGGUCAGC-3′) (Life Technologies) was used for down-regulation of RalA. The control siRNA (1022076) was purchased from Qiagen. Cells were transfected with siRNA (200 nM) using the Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer's instructions.

2.3. Induction of differentiation of L6 cells

L6 cells were induced to differentiate into multinuclear myotubes by replacement of 10% (v/v) fetal bovine serum in the culture medium with 2% horse serum (GIBCO, 16050-122) and subsequent incubation for 5 days.

2.4. Adenovirus infection

Adenoviruses for the expression of 3×HA-tagged Rac1 (WT), 3×HA-tagged Rac1(G12V), V5-tagged RalA (WT), V5-tagged RalA (G23V), and FLAG-tagged FLJ68ΔN were generated and used to infect L6-GLUT4 cells by using the Adenovirus Cre/loxP kit (Takara Bio, Japan) according to the manufacturer's instructions. A recombinant adenovirus for the N-terminally myristoylated catalytic subunit of bovine PI3Kα (Myr-p110) was kindly provided by Dr. Wataru Ogawa (Kobe University Graduate School of Medicine) [16].

2.5. Immunofluorescent microscopy and GLUT4 reporter assay

The exofacial GLUT4 reporter GLUT4*myc*7-green fluorescent protein (GFP) and an L6-derived cell line that stably expresses this reporter (termed L6-GLUT4) were previously described [7,17]. L6-GLUT4 cells were starved in serum-free medium for 2 h prior to measurement of GLUT4 translocation. For the detection of the cell surface-exposed GLUT4 reporter, formaldehyde-fixed cells were treated with an anti-Myc antibody before permeabilization. After washing three times with phosphate-buffered saline (−), cells were permeabilized with 0.1% (v/v) Triton X-100 and then counterstained with another primary antibody for the identification of the cells that express a specific ectopically expressed or endogenous protein. Images were obtained using a 40× oil objective on a confocal laser-scanning microscope (LSM 700; Carl Zeiss) and processed by the Zeiss LSM Image Browser, version 3.5. The relative amount of GLUT4*myc*7-GFP exposed at the plasma membrane was estimated from measurements of Myc and GFP fluorescence signals. Intensities of Myc and GFP fluorescence signals in regions of interest were quantified using ImageJ software, and ratios of Myc and GFP signals (Myc/GFP) were calculated [8].

2.6. Immunoblotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred on to a 0.45 μm pore size polyvinylidene difluoride membrane (GE Healthcare). The membrane was stained with the respective primary antibodies and a horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody, followed by visualization by enhanced chemiluminescence detection reagents (GE Healthcare).

2.7. Pull down assay for activated Rac1 and RalA

Proteins were expressed in L6-GLUT4 cells through adenoviral infection. Cells were harvested with a pull down assay buffer [50 mM Tris–HCl (pH 7.4), 0.5% Nonidet P-40, 0.15 M NaCl, 20 mM $MgCl_2$, and protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan)]. Lysates were centrifuged at 18,000×*g* for 1 min, and supernatants were added to glutathione S-transferase (GST)-Myc-PAK (67–150) (for Rac1) or GST-3×V5-Sec5 (1–99) (for RalA) immobilized on glutathione–Sepharose beads, and incubated at 4 °C for 30 min. Glutathione–Sepharose beads were washed twice with pull down assay buffer, and activated Rac1 or RalA bound to the beads was detected by immunoblotting using an anti-Rac1 or anti-RalA antibody.

2.8. Overlay assay (in situ detection) for activated Rac1

In situ detection of the activated form of Rac1 in paraformaldehyde-fixed cells were performed as described previously [7].

3. Results

As a step to assess the involvement of RalA in glucose uptake in muscle cells, a constitutively activated mutant of RalA (RalA (G23V)) was ectopically expressed in L6-derived myoblast (L6-GLUT4) cells, and its effect on GLUT4 translocation to the plasma membrane was examined (Fig. 1). The L6-GLUT4 cell line stably expresses an N-terminally GFP- and exofacially Myc epitope-tagged GLUT4 reporter that is detected by an anti-Myc antibody only when re-localized in the plasma membrane [7]. L6-GLUT4 cells expressing RalA(G23V) were highly positive for Myc staining whereas L6-GLUT4 cells without RalA(G23V) were not stained by an anti-Myc antibody (Fig. 1A). Fluorescent intensities of GFP and Myc staining in L6-GLUT4 cells positive or negative for RalA(G23V) expression were quantified, and the Myc/GFP ratio, which reflects the extent of cell surface translocation of GLUT4, was calculated (Fig. 1B). Ectopic expression of RalA(G23V) in fact induced an approximately 2.5-fold increase in the level of GLUT4 translocated to the plasma membrane. Therefore, RalA is likely to be implicated in signaling that leads to glucose uptake in muscle cells.

We and others have demonstrated that the Rho family GTPase Rac1 plays an important role in insulin-dependent GLUT4 translocation to the plasma membrane in cultured muscle cells [7,9] and mouse skeletal muscle [8]. However, the mechanism by which Rac1-dependent signaling causes the plasma membrane translocation of GLUT4 remains obscure. By using the GLUT4 reporter assay

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