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Role of the guanine nucleotide exchange factor in Akt2-mediated plasma membrane translocation of GLUT4 in insulin-stimulated skeletal muscle

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ABSTRACT

The small GTPase Rac1 plays a key role in insulin-promoted glucose uptake mediated by the GLUT4 glucose transporter in skeletal muscle. Our recent studies have demonstrated that the serine/threonine protein kinase Akt2 is critically involved in insulin-dependent Rac1 activation. The purpose of this study is to clarify the role of the guanine nucleotide exchange factor FLJ00068 in Akt2-mediated Rac1 activation and GLUT4 translocation in mouse skeletal muscle and cultured myocytes. Constitutively activated FLJ00068 induced GLUT4 translocation in a Rac1-dependent and Akt2-independent manner in L6 myocytes. On the other hand, knockdown of FLJ00068 significantly reduced constitutively activated Akt2-triggered GLUT4 translocation. Furthermore, Rac1 activation and GLUT4 translocation induced by constitutively activated phosphoinositide 3-kinase were inhibited by knockdown of FLJ00068. In mouse gastrocnemius muscle, constitutively activated FLJ00068 actually induced GLUT4 translocation to the sarcolemma. GLUT4 translocation by constitutively activated FLJ00068 was totally abolished in *rac1* knockout mouse gastrocnemius muscle. Additionally, we were successful in detecting the activation of Rac1 following the expression of constitutively activated FLJ00068 in gastrocnemius muscle by immunofluorescence microscopy using an activation-specific probe. Collectively, these results strongly support the notion that FLJ00068 regulates Rac1 downstream of Akt2, leading to the stimulation of glucose uptake in skeletal muscle.

1. Introduction

Insulin promotes glucose uptake in skeletal muscle by inducing the plasma membrane translocation of the glucose transporter GLUT4 [1–3]. GLUT4 is a 12-transmembrane protein that mediates ATP-independent facilitated diffusion of circulating glucose into the cell. GLUT4 is sequestered in GLUT4 storage vesicles in unstimulated cells. When skeletal muscle cells are stimulated by insulin, multiple steps of exocytosis of GLUT4 are enhanced, leading to the net accumulation of GLUT4 on the cell surface. It is important to understand the mechanism for this insulin action considering that defects in insulin-dependent glucose uptake are the major cause of type 2 diabetes.

Following insulin stimulation, a signaling cascade consisting of phosphoinositide 3-kinase (PI3K) and serine/threonine protein kinases PDK1 and Akt2 is activated, leading to enhanced glucose uptake in skeletal muscle. In particular, Akt2 has been implicated as a key regulator of insulin-triggered glucose uptake. Mice deficient in the *akt2* gene exhibited glucose intolerance and hyperglycemia due to reduced total body insulin-dependent glucose disposal [4,5]. Glucose uptake following stimulation with insulin at a low concentration in extensor digitorum longus and soleus muscles was in fact impaired in these mice [4,5].

Recently, the Rho family GTPase Rac1 has been identified as another key regulator of insulin-dependent GLUT4 translocation in skeletal muscle [6,7]. Insulin-dependent GLUT4 translocation was severely impaired when Rac1 was knocked down in cultured myocytes [8–11] and mouse skeletal muscle [12]. Correspondingly, a significant decrease in insulin-stimulated glucose uptake in skeletal muscle was observed in muscle-specific *rac1* knockout (m-*rac1*-KO) mice, and these mice actually exhibited impaired glucose tolerance [13]. GLUT4 translocation stimulated by ectopic expression of a constitutively activated Rac1 mutant was also observed in cultured myocytes [11,14] and mouse skeletal muscle [12]. Furthermore, insulin-dependent activation of Rac1 was detected in cultured myocytes [11,14] and mouse skeletal muscle [12,13].

Abbreviations: BSA, bovine serum albumin; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; HSA, human skeletal actin; m-*rac1*-KO, muscle-specific *rac1* knockout; Myr-p110α, myristoylated p110α; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA.

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However, the mechanisms whereby insulin activates Rac1, leading to GLUT4 translocation, remain largely unknown. We demonstrated that insulin activates Rac1 primarily by a PI3K-dependent mechanism, but in part by a PI3K-independent mechanism, in L6 myocytes [11,15]. PI3K-dependent Rac1 activation occurred near the cell surface, particularly in ruffling membranes, whereas PI3K-independent Rac1 activation was observed inside the cell [15]. A constitutively activated mutant of PI3K, when ectopically expressed, induced Rac1 activation, which was diminished by Akt2 knockdown [16,17]. Moreover, constitutively activated Akt2 induced Rac1 activation and GLUT4 translocation [17]. The Rac1 dependency of GLUT4 translocation stimulated by constitutively activated mutants of PI3K and Akt2 was confirmed in both L6 myocytes and mouse skeletal muscle [16,17]. Taken together, these results strongly suggest that Rac1 is regulated downstream of Akt2 in muscle insulin signaling mediated by PI3K. In marked contrast to the above-mentioned model for the regulation of Rac1, the mechanism in which Akt2 and Rac1 are independently regulated downstream of PI3K, directing different signaling events in bifurcated pathways, has also been proposed [6,14,18].

FLJ00068 (also termed PLEKHG4 and puratrophin-1) belongs to the Dbl family of guanine nucleotide exchange factors (GEFs), and is expressed in various tissues including skeletal muscle. The Dbl homology domain of FLJ00068 showed GEF activity toward Rac1 and Cdc42 [11,19]. On the basis of the analysis of insulin-stimulated signaling in L6 myocytes, FLJ00068 has been identified as a GEF that is involved in the regulation of Rac1-dependent glucose uptake [11]. In fact, knockdown of FLJ00068 significantly suppressed insulin-dependent GLUT4 translocation, and a constitutively activated mutant of FLJ00068 induced Rac1 activation [11].

In this study, the role of FLJ00068 in PI3K and Akt2-dependent activation of Rac1 in insulin signaling is investigated not only in cultured myocytes, but also in mouse skeletal muscle. We conclude that the GEF FLJ00068 plays a crucial role in Akt2-dependent activation of Rac1, which in turn directs diverse signaling pathways, leading to glucose uptake in skeletal muscle.

2. Materials and methods

2.1. Materials

An antibody against the Myc epitope tag (mouse monoclonal (05-724)) was purchased from Millipore (MA, USA). An antibody against the V5 epitope tag (goat polyclonal (A190-119A)) was purchased from Bethyl (TX, USA). An antibody against the hemagglutinin (HA) epitope tag (rat monoclonal (11 867 423 001)) was purchased from Roche Applied Science (Germany). An antibody against Rac1 (mouse monoclonal (610650)) was purchased from BD Biosciences (CA, USA). Antibodies against the FLAG epitope tag (rabbit polyclonal (F7425)) and α-tubulin (mouse monoclonal (T9026)) were purchased from Sigma-Aldrich (MO, USA). Antibodies against Akt (rabbit polyclonal (9272)) and phospho-(Ser473) Akt (mouse monoclonal (4051)) were purchased from Cell Signaling Technology (MA, USA). Antibodies against goat IgG, mouse IgG, rabbit IgG, and rat IgG conjugated with CF™ 350/543/647 were purchased from Biotium (CA, USA). Antibodies against mouse IgG (NA9310) and rabbit IgG (NA9340) conjugated with horseradish peroxidase were purchased from GE Healthcare (UK). Insulin was purchased from Eli Lilly (IN, USA).

2.2. Plasmid construction

The cDNA for N-terminally double HA-tagged human FLJ00068 (723-1191) (termed FLJ68ΔN) was synthesized and cloned into the mammalian expression vector pCAGGS (kindly provided by Jun-ichi Miyazaki, Osaka University, Osaka, Japan) [20] to generate pCAGGS-HA × 2-FLJ68ΔN.

2.3. Cell culture, transfection, and RNA interference

The L6-GLUT4 cell line was established from L6 rat myoblasts, and stably expresses the GLUT4 reporter GLUT4*myc*7-green fluorescent protein (GFP) [11,21]. L6-GLUT4 cells were cultivated in Dulbecco's modified Eagle's medium (2-6053-01, Corning (NY, USA)) supplemented with 10% (v/v) fetal bovine serum (Biowest (France)), 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were starved in serum-free medium for 2 h prior to measurement of GLUT4 translocation. L6-GLUT4 cells were transfected with the expression plasmid pCMV5-Myr-Akt2-HA × 3 [17] using the Fugene® HD transfection reagent (Promega (WI, USA)) according to the manufacturer's instructions. A mixture of three small interfering RNA (siRNA) duplexes against rat Rac1 (duplex-1, 5′-AGACGGAGCCGUUGGUAAATT-3′; duplex-2, 5′-CCUGUUAAGAAGAGGAAGATT-3′; duplex-3, 5′-CCAAUGAACCAGUCAGUAATT-3′) was purchased from Cosmo Bio (Japan). The siRNA against rat Akt2 (5′-CGACUUCGAUUAUCUCAAATT-3′) and the control siRNA (1022076) were purchased from Qiagen (Netherlands). A mixture of two siRNA duplexes against rat FLJ00068 (duplex-1, 5′-GUGAGUAUGUCCGGGCCCU-3′; duplex-2, 5′-GCAACUAUGGCCACACCUU-3′) was purchased from Sigma-Aldrich. L6-GLUT4 cells were transfected with each of the above siRNAs (200 nM) using the Lipofectamine RNAiMAX transfection reagent (Life Technologies (CA, USA)) according to the manufacturer's instructions.

2.4. Adenovirus infection

A recombinant adenovirus for FLAG-tagged FLJ68ΔN was previously described [11]. A recombinant adenovirus for the N-terminally myristoylated catalytic subunit of bovine PI3K (Myr-p110α) was kindly provided by Wataru Ogawa (Kobe University Graduate School of Medicine), and previously described [16,17,22].

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total cellular RNA was isolated from L6-GLUT4 cells using the TRIzol® RNA isolation reagent (Life Technologies) according to the manufacturer's instructions. cDNAs were synthesized using the SuperScript III first-strand synthesis system for RT-PCR (Life Technologies) and then amplified using KOD FX neo (Toyobo (Japan)) and specific primers (Life Technologies) (5′-CAGCGTGTTAATGCTGCCAC-3′ and 5′-GATAGCCCGTATCCACTCTT-3′ for Akt2; 5′-GGCAGTTGGTACGACAGGAT-3′ and 5′-CGAAGCGCAGTTTACTTTCC-3′ for FLJ00068; and 5′-CTACAATGAGCTGCGTGTGG-3′ and 5′-CAACGTCACACTTCATGATGG-3′ for β-actin) according to the manufacturer's instructions.

2.6. Immunoblot analysis

Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred on to a 0.45 μm pore size polyvinylidene difluoride membrane (GE Healthcare). Membranes were incubated with primary and horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized by Chemi-Lumi One Ultra (Nacalai (Japan)). Images were captured, and densitometric analysis was carried out by using a chemiluminescence imaging system (Ez-Capture MG, Atto (Japan)).

2.7. GLUT4 reporter assay in L6-GLUT4 cells

A GLUT4 reporter with exofacial Myc and C-terminal GFP tags, termed GLUT4*myc*7-GFP, was previously described [21]. For the detection of the GLUT4 reporter translocated to the plasma membrane, cells were fixed with 4% (w/v) paraformaldehyde and incubated with an antibody against the Myc tag prior to permeabilization. After washing three times with phosphate-buffered saline (PBS), cells were permeabilized with 0.1% (v/v) Triton X-100. Permeabilized cells were further