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Regulation of insulin signaling in skeletal muscle by PIP_3 phosphatase, SKIP, and endoplasmic reticulum molecular chaperone glucose-regulated protein 78

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ABSTRACT

Insulin resistance is characterized as a pathogenic factor in type 2 diabetes. Despite skeletal muscle being primarily responsible for systemic glucose disposal, the mechanisms underlying the induction of insulin resistance in skeletal muscle have not been fully elucidated. A number of studies have shown that it is characterized by the inhibition of the phosphatidylinositol (PI) 3-kinase signaling pathway. Here, we show that skeletal muscle- and kidney-enriched inositol polyphosphate phosphatase (SKIP), a phosphatidylinositol-3,4,5-trisphosphate (PIP_3) phosphatase, and glucose-regulated protein 78 (GRP78) are implicated in the inhibition of insulin-dependent PI 3-kinase signaling in skeletal muscle. Mechanistically, under resting conditions, SKIP forms a complex with GRP78 at the endoplasmic reticulum (ER). Insulin stimulation facilitates the dissociation of SKIP from GRP78 and its binding to the activated form of Pak1. GRP78 is necessary for membrane localization and Pak1-binding of SKIP, which facilitates inactivation of the insulin signaling pathway. These findings underscore the specific and prominent role of SKIP and GRP78 in the regulation of insulin-dependent PI 3-kinase signaling in skeletal muscle.

1. Introduction

In type 2 diabetes mellitus, skeletal muscle cells develop resistance to insulin and show inhibition of glucose transport [1–3], which play a major role in the pathogenesis of the disease. Although defective insulin secretion from β-cells is one of the major features, skeletal muscle insulin resistance is considered an initial metabolic defect in the development of the disease [4,5]. A number of studies have suggested a relationship between human skeletal muscle insulin resistance and the pathogenesis of type 2 diabetes [6–8]. A marked decrease in insulin-induced Akt phosphorylation in skeletal muscle in the normal-glucose-tolerance offspring of type 2 diabetes parents was observed in comparison to that in healthy subjects [8]. Therefore, identification of the key molecules that contribute to the suppression of insulin signaling should provide important insights for treatment of the disease.

In skeletal muscle, insulin binds to the insulin receptor, which leads to activation of phosphatidylinositol (PI) 3-kinase and an increase in the level of intracellular phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 activates Akt2 at the plasma membrane [9], which is necessary for subsequent glucose uptake into the skeletal muscle [10]. Incorporated glucose is converted to glycogen for storage or enters the glycolytic pathway where it is oxidized for energy production. PIP_3 phosphatases hydrolyze PIP_3 to PIP_2 and attenuate the PI 3-kinase signaling pathway. *S*keletal muscle- and *k*idney-enriched *i*nositol polyphosphate 5-*p*hosphatase (SKIP) is one of the PIP_3 phosphatases that negatively regulate insulin signaling and subsequent glucose incorporation [11–14]. *Pps*, a mouse ortholog of *SKIP*, heterozygous knockout mice exhibit higher systemic glucose uptake and insulin sensitivity than wild-type mice, with increased Akt phosphorylation and glucose uptake in skeletal muscle but not in adipose tissue, and they are resistant to diet-induced insulin resistance [13]. A hyperinsulinemic-euglycemic clamp study showed increased rates of glucose infusion and insulin-stimulated glucose disposal in these mice without any abnormalities in hepatic glucose production [13]. Rat L6 and mouse C2C12 muscle cell lines have been extensively used to study insulin signaling in skeletal muscle. Importantly, upon insulin stimulation, SKIP translocates from the ER to the membrane ruffles in C2C12 cells, where it binds to the activated form of p21-activated kinase 1 (Pak1) and forms a protein complex with

Abbreviations: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; SKIP, skeletal muscle- and kidney-enriched inositol polyphosphate 5-phosphatase; GRP78, glucose-regulated protein 78; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; UPR, unfolded protein response.

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Akt2 and PDK1, two PIP_3-binding proteins [3]. Knockdown of *SKIP* or *Pak1* in C2C12 cells increases insulin-induced Akt2 phosphorylation [3]. The location of SKIP proximal to these PIP_3 effectors determines the efficiency and specificity of the negative regulation of insulin signaling. Rat L6 cells are widely used to analyze insulin-mediated glucose uptake because they express more glucose transporter 4 (GLUT4) protein than C2C12 cells. Knockdown of *SKIP* in these cells markedly increases 2-deoxy glucose uptake in response to insulin stimulation [3]. Therefore, it is likely that SKIP is the specific regulator for diet-induced insulin resistance in skeletal muscle among PIP_3 phosphatases [14]. In addition to regulation of insulin action, SKIP mediates skeletal muscle differentiation [15]. Knockdown of *SKIP* increases insulin-like growth factor-II (IGF-II) transcription and potentiates the IGF-II–PI3-kinase–Akt–mTOR auto-regulation loop during myogenesis. Expression of *SKIP* is markedly increased during differentiation, which leads to inhibition of IGF signaling and termination of differentiation. Further, SKIP likely is a specific regulator of myogenesis.

Previous reports have shed light on the link between endoplasmic reticulum (ER), insulin signaling, and type 2 diabetes [16–18], and the molecular mechanism of this link has been identified. Several pathological stresses disrupt ER homeostasis, named ER stress, which leads to the accumulation of unfolded proteins in the ER lumen. GRP78 is a molecular chaperone in the luminal ER [19] that plays a pivotal role in these ER functions. Recent evidence suggests that GRP78 directly regulates systemic insulin sensitivity and glucose homeostasis [20]. *GRP78* haploinsufficiency in mice subjected to ER stress induced by a high-fat diet (HFD) activates the adaptive unfolded protein response (UPR) and improves insulin sensitivity with increased insulin-induced Akt phosphorylation and glucose uptake in the adipose tissue and liver [21]. Heterozygous *SKIP* knockout mice exhibit a normal blood glucose level, but they also exhibit increased insulin signaling and resistance to HFD-fed conditions, similar to *GRP78* heterozygous mice [13]. Furthermore, embryonic fibroblast cells isolated from these mice exhibit increased insulin-dependent Akt phosphorylation [21]. Taken together, GRP78 is likely to negatively regulate PI 3-kinase signaling. However, there are conflicting reports on the role of GRP78 in the regulation of PI 3-kinase signaling. For example, Akt activation is blocked by targeted knockout of *GRP78* in the *Pten* null prostate epithelium [22]. Therefore, the molecular mechanisms by which GRP78 regulates PI 3-kinase signaling and insulin action in the skeletal muscle remain to be elucidated. In this study, we investigated the molecular mechanisms of how GRP78 and SKIP regulate insulin signaling in skeletal muscle. We found that insulin stimulation facilitates the dissociation of SKIP from GRP78, which allows SKIP to bind to Pak1 and leads to inactivation of insulin signaling. These results identified the regulatory role of GRP78 and SKIP insulin-dependent PI 3-kinase signaling in the skeletal muscle.

2. Materials and methods

2.1. Materials

Antibodies raised against the C-terminus of SKIP were developed in our laboratory for immunoblotting. Antibodies specific for Akt, Akt2, phospho-Akt (Ser-473), phospho-Akt (Thr-308), and Pak1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-GRP78 antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-FLAG M2 monoclonal antibody, anti-FLAG M2 agarose affinity gel, insulin, and LY294002 were purchased from Sigma Aldrich (St. Louis, MO, USA). Methyl-β-cyclodextrin and cytochalasin D were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Constructs

SKIP expression vectors were generated by introducing cDNAs encoding human SKIP into the pGEX (GE Healthcare, Buckinghamshire, UK), pAcGFP-C1 (Clontech Laboratories, Mountain View, CA, USA), and p3 × FLAG-CMV8 (Sigma Aldrich) vectors. A phosphatase-negative D310G mutant (D310G), a *SKIP C*-terminal *h*omology (SKICH) domain alone mutant (SKIP 318–448), and a mutant lacking SKICH domain position 1 to 359 (SKIP 1–359) were generated by PCR and were cloned into expression vectors. pDsRed2-ER expression vectors were purchased from Clontech.

2.3. Cell culture, transfection of plasmids, and RNA interference (RNAi)

The C2C12 murine and L6 rat myoblast cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Wako Pure Chemical Industries) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS), and antibiotics (100 μg/mL streptomycin and 100 units/mL penicillin) at 37 °C under an atmosphere of 5% CO_2 [15]. For transfection, cells were cultured to 50% confluence and 1 μg of plasmid DNA was transfected into the cells. Small interfering RNA (siRNA) duplexes were purchased from Life Technologies (Carlsbad, CA, USA). The following oligonucleotides were used in this study: control, 5′-GAGTCAACGTCTGCCTGAAGCTTTA-3′; mouse GRP78 #1, 5′-TCTCAAGAACCAGTTGGAGATAAA-3′; mouse GRP78 #2, 5′-CCCAACTGGTGAAGAGGATACATCA-3′; rat GRP78 #1, 5′-TCTTAAGAACCAGATCGGAGATAAA-3′; rat GRP78 #2, 5′-CCCAACTGGTGAAGAGGATACATCA-3′; mouse SKIP, 5′-GAGTCAACGTCTGCCTGAAGCTTTA-3′; rat SKIP, 5′-CCATGGAGCAGTTTCTTCATGGATA-3′; mouse Pak1, 5′-CAAGGTGCTTCAGGCACAGTGTATA-3′. Twenty nanomoles of control, SKIP, GRP78, or Pak1 siRNA duplexes were transfected into mouse C2C12 and rat L6 myoblasts. The cells were cultured in DMEM containing 10% FBS for 48 h and then serum-starved for another 24 h.

2.4. Immunoblotting

Forty-eight hours after transfection, the C2C12 cells were serum-starved in serum-free medium for 24 h and then stimulated with insulin (100 nM) at various times. The cells were washed with PBS and lysed with cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM PMSF, and 1% Triton X-100. The cell lysates were centrifuged at 14,000 × *g* for 10 min and the supernatants were solubilized in sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane. For Western blot analysis, the membrane was incubated with the primary antibodies at room temperature for 2 h followed by washing with Tris-buffered saline (25 mM Tris–HCl [pH 7.5], 150 mM NaCl) containing 0.05% Tween-20. Then, the membrane was incubated with secondary antibodies at room temperature for 2 h. Densitometry was used to quantify the protein levels.

2.5. Immunoprecipitation

Cell lysates were subjected to immunoprecipitation in cell lysis buffer with various antibodies at 4 °C for 4 h. Twenty-five micrograms of protein A-agarose beads (Thermo Fisher Scientific, Waltham, MA, USA) were added during the last 2 h of incubation. The beads were washed 5 times with wash buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, and 1% Nonidet P-40) before the immunoprecipitates were subjected to SDS-PAGE. For immunoprecipitation by the FLAG antibody, the lysates were incubated with anti-FLAG M2 agarose affinity gel beads for 4 h at 4 °C in cell lysis buffer. Then, they were washed 5 times with wash buffer before immunoprecipitates were applied to immunoblotting.

2.6. Measurement of Akt2 phosphorylation

Phosphorylation of Akt2 at Thr-309 and Ser-474 was analyzed and quantified as described previously [3]. Lysates from C2C12 cells were