Biochemical and Biophysical Research Communications 456 (2015) 41–46

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Improvement of insulin signaling in myoblast cells by an addition of SKIP-binding peptide within Pak1 kinase domain

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ARTICLE INFO

Article history:
Received 7 November 2014
Available online 18 November 2014

Keywords:
SKIP
Pak1
Insulin signaling
Skeletal muscle

ABSTRACT

Abnormalities in insulin-induced glucose incorporation in skeletal muscle were observed in Type 2 diabetes. Our previous studies revealed that the binding between skeletal muscle and kidney-enriched inositol polyphosphate phosphatase (SKIP) and p21-activated protein kinase (Pak1) at the plasma membrane is induced insulin-dependently and that this binding mediated a rapid and efficient termination of insulin signaling and a subsequent glucose uptake into skeletal muscle cells. Here, we identified 11-amino-acids peptide within kinase domain of Pak1, necessary and sufficient for SKIP binding. Expression of this region in C2C12 cells resulted in an increase in insulin signaling. Supplementation of a synthetic peptide of this sequence increased insulin signaling and insulin-induced glucose uptake into skeletal muscle cell lines. These findings suggest the physiological role of Pak1–SKIP binding in the regulation of insulin signaling in skeletal muscle.

1. Introduction

Skeletal muscle is one of the major peripheral tissues that contributes to systemic glucose uptake by insulin stimulation. In Type 2 diabetes mellitus, insulin resistance is well characterized by an impairment of glucose uptake in this tissue [1,2]. Type 2 diabetic or obesity patients showed an approximately 50% decrease in whole body glucose uptake and skeletal muscle glucose incorporation [3,4]. Insulin stimulation leads to an activation of phosphatidylinositol (PI) 3-kinase which generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 binds to and fully activates Akt and 3-phosphoinositide-dependent protein kinase (PDK1), both of which are required for insulin-dependent translocation of glucose transporter 4 (GLUT4) to plasma membrane [5,6]. An ablation of Akt in mice exhibited metabolic defects such as insulin resistance and diabetic phenotype [7,8]. In addition, multiple defects in skeletal muscle insulin signaling were found in Type 2 diabetes [9–11]. Therefore, increase in insulin signaling in skeletal muscle may be an effective way for an improvement from insulin resistance and Type 2 diabetes mellitus.

Skeletal muscle and kidney-enriched inositol polyphosphate phosphatase (SKIP) is a PIP_3 phosphatase abundantly expressed in skeletal muscle [12]. An attenuation of endogenous SKIP in L6 myoblast cells resulted in an increase in insulin-induced Akt phosphorylation and glucose incorporation [12]. Heterozygous knockout mice for SKIP exhibited improved insulin sensitivity and systemic glucose disposal. Our recent study identified an underlying mechanism by which SKIP suppresses insulin signaling, that upon insulin stimulation SKIP translocates from endoplasmic reticulum to plasma membrane, where SKIP binds to an activated form of p21-activated kinase 1 (Pak1) and formed a complex with PIP_3 effectors including Akt2 and PDK1 [13]. SKIP consists of a phosphatase domain and a SKIP C-terminal homology (SKICH) domain that mediates its localization at the membrane ruffles [1]. This domain is conserved among 5 proteins including 2 phosphoinositide phosphatase, SKIP and proline rich inositol polyphosphate phosphatase (PIPP), and 3 calcium binding proteins, NDP52, TAX1BP1, and CALCOCO1. PIPP is a PIP_3 phosphatase implicated in oncogenic transformation [14]. An accumulating evidences reported the regulation of autophagy by NDP52 and TAX1BP1 [15–17], however, little is known about the role of this domain.

Our previous results show that SKIP SKICH domain (SKIP 318–448) is necessary and sufficient for membrane localization and SKIP D361A mutant, that possesses a mutation within this domain, does not bind to Pak1 [13]. An attenuation of Pak1 abolished membrane localization of SKIP, so that increased insulin signaling and glucose uptake in skeletal muscle cells. Pak1 acts as a scaffolding protein, which forms a large protein complex including insulin receptor, Akt, Rac1, all of which are necessary for insulin-induced glucose

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http://dx.doi.org/10.1016/j.bbrc.2014.11.031
0006-291X/

uptake. Based on these results, we consider Pak1 as a negative regulator of insulin action. However, the role of Pak1 in glucose homeostasis remains controversial. An accumulating evidence imply crucial role of Pak1 in whole body glucose disposal and insulin-dependent glucose uptake [18], skeletal muscle isolated from Pak1-null mice showed normal insulin-stimulated activation of Akt and ERK [18]. Therefore, we hypothesized that expression of minimal essential SKIP-binding region within Pak1 can improve insulin action. In this study, we found that Pak1-binding is necessary for membrane localization of SKIP. Inhibition of insulin-induced membrane localization of SKIP resulted in an increase in insulin-dependent glucose uptake.

2. Materials and methods

2.1. Materials

Anti-SKIP rabbit polyclonal antibody was purchased from LS Bio. Antibodies against Akt, phospho-Akt (Thr-308), phospho-Akt (Ser-473), and Pak1 were purchased from Cell Signaling Technology (Beverly, MA). Peptides were synthesized by MBL (Nagoya, Japan).

2.2. Constructs

cDNAs encoding SKIP, Rac1, and Pak1 were obtained by reverse transcription-PCR (RT-PCR). Expression vectors of Pak1 and SKIP were generated by mouse Pak1 cDNA into mCherry-C1 and pEGFP-C1 vector (BD Clontech, Franklin Lakes, NJ). Glutathione S-transferase conjugated expression vectors were generated by introducing cDNAs into pGEX-6P vectors (GE healthcare, UK).

2.3. Cell culture and transfection

C2C12 and L6 cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum. Cells were transfected with these constructs for 24 h and then serum-deprived for 24 h. Then cells were stimulated with insulin (100 nM) for 60 min. Cells were washed twice with phosphate-buffered saline and then lysed with cell lysis buffer (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 phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100). Lysates were then applied to Western blot analysis or immunoprecipitation.

2.4. In vitro binding assay

Recombinant proteins were purified from *Escherichia coli*. 10 μg of immobilized glutathione-S-transferase (GST)-conjugated proteins were incubated with 50 μg of these recombinant proteins for 2 h at 4 °C in a binding buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, and 2 mM EDTA).

2.5. Surface plasmon resonance

A BIAcore 2000 (GE Healthcare Biosciences, Piscataway, NJ, USA) was used to measure surface plasmon resonance. The carboxymethyl dextran matrix of the CM5 research grade sensor chips was used according to the manufacturer's protocol. Recombinant SKIP protein was captured onto the surface by injection at 20 μl/min. For analyte, recombinant proteins in various concentrations were injected at a flow rate of 10 μl/min.

2.6. Supplementation of synthetic peptides into cultured cell

Synthetic peptides were purchased from MBL Co. Ltd. (Nagoya, Japan). Peptides were dissolved in PBS and were supplemented into cells at various concentrations (0–10 μg/ml) for 24 h by using BioPORTER Protein Delivery Reagent (Genlantis, San Diego, CA, USA) according to the manufacturer's protocol.

2.7. Glucose incorporation assay

Measurement of glucose incorporation into cells was measured by 2-deoxyglucose uptake measurement kit (Cosmo Bio. Co. Ltd., Tokyo, Japan).

2.8. Immunofluorescence

C2C12 cells were fixed with 3.7% formaldehyde and then visualized by immunofluorescence. The cells were permeabilized with PBS containing 0.2% Triton X-100 for 10 min and incubated with 1% FBS in PBS for 1 h to block nonspecific binding. The cells were incubated with first antibody for 1 h and then with fluorescein-labeled secondary antibodies. F-actin was visualized with Alexa Fluor 647-labeled phalloidin (Life Technologies). The cells were observed under a FluoView 1000-D confocal microscope (IX81; Olympus) equipped with 473-, 559-, 635-nm diode lasers through an objective lens (60-Å oil immersion objective; numerical aperture [NA], 1.35; Olympus). Acquired images were processed with Photoshop CS5 software (Adobe).

3. Results and discussion

SKIP consists of N-terminal 5-phosphatase domain and C-terminal SKICH domain (Fig. 1A), and SKIP accumulates at the membrane ruffles in response to insulin, where Pak1 also concentrates (Fig. 1B). We have reported that SKIP SKICH domain (SKIP 318–448) is sufficient for the binding with Pak1 in insulin-stimulated C2C12 cells [19], this domain is sufficient for the co-localization with Pak1 at the membrane ruffles upon insulin stimulation (Fig. 1C). A BIAcore analysis conducted by immobilized recombinant SKIP protein onto and Pak1 protein was applied over the sensor chip indicated that an activated form (Pak1 + GTP form of Rac1) binds to SKIP, with the typical kinetic of this binding has a Kd value of 45.9 ± 12.3 nM (Fig. 2A). In contrast, binding between SKIP and an inactivated form (Pak1 + GDP form of Rac1) of Pak1 was hardly detected (Fig. 2B). These results implicate that SKIP directly and specifically binds to an activated form of Pak1 through SKICH domain.

SKIP binds to kinase domain of Pak1 (amino acid 247–520) through SKIP C-terminal homology (SKICH) domain [19]. Based on a structural studies, kinase domain of human Pak1 is mainly composed of two domain, minor-lobe (amino acid 248–347) and major-core-lobe (amino acid 348–520) (Fig. 3A). In an attempt to find out the SKIP-binding site within kinase domain of Pak1, in vitro binding assay revealed that only C-lobe binds to recombinant SKIP (Fig. 3B). This region was further divided into three regions (Fig. 3C), N-terminal region (amino acid 348–403), an activation loop (amino acid 404–429), and C-terminal regions (amino acid 430–464 and amino acid 464–520). In vitro binding analysis showed that only C-terminal 25 amino acid peptide region, Pak1 430–464, binds to SKIP SKICH domain (Fig. 3D). An interaction of SKIP with Pak1 463–520 was also observed, with a very little amount.

Further analysis identified 11-amino acids within Pak1 kinase domain (amino acid 446–456) as minimal SKIP-binding sites (Fig. 4A). Based on a structural data, SKIP-binding region is a part of an activation loop which includes helix αF [20]. Although major

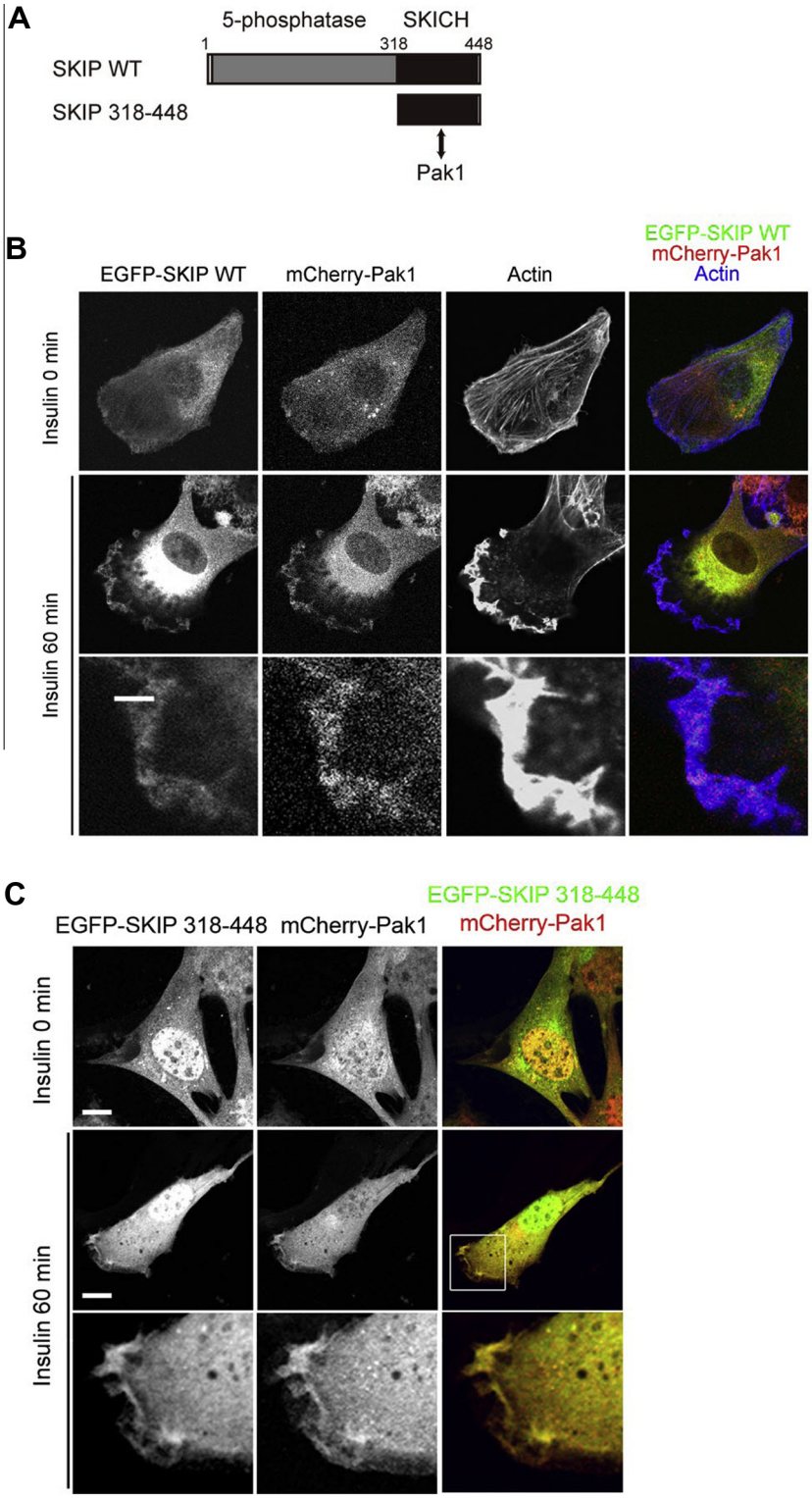

Fig. 1. SKICH domain is sufficient for insulin-induced membrane localization of SKIP. (A) Schematic representation of the SKIP constructs used in this study. (B) C2C12 cells transfected with pEGFP-SKIP WT and mCherry-Pak1 are stimulated with insulin (100 nM) for 60 min. F-actin is visualized by AlexaFluor647-phalloidin. Scale bar, 20 μm. (C) C2C12 cells transfected with pEGFP-SKIP 318–448 and mCherry-Pak1 are stimulated with insulin (100 nM) for 60 min. Yellow indicates the region of co-localization between SKIP 318–448 and Pak1. Scale bar, 20 μm.

grove is conserved among fundamentally all protein kinases, this region (activation loop) has an important variability [21]. Especially, Glu455 residue is important for the substrate preference and single mutation of this amino acid (E455I) almost completely abolished phosphorylation of Pak1. Although helix αF does not show significant conformational change between inactive dimer and active monomer, it is surrounded by dimerization interface residues [20]. Conformational difference of this interface between monomer and dimer might explain the specific binding of SKIP to active Pak1 monomer. In C2C12 cells, endogenous SKIP localized throughout

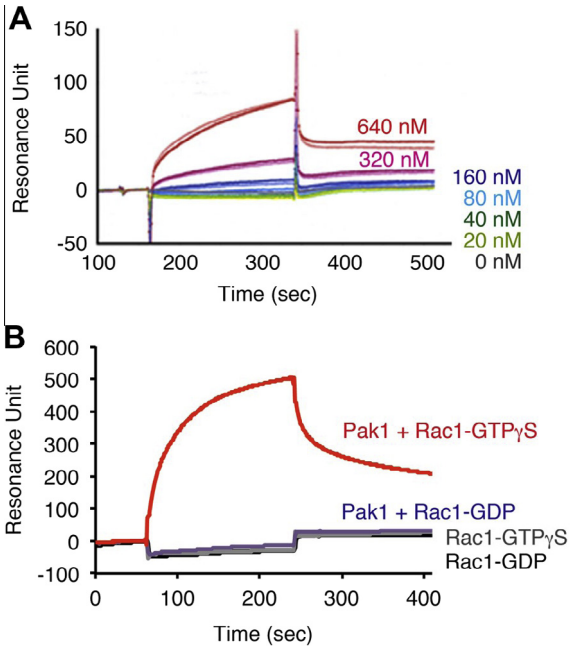

Fig. 2. SKIP specifically binds to the activated form of Pak1. (A) BIAcore analysis of immobilized SKIP binding to Pak1 protein. Recombinant SKIP proteins were captured onto the surface of CM5 chips. Pak1 protein was incubated with Rac1-GTPγS for 30 min prior to the injected across the surface, and binding was monitored by surface plasmon resonance. Concentrations of Pak1 protein injected were 640, 320, 160, 80, 40, and 20 nM. (B) BIAcore analysis of the interactions between SKIP and Pak1 protein in the presence of active Rac1 or inactive Rac1. Pak1 protein was incubated with GTPγS- or GDP-loaded Rac1 at room temperature for 30 min and then injected across the surface. Binding was monitored by surface plasmon resonance.

endoplasmic reticulum (ER), and upon insulin stimulation, it binds to Pak1 at plasma membrane, especially at the membrane ruffles. This sequence is well-conserved among STE20 family kinases including p38 MAPK, and Ask1 (Fig. 4B), and especially Met452 is conserved among Pak1 kinases (Pak1–6). In vitro binding assay showed that GST-Pak1 446–456, M452T could no longer bind to recombinant SKIP (Fig. 4C). Supplementation of synthetic peptides of this region Pak1 446–456 WT in C2C12 cells increased insulin-induced phosphorylation of Akt at Thr-308 and Ser-473 by an approximately 30% (Fig. 4D), and also increased insulin-induced glucose uptake into rat L6 myoblast cells by an approximately 29% (control: 1.23 ± 0.08 mg/min; Pak1 446–456 WT: 1.59 ± 0.09 mg/min; Pak1 446–456 M452T: 1.29 ± 0.08 mg/min) (Fig. 4E). However, these increases were not observed by an addition of Pak1 446–456, M452T peptides (Fig. 4D and E).

Finally, our results show that the supplementation of this 11-amino acid peptide in myoblast cells achieved an increase in insulin signaling and insulin-induced glucose uptake. Expression of SKIP-binding region in C2C12 cells showed co-localization with SKIP at the endoplasmic reticulum and inhibited re-localization of endogenous SKIP to the membrane ruffles upon insulin stimulation. Our previous study has shown that an attenuation of endogenous Pak1 markedly increased insulin-dependent Akt phosphorylation [13]. Supplementation of Pak1 446–456 peptides into myoblast cells likely to inhibit an interaction of SKIP to endogenous Pak1 at the membrane ruffles, and as a result, it increases in insulin-induced Akt activation and glucose uptake. In these cells, approximately by 29% of increase in insulin-mediated glucose uptake was observed by an addition of this peptide. Since skeletal muscle is responsible for more than 70% of systemic glucose uptake, and an 50% decrease in skeletal muscle glucose incorporation results in Type 2 diabetic subjects. Therefore an approximately 30% increase in insulin signaling in this tissue might be effective

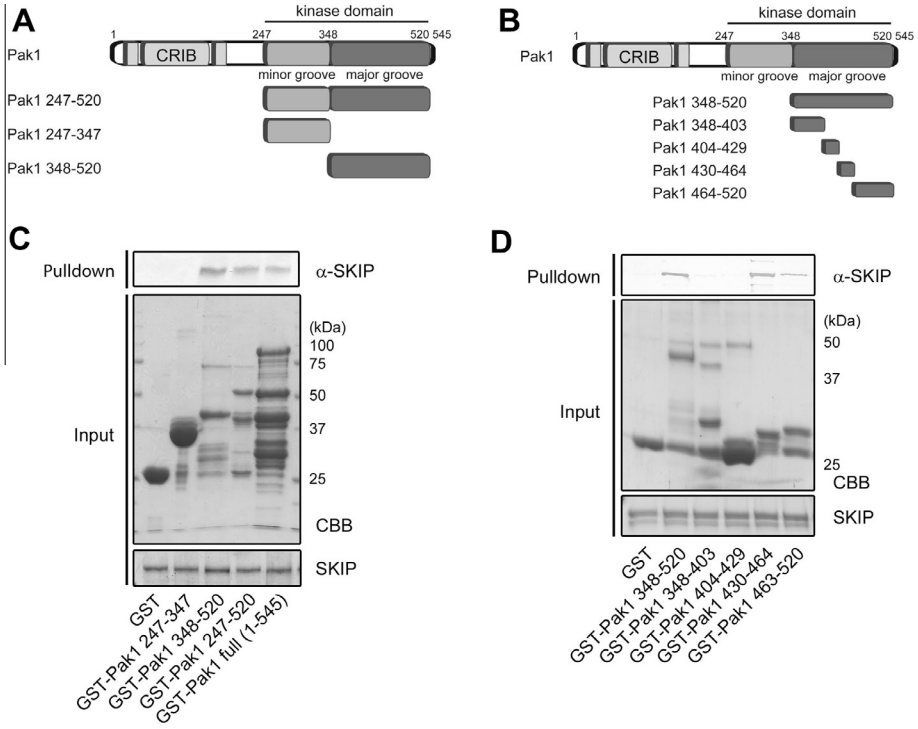

Fig. 3. Identification of SKIP binding region within Pak1 kinase domain. (A) Domain structure of Pak1. Pak1 is composed of N-terminal regulatory domain and C-terminal kinase domain. (B) In vitro binding assay between GST-conjugated Pak1 kinase domain contracts (Pak1 N-lobe, Pak1 C-lobe) and SKICH domain of SKIP. Binding was analyzed by Western blot analysis. (C) Constructs of the Pak1 C-lobe region used to in vitro binding assay in D. (D) In vitro binding assay SKIP SKICH domain and subdomain of Pak1 C-lobe region. The binding was analyzed by Western blot analysis.

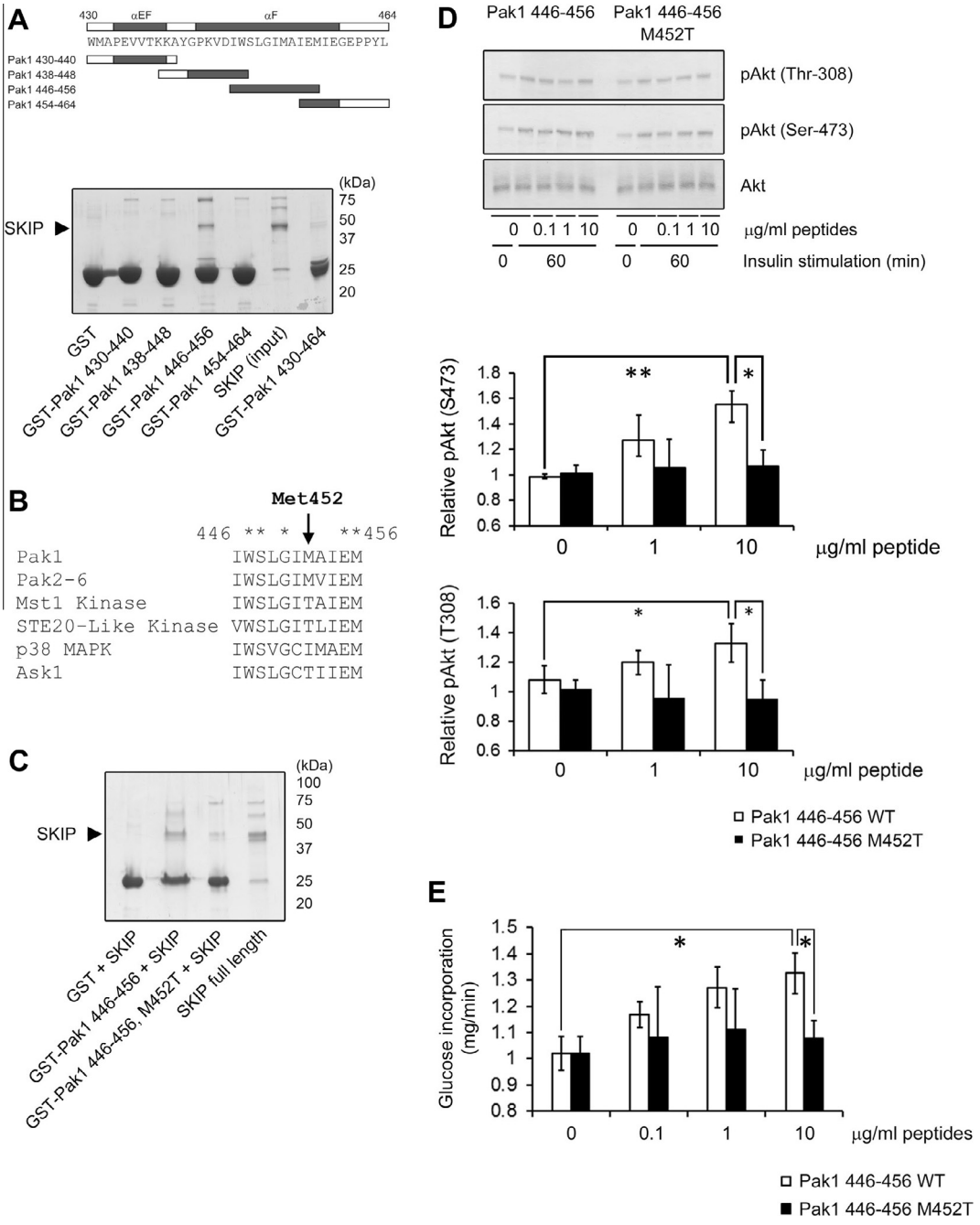

Fig. 4. Supplementation of SKIP-binding peptide increased insulin action. (A) In vitro binding assay using GST-conjugated 11 amino acid regions within Pak1 C-lobe region (Pak1 430–440, 438–448, 446–456, and 454–464) and recombinant SKIP. The result of Coomassie Brilliant Blue staining is shown. The position of recombinant SKIP is shown on the left side and the position of molecular weight markers are indicated on the right side. (B) Amino acid sequence of SKIP-binding region of Pak (amino acid 446–456) and alignment of region with other STE20 family protein kinases. (C) In vitro binding assay between GST-conjugated wild-type Pak1 446–456 or its M452T mutant and recombinant SKIP. The result of Coomassie Brilliant Blue staining is shown. The positions of recombinant SKIP and the molecular weight markers are indicated. (D) C2C12 cells were supplemented with the indicated peptides (10 μg/ml) for 24 h before cells were stimulated with insulin for 60 min. Lysates were applied to Western blot analysis to detect insulin-induced phosphorylation of Akt. (E) L6 myoblast cells were supplemented with the indicated peptides for 24 h before cells were stimulated with insulin for 60 min. Insulin-induced glucose uptake was measured by 2-DG assay kit. Results of 4 independent experiments were shown.

for an improvement from insulin resistance, a major characteristic of Type 2 diabetes. Our results show that an incorporation of Pak1 446–456 peptide into skeletal muscle might be a novel tool for an intervention from insulin resistance and Type 2 diabetes mellitus.

Acknowledgments

This work was supported in part by Grant to T.I. from Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 25460365, Novo Nordisk Pharma Research Foundation, the Japan Diabetes Foundation and by a Grant-in-aid for scientific research to T.T. by Ministry of Education, Culture, Sports, Science, and Technology (MEXT) KAKENHI Grant Number 23227005, Japan.

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