

Tumor Necrosis Factor (TNF)- α -induced Repression of GKAP42 Protein Levels through cGMP-dependent Kinase (cGK)-I α Causes Insulin Resistance in 3T3-L1 Adipocytes*

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Background: IRS-1-associated proteins play roles in modulation of insulin-induced IRS-1 tyrosine phosphorylation.

Results: A novel IRS-1-associated protein, GKAP42, is required to maintain availability of IRS-1 to the insulin receptor. TNF- α treatment suppressed the GKAP42 protein level.

Conclusion: TNF- α -induced insulin resistance is at least partially caused by GKAP42 protein level suppression.

Significance: We identified a novel TNF- α -induced pathway involved in insulin resistance.

Insulin receptor substrates (IRSs) have been shown to be major mediators of insulin signaling. Recently, we found that IRSs form high-molecular weight complexes, and here, we identify by yeast two-hybrid screening a novel IRS-1-associated protein: a 42-kDa cGMP-dependent protein kinase-anchoring protein (GKAP42). GKAP42 knockdown in 3T3-L1 adipocytes suppressed insulin-dependent IRS-1 tyrosine phosphorylation and downstream signaling, resulting in suppression of GLUT4 translocation to plasma membrane induced by insulin. In addition, GLUT4 translocation was also suppressed in cells overexpressing GKAP42-N (the IRS-1 binding region of GKAP42), which competed with GKAP42 for IRS-1, indicating that GKAP42 binding to IRS-1 is required for insulin-induced GLUT4 translocation. Long term treatment of 3T3-L1 adipocytes with TNF- α , which induced insulin resistance, significantly decreased the GKAP42 protein level. We then investigated the roles of cGMP-dependent kinase (cGK)-I α , which bound to GKAP42, in these changes. cGK-I α knockdown partially rescued TNF- α -induced decrease in GKAP42 and impairment of insulin signals. These data indicated that TNF- α -induced repression of GKAP42 via cGK-I α caused reduction of insulin-induced IRS-1 tyrosine phosphorylation at least in part. The present study describes analysis of the novel TNF- α -induced pathway, cGK-I α -GKAP42, which regulates insulin-dependent signals and GLUT4 translocation.

Insulin is a major anabolic hormone whose action plays pivotal roles in tissue development, growth, and the maintenance of glucose homeostasis. Insulin regulates glucose metabolism at several levels. It suppresses hepatic gluconeogenesis and promotes the rate of glucose uptake in muscle and adipose tissue. Glucose uptake is central to the regulation of postprandial glucose clearance from plasma, and the insulin-responsive signaling system is known to be responsible for glucose uptake into these tissues (1). Inability of the insulin signaling system to stimulate glucose uptake results in states of insulin resistance that are major causes of type 2 diabetes mellitus (2).

In general, insulin binding to insulin receptors (IRs),² leads to receptor autophosphorylation on tyrosine residues, resulting in receptor activation (3, 4). Activated receptor tyrosine kinases phosphorylate several intracellular substrates, including insulin receptor substrates (IRSs) and Shc (5, 6). Tyrosine phosphorylation of these substrates leads to binding of several Src homology 2 domain-containing signaling molecules, including the p85 PI 3-kinase regulatory subunit and Grb2 (7, 8). Binding allows activation of distinct signaling cascades, including Ras-MAPK and PI 3-kinase (6, 9). In particular, PI 3-kinase cascade activation is well known to be required for insulin-dependent glucose transporter 4 (GLUT4) translocation to plasma membrane and for subsequent glucose uptake in adipose and muscle tissues (10–12). Once PI 3-kinase is activated, it produces the lipid second messenger phosphatidylinositol 3,4,5-triphosphate, which then activates the downstream serine/threonine kinase, Akt. Subsequent phosphorylation of Akt substrate, AS160, leads to insulin stimulation of GLUT4 translocation and glucose uptake (13–16). Thus, the IRSs play important roles as mediators of the insulin signal transduction pathway.

Adipose tissues are involved in energy balance regulation not only by lipid storage but also by secretion of various factors,

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² The abbreviations used are: IR, insulin receptor; IRS, insulin receptor substrate; IGF, insulin-like growth factor; GKAP42, 42-kDa cGMP-dependent protein kinase-anchoring protein; GLUT4, glucose transporter 4; cGK, cGMP-dependent kinase; PI, phosphatidylinositol; 53BP2S, p53-binding protein 2; HEK, human embryonic kidney; iNOS, inducible nitric-oxide synthase; TES, Tris-HCl, EDTA, and sucrose; GM3, NeuAc α 2,3Gal β 1,4Glc-ceramide.

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including cytokines, that modulate numerous physiological and pathological processes. For example, tumor necrosis factor (TNF)- α is a well known cytokine, that induces insulin resistance (17–19). In 3T3-L1 adipocytes, the cell line widely used as the model of insulin resistance, pretreatment with TNF- α impaired insulin-induced glucose uptake (20–22).

There are accumulated reports elucidating the mechanism of TNF- α -induced insulin resistance. TNF- α pretreatment decreased IRS-1 protein level as well as repressed insulin-induced IRS-1 tyrosine phosphorylation (20). *In vitro* phosphorylation assays indicated that IRS-1 derived from 3T3-L1 adipocytes pretreated with TNF- α showed impaired availability to the insulin receptor (23). More detailed analyses indicated that there were multiple putative serine/threonine (Ser/Thr) phosphorylation sites in IRS-1 and that increased Ser/Thr phosphorylation of IRS-1 impaired the ability of IRS-1 to associate with the insulin receptor, inhibiting subsequent insulin-stimulated tyrosine phosphorylation (24). For instance, Ser³⁰⁷ was identified as a site for TNF- α -induced phosphorylation of IRS-1, with activation of c-Jun N-terminal kinase (JNK) involved in the phosphorylation of this residue (25, 26).

Recently, we found that IRS-1 formed high-molecular mass complexes in 3T3-L1 adipocytes not through recognition of tyrosine phosphorylation and that the amount of IRS-associated proteins was dramatically changed by TNF- α pretreatment (23). *In vitro* phosphorylation analysis washing out IRS-1-associated proteins indicated that IRS-1-associated proteins could regulate the availability of IRS-1 for the insulin receptor kinase. Previously, we and others have shown that PHIP, Nexilin, 53BP2S, or HSP90 β modulated insulin/insulin-like growth factor-1 (IGF-I)-dependent tyrosine phosphorylation of IRS-1 or IRS-2 in insulin/IGF-targeted cells (27–30). Thus, the identification of additional IRS-associated proteins, which serve to modulate IRS tyrosine phosphorylation, is an essential prerequisite for understanding the alternative mechanisms of IRS tyrosine phosphorylation.

In this study, we show that the novel IRS-1-binding protein GKAP42 is required to maintain insulin-induced IRS-1 tyrosine phosphorylation and that GKAP42 protein level repression by TNF- α through cGK-I α causes TNF- α -induced insulin resistance at least in part.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Hanks' buffered salt solution were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Calf serum, fetal bovine serum (FBS), recombinant mouse TNF- α , and bovine insulin were obtained from Sigma-Aldrich. Penicillin and streptomycin were obtained from Banyu Pharmaceutical Co. (Ibaraki, Japan). Polyclonal anti-IRS-1 antibody was raised in rabbits as described previously (31). Polyclonal anti-GKAP42 antibody was kindly provided by Dr. Noriyuki Yanaka (University of Hiroshima University, Hiroshima, Japan). Anti-IR β antibody, anti-GLUT4 antibody, and anti- β -actin antibody were obtained from Santa Cruz Biotechnology, Inc. Anti-PI 3-kinase p85 subunit antibody, anti-Myc antibody, and anti-phosphotyrosine antibody (clone 4G10) were obtained from Millipore (Billerica, MA). Anti-phospho-Akt (Ser-

473) antibody, anti-Akt antibody, anti-phospho-ERK antibody, and anti-ERK antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-FLAG antibody and anti-FLAG antibody-conjugated agarose beads were obtained from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit and anti-mouse IgG antibody were obtained from GE Healthcare. Enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Science. Alexa Fluor 594-conjugated secondary anti-mouse IgG antibody was obtained from Invitrogen. Protein A-Sepharose and 2-deoxy-D-[2,6-³H]glucose (1 mCi/ml) were purchased from Amersham Bioscience. Control and GKAP42- and cGK-I α -specific siRNAs were purchased from RNAi Co. (Tokyo, Japan). The sequence of the GKAP42 siRNA used was 5'-CGG UGU CAC UCA AAG ACU UCC-3' or 5'-GUA GGU CUA AAA GAU GGA AGA-3'. The sequence of the cGK-I α siRNA used was 5'-CUU UCU GAU CGG UUC UAA ACA-3' or 5'-GUG CUU AUC CUA UCG AAA UAC-3'. The nonrelevant control siRNA sequence was GUA CCG CAC GUC AUU CGU AUC. Other chemicals were of the reagent grade available commercially.

Plasmids—pAS-IRS-1 was prepared as described previously (28) and used for two-hybrid screening as bait. The full-length FLAG-tagged GKAP42 was a gift from Dr. Noriyuki Yanaka (Graduate School of Biosphere science, Hiroshima University, Hiroshima, Japan). Deletion mutants of FLAG-GKAP42 were constructed as follows. GKAP42 fragment encoding the amino acid residues 1–95, 1–197, 198–366, and 96–366 were generated by PCR using GKAP42 cDNA as a template. Amplified fragments were digested by BamHI and Sall and inserted into pCMV-FLAG-2 or pEGFP-C1 vector in-frame.

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed as described previously (28).

Cell Cultures—CHO-C400 cells and HEK293T cells were cultured as described previously (32). Murine 3T3-L1 preadipocytes were purchased from the American Type Tissue Culture Collection. 3T3-L1 preadipocytes were cultured in DMEM containing 10% calf serum at 37 °C in 5% CO₂ atmosphere and induced to differentiate into adipocytes as described previously (33).

Transient Transfection of HEK293T CHO-C400 or 3T3-L1 Adipocytes—HEK293T cells and CHO-C400 cells were transiently transfected with expression plasmids by the calcium phosphate precipitation method as described previously (32). Transient transfection of 3T3-L1 adipocytes was described previously (34). DMEM containing 10% FBS was added to the electroporated cells; the cells were then allowed to adhere to tissue culture dishes for 3 days, and the adipocytes were then serum-starved for 24 h before experiments. In some experiments, the electroporated adipocytes were seeded on coverslips.

Production of Recombinant Lentiviruses and Lentiviral Transduction—The green fluorescent protein (GFP) and GFP-tagged GKAP42 (GFP-GKAP42) were generated by PCR, and the amplified fragments were inserted into the BamHI and XhoI site of CSII-EF-MCS lentiviral vector. The GFP-GKAP42, pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev plasmids were cotransfected into HEK293T cells by using the calcium phosphate method. After 12 h postinfection, the culture medium was replaced with fresh medium containing 10 μ M forskolin. At

72 h postinfection, the viral supernatant were harvested, passed through 0.45- μm filters, and stored at -80°C . For the lentiviral transduction, the mature 3T3-L1 adipocytes (after 8 days of differentiation) were incubated with the viral supernatant containing 10 $\mu\text{g}/\text{ml}$ Polybrene for 12 h. After 12 h post-transduction, the medium was replaced with fresh culture medium. All experiments were performed at 3 days post-transduction.

Subcellular Fractionation—Fractionation was performed as described previously (35). Briefly, 3T3-L1 adipocytes were collected in a TES buffer (20 mM Tris-HCl, 1 mM EDTA, 255 mM sucrose, 500 μM Na_3VO_4 , 10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin, 20 $\mu\text{g}/\text{ml}$ PMSF, 100 kallikrein-inactivating units/ml aprotinin, 10 mg/ml *p*-nitrophenyl phosphate (pH 7.4)). The lysates were fractionated with ultracentrifugation in an Optima TLX ultracentrifuge with a TLA-55 rotor (Beckman Coulter). The resulting pellets were resuspended in radioimmune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin, 20 $\mu\text{g}/\text{ml}$ PMSF, 100 kallikrein-inactivating units/ml aprotinin, 10 mg/ml *p*-nitrophenyl phosphate (pH 8.0)). Membrane fractions and cytosolic supernatants were analyzed by immunoblotting.

Immunofluorescence Analysis—CHO cells transfected with pGFP-GKAP42 and pMyc-IRS-1 were grown on coverslips and serum-starved for 12 h. The cells were fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature. Cells were then washed with PBS and permeabilized with PBS containing 0.25% Triton X-100 for 5 min at room temperature. Next, cells were incubated with blocking buffer (3% BSA and 0.025% NaN_3 in PBS) for 1 h at room temperature, and primary antibody was added overnight at 4°C . The samples were again washed with PBS, incubated with a secondary antibody diluted in blocking buffer for 1 h at 37°C , and washed. Coverslips were mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA) for visualization using an FV500 confocal microscope (Olympus, Tokyo, Japan).

Glucose Uptake Assay—3T3-L1 adipocytes were incubated with the indicated concentrations of insulin in Krebs-Ringer phosphate buffer (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO_4 , 1 mM CaCl_2 , 1% BSA, pH 7.4) for 20 min at 37°C . Then 0.1 mM 2-deoxy-D-glucose containing 0.5 $\mu\text{Ci}/\text{ml}$ 2-deoxy-D-[2,6- ^3H]glucose was added, and cells were incubated for 4 min at 37°C . The reaction was terminated by the addition of ice-cold PBS containing 10 mM D-glucose. Cells were lysed with 0.1 N NaOH, and radioactivity taken up by cells was measured by a liquid scintillation counter.

GLUT4 Translocation Assay—Differentiated 3T3-L1 adipocytes electroporated with siRNA or plasmid along with pGLUT4-Myc-green fluorescent protein (GFP) were grown on coverslips. Twenty-four hours after electroporation, cells were serum-starved for 4 h and treated with or without insulin for 20 min. Cells were then fixed using 4% paraformaldehyde for 10 min without permeabilization and blocked with 3% bovine serum albumin for 1 h at room temperature. Coverslips were immunostained with anti-Myc antibody for 1 h at 37°C . After incubation, the coverslips were washed three times with PBS solution, followed by incubation in secondary antibody for 1 h at 37°C . Coverslips were again washed three times with PBS and mounted in VECTASHIELD medium. Plasma membrane

localization was determined by using confocal fluorescence microscopy (OLYMPUS, Tokyo, Japan) to score 20 representative cells per condition for the appearance of a continuous plasma membrane ring of GLUT4.

Immunoprecipitation Followed by Immunoblotting—Cells were lysed at 4°C with ice-cold Tris/Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride (PMSF), 5 $\mu\text{g}/\text{ml}$ pepstatin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 kallikrein-inactivating units/ml aprotinin, 1 mM Na_3VO_4 , and 10 mg/ml *p*-nitrophenyl phosphate). Insoluble materials were removed by centrifugation at $15,000 \times g$ for 10 min at 4°C , and supernatant was prepared as total cell lysates. For immunoprecipitation, 1 mg of protein of total cell lysates was incubated with the indicated antibody for 2 h at 4°C , and the immunocomplexes were precipitated with 10 μl of protein A-Sepharose for polyclonal antibody. These precipitates were washed three times with ice-cold lysis buffer. These precipitates or total cell lysates were subjected to SDS-PAGE and immunoblotted with the indicated antibody.

PI 3-Kinase Activity Assay—PI 3-kinase activity assay was carried out as described previously (36). 3T3-L1 adipocytes were electroporated with control or GKAP42 siRNA. Three days after electroporation, cells were serum-starved for 24 h in DMEM containing 0.1% bovine serum albumin and then treated with or without 100 nM insulin for 5 min. Cells were lysed by Tris/Triton X-100 lysis buffer, and 1 mg of whole cell lysates was immunoprecipitated with anti-IRS-1 antibody.

In Vitro Tyrosine Phosphorylation Assay—An *in vitro* tyrosine phosphorylation assay was carried out as described previously (23). Briefly, insulin receptors were semipurified by wheat germ agglutinin-agarose from NIH-3T3 cells overexpressing insulin receptor. On the other hand, immunoprecipitations with anti-IRS-1 antibody were carried out in control or GKAP42 knockdown 3T3-L1 adipocytes, and the immunoprecipitates were mixed with 10 μl of receptor solution, ATP (final concentration 500 μM), and insulin (final concentration 100 nM) or GST-GKAP42 (final concentration 10 nM) in reaction buffer (50 mM Tris-HCl pH 7.4, 5 mM MgCl_2 , 5 mM MnCl_2). They were incubated at 25°C for 30 min, and the reaction was quenched by the addition of one-third volume of $3 \times$ Laemmli's buffer and boiled for 5 min. Samples were then separated by SDS-PAGE.

In Vitro cGK Kinase Assay—Differentiated 3T3-L1 adipocyte cells were serum-starved for 3 h in DMEM containing 0.1% bovine serum albumin, and then cells were pretreated with or without 2.0 nM TNF- α for 24 h. Cells were lysed by Tris/Triton X-100 lysis buffer, and 1 mg of whole cell lysates was immunoprecipitated with anti-cGK-I α antibody. Immunoprecipitates were washed three times with Tris/Triton X-100 buffer and finally resuspended in 40 μl of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 0.2 mM [γ - ^{32}P]ATP, 2 mM protein kinase A inhibitor peptide, 5 mM glycerophosphoric acid, 5 mM cGMP, and 1 mM sodium orthovanadate) in the presence of 100 mM BPDEide (Calbiochem), a synthetic substrate selective for cGK. Assays were conducted at 30°C for various times and terminated by centrifuging and aliquoting the peptide onto phosphocellulose P-81

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paper (Whatman). The phosphocellulose pads were washed five times with 0.5% phosphoric acid and counted using a liquid scintillation counter.

Statistical Analysis—Data are expressed as mean \pm S.E. Comparisons between two groups were performed using Student's *t* test, whereas comparisons between more than two groups were analyzed by one-way or two-way analysis of variance and the Tukey post hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

GKAP42 Was Identified as an IRS-1-interacting Protein in Adipocytes—To identify proteins that interact with IRS-1, full-length rat IRS-1 fused with the Gal4 DNA binding domain was utilized as bait for yeast two-hybrid screening. We screened a 3T3-L1 adipocyte cDNA library fused with the Gal4 activation domain in pACT2. From 3×10^5 clones, 21 IRS-1-interacting candidates were identified. Seven clones included cDNAs encoding 14-3-3 isoforms (β , ϵ , and ξ), which were previously shown to interact with IRS-1 or IRS-2 (37). We also obtained a partial cDNA sequence of GKAP42. GKAP42 is a 366-amino acid protein that consists of four coiled-coil domains and was originally isolated as an anchoring protein for cGMP-dependent kinase- α to regulate its intracellular localization and access to substrates (38). Although GKAP42 was reported as a testis-specific protein, we detected its expression also in adipose tissues (Fig. 1A).

After confirming the interaction of GKAP42 with IRS-1 in a yeast two-hybrid assay, HEK293T cells were co-transfected with Myc-tagged IRS-1 and FLAG-tagged GKAP42. Cell lysates were immunoprecipitated with anti-FLAG antibody, and immunoprecipitates were separated by SDS-PAGE followed by immunoblotting using anti-Myc antibody. Myc-IRS-1 associated with FLAG-GKAP42 but not with FLAG alone, indicating that GKAP42 specifically associates with IRS-1 in a co-immunoprecipitation assay (Fig. 1B). We then investigated the interaction of endogenously expressed GKAP42 with IRS-1 in 3T3-L1 adipocytes. The co-immunoprecipitation assay revealed that endogenously expressed IRS-1 also interacted with endogenously expressed GKAP42 (Fig. 1C). Next the subcellular localization of IRS-1 and GKAP42 were examined. GFP-GKAP42 and Myc-IRS-1 was exogenously expressed in CHO-C400 cells, and immunostaining analysis was performed. As shown in Fig. 1D, GFP-IRS-1 and Myc-GKAP42 were well colocalized with each other. Furthermore, 3T3-L1 adipocytes were fractionated into plasma membrane, LDM, HDM, and Cyt, as described previously (35). Each fraction was subjected to immunoblotting analysis with anti-IRS-1 or anti-GKAP42 antibody. As shown in Fig. 1E, both IRS-1 and GKAP42 were mainly fractionated into the LDM fraction, and the fractionation pattern was quite comparable.

GKAP42 Knockdown Suppressed Insulin Signaling and Glucose Uptake in 3T3-L1 Adipocytes—To investigate the effect of GKAP42 on insulin signaling pathways, we suppressed GKAP42 protein level by RNA interference-mediated gene silencing. Fully differentiated 3T3-L1 adipocytes were electroporated with control or GKAP42 siRNA, resulting in a marked reduction in targeted GKAP42 protein level without any signif-

icant effect on the levels of other proteins (Fig. 2A). Cell lysates from GKAP42 knockdown 3T3-L1 adipocytes were immunoprecipitated with anti-IR antibody or anti-IRS-1 antibody and assessed for insulin-dependent IR or IRS-1 tyrosine phosphorylation or for binding of the p85 regulatory subunit of PI 3-kinase to IRS-1. As a result, we found that GKAP42 knockdown did not affect insulin receptor autophosphorylation induced by insulin, which is known to reflect tyrosine kinase activation (Fig. 2A). In contrast, insulin-dependent tyrosine phosphorylation of IRS-1 and binding of the p85 regulatory subunit of PI 3-kinase to IRS-1 were decreased (Fig. 2A). We then measured PI 3-kinase activity bound to IRS-1. Insulin-dependent activation of PI 3-kinase associated with IRS-1 was reduced by GKAP42 knockdown, as shown in Fig. 2B. It is well known that phosphorylation of Akt on two of its amino acid residues, threonine 308 and serine 473, is required for full activation (39). Consistent with a decrease in PI 3-kinase activity associated with tyrosine-phosphorylated IRS-1, insulin-stimulated Akt serine 473 phosphorylation and threonine 308 phosphorylation were both decreased in GKAP42 knockdown cells without any change in ERK activation (Fig. 2A).

We then investigated insulin-dependent GLUT4 translocation to the plasma membrane and glucose uptake. Fully differentiated 3T3-L1 adipocytes were electroporated with control or GKAP42 siRNA along with pGLUT4-Myc-eGFP-expressing plasmid. Twenty-four hours later, cells were serum-starved for 4 h, followed by stimulation with insulin for 20 min. Cells were fixed without permeabilization and incubated with anti-Myc antibody. We calculated the relative Myc fluorescence in comparison with the total cell GFP fluorescence. Insulin stimulation induced GLUT4 translocation to the plasma membrane in control cells. In contrast, GKAP42 knockdown significantly inhibited insulin-stimulated GLUT4 translocation to the plasma membrane (Fig. 2C), and insulin-dependent glucose uptake was impaired (Fig. 2D).

IRS-1 availability to IR tyrosine kinase was then assessed in GKAP42 knockdown cells. The same amounts of IRS-1 prepared from GKAP42 knockdown or control 3T3-L1 adipocytes were immunoprecipitated, and an *in vitro* tyrosine phosphorylation assay was performed. The results indicated that *in vitro* tyrosine phosphorylation of IRS-1 prepared from GKAP42 knockdown cells was suppressed compared with that of IRS-1 from control cells (Fig. 2E), indicating that IRS-1 availability for IR tyrosine kinases is decreased by GKAP42 knockdown. Moreover, GST or GST-GKAP42 protein purified from *Escherichia coli* was added into the *in vitro* phosphorylation assay. The addition of GST-GKAP42 could not recover the impaired availability of IRS-1 to IR in GKAP42 knockdown cells (Fig. 2F), suggesting that IRS-1 derived from GKAP42 knockdown cells has already been modified to have impaired availability to IR.

Overexpression of the N-terminal Coiled-coil Domain of GKAP42 Impaired Insulin-dependent GLUT4 Translocation—To identify the region of GKAP42 responsible for the interaction with IRS-1, plasmids expressing a GKAP42 deletion series fused to a FLAG tag were constructed (D1–D4), and co-immunoprecipitation assays were carried out (Fig. 3A). The GKAP42 mutants that contain the coiled-coil domain in the N-terminal region (D1 and D2) interacted with IRS-1 but not a version

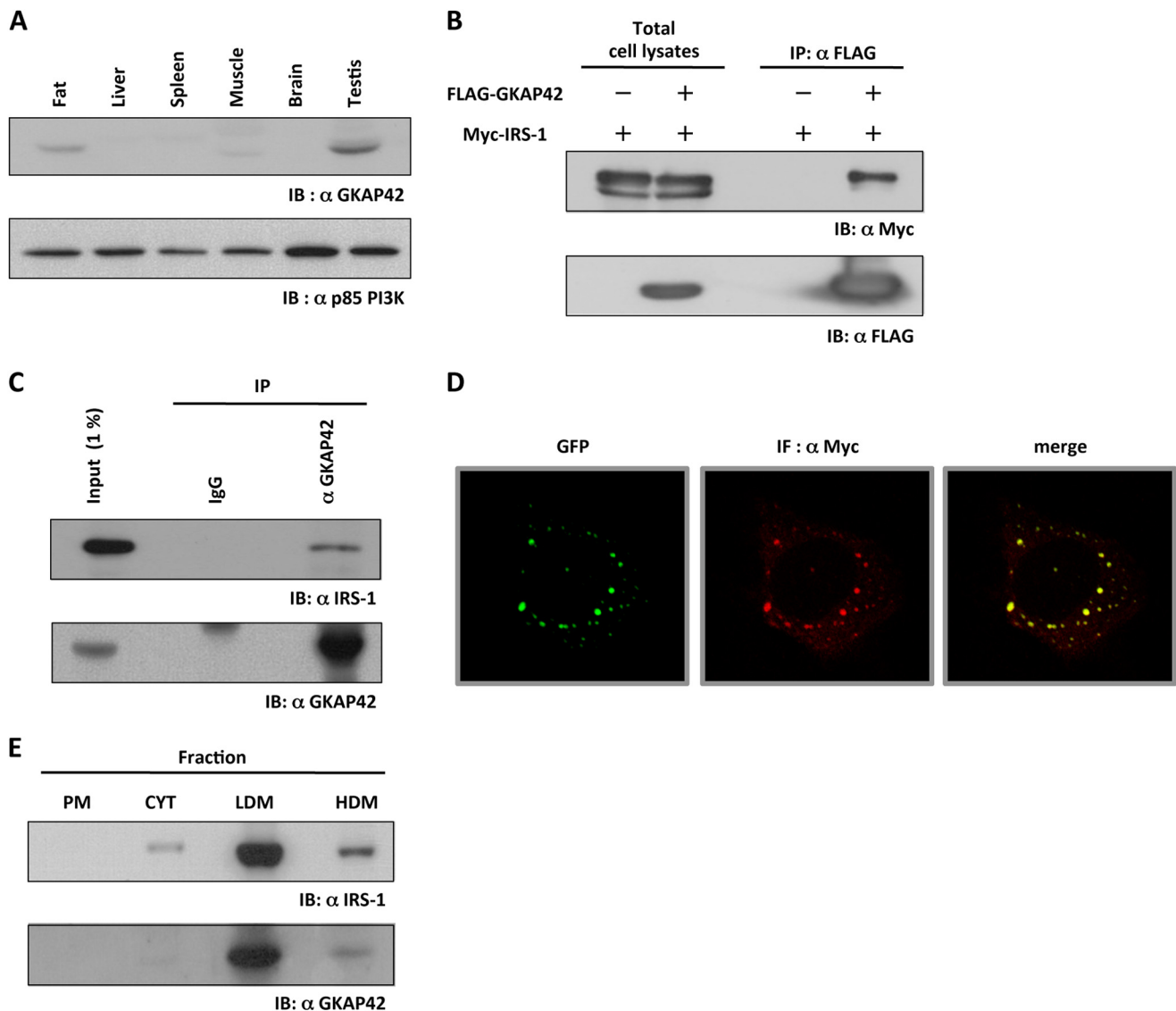


FIGURE 1. GKAP42 interacted with IRS-1. *A*, the indicated mouse tissues were collected, and the lysates were subjected to SDS-PAGE followed by immunoblotting (IB) with antibodies against GKAP42 or the p85 regulatory subunit of PI 3-kinase. *B*, HEK293T cells were co-transfected with pFLAG or pFLAG-GKAP42 in combination with pMyc-IRS-1. Cell lysates were immunoprecipitated by anti-FLAG antibody and immunoblotted with the indicated antibodies. *C*, cell lysates of fully differentiated 3T3-L1 adipocytes were subjected to immunoprecipitation with anti-GKAP42 antibody and immunoblotted with the indicated antibodies. Input represents an aliquot corresponding to 1% of lysate. Shown are representative immunoblots independently performed at least three times. *D*, CHO-C400 cells were transfected with Myc-GKAP42 and GFP-IRS-1 by Lipofectamine 2000 reagent. Cells were fixed, permeabilized, and immunostained with anti-Myc antibody. Transfected GFP-IRS-1 was visualized in the confocal microscopy in *green*, and Myc-GKAP42 protein was visualized by Alexa594 in *red*. *IF*, immunofluorescence. *E*, 3T3-L1 adipocytes were subjected to subcellular fractionation. Fractions (cytosol (CYT), plasma membrane (PM), high-density microsome (HDM), and low-density microsome (LDM)) were analyzed by immunoblotting with the indicated antibodies.

containing the coiled-coil domains of the C-terminal region (D3) (Fig. 3A). These data indicate that the N-terminal region (amino acid residues 1–95) of GKAP42 (GKAP42-N) is sufficient for its interaction with IRS-1. Weak interaction could be observed in a mutant containing just the second coiled-coil domain of the N-terminal region (D4) (Fig. 3A), suggesting that the second coiled-coil domain in the N-terminal region possibly plays some roles in the interaction between GKAP42 with IRS-1.

Next we assayed the effect of overexpression of GKAP42-N on the interaction between IRS-1 and GKAP42. GKAP42-N was overexpressed in HEK293T cells, and the interactions between IRS-1 and GKAP42 were studied by co-immunoprecipitation assays. In cells overexpressing GKAP42-N, decreased binding of

IRS-1 to GKAP42 was observed, indicating that overexpressed GKAP42-N outcompeted GKAP42 for IRS-1 (Fig. 3B).

To examine the role of GKAP42 binding to IRS-1, we assessed the effect of GKAP42-N overexpression on insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were transfected with pFLAG or pFLAG-GKAP42-N along with pGLUT4-Myc-eGFP by electroporation. Cells were serum-starved for 4 h, followed by stimulation with insulin for 20 min. We calculated the GLUT4 translocation ratio as described under “Experimental Procedures” and found that overexpression of GKAP42-N significantly inhibited insulin-stimulated GLUT4 translocation to the plasma membrane (Fig. 3C).

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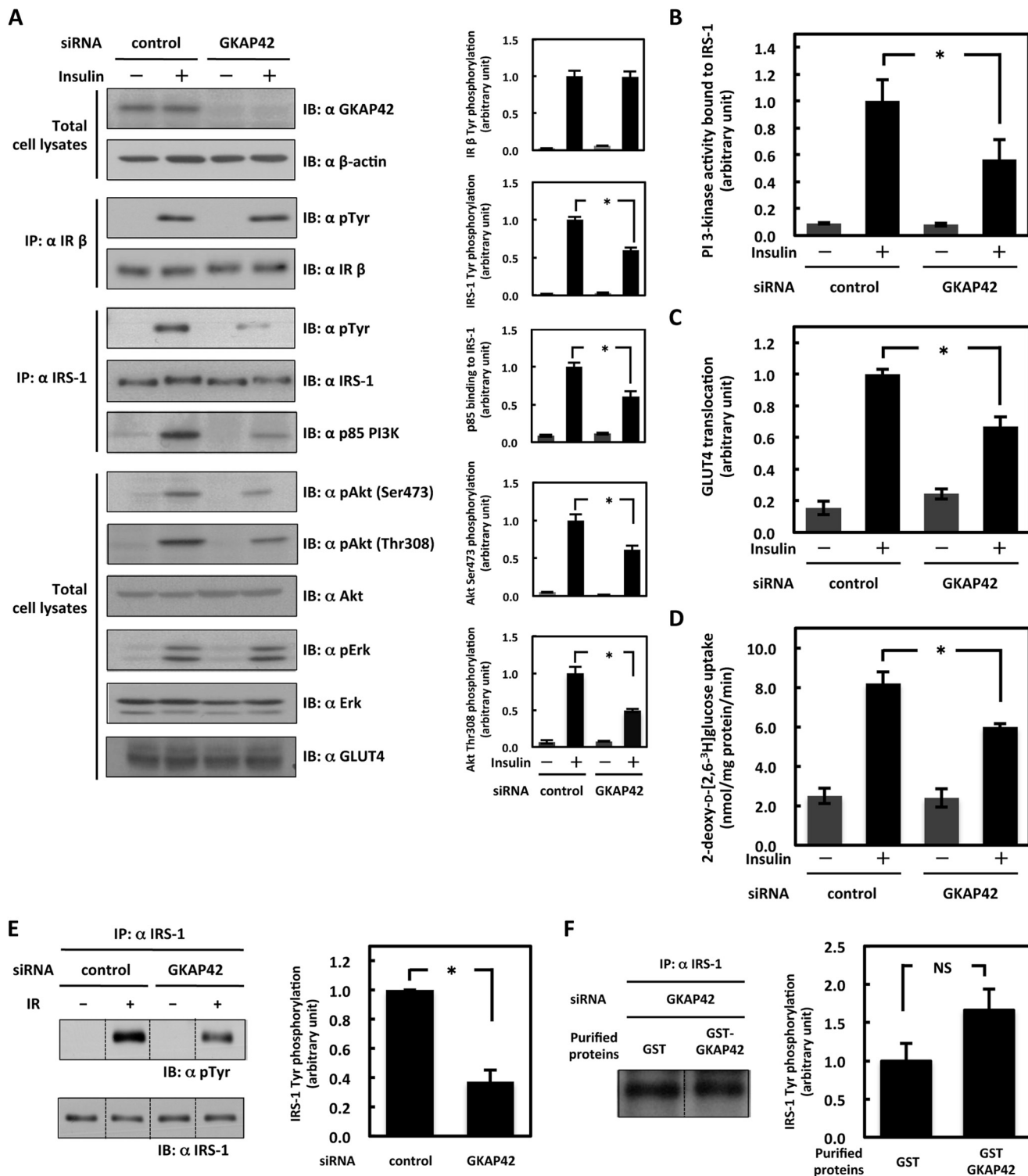


FIGURE 2. Effects of GKAP42 knockdown on insulin signaling and glucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes electroporated with control or GKAP42 siRNA were serum-starved, followed by stimulation with 100 nM insulin. *A*, cell lysates were immunoprecipitated with the indicated antibodies, and immunoprecipitates (IP) or total cell lysates (TCL) were immunoblotted (IB) with the indicated antibodies. Bands were quantified from each blot by ImageJ software (National Institutes of Health). Protein amounts of phosphorylated IR, phosphorylated IRS-1, p85 associated with IRS-1, and phosphorylated Akt were calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. (error bars) of triplicate determinations. *B*, PI 3-kinase activity associated with IRS-1 immunocomplex was measured by *in vitro* kinase assay. The results are presented as the means \pm S.E. of triplicate determinations. *C*, 3T3-L1 adipocytes were electroporated with control or GKAP42 siRNA along with pGLUT4-Myc-GFP. Cells were then fixed without permeabilization and immunostained with anti-Myc antibody. The amount of exofacially exposed Myc epitope was quantified. The results are presented as the means \pm S.E. of 20 cells. *D*, cells were assayed for glucose uptake. The results are presented as the means \pm S.E. of five wells. *, the difference between control siRNA and GKAP42 siRNA with insulin stimulation is significant, with $p < 0.05$ (*A–D*). *E* and *F*, cell lysates were subjected to immunoprecipitation with anti-IRS-1 antibody. Precipitates were subjected to an *in vitro* tyrosine phosphorylation assay using insulin receptor kinase. Bands were quantified from each blot by ImageJ software. The protein amount of phosphorylated IRS-1 was calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. of three different experiments. *, the difference between control siRNA and GKAP42 siRNA with the IR addition is significant, with $p < 0.05$ (*E*). Shown are representative data from experiments independently performed three times.

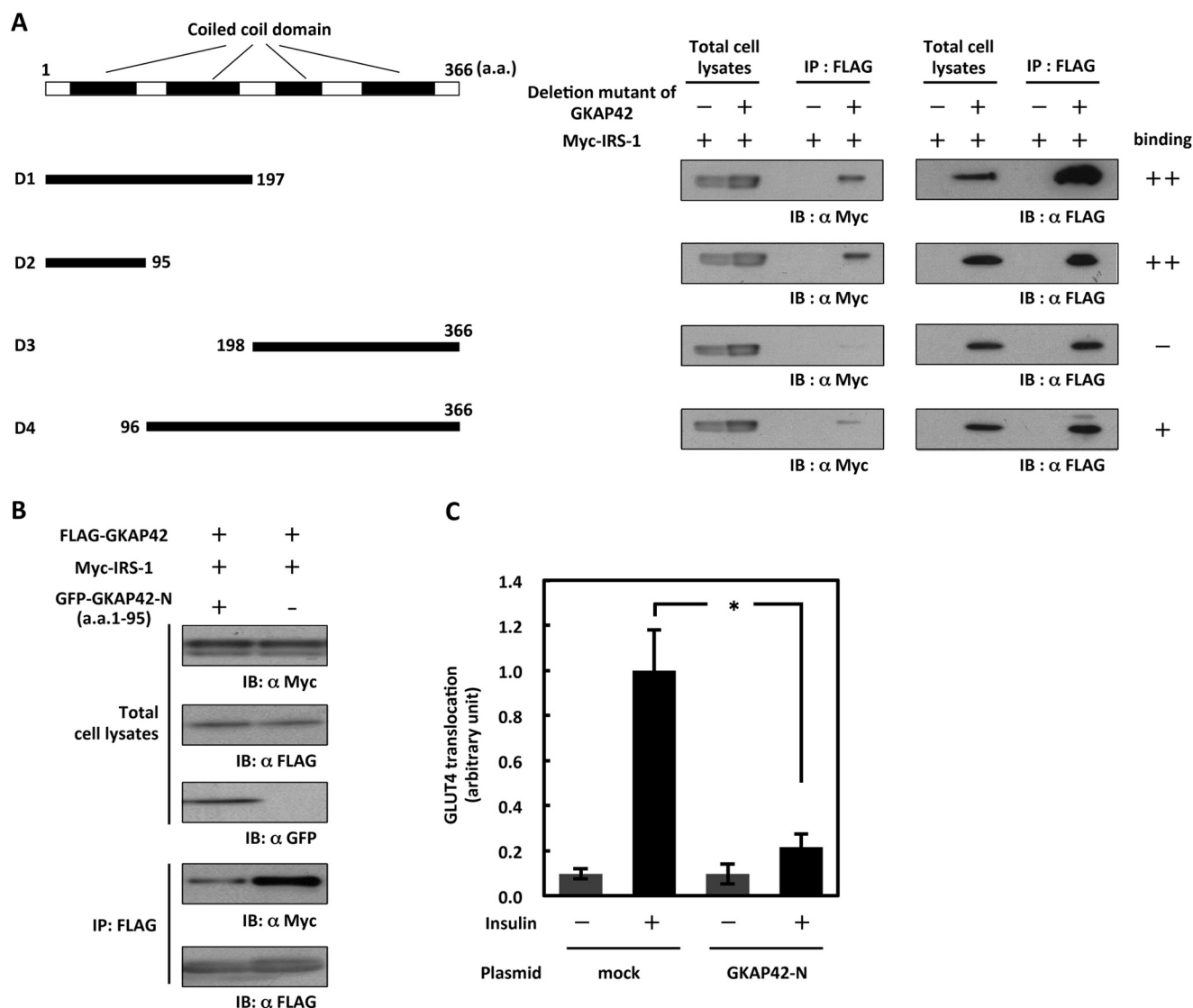


FIGURE 3. Effects of N-terminal coiled-coil domain of GKAP42 overexpression on insulin-dependent GLUT4 translocation. *A*, diagrams of FLAG-tagged GKAP42 deletion constructs used for experiments are shown. The number indicates the amino acid (a.a.) position number of GKAP42. Coiled-coil domains are shown in black. Below the GKAP42 structure, constructs of some deletion mutants (D1–D4) are shown. Plasmids expressing each deletion mutant of GKAP42 tagged with FLAG and pMyc-IRS-1 were co-transfected in HEK293T cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody-conjugated agarose and immunoblotted (IB) with the indicated antibodies. Total cell lysates, an aliquot corresponding to 1% of the lysates. *B*, HEK293T cells were co-transfected with pMyc-IRS-1 and pFLAG-GKAP42 following transfection with or without pGFP-GKAP42-N. Cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with the indicated antibodies. *C*, 3T3-L1 adipocytes were electroporated with plasmid pFLAG-GKAP42-N or pFLAG along with pGLUT4-Myc-GFP. Cells were then fixed without permeabilization and immunostained with anti-Myc antibody. The amount of exofacially exposed Myc epitope was quantified. The results are presented as the means \pm S.E. (error bars) of 20 cells. *, the difference between mock and GKAP42-N expression with insulin stimulation is significant, with $p < 0.05$. Shown are representative data from experiments independently performed at least three times.

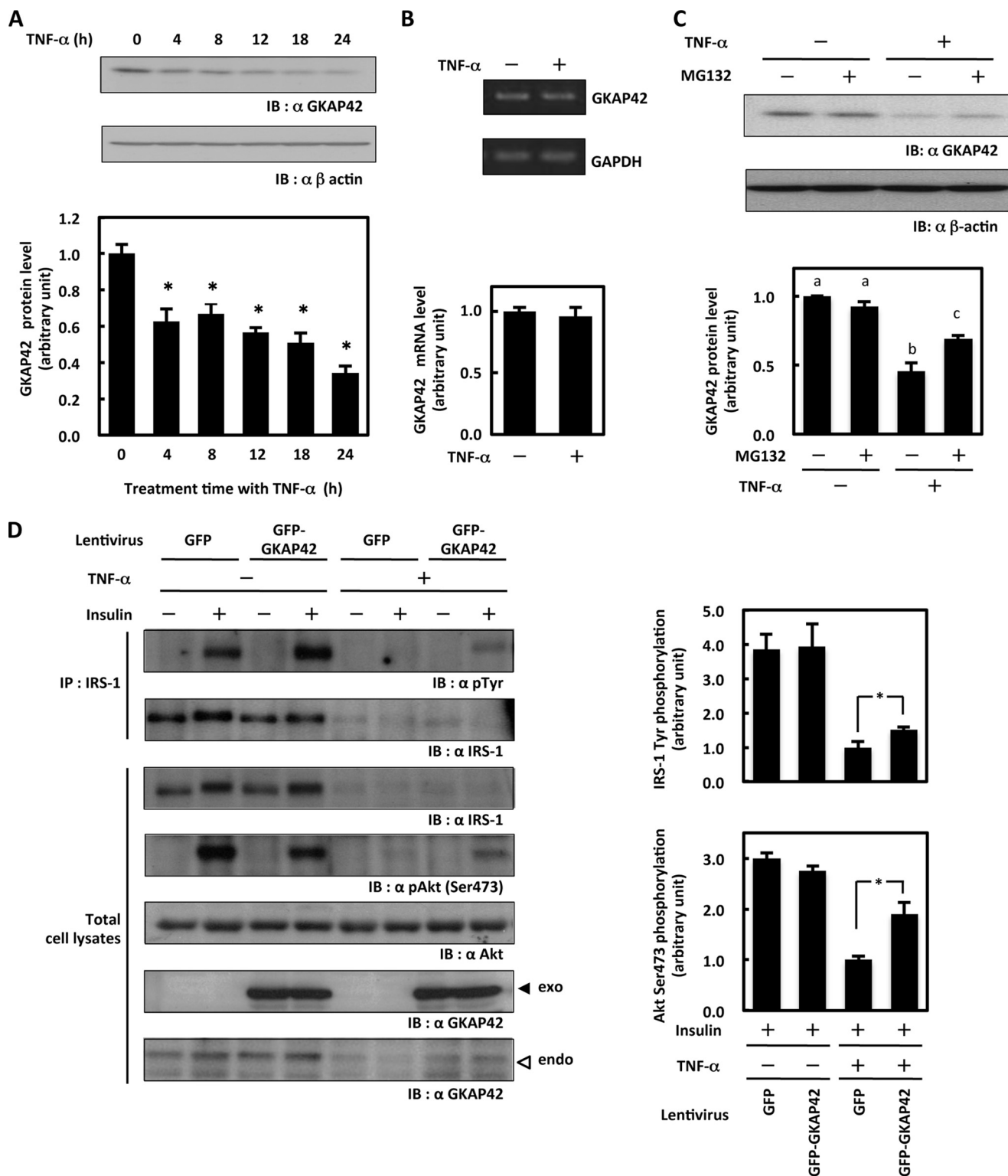
GKAP42 Protein Level Is Decreased by TNF- α Treatment and Rescued in the Presence of a Proteasome Inhibitor—It has been reported that TNF- α pretreatment inhibits insulin-induced IRS-1 tyrosine phosphorylation, resulting in inhibition of insulin signaling and glucose uptake in 3T3-L1 adipocytes (20–22). To evaluate whether GKAP42 is involved in TNF- α -induced insulin resistance, we measured GKAP42 protein in 3T3-L1 adipocytes treated with TNF- α for the indicated periods. Our results showed that TNF- α pretreatment suppressed the levels of GKAP42 protein in a time-dependent manner (Fig. 4A). Under the same conditions, TNF- α treatment did not decrease mRNA expression of GKAP42 (Fig. 4B). Treatment with an inhibitor of the proteasome (MG-132) rescued TNF- α -induced

reduction in GKAP42 protein expression (Fig. 4C), suggesting that GKAP42 was degraded in response to TNF- α through the proteasome in 3T3-L1 adipocytes. To examine whether TNF- α -induced decrease in GKAP42 protein level causes impairment of IRS-1 tyrosine phosphorylation, we overexpressed GFP-GKAP42 in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were infected with lentivirus expressing GFP or GFP-GKAP42. Cells were pretreated with TNF- α followed by stimulation with insulin, and then insulin signal activation was measured. As shown in Fig. 4D, GKAP42 overexpression could partially rescue TNF- α -induced impairment of insulin-induced IRS-1 tyrosine phosphorylation and Akt serine 473 phosphorylation.

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TNF- α Treatment Repressed GKAP42 Protein Level via cGK-I α —GKAP42 has been shown to interact with cGK-I α (38). To investigate the role, if any, of cGK-I α in TNF- α -induced repression of GKAP42 protein, we measured cGK-I α kinase activity. cGK-I α kinase activity was confirmed to be slightly enhanced by TNF- α treatment (Fig. 5A). Next, we knocked down cGK-I α in 3T3-L1 adipocytes. TNF-

α -induced repression of GKAP42 protein level was partially rescued by cGK-I α knockdown (Fig. 5B). At the same time, TNF- α -induced repression of insulin-dependent IRS-1 tyrosine phosphorylation, p85 associated with IRS-1, and Akt serine 473 phosphorylation were also partially rescued by cGK-I α knockdown (Fig. 5C). Next we assessed insulin-induced glucose uptake. As shown in Fig. 5D, cGK-I α knock-



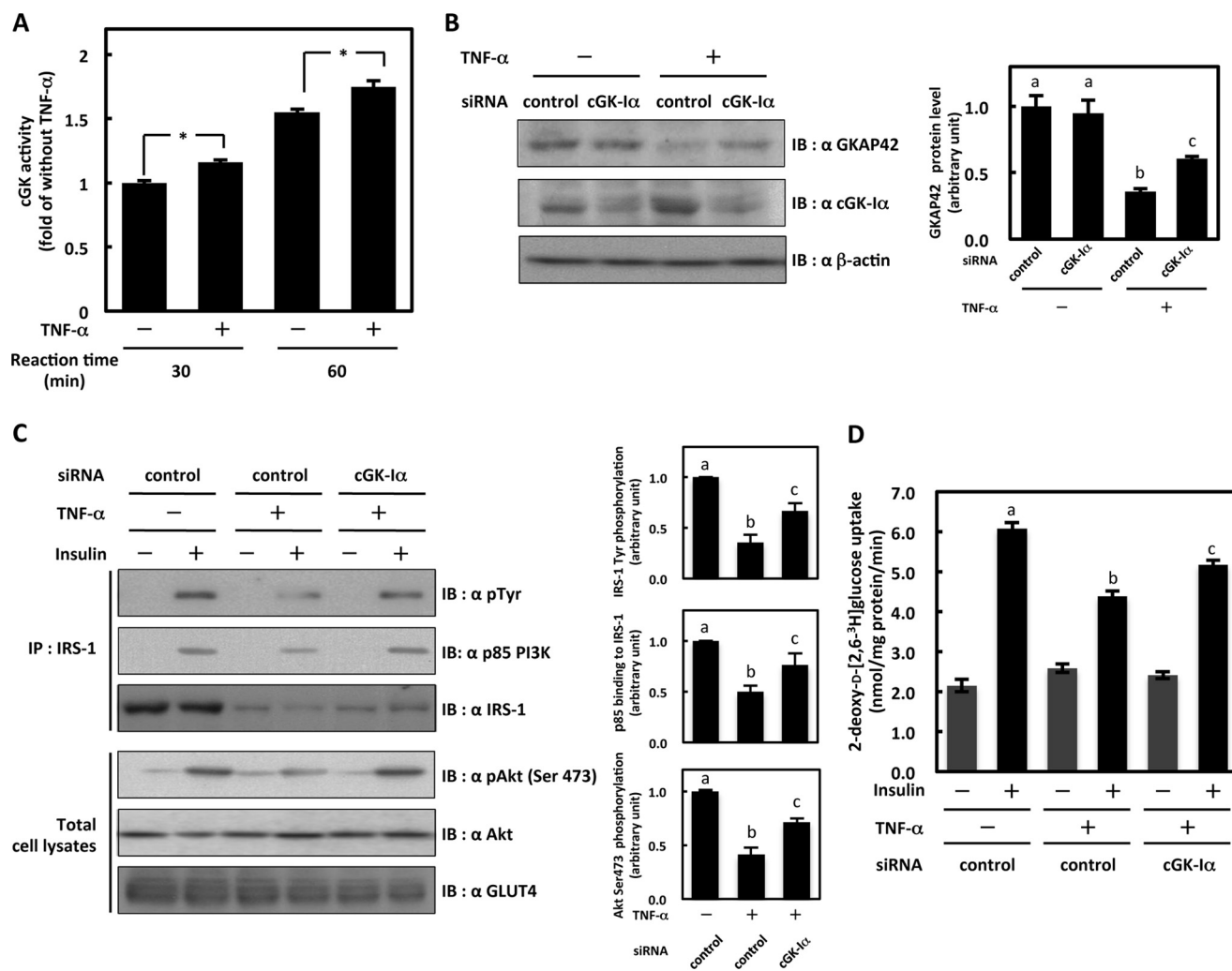


FIGURE 5. Effects of cGK-I α knockdown on TNF- α -induced repression of GKAP42 protein level. *A*, after being serum-starved for 3 h, 3T3-L1 adipocytes were treated with 2 nM TNF- α for 24 h. cGK activity was measured in the presence of 5 mM cGMP using the synthetic peptide BPDEide. *, the difference between without TNF- α and with TNF- α is significant, with $p < 0.05$. *B–D*, 3T3-L1 adipocytes were electroporated with cGK-I α or control siRNA and then treated with 2 nM TNF- α . *B*, cell lysates were analyzed by immunoblotting (IB) with the indicated antibodies. Bands were quantified from each blot by ImageJ software. The protein amount of GKAP42 was calculated, and values were shown in the graphs. The results are presented as the means \pm S.E. (error bars) of three different experiments. *C*, cell lysates were subjected to immunoprecipitation (IP) with anti-IRS-1 antibody and immunoblotted with the indicated antibodies. Bands were quantified from each blot by ImageJ software. The protein amounts of phosphorylated IRS-1 and p85 associated with IRS-1 were calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. of three different experiments. Shown are representative immunoblots independently performed at least three times. *D*, cells were assayed for glucose uptake. The results are presented as the means \pm S.E. of five wells. There are significant differences between values with different letters ($p < 0.05$).

down partially rescued TNF- α -induced impairment of insulin-dependent glucose uptake.

cGK-I α Knockdown Completely Rescued the Defect of Insulin Signals and Insulin-induced Glucose Uptake in GKAP42 Knockdown Cells—Finally, we examined the effect of cGK-I α knockdown on insulin signals and insulin-induced glucose uptake in

GKAP42 knockdown cells. As shown in Fig. 2, insulin-induced IRS-1 tyrosine phosphorylation or Akt phosphorylation was impaired in GKAP42 knockdown cells. This repression was completely rescued by cGK-I α knockdown (Fig. 6A). Furthermore, we assessed insulin-induced glucose uptake. As shown in Fig. 6B, cGK-I α knockdown completely rescued impairment of

FIGURE 4. Effects of TNF- α treatment on GKAP42 protein level. *A*, after being serum-starved for 3 h, 3T3-L1 adipocytes were treated with 2 nM TNF- α for the indicated periods. Cell lysates were analyzed by immunoblotting (IB) with the indicated antibodies. Bands were quantified from each blot by ImageJ software. Protein amount of GKAP42 was calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. (error bars) of triplicate determinations. *, the difference versus 0 h of TNF- α treatment is significant, with $p < 0.05$. *B*, total RNA was extracted from 3T3-L1 adipocytes. Using this total RNA, first strand cDNA was synthesized, and PCR was carried out using these first strand cDNA as templates. Shown are representative data of independent triplicate determinations. *C*, 3T3-L1 adipocytes were serum-starved for 3 h and treated with MG-132, and cells were then treated with 2 nM TNF- α for 24 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies. Bands were quantified from each blot by ImageJ software. The protein amount of GKAP42 was calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. of three different experiments. There are significant differences between values with different letters ($p < 0.05$). *D*, 3T3-L1 adipocytes were infected with recombinant lentivirus expressing GFP or GFP-GKAP42. Then cells were pretreated with or without 2 nM TNF- α for 24 h and treated with or without 100 nM insulin for 5 min. Cell lysates were immunoprecipitated with anti-IRS-1 antibody, and immunoprecipitates (IP) or total cell lysates (TCL) were immunoblotted with the indicated antibodies. Bands were quantified from each blot by ImageJ software. Protein amounts of phosphorylated IRS-1 and phosphorylated Akt were calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. of three different experiments. *, the difference between GFP and GFP-GKAP42 expression with TNF- α treatment is significant, with $p < 0.05$.

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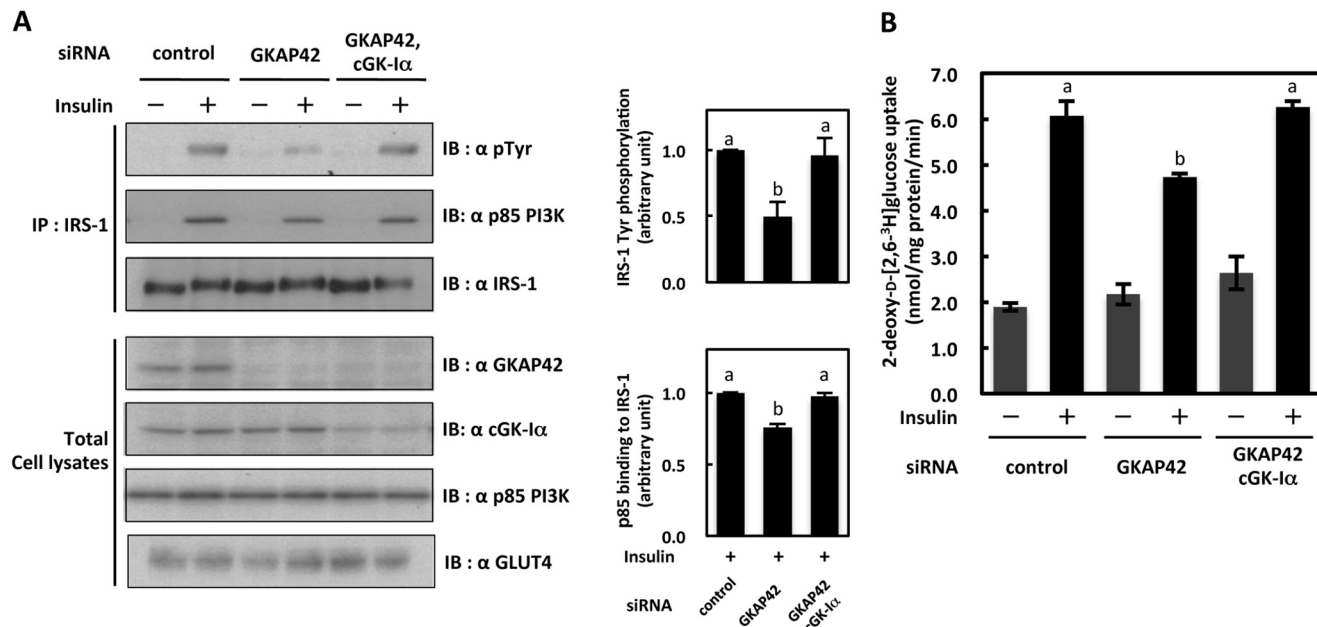


FIGURE 6. Effects of GKAP42 and cGK-I α double knockdown on insulin signaling and glucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes electroporated with control, GKAP42, or GKAP42 and cGK-I α siRNA were serum-starved, followed by stimulation of 100 nM insulin. *A*, cell lysates were immunoprecipitated with anti-IRS-1 antibody, and immunoprecipitates (*IP*) or total cell lysates (*TCL*) were immunoblotted (*IB*) with the indicated antibodies. Bands were quantified from each blot by ImageJ software. Protein amounts of phosphorylated IRS-1 and p85 associated with IRS-1 were calculated, and values are shown in the graphs. The results are presented as the means \pm S.E. (error bars) of triplicate determinations. *B*, cells were assayed for glucose uptake. The results are presented as the means \pm S.E. of five wells. There are significant differences between values with different letters ($p < 0.05$).

insulin-dependent glucose uptake in GKAP42 knockdown cells.

DISCUSSION

Recently, we have shown that IRSs form high-molecular weight complexes through association with various proteins in a phosphotyrosine-independent manner and that the IRS-associated proteins modulate tyrosine phosphorylation of IRSs by IR/IGF-I receptor tyrosine kinases (23). In this study, we demonstrated that GKAP42, a novel binding partner of IRS-1, is required to maintain insulin-dependent IRS-1 tyrosine phosphorylation and plays important roles in TNF- α -induced impairment of insulin-dependent IRS-1 tyrosine phosphorylation.

Knockdown of GKAP42 decreased insulin-dependent IRS-1 tyrosine phosphorylation without affecting insulin receptor phosphorylation in 3T3-L1 adipocytes (Fig. 2*A*). *In vitro* phosphorylation analysis revealed that IRS-1 from GKAP42 knockdown cells had impaired availability to insulin receptor (Fig. 2*E*). These data indicated that GKAP42 plays important roles to regulate IRS-1 availability to the insulin receptor. These results, taken together with the data showing that interfering with the interaction between IRS-1 and GKAP42 by overexpressing GKAP42-N suppressed insulin-dependent GLUT4 translocation to plasma membrane in 3T3-L1 adipocytes, suggest that association of IRS-1 with GKAP42 is required to maintain insulin signaling through retention of IRS-1 tyrosine phosphorylation induced by insulin.

How is insulin-dependent IRS-1 phosphorylation affected by association of GKAP42 with IRS-1? *In vitro* phosphorylation assays showed that IRS-1 from GKAP42 knockdown cells had impaired availability to IR, suggesting that GKAP42-associated IRS-1 increased availability to IR tyrosine kinase. However,

GST-GKAP42 addition in *in vitro* phosphorylation assays could not recover decreased availability to IR in GKAP42 knockdown cells. This suggested that IRS-1 from GKAP42 knockdown cells has already been post-translationally modified. Multiple studies have suggested that post-translational modification of IRS-1, such as Ser/Thr phosphorylation by various kinases, plays an important role in decreasing insulin-dependent tyrosine phosphorylation (40, 41). Particularly, specific serine residues of IRS-1, such as Ser-307, Ser-408, Ser-612, and Ser-632, were reported to be phosphorylation sites responsible for inhibition of IRS-1 tyrosine phosphorylation by IR tyrosine kinase (25, 42–44). We identified a cGK-I α candidate phosphorylation motif RKX(S/T) surrounding residue 520, RKRT⁵²⁰, in IRS-1. cGK-I α knockdown completely rescued impairment of IRS-1 availability to IR in GKAP42 knockdown cells. It is possible that GKAP42 knockdown allows cGK-I α kinase access to IRS-1 and that it then phosphorylates IRS-1, resulting in impairment of IRS-1 tyrosine phosphorylation. GKAP42 possibly protects IRS-1 from Ser/Thr phosphorylation by cGK-I α and other kinases.

How is GKAP42 protein level reduced in response to TNF- α treatment? There are several reports showing that TNF- α increases the expression of inducible nitric-oxide synthase (iNOS) and NO production (45, 46). Enhanced iNOS expression by TNF- α can increase the production of cGMP, resulting in activation of cGK-I α (47). We showed that cGK-I α activity was slightly enhanced by TNF- α treatment (Fig. 5*A*). In addition, knockdown of cGK-I α partially rescued TNF- α -induced repression of GKAP42 protein levels (Fig. 5*B*), suggesting that cGK-I α is required for TNF- α -induced GKAP42 protein degradation at least in a part. It is

well known that IRS-1 is also degraded in response to TNF- α treatment by ubiquitin ligases, such as SOCS1 or SOCS3 (48, 49). Our data showed that cGK-I α knockdown did not affect IRS-1 protein level (Fig. 5C). These data strongly suggested that the degradation mechanisms of IRS-1 and GKAP42 could be quite different. It is possible that active cGK-I α induces the activation of a different type of ubiquitin ligase from SOCS1/3. Analysis of the mechanism of GKAP42 degradation by cGK-I α is in progress.

Fig. 5 showed that knockdown of cGK-I α only partially recovered TNF- α -induced insulin resistance. Lentivirus infection of GFP-GKAP42 could also only partially recover TNF- α -induced impairment of insulin-dependent IRS-1 tyrosine phosphorylation (Fig. 4D). TNF- α is shown to activate various kinases, including Jun kinases, mitogen-activated protein kinases, and protein kinase Cs, to phosphorylate IRS-1, resulting in repression of its tyrosine phosphorylation in response to insulin and insulin bioactivities (25, 50, 51). TNF- α also increases lipids, such as diacylglycerols, ceramides, and ganglioside GM3, resulting in inhibition of insulin-dependent IR activation and its proximal signaling (52–55). In addition, knockdown of cGK-I α could only restore GKAP42 protein level and not IRS-1 protein level, indicating that a degradation system other than cGK-I α was induced by TNF- α treatment. Such TNF- α multiple effects might be a reason why knockdown of cGK-I α or GKAP42 overexpression only partially recovered TNF- α -induced insulin resistance.

In this study, we have shown that long term treatment with TNF- α inhibited insulin-induced GLUT4 translocation. As shown in Fig. 2C, knockdown of GKAP42 impaired GLUT4 translocation induced by insulin. In addition, long term treatment with TNF- α repressed GKAP42 protein level (Fig. 4A), indicating that TNF- α -induced GKAP42 protein repression caused insulin resistance at least in part. We also tested whether GKAP42 overexpression restored TNF- α -induced impairment of GLUT4 translocation. Interestingly, overexpression of GKAP42 itself markedly enhanced GLUT4 translocation without insulin treatment (data not shown), suggesting that GKAP42 has some other function to directly regulate GLUT4 translocation in addition to sustaining IRS-1 tyrosine phosphorylation. On the contrary, cGK-I α knockdown could partially restore TNF- α -induced impairment of glucose uptake (Fig. 5D). These partial rescue effects could be observed because of adequate recovery of GKAP42 protein level.

In summary, we identified GKAP42 as a novel IRS-1-interacting protein and showed that GKAP42 is required for maintenance of availability of IRS-1 to IR tyrosine kinase, resulting in maintenance of insulin-dependent glucose uptake in 3T3-L1 adipocytes. In addition, we showed that the GKAP42 protein level was suppressed by long term TNF- α treatment through cGK-I α . Taken together, we concluded that TNF- α -induced GKAP42 protein repression caused insulin resistance at least in part. The present study demonstrated that cGK-I α and GKAP42 are the novel key molecules that cause insulin resistance induced by TNF- α . Our results reveal unrecognized cross-talk between cGK-I α -GKAP42 and insulin pathways that enable modulation of glucose uptake.

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