FIGURES

Figure 1

Identification and expression profile of GPC3

A. Interaction between GLUT4 and GPC3 was confirmed by the yeast two-hybrid system. Full-length cDNAs of GLUT4 and GPC3 were used as bait and prey, respectively.

B. Lysates of 3T3-L1 adipocytes and HepG2 cells were prepared, and separated by SDS-PAGE. The membrane was then blotted with anti-GPC3 antibody. HepG2 lysate was used as positive control. Arrow head indicates the non-glycosylated full-length GPC3 core protein. Arrow indicates a cleavage product containing the N terminus of GPC3.

Figure 2

Intracellular localization of GPC3

A. Primary hepatocytes and HepG2 cells were plated on cover slips. These cells were fixed with formaldehyde and then stained using anti-GPC3 antibody followed by FITC-labeled secondary antibody. Rhodamine conjugated wheat germ agglutinin (WGA) was used as counter staining of cell membrane and Golgi system.

B. 3T3-L1 fibroblasts and adipocytes were serum-starved for 3-4 h and treated with or without 100 nM of insulin for 20 min. 3T3-L1 adipocytes were fixed with formaldehyde and stained using anti-GPC3 and anti-GLUT4 antibodies followed by appropriate FITC or Cy3 labeled secondary antibodies. These cells were observed by laser confocal microscopy.

C. 3T3-L1 adipocytes cultured on glass coverslips were treated with or without 100 nM of insulin for 15 min. At the end of each experiment, cells were rapidly washed in PBS followed by a 40 sec treatment in PBS containing 0.5 mg/ml poly-L-lysine (Sigma). The cells were subsequently swollen using hypotonic buffer and sonicated to generate a lawn of plasma membrane fragments. The membranes were immunostained with anti-GPC3 and anti-GLUT4 antibody. Stained cells were observed using the confocal microscopy system as described above. Fluorescence intensity was quantified using Adobe Photoshop software (Adobe Systems Inc.).

Figure 3

GPC3 binds to GLUT4

A. 3T3-L1 adipocytes were stimulated with 100 nM of insulin for 15 min. The lysates were incubated with or without anti-GLUT4 rabbit antibody followed by precipitation with protein A sepharose beads. Precipitates were separated by SDS-PAGE and immunoblotted with anti-GPC3 mouse monoclonal and anti-GLUT4 goat antibodies. Protein signals were visualized using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence substrate kit (GE Healthcare Biosciences). Arrow head indicates GPC3 core protein.

B. 3T3-L1 adipocytes were stimulated with 100 nM of insulin for 15 min. Then, lysates of these adipocytes were immunoprecipitated using anti-GPC3 antibody or beads only. Precipitates were immunoblotted with anti-GLUT4 and anti-GPC3 antibodies for determination of GPC3 binding proteins.

C. GST-GPC3 and GST alone were bacterially expressed and purified by glutathione sepharose beads. 4×Myc-tagged GLUT4-eGFP protein was expressed in 293 cells and purified using anti-Myc antibodies. Purified GST-GPC3 or GST proteins and GLUT4 protein were mixed and pulled down with gluthatione sepharose beads. The precipitates were separated by SDS-PAGE and analyzed by Western blotting using anti-GLUT4 and anti-GST antibodies. Arrow head indicated full-length GST-GPC3.

Figure 4

Effect of GPC3 expression on glucose uptake

3T3-L1 adipocytes were infected with recombinant adenovirus vectors encoding FLAG-GPC3 or eGFP as a control at a m.o.i. of 50. The cells were serum starved for 3h and treated with or without 100 nM of insulin for 15min. Glucose uptake was determined by 2-deoxy-D-[2, 6 ^{3}H] glucose incorporation. Nonspecific glucose uptake was measured in the presence of 20 μM cytochalasin B and subtracted from each determination to obtain the specific uptake. Statistical analyses were performed using student's t-test. Experiments were repeated 6 times. Values are expressed as means ± S.E., as indicated.

Articles in PresS. Am J Physiol Endocrinol Metab (February 12, 2008). doi:10.1152/ajpendo.00253.2007

Title

Hepatic overexpression of a dominant negative form of Raptor enhances Akt phosphorylation and restores insulin resistance in K/KAy mice.

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Running Head : mTOR signaling and insulin resistance

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ABSTRACT

Several serine/threonine kinases reportedly phosphorylate serine residues of IRS-1, and thereby induce insulin resistance. In this study, to investigate the effect of mTOR/Raptor on insulin signaling and metabolism in K/KAy mice with genetic obesity-associated insulin resistance, a dominant-negative Raptor, C terminally deleted Raptor (Raptor-ΔCT), was overexpressed in the liver via injection of its adenovirus into the circulation. Hepatic Raptor-ΔCT expression levels were 1.5-4 fold that of endogenously expressed Raptor. Glucose tolerance in Raptor-ΔCT overexpressing mice improved significantly as compared with that of LacZ overexpressing mice. Insulin-induced activation of p70S6K was significantly suppressed in the livers of Raptor-ΔCT overexpressing mice. In addition, insulin-induced IRS-1, Ser307 and Ser636/639 phosphorylations were significantly suppressed in the Raptor-ΔCT overexpressing liver, whereas tyrosine phosphorylation of IRS-1 was increased. PI 3-kinase activation in response to insulin stimulation was increased approximately two-fold, and Akt phosphorylation was clearly enhanced under both basal and insulin-stimulated conditions in the livers of Raptor-ΔCT mice. Thus, our data indicate that suppression of the mTOR/p70 S6 kinase pathway leads to improved

glucose tolerance in K/KAy mice. These observations may contribute to the development of novel anti-diabetic agents.

Keywords: Insulin resistance; Raptor; IRS-1

INTRODUCTION

THE MAMMALIAN TARGET OF RAPAMYCIN (mTOR) is a Ser/Thr kinase that belongs to the phosphatidylinositol (PI) kinase-related protein kinase family, which regulates cell growth and metabolism(7, 21). The mTOR signaling network consists of two major branches, each mediated by a specific mTOR complex (mTORC) (27). The rapamycin-sensitive mTORC1 consists of mTOR, raptor and mLST8 (also known as GβL) and regulates cell growth through effectors such as S6K1 and 4E-BP1(4, 10). The rapamycin-insensitive mTORC2 contains mTOR, rictor and mLST8, and regulates cellular proliferation through Akt (22), cytoskeleton organization through protein kinase Cα(20) and the small GTPases Rho and Rac (9).

Raptor is a large protein (150 kDa) containing a highly conserved, amino-terminal domain followed by several HEAT repeats and seven carboxy-terminal WD40 repeats (4). A number of groups have proposed that Raptor acts as an adaptor to recruit substrates, p70S6K and 4E-BP1, to mTOR (2, 12, 23). Recent studies have shown the existence of a negative feedback loop from the nutrient-sensitive TSC-mTOR-S6K1 pathway to the upstream, insulin-responsive IRS-PI3K-PDK1-Akt pathway (6, 24, 26). S6K1 knockout mice were shown to be hypoinsulinemic with a decrease in β-cell mass (17). Moreover,

S6K1-deficient mice are hypersensitive to insulin due to loss of the negative feedback loop from S6K1 to IRS1, and are protected from age- and diet-induced obesity (26). Meanwhile, in genetic models of obesity such as *K/KAy* and *ob/ob* mice, insulin signaling is suppressed with increased phosphorylation of Ser307 and Ser636/639 in IRS-1 (26). In such mice, the activities of JNK and mTOR/S6K1 which can phosphorylate serine residue(s) of IRS-1 are reportedly elevated (8, 26).

In this study, to elucidate the contribution of mTORC1, we overexpressed a dominant-negative Raptor, C terminally deleted Raptor (Raptor-ΔCT), using adenovirus gene transfer into the livers of K/KAy mice. Since Raptor-ΔCT binds S6K but not mTOR, Raptor-ΔCT overexpression inhibits mTOR/S6K signaling (12, 25). Under these conditions, we were able to evaluate the contribution of the mTORC1 pathway to glucose tolerance as well as signal transduction. Herein, we present data suggesting inhibition of mTORC1 to significantly enhance insulin signaling, particularly Akt activation, and thereby to ultimately improve glucose tolerance in K/KAy mice.

MATERIALS AND METHODS

Materials. Affinity-purified antibodies against insulin receptor substrate (IRS)-1, IRS-2, phosphorylated tyrosine (4G10), S6 kinase (S6K) and Akt/protein kinase B were prepared as previously described (11). Anti-flag tag antibody was purchased from Sigma-Aldrich Corporation (St. Louis, MO). The antibodies against Raptor, phospho-Thr389 of S6K, phospho-Ser307 and phospho-Ser636/639 of IRS-1, phospho-Thr 37/46 and phospho-Thr 70 of 4E-BP1 and phospho-Ser473 and Thr308 of Akt, were purchased from Cell Signaling Technology.

Adenoviruses and Animals. Raptor-ΔCT (amino acids 1-905), a dominant-negative Raptor, was constructed by deleting the C terminus of Raptor. Polymerase chain reaction (PCR) was performed to amplify human Raptor cDNA using a cDNA library obtained from HEK293 as a template and oligonucleotides based on its reported sequence (4) as primers, yielding Raptor cDNA encompassing the entire coding region. Raptor-ΔCT (Δ;906-1335) was generated by standard PCR-based strategies. The construct was designed to contain a Myc tag and a Flag tag at the N terminus. Recombinant adenovirus expressing

beta-galactosidase (i.e., the *E.coli* beta-galactosidase gene [LacZ]) and C terminally deleted Raptor (Raptor-ΔCT) were generated, purified and concentrated using cesium chloride ultracentrifugation as reported previously(19). Adenovirus encoding LacZ served as a control. Male K/KAy mice, 9 weeks of age, were obtained from Nippon Bio-Supp.Center (Tokyo, Japan). They were injected, via the tail vein, with adenovirus at a dose of 2.5 x 10^7 plaque-forming units/g body wt. Four days after adenovirus injection, the following experiments were performed.

Serum Glucose and Lipid Profiles. Blood glucose was measured with a portable blood glucose monitor, Glutest-Ace R (Sanwa Kagaku Kenkyusho, Nagoya Japan). The plasma insulin level was determined with an enzymatic immunoassay kit (SHIBAYAGI). Serum triglyceride, cholesterol and free fatty acids were assayed with the Triglyceride E test, Cholesterol E test and NEFA C test (all from Wako Chemicals, Japan), respectively.

Intraperitoneal glucose tolerance tests. Mice were fasted for 14h followed by blood sampling and intraperitoneal injection of glucose (2g per kg body weight). Whole venous blood was obtained from the tail vein at the indicated time points after the glucose load.

Blood glucose was measured with a portable blood glucose monitor as described above. We calculated the AUCs (areas under the curve) for glucose for each group and then compared the values obtained using the t-test.

In vivo Insulin Stimulation. In vivo insulin stimulation was performed, as previously described(15). Mice were anesthetized with pentobarbital sodium and 0.2 ml of blood were collected from the heart and the same amount of normal saline (0.9% NaCl), with or without insulin (1 unit/kg body wt), was then injected into the heart. The livers were removed 5 min or 20 min later, and immediately homogenized with a Polytron homogenizer in 10 volumes of solubilization buffer ([A:1% Triton X-100, 20 mM Tris (pH 7.5), 150mM NaCl, 10% glycerol, 1 mM EDTA, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 20 mM beta glycerophosphate, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02 mg/ml aprotinin], [B: 137 mM NaCl, 20mM Tris (pH 7.5), 1mM $MgCl_2$ 1mM $CaCl_2$, 10 % glycerol, 1 % NP-40, 0.05 mM sodium vanadate, 1 mM PMSF], [C: 20 mM Tris (pH 7.5), 20 mM NaCl, 1 mM EDTA, 5 mM EGTA, 1 % CHAPS, 20 mM beta glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 1 mM DTT]). The extract was centrifuged at 20,000 *g* for 15 min at 4 degrees C,

and the supernatants were used as samples for immunoprecipitation, immunoblotting (buffer A), or kinase assay of PI 3-kinase (buffer B) and S6 kinase (buffer C).

Immunoprecipitation and immunoblotting. Supernatants containing equal amounts of protein (10 mg) were incubated with anti-IRS-1 and anti-S6K antibodies (3 μg/ml each) and then incubated with 45 μl of protein A- and G-Sepharose. The samples were washed and then boiled in Laemmli sample buffer containing 100 mM dithiothreitol. SDS-PAGE and immunoblotting were carried out using enhanced chemiluminescenece (ECL Detection Kit, Amersham), and representative blots were obtained by exposing the films. The bands were quantitatively analyzed using Molecular Imager FX (Biorad) without exposure of the films.

Measurement of PI 3-Kinase. For PI 3-kinase assay, the supernatants containing equal amounts of protein were immunoprecipitated for 2 h at 4 degrees C with anti-IRS-1 or 4G10 antibody and protein A- or G-Sepharose. PI 3-kinase activities in the immunoprecipitates were assayed as previously described (14).

P70 S6 Kinase Assay. For p70 S6K assay, the supernatants containing equal amounts of protein (10 mg) were immunoprecipitated for 2 h at 4 degrees C with anti-S6K antibody and protein A-Sepharose. Kinase activity was analyzed using an S6 kinase assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) and the assay was carried out according to the manufacturer's instructions. In brief, the reaction mixture, containing 50 μM substrate peptide (KKRNRTLTK), inhibitor mixture (20 μM protein kinase C inhibitor peptide, 2 μM protein kinase A inhibitor peptide, and 20 μM Compound R24571, an inhibitor of brain calmodulin-dependent phosphodiesterase in Assay Dilution Buffer I (20 mM MOPS, pH 7.2, 25 mM beta glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol)), p70 S6 kinase (immunoprecipitates) and diluted [γ-^{32}P]ATP mixture, were incubated for 10 min at 30 degrees C. Then, 25 μl of the reaction mixture were spotted onto p81 phosphocellulose squares. Intensities of the resultant bands were determined using BAS2000 (Fuji film, Japan).

Statistical Analysis. Results are expressed as means ± S.E.M. Comparisons were made using one-way ANOVA followed by the Tukey test and the unpaired Student's *t* test. Values of $p < 0.05$ were considered statistically significant

RESULTS

Overexpression of Raptor-ΔCT markedly suppressed insulin-induced activation of p70S6K in the livers of K/KAy mice. To examine levels of endogenously expressed Raptor and Raptor-ΔCT in the liver, we carried out immunoblotting with anti-flag tag or anti-Raptor antibody. Raptor-ΔCT expressions were identified by immunoblotting with anti-flag tag antibody in Raptor-ΔCT overexpressing mice but not in controls (Fig. 1). Immunoblotting with the anti-Raptor antibody detected both endogenous Raptor and overexpressed Raptor-ΔCT, and the levels of Raptor-ΔCT were approximately 1.5-4 fold that of endogenously expressed Raptor (Fig.1 A). In addition, overexpression of Raptor-ΔCT was limited to the liver, i.e. none was detected by immunoblotting of other tissues (Fig.1 B). (Faint bands in the lung, heart and kidney were nonspecific.) Next, we investigated the associations of wild-type Raptor and Raptor-ΔCT with mTOR or IRS-1. As shown in Fig 2, IRS-1 and mTOR were detected in the Flag-tagged Raptor immunoprecipitates. In contrast, it was revealed that Raptor-ΔCT had lost the ability to associate with IRS-1, and the association of Raptor-ΔCT with mTOR was also much weaker than that of wild-type Raptor. Thus, it was suggested that Raptor-ΔCT functions

as a dominant negative construct.

Subsequently, the effect of Raptor-ΔCT overexpression on p70S6K activity was investigated in the liver. S6 kinase assay and immunoblotting of liver lysates with S6K and phospho-S6K(Thr389) antibodies revealed insulin-induced activation of p70S6K to be markedly suppressed in the livers of Raptor-ΔCT overexpressing mice (Fig. 3 A, B). However, surprisingly, 4E-BP1 phosphorylations of both Thr37 and 46 were significantly increased by Raptor-ΔCT overexpression under both basal and insulin-stimulated conditions, and that of Thr70 was also increased in the insulin-stimulated state (Fig. 3 C).

Weights and Metabolic Profiles of control(LacZ) and Raptor-ΔCT overexpressing mice. The body weights, major organ's weights, blood glucose levels, and lipid concentrations of Raptor-ΔCT mice did not differ from those of control mice, either before or 4 days after adenovirus injection. Fasting serum insulin levels of Raptor-ΔCT mice were lower but not significantly (Table 1).

Hepatic Raptor-ΔCT overexpressing mice showed a profound increase in glucose tolerance. To investigate the effect of hepatic Raptor-ΔCT overexpression on glucose

tolerance, we performed intraperitoneal glucose tolerance tests (IPGTT) (Fig. 4A). Blood glucose levels of Raptor-ΔCT mice were significantly lower than those of control mice (Fig. 4B).

Hepatic Raptor-ΔCT overexpression enhanced insulin signaling, associated with decreased IRS-1 Ser307 and Ser636/639 phosphorylation in K/KAy mice. As shown in Fig. 5A, there were no differences in hepatic expression levels of IRS-1 protein between Raptor-ΔCT and control mice. Insulin-induced IRS-1 tyrosine phosphorylation was significantly increased in hepatic Raptor-ΔCT overexpressing mice (Fig, 5B), whereas insulin-induced IRS-1 Ser307 and Ser636/639 phosphorylation were markedly depressed in Raptor-ΔCT mice (Fig. 5C and D). Moreover, we performed PI3K assays of the liver in order to investigate PI3K activity. Fig.6 presents insulin-induced tyrosine phosphorylation-associated PI3K activity and IRS-1-associated PI3K activity, both of which were increased approximately two-fold as compared to those of LacZ mice. Insulin-induced Akt Ser473 and Thr308 phosphorylations were markedly increased in Raptor-ΔCT mice (Fig.7 B and C), as shown by immunoblotting of liver lysates with Akt and phospho-Akt Ser473 and Thr308 antibodies, but there was no difference between these

mice in Akt protein expression (Fig.7A). In addition, basal Akt Ser473 and Thr308 phosphorylations were also markedly increased in Raptor-ΔCT mice (Fig.7 B and C).

DISCUSSION

Insulin resistance is induced by many factors including obesity, high-fat diet, insufficient exercise, hypertension(13) and various hormones. Among these factors, obesity induced by excessive caloric intake is considered to be the most common and important factor leading to the occurrence of diabetes mellitus. In obese animals, PI 3-kinase activation via the association with IRS-proteins is impaired, and increased serine phosphorylation in IRS-1 is reportedly involved in this impaired insulin-induced PI 3-kinase activation. Phosphorylation of serine residues of IRS-1 is also reportedly involved in IRS-1 degradation (1, 18, 25). To date, several serine/threonine kinases have been reported to phosphorylate serine residues of IRS-1.

IRS-1 phosphorylation mechanisms under insulin-resistant conditions can essentially be divided into two major categories; one involves adipocyte-derived factors such as TNF-α, resistin and free fatty acids, which activate JNK and/or ERK and thereby increase the serine phosphorylation of IRS-1. The other operates in response to intracellular nutrient conditions. The nutritional status of the cell directly regulates the AMPK/mTOR pathway, independently of proteins secreted by adipocytes, and mTOR and S6 kinase