Hepatic overexpression of a dominant negative form of raptor enhances Akt phosphorylation and restores insulin sensitivity in K/KAy mice

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1Department of Internal Medicine, Graduate School of Medicine, University of Tokyo; 2Institute for Adult Disease, Asahi Life Foundation; 3Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, Tokyo; and 4Department of Medical Science, Graduate School of Medicine, University of Hiroshima, Hiroshima, Japan

Submitted 28 August 2007; accepted in final form 11 February 2008

Koketsu Y, Sakoda H, Fujishiro M, Kushiyama A, Fukushima Y, Ono H, Anai M, Kikuchi T, Fukuda T, Kamata H, Horike N, Uchijima Y, Kurihara H, Asano T. Hepatic overexpression of a dominant negative form of raptor enhances Akt phosphorylation and restores insulin sensitivity in K/KAy mice. Am J Physiol Endocrinol Metab 294: E719–E725, 2008. First published February 12, 2008; doi:10.1152/ajpendo.00253.2007.—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, COOH-terminally deleted raptor (raptor-ΔCT), was overexpressed in the liver via injection of its adeno virus into the circulation. Hepatic raptor-ΔCT expression levels were 1.5- to 4-fold that of endogenously expressed raptor. Glucose tolerance in raptor-ΔCT-overexpressing mice improved significantly 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 twofold, 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/p70S6k pathway leads to improved glucose tolerance in K/KAy mice. These observations may contribute to the development of novel antidiabetic agents.

THE MAMMALIAN TARGET OF RAPA MYCIN (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 of which are mediated by a specific mTOR complex (mTORC) (27). The rapamycin-sensitive mTORC1 consists of mTOR, raptor, and mLST8 (also known as GBL) and regulates cell growth through effectors such as ribosomal protein S6 kinase (S6K)1 and eukaryotic translation initiation factor 4E-binding protein-1 (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 (2, 12, 23) have proposed that raptor acts as an adaptor to recruit substrates p70S6k (p70S6k) and 4E-BP1 to mTOR. Recent studies (6, 24, 26) have shown the existence of a negative feedback loop from the nutrient-sensitive TSC-mTOR-S6K1 pathway to the upstream, insulin-responsive insulin receptor substrate (IRS)-PI 3-kinase-PDK1-Akt pathway. 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 IRS-1 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 the present study, to elucidate the contribution of mTORC1, we overexpressed a dominant negative raptor, COOH-terminally deleted raptor (raptor-ΔCT), using adeno virus 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 IRS-1, IRS-2, phosphorylated tyrosine (4G10), S6K, and Akt/protein kinase B were prepared as previously described (11). Anti-Flag tag antibody was purchased as previously described (11). Anti-Flag tag antibody was purchased from Cell Signaling Technology.

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We calculated the areas under the curve for glucose for each group at the indicated time points after the glucose load. Blood glucose was measured with an enzymatic immunoassay kit (Shibayagi). Serum triglyceride, cholesterol, and free fatty acids were assayed as described previously (14). The plasma insulin level was determined with a portable blood glucose monitor, Glutest-Ace R (Sanwa Kagaku Kenkyusho, Nagoya, Japan). The expression levels of endogenous raptor and overexpressed raptor-dCT in the livers of K/KAy mice and controls, B: immunoblotting (IB) of overexpressed raptor-dCT in various tissues with anti-Flag tag antibody. Each tissue (30 μg), from raptor-dCT-overexpressing mice, was electrophoresed and immunoblotted with anti-Flag tag antibody. Blot 1: brain; blot 2: lung; blot 3: heart; blot 4: spleen; blot 5: pancreas; blot 6: kidney; blot 7: fat; blot 8: muscle; blot 9: testis; blot 10: liver.

Adenoviruses and animals. Raptor-dCT (amino acids 1–905), a dominant negative raptor, was constructed by deleting the COOH terminus of raptor. PCR was performed to obtain human raptor cDNA using a cDNA library obtained from HEK293 as a template and oligonucleotides on the basis of its reported sequence (4) as primers, yielding raptor cDNA encompassing the entire coding region. Raptor-dCT (∆, 906–1335) was generated by standard PCR-based strategies. The construct was designed to contain a Myc tag and a Flag tag at the NH2 terminus. Recombinant adenovirus expressing β-galactosidase [i.e., the E. coli β-galactosidase gene (LacZ)] and COOH-terminally deleted raptor (raptor-dCT) 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 wk 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 × 106 plaque-forming units/g body wt. Four days after the 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), respectively.

Intraperitoneal glucose tolerance tests. Mice were fasted for 14 h, followed by blood sampling and intraperitoneal injection of glucose (2 g/kg body wt). 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 areas under the curve for glucose for each group and then compared the values obtained using Student’s t-test.

In vivo insulin stimulation. In vivo insulin stimulation was performed as described previously (15). Mice were anesthetized with pentobarbital sodium, 0.2 ml of blood was 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 or 20 min later and immediately homogenized with a Polytron homogenizer in 10 volumes of solubilization buffer [buffer A: 1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 20 mM β-glycerophosphate, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02 mg/ml apro- tinin; buffer B: 137 mM NaCl, 20 mM Tris (pH 7.5), 1 mM MgCl2 1 mM CaCl2, 10% glycerol, 1% NP-40, 0.05 mM sodium vanadate, 1 mM PMSF; buffer C: 20 mM Tris (pH 7.5), 20 mM NaCl, 1 mM EDTA, 5 mM EGTA, 1% CHAPS, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 1 mM DTT]. The extract was centrifuged at 20,000 g for 15 min at 4°C, and the supernatants were used as samples for immunoprecipitation, immunoblotting (buffer A), or kinase assay of PI 3-kinase (buffer B) and S6K (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 DTT. SDS-PAGE and immunoblotting were carried out using enhanced chemiluminescence (ECL detection kit; Amersham), and representative blots were obtained by exposing the films. The bands were quantitatively analyzed using Molecular Imager FX (Bio-Rad) 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°C with anti-IRS-1 or 4G10 antibody and protein A- or G-Sepharose. PI 3-kinase activities in the immunoprecipitates were assayed as described previously (14).

Fig. 2. COOH terminus of raptor is essential for binding with mammalian target of rapamycin (mTOR) and insulin receptor substrate-1 (IRS-1). For wild-type raptor, raptor-dCT, and control (LacZ) gene transfer into HepG2 cells, the cells were incubated for 1 h in DMEM containing recombinant adenovirus. Two days later the cells were collected, and cell lysates were immunoprecipitated with Flag-tag antibody. Cell lysates and anti-Flag tag immunoprecipitates were immunoblotted with each (IRS-1, mTOR, and Flag) antibody as a probe. Representative results are shown. LacZ, n = 3; raptor: n = 3; raptor-dCT: n = 3.
**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-CT-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

<table>
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<tr>
<th>LacZ</th>
<th>Raptor-ΔCT</th>
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<tr>
<td>BW, g (day 1)</td>
<td>36.3 ± 1.04</td>
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<tr>
<td>BW, g (day 5)</td>
<td>36.9 ± 1.1</td>
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<tr>
<td>Liver/BW × 10^7 (day 5)</td>
<td>4.87 ± 0.44</td>
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<tr>
<td>Fat/BW × 10^7 (day 5)</td>
<td>4 ± 0.62</td>
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<td>Heart/BW × 10^7 (day 5)</td>
<td>0.37 ± 0.25</td>
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<tr>
<td>Kidney/BW × 10^7 (day 5)</td>
<td>0.94 ± 0.62</td>
</tr>
<tr>
<td>FBS, mg/dl (day 1)</td>
<td>122 ± 7.48</td>
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<tr>
<td>FBS, mg/dl (day 5)</td>
<td>160 ± 6.31</td>
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<tr>
<td>T-cho, mg/dl (day 5)</td>
<td>123 ± 10.8</td>
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<td>TG, mg/dl (day 5)</td>
<td>197 ± 83.2</td>
</tr>
<tr>
<td>NEFA, mEq/l (day 5)</td>
<td>0.79 ± 0.13</td>
</tr>
<tr>
<td>Insulin, mg/ml (day 5)</td>
<td>2.36 ± 1.78</td>
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Values are means ± SE. LacZ, control; raptor-ΔCT, COOH-terminally deleted raptor; BW, body weight; FBS, fasting blood sugar; T-cho, total cholesterol; TG, triglyceride; NEFA, nonesterified fatty acid. The body weights, major organ weights, blood glucose levels, and lipid concentrations of LacZ and Raptor-ΔCT mice, before and 4 days after adenovirus injection, are shown. LacZ: n = 8; raptor-ΔCT: n = 8.
raptor-ΔC_T were 1.5- to fourfold that of endogenously expressed raptor (Fig. 1A). In addition, overexpression of raptor-ΔC_T was limited to the liver; i.e., none was detected by immunoblotting of other tissues (Fig. 1B). (Faint bands in the lung, heart, and kidney were nonspecific.) Next, we investigated the associations of wild-type raptor and raptor-ΔC_T 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-ΔC_T had lost the ability to associate with IRS-1, and the association of raptor-ΔC_T with mTOR was also much weaker than that of wild-type raptor. Thus, it was suggested that raptor-ΔC_T functions as a dominant negative construct.

Subsequently, the effect of raptor-ΔC_T overexpression on p70S6k activity was investigated in the liver. S6K 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-ΔC_T-overexpressing mice (Fig. 3A and B). However, surprisingly, 4E-BP1 phosphorylations of both Thr37 and Thr46 were significantly increased by raptor-ΔC_T overexpression under both basal and insulin-stimulated conditions, and that of Thr70 was also increased in the insulin-stimulated state (Fig. 3C).

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

Hepatic raptor-ΔC_T 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. 5, C and D). Moreover, we performed PI 3-kinase assays of the liver to investigate PI 3-kinase activity. Figure 6 presents insulin-induced tyrosine phosphorylation-associated PI 3-kinase activity and IRS-1-associated PI 3-kinase activity, both of which were increased approximately twofold compared with 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

Fig. 6. Insulin-induced phosphatidylinositol (PI) 3-kinase activity in hepatic raptor-ΔCT mice. For PI 3-kinase assay, supernatants containing equal amounts of protein were immunoprecipitated for 2 h at 4°C with anti-IRS-1 or phosphorylated tyrosine antibody and protein A- or G-Sepharose. PI 3-kinase activities in the immunoprecipitates were measured. A and B: insulin-induced tyrosine phosphorylation-associated PI 3-kinase activity and IRS-1-associated PI 3-kinase activity were both increased to approximately double those of LacZ mice. LacZ: n = 8 (insulin−: n = 4; insulin+: n = 4); ΔCT: n = 8 (insulin−: n = 4; insulin+: n = 4). *P < 0.05; **P < 0.01.

Fig. 7. Insulin-induced Akt phosphorylation in hepatic raptor-ΔCT mice. Liver lysates were immunoblotted with Akt and phospho-Akt Ser473 and Thr308 antibody. A: there was no difference in Akt protein expression levels between these mice. B and C: basal Akt Ser473 and Thr308 phosphorylation as well as insulin-induced Akt Ser473 and Thr308 phosphorylation were also markedly increased in raptor-ΔCT mice. LacZ: n = 8 (insulin−: n = 4; insulin+: n = 4); ΔCT: n = 8 (insulin−: n = 4; insulin+: n = 4). *P < 0.05; **P < 0.01.

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 TNFα, 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 S6K reportedly enhance phosphorylation
of serine residues of IRS-1 (3, 5, 16). Although S6K1-deficient mice were shown to be resistant to age- and diet-induced obesity and insulin resistance (26), we investigated the acute effect of transient inhibition of raptor on the impaired insulin signaling and glucose intolerance of K/Kay mice with genetic obesity-associated insulin resistance. In the K/Kay mice, one of the obese rodent models, IRS-1 Ser307 and IRS-1 Ser653/659 phosphorylations were increased (26).

Raptor contains a highly conserved amino-terminal domain, followed by several HEAT repeats and seven carboxy-terminal WD40 repeats (4), and acts as an adaptor to recruit substrates p70S6k and 4E-BP1 to mTOR (2, 12, 23). The domains in raptor and mTOR that interact with each other have been clearly demonstrated and suggest multiple contact sites between these two proteins (4, 10), in contrast with the selective binding of p70S6k to the NH2-terminal portion of raptor (12). We were unable to detect the associations of raptor and COOH-terminally deleted raptor (raptor-ΔCT) with endogenous S6K (data not shown). However, it was demonstrated that raptor-ΔCT binds to a far smaller amount of mTOR but not to IRS-1, whereas wild-type raptor binds to both. Indeed, IRS-1 phosphorylation at Ser653/659 was markedly decreased by raptor-ΔCT overexpression. These findings suggest that raptor-ΔCT functions as a dominant negative protein for mTOR/S6K or mTOR/IRS-1 signaling.

Interestingly, we found that 4E-BP1 phosphorylations of both Thr37/46 and Thr70 in the liver were significantly increased by raptor-ΔCT overexpression. Thus, the inhibitory effect of raptor-ΔCT is specific for S6K. This result was unexpected, but it is hoped that it will provide useful information regarding how the raptor-mTOR complex recognizes individual downstream molecules. We speculate that S6K, but not 4E-BP1, preferentially associates with raptor-ΔCT to full-length raptor. If so, raptor-ΔCT overexpression would inhibit S6K binding, but not that of 4E-BP1, with the mTOR/raptor complex. It is also possible that some unidentified molecule is required for this association between S6K and the raptor-mTOR complex and that raptor-ΔCT binds to this as yet unknown molecule. In this case, S6K cannot bind the mTOR complex in the raptor-ΔCT-overexpressing cells, whereas 4E-BP1 phosphorylation is unaffected. Further study is necessary to resolve this issue.

In the present study, hepatic overexpression of raptor-ΔCT strongly inhibited insulin-induced p70S6k activation and improved glucose intolerance and hyperinsulinemia. Importantly, Akt phosphorylation was markedly enhanced not only under insulin-stimulated but also basal conditions. Decreased IRS-1 Ser307 and Ser653/659 phosphorylations and the resulting increases in tyrosine phosphorylation of IRS-1 and subsequent PI3K activity can account for the increased Akt phosphorylation under insulin-stimulated conditions. However, this may not fully explain the mechanism leading to markedly increased basal Akt phosphorylation since basal PI3-kinase activity was not altered by raptor-ΔCT. Thus, it is possible that other mechanisms, such as increased PKD and/or rictor activity, or even suppression of Akt dephosphorylation, are involved in the increased basal Akt phosphorylation. Indeed, it has been reported (22) that raptor-mTOR and rictor-mTOR complexes regulate Akt phosphorylation in a reverse manner. Further study is necessary to clarify whether suppression of the raptor-mTOR complex via overexpression of raptor-ΔCT leads to elevated rictor-mTOR activity or suppressed Akt dephosphorylation.

In summary, we demonstrated that hepatic p70S6k inhibition in diabetic mice improves glucose tolerance by enhancing both basal and insulin-stimulated Akt phosphorylations. Although further experiments are needed to clarify the molecular mechanisms of increased basal Akt phosphorylation, our results suggest that mTORC1 inhibition is a potential treatment strategy for obesity-related insulin resistance.

REFERENCES


