The Direct Involvement of SirT1 in Insulin-induced Insulin Receptor Substrate-2 Tyrosine Phosphorylation

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NAD+-dependent Sir2 family deacetylases and insulin signaling pathway are both conserved across species to regulate aging process. The interplay between these two genetic programs is investigated in this study. Protein deacetylase activity of SirT1, the mammalian homologue of Sir2, was suppressed through either nicotinamide treatment or RNA interference in several cell lines, and these cells displayed impaired insulin responses. Suppression of SirT1 activity also selectively inhibited insulin-induced tyrosine phosphorylation of insulin receptor substrate 2 (IRS-2), whereas it had minimal effect on that of IRS-1. Further analyses showed that both IRS-1 and IRS-2 interacted with SirT1, and the acetylation level of IRS-2 was down-regulated by insulin treatment. Inhibition of SirT1 activity prevented deacetylation and insulin-induced tyrosine phosphorylation of IRS-2. Mutations of four lysine residues to alanine in IRS-2 protein, on the other hand, led to its reduced basal level acetylation and insulin-induced tyrosine phosphorylation. These results suggest a possible regulatory effect of SirT1 on insulin-induced tyrosine phosphorylation of IRS-2, a vital step in insulin signaling pathway, through deacetylation of IRS-2 protein. More importantly, this study may imply a pathway through which Sir2 family protein deacetylases and insulin signaling pathway jointly regulate various metabolic processes, including aging and diabetes.

Calorie restriction, the only physiological method known so far to extend lifespan in animals ranging from Caenorhabditis elegans to mammals, is associated with global metabolic changes, including reduced insulin level, increased gluconeogenesis, and reduced lipid metabolism. The identification of Sir2 as the mediator of calorie restriction on lifespan regulation is a major breakthrough in understanding the molecular mechanism underlying the aging process (1, 2).

Sir2 and its mammalian counterpart, SirT1, belong to a family of NAD+-dependent protein deacetylases. Sir2 is actively involved in lifespan regulation in C. elegans, yeast, and Drosophila. Disruption of Sir2 gene leads to a reduced lifespan, whereas overexpression of Sir2 leads to significant lifespan extension in these species (2). In the mammalian system, SirT1 has been involved in multiple metabolic processes including glucose homeostasis (3), insulin secretion (4, 5), and lipid mobilization (6). However, its role in lifespan regulation remains ambiguous.

Insulin signaling pathway is also actively involved in the aging process (7). As a critical component of overall energy homeostasis, insulin signaling pathway has been well studied, and the key steps have been characterized (8). Insulin signaling pathway is initiated by auto tyrosine phosphorylation of insulin receptor upon insulin binding, and subsequently tyrosine phosphorylations of several key adaptors proteins including insulin receptor substrate 1 (IRS-1) and IRS-2. The phosphorylated IRS proteins further transmit insulin signaling to downstream events, mainly through two kinase cascades, the mitogen-activated protein kinase cascade (MAPK) and phosphatidylinositol 3-kinase-Akt cascade.

Genetic studies using C. elegans as a model system demonstrate a tight connection between insulin signaling pathway and aging. Mutations at several key components of insulin signaling pathway, including daf-2 (Insulin receptor), Age-1 (phosphatidylinositol 3-kinase), and daf-16 (Foxo proteins), all significantly extend the lifespan of C. elegans (7). Likewise, inactivation of insulin receptor in adipose tissue leads to extended lifespan in mice (9), and deletion of Klotho, a protein potently inactivating insulin receptor activity in cell culture studies, accelerates aging process in mutant mice (10). In this laboratory resveratrol, a chemical compound known to extend lifespan in several species including C. elegans, yeast, and Drosophila, has also been demonstrated to inhibit insulin signaling pathway in cell culture studies (11). These observations support a vital role of insulin signaling pathway in aging process.

The associations of both the insulin signaling pathway and the Sir2 family protein deacetylases with the aging process raise an important question; do these two pathways independently or jointly regulate aging process? In other words, is there any direct association between these two pathways? This question is explored in this study, and the results provide direct evidence supporting a novel role of SirT1 in regulating insulin-signaling pathway in mammalian cells.

EXPERIMENTAL PROCEDURES

General Reagents—Various cell culture reagents, including Dulbecco’s modified Eagle’s medium, M199 medium, penicil-
lin/streptomycin, and fetal bovine serum were obtained from Fisher. Insulin was obtained from Sigma. Antibodies against p44/42 MAPK, phospho-AKT (serine 473 and threonine 308), AKT, and acetylated lysine polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal FLAG antibody (M2) and phospho-p44/42 MAPK monoclonal antibody were obtained from Sigma. Anti-acetyl lysine 4G12 and anti-phosphotyrosine 4G10 antibodies were obtained from Millipore (Lake Placid, NY). Antibodies against insulin receptor β, retinoblastoma, were obtained from BD Biosciences. Antibodies against SirT1, cAMP-responsive element-binding protein-1, IRS-1, IRS-2, normal mouse IgG, normal rabbit IgG, and protein A/G plus beads were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Cell Culture**—H4IIE, HEK293 cells, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Rat primary hepatocytes were isolated as described previously (11) and set up in M199 medium supplemented with 100 nM dexamethasone, 100 nM triiodothyronine, and 1 nM insulin overnight before they were changed into M199 medium supplemented with 100 nM dexamethasone for further treatments.

**Cell Fractionation**—HEK293 cells were seeded at 3 × 10^6/100-mm dish, and grew in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Rat primary hepatocytes were isolated as described previously (11) and set up in M199 medium supplemented with 100 μm dexamethasone, 100 μm triiodothyronine, and 1 μm insulin overnight before they were changed into M199 medium supplemented with 100 μm dexamethasone for further treatments.

**Cell Fractionation**—HEK293 cells were seeded at 3 × 10^6/100-mm dish, and grew in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Cells were serum-starved overnight, and 3 dishes of cells were pooled together and resuspended in 0.2 ml of hypotonic buffer A (10 mM Hepes-KOH, pH 7.4, 1.5 mM MgCl_2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose supplemented with protease inhibitors) and passed through a 22.5-gauge needle 15 times before they were centrifuged at 1000 × g for 7 min at 4 °C. The 1000 × g pellet was resuspended in 0.1 ml of buffer B (20 mM Hepes-KOH, pH 7.4, 2.5% glycerol, 0.42 mM NaCl, 1.5 mM MgCl_2 supplemented with protease inhibitors), rotated at 4 °C for 1 h, and centrifuged at 1 × 10^5 × g for 30 min in a Beckman TLA 100.2 rotor. The resulted supernatant was designated as the nuclear fraction. In the meantime, the supernatant from original 1000 × g centrifugation was re-centrifuged at 8000 × g for 1 min using microcentrifugation at 8000 × g for Western blot analysis. For immunoprecipitation, equal amount of cell lysates were precipitated with protein A/G plus beads for 30 min at 4 °C under constant agitation, and the resulting supernatants were transferred into fresh tubes and incubated with primary antibody for another hour followed by the addition of 60 μl of protein A/G plus beads for an additional 1 h. Protein A/G plus beads were collected at 1000 × g for 1 min using microcentrifugation, washed several times with lysis buffer, and subjected to Western blot analysis. For Western blot analysis, immunoprecipitates or total cell lysates were resuspended in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol supplemented with bromphenol blue) and heated for 5 min at 75 °C. Proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

**Northern Blot Analysis**—Total RNA was extracted using TRIzol reagent from Invitrogen following the manufacturer’s instructions. An equal amount of RNA was resuspended into RNA loading buffer (Sigma), separated by 1% agarose-formaldehyde gel, and transferred to a nylon membrane (Hybond-N+). The cDNA probes were labeled with [α-32P]dCTP using a random primer labeling kit from Amersham Biosciences. Hybridization was performed at 65 °C in ExpressHyb hybridization solution (BD Biosciences Clontech) according to the manufacturer’s instructions. Membranes were exposed to x-ray film at either room temperature or −80 °C for 16 h.

**Establishing Stable Cell Lines**—H4IIE cells were transfected with 0.1 μg/100-mm dish pSIREN-RetroQ plasmid constitutively producing small interfering RNA against either luciferase (pSIREN-Luciferase) or the SirT1 coding sequence (pSIREN-SirT1) using FuGENE 6 transfection reagent for 48 h. Stable clones were isolated by puromycin selection at 6 μg/ml. The stable clones were isolated based on Northern blot analysis using specific probe against SirT1. For establishing HEK293 stable clones, cells were transfected with pSIREN-Luciferase or
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pSIREN-SirT1 for 48 h using FuGENE 6 transfection reagent, and stable clones were isolated using puromycin selection at 1 μg/mL. The stable clones were isolated based on Western blot analysis using SirT1 antibody from Santa Cruz.

In Vitro Deacetylation—HEK293 cells were transfected with 0.5 μg/60-mm dish FLAG-IRS-2 construct using FuGENE 6 reagent according to the manufacturer’s instructions. After 48 h, cells were serum-starved overnight before total cell lysates were prepared for immunoprecipitation using anti-FLAG antibody. Meanwhile, untransfected HEK293 cells were also serum-starved overnight and treated with insulin for 10 min. Endogenous SirT1 protein was immunoprecipitated using rabbit anti-SirT1 antibody from total cell lysates and washed several times in the lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, and 0.4% Triton X-100). The immunoprecipitated agarose beads were separated into deacetylation buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 4 mM MgCl₂, 10% glycerol, and 1 mM dithiothreitol), treated with and without β-NAD⁺, and incubated for 2 h at 37 °C. Total samples were quickly spun using a microcentrifuge and resuspended in sample buffer for Western blot analysis.

RESULTS

Several small chemical compounds, including nicotinamide and sirtuin inhibitors, have been demonstrated to potently inhibit protein deacetylase activity of SirT1 (12). These compounds were first used to treat H4IE cells to investigate their effects on insulin-induced MAPK and Akt activations (Fig. 1A). Insulin treatment alone induced strong Akt activation, demonstrated here by phosphorylations at both threonine 308 and serine 473 sites of Akt protein. Coincubation of either nicotinamide at 6 mM or splitomycin at 100 μM for 6 h significantly inhibited insulin-induced Akt phosphorylations at both sites. However, under the same condition, these compounds had minimal effects on insulin-induced MAPK activation, demonstrated here by phosphorylations of p42/p44 extracellular signal-regulated kinase (ERK) proteins.

A dose response study of the nicotinamide effect on insulin-induced MAPK and Akt activations was shown in Fig. 1B. Cells were treated with nicotinamide ranging from 0.3 to 10 mM for 6 h, and Western blot analysis showed that although MAPK activation was not affected by nicotinamide treatment at all, insulin-induced Akt activation was slightly inhibited with 3 mM nicotinamide and significantly inhibited with 10 mM nicotinamide. To rule out the possibility that these results were due to cell line specificity, similar experiments were also conducted using rat primary hepatocytes (Fig. 1C) and HeLa cells (data not shown). In these cells nicotinamide significantly inhibited insulin-induced Akt activation but had minimal impact on insulin-induced MAPK activation.

The nicotinamide effect on the insulin regulation of downstream target gene expressions at the transcriptional level was also investigated (Fig. 1D). Consistent with what was observed in SirT1 null cells (13), nicotinamide treatment led to a significantly increased mRNA level of phosphoenolpyruvate carboxykinase at the basal state but had little effect on the suppressive effect of insulin on phosphoenolpyruvate carboxykinase expression. Nicotinamide treatment also clearly suppressed insulin-induced up-regulation of fatty acid synthase mRNA level. The mRNA level of cyclophilin was measured as the loading control in this experiment. These results suggest that inhibition of SirT1 activity directly interferes with insulin signaling pathway at both protein and mRNA levels.

SirT1 activity was also inhibited through reducing SirT1 expression level by RNAi, and its effect on insulin signaling pathway was examined to avoid any possible side effects of chemical compound treatments. Several H4IIE stable clones carrying pSIREN-RetroQ plasmid producing either small interfering RNA against luciferase (pSIREN-Luciferase) or SirT1 (pSIREN-SirT1) were isolated through puromycin selection, and insulin-induced MAPK and Akt activations were examined in these cells. One typical experiment was shown in Fig. 2A. Clone Luc was isolated from cells transfected with pSIREN-Luciferase, whereas clones 1 and 2 were derived from cells transfected with pSIREN-SirT1. In all three clones insulin treatment led to potent MAPK activation. Akt kinase was also potently activated by insulin treatment in Luc and clone 1, but not clone 2, as quantified in Fig. 2A. Northern blot analysis showed that the SirT1 mRNA level was only reduced in clone 2, but not in clone 1, thus further supporting the observation that
SirT1 activity was necessary for insulin-induced Akt activation. The expression level of cyclophilin was used as the loading control. For the convenience of description, the stable cells carrying pSIREN-Luciferase were named SC (stable control) cells, whereas clone 2 or any clone where SirT1 mRNA levels was significantly reduced was named SS (Stable SirT1) cells.

Insulin signaling pathway was further dissected to identify the underlying mechanism of the suppressive effect of reduced SirT1 activity on insulin-induced Akt activation. One critical step in insulin signaling pathway is the tyrosine phosphorylation of insulin receptor (IR), which further phosphorylates tyrosine residues in both IRS-1 and IRS-2. Therefore, the tyrosine phosphorylation statuses of IR, IRS-1, and IRS-2 remain to be strongly tyrosine-phosphorylated, and IRS-1- and IRS-2-coding sequences were PCR-amplified from mouse liver cDNA and were used to create expression constructs with N-terminal FLAG epitope. These DNA constructs were overexpressed in HEK293 cells (Fig. 3, B and C). The transfected cells were serum-starved overnight with or without co-incubation of 6 mM nicotinamide and treated with insulin for 10 min. Consistent with the result in H4IIE cells, insulin treatment clearly led to potent tyrosine phosphorylations of both FLAG-IRS-1 and FLAG-IRS-2 proteins. However, although nicotinamide had minimal effect on insulin-induced IRS-1 tyrosine phosphorylation (Fig. 3B), it reduced insulin-induced tyrosine phosphorylation of IRS-2 protein significantly (Fig. 3C). Thus, FLAG-tagged IRS-2 in HEK293 cells responded similarly to nicotinamide treatment as endogenous IRS-2 protein.

The requirement of SirT1 deacetylase activity for insulin-induced tyrosine phosphorylation of IRS-2 raises a possibility that acetylation and tyrosine phosphorylation of IRS-2 might be inversely related, and deacetylation of IRS-2 by SirT1 might be a prerequisite for insulin-induced tyrosine phosphorylation of IRS-2.
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IRS-2. An obstacle to this hypothesis is that SirT1 protein is generally regarded as a nuclear protein, whereas IRS-2 protein is reported to be a cytoplasmic protein (14). The reported substrates of SirT1 are all but one cytoplasmic proteins (13, 15–21). Therefore, cellular localization of SirT1 was first examined in HEK293 cells. Cell fractionation studies showed that SirT1 protein was presented in both nuclear and cytosolic fractions (Fig. 4A). As experimental controls, two nuclear proteins, retinoblastoma protein and cAMP responsive element binding protein 1 (CREB-1), and tubulin was also examined in equal portion using their respective antibodies. B, interaction of SirT1 and IRS-2 in transfected HEK293 cells. FLAG-tagged IRS-2 construct (0.5 μg/60-mm dish) was transfected to HEK293 cells with and without co-transfection of SirT1-Myc construct (0.5 μg/60 mm dish), as indicated in the figure. Cells were transfected for 48 h before they were serum-starved overnight and harvested for total cell lysates. Immunoprecipitation (IP) was performed using either FLAG or Myc monoclonal antibody and analyzed using FLAG and Myc antibodies, respectively. WB, Western blot. C, in vivo interaction of SirT1 and IRS-2 in HEK293 cells. HEK293 cells were serum-starved overnight. Cells from three dishes were pooled together for each treatment. Endogenous IRS-2 protein was immunoprecipitated using a polyclonal IRS-2 antibody, and normal rabbit IgG was used as negative control. The SirT1 protein level in half of the immunoprecipitate was analyzed by Western blot analysis using a polyclonal anti-SirT1 antibody from Santa Cruz. The IRS-2 protein level was also examined in 1/8 of total immunoprecipitate using polyclonal anti-IRS-2 antibody. D, IRS-1 interacted equally well with SirT1 as IRS-2. HEK293 cells were transfected with FLAG-IRS-1 or FLAG-IRS-2 with and without co-transfection of SirT1-Myc construct, as indicated in the figure. FLAG-IRS-1 and FLAG-IRS-2 were immunoprecipitated from total cell lysates using FLAG antibody. The immunoprecipitates were analyzed by Western blot analysis using Myc and FLAG antibody, respectively. The expression level of SirT1-Myc was also analyzed in whole cell lysates (WCL) using Myc antibody.

To investigate the acetylation status of IRS-2 protein, FLAG-tagged IRS-2 protein was expressed in HEK293 cells (Fig. 5A) and serum-starved overnight with and without co-treatment of 10 μM nicotinamide. These cells were treated with insulin for 10 min and harvested for immunoprecipitation using monoclonal anti-FLAG antibody. Normal mouse IgG was used as negative control. Western blot analysis showed that immunoprecipitated FLAG-IRS-2 protein was acetylated at the basal level (compare lane 1 with lane 2), and treatment of insulin significantly reduced the acetylation level of FLAG-IRS-2 (compare lane 2 and lane 3). Treatment of nicotinamide overnight, on the other hand, prevented insulin-induced deacetylation of IRS-2 protein (compare lane 3 with lane 5). Next, endogenous IRS-2 protein was immunoprecipitated from H4IIE cells with and without insulin treatment using polyclonal anti-IRS-2 antibody alongside normal rabbit IgG as negative control. The acetylation status of IRS-2 protein was examined, and again, IRS-2 was acetylated at the basal level, and insulin treatment led to reduced acetylation level of IRS-2 protein. Overall, these results suggest that IRS-2 protein is acetylated at basal state, and its acetylation level is down-regulated by insulin.

Both IRS-1 and IRS-2 interacted equally well with SirT1. To understand why insulin-induced tyrosine phosphorylations of IRS-1 and IRS-2 responded differently to reduced SirT1 activity (Fig. 3), the acetylation status of IRS-1 was also examined alongside with that of IRS-2 in HEK293 cells using FLAG-tagged proteins (Fig. 5C). Consistent with previous results, FLAG-IRS-2 protein was acetylated at the basal level, and insulin treatment led to reduced acetylation level of FLAG-IRS-2. On the other hand, although the protein amount of IRS-1 is higher than that of IRS-2, the acetylation level of FLAG-IRS-1 protein was too low to be detected under the same conditions. Longer exposure showed that IRS-1 was weakly acetylated, and its acetylation was not regulated by insulin (data not shown). These results strongly suggest that insulin-induced IRS-2 deacetylation is accountable for the regulatory effect of SirT1 on insulin-induced IRS-2 tyrosine phosphorylation.

The direct involvement of SirT1 in insulin-induced IRS-2 deacetylation was also investigated in an in vitro deacetylation assay (Fig. 5D). FLAG-tagged IRS-2 protein was expressed in HEK293 cells and immunoprecipitated from total cell lysates using monoclonal anti-FLAG antibody. Endogenous SirT1 protein was also immunoprecipitated from insulin-treated HEK293 cells. The immunoprecipitated FLAG-IRS-2 protein and SirT1 protein were set up as indicated in Fig. 5D for 2 h at 37 °C, and the acetylation level of FLAG-IRS-2 protein was examined. The addition of SirT1 protein alone had a minimal
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A. FLAG-IRS-2 acetylation was down-regulated by insulin. HEK293 cells were transfected with FLAG-IRS-2 and serum-starved overnight with and without co-incubation of 10 mM nicotinamide (NAM). Cells were treated with and without insulin for 10 min, and FLAG-IRS-2 protein was immunoprecipitated (IP) from total cell lysates by FLAG antibody. Mouse normal IgG was used as a negative control. The acetylation level (Ac-K) of FLAG-IRS-2 protein was examined using monoclonal anti-Acetyl-Lysine antibody from Millipore (4G12). B. endogenous IRS-2 protein was acetylated at basal level. HEK293 cells were serum-starved overnight before they were treated with and without insulin for 10 min. Endogenous IRS-2 protein was immunoprecipitated from total cell lysates using a polyclonal IRS-2 antibody from Santa Cruz. The acetylation level (Ac-K) of FLAG-IRS-2 protein was examined using monoclonal anti-Acetyl-Lysine antibody from Millipore (4G12). Rabbit normal IgG was used as a negative control. The acetylation and tyrosine phosphorylation levels (WB: αAc-K) of FLAG-IRS-1 and FLAG-IRS-2 were examined by Western blot analysis using monoclonal anti-acetyl-lysine antibody (4G12). Mouse normal IgG was used as a negative control. The acetylation level (Ac-K) and tyrosine phosphorylation levels (WB: αAc-K) of FLAG-IRS-1 and FLAG-IRS-2 were examined by Western blot analysis using monoclonal anti-acetyl-lysine antibody (4G12). Mouse normal IgG was used as a negative control.

The inverse relationship between IRS-2 acetylation and tyrosine phosphorylation indicates that one or more acetylated lysine residues in IRS-2 protein may be critical for SirT1 regulation of insulin-induced tyrosine phosphorylation of IRS-2. However, to identify these acetylation sites in IRS-2 protein may be difficult, since there is a total of 55 lysine residues in the IRS-2 coding sequence. On the other hand, considering the fact that nicotinamide significantly prevented insulin-induced deacetylation and tyrosine phosphorylation of IRS-2 protein (Fig. 3B), it is reasonable to assume that insulin-induced tyrosine phosphorylations of those IRS-2 fragments carrying putative acetylated lysine residues would be suppressed by nicotinamide. Therefore, a series of deletion mutants of FLAG-IRS-2 were created to narrow down IRS-2 fragment carrying these putative lysine residues.
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| FLAG tagged Construct | WT | K4A |
|-----------------------|----|-----|
| Insulin               | -  | +   | -  | +  |
| Lane                  | 1  | 2   | 3  | 4  |

**FIGURE 6. Identification of the critical lysine residues of IRS-2 in sirT1 regulation of insulin-induced IRS-2 tyrosine phosphorylation.** A mutated FLAG-IRS-2 construct was created where four lysines at Lys-118/289/292/412 were mutated to alanines using site-directed mutagenesis and named FLAG-IRS-2 (K4A). This mutated construct was used alongside with FLAG-IRS-2 to transfect HEK293 cells, and the transfected cells were serum-starved overnight before they were treated with and without insulin (100 nM) for 10 min. Wild type (WT) and mutated proteins were immunoprecipitated (IP) using FLAG antibody, and their acetylation and tyrosine phosphorylation levels were analyzed by Western blot (WB 4G10) analysis using anti-acetyl-lysine (4G12) and anti-tyrosine phosphorylation antibodies.

residues. Using this method, the potential acetylation residues of IRS-2 was narrowed down to the first 590 amino acids of IRS-2 protein.

IRS-1 and IRS-2 share high homology at the N-terminal region (22), yet compared with IRS-2, IRS-1 is hypo-acetylated, and its acetylation was not regulated by insulin (Fig. 4C). These observations suggest that the acetylation residues critical to insulin-induced tyrosine phosphorylation of IRS-2 may be unique to IRS-2 protein. Mouse IRS-1 and IRS-2 sequence was compared using the MegAlign program of DNAStar. For the first 590 amino acids of mIRS-2 coding sequence, there were only four lysine residues presented solely in the IRS-2 sequence (Lys-118, -289, -292, and -412). These four lysine residues are also conserved in human IRS-2 sequence. A series of site-directed mutagenesis were conducted to mutate these four lysine residues to alanine, and the resulting mutant is named FLAG-IRS-2 (K4A). The acetylation and tyrosine phosphorylation levels of this mutant in response to insulin treatment was compared with those of FLAG-IRS-2 in Fig. 6. Consistent with the hypothesis, the basal acetylation level of this mutant was significantly reduced compared with that of the wild type protein along with its tyrosine phosphorylation level upon insulin treatment. These results further support that insulin-induced deacetylation of IRS-2 by SirT1 is a critical step for insulin-induced IRS-2 tyrosine phosphorylation.

**DISCUSSION**

In this study reduced SirT1 deacetylation activity, either through direct inhibition or through reduced protein expression, led to reduced Akt activation and insulin-induced IRS-2 tyrosine phosphorylation; IRS-2 was acetylated at basal state, and the acetylation of IRS-2 was down-regulated by insulin; inhibition of SirT1 activity prevented insulin-induced IRS-2 deacetylation; an IRS-2 mutant was created where four lysine residues were mutated to alanine, and the basal level acetylation of this mutant protein was significantly inhibited along with its tyrosine phosphorylation upon insulin treatment. Based on these results, the underlying molecular mechanism of the regulatory effect of SirT1 on the insulin signaling pathway is proposed in Fig. 7. In this model insulin-induced tyrosine phosphorylation of insulin receptor and activation of SirT1 deacetylase are suggested to be two separate events in insulin signaling pathway. IRS-2 protein is acetylated at the basal state. Although insulin treatment leads to tyrosine phosphorylation of IR, which further recruits insulin receptor substrates including IRS-1 and IRS-2 to its kinase domain, the acetylated lysine residues in IRS-2 protein prevents IR kinase from further phosphorylating tyrosine residues in IRS-2 protein. Continuing phosphorylation of the tyrosine residues in IRS-2 protein requires removal of the acetyl group from acetylated lysine residues in IRS-2 protein by insulin-activated SirT1 protein deacetylase, and the phosphorylated IRS-2 protein then serves as an adaptor protein to further transmit insulin signaling to downstream targets. Undoubtedly, there are many unanswered questions in this model, and further experiments are required to fully vindicate or rectify this model. In addition, this model is based on cell culture studies. Animal studies are urgently needed to verify this model and to investigate its physiological significance.

How deacetylation of IRS-2 protein facilitates its tyrosine phosphorylation is still unclear, although a similar observation with retinoblastoma protein has been made in a recent study (23). One consequence of deacetylation of acetylated lysine residue is to reveal its positive charge. These exposed positive
chages of lysine residues after deacetylation may play critical role in insulin-induced tyrosine phosphorylation of IRS-2 protein, as neutralization of these positive charges either through acetylation or mutating to alanine (Fig. 6) all associate with reduced tyrosine phosphorylation of IRS-2 protein. Mutation of lysine to arginine (K412R) renders this protein unstable (data not shown), which is consistent with the observations that insulin treatment leads to IRS-2 tyrosine phosphorylation and degradation in vivo (24, 25).

Although the full impact of this regulation on overall energy homeostasis is still unclear, the physiological significance of this regulation has been suggested in several animal studies. The SirT1 protein level has been shown at a reduced level in mice subjected to high fat feeding (26) and in two aging models (27). Both aging and high fat-feeding are known to be associated with insulin resistance in vivo. Reduced SirT1 expression under these conditions through interfering with the insulin signaling pathway may directly cause or at least significantly contribute to overall insulin resistance in vivo.

Both the insulin signaling pathway and Sir2 family deacetylases have been tightly associated with aging, and there is some evidence suggesting that these two pathways may converge to regulate aging process in C. elegans. Overexpression of Sir2.1, the C. elegans counterpart of Sir2, significantly increases lifespan in C. elegans, and this effect is suppressed by mutation at Daf-16, the C. elegans homologue of forkhead proteins, and a key component of insulin signaling pathway (28). In a mammalian system, SirT1 is suggested to modulate insulin secretion in pancreas (4, 5), and it also directly regulates the transcriptional activity of forkhead proteins at the cellular level (13, 29). This study complements well with these reports, implying a common pathway through which both the insulin signaling pathway and Sir2 family deacetylases act on to regulate the aging process.

Although many components of the insulin signaling pathway have been demonstrated to be involved in the aging process ranging from C. elegans to mammals, the putative role of IRS proteins in this process is still unclear. There is little information about the role of IST-1 (C. elegans homologue of IRS protein) in the aging process (30). On the other hand, loss of Chico, the Drosophila homologue of IRS proteins, leads to significant lifespan extension (31). In the mammalian system, there is a family of four IRS proteins with divergent functions. The specific effect of SirT1 on IRS-2 tyrosine phosphorylation indicates that IRS-2 rather than other members of IRS family proteins may play critical role in the aging process.

This study also provides further insight into the molecular mechanism underlying the regulatory effect of SirT1 protein deacetylase on aging and other metabolic processes. The regulatory effect of Sir2 family protein deacetylases in the aging process has been well established in C. elegans, yeast, and Drosophila (2). However, the molecular mechanism underlying this regulation is still unclear. The identification of IRS-2 as another putative substrate of SirT1 suggests that SirT1 may also be through regulating IRS-2-mediated signaling pathway to control aging and other biological processes.

In fact, animal models with genetically mutated SirT1 and IRS-2 genes strongly support this hypothesis (32, 33). The female null mice of both IRS-2 and SirT1 showed smaller ovaries with very few follicles and reduced luteinizing hormone, prolactin, and sex steroids (33, 34). The remarkable similar phenotypes in female infertility between SirT1 and IRS-2 null mice strongly imply an intrinsic connection between SirT1 and IRS-2-mediated signaling pathway in female reproductivity.

One unexpected observation in this study is the cytosolic localization of SirT1 protein. SirT1 protein has been demonstrated as a nuclear protein (35). However, this study relied heavily on fluorescence microscopy technique. Although this technique can convincingly demonstrate the presence of SirT1 protein in nucleus, it cannot rule out the presence of this protein in cytosol. Furthermore, the presence of SirT1 protein in cytosol was also suggested in several other studies, including the phosphatidylinositol 3-kinase regulation of nucleocytoplasmic shuttling of SirT1 protein (36) and the putative SirT1 deacetylation of Acetyl-CoA synthase, a known cytosolic protein (21).

In addition, this study also reveals useful information about the signaling pathway leading to the activation of MAPK and Akt kinases. MAPK and Akt kinase cascades are important components of insulin signaling pathway, mediating a variety of downstream events. In this study reduced IRS-2 tyrosine phosphorylation in cells with impaired SirT1 activity led to significantly reduced Akt activation yet had minimal effect on MAPK activation, suggesting that IRS-2 tyrosine phosphorylation may be exclusively linked with Akt activation in these cells. This conclusion is consistent with a study where mouse ovaries from wild type and IRS-2 null mice were used. Compared with that of wild type mice, insulin-induced Akt activation was significantly attenuated in IRS-2 null mice, whereas insulin-induced MAPK activation was minimally affected (37). However, in other studies, reduced IRS-2 protein levels through RNAi led to diverse effects on AKT and MAPK activations, including appreciable increased Akt activation in mouse liver (38) or reduced activations of both MAPK and Akt kinases in muscle cells (39, 40). The molecular mechanism underlying these discrepancies is still unclear. The probable explanations may include tissue specificity and that fact that in this study the overall tyrosine phosphorylation level rather than the protein level of IRS-2 protein was significantly reduced.

In summary, the current study suggests that SirT1 protein may, through regulation of the acetylation level of IRS-2 protein, directly regulate insulin-induced IRS-2 tyrosine phosphorylation and its downstream Akt activation. This study also suggests that deacetylation of IRS-2 protein is a critical component of insulin-induced IRS-2 tyrosine phosphorylation. The identification of a novel regulatory step in insulin-induced IRS-2 tyrosine phosphorylation and the novel role of SirT1 in regulation of insulin signaling pathway also have direct implications in our understanding of aging, diabetes, and other metabolic processes associated with insulin signaling pathway.

Acknowledgments—I thank Dr. Scott Grundy (University of Texas Southwestern Medical Center) for constant support and Dr. Mike Brown (University of Texas Southwestern Medical Center) for support and invaluable advice.
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