Salt-inducible kinase (SIK), first cloned from the adrenal glands of rats fed a high salt diet, is a serine/threonine protein kinase belonging to an AMP-activated protein kinase family. Induced in Y1 cells at an early stage of ACTH stimulation, it regulated the initial steps of steroidogenesis. Here we report the identification of its isoform SIK2. When a green fluorescent protein-fused SIK2 was expressed in 3T3-L1 preadipocytes, it was mostly present in the cytoplasm. When coexpressed in cAMP-responsive element-reporter assay systems, SIK2 could repress the cAMP-responsive element-dependent transcripion, although the degree of repression seemed weaker than that by SIK1. SIK2 was specifically expressed in adipose tissues. When 3T3-L1 cells were treated with the adipose differentiation mixture, SIK2 mRNA was induced within 1 h, the time of induction almost coinciding with that of c/EBPβ mRNA. Coexpressed with human insulin receptor substrate-1 (IRS-1) in COS cells, SIK2 could phosphorylate Ser794 of human IRS-1. Adenovirus-mediated overexpression of SIK2 in adipocytes elevated the level of phosphorylation at Ser794, the mouse equivalent of human Ser794. Moreover, the activity and content of SIK2 were elevated in white adipose tissues of db/db diabetic mice. These results suggest that highly expressed SIK2 in insulin-stimulated adipocytes phosphorylates Ser794 of IRS-1 and, as a result, might modulate the efficiency of insulin signal transduction, eventually causing the insulin resistance in diabetic animals.

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Salt-inducible kinase (SIK), first cloned from the adrenal glands of rats fed a high salt diet, is a serine/threonine protein kinase belonging to an AMP-activated protein kinase family. Induced in Y1 cells at an early stage of ACTH stimulation, it regulated the initial steps of steroidogenesis. Here we report the identification of its isoform SIK2. When a green fluorescent protein-fused SIK2 was expressed in 3T3-L1 preadipocytes, it was mostly present in the cytoplasm. When coexpressed in cAMP-responsive element-reporter assay systems, SIK2 could repress the cAMP-responsive element-dependent transcription, although the degree of repression seemed weaker than that by SIK1. SIK2 was specifically expressed in adipose tissues. When 3T3-L1 cells were treated with the adipose differentiation mixture, SIK2 mRNA was induced within 1 h, the time of induction almost coinciding with that of c/EBPβ mRNA. Coexpressed with human insulin receptor substrate-1 (IRS-1) in COS cells, SIK2 could phosphorylate Ser794 of human IRS-1. Adenovirus-mediated overexpression of SIK2 in adipocytes elevated the level of phosphorylation at Ser794, the mouse equivalent of human Ser794. Moreover, the activity and content of SIK2 were elevated in white adipose tissues of db/db diabetic mice. These results suggest that highly expressed SIK2 in insulin-stimulated adipocytes phosphorylates Ser794 of IRS-1 and, as a result, might modulate the efficiency of insulin signal transduction, eventually causing the insulin resistance in diabetic animals.

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The lipid metabolism in adipose tissues is under the control of two hormonal signaling pathways; insulin stimulates glucose uptake and lipogenesis, whereas cAMP, generated by exogenous stimuli like adrenalin and glucagon, stimulates lipolysis. If the balance between the two signaling systems becomes lost and the adipose tissues are exposed to hyperinsulinemia for a prolonged time, they gradually become resistant to insulin stimulation (1, 2). The insulin resistance occurring in tissues involved in biological fuel metabolism, such as adipose tissues, liver, and skeletal muscles, would finally cause disorders in energy metabolism of the whole body, such as obesity and type 2 diabetes (3, 4). Insulin receptor substrate (IRS) proteins are key molecules of the insulin-signaling cascade (5); they are phosphorylated on tyrosine residues by the action of insulin-dependently activated insulin receptor kinase, and the tyrosine-phosphorylated IRS proteins trigger further intracellular cascades. Several investigators recently reported (6, 7) that IRS proteins, under certain non-physiological conditions, were phosphorylated on serine residues. The serine phosphorylation of IRS proteins would modulate the efficiency of the insulin-signaling cascade (8, 9) and eventually render the animals resistant to insulin stimulation (10, 11). Molecular identification of several protein kinases responsible for the serine phosphorylation of IRS proteins has been reported (12–24).

Salt-inducible kinase (SIK) was first cloned from the adrenal glands of rats fed a high salt diet (25, 26). It is a serine/threonine protein kinase that belongs to a sucrose-nonfermenting-1 protein kinase/AMP-activated protein kinase (AMPK) family. It was induced in adrenocortical cells at a very early phase of ACTH stimulation, and its molecular properties have been investigated in detail (27–29). Its presence in tissues other than the adrenal cortex was also reported (27). Examining the genomic data base, we noticed the presence of an isoform of SIK. A cDNA clone of the isoform was isolated; the newly identified enzyme was named SIK2, and the previously reported isoform was renamed SIK1. We demonstrate here that...
SIK2 was abundantly expressed in adipose tissues and was induced at a very early stage during the process of preadipocyte-adipocyte differentiation. When expressed together with human IRS-1 in COS-7 cells, it phosphorylated Ser<sup>794</sup> of human IRS-1. Moreover, its activity and content in white adipose tissues of diabetic animals were elevated.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—Male ddY, C57BL/6Cr, and C57BLKsJ db/db (30 mice (10 weeks old) were purchased from SLC Co. Ltd. (Shizuoka, Japan) and maintained under standard conditions of light and temperature. All experiments were carried out in accordance with guidelines for the care of Osaka University Medical School.

**Cloning of Mouse SIK2 cDNA**—By searching human genome data base GenBank<sup>TM</sup>EMBL/DDJB for SIK homolog, we found that the putative protein kinase KIAA0781 (GenBank<sup>TM</sup> accession number AB018824) and SIK1 were rather similar to each other, although the COOH-terminal half of KIAA0781 seemed completely different from SIK1. We examined the chromosomal loci of human SIK1 and KIAA0781 genes; the SIK1 gene was found on chromosome 21 (GenBank<sup>TM</sup> accession numbers AP001046 and AP001047), whereas the KIAA0781 gene was found on chromosome 11 (GenBank<sup>TM</sup> accession number AP000925), indicating that the two genes were similar but distinct. Because the KIAA0781 protein predicted from the data base lacked an NH<sub>2</sub>-terminal part, we decided to isolate a full-length cDNA of KIAA0781 protein. However, the available knowledge of expressed human cDNAs was poorer than that of mouse cDNAs. Therefore, a mouse expression tag (EST) clone data base was searched for mouse homologs of the two genes. As the result, a 3′-non-coding cDNA fragment of mouse SIK1 (GenBank<sup>TM</sup> accession number U11494) and that of mouse KIAA0781 protein (GenBank<sup>TM</sup> accession number AA889086) were identified. Based on the cDNA structure, a 3′-non-coding region of mouse SIK1 cDNA was amplified by PCR by using primers, 5′-TTGCTCATGCCCCTTGAGTAGT and 5′-TTGCTCAGCTGTCAGGAAGTAA. An EST clone containing a 3′-non-coding region of mouse KIAA0781, IMAGE:1230878, was purchased from Invitrogen. By using these cDNA fragments as probes, we found that mouse KIAA0781 protein was expressed as a single transcript. The resultant full-length human IRS-1 cDNA was cloned into the pET28a (Novagen). cDNA probes for c/EBPs and PPAR<sub>α</sub> were were described previously (32, 33).

**Immunoprecipitation**—Mouse tissues (0.3–0.5 g) were homogenized in 2 ml of lysis buffer (29). The homogenates were preincubated with 100 μl of protein-G-Sepharose at 4°C for 30 min to remove nonspecifically bound proteins. The mixture was centrifuged at 15,000 rpm for 15 min, and the resulting infranatant was recovered, and the protein concentration was determined with Protein Assay (Bio-Rad). The protein solutions, 3 mg obtained from white adipose, 3 mg from brown adipose, 18 mg from liver, and 18 mg from skeletal muscles, were incubated with 10 μl of anti-SIK2-specific antiserum and 50 μl of protein-G-Sepharose at 4°C for 3 h. The SIK2-IgG-protein-G-Sepharose complex was precipitated by centrifugation at 3000 × g for 5 s, washed three times with lysis buffer, and washed once with 1 ml of wash buffer (27). The final precipitate was suspended in the SIK-reaction buffer with a final volume of 50 μl. Two aliquots of the SIK2-IgG suspension were used for the immunoblot analysis and in vitro kinase assay.

To prepare the samples from cultured cells, fully differentiated 3T3-L1 adipocytes were lysed in 1 ml of lysis buffer, and aliquots of the lysate were incubated with anti-IRS-1 antibody (5 μl), anti-phospho-Ser<sup>794</sup> IRS-1 antiserum (10 μl), or anti-SIK2 antisem (10 μl) in the presence of 50 μl of protein-A-Sepharose for 3 h.

**Cells**—3T3-L1 cells, obtained from Japan Health Sciences Foundation, were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) containing 10% fetal calf serum and antibiotics, at 37°C under an atmosphere of 5% CO<sub>2</sub> and 95% air. After reaching confluence, the cells were treated with a differentiation mixture (36), MIX (0.5 mM 3-methyl-D-fucose), 1 μg/ml insulin, and 10 μg/ml dexamethasone in DMEM containing high concentration of glucose (2.5 g/liter). After the 2 days of treatment, the cells were transferred into DMEM containing high glucose, and the medium was changed every 2nd day. COS-7 cells were also maintained in DMEM. For transfection of expression vectors, LipofectAMINE 2000 (Invitrogen) was used in this study.

**Phosphorylation of Human IRS-1**—The phosphorylation of Ser<sup>794</sup> in human IRS-1 by SIK2 in COS-7 cells was performed by a method described previously (29) with some modification. COS-7 cells, after having been transformed with pTarget-SIK2 and pEBG IRS-1, were incubated with <sup>32</sup>PPO<sub>4</sub> (0.05 μCi, 1.85 MBq) for 12 h in phosphate/serum-free medium. GST-human IRS-1 was expressed in the cells by purifying using a glutathione-conjugated column (MicroSpin<sup>™</sup> GST Purification Module, Amersham Biosciences) and subjected to SDS-PAGE followed by autoradiography.

**Adenovirus-mediated Expression of SIK2**—To overexpress SIK2 protein in 3T3-L1 adipocytes, ViraPower<sup>™</sup> adenoviral expression system (Invitrogen) was used. cDNA fragments of wild-type SIK2, SIK2(SS87A), and SIK2(K49M) were prepared by BamHI/NotI digestion and ligated into the BamHI/NotI site of pBENTR-1A vector (Invitrogen). pAd/CMV/V5-DEST vector and the Gateway system were used to pAd/CMV/V5-DEST vector and the Gateway system were used to construct adenovirus DNAs. About 1 × 10<sup>1</sup> plaque-forming units of virus were used for infection of 3T3-L1 adipocytes in 6-well dishes.

**Other Procedures**—Expression of GST-tagged proteins in E. coli and COS-7 cells and their purification was described before (27, 29). Procedures for Northern blot analysis, in vitro kinase assays, and reporter assays were described in Ref. 27.
RESULTS

Primary Structure of SIK2—Mouse SIK2 is a 931-amino acid protein, with a molecular mass about 120 kDa. Fig. 1A shows the sequence similarity between mouse SIK2 and rat SIK1, highlighting three highly conserved domains. Domain 1, a serine/threonine protein kinase domain, was found near the NH2-terminus of SIK2, at the region 20–271 residues. Amino acid residues in the SIK2 and SIK1 kinase domains were 78% identical to each other. Domain 2, a 54-amino acids stretch, with 70% residues identical between SIK2 and SIK1, was found in the central part of the protein. A computer-assisted data base search (www.sanger.ac.uk/Software/Pfam/) revealed that this domain contained a ubiquitin-associate motif, a motif found in several proteins having connections with ubiquitin-dependent intracellular protein degradation. Whether domain 2 plays a role for the intracellular turnover of SIK protein remains to be explored. Domain 3, present one-third from the COOH terminus, contained a protein kinase A-dependently phosphorylatable Ser587, a SIK2 equivalent for Ser577 of SIK1. Serine Kinase Activity of SIK—Full-length SIK2 and SIK1 were expressed as GST-fused proteins in COS-7 cells and purified by using a glutathione-Sepharose column. The purified GST-SIKs were subjected to SDS-PAGE followed by immunoblot analyses using the anti-GST antibody. The expressed GST-SIK2 had an apparent molecular mass of about 150 kDa, the sum of GST and SIK2 (left panel in Fig. 1B). The purified GST-SIK2 was next incubated with GST-Syntide 2 in the presence of [32P]-labeled ATP, and the reaction mixture was subjected to SDS-PAGE followed by autoradiography (right panel in Fig. 1B). Two radioactive bands appeared on an x-ray film, and the band with the lower molecular weight was that of the phosphorylated Syntide 2, whereas that with the higher molecular weight was that of the autophosphorylated SIK2. Lys49 of SIK2, present in the ATP-binding loop (shown in Fig. 1A), is an SIK2 equivalent to Lys56 of SIK1 that was essential for the kinase activity. A mutant SIK2 having Met49 instead of Lys was assayed for the kinase activity, with the expected results of the mutant being inactive. The similar experiments performed with SIK1 provided the results completely consistent with those reported previously.

Intracellular Distribution and CRE-repressing Activity of SIK2—Our previous study of SIK1 demonstrated that it was present in both nuclear and cytoplasmic compartments of resting Y1 cell, with the higher content in the nucleus (29). Also shown was that when the cells were stimulated with ACTH, Ser377 was protein kinase A-dependently phosphorylated, and the phosphorylated SIK1 was translocated to the cytoplasm. We examined the subcellular distribution of SIK1 and SIK2 by expressing GFP-fused SIKs in 3T3-L1 cells (Fig. 1C). GFP-SIK1, expressed in 3T3-L1 cells, like that expressed in Y1 cells, was mostly present in the nucleus, and the protein was translocated to the cytoplasm after the cells were stimulated with a differentiation mixture (MIX). In contrast, GFP-SIK2 seemed to exist mostly in the cytoplasmic compartment of both resting and stimulated cells, although weak green fluorescence signal could be detected in the nucleus as well. That SIK2 was in fact present in the nucleus was more clearly shown in the cells expressing GFP-SIK2(SS577A), whose phosphorylatable Ser577 had been replaced with Ala.

SIK1, when coexpressed with CRE-reporter gene in Y1 cells, could repress the PKA-dependently activated reporter activity (28). We examined the effect of SIKs on CRE-dependent transcription efficiency in 3T3-L1 cells by using CRE-reporter cotransfection assays. The extent of SIK2-mediated inhibition of the forskolin-dependent activation of CRE-reporter was about 40% weaker (lower panel in Fig. 1D), compared with about 80% obtained by SIK1 (upper panel in Fig. 1D). As expected, SIK2(SS577A), whose PKA-dependent phosphorylation site had been abolished, could repress the CRE activity more prominently than the wild-type SIK2. The SIK kinase activity was indispensable for this repressive effect, because the enzymatically inactive SIK2(K49M) did not seem to be repressive.

Tissue Distribution of SIK2—The tissue distribution of SIK2 was examined by Northern blot analyses of various tissues taken from 10-week-old ddY mice (Fig. 2A). Large amounts of SIK2 transcripts were found in both white adipose and brown adipose tissues, and a little was found in the testis. Two SIK2 mRNA bands, 4 and 6 kb long, were consistently detected; this seemed to result from an alternative splicing. As reported before (27), SIK1 mRNA was present mainly in adrenal gland and in smaller amounts in ovary, brain, testis, and skeletal muscle in the diminishing order.

SIK2 Is Expressed during Differentiation of Adipocytes—The high expression of sik2 genes in adipose tissues prompted us to examine the level of SIK2 mRNA during the process of preadipocyte-adipocyte differentiation. Thus, 3T3-L1 fibroblasts were cultured for 4 days in DMEM to attain confluence. Then they were treated with the differentiation mixture to initiate the differentiation. At several time points after the treatment, the cells were harvested to examine mRNA levels of SIK2 and several other adipocyte differentiation markers. As shown in Fig. 2B, SIK2 mRNA was prominently expressed after the first 24-h incubation, and the level remained high until day 7, when the preadipocytes were mostly differentiated into mature adipocytes (judged by oil Red-O staining; data not shown). On the other hand, the level of SIK1 mRNA, although quite lower compared with that of SIK2 (see the difference in film exposure times given in the legend), seemed to rise a little during the first 24 h and remained at the elevated level until day 7. The level of Pref-1 mRNA, a marker of preadipocytes (37), began to decline after the day 2. The mRNA levels of c/EBPβ and c/EBPδ, transcription factors known to appear early in the adipocyte differentiation (36), rose during the first 24 h in a manner similar to that of SIK2. The mRNA levels of known late response genes of adipogenesis, such as SREBP-1, c/EBPα, PPARγ, and aP2 (38, 39), began to rise after day 2 or day 4. We further examined the expression of mRNAs during the first 12 h after the stimulation (Fig. 2C). The SIK2 mRNA level rose within 1 h after the stimulation, and the elevation almost coincided with that of c/EBPβ mRNA. The elevation of c/EBPδ mRNA level occurred within 1 h as well, but the level gradually declined after a few hours.

In order to examine which agent in the mixture of three hormones, insulin, cAMP, and dexamethasone, was required to stimulate the transcription of sik2 gene, the preadipocytes were incubated for 2 h with a single hormone or a mixture of the three, and the mRNA levels were examined (Fig. 2D). The transcription of sik2 gene seemed to be activated by dexamethasone alone to the similar degree as by the mixture, although even insulin alone could also substantially activate the transcription.

SIK2 Phosphorylates Ser794 of Human IRS-1—The above findings strongly suggest that SIK2 may be involved in the signal transduction pathways in adipose tissues. But nothing more could be added to this issue until we could identify the intracellular target molecule(s) of SIK2 action. In our attempt to search for the target molecule, we noticed that several serine/threonine kinases, including AMPK, to whose family SIK2 belongs, mitogen-activated protein kinase, and c-Jun NH2-terminal kinase, are known to play important roles in modulating the insulin-dependent stimulus-response coupling (6, 7).
Therefore, we surmised that the target molecules(s) of SIK2 might be found somewhere in the insulin-stimulated signaling pathways of adipose tissues. Our in vitro kinase assays using various synthetic peptide substrates suggest that the canonical phosphorylation motif of SIK is (Hy)((B)X or X(B))XX(S/T)XXX(Hy), where S/T is the phosphorylatable Ser or Thr, and (Hy) and (B) are hydrophobic and basic residues, respectively. Because a peptide stretch, Leu-Arg-Leu-Ser-Thr-Ser 794-Ser-Gly-Arg-Leu, in human IRS-1 seemed consistent with the canonical SIK motif, 

Y. Katoh, H. Takemori, and M. Okamoto, unpublished results.
Fig. 2. Adipose-specific expression of SIK2. A, total RNAs (10 μg), each prepared from the indicated organs or cell lines (except for the case of ovary) ddY mice (10 weeks old), were electrophoresed in 1% agarose gel and subjected to Northern blot analysis. cDNA fragments of mouse SIK2 (top panel), mouse SIK1 (2nd panel), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (3rd panel) were used as probes (see “Experimental Procedures”). Ethidium bromide staining of ribosomal RNA was shown in the 4th panel. WAT, epididymal white adipose tissue; BAT, brown adipose tissue; SK Muscle, skeletal muscles. B, SIK2 is induced during adipocyte differentiation of 3T3-L1 cells. 3T3-L1 cells, having been cultured in 10-cm dishes, were harvested for RNA extraction before (Growth) or after (Confluent) confluency or during the adipocyte differentiation (Day 1 to Day 7). To induce adipocyte differentiation, cells were incubated with the differentiation MIX in the DMEM containing high glucose (2.5 g/liter) for 2 days. After incubation, medium was changed to fresh medium with high glucose every 2nd day. The cells harvested at the indicated days were subjected to Northern blot analyses by using cDNA probes indicated. C, 3T3-L1 cells that reached confluency or during the differentiation mixture for short periods. Dibutyryl cAMP (1 mM) instead of methylisobutylxanthine was used as a component of the differentiation mixture. D, each of the three hormones in the differentiation mixture was added alone, or as the mixture, to 3T3-L1 cells for 2 h. The vertical bar denotes “without hormone.” Autoradiographs shown in A–D are the representative data from triplicate experiments. B–D, the filters were exposed to X-ray films for 16 h to visualize SIK2 mRNA, and for 3 days to visualize SIK1 mRNA. DX, dexamethasone.

Mononuclear motif, we first tested whether or not several synthetic peptides having sequences similar to this stretch could serve as substrates of E. coli-expressed SIK2. As shown in Fig. 3A, a rat IRS-1-derived peptide (784–793 residues), Leu784–Arg-Leu-Ser-Ser-Ser793, was strongly phosphorylated, and the Ser789-disrupted mutant peptide was weakly phosphorylated by SIK2. Because the rat IRS-1 peptide contained four successive serine residues, we surmised that one, or several, of the neighboring three residues, Ser787, Ser788, or Ser790, in the Ser789-disrupted peptide (S789A) might be weakly phosphorylated by SIK2. In any case, this result clearly indicated that Ser789 in rat IRS-1, an equivalent for Ser794 in human IRS-1, was a possible candidate for phosphorylation by SIK2 in vitro.

Next, GST-linked full-length human IRS-1 or its Ser794-disrupted derivative was coexpressed with SIK2 or Lys97-disrupted inactive SIK2 in H1299PO4-preincubated COS-7 cells. As seen in the upper panel of Fig. 3B, the wild-type IRS-1 was prominently phosphorylated in the wild-type SIK2-expressed cells, although a weakly phosphorylated IRS-1 was found in the inactive SIK2(K49M)-expressed cells, indicating that IRS-1 could also be phosphorylated in COS-7 cells by a protein kinase(s) other than SIK2. In contrast, the Ser794-disrupted IRS-1 could only weakly, if at all, be phosphorylated in either the wild-type SIK2- or the inactive SIK2-expressed cells. This result suggests that Ser794 in human IRS-1 could be phosphorylated by SIK2 in the cells.

To confirm that the phosphorylated residue in human IRS-1 was indeed Ser794, we analyzed the cell homogenates by using a specific antibody raised against the phosphorylated Ser794 of rat IRS-1 (middle panel of Fig. 3B). The phosphorylated Ser794 was clearly seen in the homogenates prepared from the wild-type IRS-1- and the enzymatically active SIK2-transfected cells. A very faint immunoreactive product was seen in the lane of the homogenate prepared from the wild-type IRS-1- and the inactive SIK2-transfected cells; this occurred probably because the cells contained a serine kinase(s) other than SIK2 that could phosphorylate Ser794. As expected, the antibody could not detect the phosphorylation product in the homogenate prepared from the Ser794-disrupted IRS-1- and SIK2-transfected cells.

Whether or not SIK2 phosphorylates the Ser789 in mouse adipose cells could be determined by overexpressing SIK2 in 3T3-L1 cells. But in order to do so, the phosphorylation must be determined by using the samples taken from the fully differentiated adipocytes rather than preadipocytes, because the level of IRS-1 protein in the latter is rather low. On the other hand, the efficient transformation of adipocytes by naked plasmids would be rather difficult. To overcome these difficulties, we attempted to employ an adenovirus-mediated transformation system for the SIK2 expression in adipocytes. Adenoviruses containing various kinds of constructs for SIK2 were used to infect the fully differentiated 3T3-L1 cells (Fig. 3C), signals for anti-SIK2-immunoreactive proteins increased by 5–10-fold the control, shown in the lane labeled lacZ in the lower panel. The total amount of IRS-1 present in the cell homogenates was not changed by SIK2 overexpression (middle panel in Fig. 3C). When the immunoblot analyses were carried out by using anti-phospho-Ser789 antibody (upper panel in Fig. 3C), weak, but significant, immunoreactive bands having the molecular weight of IRS-1 appeared in the samples prepared from the wild-type-SIK2- or PKA-resistant SIK2(S587A)-overexpressing adipocytes but not in those prepared from the lacZ-infected control cells or the inactive SIK2(K49M)-expressing cells.

The finding that either the wild-type SIK2 or the PKA-resistant SIK2(S587A) could phosphorylate Ser789 of IRS-1 led us to examine whether the Ser-phosphorylated IRS-1 could influence the forskolin-dependent CRE activation and the SIK2-dependent suppression of CRE activity. Thus, the human IRS-1 or its phosphorylation site-disrupted mutant IRS-1(S794A) was coexpressed in the CRE reporter assay system (Fig. 3D). The forskolin-dependently activated CRE activity was further elevated in the presence of wild-type human IRS-1. However, this elevation seemed not to occur in the presence of IRS-1(S794A),
suggesting that IRS-1 was indeed phosphorylated at Ser 794 in the PKA-stimulated adipocytes, and the phosphorylated IRS-1 could up-regulate the forskolin-induced CRE activation. Whether or not a kinase capable of this phosphorylation was the endogenously expressed SIK2 was unclear in this experiment. When the PKA-resistant SIK2(S587A) was overexpressed in this IRS-1/CRE system, the forskolin-dependently activated CRE activity was completely abolished either in the presence or the absence of IRS-1, suggesting that the ability of SIK2 to repress the CRE-reporter transcription might overwhelm the Ser-phosphorylated up-regulation capability of IRS-1.

SIK2 Is Activated in White Adipose Tissues of Diabetic Mice—The phosphorylation of Ser789 in rat IRS-1 by AMPK has been reported to occur in C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside (20). More recently, Qiao et al. (35) reported that the Ser 789 could be phosphorylated in the liver of diabetic rats by a serine kinase that was different from AMPK. These reports prompted us to measure the level of SIK2 in tissues of genetically diabetic mice. Thus, white adipose tissue, brown adipose tissue, liver, and skeletal muscle were taken from db/db mice (30) or the wild-type lean mice and homogenized. Northern blot analyses (Fig. 4A) indicated that SIK2 mRNA was present in the white as well as brown adipose tissues of both diabetic and wild-type animals at the similar levels. As expected, the SIK2 mRNA contents in liver and skeletal muscles were quite lower compared with those in the adipose tissues. Again, there seemed to be no difference in the mRNA contents in these tissues between diabetic and wild-type animals. However, to our surprise, the levels of SIK1 mRNA in brown adipose tissue, liver, and skeletal muscle of diabetic mice were significantly elevated compared with those of wild-type mice.

Cytosolic fractions of the respective tissues were subjected to immunoprecipitation using an antibody raised against the COOH-terminal peptide of SIK2. The immunoprecipitates were then subjected to SDS-PAGE followed by the immunoblot analyses using the anti-SIK2 antibody (Fig. 4B). The results clearly showed that the content of SIK2 protein in white adipose tissue of diabetic animals was higher than that of wild-type animals. The SIK2 content in brown adipose tissue of diabetic animals, on the other hand, was lower than that of wild-type animals. Because the SIK2 contents in liver and skeletal muscle were low, the immunoreactive bands were

Fig. 3. SIK phosphorylates IRS-1 in vivo and in vitro. A, peptide (5 μg) of rat IRS-1, 784–793 residues, and its mutant (S789A) were expressed as GST fusion proteins in E. coli expression systems, purified, and used for substrates of GST-liked SIK2 produced in COS-7 cells in the in vitro kinase assays. Upper panel shows the incorporation of 32P into the synthetic substrates, and the lower panel shows the Coomassie Brilliant Blue (CBB) staining of the substrates. GST-Syntide2 was used as positive control. B, COS-7 cells, cotransformed with mammalian GST-expression vectors (pEBG, 1.5 μg) for GST-human IRS-1 (full-length and its mutant S794A) and SIK2 and its kinase-defective mutant, K49M, (pTarget; 1.5 μg), were incubated with 32PO4 (0.05 mCi, 1.85 MBq) for 12 h in phosphate/serum-free medium and lysed in 700 μl of lysis buffer. The GST-human IRS-1s were purified by glutathione columns (CP, column purification) and subjected to SDS-PAGE (10%), and the levels of phosphorylation were visualized by autoradiography (upper panel). Similar experiments were done without isotope labeling and subjected to Western blot (WB) analyses using anti-phospho-Ser789 of rat IRS-1 (middle panel) and anti-IRS-1 antibodies (lower panel). C, adenovirus-mediated overexpression of SIK2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes, fully differentiated after 10 days, were infected with adenovirus that could express lacZ (control), wild-type SIK2 (WT), SIK2(S587A), and SIK2(K49M). After 48 h of incubation, cells were lysed in 1 ml of lysis buffer, and IRS-1, phospho-IRS-1, and SIK2 proteins were purified by immunoprecipitation (IP) followed by Western blotting with respective antibodies as described under “Experimental Procedures.” D, IRS-1 was phosphorylated at Ser794 in preadipocytes, but the SIK2-dependent CRE suppression overwhelmed the phosphorylated IRS-1-mediated up-regulation of CRE activity. Wild-type IRS-1 up-regulates forskolin-induced CRE activity in 3T3-L1 preadipocytes. The procedure for reporter assay is given in Fig. 1 legend. In addition to plasmids denoted in Fig. 1D, 0.2 μg of pEBG, pEBG-hIRS-1, and pEBG-IRS-1(S794A) were transformed into the cells.
scarcely detected in these tissues, showing no or little difference between the diabetic and wild-type animals.

The respective immunoprecipitate was next assayed for protein kinase activity using Syntide-2 as a substrate. As shown in Fig. 4A, the precipitate of white adipose tissue of diabetic mice had distinctly higher SIK2 kinase activity than that of wild-type animals. These results seemed to indicate that SIK2 kinase activity was increased in the white adipose tissue of diabetic mice.

The liver homogenate had substantially higher SIK2 kinase activity than that of diabetic animals. These results seemed consistent with the difference in SIK2 protein content between the two animals (Fig. 4B). The liver homogenate had substantially higher SIK2 activity and that from diabetic animals had a little lower activity than that of the wild-type animals. The homogenate of skeletal muscle taken from diabetic animals showed barely discernible SIK2 activity, whereas that from wild-type animals showed no activity.

Together, these results strongly suggest that SIK2 protein as well as its activity were elevated in the white adipose tissue of diabetic mice.

DISCUSSION

We herein for the first time described the enzymatic properties of SIK2. In general, they are similar to those of SIK1. However, several properties different between the two kinases should be noted. Although SIK1 was found in the nuclear compartment of resting cells, SIK2, as visualized by green fluorescence signal of the expressed GFP-fused protein, was mostly present in the cytoplasm of 3T3-L1 cells. However, the possible presence of a small amount of endogenous SIK2 in the nucleus could not be excluded, because the immunocytochemical technique could only detect the intracellular localization of the expressed protein. The precise subcellular distribution of SIK2, therefore, must be established in the future by immunocytochemical methods using a highly purified antibody raised against SIK2. That SIK2 could substantially repress the forskolin-dependent transcriptional activation of CRE-reporter gene may indicate the intranuclear presence of SIK2. Almost complete repression obtained by the PKA-resistant SIK2-(S587A) clearly demonstrated that SIK2 could act as a repressor for the CRE-mediated gene transcription, as was the case with SIK1 (29).

The marked expression of s/s gene in the adipose tissues and its involvement in the insulin-mediated adipogenic signal transduction must be emphasized here. SIK2 appeared to be one of the very early response genes during preadipocyte- adipocyte differentiation. The time-dependent elevation of SIK2 mRNA level almost coincided with that of the adipocyte-specific transcription factors, such as c/EBPβ and c/EBPα. The role played by SIK2 during the initial stage of adipocyte differentiation has yet to be clarified. However, it should be noted that the overexpression of SIK2 could repress the CRE-reporter activity. Besides, based on our previous investigation on SIK1 (40), SIK2 is likely to repress the transcriptional activity of both constitutive active and dominant negative CREBs. On the other hand, the activation of CREB/CRE signaling has been reported (41, 42) to be an essential step of adipocyte differentiation. When the CRE activation was disturbed by dominant negative CREB or by addition of TNF-α, the adipogenesis of 3T3-L1 cells was inhibited (41). The overexpression of constitutive active CREB in 3T3-L1 cells rescued the apoptotic action of TNF-α (43) and induced the adipogenesis (44). These considerations may suggest that SIK2 might be involved in the regulation of the CRE-mediated transcription of genes essential for the initiation of adipogenesis. However, a possibility that SIK2 may also act in the cytoplasm by regulating the signal transduction occurring in the early stage of adipogenesis must be considered as well.

Numerous recent investigations suggest the regulatory roles played by serine/threonine kinase(s) in the insulin signaling (6, 7). Several serine kinases, including mitogen-activated protein kinase, glycogen-synthase kinase 3 (14), casein kinase (12), phosphatidylinositol 3-kinase (23), mTOR (16, 19, 22), c-Jun NH2-terminal kinase (21, 45), Akt (16), and AMPK (20), are known to phosphorylate IRS-1. The activation of AMPK in mouse C2C12 myotubes resulted in phosphorylation of Ser789 in IRS-1 (20). The Ser789-phosphorylated IRS-1 was also shown to be tyrosine-phosphorylated. The resulting IRS-1 had a high affinity to p85/p110 and thus activated the downstream signaling, such as phosphatidylinositol 3-kinase (20). In general, the tyrosine phosphorylation of IRS-1 and the up-regulation of the downstream signaling are thought to enhance the insulin sensitivity and the resistance to a high plasma level of glucose (3). However, in the liver of high salt-induced type 2 diabetic rats, the similar up-regulation of insulin signaling through IRS-1 occurred, and in this case, the serine phosphorylation of IRS-1 was thought to eventually generate the insulin resistance (46,

FIG. 4. Expression of SIK1 and SIK2 in diabetic and wild-type animals. A, total RNAs were extracted from 10-week-old male mice of C57BL/6Cr (used as wild-type (WT)) and C57BLKs/J db/db (diabetic animal (db)). To estimate the amounts of SIK1 and SIK2 mRNAs in various tissues under the similarly visualized conditions, 5 µg of RNAs each from WAT and BAT and 30 µg of RNAs each from liver and skeletal muscle (Sk M) were subjected to Northern blot analyses. B, SIK2 proteins were purified by immunoprecipitation from lysates containing 3 mg of proteins each from WAT and BAT and 18 µg of proteins each from liver and skeletal muscle, and subjected to Western blot analyses. C, immunopurified SIK2 from various tissues was subjected to in vitro kinase assay using GST-Syntide2 as the substrate. The results shown are the representative data from triplicate experiments.
47). Recently, Qiao et al. (35) reported the presence in the hepatic cytosols of a genetically diabetic rat of a kinase responsible for the Ser789 phosphorylation of IRS-1, which was distinct from AMPK. In viewing these reports, we hypothesized that SIK2 might be involved in the serine phosphorylation of insulin-signaling molecule(s) and regulate the signaling pathway in adipose tissues. We found that Ser789 in human IRS-1, an equivalent of Ser794 in rat IRS-1, could be phosphorylated by SIK2 in the in vitro kinase assays as well as in COS-7 cells or 3T3-L1 adipocytes. Then we tested whether or not SIK2 was activated in tissues prepared from diabetic animals. SIK2 was indeed found activated in the white adipose tissue of diabetic mice. Therefore, SIK2, by phosphorylating Ser789 in IRS-1, might be involved in regulation of insulin sensitivity of white adipose tissues of diabetic mice.

The case was different for the liver of diabetic mice, whose SIK2 level seemed similar to that of wild-type animals. However, the levels of SIK1 mRNA were found markedly elevated in the brown adipose tissues, livers, and skeletal muscles of diabetic animals. Because our anti-SIK1 antibody cross-reacted with SIK2 protein, we could not determine the content and activity of SIK1 protein in these tissues. However, a possibility remains that SIK1, which was originally described as “salt-inducible kinase” (26), might be the kinase responsible for phosphorylation of Ser789 in IRS-1 in the livers of diabetic animals. Taken together, we propose here that in the diabetic animals, SIK2 in white adipose tissues and SIK1 in brown adipose tissues, livers, and skeletal muscles might be involved in the disturbance of the insulin signaling occurring in these tissues and therefore might play important pathogenic roles in the development of type 2 diabetes.

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