Cloning of a novel kinase (SIK) of the SNF1/AMPK family from high salt diet-treated rat adrenal[1]

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Abstract PCR-coupled cDNA subtraction hybridization was adapted to identify the genes expressed in the adrenocortical tissues from high salt diet-treated rat. A novel cDNA clone, termed salt-inducible kinase (SIK), encoding a polypeptide (776 amino acids) with significant similarity to protein serine/threonine kinases in the SNF1/AMPK family was isolated. An in vitro kinase assay demonstrated that SIK protein had autophosphorylation activity. Northern blot revealed that SIK mRNA levels were markedly augmented by ACTH treatment both in rat adrenal glands and in Y1 cells. SIK may play an important role in the regulation of adrenocortical functions in response to high plasma salt and ACTH stimulation.

Key words: Salt inducible kinase; Protein serine/threonine kinase; SNF1/AMPK; Adrenocorticotropic hormone; Y1 mouse adrenocortical tumor cell; Adrenocortical function

1. Introduction

The Na^+/K^+ balance in plasma is one of the major factors to regulate the function of the adrenal cortex. High plasma Na^+/K^+ modulates cell proliferation, mitochondrial density and biosynthesis of aldosterone in the adrenal cortex [1–4]. However, the molecules involved in the regulation of the biosynthesis of steroidogenic enzymes in the adrenal cortex when stimulated by angiotensin II (AII), K^+, Na^+, or adrenocorticotropic hormone (ACTH) remain to be identified, since the molecular events taking place between these agents' binding to the cell surface and the stimulated biosynthesis of steroidogenic enzymes are still unclear [3,5].

To understand the detailed mechanism(s) underlying the regulation of adrenocortical gene expression under the influence of high plasma Na^+ or K^+ concentration, the PCR-coupled cDNA subtraction hybridization method was adapted to identify the genes expressed in the adrenal cortex of rats treated with a high Na^+ or high K^+ diet. We report here the molecular cloning and characterization of a novel protein kinase named salt-inducible kinase (SIK) from rat adrenal gland.

2. Materials and methods

2.1. Experimental animals

Sprague-Dawley rats were purchased from SLC Co. Ltd., Shizuoka, Japan, and maintained under standard conditions of light and temperature. All experimental procedures were carried out in accordance with the guidelines for animal care of Osaka University Medical School.

2.1.1. High Na^+ and high K^+ diet treatment. Five-week-old male rats were divided into three groups each comprising three rats. Group one received Na^+-enriched diet (containing 8% NaCl, Oriental Co. Ltd., Chiba, Japan) with tap water, group two K^+-loaded water (150 mM KCl) with a low Na^+ diet (0.3% NaCl), and group three a normal diet. After 7 days treatment the rats were killed under anesthesia, and their adrenal glands were removed rapidly and kept at −80°C for further studies.

2.1.2. ACTH treatment. Five-week-old male rats were injected intraperitoneally with daily doses of 0.1 mg ACTH (Daiichi Seiyaku Co., Tokyo, Japan) dissolved in 0.6% NaCl. On day 1, 2, 4 or 7 the rats were killed 24 h after the last ACTH injection, and their adrenal glands were removed rapidly and kept at −80°C for further studies.

2.2. Isolation of a novel cDNA clone

An adrenal gland removed from high Na^+- or high K^+-treated rat was separated into two portions: the capsular portion comprising mainly zona glomerulosa tissue, and the decapsulated portion composed of zona fasciculata/reticularis and medullary tissues. Modified PCR-coupled cDNA subtraction hybridization was performed between two cDNA populations prepared from the high K^+-treated capsular portion and from the high Na^+-treated decapsulated portion as described previously [6–8]. As the result of this subtraction hybridization, several independent cDNA fragments were isolated, cloned into the *Bam*HI site of pUC18, and sequenced. A novel cDNA clone of ~400 bp in length, named LF20 (later designated SIK), showed interesting features and was used for isolation of the full-length cDNA.

A rat adrenal cDNA library [6,9] was plated at a density of 1×10^5 plaques per 15-cm agar plate. Replicas of the plaques were screened using a ^{32}P-labeled cDNA fragment of LF20. All procedures for the screening of the cDNA library were basically described by Maniatis et al. [10]. As the result, a positive cDNA clone of ~1.4 kb was isolated and sequenced. The sequence revealed that this cDNA clone had a region of ~100 bp nucleotides overlapping with LF20, but lacked 5′- and 3′-termini.

2.3. Isolation of 5′- and 3′-terminal cDNA fragments

The rapid amplification of cDNA ends (RACE) method was employed to isolate terminal cDNA fragments of LF20 with a RACE-PCR kit (Takara Shuzo, Kyoto, Japan). Briefly, total RNA (~3 μg) prepared from the adrenal gland of high K^+-treated rat was reverse-transcribed into cDNA. The sequence information of LF20 was used to design primers according to the manufacturer's instructions. For the 5′-RACE-PCR, a 5′-phosphorylated LF20-specific primer (5′-P-CAGCACTGATGTGCAAGTG-3′) was used to synthesize the first-

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB020480.

Abbreviations: AII, angiotensin II; ACTH, adrenocorticotropic hormone; SIK, salt-inducible kinase; SNF1, sucrose-non-fermenting 1 protein kinase; AMPK, AMP-activated protein kinase; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; PKC, calcium/phospholipid-dependent protein kinase

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strand cDNA. Hybrid DNA-RNA was digested by RNase H. The first-strand cDNA was self-ligated with T4 RNA ligase. Two sets of nested LF20-specific primers were used in the 1st and 2nd PCRs (1st PCR: 5′-TGAGGGCTGGTGCTTCGAT-3′ and 5′-GGATGAGTACCTTCCACAC-3′; 2nd PCR: 5′-CGCTCGAGCAGGAGGTAGTA-3′ and 5′-AGCCGTCTCATCTGACTAC-3′). The 1st and 2nd PCRs were carried out at 94°C, 40 s; 56°C, 40 s; and 72°C, 3 min for 30 cycles with a final extension at 72°C for 5 min. The second PCR products were purified by a spin column (Qiagen Inc.), cloned into pT7BlueR vector (Novagen, Inc.), and sequenced. For the 3′-RACE-PCR, an oligo(dT)-tailed primer (5′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTT-3′) provided in the RACE kit was used to synthesize the first-strand cDNAs. Using the same oligonucleotide as a 3′-terminal primer and two upstream staggered LF20-specific primers (1st PCR: 5′-CTCTCCAGTCAGATTGGC-3′; 2nd PCR: 5′-AGCCGTCTCATCTGACTAC-3′), the 1st and 2nd PCR amplifications were performed under the same conditions as for the 5′-RACE-PCR. The second PCR products were processed as described for 5′-RACE-PCR.

To generate a full-length cDNA clone of SIK, the first-strand cDNAs described for 3′-RACE were used as templates for PCR amplification with a SIK 5′-specific primer (5′-CCGGATCC**ATG**GTGATCATGTCGGAGTTC-3′) to introduce a *Bam*HI site immediately before the putative first ATG, and a SIK 3′-specific primer (5′-CCGAATTCTTATCATTGAGGTCCTCAG-3′) containing a *Eco*RI site. The PCR product, a fragment of 2.4 kb, was purified and cloned into pT7BlueR vector.

2.4. DNA sequencing

The nucleotide sequence of the full-length cDNA clone of SIK in pT7BlueR vector was determined using the ABI Prism Dye Terminator Cycle Sequencing kit (PE Applied Biosystems, CA) and an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) according to the manufacturer's instructions. Both forward and reverse strands of three independent clones were sequenced with SIK-specific primers to avoid possible mistakes generated by PCR amplifications.

2.5. Expression and purification of GST-SIK fusion protein

SIK-encoded protein was expressed as a glutathione *S*-transferase (GST)-fused protein GST-SIK. Briefly, the 2.4-kb cDNA fragment containing the entire open reading frame of rat SIK was cloned into the *Bam*HI and *Eco*RI sites of pGEX2T vector (Pharmacia Biotech Inc.). The recombinant plasmid pGEX2T-SIK was transformed into *Escherichia coli* BL21 cells (Stratagene, La Jolla, CA). The transformed *E. coli* cells were cultured until OD_{600} reached 0.6, and induced with isopropylthiogalactopyranoside at a final concentration of 0.5 mM at 27°C for 3 h. The cells were lysed by sonication at 4°C, and centrifuged at $12\,000 \times g$ for 30 min. GST-fused proteins in the supernatant were purified by affinity adsorption to glutathione-Sepharose 4B beads and eluted with glutathione as described by the manufacturer (Pharmacia). Affinity-purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting using anti-GST antibody (Pharmacia).

2.6. Assay for protein kinase activity in vitro

Kinase activity of SIK protein was determined in terms of its autophosphorylation activity in vitro. An aliquot (~10 μg) of the affinity-purified GST-SIK fusion protein was incubated with 370 kBq [γ-^{32}P]ATP in 30 μl kinase buffer (25 mM HEPES, pH 7.5, 50 mM Tris, 50 mM $MgCl_2$, 5 mM $MnCl_2$, and 5 mM dithiothreitol) at 30°C for 30 min. Reactions were terminated by heating at 100°C for 5 min. The reaction mixture was filtered through a microconcentrator (MICROCON 30, Amicon, Inc., USA) by spinning at 11 000 rpm for 10 min to remove the unincorporated [γ-^{32}P]ATP, and then resolved by 12% SDS-PAGE. After electrophoresis, the gel was dried and autoradiographed using Kodak X-ray film at −70°C for 12 h. Autophosphorylation activities of the same amount of heat-denatured GST-SIK or GST-SIK incubated with an excess amount of cold ATP (1 mM) before addition of radio-labeled ATP were also examined in the same way.

2.7. Northern blot analyses

Total RNAs were extracted from adrenal glands of differently treated rats as mentioned in Section 2.1 using the guanidine thiocyanate method [7]. Equal amounts of total RNAs (10 μg) of each sample were denatured with 50% formamide/6% formaldehyde and fractionated by electrophoresis on 1% agarose gels containing 6% formaldehyde. The gel-separated RNAs were transferred to a Hybond-N nylon membrane (Amersham) and hybridized with the ^{32}P-labeled cDNA fragment of LF20, which corresponded to the nucleotides 1901–2300 bp of SIK cDNA. The cDNA probes specific for rat P450(11β)s and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were prepared as described previously [6].

2.8. Analysis of SIK mRNA levels in ACTH-treated Y1 cells

Y1 mouse adrenocortical tumor cells were plated at 3×10^5 cells per 60-mm dish in DMEM containing 10% FCS (Gibco BRL) with penicillin and streptomycin, and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. ACTH was added at the semi-confluent stage of growth at a concentration of 1 μM. The cells were collected at different time points after ACTH treatment, and the total RNAs were extracted using the method of Chomczynski and Sacchi [11]. Northern blot analysis was performed on ~10 μg total RNAs using a ^{32}P-labeled *Xho*I/*Eco*RI cDNA fragment of SIK.

3. Results and discussion

3.1. Molecular cloning and sequence analysis of SIK

To investigate the effects of high salt diet on the gene expression in the adrenal cortex, we performed PCR-coupled cDNA subtraction hybridization between mRNAs prepared from the adrenal capsular portion (containing mainly zona glomerulosa cells) of high K^+-treated rat and those from the adrenal decapsulated portion (containing zona fasciculata/reticularis and medulla) of high Na^+-treated rat. This subtraction hybridization should result in isolation of the specific cDNAs in the high K^+-treated zona glomerulosa. As a result, a novel cDNA fragment was isolated and named LF20. Northern blot analysis showed that LF20 was expressed at a higher level in the K^+-treated adrenal compared to the non-treated adrenal without zone specificity. To examine whether or not LF20 mRNA is suppressed in the high Na^+-treated adrenal, total RNAs of rat adrenal gland were tested under the high Na^+-feeding conditions. The level of LF20 mRNA was unexpectedly higher in either high Na^+ or high K^+ diet than that in normal diet (Fig. 1). Transcripts for LF20 looked smeared, indicating that LF20 mRNA may be

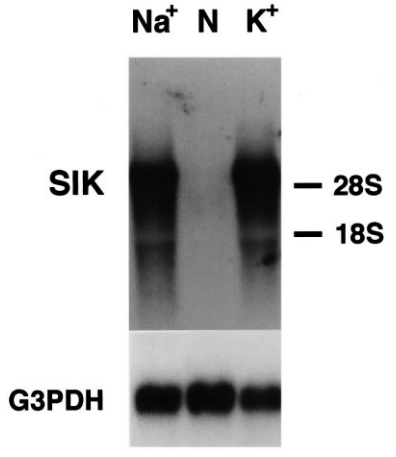

Fig. 1. Northern blot analysis of SIK mRNA in rat adrenal. Total RNAs (~10 μg) prepared from adrenal glands of rats treated with high sodium (Na^+), normal (N) or high potassium (K^+) diet were probed with a ^{32}P-labeled cDNA fragment of LF20 (nucleotide positions 1901–2300 bp of SIK cDNA). The same membrane was reprobed with a ^{32}P-labeled cDNA fragment of rat G3PDH. Migration of 18S and 28S rRNA is indicated.

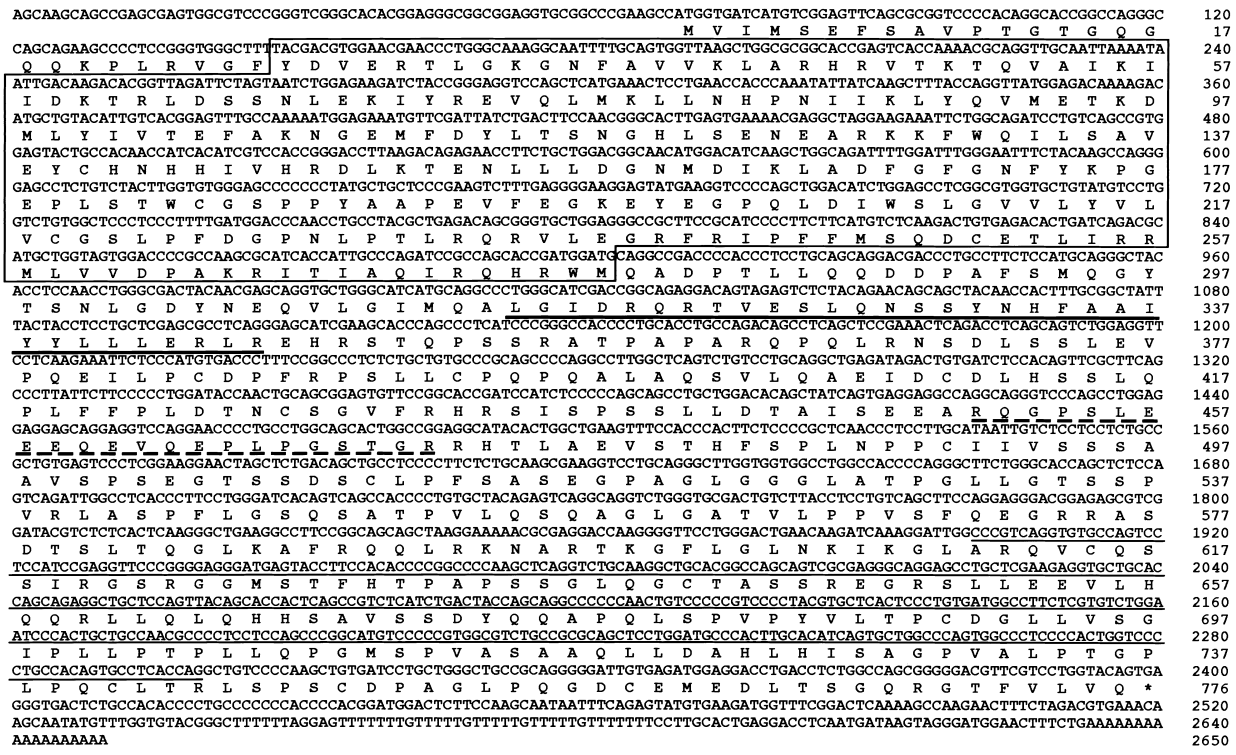

Fig. 2. Nucleotide and deduced amino acid sequences of SIK from rat adrenal. The deduced amino acid sequence of SIK is shown in single-letter code under the nucleotide sequence. Both are numbered on the right. The protein serine/threonine kinase catalytic domains are boxed. The SNH domain is underlined in bold. The PEST region is indicated with a broken line. The nucleotide sequence of LF20 is underlined with a thin line.

labile, or alternatively spliced. Using the LF20 fragment as a probe to screen the adrenal cDNA library prepared from high K^+-treated rat, we isolated a cDNA clone, which overlapped with LF20, missing both the 5′- and 3′-ends. RACE-PCRs were carried out on the clone, and the full-length cDNA clone was obtained and termed salt-inducible kinase (SIK). The cDNA clone contained 2650 bp nucleotides with a putative open reading frame of 2331 bp nucleotides (Fig. 2). The encoded protein contained 776 amino acids with a predicted molecular mass of 85.8 kDa. Comparison of its deduced ami-

Fig. 3. Alignment of the deduced amino acid sequences of rat SIK and other members of the SNF1/AMPK family. Partial amino acid sequences of SIK, p78, emk, SNRK, AMPK and SNF1 were aligned using the CLUSTAL W algorithm. Protein kinase catalytic domains are boxed. The SNH domain is boxed with a bold line. Identical residues are indicated by asterisks, and conservative substitutions by dots under the sequences. Numbers on the right refer to the amino acid residues of each sequence. Data base accession numbers of the respective sequences are: SIK (gb, AB020480), p78 (PIR, s27966), emk (PIR, s31333), SNRK (gb, x89383), AMPK (gb, z29486) and SNF1 (PIR, a26030).

no acid sequence with other known proteins revealed that SIK had significant homology to the protein kinases in SNF1/AMPK family [12–14] (Fig. 3). The N-terminal part (amino acid residues 27–278) of SIK contains all 11 catalytic subdomains conserved in protein serine/threonine kinases [15,16]. The C-terminal part of SIK is unique, its sequence distinguishing it from other proteins. However, there are several characteristics in the primary structure of the C-terminal part: (1) A so-called SNH (SNF1 homology) domain. This highly conserved domain is known to exist only in SNF1-related protein kinases [17]. (2) With the aid of the PROSITE program, a number of potential phosphorylation sites for different protein kinases were detected, including three for cAMP-dependent protein kinase (PKA), five for calcium/phospholipid-dependent protein kinase (PKC), and one for tyrosine kinase. (3) Using the computer program PESTFIND, a hydrophilic stretch of 22 amino acid residues enriched in proline, glutamic acid, serine and threonine was found at amino acid residues 451–473 (the PEST score [18,19] calculated for this stretch is 8.19). This structural information suggests that SIK protein might be a rapid turnover protein with a short intracellular half-life and could be phosphorylated by PKA, PKC and tyrosine kinase, playing intricate roles in transmitting the signals from multiple physiological stimuli to the downstream genes.

3.2. SIK protein is an active protein kinase

To understand the function of SIK protein, SIK-encoded protein was expressed as a GST-fused protein GST-SIK in *E. coli* cells. The expressed fusion proteins in the supernatant (solubilized fraction) of the cell lysates were purified by affinity adsorption with glutathione-Sepharose 4B beads, and analyzed by SDS-PAGE followed by immunoblotting using anti-GST antibody. An immunostained protein having a molecular mass of approximately 112 kDa was observed (Fig. 4A). It may represent the intact form of fusion protein GST-SIK, since its molecular mass coincided with the predicted molecular mass of GST-SIK. Several other lower molecular mass proteins on the same filter might be the proteolytic degradation products of GST-SIK.

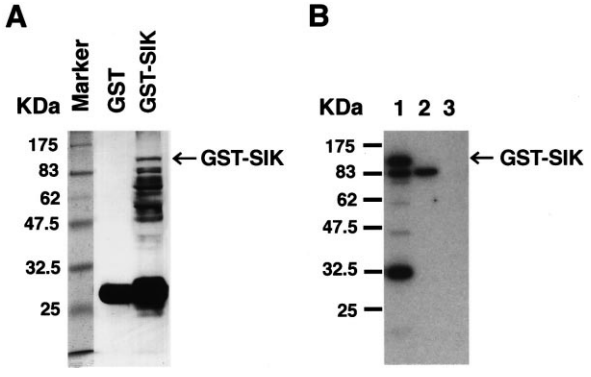

Fig. 4. Expression and autophosphorylation of recombinant protein GST-SIK. A: GST and fusion protein GST-SIK were expressed in *E. coli* BL21 cells, and purified by affinity adsorption to glutathione-Sepharose 4B beads. Partially purified GST and GST-SIK were separated by 12% SDS-PAGE and analyzed by Western blot using anti-GST antibody. B: In vitro kinase assay. An aliquot (~10 μg) of the partially purified GST-SIK was incubated with kinase buffer in the presence of 370 kBq [γ-^{32}P]ATP. The reaction mixture was resolved by 12% SDS-PAGE and visualized by autoradiography for 12 h. Lane 1, GST-SIK. Lane 2, heat-denatured GST-SIK. Lane 3, GST-SIK incubated with an excess amount (1 mM) of cold ATP before incubation with [γ-^{32}P]ATP. Positions of protein standards (kDa) are indicated on the left.

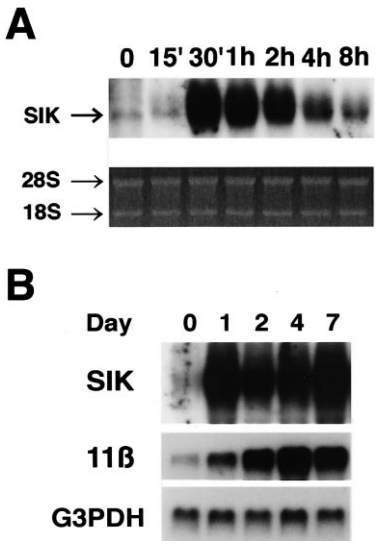

Fig. 5. Effects of ACTH on SIK mRNA levels. A: Y1 mouse adrenocortical tumor cells were treated with ACTH (1 μM). The total RNAs (10 μg) extracted at different time points (as indicated) after addition of ACTH were hybridized with a ^{32}P-labeled *Xho*I/*Eco*RI cDNA fragment of SIK. Ethidium bromide staining is placed below, showing that an equivalent amount of total RNAs was loaded in each lane. B: Rats were treated with daily injections of 0.1 mg ACTH for 1, 2, 4 or 7 days, or without ACTH treatment (0 day). Total RNAs (10 μg) extracted from their adrenal glands were hybridized with a ^{32}P-labeled *Xho*I/*Eco*RI cDNA fragment of SIK, or cDNA probes specific for rat P450(11β)s, or rat G3PDH.

To determine whether SIK protein has kinase activity, an in vitro kinase assay was performed using GST-SIK fusion protein as described in Section 2. Three major proteins were phosphorylated in the incubation mixture of GST-SIK with γ-^{32}P-labeled ATP (Fig. 4B). The largest one with a molecular mass of approximately 112 kDa appeared to be the phosphorylated intact GST-SIK fusion protein. In contrast, the incubation mixture of heat-denatured GST-SIK with γ-^{32}P-labeled ATP did not present the radiolabeled protein of the same molecular mass, nor the incubation mixture of GST-SIK in the presence of excess amount of cold ATP. These results suggest that SIK protein could be enzymatically phosphorylated by its autophosphorylation activity.

Northern blot analysis of SIK mRNA expression in various tissues from high K^+-treated rat did not present clear bands in the tissues other than adrenal. This may be due to a poor content of the message or the unstable nature of SIK mRNA. However, the expression of SIK mRNA was detected by RT-PCR in a number of high K^+-treated rat tissues other than adrenal glands such as heart, kidney, stomach, lung, testis and ovary (data not shown). This result suggests that SIK may be a ubiquitously expressed protein.

3.3. ACTH stimulates SIK mRNA expression both in vitro and in vivo

It is well known that ACTH stimulates steroidogenesis and/or cell proliferation in adrenal cortex. To examine whether or not SIK mRNA expression could be influenced by ACTH,

SIK mRNA levels were analyzed by Northern blotting on both ACTH-treated Y1 mouse adrenocortical tumor cells (in vitro) and ACTH-treated rat adrenal glands (in vivo). When Y1 cells were treated with ACTH (1 μM), SIK mRNA levels were significantly increased and reached a maximum within 30 min, then returned to the original level after 8 h (Fig. 5A). It should be noted that the ACTH-stimulated transcription of SIK occurred much earlier than that of steroidogenic enzymes. Considering that SIK protein may be a rapid turnover protein as suggested by the existence of a PEST region in its primary structure, it is possible that SIK may play a role in regulating the ACTH-stimulated acute steroidogenic response.

When rats were treated with daily injections of 0.1 mg ACTH for 1, 2, 4, or 7 days, SIK mRNA levels in the adrenal glands were markedly elevated and maintained a significantly high level as long as ACTH was present (Fig. 5B). These results suggest that SIK protein might be involved in both acute and chronic ACTH actions on adrenal cortex.

High Na^+ or high K^+ intake disturbs the animal's plasma salt balance, resulting in the modulation of its adrenocortical functions [20]. The phenomenon that SIK mRNA in the rat adrenal glands was significantly induced by a high salt diet may be explained by the assumption that SIK is involved in the salt-mediated modulation of adrenocortical function. An alternative possibility is that SIK is induced in the adrenal cortex by plasma ACTH that may be elevated as a stress response caused by the high salt feeding [21,22].

Members of the SNF1/AMPK family, which are highly conserved between fungi, plants and animals, act as metabolic sensors that monitor cellular AMP and ATP levels, playing important roles in the response of cells to environmental and/or nutritional stresses such as heat shock, hypoxia, arsenite, deprivation of cellular energy charge [23]. The physiological functions of these enzymes are believed to protect the cell against cellular stresses that deplete ATP by switching off ATP-consuming pathways such as fatty acid synthesis and sterol synthesis, and switching on alternative ATP-producing pathways such as fatty acid oxidation. One of the target substrates of SNF1/AMPK family enzymes is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). HMG-CoA reductase is phosphorylated and inactivated by activation of SNF1/AMPK, resulting in the inhibition of sterol synthesis [24–26]. Recent studies in our laboratory have shown that expression of P450scc mRNA in ACTH-treated Y1 cells is weakened by overexpression of SIK in the cells (Lin et al., unpublished data). Taken together, it is possible that SIK may play a negative regulatory role in ACTH-induced steroidogenesis in adrenocortical cells.

The direct evidence is not sufficient at present to draw conclusions on the physiological role of SIK. Whether the activation of SIK is related to ACTH-stimulated steroidogenesis remains to be established. Nevertheless, the activation of SIK is an important component in the adrenocortical responses to high plasma Na^+, K^+, ACTH or stress.

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