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Salt-inducible kinase-mediated regulation of steroidogenesis at the early stage of ACTH-stimulation☆

Hiroshi Takemori[a,*], Junko Doi[b], Nanao Horike[a], Yoshiko Katoh[a], Li Min[a], Xing-zi Lin[a], Zin-nong Wang[a], Masaaki Muraoka[a,c], Mitsuhiro Okamoto[a,d]

[a] *Department of Biochemistry and Molecular Biology, Graduate School of Medicine (H-1), Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

[b] *Department of Life Science, Kinran College, Suita, Osaka 565-0873, Japan*

[c] *Protein Express Co., Ltd., 2–11 Chuo-cho, Choshi, Chiba 288-0041, Japan*

[d] *Laboratories for Biomolecular Networks, Graduate School of Frontier Biosciences, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

Abstract

Salt-inducible kinase (SIK), expressed in Y1 mouse adrenocortical tumor cells at an early stage of adrenocorticotropic hormone (ACTH)-stimulation, represses the cAMP-responsive element (CRE)-dependent gene expression of CYP11A and StAR by acting on bZIP domain of CRE-binding protein. ACTH induced the SIK's nuclear to cytosolic translocation in a PKA-dependent manner. A mutant SIK in which the PKA-dependently phosphorylatable Ser577 had been replaced with Ala could not move out of the nucleus. The degree of CRE-reporter repression by SIK was strong as long as SIK was present in the nucleus. These indicated that intracellular translocation of SIK might be an important factor to determine the time-dependent change in the level of steroidogenic gene expression in ACTH-stimulated cells. Promoter analyses suggested that SIK repressed gene expressions not only of CYP11A and StAR but also of CYP11B1, CYP11B2 and SIK itself. We propose here that SIK is one of important molecule regulating expression of steroidogenic genes in the early phase of ACTH treatment.

Keywords: SIK; ACTH; CYP11A; StAR; Nuclear cytoplasmic shuttling

1. Introduction

Adrenocorticotropic hormone (ACTH) is the major stimulant for biosynthesis of steroid hormones in the adrenocortical cells [1]. The ACTH-induced steroidogenesis occurs in two consecutive phases. Cholesterol-ester stored in the cytosol is hydrolyzed immediately after the ACTH-stimulation, and then, the freed cholesterol, transported to the mitochondria and translocated from the outer to inner membranes, is subjected to the side-chain cleavage reaction. This phase proceeds by the mechanism not related to gene transcription. The late phase of ACTH-stimulation results from the transcriptional activation of steroidogenic genes [2,3]. Although numerous studies have focused on the structure and function of these genes, the mechanism underlying the delayed initiation of gene transcription is still unclear. ACTH, by means of the signal transduction machinery including the specific receptor and G-protein, activates membrane-associated adenylate cyclase. The activated adenylate cyclase generates cAMP, which then activates cAMP-dependent protein kinase (PKA). The delayed response to ACTH-stimulation is thought to occur as the result of PKA activation [4].

Salt-inducible kinase (SIK) was identified as a specific kinase induced in the adrenal glands of rats fed a high-salt diet [5,6]. SIK belongs to a family of AMP-activated protein kinase, a serine/threonine protein kinase which plays important roles in regulating metabolism of cells under the stress [7]. The levels of SIK mRNA, protein and kinase

Abbreviations: SIK, salt-inducible kinase; ACTH, adrenocorticotropic hormone; cAMP, cyclic AMP; CRE, cAMP-responsive element; CYP11A, cholesterol side-chain cleavage cytochrome P450; StAR, steroidogenic acute regulatory; PKA, cAMP-dependent protein kinase; CREB, cAMP response element-binding protein; 8Br-cAMP, 8brom-cyclic AMP; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione-*S*-transferase; pS577, phospho-Ser577; bZIP, basic leucine zipper.

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* Corresponding author. Tel.: +81-6-6879-3286; fax: +81-6-6879-3289.
E-mail address: takemori@mr-mbio.med.osaka-u.ac.jp (H. Takemori).

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activity in Y1 mouse adrenocortical tumor cells were elevated within 30 min after the ACTH-stimulation, and returned to the initial levels after a few hours [8]. The initiation of transcription of StAR protein- and CYP11A-genes appeared to coincide with the decline of the SIK levels. Overexpression of SIK in Y1 cells lowered the level of the ACTH-induced transcription of CYP11A gene [8]. Reporter analyses of the human CYP11A promoter revealed that SIK repressed the PKA-induced activation of the CYP11A gene promoter by acting on cAMP-responsive element-binding protein (CREB) bound to a cAMP-responsive element (CRE) [9].

The suppression of ACTH-induced transcription of the StAR gene in SIK-overexpressing cells was also observed, but the time course of its manifestation seemed to differ from that of the CYP11A gene [8,10]. Two hours after the addition of ACTH, the level of StAR mRNA in the SIK-overexpressing cells was elevated to a similar level as that in the control cells, but the level was markedly suppressed after 12 h. This may imply that the time course of StAR gene expression during the ACTH-activation might be divided into two consecutive phases; the first in which SIK seemed not capable of exerting its repressive effect on the StAR gene, and the second in which SIK exerted its repressive activity.

2. Nucleocytoplasmic shuttling of SIK occurs through cAMP/PKA signaling

Immunocytochemical analyses demonstrated that SIK was localized both in the nuclear and cytoplasmic compartments of Y1 cells, but, after cells were stimulated by ACTH, the nuclear SIK rapidly moved to the cytoplasm. This nucleocytoplasmic re-distribution was also observed when SIK was expressed as a green fluorescence (GFP) fusion protein. As shown in Fig. 1, 10^{-11} M of ACTH could induce nucleocytoplasmic translocation of GFP-SIK, which coincided with the activation of cAMP/PKA signaling [8]. Moreover, the overexpression of PKA [10] induced nucleocytoplasmic re-distribution of GFP-SIK even in PKA-less Kin-7 cells [4], suggesting that ACTH-induced nucleocytoplasmic shuttling of SIK resulted from the activation of cAMP/PKA signaling.

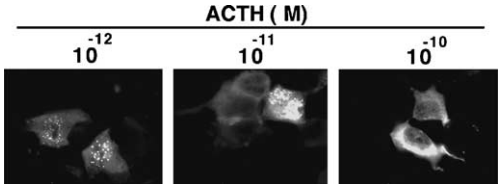

Fig. 1. Nucleocytoplasmic shuttling of SIK in Y1 mouse adrenocortical tumor cells. Y1 cells, transformed with overexpression vector for GFP-fused SIK protein, were stimulated with ACTH (10^{-12} to 10^{-10} M) for 30 min and fixed.

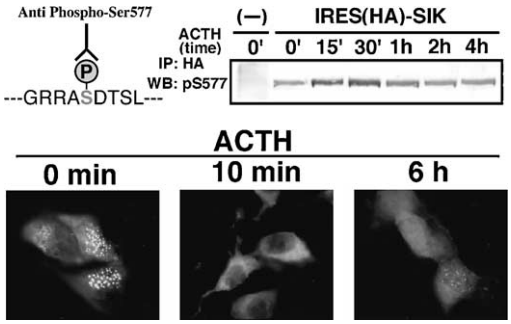

Fig. 2. Time-dependent change of the level of phospho-Ser577 and intracellular distribution of GFP-SIK. Y1 cells transformed with an expression plasmid for HA-tagged SIK were treated with or without ACTH (10^{-6} M) for indicated periods, and then lysed. HA-tagged SIK protein was immunoprecipitated using anti-HA-tag IgG and protein G-sepharose. The aliquots of immunopurified SIK were subjected to Western blot analyses with anti-phospho-Ser577 IgG [10]. Y1 cells, transformed with overexpression vector for GFP-fused SIK protein, were stimulated with ACTH (10^{-6} M) for indicated periods and fixed (lower pictures).

3. PKA phosphorylates SIK at Ser577

There are three consensus PKA-dependent phosphorylation motifs, R/K-R/K-X-S/T, in SIK protein; the respective motif contains a phosphorylatable amino acid residue, Thr268, Thr475 or Ser577. The experiments performed by site-directed mutagenesis indicated that the disruption of Ser577 abolished the ACTH-induced nuclear export of SIK. The results of immunochemical analyses using pS577-specific IgG showed that PKA could phosphorylate Ser577 in vivo and in vitro [10]. The time-dependent change of phosphorylation level at Ser577 during the ACTH-stimulation well correlated with the time-dependent cellular distribution of SIK (Fig. 2). This suggested that the PKA-dependent phosphorylation of Ser577 was an important step for the nuclear export of SIK.

4. Correlation between SIK's nucleocytoplasmic shuttling and repression of PKA-induced CRE activity

The kinase activity of SIK was essential for the repression of PKA-induced CRE activity. In Y1 cells, however, the kinase activity was constitutive and did not change after ACTH treatment, suggesting presence of unknown mechanisms underlying the modification of the CRE-repressive activity. The correlation between the intracellular location of SIK and the SIK-dependent repression of ACTH- or forskolin-induced CRE activity was examined. The SIK's ability to suppress the forskolin-dependent CRE activation was stronger during the period when most SIK existed in the nucleus than the period when it was present in the cytoplasm (Fig. 3). The nuclear SIK, S577A mutant, almost completely repressed CRE activity at any time points during the treatment. These results suggested that SIK repressed CRE in the nucleus,

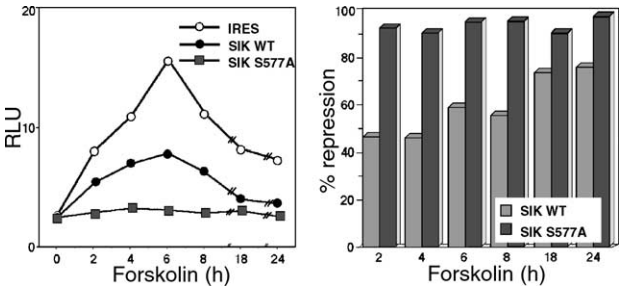

Fig. 3. Nuclear SIK represses ACTH-induced CRE activity Y1 cells were transformed with pTAL-CRE-reporter, SIK expression vectors (pIRES-SIK (WT), pIRES-SIK (S557A) or pIRES), and pRL-SV40 internal control. After 12 h forskolin (20 μM) was added to the cells, and they were incubated for indicated periods and harvested for luciferase activities in the Dual-Luciferace Reporter Assay System. Transformation efficiencies were corrected by Renilla luciferase activities (left panel). CRE-luciferase activity. Percentage repression of CRE-luciferase activity by co-expression of wild type SIK and nucleus SIK (S577A) was indicated (right panel).

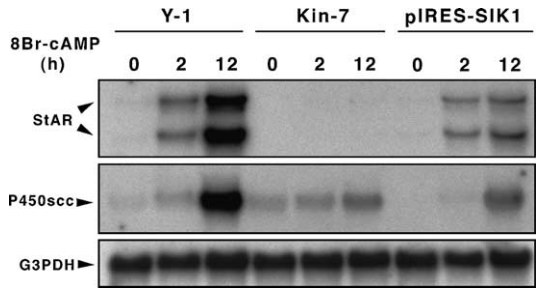

Fig. 4. Expression profiles of StAR and P450scc mRNA in wild, PKA-less and SIK-overexpressing Y1 cells. Y1, Kin-7 (PKA-less) and SIK-overexpressing Y1 (pIRES-SIK1) were treated with or without 8Br-cAMP (1 mM) for 2 or 12 h. Total RNAs (10 μg) extracted from the cells were subjected to Northern blot analyses using StAR, P450scc and G3PDH-cDNA fragments as probes.

and PKA, by phosphorylating SIK at Ser577, attenuated the SIK-mediated repression.

5. Regulation of StAR promoter activity by SIK

The cAMP-induced StAR gene expression is also regulated by CREB and related nuclear factors [11]. The effect of forced expression of SIK in Y1 cells on the cAMP-dependent elevation of StAR mRNA level was examined. Interestingly, 2 h after the cAMP-stimulation the level of StAR mRNA in the SIK-overexpressing cells appeared to be similar to that in the control cells. However, after 12 h the level in the SIK-expressing cells was clearly repressed compared to that in the control cells (Fig. 4). Moreover, the expression profiles of StAR- and CYP11A-mRNAs induced by cAMP in SIK-overexpressing Y1 cells differed from those in PKA-less Kin-7 cells. These suggest that the previously presented model for the down regulation of CREB by SIK [9] is not enough to explain these results of SIK-overexpressing Y1 cells. We, therefore, asked whether the time-dependent change in the StAR mRNA level of cAMP-stimulated SIK-expressing cells could well reflect the time-dependent change in the intracellular location of SIK. Similarly to the results obtained for CRE-reporter (Fig. 3), the human StAR promoter [12] was also affected by SIK-overexpression in a time-dependent manner, and the nuclear SIK (S577A mutant) strongly repressed PKA-induced StAR promoter activity. The disruption of the CRE-like element that overlapped with the proximal SF-1 site abolished both PKA and SIK actions [10]. These results indicate that SIK may be one of the important factors regulating the early phase of StAR gene expression induced by PKA, and PKA also modulates repressive function of SIK. Taken together, the mechanism of PKA-dependent regulation of StAR gene expression should be considered by including the action of SIK in the very early phase of ACTH-stimulation, and, therefore, would be more complicated than the mechanism proposed before [13].

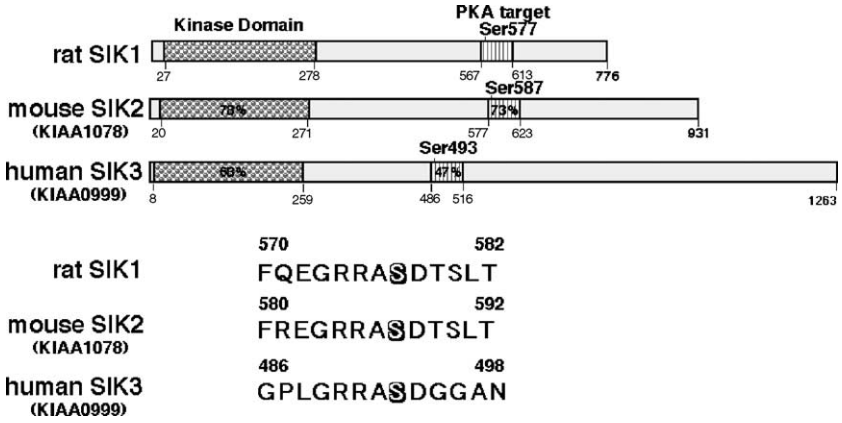

Fig. 5. Comparison of SIK family kinases. Overall structures of rat SIK1 (GenBank accession no. AB020480), mouse SIK2 (GenBank accession no. AB067780) and human SIK3 (GenBank accession no. BAA76843) were depicted. All kinases have the kinase domains at the N-terminal sides and the PKA-phosphorylation motifs in the C-terminal domains.

The generally similar results were also observed in the cases for the CYP11B1- and CYP11B2-promoters. However, the site of action of SIK on CYP11B-promoters seemed to differ from the CRE (Ad1) previously reported to be essential for the cAMP-PKA action. Moreover, we should point out here that overexpression of SIK in Y1 cells repressed the basal level of CYP11A mRNA (Fig. 4), suggesting multiple target sites of SIK on the promoters of steroidogenic genes.

6. SIK family kinases have PKA phosphorylation site in the C-terminal domain

Recent genome projects revealed at least two more human proteins having putative kinase domains highly similar to that of rat SIK, KIAA1078 (GenBank accession no. BAA83030) and KIAA0999 (GenBank accession no. BAA76843). We succeeded to clone a functionally active mouse homolog of KIAA0178 (GenBank accession no. AB067780) [14] and a human clone coding KIAA0999, and named them SIK2 and SIK3, respectively (Fig. 5). These three kinases have the PKA phosphorylation motifs in their C-terminal regions. All three kinases are present in the adrenal glands in distinct amounts. Therefore, the time-dependent regulation of steroidoigenic gene expression should be analyzed in the future by including the roles played by these SIK isoforms.

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