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cAMP-dependent proteolysis of GATA-6 is linked to JNK-signaling pathway

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ABSTRACT

A JNK inhibitor SP600125 inhibited cAMP-dependent proteolysis of GATA-6 by proteasomes around its IC50. We further examined the effects of SP600125 on the degradation of GATA-6 in detail, since an activator of JNK (anisomycin) is available. Interestingly, anisomycin immediately stimulated the export of nuclear GATA-6 into the cytoplasm, and then the cytoplasmic content of GATA-6 decreased slowly through degradation by proteasomes. Such an effect of anisomycin was inhibited by SP600125, indicating that the observed phenomenon might be linked to the JNK signaling pathway. The inhibitory effect of SP600125 could not be ascribed to the inhibition of PKA, since phosphorylation of CREB occurred in the presence of *dbc*AMP and SP600125. The nuclear export of GATA-6 was inhibited by leptomycin B, suggesting that CRM1-mediated export could be activated by anisomycin. Furthermore, it seems likely that the JNK activated by anisomycin may stimulate not only the nuclear export of GATA-6 through CRM1 but also the degradation of GATA-6 by cytoplasmic proteasomes. In contrast, A-kinase might activate only the latter process through JNK.

1. Introduction

Transcription factor GATA-6, originally named gastric GATA-GT1 [1,2], is essential for the development of and tissue-specific gene expression in mammals [3]. GATA-6 stably expressed in CHO-K1 cells is degraded by proteasomes upon elevation of the cytoplasmic cAMP concentration [4]. It has been suggested by inhibitor studies that this signaling pathway is mediated by PKA, but not by protein kinase C, MAPK (MAPK/ERK kinase and p38), Ca^{2+}/calmodulin-dependent protein kinase II, or Src kinase [5]. A similar degradation pathway has been demonstrated for the proteolysis of Sp1 in NRK cells [6,7]. Although the phenomenon of cAMP-dependent proteolysis was evidently found in mammalian cultured cells, its physiological role has not been unveiled.

The degradation of GATA-6 occurs at least in the cytoplasm, as we reported that GATA-6 fixed on the cytoplasmic face of the endoplasmic reticulum membrane is degraded similarly to that expressed in the nucleus [8]. We also obtained mutant clones in which GATA-6 is not degraded even in the presence of *dbc*AMP [9]. Although it has been suggested that PKA cross-talks with other intracellular signaling pathways [10], nothing is known about the component molecules that participate in the signaling pathway for GATA-6 degradation except cAMP and PKA.

In this study, we examined whether or not the JNK signaling pathway participates in the cAMP-dependent proteolysis of GATA-6 since a JNK inhibitor, SP600125 [11], inhibited the proteolysis. JNK is a member of the MAPK group of signaling proteins, and is known to phosphorylate and activate some activator protein-1 transcription factors and other cellular factors that regulate gene expression and cellular growth [12]. Since the activation of JNK seems to be closely related to the pathogeneses of human diseases such as inflammatory, vascular, neurodegenerative, metabolic and oncological diseases, drug discovery targeting JNK has been of keen interest [13].

Interestingly, we found that anisomycin, an activator of JNK [14,15], stimulates the nuclear export of GATA-6 through CRM1 rapidly, and then further enhances the proteolytic degradation of GATA-6 by proteasomes. Such a novel finding will be discussed from the viewpoints of both the intra-cellular localization of GATA-6 and GATA-mediated gene regulation.

2. Materials and methods

2.1. Materials

FBS was obtained from Gibco BRL. Ham F-12 and *dbc*AMP were purchased from Sigma. The SCADS inhibitor kit III was provided by

Abbreviations: CHO, Chinese hamster ovary; CREB, cAMP-response element-binding protein; CRM1, chromosome maintenance region 1; *dbc*AMP, dibutyryl cAMP; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; Ig, immunoglobulin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MG115, benzyloxycarbonyl-Leu-Leu-norvalinal; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NES, nuclear export signal; PBS, 10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl; PKA, cAMP-dependent protein kinase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SP600125, 1,9-pyrazoloanthrone.

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the Screening Committee on Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan [16]. HRP-linked donkey anti-rabbit Ig was purchased from Amersham Biosciences. Leptomycin B and MTT were obtained from Calbiochem and Nacalai Tesque, respectively. SP600125 and anisomycin were provided by Alexis Biochemicals. MG115 and oligonucleotides were from the Peptide Institute and Hokkaido System Science, respectively. All other chemicals used were of the highest grade commercially available.

2.2. Cell Culture

CHO-K1 cells (1.5×10^6 cells in a 100 mm dish) stably expressing rat GATA-6Δ50 (tc1–17a cells) [4] were cultured in Ham F-12 medium containing 7% (v/v) FBS at 37 °C. Cell viability in the presence of anisomycin was determined by means of the MTT assay as previously reported [17]. Briefly, tc1–17a cells were seeded onto a 24-well plate (5×10^4 cells/well). After 24 h, the medium was changed to fresh medium containing various concentrations of anisomycin, followed by further culture for 24 h. The cells were washed with PBS, and then 0.5 ml of an MTT solution (0.5 mg/ml) was added in each well, followed by reaction for 3 h. After washing with PBS (0.5 ml), 200 μl of DMSO was added to dissolve the formazan crystals. The obsorbance at 570 nm was measured with a microplate reader (Benchmark, BIO-RAD).

3. Knockdown of CRM1 mRNA

To construct a vector expressing CRM1 siRNA, a synthetic oligonucleotide cassette (5′-GATCCGGAGCCCAGCAAAGAATGGTTCAAGAGACCATTCTTTGCTGGGCTCCTTA-3′ and 5′-AGCTTAAGGAGCCCAGCAAAGAATGGTCTCTTGAACCATTCTTTGCTGGGCTCCG-3′) encoding siRNA targeting CRM1 [18] was inserted between the *Bam*HI and *Hin*dIII sites of the pSilencer 2.0-U6 vector (Ambion). The resulting vector, pSilencer 2.0-U6-CRM1, was introduced transiently into tc1–17a cells in the presence of Lipofectamine 2000 (Invitrogen) [19]. The molecular biological methods used for DNA manipulations were based on standard procedures [20].

4. Immunoblotting Analysis of GATA-6

Cells treated with both *dbc*AMP (2 mM) and a kinase inhibitor (1 μM) for 24 h were collected to prepare a nuclear extract by the published method [5]. Briefly, cells were washed with PBS and then scraped into 1 ml PBS with a rubber policeman. The cells were precipitated in a micro-centrifuge (3000 rpm) for 2 min at 4 °C, and then suspended in 200 μL Buffer A [10 mM HEPES-KOH (pH 7.6), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF]. After incubation for 10 min at 4 °C, the suspension was mixed with a vortex mixer, and then centrifuged (3000 rpm) for 10 min at 4 °C. The nuclear pellet was suspended for 30 min at 4 °C in 50 μl Buffer C [20 mM HEPES-KOH (pH 7.6), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 25% (v/v) glycerol]. The suspension was centrifuged (15,000 rpm) for 2 min at 4 °C, and the supernatant was stored at −80 °C until use. Protein concentrations were determined with BCA Protein Assay (Pierce) using bovine serum albumin (Fraction V, Sigma) as a standard [21].

Proteins (10 μg) were subjected to SDS–polyacrylamide gel-electrophoresis [7.5% (w/v) separation gel] [22], and then electroblotted onto an Immobilon™-P membrane (Millipore) [23], and detected with an Amersham™ ECL Western Blotting analysis system [1:2,000 dilution of anti-GATA-6 antibodies [4] and anti-CRM1 antibodies (Santa Cruz, sc-5595), and 1:5,000 dilution of HRP-linked donkey anti-rabbit Ig].

5. Results

5.1. Effect of JNK inhibitor SP600125 on cAMP-dependent proteolysis of GATA-6

We previously reported that the activation of PKA by *dbc*AMP and cholera toxin resulted in the degradation of GATA-6 by proteasomes [4,5]. However, nothing is known about the component molecules communicating between cAMP and proteasomes except for PKA. We anticipate cross-talk between PKA and other protein kinases that we have not examined. Thus, we examined the effects of kinase inhibitors (SCADS inhibitor kit III) [16] on cAMP-dependent proteolysis of GATA-6 using tc1–17a cells, which is a CHO-K1 clone stably expressing GATA-6Δ50 [4].

Among 95 kinase inhibitors [16], H-89 (PKA inhibitor) [24] exhibited an inhibitory effect, as we previously reported [5], and another PKA inhibitor, 4-cyano-3-methylisoquinoline [25], also inhibited the proteolysis (not shown). These results confirmed that PKA participates in the proteolysis of GATA-6. Interestingly, SP600125 [11], an inhibitor of serine-threonine kinase JNK [12], strongly inhibited GATA-6 degradation in the presence of *dbc*AMP since it was effective at the concentration as low as 0.01 μM around its IC50. (Fig. 1A). These results suggest that PKA together with JNK could be components responsible for signal-induced proteolysis of GATA-6, and that JNK could be located downstream of PKA.

5.2. Phosphorylation of CREB in the presence of SP600125

As SP600125 inhibited cAMP-dependent proteolysis of GATA-6 (Fig. 1A), we determined whether this inhibitor inhibited PKA *per*

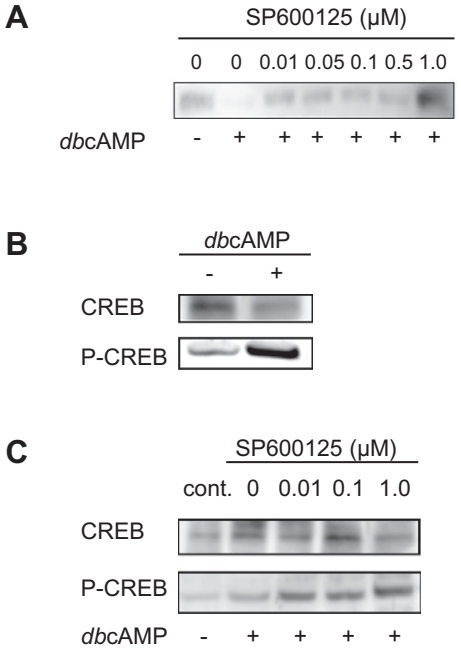

Fig. 1. Effects of SP600125 on cAMP-dependent proteolysis and the phosphorylation of CREB. (A) tc1–17a cells were treated with 2 mM *dbc*AMP and each concentration of SP600125 for 24 h. Nuclear extracts were prepared as described under Materials and Methods. GATA-6 was detected by means of Western Blotting with anti-GATA-6 antibodies [4]. (B) tc1–17a cells were treated with 2 mM *dbc*AMP to phosphorylate CREB through A-kinase for 24 h, and then phosphorylated and unphosphorylated CREB (P-CREB and CREB, respectively) in the nuclear extract were detected by means of Western Blotting using specific antibodies for both types of CREB. (C) Each concentration of SP600125 was added together with *dbc*AMP as in (B).

se or not. A typical target of PKA is CREB [26], and actually the phosphorylation of CREB increased on *db*cAMP treatment under our experimental condition (Fig. 1B). Furthermore, the phosphorylation of CREB was not inhibited even in the presence of SP600125, as shown in Fig. 1C. These results strongly suggested that the inhibitory effect of SP600125 on the cAMP-dependent proteolysis of GATA-6 could not be ascribed to the inhibition of PKA. But rather it seems to be likely that one of the components stimulated by PKA could be linked to JNK.

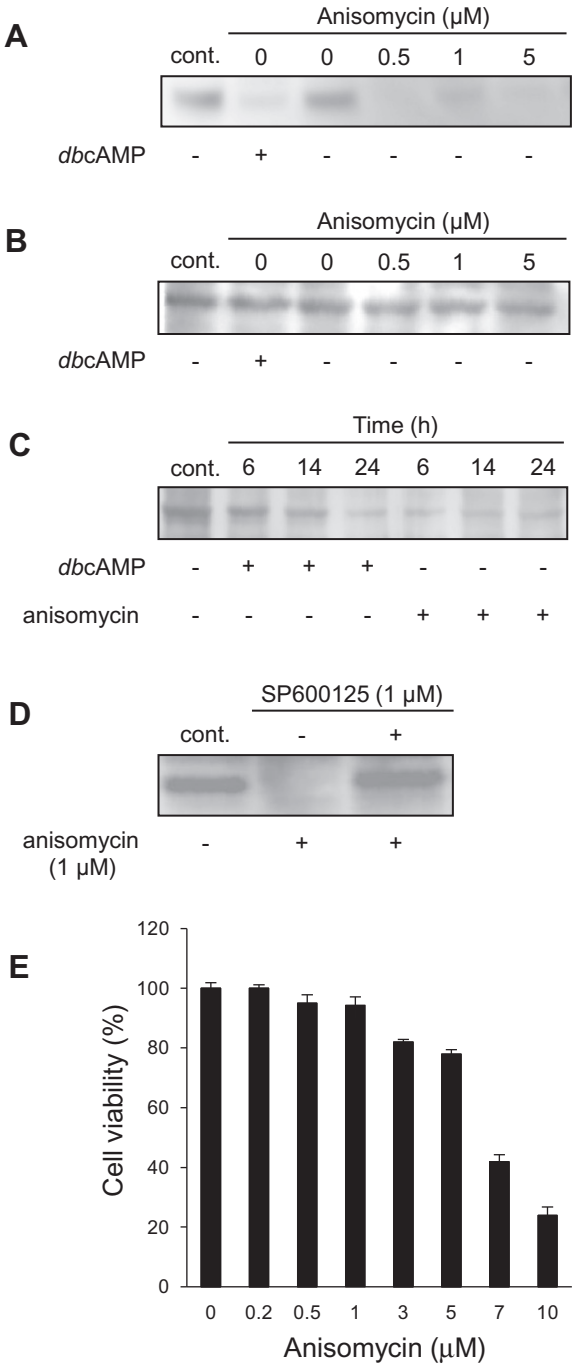

Fig. 2. Effect of anisomycin on the amount of GATA-6 in the nucleus. (A) tc1–17a cells were treated with anisomycin at the indicated concentrations for 24 h, and then GATA-6 in the nuclear extracts was detected by means of Western Blotting as described in the legend to Fig. 1. The concentration of *db*cAMP was 2 mM. (B) 37–5 cells [9] were similarly treated with anisomycin for 24 h and the nuclear GATA-6 was detected as in (A). (C) tc1–17a cells were treated with 2 mM *db*cAMP or 1 μM anisomycin for the indicated times, and then the nuclear GATA-6 was detected. (D) Anisomycin (1 μM) was added in the presence or absence of SP600125 (1 μM) for 6 h. (E) The viability of tc1–17a cells in the presence of anisomycin was determined by means of the MTT assay. The results are presented as mean values ± standard deviation ($n = 3$). The viability of 37–5 cells was essentially the same (not shown).

5.3. Stimulation of nuclear export of GATA-6 by JNK activator anisomycin

Since a JNK inhibitor inhibited the proteolysis of GATA-6, we next studied the effect of JNK activator anisomycin [14,15]. We first detected the nuclear localized GATA-6 upon adding anisomycin. After 24 h incubation of tc1–17a cells with anisomycin, the amount of nuclear GATA-6 was significantly decreased similar to in the case of *db*cAMP treatment (Fig. 2A). However, interestingly, the rate of decrease in the presence of anisomycin seemed to be different from that in the presence of *db*cAMP (Fig. 2C); anisomycin treatment rapidly reduced the amount of GATA-6 in the nucleus. Within 6 h treatment with anisomycin the level of GATA-6 became equivalent to that after 24 h treatment with *db*cAMP. Furthermore, nuclear GATA-6 decreased even 3 h after the addition of anisomycin, but it did not decrease during a similar incubation period with *db*cAMP (Fig. 3B and D left). This decrease with anisomycin was abolished in the presence of SP600125 (Fig. 2D), suggesting that JNK participated in the observed phenomenon. It must be noted that the anisomycin concentration used in this study (1 μM) did not affect the cell viability (Fig. 2E).

5.4. Effect of leptomycin B on the decrease of GATA-6 from the nucleus

To elucidate the mechanism of the decrease of nuclear GATA-6, we examined the content of GATA-6 in a whole cell upon the addition of anisomycin. As shown in (Fig. 3A and C left), the total cellular amount of GATA-6 did not change significantly during 3 h incubation with *db*cAMP or anisomycin. Thus, the initial event induced by anisomycin would be stimulation of the export of GATA-6 from the nucleus to the cytoplasm.

It is well known that CRM1-dependent nuclear export is inhibited by leptomycin B [27]. So we examined whether the rapid decrease of GATA-6 in the nucleus in the presence of anisomycin was inhibited by leptomycin B or not. As shown in Fig. 4A, leptomycin B clearly inhibited the decrease of GATA-6. However, *db*cAMP did not induce the decrease of GATA-6 in the nucleus during this short period (Fig. 4B). Furthermore, treatment of cells with an effective siRNA for CRM1 [18] inhibited the decrease of GATA-6 in the nucleus (Fig. 4C). These results indicated that anisomycin induced the CRM1-dependent nuclear export of GATA-6 through activation of JNK, while *db*cAMP, and thus PKA, did not.

5.5. Discrimination of nuclear export and proteolysis of GATA-6

We previously reported a clone (37–5 cells) that was resistant to cAMP-dependent proteolysis of GATA-6 [9]. Interestingly, this clone did not respond to anisomycin, as the nuclear content of GATA-6 did not decrease in its presence (Fig. 2B). It is evident that *db*cAMP does not induce proteolysis of GATA-6 in 37–5 cells [9] when even it activates the cytoplasmic proteasome system [8]. Thus, 37–5 cells must be defective in the process of nuclear export of GATA-6.

A proteasome inhibitor, MG115, did not inhibit the decrease of nuclear GATA-6 in the presence of anisomycin (Fig. 3D right). However, the decrease of GATA-6 in a whole cell was inhibited by MG115 (Fig. 3C right). These results suggested that the decrease of nuclear GATA-6 was not due to the action of nuclear proteasomes [28], and that the cytoplasmic GATA-6 exported from the nucleus could be degraded slowly by the cytoplasmic proteasomes. In the presence of *db*cAMP, the decreases of GATA-6 in the nucleus

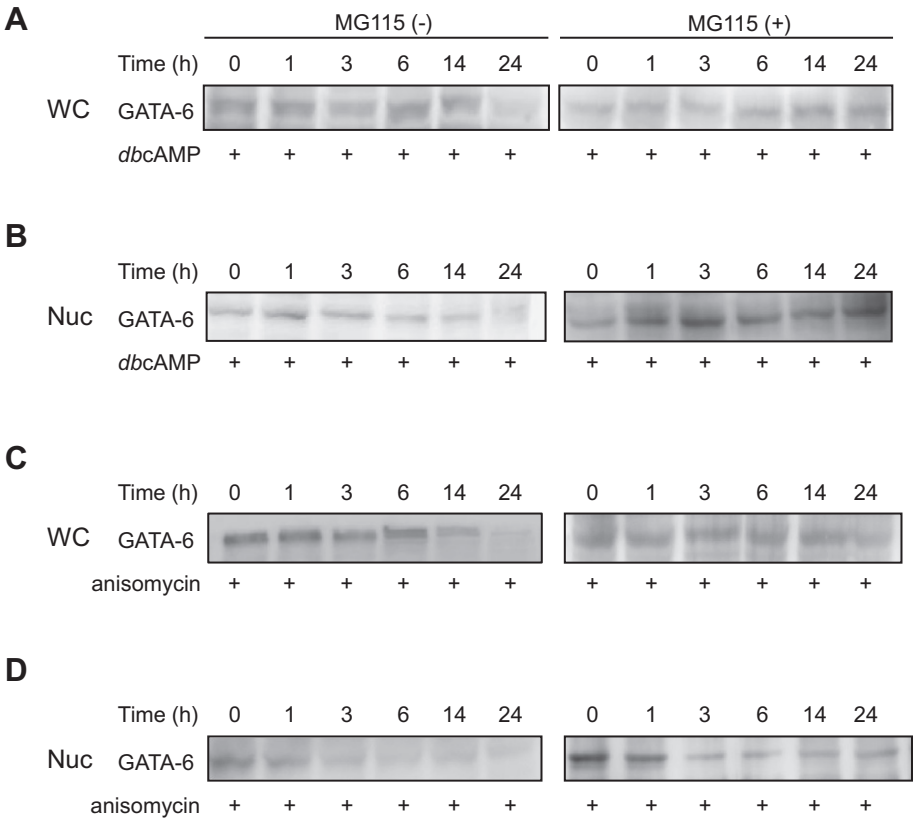

Fig. 3. Different effects of anisomycin and *dbc*AMP on the nuclear export and proteolysis of GATA-6. tc1–17a cells were pre-treated with or without MG115 (20 μM) (right and left panels, respectively), a proteasome inihibitor [4,8], for 1 h. Then, the cells were further treated with 2 mM *dbc*AMP (A, B) or 1 μM anisomycin (C, D) for the indicated times. Whole cell extracts (WC) (A, C) and nuclear extracts (Nuc) (B, D) were prepared for Western Blotting to quantitate GATA-6.

and a whole cell were not significantly different. These decreases were both inhibited by MG115 (Fig. 3A and B), indicating that the proteolysis of GATA-6 by proteasomes in the presence of *dbc*AMP could induce the exit of GATA-6 from the nucleus.

All these results suggested that anisomycin could immediately stimulate the nuclear export of GATA-6, and that a further decrease of cytoplasmic GATA-6 could occur relatively slowly. Furthermore, both the nuclear export and cytoplasmic degradation of GATA-6 are enhanced by JNK, although *dbc*AMP would primarily stimulates the latter process.

6. Discussion

JNK inhibitor SP600125 inhibited cAMP-dependent proteolysis of GATA-6 [4] at concentration around its IC50 without inhibition of phosphorylation of CREB by PKA (Fig. 1). Thus, JNK could functionally interact with PKA and participate in the GATA-6 degradation pathway. Possibly PKA could phosphorylate JNK directly or indirectly, and stimulate cytoplasmic degradation of GATA-6 by proteasomes [8].

Interestingly JNK activator anisomycin stimulated the export of nuclear-localized GATA-6 through nuclear pores since leptomycin B inhibits the process (Fig. 4). The nuclear export immediately occurred upon the addition of anisomycin (Figs. 2 and 3). Proteolysis of GATA-6 by proteasomes is also stimulated by anisomycin, since the cellular GATA-6 content decreased. Such a decrease is inhibited by a proteasome inhibitor, and rather slow compared with nuclear export. Evidently, the export *per se* is not inhibited by the proteasome inhibitor (Fig. 3).

As for the effect of *dbc*AMP, the export of GATA-6 from the nucleus was not enhanced (Figs. 2 and 3), but it seems to be rather determined by equilibration with the cytoplasmic content of GATA-6. We previously isolated mutant cells that could not degrade GATA-6 in the presence of *dbc*AMP [9]. One of these clones did not exhibit export of GATA-6 from the nucleus even in the presence of anisomycin (Fig. 2). Thus, the nuclear export and proteolysis of GATA-6 do not seem to be tightly coupled.

Leptomycin B inhibits the function of CRM1, which carries a cargo together with a Ran GTP complex [29]. It is suggested that cellular co-factors are involved in the protein nuclear export through the recruitment of the NES-CRM1-Ran GTP complex to the nuclear pore complex [30]. As for potential NES on the GATA-6 sequence, ^{328}LHGVPRPLAM^{337} in the carboxyl-terminal region of the conserved zinc finger domain could be a candidate [31], as fit with a "leucine-rich" peptide stretch harboring four hydrophobic residues, Φ^1-(x)$_{2\text{-}3}$-Φ^2-(x)$_{2\text{-}3}$-Φ^3-x-Φ^4 [29]. However, stably expressed GATA-6 is localized primarily in the nucleus [4,5], indicating that the nuclear localizing signal would be dominant. One possibility is that signal-induced exposure of the above NES would be recognized latently by the nuclear export system.

The potential phosphorylation site for JNK (nonpolar-X-Ser/Thr-Pro) [32] was found on the amino-terminal side (^{208}FETP) of the zinc-finger domain of GATA-6 [2]. It is interesting as to whether or not the phosphorylation at this site plays a role in stimulation of the accessibility of GATA-6 to the nuclear export system. Another possibility is that the export process is stimulated by the phosphorylation of some components other than GATA-6 by JNK, since GATA-6 did not show a change in mobility on a SDS–polyacrylamide gel.

Nuclear GATA-6 overexpressed in colon cancer cells contributes to silencing of the 15-lipoxygenase-1 gene and thus results in escape from apoptosis [33]. Furthermore, down regulation of

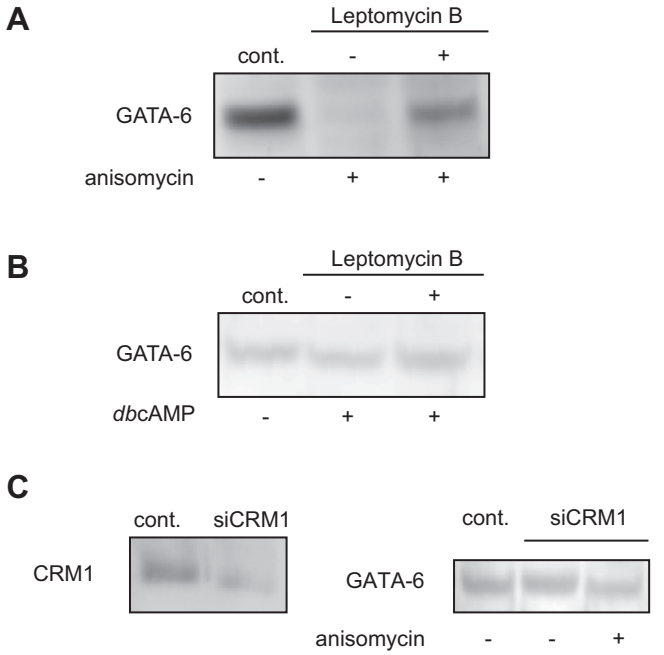

Fig. 4. Inhibition of CRM1 suppresses the decrease of GATA-6 in the nucleus in the presence of anisomycin. tc1–17a cells were pre-treated with 20 ng/mL leptomycin B for 1 h, and then incubated with 1 μM anisomycin (A) or 2 mM *dbc*AMP (B) for 3 h. The amounts of GATA-6 in the nuclear extracts were determined as described in the legend to Fig. 1. To knockdown CRM1, tc1–17a cells were transiently transfected with an expression vector of anti-sense RNA for CRM1 and cultured for 48 h (see Materials and Methods). Then the cells were further treated with 1 μM anisomycin for 3 h. The amounts of CRM1 (left) and GATA-6 (right) in the nuclear extracts were determined by means of Western Blotting as described under Materials and Methods (C).

GATA-6 expression significantly restores transcription of the 15-lipoxygenase-1 gene, and induces apoptosis in the presence of histone deacetylase inhibitor or nonsteroidal anti-inflammatory drugs [33,34]. It is interesting as to whether the GATA-6 in colon cancer cells is exported from their nuclei and degraded by proteasomes or not under these conditions, similar to our present findings in the presence of anisomycin. The nuclear export of the glucocorticoid receptor in HeLa cells and Cos-7 cells is also enhanced by JNK [32]. Thus, our results suggest that the chemicals that retain GATA-6 in the cytoplasm would be beneficial as to a therapeutic approach for colorectal cancer cells.

7. Conflict of interest

The authors have no financial conflicts of interest.

Acknowledgments

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