Gastric GATA-6 DNA-binding protein: proteolysis induced by cAMP

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Abstract The rat gastric GATA DNA-binding protein, GATA-6 (GATA-GT1), was stably expressed in CHO-K1 cells. The GATA-6 protein was localized in the nucleus but not in the cytoplasm. Interestingly, when cells were treated with dibutyryl cAMP, the GATA-6 protein was specifically degraded. Such a phenomenon was not observed in the presence of 5′-AMP or dibutyryl cGMP. The cellular level of the GATA-6 protein was restored upon removal of dibutyryl cAMP. Degradation was also induced by cholera toxin, which increased the cellular cAMP concentration, and was inhibited by a protein kinase A inhibitor. However, activators of protein kinase C did not have any effect. The degradation was inhibited by proteasome inhibitors (PSI (benzyloxycarbonyl-Ile-Glu(*O-t*-Bu)-Ala-leucinal) and MG115 (benzyloxycarbonyl-Leu-Leu-norvalinal)) but not by those of lysosomes and serine proteases. These results suggest that a kinase-mediated protein phosphorylation is the cellular signal for degradation of the GATA-6 protein. This finding constitutes a novel aspect of regulation by GATA DNA-binding proteins, which are essential for developmental processes and tissue-specific transcription.

Key words: GATA factor; DNA-binding protein; Proteolysis; Proteasome; cAMP; A kinase

1. Introduction

The GATA transcription factors in vertebrates carry tandem zinc fingers (Cys–X_2–Cys–X_{17}–Cys–X_2–Cys)–X_{29}–(Cys–X_2–Cys–X_{17}–Cys–X_2–Cys) in the central part of the molecule [1]. Previously, the primary structures of six GATA proteins were reported. They are divided into two groups: one (GATA-1, GATA-2 and GATA-3) is mainly found in blood cells [2], and the other (GATA-4, GATA-5 and GATA-6) in the heart, gastrointestinal tract and other tissues [3,4]. These GATA proteins are suggested to play important roles in tissue-specific gene expression and developmental processes: the targeted disruption of the GATA-1 [5], GATA-2 [6], GATA-3 [7], and GATA-4 [8] genes demonstrated that these genes are essential for embryogenesis. In each system, the remaining five GATA protein genes could not compensate for the deleted GATA gene, indicating that the expression program for each GATA protein could be tightly regulated in cell- and time-dependent manners.

The tandem zinc fingers are highly conserved among these GATA proteins, whereas their outside sequences are divergent [9], suggesting that they are responsible for the unique functions in transcriptional regulation of each GATA protein. We isolated GATA-4 and GATA-6 (previously named GATA-GT2 and GATA-GT1, respectively) cDNA clones from a rat stomach library in the course of a study on the cell-specific expression of the gastric proton pump [3]. The rat GATA-6 has a unique serine residue (Ser-284) potentially phosphorylated by protein kinases A and C in the tandem zinc fingers. It is interesting to determine how the kinase(s) affect the functions and cellular distribution of GATA-6. In this study, we prepared a stable cell line that expresses a truncated GATA-6 (residues 51–441), and found that this protein was degraded in response to elevation of the cellular cAMP concentration. This phenomenon was examined in detail and its biochemical significance was discussed.

2. Material and methods

2.1. A stable CHO-cell line expressing GATA-6

Rat GATA-6 cDNA (*Bst*XI fragment encoding Met-51–Ala-441) [6] was inserted into the *Eco*RV site of pBluescriptSKII(+). The *Bam*HI–*Kpn*I fragment of the resulting plasmid was substituted with the *Eco*RI–*Kpn*I fragment of mammalian expression vector pcDL-SRα (kindly provided by Dr. K. Maruyama, The Institute of Medical Science, The University of Tokyo). Deletion of the 50 amino-terminal residues did not affect the function of GATA-6, as judged with a reporter gene assay [10].

CHO-K1 cells [11] were cultured in F12 medium containing 7% (v/v) fetal bovine serum (Gibco BRL, NY) at 37°C. A stable CHO clone expressing GATA-6 (tc1-17a) was isolated by introduction of the above expression plasmid (pSRα-GT1′) together with pMAMneo (CLONTECH, CA), in the ratio of 1/10 (w/w), and selection in the presence of 1 mg/ml G418 (Sigma, St. Louis, MO). A G418 resistant clone (n-5) carrying only pMAMneo was also isolated.

2.2. Site-specific antibodies for GATA-6

A 579 bp *Hin*cII–*Eco*RI fragment encoding carboxyl terminal Thr-383–Ala-441 of rat GATA-6 was inserted between the *Sma*I and *Eco*RI sites of the pGEX-2T vector (Pharmacia Biotech, Uppsala). The construct was introduced into *E. coli* XL1-Blue and then the fusion protein with glutathione S-transferase (GST) was purified on a glutathione affinity column (Pharmacia Biotech). A rabbit was immunized with the purified protein. The site-specific polyclonal antibodies were purified by passing them through Affigel 10 (Bio-Rad, CA) conjugated with GST isolated from *E. coli* with pGEX-2T.

2.3. Detection of GATA-6 in CHO cells

Indirect fluorescence analysis was carried out by the published method [12]. Cells grown on glass coverslips were processed and then reacted with the above antibodies (1:1000 dilution), followed

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Abbreviations: CHO, Chinese hamster ovary; *db*cAMP, dibutyryl cAMP; *db*cGMP, dibutyryl cGMP; DOG, sn-1,2-dioctanoylglycerol; IP3, D-*myo*-Inositol-1,4,5-triphosphate; ECL, enhanced chemiluminescence; MG101, *N*-acetyl-Leu-Leu-norleucinal; MG115, benzyloxycarbonyl-Leu-Leu-norvalinal; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PSI, benzyloxycarbonyl-Ile-Glu(*O-t*-Bu)-Ala-leucinal

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by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:500 dilution, Zymed Laboratories, San Francisco, CA).

The whole-cell lysate and subcellular fractions (cytosol (postnuclear supernatant) and nuclear protein extracts prepared by the published method [13]) were subjected to SDS-polyacrylamide gel electrophoresis for protein staining [14] and Western blotting [15]. The proteins were electro-blotted onto nitrocellulose filters (Schleicher and Schüell, Dassel, Germany), and detected with an Amersham ECL™ Western blotting analysis system (1:2000 and 1:5000 dilutions of the GATA-6 antibodies and horseradish peroxidase-linked donkey anti-rabbit Ig, respectively). The intensities of the bands were determined using a NIH image analysis program. Gel-shift analysis involving a nuclear extract was carried out as described previously [16]. Protein was assayed with a Bio-Rad Protein Assay [17], with bovine serum albumin (fraction V, Sigma) as a standard.

2.4. Chemicals

Restriction enzymes, T4 DNA ligase, calf intestine phosphatase and the Klenow fragment were obtained from Takara Shuzo (Kyoto), Toyobo (Osaka), New England BioLabs (Beverly, MA) or Nippon Gene (Toyama). *db*cAMP and *db*cGMP were obtained from Sigma and Yamasa (Chiba, Japan), respectively. Methylamine hydrochloride and chloroquine diphosphate were from Nacalai Tesque (Kyoto). Cholera toxin and cycloheximide were from Wako (Osaka). 5′-AMP, IP3, K-252a and E-64d were supplied by Kohjin (Tokyo), Dojindo Lab. (Kumamoto), Kyowa Medex (Tokyo), and Taisho Pharmaceutical Co. (Tokyo), respectively. MG101, MG115 and PSI were purchased from Peptide Institute Inc. (Osaka). DOG and TPA were from Seikagaku-Kogyo (Tokyo) and Wako, respectively. All other chemicals used were of the highest grade commercially available.

3. Results

3.1. Expression of the GATA-6 DNA-binding protein in CHO-K1 cells

We introduced an expression plasmid for gastric GATA-6 into CHO-K1 cells and isolated several G418 resistant clones stably expressing this protein. GATA-6 was detected in the nuclear extracts from the resistant clones on gel shift analysis with site-specific antibodies (not shown). However, only one clone, tc1-17a, expressed enough GATA-6 to be detectable on Western blotting. We used this clone for further studies. GATA-6 was localized in the nucleus, as demonstrated by indirect immuno-fluorescence analysis (Fig. 1A). We also confirmed that GATA-6 was present in the nuclear protein extract, but not in the post-nuclear cytoplasmic fraction on Western blotting. The expression of GATA-6 affected neither the cell morphology nor growth behavior, such as the doubling time and saturation density. It must be noted that we could not detect endogenous GATA-6 in the control cells (n-5) on gel shift analysis (not shown) and indirect immuno-fluorescence analysis (Fig. 1C).

3.2. Effect of dbcAMP on the cellular amount of GATA-6

GATA-6 has a potential phosphorylation site (Ser-284) for a protein kinase A and a protein kinase C between the tandem zinc fingers [3,9], which prompted us to study the effects of

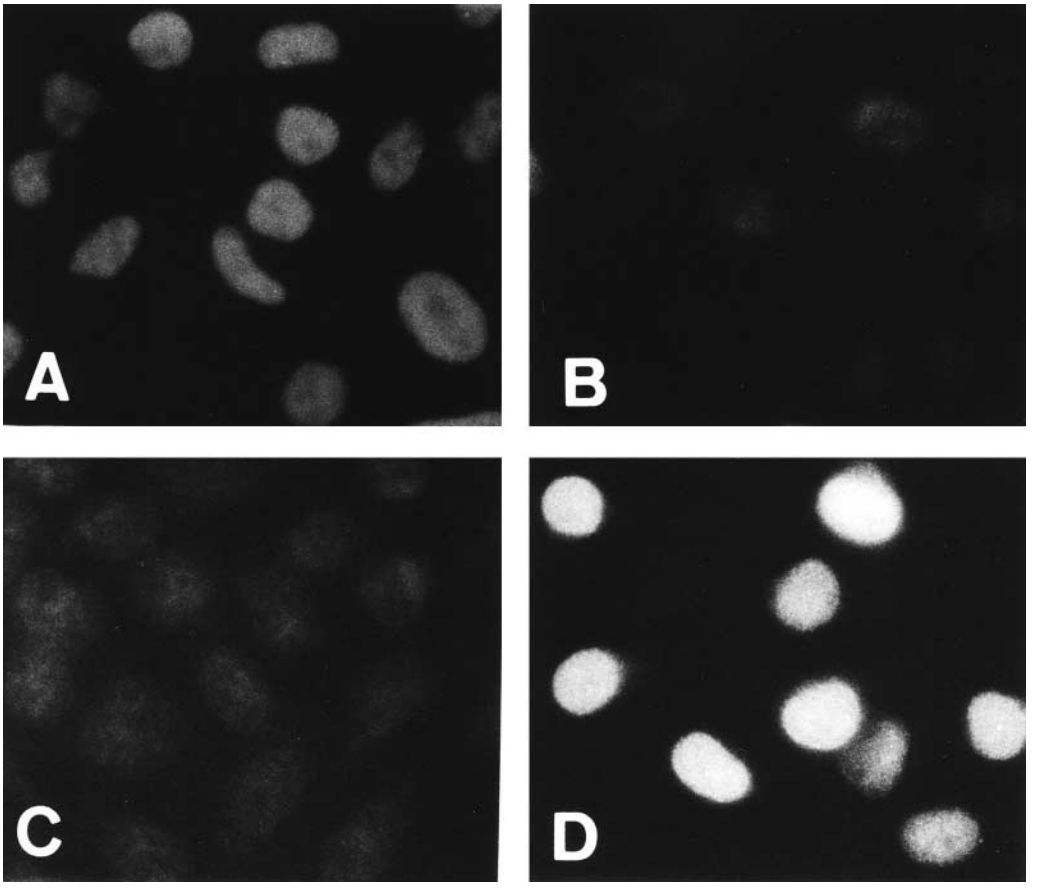

Fig. 1. Effects of *db*cAMP and a proteasome inhibitor on the nuclear localization of the GATA-6 protein. Cells, tc1-17a (A,B,D) and n-5 (C), were plated on coverslips and grown for 48 h, and then further incubated for 12 h without an addition (A,C), or with 2 mM *db*cAMP (B) or 2 mM *db*cAMP plus 50 μM MG115 (D). The cells were fixed, and the GATA-6 protein was visualized by indirect fluorescence analysis using site-specific GATA-6 polyclonal antibodies and fluorescein isothiocyanate-conjugated anti-rabbit antibodies.

*db*cAMP (a metabolically stable cAMP analogue) [18] on the function and intracellular localization of GATA-6. Surprisingly, immunochemical analysis revealed that *db*cAMP treatment decreased the cellular amount of GATA-6 (Fig. 1B). The effective concentration for disappearance of the GATA-6 was in the mM range (Fig. 2, top), and the decrease in GATA-6 was time-dependent (half-life, about 6 h) (Fig. 2, middle). The observed change was reversible, since on removal of *db*cAMP the initial expression level of GATA-6 was rapidly restored (Fig. 2, bottom).

The decrease in GATA-6 was specific for *db*cAMP, since similar concentrations of 5′-AMP and *db*cGMP did not cause such a decrease (Fig. 3, top left). Treatment with cholera toxin, which is known to increase the cellular cAMP concentration by stimulating adenylate cyclase [19], had similar effect to *db*cAMP (Fig. 3, top right). Furthermore, the A kinase inhibitor, K-252a [20], abolished the effect of *db*cAMP (Fig. 3, middle). These results suggest that GATA-6 degradation was induced by elevation of the intracellular cAMP level and activation of protein kinase A. Chemicals which activate the protein kinase C pathway, such as IP3 [21], DOG [22], and TPA [23], did not affect the cellular amount of GATA-6 (Fig. 3, bottom). The turnover of GATA-6 decreased 2-fold with *db*cAMP: the half-lives of GATA-6 in the presence and absence of *db*cAMP were 4 and 7 h, respectively, when GATA-6 degradation was followed in the presence of cycloheximide (40 μg/ml). The degradation is specific for GATA-6, since the staining patterns of cellular proteins in the presence and absence of *db*cAMP on gel electrophoresis were the same.

3.3. *Effects of protease inhibitors*

To determine what proteases participate in the observed phenomenon, we examined the effects of various protease inhibitors added to the culture medium together with *db*cAMP. E-64d [24,25], and methylamine or chloroquine [26] did not have any effect, suggesting that neither serine proteases nor lysosomal proteases are involved in the degradation (Fig. 4, top). However, proteasome inhibitors (PSI and MG115) [24,25,27] prevented the degradation of GATA-6 (Fig. 4, bottom middle and right). This was further confirmed by the nuclear localization of GATA-6 in the MG115-treated cells (Fig. 1D). MG101 only partially prevented the degradation, possibly because its major target is calpain and not proteasome [24] (Fig. 4, bottom left).

4. Discussion

Our results clearly suggested that GATA-6 was specifically degraded by proteasome. This phenomenon was induced by an intracellular signal transduction system related to a protein kinase A. Gastric GATA-6 plays a role in the transcriptional activation of the genes for proton pump subunits [9]. The acid secretion by the proton pump was stimulated by an increase in the cytoplasmic cAMP concentration through the G-protein-coupled H2 receptor [28]. Thus, the loss of GATA-6 through its degradation induced by cAMP could be a kind of negative feedback mechanism for reducing the proton pump protein and hence acid secretion.

Degradation of transcription factors by proteasome has

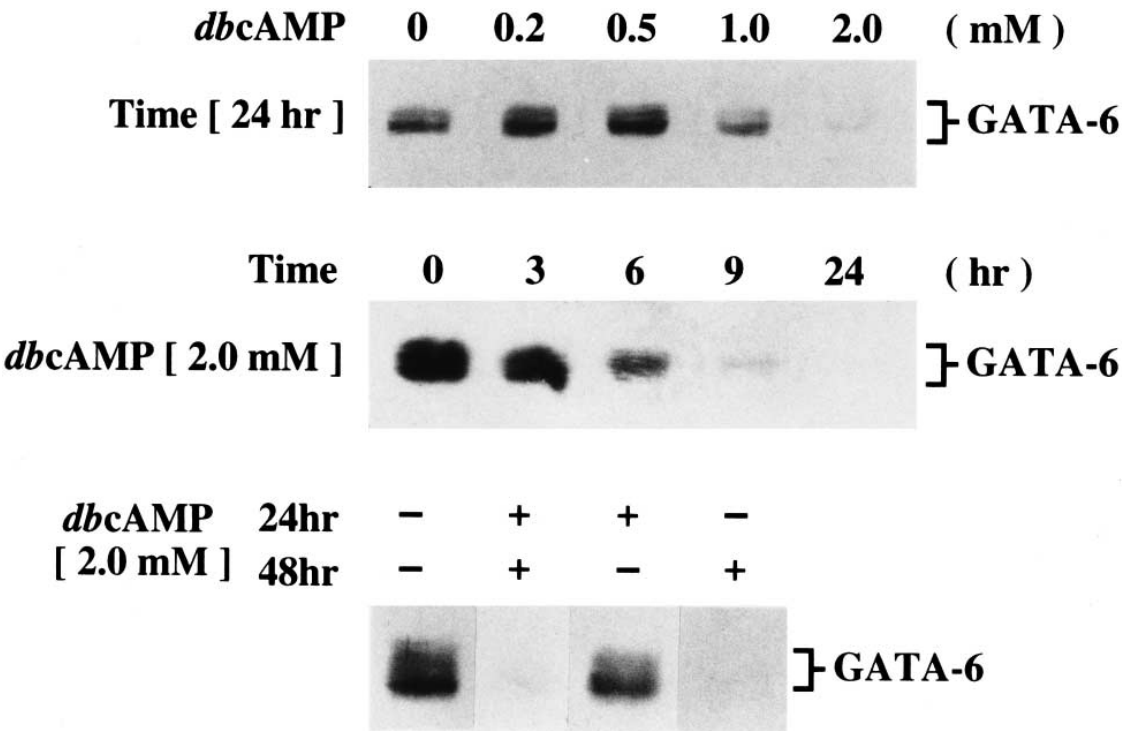

Fig. 2. Detection of the GATA-6 protein in tc1-17a cells cultured in the presence or absence of *db*cAMP. tc1-17a cells (1×10^5 cells/6 cm diameter dish) were plated and cultured for 24 h. Then they were incubated for 24 h in the presence of various concentrations of *db*cAMP (top) and for the indicated time periods with 2 mM *db*cAMP (middle). The cells incubated for 24 h with (+) or without (−) 2 mM *db*cAMP were further treated with (+) or without (−) 2 mM *db*cAMP in fresh medium for 24 h (bottom). Whole-cell lysates (10 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. The GATA-6 protein was reacted with site-specific GATA-6 polyclonal antibodies and then detected by means of ECL.

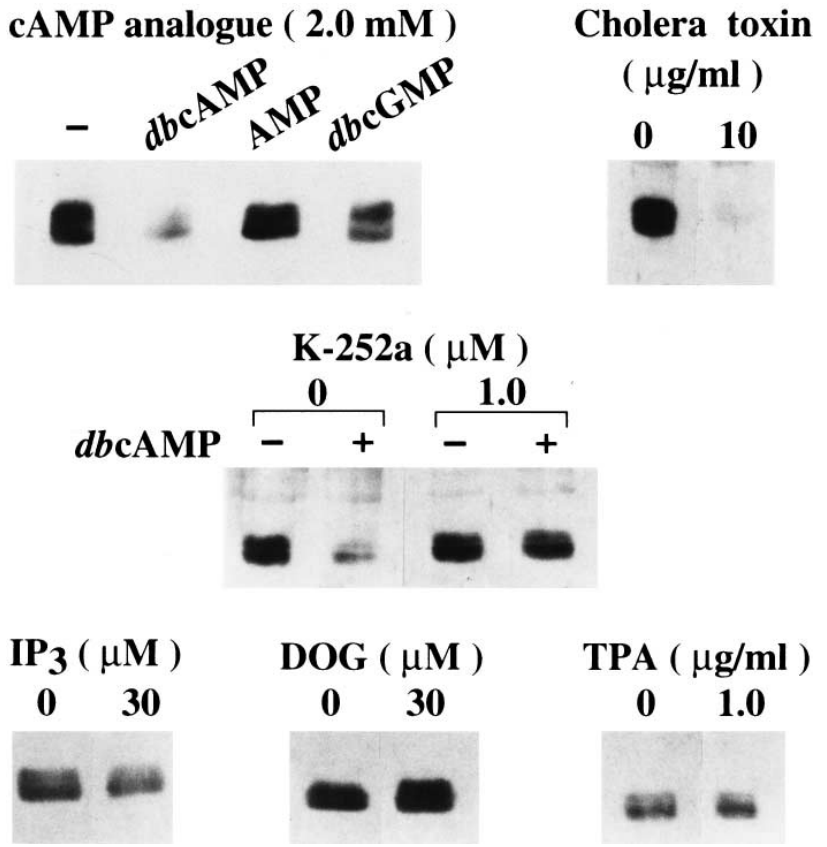

Fig. 3. Effects of various compounds related to protein kinases on the cellular behavior of GATA-6. tc1-17a cells were treated with *db*cAMP or related compounds, or cholera toxin for 24 h at the indicated concentrations (top). A protein kinase A inhibitor, K-252a, was added to the medium in the presence (+) or absence (−) of 2 mM *db*cAMP (middle). The cells were cultured with compounds related to the protein kinase C activation pathway for 24 h (bottom). The GATA-6 protein was analyzed as described in the legend to Fig. 2. The slight decrease with *db*cGMP could be due to the presence of a small amount of mono butylic cAMP in the reagent.

been reported for those playing roles in cell growth [29]. However, our results suggest that the factors (GATA proteins) involved in the processes of cell-specific gene regulation and development are another type of substrates for proteasome. This novel finding seems to be unique, and constitutes a new regulatory aspect of transcriptional factors. The contribution of proteasome to GATA-6 is different from that to NF-kB, since the phosphorylation of an inhibitory factor, IκB, triggers its degradation and the nuclear localization of an active form of NF-κB from the cytoplasm [30].

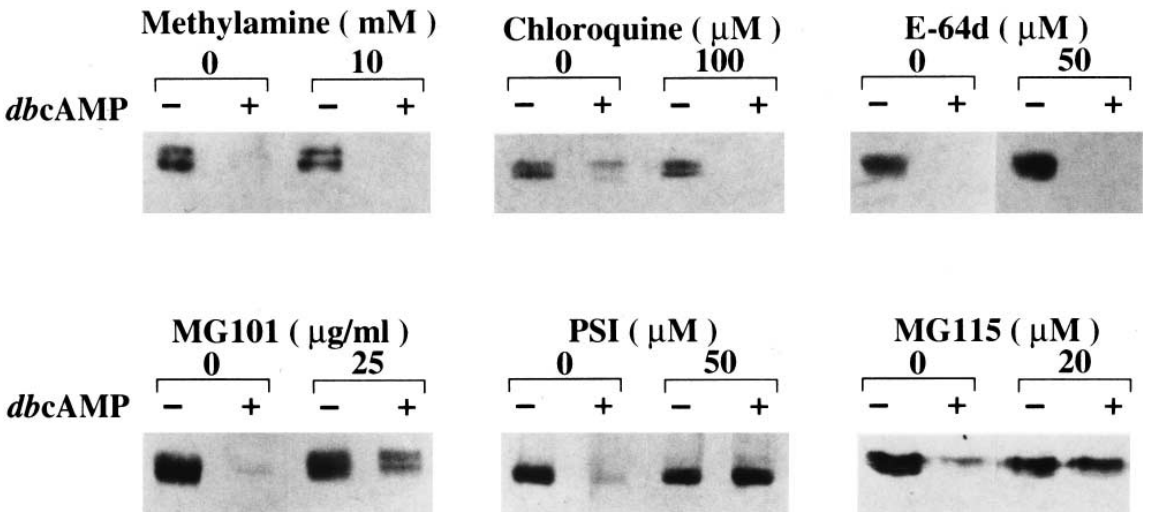

Fig. 4. Effects of various protease inhibitors on degradation of the GATA-6 protein. tc1-17a cells were treated with each protease inhibitor at the indicated concentrations for 12 h in the presence (+) or absence (−) of 2 mM *db*cAMP. The GATA-6 protein was analyzed as described in the legend to Fig. 2.

Two bands of GATA-6 were observed on gel electrophoresis in this study. However, the precursor-product relationship of the upper and lower bands was difficult to determine, since they behaved almost similarly under our experimental conditions, except that the upper one was accumulated in MG115-treated cells. These bands failed to react with specific monoclonal antibody for multi-ubiquitin chains [31], suggesting that they are not polyubiquitinated. Furthermore, the A kinase inhibitor, K-252a, did not affect the steady-state content of GATA-6 (Fig. 3, middle). Thus it would be interesting to determine the target site of protein kinase A and the degradation signal on the GATA-6 protein. Further studies are required to see whether the full-length GATA-6 and other GATA proteins (GATA-1 ~ -5) have similar properties. Experiments along such lines are now in progress.

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