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Isolation of CHO-K1 clones defective in cAMP-dependent proteolysis, as determined by the stability of exogenously expressed GATA-6☆

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Abstract

Degradation of the GATA-6(Δ50) protein expressed in a CHO-K1 clone (tc1-17a) is stimulated in the presence of *db*cAMP through proteasome without new protein synthesis [FEBS Lett. 408 (1997) 301], whereas the intrinsic GC-box-binding protein was stable. To examine the cellular mechanism responsible for this specific degradation of GATA-6(Δ50), we initially introduced the blasticidin-S deaminase gene carrying a promoter with GATA motifs that are recognized by GATA-6. The resulting cell line (tc2G2) grew in the presence of blasticidin S. However, the presence of both blasticidin S and *db*cAMP was lethal due to degradation of GATA-6. Cells resistant to such lethality were isolated by chemical mutagenesis. The GATA-6(Δ50) in these resistant cells was stable in the presence of *db*cAMP in contrast to that in the parent tc2G2 cells, as determined by gel-mobility shift analysis and Western blotting. These clones could be beneficial for identification and characterization of the components participating in the signaling pathway for both protein degradation and cAMP-dependent biological processes.

Keywords: cAMP; CHO-K1; GATA; Proteasome; Proteolysis

Sequence-specific DNA-binding proteins together with coregulators play crucial roles as transcription regulatory factors in gene expression [1]. External signals modulate the DNA-binding potential of the transcription factors and/or their ability to recruit the basal transcription machinery [2] through posttranslational modifications such as phosphorylation [3] and proteolysis [4]. Among proteolytic enzymes, proteasome seems to have the most complex structure and mode of regulation in terms of molecular assembly [5]. Protein modification with ubiquitin is a prerequisite for substrate recognition and degradation by proteasome [6]; the ubiquitin-activating enzyme (E1), ubiquitin conjugation enzymes (E2), and ubiquitin ligases (E3) function in the polyubiquitination of the degradation products. The presence of multiple forms for E2 and E3 contributes to the recognition of different and specific substrates [9].

Changes in protein activities and removal of misfolded proteins by proteasome are necessary for maintaining and/or shifting the dynamic equilibrium of cellular proteins, and thus defects in protein degradation are associated with many human diseases including neurodegenerative disorders and cancer [6]. Furthermore, protein ligation of not only ubiquitin but also ubiquitin-related proteins makes the degradation pathway much more complicated [7]. Thus, the molecular mechanisms underlying the proteasome pathway are the subject of keen interest.

Recently, we reported that nuclear GATA-6(Δ50) (truncated GATA-6 transcriptional regulatory factor

☆ *Abbreviations:* BSA, bovine serum albumin; CHO, Chinese hamster ovary; *db*cAMP, dibutyryl cAMP; DIG, digoxigenin; EDTA, ethylenediaminetetraacetic acid; EMS, ethyl methanesulfonate; Ig, immunoglobulin; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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stably expressed in CHO-K1 cells) was degraded by proteasome in a cAMP-dependent manner without new protein synthesis [8]. In this study, we established a cell line sensitive to an antibiotic only in the presence of *db*cAMP. Then mutagenesis was carried out, and resistant clones were isolated under non-permissive conditions. Expectedly, GATA-6(Δ50) was stable in these resistant clones. Thus, our system should be valuable for determining the genetic components that participate in the novel cAMP–proteasome pathway.

Materials and methods

Cell culture. All the transfectants and mutant cells used were derivatives of CHO-K1 cells [9], and cultured in Ham's F12 medium (GIBCO) supplemented with 7% (v/v) fetal bovine serum (JRH Biosciences) and antibiotics [2.5 μg/ml fungizon (Gibco), 100 U/ml penicillin G (Wako), and 100 μg/ml streptomycin sulfate (Wako)]. A plasmid carrying the GATA-responsive blasticidin S-deaminase gene was introduced into tc1-17a cells [8] by the calcium phosphate precipitation method [10], and stable transformants were isolated in the presence of 8 μg/ml blasticidin S (Kaken Pharmaceutical). The tc2G2 cells (5×10^5 to 2×10^6), grown as a monolayer in a 75 cm^2 flask, were mutagenized with EMS (Sigma) (150 μg/ml) overnight at 37 °C in the presence of 10 μg/ml thymidine [11,12], and then resistant clones were isolated in the presence of both 8 μg/ml blasticidin S and 2 mM *db*cAMP (Sigma).

The sensitivity to blasticidin S was examined by colony formation at 37 °C. 10^2 cells were seeded on a 12-well plate (19 mm in diameter). On the following day, 8 μg/ml blasticidin S and/or 2 mM *db*cAMP were added, followed by culturing for 7–10 days. Colonies on the plates were fixed with 15% (w/v) trichloroacetic acid after washing with phosphate-buffered saline [10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, and 3 mM KCl], then stained [45% (v/v) methanol, 45% (v/v) acetic acid, and 0.05% (w/v) Coomassie brilliant blue R250], and destained [45% (v/v) methanol and 45% (v/v) acetic acid]. The plates were washed with tap water and then dried. The colony-forming ability of the clones was also determined at 33 or 39 °C. Cell numbers were determined with a hemocytometer after scraping the cells off the plate (60 mm in diameter) with a rubber policeman.

Plasmid constructs. A blasticidin S-deaminase gene with a GATA-responsive promoter was constructed (p2GATA-BSD) [13]. A *Nco*I–*Apa*LI fragment derived from the rat intrinsic factor gene [14] was inserted between the *Eco*RV and *Hin*dIII sites of pBluescript SKII(+), after the *Nco*I, *Apa*LI, and *Hin*dIII sites had been treated with Klenow enzyme. The *Hin*dIII site was regenerated in the construct. Then the *Eco*RI–*Hin*dIII fragment was substituted with the corresponding fragment of pMAM2-BSD (Kaken Pharmaceutical). The DNA sequence was determined by the dideoxy method [15].

Gel-mobility shift analysis. Probe DNA with GATA motif was prepared by means of the polymerase chain reaction [16] and labeling with DIG-11-ddUTP [17]. The T22 sequence (5′-GACAGTCTCGAGGACAAGAAGTCAA**AGATAA**GACGAATTCGTTGAG-3′) was chosen, since it contains the AGATAA sequence that is the most abundant binding site for GATA-6 [21]. Primers (50 pmol each) (Gibco) hybridizing to the underlined portions and a plasmid template (0.1 μg) carrying the T22 sequence were used for the chain reaction with *Taq* polymerase (*Gene Taq*, Nippon Gene): 20 cycles of denaturation (94 °C, 1 min), annealing (45 °C, 1 min), and extension (72 °C, 0.5 min). The amplified DNA was treated with phenol/$CHCl_3$ and then ethanol-precipitated [14]. The probe DNA with GC-boxes was purchased from Gibco-BRL. The DNA (100 pmol) was labeled with DIG-11-ddUTP according to the manual supplied by the manufacturer (DIG Gel Shift Kit; Boehringer–Mannheim).

A nuclear protein extract was prepared as described previously [11]. The binding reaction was initiated by adding the nuclear extract (3 μg protein) to a mixture (total 20 μl) comprising 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–NaOH (pH 7.6), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 0.2% (w/v) Tween 20, 30 mM KCl, 0.05 ng/μl poly L-Lys, and the labeled-DNA (54 fmol), followed by incubation for 30 min on ice. Five microliters of the sample buffer [(33.5 mM Tris, 0.6 mM EDTA, and 11 mM boric acid, pH 8.0):glycerol = 6:4(v/v)] was added and the mixture was subjected to 8%(w/v) polyacrylamide gel electrophoresis. DNA was electroblotted onto a nylon membrane (BM nylon membranes positively charged; Boehringer–Mannheim), fixed, and detected with alkaline phosphatase-conjugated anti-DIG antibodies [17]. Protein was assayed with a Bio-Rad Protein Assay [18], with BSA (fraction V, Sigma) as a standard.

Detection of GATA-6 by Western blotting. A nuclear extract (10 μg protein) was subjected to SDS–polyacrylamide gel [10%(w/v), 1 mm thickness] electrophoresis [19], and then electroblotted (200 mA, 1.5 h; ATTO Model-AE6675) onto a nitrocellulose filter (Schleicher & Schuell, Dassel) [20]. The filter was blocked for 1 h with phosphate-buffered saline containing 3%(w/v) BSA. Rabbit site-specific polyclonal antibodies recognizing human GATA-6 (Leu^{59}-Gln^{217}) [21] were used as the first antibodies (400× diluted). The second antibodies were horseradish peroxidase-linked donkey anti-rabbit Ig (2000× diluted). An enhanced chemiluminescence Western blotting kit (Amersham–Pharmacia Biotech) was used for detection. Monoclonal antibodies for phosphoserine (clone PSR45, Sigma) and ubiquitin (P4D1, Santa Cruz Biotechnology) were also used as the first antibodies (500× and 400× diluted, respectively). The second antibodies were horseradish peroxidase-linked donkey anti-mouse Ig (Amersham-Pharmacia Biotech) (4000× diluted).

Chemicals. Restriction enzymes, T4 DNA ligase, and Klenow enzyme were obtained from TaKaRa (Kyoto), Toyobo (Osaka), and New England Biolabs (Beverly, MA). Colchicine and MG115 were obtained from Calbiochem and Peptide Institute, respectively. All other chemicals used were of the highest grade commercially available.

Results

Rationale for mutant cell isolation

We first determined the specificity of cAMP-dependent proteolysis. Since it is reported that cAMP stimulated the degradation of a GC-box-binding protein, SP1, in normal rat kidney cells [22], we analyzed the behavior of GC-box-binding protein(s) in a CHO-K1 derivative (tc1-17a) expressing rat GATA-6(Δ50) [8] upon adding *db*cAMP. As shown in Fig. 1, the content of GC-box-binding protein(s) was essentially the same in the nuclear extract from cells treated with and without *db*cAMP, while that of GATA-6(Δ50) disappeared in the presence of *db*cAMP due to proteolysis [8]. The shifted bands with arrows competed with non-labeled wild-type probe DNA (Competitor W), but not with mutant DNA (Competitor M), indicating that the proteins recognize GATA-motif and GC-box (Figs. 1A and B, respectively), although we could not conclude

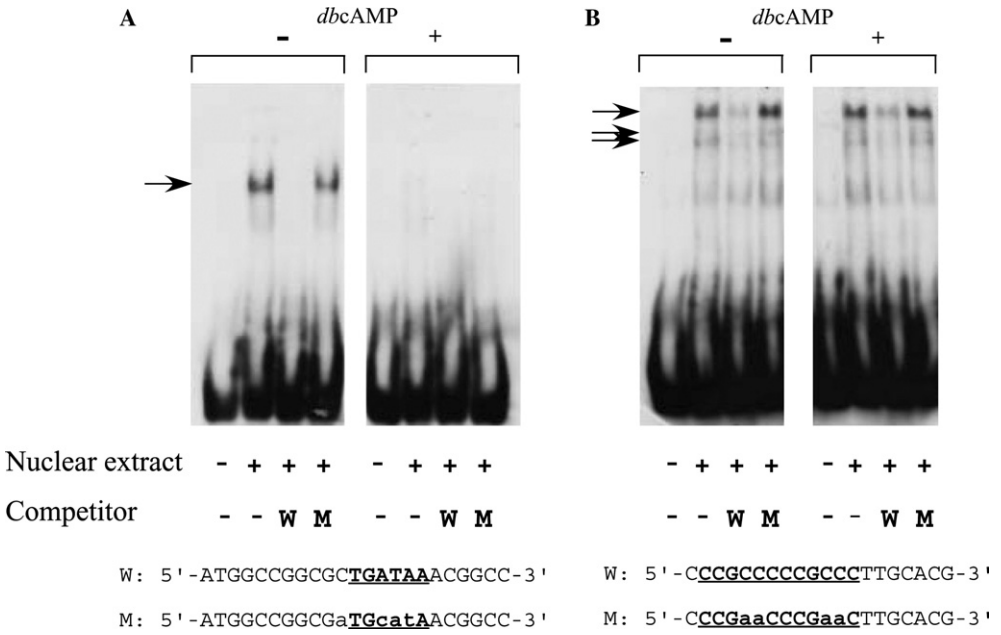

Fig. 1. Stability of intrinsic GC-box-binding protein in tc1-17a cells in the presence of *db*cAMP. The tc1-17a cells were grown for 2 days in the presence (+) and absence (−) of 2 mM *db*cAMP, and then a nuclear protein extract was prepared (see Materials and methods). The extract was subjected to gel-mobility shift analysis using a probe with GATA motif (A) or tandem GC-boxes (B) (CCGCCC [38]). The excess competitor (130 times) with wild-type (W) or mutant motif (M) was also added into the reaction mixture. Arrows indicate the positions of bound probes. The GC-box-binding protein(s) from the nuclei of CHO-K1 cells could not react with monoclonal antibody for Sp1 (Santa Cruz, 1C6), although this antibody reacted with that from HeLa cell nuclei and inhibited the gel-mobility shift using the above probe with GC-boxes (not shown). Thus, we tentatively conclude that the GC-box-binding protein(s) detected in CHO-K1 cells in (B) are not Sp1, although the species difference of primary structure would affect the antibody binding.

that the GC-box-binding protein(s) is Sp1 (see legend to Fig. 1). These results strongly suggest that GATA-6(Δ50) could be specifically degraded in tc1-17a cells in the presence of *db*cAMP.

To know a metabolic process that is difficult to identify, CHO-K1 cells seem to be advantageous because of their successful use in somatic cell genetics [11,12]. For the isolation of mutants with defective cAMP-dependent proteolysis, a key point would be the construction of a conditional lethal system. An antibiotic-resistant gene ligated downstream of the GATA-6-responsive promoter, for example, might be suitable for this purpose. The introduction of a gene encoding blasticidin S-deaminase (Fig. 2) would endow blasticidin S resistance on tc1-17a cells in the absence of *db*cAMP. However, the cells may become sensitive to this antibiotic in the presence of *db*cAMP due to the degradation of GATA-6(Δ50), which could act as a transcriptional activator of the blasticidin S-deaminase gene (Fig. 3). It should be noted that GATA-6(Δ50) showed essentially the same transcriptional activation competency as that of GATA-6 (not shown), as determined by means of the luciferase assay using common reporter gene constructs (see Figs. 3 and 4 in our previous report [23]). Thus, it seems likely that somatic mutations inhibiting the cAMP-dependent proteolytic pathway could suppress the lethal effect of *db*cAMP.

Isolation of cells grown in the presence of both blasticidin S and dbcAMP

Such an expectation prompted us to construct a promoter with GATA-binding sites. The 5′-upstream sequence of the rat intrinsic factor gene [14] contains two GATA motifs [17] conserved in rodents in front of the TATA-box (Fig. 2A). Then we substituted the promoter region of an expression plasmid for the blasticidin S-deaminase gene (pMAM2-BSD), and the resulting plasmid (p2GATA-BSD) (Fig. 2) was introduced into tc1-17a cells expressing GATA-6(Δ50). The colonies formed in the presence of blasticidin S were isolated. One of the clones isolated (tc2G2) as well as tc1-17a cells was viable in the presence of *db*cAMP. However, tc2G2 (Fig. 4) was sensitive to blasticidin S in the presence of *db*cAMP. These results suggest that tc2G2 cells fulfill our initial rationale. It should be pointed out that n-5 cells (Fig. 4) [8], which do not express GATA-6(Δ50), did not produce blasticidin S-resistant clones. We further confirmed that GATA-6(Δ50) in tc2G2 cells was degraded in the presence of *db*cAMP, as shown later (Fig. 5).

To isolate clones having defects in the components of the cAMP-dependent proteolytic pathway, we treated tc2G2 cells with alkylating reagent EMS as described under Materials and methods. The cells were further

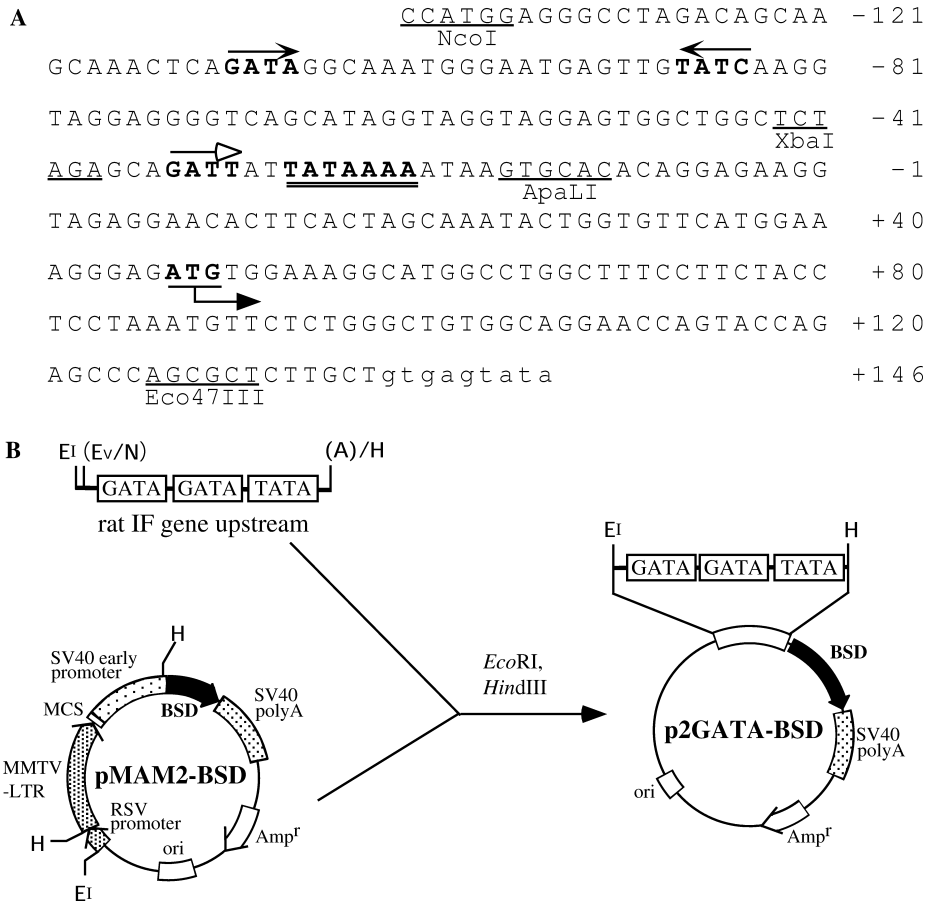

Fig. 2. Construction of a promoter with GATA-6-binding sites. (A) The nucleotide sequence for upstream of the rat intrinsic factor gene [14] is shown. The conserved GATA motifs and potential TATA-box are shown by bold letters with arrows above and double underlining, respectively. The GATT sequence, a potential GATA-6-binding site [17], is indicated by an open arrow above. The translational start site is shown by underlining with an arrow. The restriction enzyme sites are shown by underlinings with the enzyme names. The sequence of intron 1 is indicated by small letters. (B) The sequence between the *Nco*I–*Apa*LI sites in (A) was ligated to the *Eco*RV site of pBluescriptSKII(+), and then the *Eco*RI–*Hin*dIII fragment was substituted with the corresponding fragment of pMAM2-BSD in front of the blasticidin S-deaminase gene, as described under Materials and methods. The abbreviations for restriction enzymes are: A, *Apa*L1; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nco*I, and those for others are: BSD, blasticidin S-deaminase; IF, intrinsic factor; and MCS, multi-cloning site, respectively.

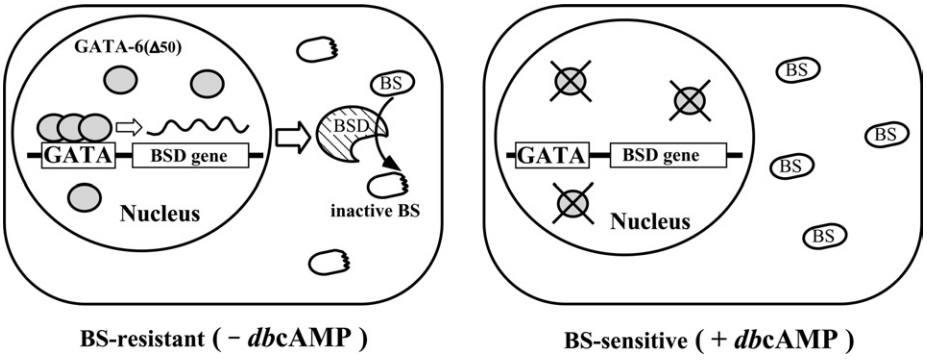

Fig. 3. A conditional lethal system for the isolation of mutants with defective cAMP-dependent proteolysis. To isolate mutants with defective cAMP-dependent proteolysis, we initially constructed a conditional lethal system by introducing the GATA-6-responsive blasticidin S-deaminase gene into tc1-17a (a derivative of CHO-K1 cells expressing rat GATA-6(Δ50) [8]). The cells are viable in the presence of blasticidin S (left). However, together with *db*cAMP (a cAMP analogue), the cells are expected to be lethal because of the proteolytic degradation of GATA-6(Δ50).

grown in the absence of both blasticidin S and *db*cAMP to fix the mutation and then cultured in the medium containing these two reagents. On a separate plate, the same alkylating reagent-treated cells were cultured in the presence of 2 mM ouabain. From 1×10^7 cells, 78 resistant colonies were obtained, suggesting that the

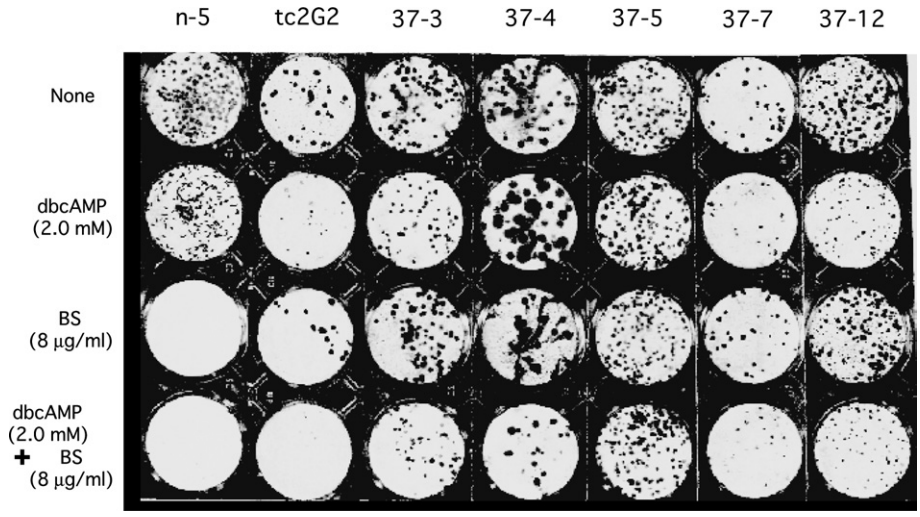

Fig. 4. Colony formation by parent and resistant cells. To examine the growth behavior of the resistant cells, they were seeded onto a plastic dish (19 mm in diameter) and grown for 8 days at 39 °C in the presence or absence of *db*cAMP and/or BSD. Colonies were fixed and stained as described under Materials and methods.

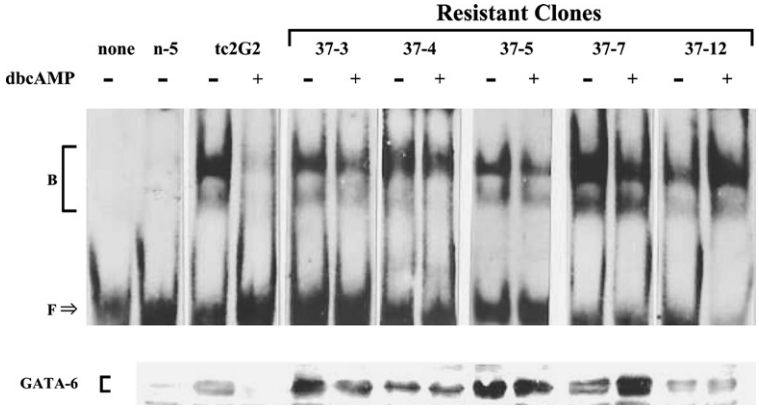

Fig. 5. Behavior of GATA-6(50) in parent and resistant cells in the presence and absence of *db*cAMP. Parent (tc2G2) and resistant cells were grown for 2 days in the presence of 2 mM *db*cAMP. The nuclear extract was subjected to gel-mobility shift analysis (upper) and Western blotting after SDS–polyacrylamide gel electrophoresis.

frequency of mutation seems to be increased in the presence of EMS [24]. The single colonies formed in the presence of both blasticidin S and *db*cAMP were isolated. Among the 20 clones isolated, five (37-3, 37-4, 37-5, 37-7, and 37-12) showed apparent resistant growth, as shown in Fig. 5. Furthermore, the growth curves at 37 °C of the isolated clones were similar to those of the parent tc2G2 cells (not shown), suggesting that these clones should be useful for biochemical analysis.

Stability of GATA-6(Δ50) in resistant cells.

To determine whether or not *db*cAMP treatment affects the behavior of the GATA-6 protein in resistant cells, nuclear extracts of parent and resistant cells were subjected to gel-mobility shift analysis. As shown in Fig. 5 (upper panel), the mobility of the DIG-labeled probe with a GATA-protein-binding site (none) was retarded on addition of the control extract for each cell type (minus lanes for tc2G2, 37-3, 37-4, 37-5, 37-7, and 37-12), whereas the cells (n-5) without forced expression of GATA-6 did not produce a shifted band. The extract of parent cells treated with *db*cAMP (plus lane for tc2G2) gave almost no retarded bands, as was demonstrated previously for tc1-17a cells [8], from which tc2G2 was derived. However, essentially similar shifted bands were observed with nuclear extracts of *db*cAMP-treated resistant cells (plus lanes). Consistent with such an observation, Western blotting analysis of the GATA-6 protein demonstrated that the parent cells lost GATA-6 in the presence of *db*cAMP, whereas the protein was present with and without *db*cAMP treatment of resistant cells (lower panel in Fig. 5). These results suggest that the GATA-6 protein in the resistant cells is stable in the presence of *db*cAMP, possibly due

to a defect in the activation process that links cAMP to the proteasome.

Discussion

Since our finding of cAMP-dependent proteolysis of stably expressed GATA-6(Δ50) in CHO-K1 cells [8], a similar cAMP-signaling pathway coupled with proteasome function has been reported [22,25,26], although the coupling of cell signaling and elevated proteasome function is still not well known. The induced proteolysis of GATA-6(Δ50) was reproducibly observed with clones isolated from different batches of CHO-K1 cells (not shown). Furthermore, intrinsic GC-box-binding protein(s) did not respond to *db*cAMP, suggesting that the cAMP-dependent proteolysis could be specific to GATA-6 (and potentially also unidentified groups of intrinsic proteins) at least in CHO-K1 cells. The fact that the intact GATA-6 also responded to *db*cAMP excludes the possibility that a cryptic signal would be artificially exposed in the amino-terminal truncated GATA-6(Δ50) (not shown).

In combination with somatic genetics [12], CHO-K1 cells have been successfully used to study biological processes such as cholesterol metabolism [27,28] and vesicular transport [29,30]. Actually, introduction of the blasticidin-S deaminase gene with the GATA-dependent promoter and subsequent mutagenesis gave resistant clones in the presence of both *db*cAMP and blasticidin S. The rationale that stable GATA-6(Δ50) in the presence of *db*cAMP would transcribe the blasticidin-S deaminase gene and detoxify blasticidin S seems to be reasonable, since GATA-6(Δ50) in the resistant clones did not decrease during the incubation period with *db*cAMP in contrast to that in the sensitive parent (tc2G2). Furthermore, great attention has been paid to cAMP from the viewpoint of human diseases [31]. Thus, our system should become one of the model systems for specific protein degradation and cAMP signaling.

Polyubiquitinated proteins were accumulated in the nucleus in the presence of both *db*cAMP and proteasome inhibitor MG115, suggesting that the ubiquitin–proteasome pathway in the resistant clones is functional. Actually, resistant cells showed essentially the same colony formation under the growth conditions at both 33 and 39 °C (not shown), in contrast to the thermolability phenotype of the E1 mutation [32]. We could not detect apparent ubiquitinated GATA-6(Δ50), possibly due to the very low concentration of ubiquitinated GATA-6(Δ50) in the cells and/or tight coupling of ubiquitination and degradation of GATA-6(Δ50), although the possibility cannot be excluded that ubiquitination of the protein associated with GATA-6(Δ50) could induce the degradation of GATA-6(Δ50). We also found that the phosphoserine level was essentially the same in the parent and resistant clones, suggesting that the A-kinase pathway could work in these clones. We further demonstrated that all these clones, as well as tc2G2 cells, showed similar colchicine sensitivity (not shown), suggesting that the resistant phenotype of clones could not be ascribed to the enhancement of the excretion system linked to multi-drug resistance [33].

Mutation of the regulatory subunits of cAMP-dependent protein kinase affected the *db*cAMP-sensitive growth of S49 mouse lymphoma cells [34]. The presence of multiple protein kinase A catalytic and regulatory subunits as well as A-kinase anchoring proteins [35] could modulate the effects of cAMP in a cell type-specific manner. Although the defective sites in the resistant clones are difficult to identify at present, the gene trapping method with retrovirus [36,37] could be directly applied to identify defects in the relevant genes, since the present study demonstrated that resistant clones could be isolated by treatment with a mutagen. Experiments along these lines are currently in progress.

Acknowledgments

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