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Ubiquitin-dependent degradation of adenovirus E1A protein is inhibited by BS69

Tomoyasu Isobe, Chiharu Uchida, Takayuki Hattori, Kyoko Kitagawa, Toshiaki Oda, Masatoshi Kitagawa *

Department of Biochemistry 1, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan

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Abstract

Adenovirus E1A protein perturbs the cell cycle and promotes cell transformation. Although E1A is relatively unstable, regulation of E1A stability has not been fully elucidated. Here, we showed that E1A was ubiquitinated and degraded using a proteasome in vivo system. Interestingly, we found that BS69, one of the E1A-binding proteins, inhibited ubiquitination of E1A. BS69 mutants lacking the MYND domain could not bind to E1A and did not inhibit ubiquitination of E1A. Moreover, we demonstrated that overexpression of BS69 stabilized E1A in vivo. These results suggest that BS69 controls E1A stability via inhibition of ubiquitination.

Keywords: E1A; Ubiquitin; Proteasome; BS69

The protein encoded by the early region 1A (E1A) of human adenovirus type 5 plays an important role in viral replication [1]. E1A promotes immortalization of primary rodent cells, and full transformation cooperating with other oncogene products such as activated-Ras or E1B [2]. E1A protein interacts with various cellular proteins including pRB family proteins and p300/CBP, and perturbs cell proliferation and differentiation [3,4]. pRB controls G1/S progression by interacting with E2F family transcription factors [5,6]. Interaction of E1A with pRB releases E2F from pRB and stimulates E2F-dependent transcription of several growth promoting genes [7]. Therefore, E1A promotes an irregular S-phase entry and perturbs the cell cycle. p300 and CBP are transcriptional coactivators that are recruited to various promoters by interacting with a number of transcription factors [4]. Interaction of E1A with p300/CBP modulates p300-mediated transcriptional activation and chromatin remodeling [8], and multiple cellular events are abrogated. Because E1A shows not only oncogenic activity but also tumor suppressive properties, it plays multifunctional roles in adenovirus-infected cells [9,10].

BS69 was also originally identified as a nuclear protein that binds to E1A [11]. BS69 binds to the PXLXP motif of E1A protein via its C-terminal MYND domain and inhibits E1A-mediated transcriptional activation [12]. In addition, PXLXP-containing proteins such as N-CoR [13], EBNA1/2 [12], EMSY [14], c-Myb [15], and MGA [12] were reported to also bind to the MYND domain of BS69. Although it is suggested that BS69 plays a role in transcriptional regulation through association with these proteins, physiological functions of BS69 have not been fully elucidated.

Abundance of several cellular proteins, particularly short-lived regulatory proteins such as cyclins, p53, IκBα, β-catenin, $p27^{Kip1}$, and Myc, is controlled by the ubiquitin–proteasome system [16–18]. Aberrations in this system directly or indirectly underlie pathogenesis of cancers and many other diseases [17,19]. Poly-ubiquitin conjugations of these proteins are putative signals specifying selective

* Corresponding author. Fax: +81 53 435 2322.
E-mail address: kitamasa@hama-med.ac.jp (M. Kitagawa).

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recognition and hydrolysis by the 26S proteasome [16]. Some studies reported that E1A modulated proteasome-dependent degradation of cellular proteins [20]. In addition, E1A protein is unstable in host cells [21,22], and previous studies showed that degradation of E1A protein in vitro was mediated by the ubiquitin system in an energy-dependent manner [23]. However, it has not been demonstrated whether E1A protein was ubiquitinated in

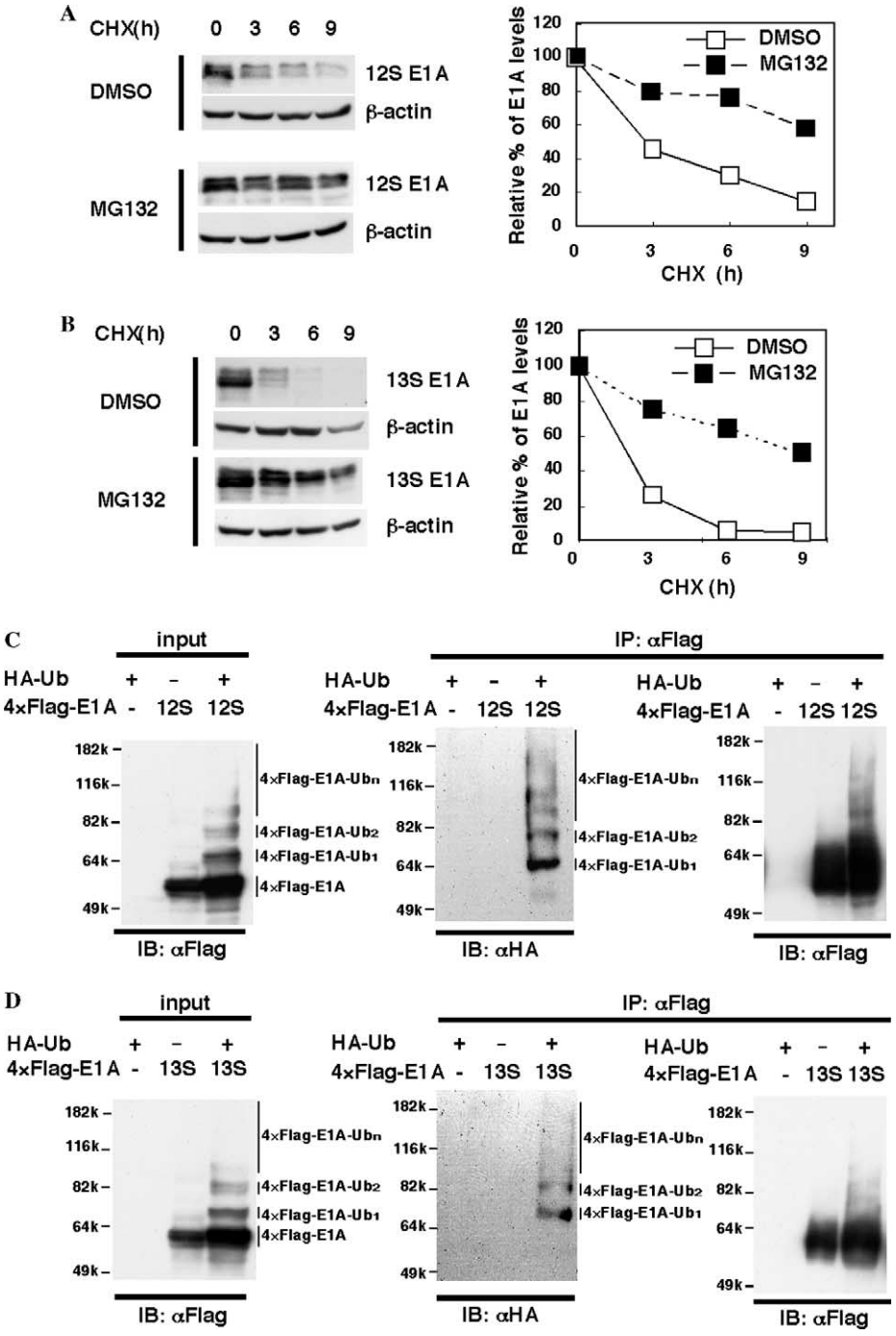

Fig. 1. Ubiquitin-dependent degradation of transfected E1As. (A,B) Proteasome-dependent degradations of transfected 12S and 13S E1As. In vivo degradation assay was performed as described in Materials and methods. HepG2 cells transiently transfected with the 12S E1A expression plasmid (A) or 13S E1A expression plasmid (B) were treated with 10 μg/ml cycloheximide (CHX) for appropriate periods with or without 20 μM MG132. Cell extracts were analyzed by immunoblotting using anti-E1A or anti-β-actin antibodies. E1A signals were quantified and normalized against β-actin, and values relative to the value at time 0 are plotted. (C,D) Ubiquitinations of transfected 12S and 13S E1As. 293 cells were transiently transfected with 4× Flag-tagged 12S E1A (C) or 4× Flag-tagged 13S E1A (D) expression plasmids together with or without the HA-tagged ubiquitin (HA-Ub) expression plasmid. Cell lysates were immunoblotted with an anti-Flag antibody (input), or subjected to immunoprecipitation with an anti-Flag antibody (IP: αFlag), and immunoblotted with anti-HA or anti-Flag antibodies. Positions of free E1A, mono-ubiquitinated E1A, di-ubiquitinated E1A, and polyubiquitinated E1A are indicated on the right of each panel.

mammalian cell lines. Moreover, mechanisms of regulation of E1A turnover including ubiquitin ligase(s) for E1A are still unclear. In the present study, we demonstrated that two types of E1A proteins 12S- and 13S-E1A were ubiquitinated and degraded in vivo in a proteasome-dependent manner. Moreover, we found that BS69 inhibited E1A ubiquitination and stabilized E1A in a MYND domain-dependent manner in cultured mammalian cells.

Materials and methods

Cell culture, reagents, and antibodies. 293 cells and HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. MG132 and cycloheximide were commercially purchased from Peptide institute and Wako, respectively. Monoclonal antibodies against Flag-epitope (M2, Sigma), HA-epitope (12CA5, Roche), Xpress-epitope (Invitrogen), β-actin (AC-15, Sigma), ubiquitin (FK-2, Nippon Bio-Test Laboratories), and E1A (M73, Santa Cruz Biotechnologies) were used for immunoprecipitation and/or immunoblotting.

Plasmids and transfection. Expression vectors of E1A and BS69 were kindly provided by Nakajima [24] and Shibuya [25], respectively. The full-length human BS69 cDNA was recloned into pcDNA4-HisMax (Invitrogen). PCR-based site-directed mutagenesis was used to obtain cDNA fragments encoding the PHD zinc-finger mutant hBS69 (C63A/C102A). To generate expression plasmids for BS69ΔMYND, the cDNA region corresponding to the carboxyl-terminal 40 amino acids of human BS69 was removed from pcDNA4-HisMax-BS69 by PCR-based deletion. pcDNA4-HisMax-BS69ΔPHD was generated by ligation of two segments within the BS69 cDNA encoding amino acids 2–52 and 109–562, which were amplified by PCR. The 4× Flag-tagged BS69 and E1A expression plasmids were constructed by introducing each cDNA into pCMV-Tag2 expression vectors (Stratagene) and were annealed with 3× Flag-tag oligonucleotides. pCGN-HA-ubiquitin has been previously described [26]. Expression plasmids were transfected into 293 and HepG2 cells using the calcium phosphate method.

Analysis of ubiquitination. Immunoprecipitation and immunoblotting were performed as described previously [26,27] with some modifications. For analysis of ubiquitination of transfected E1A, 293 cells were transfected with pCMV-4× Flag-12S E1A or 13S E1A together with pCGN-HA-ubiquitin. After 36 h, cells were treated with or without 20 μM MG132 for 12 h and were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5% Triton X-100, and protease inhibitors). Cell lysates were subjected to immunoblotting with an anti-Flag antibody. To detect the ubiquitin chain conjugated to E1A proteins, immunoprecipitation followed by immunoblotting (IP-IB) was performed. Briefly, cell lysates were incubated with 2 μg anti-Flag antibody and protein G–Sepharose 4FF (Amersham Bioscience) at 4 °C for 2 h. Immunocomplexes were washed four times with lysis buffer and subjected to immunoblotting with an anti-HA antibody for detection of ubiquitin or with anti-Flag antibody for detection of E1A. For analysis of ubiquitination of endogenous E1A, IP-IB analysis was performed using anti-E1A and anti-ubiquitin antibodies.

Analysis of protein stability. 293 cells or HepG2 cells were transiently transfected with appropriate plasmids. After 36 h, cells were treated with 10 μg/ml cycloheximide (CHX) for indicated periods and with or without 20 μM MG132. Flag-tagged or un-tagged E1A proteins were detected by immunoblotting with anti-Flag or anti-E1A antibodies. E1A signals were quantified using ImageGauge (Fuji Film) and normalized against β-actin signals detected using an anti-β-actin antibody.

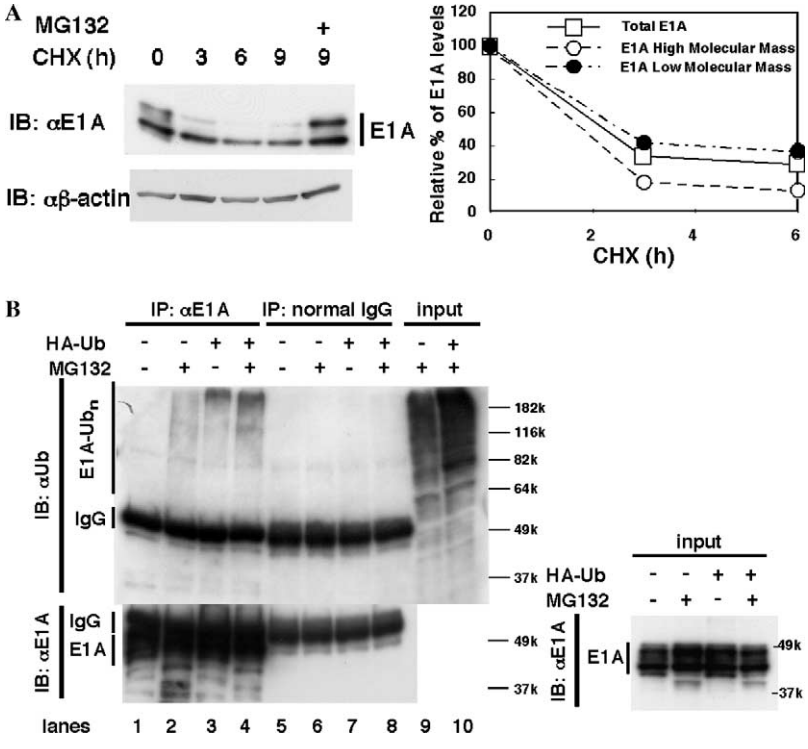

Fig. 2. Ubiquitin-dependent degradation of endogenous E1A. (A) Proteasome-dependent degradation of endogenous E1A. 293 cells were treated with 10 μg/ml cycloheximide (CHX) for appropriate periods with or without 20 μM MG132. Cell extracts were analyzed by immunoblotting using anti-E1A or anti-β-actin antibodies. E1A signals were quantified as described in Fig. 1A. (B) Ubiquitination of endogenous E1A. 293 cells were transiently transfected with or without HA-tagged ubiquitin (HA-Ub) plasmids. At 36 h after transfection, cells were incubated with or without 20 μM MG132 for 12 h. Cell lysates were immunoprecipitated with an anti-E1A antibody (IP: αE1A) or control immunoglobulin (IP: normal IgG), and immunoblotted with anti-ubiquitin or anti-E1A antibodies.

Results and discussion

Ubiquitin-dependent degradation of E1A

To assess stability of 12S and 13S E1A proteins, we measured the turnover rate of ectopically expressed E1A proteins in HepG2 cells by inhibiting de novo protein synthesis with cycloheximide (CHX). As shown in Fig. 1, 12S (A) and 13S (B) E1As were rapidly degraded with a half-life of less than 3 h, which was consistent with previous reports [21,22]. We found that a proteasome inhibitor MG132 (Figs. 1A and B) and lactacystin (data not shown) significantly decreased turnover rates of these proteins. These observations indicated that 12S and 13S E1A proteins were rapidly degraded in cells in a proteasome-dependent manner.

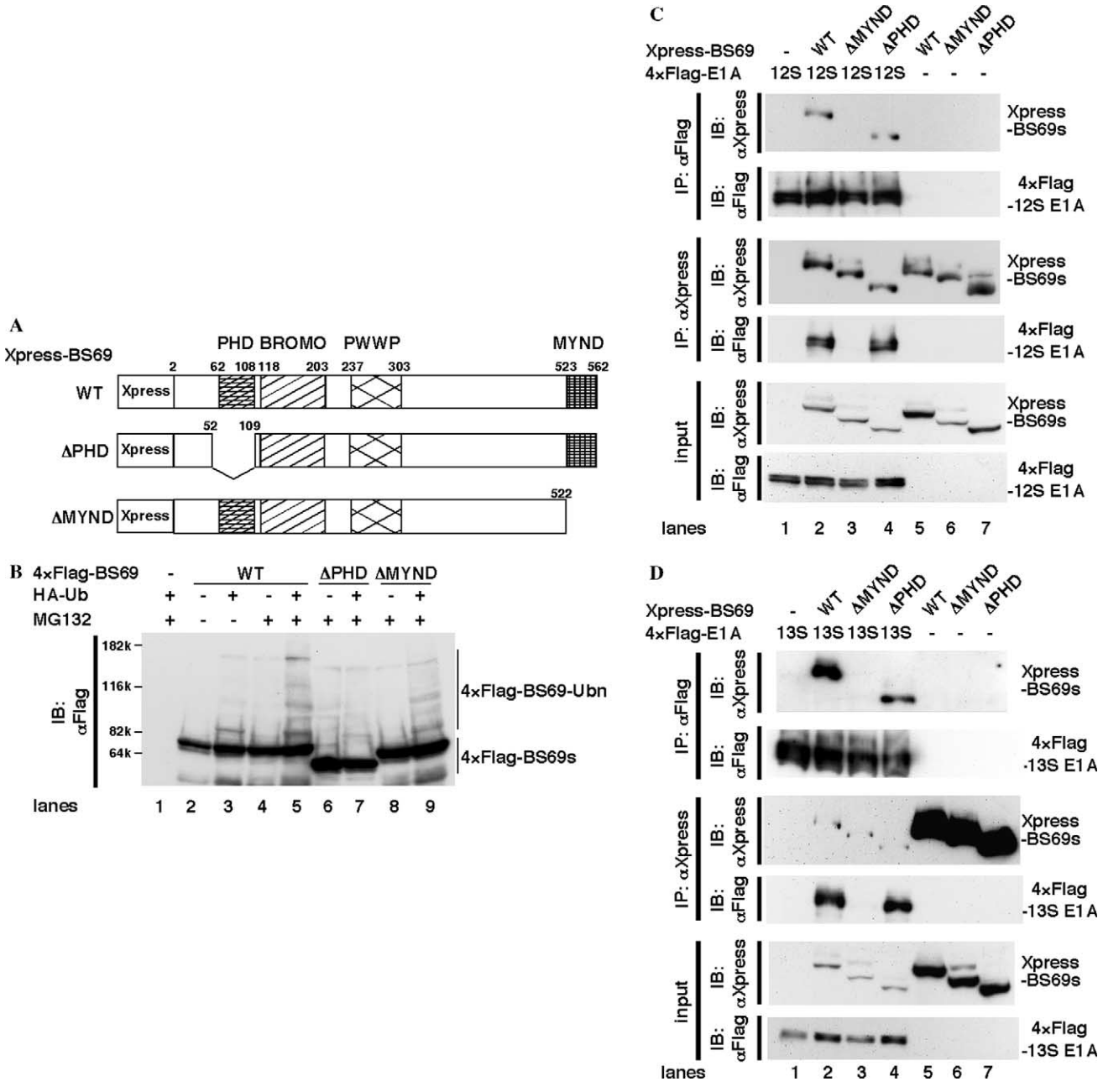

Fig. 3. Interaction between E1A and BS69. (A) Schematic diagram of BS69 constructs. PHD, plant homeo domain zinc-finger structure; BROMO, bromodomain; PWWP, PWWP domain; MYND, Mynd domain. Numbers represent amino acid positions. (B) Auto-ubiquitination of BS69. 4× Flag-tagged BS69 wild-type (WT) or deletion mutant (ΔPHD or ΔMYND) expression plasmids were transfected with or without HA-tagged ubiquitin expression plasmids into HepG2 cells. At 36 h after transfection, cells were incubated in the presence or absence of 20 μM MG132 for 12 h. Cell lysates were immunoblotted with an anti-Flag antibody. (C) Interaction between 12S E1A and BS69. 293 cells were co-transfected with the indicated expression plasmids, and cell lysates were immunoprecipitated (IP) and immunoblotted (IB). (D) Interaction between 13S E1A and BS69. Coimmunoprecipitation assay was performed as described in (C).

Polyubiquitin conjugation is a key signal marking target proteins for proteasome recognition and degradation [16]. We determined whether ubiquitin was conjugated to E1A proteins in vivo. When 4× Flag-tagged 12S E1A was coexpressed with HA-tagged ubiquitin in 293 cells, immunoblots with an anti-Flag antibody revealed ladders with mobility shifts identical to the molecular mass of an HA-tagged ubiquitin chain (Fig. 1C input). To confirm that high-molecular-mass species recognized by the anti-Flag antibody were ubiquitin-conjugated 12S E1As, cell lysates were immunoprecipitated with an anti-Flag antibody following immunoblotting with an anti-HA antibody or anti-Flag antibody (Fig. 1C, IP: αFlag). We demonstrated that high-molecular-mass bands and smears detected in immumoblotting (input, Fig. 1C left) and IP-IB (right) were due to polyubiquitination of E1A proteins. These results indicated that 12S E1A was ubiquitinated in vivo. We confirmed that 13S E1A and 12S E1A were ubiquitinated in vivo (Fig. 1D).

As shown in Fig. 2A, endogenous E1A proteins with different molecular masses including 12S and 13S were also rapidly degraded ($T_{1/2} = 2$–3 h) in 293 cells that were transformed with adenoviruses and degradation was blocked by MG132. Next, we investigated whether endogenous E1A proteins were ubiquitinated in cells. Cell lysates prepared from 293 cells treated with or without MG132 were subjected to immunoprecipitation with an anti-E1A antibody or normal IgG as a control, followed by immunoblotting with anti-Ub or anti-E1A antibodies. As shown in Fig. 2B (lane 2), a smeared signal was detected in immunoprecipitates of MG132-treated cells using the anti-E1A antibody, but not in control precipitation, and this pattern was enhanced by transfection of HA-Ub (lanes 3 and 4). These results indicated that endogenous E1A proteins were ubiquitinated in 293 cells. Taken together, our findings showed that the ubiquitin–proteasome pathway regulated E1A protein levels in 293 cells and in E1A-transfected HepG2 cells. Thus, our results clearly demonstrated ubiquitination and proteasome-dependent degradation of E1A, as it was predicted in previous studies [21–23].

Binding ability of BS69 to E1A

We attempted to identify the ubiquitin ligase for E1A. We were interested in BS69, an E1A binding protein [11], because it showed the typical PHD finger domain as shown in Fig. 3A. It has been reported that the PHD finger motif in some ubiquitin ligases can act as an active center for ubiquitin-conjugation [28], but it is unknown whether BS69 shows ubiquitin ligase activity. Therefore, we first examined auto-ubiquitination of BS69, because ubiquitin ligases usually show auto-ubiquitination activity. 4×; Flag-BS69 (WT) or deletion mutants of 4 × Flag-BS69 (ΔPHD or ΔMYND) were transiently coexpressed with or without HA-Ub in HepG2 cells (Fig. 3B). Wild-type and ΔMYND BS69 showed low mobility ladders of auto-ubiquitinated BS69 detected by immunoblotting with an anti-Flag antibody, whereas ΔPHD did not show such ladders. Moreover, auto-ubiquitination activity was missing in a BS69-mutant with substitutions of both cysteine 63 and 102 to alanine in the PHD finger domain (data not shown). These results suggested that BS69 showed ubiquitin ligase activity in a PHD finger-dependent manner.

Next, we examined the binding ability of BS69 to two types of E1A using an in vivo-coimmunoprecipitation assay. ΔPHD BS69 and wild-type BS69 coimmunoprecipitated with 12S and 13S E1A proteins, but ΔMYND mutants did not (Figs. 3C and D, IP: αFlag and IB:

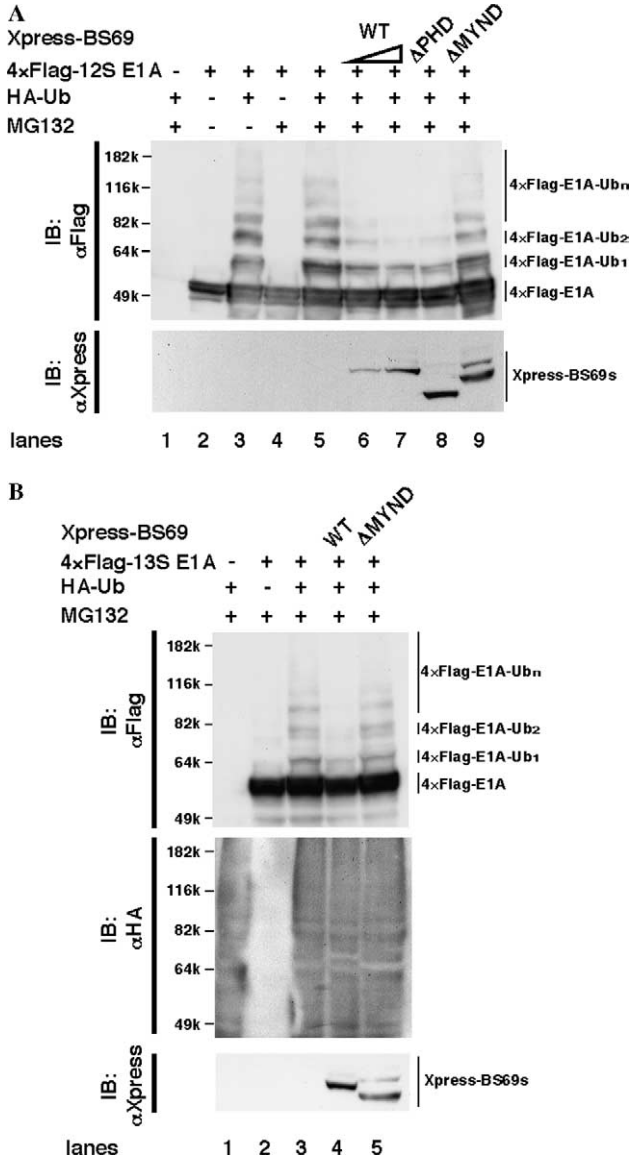

Fig. 4. Inhibition of E1A ubiquitination by BS69. 4× Flag-tagged 12S E1A (A) or 13S E1A (B) expression plasmids were transfected into 293 cells together with other indicated expression plasmids. At 36 h after transfection, cells were incubated with or without 20 μM MG132 for 12 h. Cell lysates were immunoblotted with an anti-Flag antibody for detection of ubiquitinated E1A, with an anti-HA antibody for detection of bulk ubiquitination of cellular proteins, or with an anti-Xpress antibody for detection of exogenous BS69.

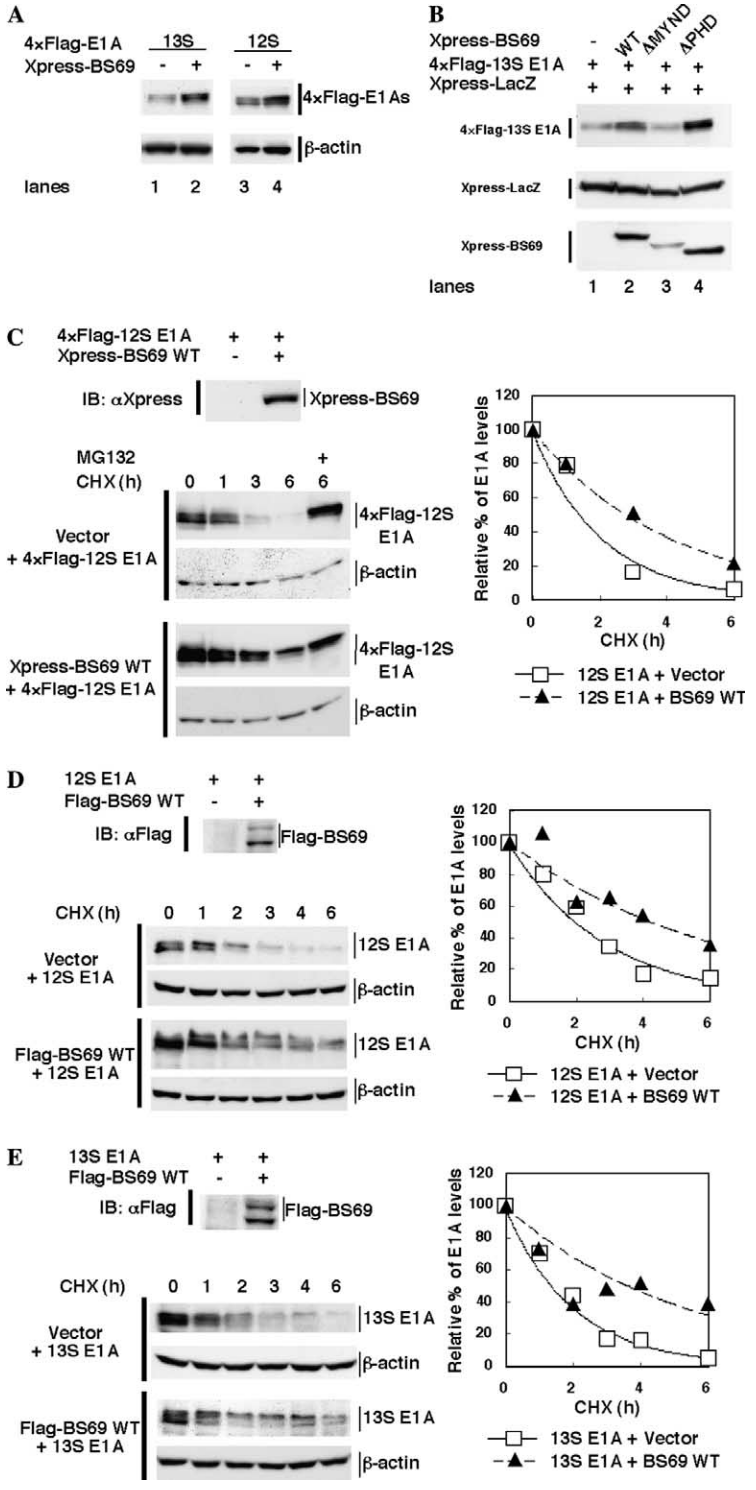

Fig. 5. Stabilization of E1A by overexpression of BS69. (A,B) Accumulation of E1A protein by co-transfection of BS69. 293 cells were transfected with 4× Flag-tagged E1A expression plasmids together with a control or Xpress-tagged BS69 wild-type (A) or deletion mutant (B) expression plasmids. Steady state levels of exogenous E1A were assessed by immunoblotting using an anti-Flag antibody. (C) 293 cells transfected as described in (A) were treated with 10 μg/ml cycloheximide for appropriate periods with or without 20 μM MG132. Cell extracts were analyzed by immunoblotting using anti-Flag or anti-β-actin antibodies. E1A signals were quantified, and normalized against β-actin, and values relative to the value at time 0 were plotted. Expression of exogenous BS69 was confirmed by immunoblotting using an anti-Xpress antibody. (D,E) HepG2 cells transiently transfected with 12S E1A (D) or 13S E1A (E) expression plasmids together with a control or the Flag-tagged BS69 expression plasmid were treated with 10 μg/ml cycloheximide for appropriate periods. Cell extracts were analyzed by immunoblotting with anti-E1A, anti-β-actin or anti-Flag antibodies.

αXpress). Alternatively, both 12S and 13S E1A coimmunoprecipitated with WT and ΔPHD BS69, but not with ΔMYND (Figs. 3C and D, IP: αXpress, IB: αFlag). These results indicated that BS69 bound to E1A proteins via the MYND domain of BS69, whereas the PHD finger was not required for interaction with E1A proteins.

Inhibition of E1A ubiquitination by BS69

We examined whether BS69 ubiquitinated E1A. 4× Flag-12S E1A and HA-ubiquitin were coexpresssed in 293 cells together with Xpress-BS69 or Xpress-BS69 mutants (ΔPHD or ΔMYND). Then, cell lysates were immunoblotted with an anti-Flag antibody to assess ubiquitination of E1A. Fig. 4A shows that E1A was efficiently ubiquitinated in a ubiquitin-dependent manner (lanes 1–5). Surprisingly, coexpression of BS69 reduced ubiquitination of E1A in a dose-dependent manner (Fig. 4, lanes 5–7). ΔPHD BS69 lacking auto-ubiquitination activity retained inhibitory effects on E1A ubiquitination, but ΔMYND BS69 lacking binding activity to E1A did not suppress ubiquitination (Fig. 4A, lanes 8–9). We also demonstrated that BS69 inhibited ubiquitination of 13S E1A (Fig. 4B). Additionally, we confirmed that ubiquitination of cellular bulk proteins detected by reprobing with anti-HA antibody was unaffected by coexpression of BS69, suggesting that BS69 specifically inhibits E1A ubiquitination (Fig. 4B, middle panel).

Stabilization of E1A by BS69

The above results prompted us to examine whether BS69 regulated stability of E1A in cells. 4× Flag-E1A plasmids were transfected with plasmids of Xpress-BS69 or empty vectors into 293 cells, and levels of E1A proteins were analyzed by immunoblotting with an anti-Flag antibody. Levels of both 12S and 13S E1A increased by coexpression of BS69 in 293 cells (Fig. 5A), and coexpression of BS69 ΔPHD also increased 13S E1A level, but coexpression of BS69 ΔMYND did not (Fig. 5B). Then, we performed a chase experiment with CHX treatment to test effects of BS69 expression on degradation rate of E1A. As shown in Fig. 5C, degradation rate of 4× Flag-12S E1A protein decelerated by coexpression of BS69 in 293 cells. Coexpression of BS69 ΔMYND hardly interfered with degradation of this protein (data not shown). BS69-mediated stabilization of E1A was also observed in HepG2 cells (Figs. 5D and E). Turnover rates of 12S (Fig. 5D) and 13S (Fig. 5E) E1A proteins were lower in BS69-transfected cells than in control cells. These results indicated that BS69 stabilized E1A proteins in vivo. Although BS69 sequence has less homology with deubiquitinating enzymes such as HAUSP [29], we cannot exclude the possibility that BS69 has deubiquitinating activity. Our results suggested that BS69 inhibited ubiquitination of E1A proteins through direct interaction of E1A in a MYND domain-dependent manner. Ansieau and Leutz [12] reported that BS69 bound to PXLXP motif between CR2 and CR3 domains of E1A through the MYND domain of BS69. Ubiquitin ligase for E1A may bind to the PXLXP motif, and its interaction with E1A may compete with BS69. There are some MYND-containing proteins with ubiquitin ligase motifs including RING finger in the NCBI database. They are candidates for the ubiquitin ligase for E1A.

This is the first study reporting that BS69 inhibited ubiquitin-dependent degradation of E1A. Although previous reports showed that BS69 inhibited E1A-mediated transactivation in a CR3 domain dependent manner [11], our results indicated that effects of BS69 on E1A ubiquitination were independent of CR3, because BS69 inhibited ubiquitination of not only 13S E1A but also 12S E1A, which lacks the CR3 domain. BS69 may have at least two different functions in regulation of E1A-mediated transactivation and protein stability of E1A. Further studies are required to investigate mechanisms of BS69-mediated downregulation of E1A.

Acknowledgments

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