Adenovirus E1A Inhibits SCF<sup>Fbw7</sup> Ubiquitin Ligase<sup>*§</sup>  

Tomoyasu Isobe<sup>1</sup>, Takayuki Hattori<sup>1,1</sup>, Kyoko Kitagawa<sup>1</sup>, Chiharu Uchida<sup>1,1</sup>, Yojiro Kotake<sup>8</sup>, Isao Kosugi<sup>15</sup>, Toshiaki Oda<sup>4</sup>, and Masatoshi Kitagawa<sup>2,2</sup>  

From the<sup>1</sup>Department of Biochemistry 1 and<sup>2</sup>Second Department of Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan

The SCFFbw7 ubiquitin ligase complex plays important roles in cell growth, survival, and differentiation via the ubiquitin-proteasome-mediated regulation of protein stability. Fbw7 (also known as Fbxw7, Sel-10, hCdc4, or hAgo), a substrate recognition subunit of SCFFbw7 ubiquitin ligase, facilitates the degradation of several proto-oncogene products by the proteasome. Given that mutations in Fbw7 are found in various types of human cancers, Fbw7 is considered to be a potent tumor suppressor. In the present study, we show that E1A, an oncogene product derived from adenovirus, interferes with the activity of the SCF<sup>Fbw7</sup> ubiquitin ligase. E1A interacted with SCF<sup>Fbw7</sup> and attenuated the ubiquitylation of its target proteins in vivo. Furthermore, using in vitro purified SCF<sup>Fbw7</sup> component proteins, we found that E1A directly bound to Roc1/Rbx1 and CUL1 and that E1A inhibited the ubiquitin ligase activity of the Roc1/Rbx1-CUL1 complex but not that of another RING-type ubiquitin ligase, Mdm2. Ectopically expressed E1A interacted with cellular endogenous Roc1/Rbx1 and CUL1 and decelerated the degradation of several protooncogene products that were degraded by SCF<sup>Fbw7</sup> ubiquitin ligase. Moreover, after wild-type adenovirus infection, adenovirus-derived E1A interacted with endogenous Roc1/Rbx1 and decelerated degradation of the endogenous target protein of SCF<sup>Fbw7</sup>. These observations demonstrated that E1A perturbs protein turnover regulated by SCF<sup>Fbw7</sup> through the inhibition of SCF<sup>Fbw7</sup> ubiquitin ligase. Our findings may help to explain the mechanism whereby adenovirus infection induces unregulated proliferation.

Ubiquitylation is a post-translational modification that modulates stability, localization, and function of proteins (1). Ubiquitin forms a polyubiquitin chain via an isopeptide linkage to a lysine of the target protein, which then efficiently targets the proteins for degradation via the 26S proteasome. The abundance of several proteins is regulated by the ubiquitin-dependdent proteolytic pathway (2). Protein ubiquitylation is catalyzed by a cascade reaction involving three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The E3 components are primarily involved in the specific recognition of their target proteins (3). The RING finger is one of the most characterized motifs and is essential for E3 ubiquitin ligase activity (4). The SCF complex is a multisubunit RING finger type ubiquitin ligase that catalyzes the ubiquitylation of various proteins involved in cell cycle control and signal transduction. The SCF complex consists of three invariable components, Roc1/Rbx1 (RING finger protein), Skp1 (adapter protein), and CUL1 (scaffold protein), as well as one variable F-box protein that serves as a receptor for the target proteins (5–7).

Fbw7 (F-box and WD repeat-domain-containing 7, also known as Fbxw7, Sel-10, hCdc4, or hAgo) is an F-box protein that binds to and ubiquitylates some key regulators of cell growth, including cyclin E (8), c-Myc (9, 10), c-Myb (11, 12), c-Jun (13), and Notch (14). Given that Fbw7 is responsible for degradation of several protooncogene products, Fbw7 can be considered to be a tumor suppressor. Indeed, mutations in Fbw7 were found in various types of cancers, and Fbw7<sup>−/−</sup> mice have greater susceptibility to radiation-induced tumorigenesis than wild-type mice (15–18). It has been proposed that F-box proteins often recognize a distinct consensus amino acid (aa) sequence that contains a phosphorylation site. The consensuss phospo-binding motif for Fbw7, termed the Cdc4 phosphodegron, is found in its substrates (19). Substrate phosphorylation plays an important role in interaction with an F-box protein for ubiquitylation. It has been reported that neddylation of CUL1 is necessary for the ubiquitin ligase activity of the SCF complex, and CAND1 binds to unneddylated CUL1 to form an inactive complex (20–23). However, other mechanisms that regulate the activity of the SCF complex are poorly understood.

The proteins encoded by E1A (early region 1A) of human adenovirus type 5 play an important role in viral replication (24). E1A promotes DNA synthesis and immortalization of primary rodent cells, and it cooperates in cell transformation with other oncogene products, including E1B encoded by E1B (early region 1B) of human adenovirus (25). The E1A gene generates two major species of mRNA, 13S and 12S, that encode proteins of 289 and 243 aa, respectively. Although they differ in that the
larger protein contains an extra internal 46-aa sequence, both isoforms do possess potent cell transforming activities. E1A protein interacts with various cellular proteins, including the pRb family proteins and p300/CBP, and perturbs cell proliferation and differentiation (26, 27). pRb controls G1/S progression by interacting with E2F family transcription factors. Interaction of E1A with pRb stimulates E2F-dependent transcription of several growth-promoting genes (28). Therefore, E1A promotes an unregulated S-phase entry and perturbs the cell cycle. p300 and CBP are transcriptional coactivators, which are recruited to various promoters by interacting with diverse transcription factors. E1A modulates p300/CBP-mediated transcriptional activation and chromatin remodeling through direct interaction (29). However, additional uncharacterized cellular polypeptides are found in E1A immunoprecipitates of adenovirus-transformed or adenovirus-infected cells (30). Thus, the mechanism by which E1A exerts its biological activity is not fully understood.

In the present study, we found that E1A interacts with SCF<sup>Fbw7</sup> in mammalian cells. Given that E1A and Fbw7 possess opposite functions in cell cycle regulation, we investigated whether E1A and Fbw7 interfere with each other. Although Fbw7 did not affect the stability of the E1A protein, ubiquitylation of the target proteins of SCF<sup>Fbw7</sup> was attenuated by coexpression with E1A. Using <i>in vitro</i> purified components, we found that E1A directly targeted Roc1/Rbx1 and Cul1. Indeed, we observed that the ubiquitin ligase activity of the Roc1/Rbx1-Cul1 complex was disturbed by E1A <i>in vitro</i>. Furthermore, we observed a decelerated turnover of SCF<sup>Fbw7</sup> target proteins. These results suggest that E1A can interfere with the turnover of proteins whose stability is regulated by SCF<sup>Fbw7</sup> ubiquitin ligase through the inhibition of the SCF<sup>Fbw7</sup> complex. Given that loss of function of Fbw7 promotes unregulated cell growth (18, 31), our findings have the potential to help explain the mechanism whereby adenovirus infection induces unregulated proliferation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Mammalian expression plasmids for full-length E1A, full-length Fbw7, and its deletion mutants, Skp2, Fbw1, c-Myb, ubiquitin, cyclin E, c-Myc, GSK3β, IκBα, and IKK were described in previous reports (9, 11, 32–34). Expression plasmids for Roc1/Rbx1 and its deletion mutants, Cul1–5, and Cul1 deletion mutants were kindly provided by Drs. M. Furukawa, T. Ohta, and Z. Q. Pan, respectively (35–37). To generate expression plasmids for E1A ΔN39 and ΔC104, the cDNA regions corresponding to the N-terminal 40 amino acids or C-terminal 104 amino acids were removed from pcDNA4–125 E1A by PCR-based deletion. pcDNA4-E1A ΔCR1, Δ80–119, and ΔCR2 were generated by ligation of two segments within the 12S E1A cDNA encoding amino acids 1–39 and 85–243, 1–79 and 120–243, and 1–118 and 147–243, respectively, which were each amplified by PCR. For the retroviral vectors, cDNA for 12S E1A or E1AΔN39 were recloned into the pBabe-puro plasmid. To generate bacterial expression plasmids for glutathione S-transferase (GST)-fused E1A, E1AΔN39, T7-Roc1/Rbx1, Myc-CUL1, HA-Skp1, and FLAG-Fbw7, each portion of cDNA was recloned into pGEX6P (GE Healthcare). For Strep-Tag-fused E1A, E1A cDNA was recloned into pET52 (Novagen). pGEX-4T3/pET-15b-(GST-HA-Roc1/Rbx1)-(His-FLAG-CUL1-(324–776)) was kindly provided by Dr. Z. Q. Pan (38).

**Antibodies and Reagents**—For immunoprecipitation or immunoblotting of His<sub>6</sub>-Xpress-tagged proteins, an Omni probe (Santa Cruz Biotechnology) or anti-Xpress antibody (Invitrogen) was used. Full-length c-Myc was immunoprecipitated using a polyclonal antibody (N-262; Santa Cruz Biotechnology) and detected using a monoclonal antibody (C-33; Santa Cruz Biotechnology). Antibodies against the HA epitope (3F10; Roche Applied Science), FLAG epitope (M2; Sigma), Myc epitope (9E10; Roche Applied Science) and 9B11 (Cell Signaling)), GST (B-14; Santa Cruz Biotechnology), E1A (M73; Santa Cruz), c-Myb (1-1; Upstate), α-tubulin (DM1A; Sigma), ubiquitin (Dako), Mdm2 (SMP-14; Santa Cruz Biotechnology), cyclin D1 (MBL International), p27 (57; BD Bioscience), and IκBα (C21; Santa Cruz Biotechnology) were commercially purchased. Anti-Roc1/Rbx1 antiserum (36) was kindly provided by Dr. T. Ohta. HA peptide (Sigma), MG132 (Peptide Institute) and cycloheximide (Wako) were commercially purchased.

**Cell Culture, Plasmid Transfection, and Adenovirus Infection**—All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. HEK293 cells were transfected using the calcium phosphate method, whereas HeLa cells and U2OS cells were transfected using FuGENE 6 reagent (Roche Applied Science) or Lipofectamine 2000 reagent (Invitrogen). Adenovirus type 5 was propagated in HEK293 cells. The infectious units for each cell type were determined by immunofluorescence with the anti-E1A antibody. HeLa and U2OS cells were infected at 5 infectious units/cell.

**Immunoprecipitation and Immunoblotting**—Cells were lysed in immunoprecipitation lysis buffer (0.5% Triton X-100, 300 mM NaCl, 25 mM Tris-HCl (pH 7.5), and protease inhibitor mix). Immunoprecipitation and immunoblotting were done as described previously with some modifications (32, 39).
vivo ubiquitylation assays, cell lysates were denatured by treatment with 2% SDS and 5% 2-mercaptoethanol at 100 °C to dissociate non-covalent protein associations prior to immunoprecipitation.

Retroviral Production and Transduction—For retrovirus production, pBabe-puro-based individual expression plasmids were co-transfected with gag, pol, and VSV-G envelope expressing helper plasmids into HEK293T cells using the FuGENE 6 reagent. At 18 h after transfection, the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum and incubated for an additional 24 h. Viral supernatant was collected and filtered using a 0.45-

Recombinant Proteins and in Vitro Binding Assay—GST and GST-fused Roc1/Rbx1, CUL1, Skp1, and Fbw7 proteins were expressed in Escherichia coli BL21 (DE3) and affinity-purified with glutathione-Sepharose 4B (GE Healthcare). The proteins, with the exception of GST-fused E1A, were eluted with 50 mM glutathione and then dialyzed to remove the glutathione. In the case of GST-fused E1A, GST was removed by cleavage with PreScission Protease (GE Healthcare), and the released E1A was collected. StrepTag-fused E1A protein was expressed as described above and purified using a StrepTactin Super Flow column (Novagen). A GST pull-down assay was performed as follows. A total of 10 pmol of StrepTag-fused E1A was incubated with equal amounts (mol/mol) of GST or GST-fused protein. GST-fused proteins were pulled down with glutathione-Sepharose beads and immunoblotted with anti-E1A antibody (top) or anti-GST antibody (bottom). Each of the recombinant proteins described here was bacterially expressed and purified independently.

E1A Inhibits SCF<sub>Fbw7</sub>

OCTOBER 9, 2009• VOLUME 284 • NUMBER 41

E1A Inhibits SCF<sub>Fbw7</sub>
E1A Inhibits SCF<sub>Fbw7</sub>

A

| HA-Fbw7 | + + + |
| FLAG-CUL1 | + + |
| Xpress-12S E1A | + |

IP: α-HA
IB: α-HA
IB: α-HA
IB: α-FLAG
IB: αE1A

WCE

B

| FLAG-CUL1 | + + + |
| HA-Roc1/Rbx1 | + + + |
| Xpress-12S E1A | + |

IP: α-HA
IB: α-HA
IB: α-HA
IB: α-FLAG
IB: αE1A

WCE

C

Myc-CUL1
HA-Fbw7
4xFLAG-12S E1A

2nd IP: α-FLAG
IB: Myc-CUL1
IB: HA-Fbw7
IB: 4xFLAG-12S E1A

1st IP: α-HA, Non-denaturing elution
IB: Myc-CUL1
IB: HA-Fbw7
IB: 4xFLAG-12S E1A

WCE

D

| FLAG-CUL1 | + + + |
| HA-Roc1/Rbx1 | + + + |
| Xpress-12S E1A | + |

2nd IP: Omni Probe
IB: FLAG-CUL1
IB: HA-Roc1/Rbx1
IB: Xpress-12S E1A

1st IP: α-HA, Non-denaturing elution
IB: FLAG-CUL1
IB: HA-Roc1/Rbx1
IB: Xpress-12S E1A

WCE
In Vitro Ubiquitylation Assay—GST-HA-tagged Roc1/Rbx1 and the His6-FLAG-tagged CUL1 C terminus region (corresponding to aa 324–776) were simultaneously expressed in E. coli BL21 (DE3) and co-purified with glutathione-Sepharose 4B, as described in a previous report (38). The resultant Roc1/Rbx1-CUL1 complex was incubated with 50 ng of E1 (Boston Biochem), 300 ng of UbchH5c (Boston Biochem), and 1 μg of ubiquitin (Sigma) in a ubiquitylation buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 2 mM NaF, 2 mM ATP, and 0.6 mM dithiothreitol) together with or without recombinant E1A for 60 min at 37 °C. The reactions were terminated and denatured in Laemmli’s sample buffer at 100 °C and analyzed by immunoblotting.

RESULTS

E1A Interacts with Fbw7 in Vivo—We previously reported that adenovirus E1A gene products were polyubiquitylated and degraded in a proteasome-dependent manner in mammalian cells (32). However, the ubiquitin ligase that targets E1A for ubiquitin-dependent degradation has not been identified. We noticed that there was a possible Cdc4 phosphodegron (11, 19) within the 13S E1A gene product but not within the 12S product (aa residues 183–191 of 13S E1A). Fbw7/Cdc4 recognizes the Cdc4 phosphodegron and binds to the substrates via a WD40 repeat domain. Thus, we first hypothesized that Fbw7 mediates the ubiquitin-dependent degradation of 13S E1A protein as a ubiquitin ligase. To address this hypothesis, we investigated whether Fbw7 interacts with E1A in mammalian cells. The expression plasmid for HA-Fbw7 was cotransfected into HEK293 cells together with the plasmid for 4x-FLAG-13S or -12S E1A. When FLAG-tagged 13S E1A was immunoprecipitated with anti-FLAG antibody, Fbw7 was coprecipitated. However, unexpectedly, Fbw7 was also coprecipitated with 12S E1A (Fig. 1).

Next, to analyze the effect of Fbw7 on 13S E1A protein stability, we chased protein levels of E1A by immunoblotting after inhibiting de novo protein synthesis in the cells with cycloheximide (CHX chase assay). The result indicated that coexpression of Fbw7 has no apparent effect on the degradation of 13S E1A (supplemental Fig. S1). Taken together, Fbw7 physically interacts with E1A without affecting the turnover of E1A.

E1A Attenuates Ubiquitylation of SCF<Fbw7> Target Proteins in Mammalian Cells—Although E1A usually affects the level of protein expression by its action at the transcriptional level through the interaction with pRb and p300/CBP (21, 40, 41), it has been shown that E1A alters certain protein expression levels by affecting regulation mechanisms of the protein stability (42, 43). Thus, we supposed that the ubiquitylation activity of the SCF<Fbw7> complex is affected by E1A. To test this hypothesis, we assessed the effect of E1A on c-Myc ubiquitylation, which is mediated by the SCF<Fbw7> complex and then followed by proteasomal degradation (11, 12) in mammalian cells (in vivo ubiquitylation assay). Myc epitope-tagged c-Myc (c-Myc-Myc), FLAG-ubiquitin, and HA-Fbw7 expression plasmids were cotransfected into HEK293 cells with or without a His6-Xpress-tagged E1A (hereafter referred to as Xpress-E1A) expression plasmid. To dissociate proteins noncovalently associated with c-Myc, cell lysates were incubated under denaturing conditions, and subsequently c-Myc-Myc was immunoprecipitated with anti-Myc antibody. The immunoprecipitates were subjected to immunoblotting using an anti-FLAG antibody to evaluate the ubiquitylation levels of c-Myc (Fig. 2A, Denature, IP: αMyc). Consistent with previous studies, coexpression of Fbw7 enhanced c-Myc ubiquitylation (lanes 3 and 4). Importantly, when E1A was coexpressed, c-Myc ubiquitylation was markedly diminished (lane 5).

Furthermore, in vivo ubiquitylation assays for cyclin E (Fig. 2B) and c-Myc (Fig. 2C), which are well characterized targets of SCF<Fbw7> (8–10, 16), were also performed. Similar to the ubiquitylation of c-Myc, ubiquitylation of cyclin E (Fig. 2B, lanes 3 and 4) and c-Myc (Fig. 2C, lanes 3 and 4) were facilitated by Fbw7, and E1A attenuated Fbw7-induced ubiquitylation of both proteins (Fig. 2, B, lanes 5 and 6, and C, lane 5).

We also confirmed that the ubiquitylation level of c-Myc protein was attenuated by coexpression of E1A in U2OS cells, which do not express any viral oncoproteins (supplemental Fig. S2).

Substrate specificity of SCF ubiquitin ligase is altered by the substrate recognition subunit F-box protein. To verify whether E1A specifically affects SCF<Fbw7>, we assessed the effect of E1A on IkBa ubiquitylation, which is mediated by the SCF<Fbw7>/βTrCP complex (34, 44). Although the ubiquitylation of IkBa is facilitated by Fbw1 (Fig. 2D, lanes 3 and 4), E1A did not affect it (Fig. 2D, lane 5). Taken together, these results suggested that E1A specifically inhibited the E3 ubiquitin ligase activity of SCF<Fbw7>.

E1A Directly Targets Roc1 and CUL1—Fbw7 recognizes phosphorylated substrates via a WD40 repeat domain and binds to Skp1 through the F-box to recruit the remainder of an SCF ubiquitin ligase complex (45). To determine whether these domains are required for the interaction between Fbw7 and E1A, expression plasmids for the WD40 repeat deletion (ΔWD) or the F-box deletion (ΔF) mutant Fbw7 (Fig. 3A) were transfected into HEK293 cells, and a coimmunoprecipitation assay...
was performed. Because HEK293 cells harbor 11% of the adenovirus 5 DNA sequence in a chromosome-integrated fashion, they persistently express E1A and E1B gene products (46). When the HEK293 genome-derived E1A was immunoprecipitated with anti-E1A antibody, wild-type and ΔWD Fbw7 but not ΔF were coprecipitated with E1A (Fig. 3B).

Because Fbw7 interacts with E1A in an F-box-dependent manner, it is likely that E1A directly targets the F-box and competes with Skp1 for binding to Fbw7. However, interaction between Fbw7 and CUL1 were still observed when E1A was overexpressed in HEK293 cells (supplemental Fig. S3), suggesting that E1A does not disrupt the association of Fbw7 with Skp1. To determine the direct target of E1A, Strep-Tag-fused E1A and GST-fused Roc1/Rbx1, Skp1, CUL1, and Fbw7 were expressed in E. coli and purified independently. Binding of E1A to each GST-fused protein was examined using the GST pull-down assay. As shown in Fig. 3C, Roc1/Rbx1 and CUL1 still coprecipitated with E1A in a purified cell-free system, whereas Skp1 and Fbw7 as well as the control GST did not. In addition, Roc1/Rbx1 and CUL1 exogenously expressed in HEK293 cells were coprecipitated with E1A (data not shown). Thus, we concluded that Roc1/Rbx1 and CUL1 are direct targets of E1A and that E1A indirectly interacts with Fbw7 via the Roc1/Rbx1-CUL1 complex.

**Interaction of E1A with Endogenous Roc1 and CUL1**—To confirm the interaction of E1A with endogenous Roc1/Rbx1 and CUL1 in mammalian cells, cell lysate prepared from HEK293 cells was subjected to immunoprecipitation using an antibody against E1A or control immunoglobulin, followed by immunoblotting with antibodies against CUL1 and Roc1/Rbx1. Both CUL1 and Roc1/Rbx1 were coprecipitated with E1A in the case of precipitation with anti-E1A but not with the control (Fig. 3D), indicating that 293 genome-derived E1A interacts with endogenous CUL1 and Roc1/Rbx1.

It was indicated that CUL1 was detected as double bands by immunoblotting and that the slower migrated band corresponded to the neddylylated form of CUL1 (47–49). To examine whether E1A preferentially binds to the neddylylated form of CUL1, HEK293 cells were transfected with control or Xpress-E1A expression plasmid. Cell lysates were subjected to immunoprecipitation using the Omni probe, which reacts with the His6-Xpress tag, followed by immunoblotting with an antibody (Fig. 3D), indicating that 293 genome-derived E1A interacts with endogenous CUL1 and Roc1/Rbx1.

**Effect of E1A on SCF Complex Formation**—To explain the mechanism by which E1A negatively regulates the SCF<sup>Fbw7</sup> complex or how the specificity was determined, it is important to determine whether E1A interferes with the formation of the SCF complex. As mentioned above, coexpression of E1A did not interfere with the interaction of CUL1 with Roc1/Rbx1, Fbw7, and Skp1 in HEK293 cells (supplemental Fig. S3). Using U2OS cells that do not naturally express E1A, we confirmed this result more robustly. As shown in Fig. 4A, immunoprecipitation with anti-FLAG antibody coprecipitated HA-Fbw7 in the presence of FLAG-CUL1 (lanes 1 and 2). Coprecipitation of HA-Fbw7 was still observed under conditions when E1A was coexpressed (lane 3). Thus, it was believed that E1A did not
interfere with the interaction between CUL1 and Fbw7 in U2OS cells. Based on the results from a similar coimmunoprecipitation experiment, it was also believed that E1A did not interfere with the interaction between CUL1 and Roc1/Rbx1 (Fig. 4B).

To probe more deeply into this matter, we examined whether CUL1, Fbw7, and E1A were contained in an identical complex. Myc-CUL1, HA-Fbw7, and 4×FLAG-E1A were coexpressed in HEK293 cells (Fig. 4C, bottom). HA-Fbw7 was immunoprecipitated with anti-HA antibody and was eluted under native conditions so as not to disrupt protein-protein interactions. Coprecipitations of Myc-CUL1 and 4×FLAG-E1A were confirmed as shown in Fig. 4C, middle. Then the “4×FLAG-E1A interacting with HA-Fbw7” was immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4C, top, Myc-CUL1 was coprecipitated, indicating that CUL1, Fbw7, and E1A were contained in an identical complex. A similar coimmunoprecipitation experiment also indicated that CUL1, Roc1/Rbx1, and E1A were contained in an identical complex (Fig. 4D). Thus, both CUL1-Fbw7-E1A and CUL1-Roc1/Rbx1-E1A, respectively, were detected as a ternary complex. Taken together, these results suggested that E1A binds to an intact SCF<sup>Fbw7</sup> complex.

### Analysis of the Region within E1A Required for Interaction with Roc1 and CUL1

We next determined the region within E1A that was necessary for interaction with Roc1/Rbx1 and CUL1. For this purpose, we constructed expression plasmids for deletion mutants of E1A (Fig. 5A). Individual E1A mutants or wild-type E1A were coexpressed with HA-Roc1/Rbx1 in HEK293 cells. When HA-Roc1/Rbx1 was immunoprecipitated with the anti-HA antibody, wild-type E1A but not ΔN39 was coprecipitated (Fig. 5C). This result indicated that the N-terminal por-
tion of E1A was required for the association with the SCF complex. Indeed, the E1A ΔN39 mutant failed to attenuate the ubiquitylation of c-Myb induced by Fbw7 in vivo, implying that E1A attenuated the ubiquitylation through direct interaction with the SCF<sup>Fbw7</sup> complex (Fig. 2A, lane 6).

**E1A Interferes with Roc1-CUL1 Complex-mediated Ubiquitin Polymerization in Vitro**—The observation that overexpressed E1A attenuates ubiquitylation of SCF<sup>Fbw7</sup> target proteins in vivo (Fig. 2) prompted us to investigate whether E1A directly inhibits the ubiquitin ligase activity of the SCF<sup>Fbw7</sup> complex. We performed an in vitro ubiquitylation assay mediated by Mdm2, a well characterized RING finger type ubiquitin ligase (51). Similar to the Roc1/Rbx1-CUL1 complex, when purified GST-Mdm2 (39) was incubated with E1, E2, ubiquitin, and ATP, formation of a polyubiquitin chain was observed (Fig. 6B, lanes 1–3). The presence of E1A did not impact the polyubiquitin chain formation (Fig. 6B, lanes 4–6), the molar ratios of E1A to Mdm2 were 0.2, 2, and 20, respectively. Consistent with the binding activity (Fig. 5B), the addition of E1A AN39 resulted in a remarkably reduced effect on Roc1/Rbx1-CUL1-mediated polyubiquitin chain elongation (lanes 8–10). In addition, we found that activation of E2 by E1 (50) was not affected by E1A (supplemental Fig. S6). Taken together, these results suggested that E1A directly inhibits the ubiquitin ligase activity of the Roc1/Rbx1-CUL1 complex. To evaluate whether E1A targets other cullin-based ubiquitin ligase complexes, Myc-tagged CUL1, CUL2, CUL3, CUL4A, and mouse CUL4A expression plasmids. E1A protein derived from HEK293 genome was immunoprecipitated with anti-E1A antibody and exerted ubiquitin polymerization activity (38). Using this system with minor modifications (see “Experimental Procedures” and supplemental Fig. S5), we examined the effect of E1A on the polyubiquitin chain elongation activity of the Roc1/Rbx1-CUL1 complex. As shown in Fig. 6A, incubation of the purified Roc1/Rbx1-CUL1-(324–776) complex with E1, E2, ubiquitin, and ATP resulted in the formation of a polyubiquitin chain (compare lane 3 with lane 4). This polyubiquitin chain formation was inhibited by recombinant E1A in a dose-dependent manner (lanes 5–7; the molar ratios of E1A to Roc1/Rbx1-CUL1 were 0.2, 2, and 20, respectively). These results suggested that E1A preferably interacts with CUL1 rather than with other cullins. Next, to examine whether the inhibition of ubiquitin ligase activity is specific for the Roc1/Rbx1-CUL1 complex, we performed an in vitro ubiquitylation assay mediated by Mdm2, a well characterized RING finger type ubiquitin ligase (51). Similar to the Roc1/Rbx1-CUL1 complex, when purified GST-Mdm2 (39) was incubated with E1, E2, ubiquitin, and ATP, formation of a polyubiquitin chain was observed (Fig. 6B, lanes 1–3). The presence of E1A did not impact the polyubiquitin chain formation (Fig. 6B, lanes 4–6), the molar ratios of E1A to Mdm2 were 0.2, 2, and 20, respectively. In addition, ubiquitylation of p53 by E1A was also not affected by E1A (supplemental Fig. S7).

**E1A Selectively Interacts with CUL1 among the Cullin Family Proteins**—CUL1 represents a multigene family that includes seven members: cullin 1, 2, 3, 4A, 4B, 5 and 7 in mammalian cells. Roc1/Rbx1 associates with all cullins, and each cullin forms a separate class of ubiquitin ligase complex together with distinct adaptors and/or substrate recognition subunits (36, 52). To evaluate whether E1A targets other cullin-based ubiquitin ligase complexes, Myc-tagged CUL1, CUL2, CUL3, CUL4A, and CUL5 were expressed in HEK293 cells. Immunoprecipitation with anti-E1A antibody revealed that only CUL1 was coprecipitated with E1A (Fig. 7A), whereas Roc1/Rbx1 bound to all cullins tested (Fig. 7B). Taken together, these results suggest that although Roc1/Rbx1 commonly interacts with different cullins, E1A associates with the CUL1-based ubiquitin ligase complex rather than other cullin-based ubiquitin ligase complexes.

**E1A Alters the Protein Stabilities Regulated by SCF<sup>Fbw7</sup> in Mammalian Cells**—The above results prompted us to investigate whether E1A alters the protein stabilities that are regulated by SCF<sup>Fbw7</sup>. We first examined the degradation rate of c-Myb exogenously expressed in HeLa cells by the CHX chase assay (Fig. 8A). The degradation rate of c-Myb was accelerated by coexpression of Fbw7 (Fig. 8A, top and second from the top panels; half-lives under these conditions were >240 min and 9.3 min, respectively). Coexpression of E1A with Fbw7 attenuated the acceleration of c-Myb degradation (Fig. 8A, third from the top panel; half-life under this condition was 28 min), suggesting that E1A inhibits the facilitation of the protein degradation by Fbw7. Similar results were observed in the degradation of cyclin E (Fig. 8B; half-lives when cotransfected with control, Fbw7, and both Fbw7 and E1A plasmids were >60, 36, and 59 min, respectively) and c-Myc (Fig. 8C; half-lives when
cotransfected with control, Fbw7, and both of Fbw7 and E1A plasmids were 26, 13, and 25 min, respectively. To clarify the role of E1A in a more physiological setting, E1A was ectopically expressed with a retrovirus vector in HeLa cells, and the effect on the degradation rate of endogenous c-Myc was assessed by the CHX chase assay. We found that the degradation of c-Myc protein was prominently retarded by the infection with the E1A expression retrovirus compared with the infection with a control virus. In contrast, degradation of cyclin D1 and p27, targets of SCF<sup>Fbw7</sup>/H9251 B-crystallin and SCF<sup>Skp2</sup> (53–55), respectively, were not retarded by E1A (Fig. 9A, top). A similar result was observed in U2OS cells (Fig. 9A, bottom). We also confirmed that infection of U2OS with retrovirus expressing E1A resulted in accumulation of endogenous c-Jun and cyclin E, other targets of SCF<sup>Fbw7</sup>. This effect seemed to be, at least partially, independent of the pRb-E2F pathway, because a similar effect was observed in pRb-inactive Saos-2 cells (Fig. 9B). In addition, SCF<sup>Fbw7/βTrCP</sup>-mediated degradation of IkBα in response to TNFα occurred normally in the presence of E1A (Fig. 9C). These observations suggest that E1A specifically alters the turnover rates of the target proteins of SCF<sup>Fbw7</sup> in mammalian cells.

Finally, we tried to determine whether infection of wild-type adenovirus 5 affects the stability of c-Myc. As shown in Fig. 9D, the expression of E1A protein was detected in HeLa cells infected with human adenovirus 5. We confirmed that E1A associated with endogenous Roc1/Rbx1. Infection with adenovirus 5 decelerated the degradation rate of endogenous c-Myc protein in both HeLa cells and U2OS cells (Fig. 9E). Taken together, these observations suggest that adenovirus perturbed the regulation of several oncogenic protein stabilities through the inhibition of the SCF<sup>Fbw7</sup> ubiquitin ligase complex by E1A.

**DISCUSSION**

In studies of protein degradation systems mediated by SCF ubiquitin ligase, a large number of analyses have been made on the modifications within the substrate in response to degradation signals and their recognition by F-box proteins. However, regulation of the activity of SCF ubiquitin ligase itself is poorly understood.

This is the first study to report that E1A inhibits SCF ubiquitin ligase. However, two major issues, the mechanism by which E1A inhibits the activity of ROC1/Rbx1 complex, at first, we neglected the possibility that E1A inhibited the E1-mediated activation of E2 in an E3-independent manner. As shown in supplemental Fig. S6, ubiquitin was transferred to E2 in an E1-dependent manner.

**FIGURE 8.** E1A decelerates degradation rate of Fbw7 target proteins. A, degradation rate of c-Myb. HeLa cells were cotransfected with Myc-tagged c-Myb and control or Fbw7 expression plasmids together with or without the E1A expression plasmid, as indicated. The cells were treated with 10 μg/ml CHX for the indicated periods. Cell extracts were analyzed by immunoblotting using anti-c-Myb or anti-α-tubulin antibodies. Expression levels of α-tubulin are indicated as an internal control (top). Expression levels of HA-Fbw7 and Xpress-E1A were compared by immunoblotting with anti-HA and anti-E1A antibodies, respectively (bottom). B and C, degradation rates of exogenous Myc-tagged cyclin E (B) and c-Myc (C) were assessed as described in A. Myc-cyclin E and c-Myc were detected using anti-Myc (tag) and anti-c-Myc antibodies, respectively.
either in the presence or absence of E1A. Therefore, E1A did not affect the E1-mediated activation of E2 in vitro. This observation is in accordance with the fact that E1A had no effect on the ubiquitin ligase activity of Mdm2. Thus, it seemed that E1A affected the process posterior to E2 activation.

Neddylation of CUL1 is a known modification that is required for activation of the SCF ubiquitin ligase complex (20). It was of interest to determine whether E1A inhibited the neddylation of CUL1. As shown in supplemental Fig. S8, coexpression of E1A did not alter the neddylation level of CUL1 in HEK293 cells. This result is consistent with the finding that E1A inhibited the ubiquitin ligase activity of the Roc1/Rbx1-CUL1 complex in an in vitro reconstitution assay that was absent from the neddylation system. Thus, we believe that E1A represses the ubiquitin ligase activity without affecting the neddylation of CUL1. In addition, E1A interacts with CUL1 without remarkable selectivity between the neddylated and unneddylated forms of CUL1.

Furthermore, we examined the effects of E1A on the formation of the SCF complex. Evidence from the coimmunoprecipitation assay indicated that E1A did not disrupt the SCF complex. E1A was contained in an identical complex between CUL1 and Roc1/Rbx1 as well as between CUL1 and Fbw7. Therefore, E1A binds to the intact SCF<sub>Fbw7</sub> complex and inhibits ubiquitylation of the substrate, whereas E1A does not interfere with the formation of the SCF complex.

Next, we assumed that E1A would repress the recruitment of E2 to the Roc1/Rbx1-CUL1 complex and examined it using the coimmunoprecipitation assay. Probably because the interaction between E2 and Roc1/Rbx1-CUL1 was transient and unstable (47, 56), we failed to detect its presence (data not shown). Recently, Saha and Deshaies (56) displayed this transient interaction by taking advantage of a developed assay based on the formation of the SCF complex. Evidence from the coimmunoprecipitation assay indicated that E1A did not disrupt the SCF complex but not with that of the other cullin-Roc1/Rbx1 complexes. Although purified recombinant Roc1/Rbx1 interacted with E1A in vitro, Roc1/Rbx1 coprecipitated with CUL2, CUL3, CUL4A, and CUL5 did not interact with E1A in the cells. We speculate that except for CUL1, the E1A binding sequence within Roc1/Rbx1 might be masked by other cullins that associate with Roc1/Rbx1. Alternatively, as shown in supplemental Fig. S9, C and D, the C-terminal 131 aa residues of CUL1 were required for interaction with E1A. This observation is consistent with the fact that aa 650–776 of the E1A-binding domain in CUL1 is only 40% conserved in other cullins, such as CUL2 and CUL3. Therefore, E1A selectively binds to CUL1 and inhibits SCF-type ubiquitin ligase.

Most importantly, E1A specifically inhibits the activity of SCF<sub>Fbw7</sub> but not the other SCF ubiquitin ligases, including SCF<sub>Fbw1/TrCP</sub>. SCF ubiquitin ligase contains the growth-promoting subtype SCF<sub>Skp2</sub> (6). Adenovirus may acquire the more preferable form of the E1A gene to replicate the viral genome during the process of natural selection.

Tworkowski et al. (60) reported that E1A stabilized c-Myc protein via interaction with p400. Since the N-terminal region of E1A is required for interaction with p400 (61), it is not easy to experimentally distinguish between the effect of E1A on SCF<sub>Fbw7</sub> and on p400. Because several substrates for SCF<sub>Fbw7</sub>, such as c-Myc, c-Myb, c-Jun, and cyclin E, were stabilized by E1A, we believe that E1A has inhibitory activity against SCF<sub>Fbw7</sub>. However, we cannot deny the involvement of p400 on the stabilization of c-Myc through their binding with each other.

**FIGURE 9.** E1A interferes with the degradation of endogenous c-Myc protein. A, ectopic expression of E1A decelerates the degradation rate of endogenous c-Myc. HeLa or U2OS cells were infected with control or E1A expression retrovirus vectors. The cells were treated with 10 μg/ml cycloheximide for the indicated periods. The degradation rates of endogenous c-Myc, cyclin D1, and p27 were analyzed by immunoblotting with anti-c-Myc, cyclin D1, and p27 antibodies, respectively. Pictures of immunoblotting shown at the left are representative of three independent experiments. For representative purposes, c-Myc and cyclin D1 levels were quantified and normalized against α-tubulin, and mean values relative to the value at zero time are plotted to the right. The error bars show S.D. B, accumulation of c-Jun and cyclin E proteins by ectopic expression of E1A. U2OS or Saos-2 cells were infected with control or E1A expression retrovirus vectors. Steady-state c-Jun and cyclin E protein levels were analyzed by immunoblotting with anti-c-Jun and with anti-cyclin E antibodies. C, E1A does not affect the degradation of IκBα. HeLa cells were infected with control or E1A expression retrovirus vectors. The cells were treated with 10 ng/ml TNFα for the indicated periods. The degradation rates of endogenous IκBα were analyzed by immunoblotting with anti-IκBα antibody. Pictures of immunoblotting shown at the top are representative of three independent experiments. The results were plotted after quantitation as described in A (bottom). D, E1A interacts with endogenous Roc1/Rbx1. HeLa cells were infected with adenovirus type 5 (Ad5). After 24 h, cells were lysed, and E1A was immunoprecipitated with anti-E1A antibody. Immunoprecipitates were immunoblotted with the indicated antibodies. Uninfected cells were used as a control. E, effect of adenovirus 5 infection on the degradation rate of endogenous c-Myc. HeLa or U2OS cells were infected with adenovirus 5 as described in D. After 16 h, cells were treated with cycloheximide, and the degradation rates of endogenous c-Myc were assessed by immunoblotting.
E1A Inhibits SCF^{Fbw7}

Based on the observation that E1A forms a ternary complex with CUL1 and Fbw7, it was believed that specific competition against F-box protein could not explain the specificity of the negative effect of E1A. As shown in supplemental Fig. S9, we found that E1A bound to the N-terminal portion (aa 23–42) of Roc1/Rbx1 and the C-terminal portion (aa 650–776; from helix 28 to the C-terminal end) of CUL1 to form the ternary complex. CUL1 binds to the S1 (β-strand 1; aa 1–23) region of Roc1/Rbx1 (62, 63). E1A bound to loop 1 (aa 23–42) behind the S1 region of Roc1/Rbx1. Because E1A bound to the C-terminal portion (aa 650–776; from helix 28 to the C-terminal end) of CUL1, the E1A binding region of CUL1 is believed to be helices 29–31 of CUL1. When E1A binds to SCF^{Fbw7}, E1A could effectively inhibit the ubiquitin ligase activity of Roc1/Rbx1. In contrast, we speculate, because of the conformation of the complex, E1A may not possess effective interactions with SCF ligases with other F-box proteins, such as Fbw1, to inhibit their activity. Namely, in the case of attachment of ubiquitin to the substrates via SCF^{Fbw7}, it seems that the C-terminal portion of E1A inserts in the space between E2 and the substrate to interfere with the ubiquitin transfer from E2 to the substrate. On the contrary, in the case of SCF^{Fbw4}, E1A could not inhibit ubiquitin transfer from E2 to its specific substrates, whereas aa 1–40 of E1A also binds to Roc1/Rbx1 and CUL1. Because there may be some differences in the three-dimensional space containing the F-box protein, substrates, and E2, E1A may not be able to locate in the region between E2 and the substrates. E1A inhibited the substrate-independent polyubiquitin chain elongation activity of the CUL1-Roc1/Rbx1 complex without Skp1 and the F-box protein. We speculate the reason for this is that there are no three-dimensional spatial obstacles. Analysis of the high order structure of the E1A-SCF^{Fbw7} complex may provide an answer as to why the specificity is yielded.

Although we investigated effects of E1A on some kinds of SCF complexes, further studies are required in order to better understand the specificity of the inhibitory activity of E1A. However, we conclusively demonstrated that E1A has selective inhibitory activity against SCF^{Fbw7}.

Biological Significance of Inhibition of SCF^{Fbw7}—Small DNA tumor viruses, such as human adenoviruses, papillomaviruses, and SV40, rely on the host cell DNA replication machinery for replication of the viral genomes. Because the host DNA replication machinery becomes available during S phase, productive infection depends upon the ability of the virus to induce S phase entry of the host cell from a quiescent state. The E1A gene is essential for the replication of human adenovirus. E1A promotes unregulated DNA synthesis and possesses potent cell transforming activity, so repression of Fbw7, which down-regulates several oncogenic proteins and is considered to be a potent tumor suppressor, is consistent with the function of the E1A gene. Indeed, conditional inactivation of Fbw7 in the T cell lineage of mice caused thymic hyperplasia as a result of c-Myc accumulation, and the mice eventually developed thymic lymphoma (31). In the present study, we proved that, after adenovirus infection, adenovirus-derived E1A bound to endogenous Roc1/Rbx1 and attenuated degradation of the endogenous target protein of SCF^{Fbw7}. This is direct evidence that SCF^{Fbw7} is a bona fide target of adenovirus in the host cell.

E1A induces the accumulation of p53 protein and apoptosis through the activation of the ARF-Mdm2-p53 pathway in the host cell (64–66). Thus, destabilization of p53 is also an important process to utilize the host cell replication machinery. Querido et al. (67) reported that adenovirus E1B 55K and E4orf6 interacted with the Roc1/Rbx1-CUL5 ubiquitin ligase complex in a coordinated fashion, behaving as a substrate recognition subunit so that polyubiquitination and degradation of p53 was facilitated. Since E1A did not interact with the Roc1/Rbx1-CUL5 complex, we consider that inhibition of SCF^{Fbw7} is concomitant with the utilization of the Roc1/Rbx1-CUL5 complex to degrade the growth-suppressive protein p53.

The SCF complexes, which are mainly active from late G1 to G2 phase, are involved in the proteolysis of the core components of the cell cycle machinery. The anaphase-promoting complex/cyclosome (APC/C), which is active from the M to G1 phase, is another macromolecular ubiquitin ligase complex that targets various proteins important for the cell division process. Turnell et al. (43) showed that APC/C interacted with a histone acetyltransferase p300/CBP and stimulated its activities. They proposed that E1A interfered with the activity of APC/C through the interaction with p300/CBP. Interestingly, E1A that lacked the N-terminal region failed to interact with p300/CBP (68). These observations suggest that the N-terminal region is essential for interference with both the SCF complex and APC/C.

It has been shown that SV40 large T antigen, an oncprotein that is produced by SV40 DNA tumor virus, interacts with Fbw7 and interferes with Fbw7-driven substrate turnover by competition with the substrate for Fbw7 binding (69). Since direct binding of E1A to Fbw7 was not observed, it is unlikely that E1A competes with the substrates for binding to Fbw7. However, repression of Fbw7 may be a common event to stimulate the DNA replication machinery of host cells by such DNA tumor viruses. Thus, it implies that regulation of several proto-oncogene products by SCF^{Fbw7} at the post-translational level is essential for the maintenance of normal cell cycle regulation.

Acknowledgments—We thank Drs. Zhen-Qiang Pan, Tomoki Chiba, Manabu Furukawa, and Keiichi I. Nakayama for providing plasmids and Sayuri Suzuki, Harumi Shiratori, Yuki Fujimoto, Daisuke Aka-hori, Kensuke Kitsugi, and Azusa Mori for technical assistance.

REFERENCES
1. Hicke, L., Schubert, H. L., and Hill, C. P. (2005) Nat. Rev. Mol. Cell Biol. 6, 610–621
2. Pickart, C. M. (2000) Trends Biochem. Sci. 25, 544–548
3. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
4. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 11364–11369
5. Cardozo, T., and Pagano, M. (2004) Nat. Rev. Mol. Cell Biol. 5, 739–751
6. Nakayama, K. I., and Nakayama, K. (2006) Nat. Rev. Cancer 6, 369–381
7. Peterski, M. D., and Deshaies, R. J. (2005) Nat. Rev. Mol. Cell Biol. 6, 9–20
8. Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Science 294, 173–177
9. Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., and Nakayama, K. I. (2004) EMBO J. 23, 2116–2125
10. Welcker, M., Orian, A., Jin, J., Grim, J. A., Harper, J. W., Eisenman, R. N.,
