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Adenoviral E1A Targets Mdm4 to Stabilize Tumor Suppressor p53

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ABSTRACT

The adenoviral protein E1A associates with multiple anticancer activities, including stabilization of p53 tumor suppressor, and has been tested through gene therapy approaches in clinical trials. To identify potential E1A-binding proteins involved in E1A's anticancer activities, we screened a yeast two-hybrid library and identified Mdm4, an Mdm2-related p53-binding protein, as a novel E1A-binding protein. The NH_2-terminal region of Mdm4 and the CR1 domain of E1A were required for the interaction between E1A and Mdm4. E1A preferentially bound to Mdm4 rather than Mdm2 and formed a complex with p53 in the presence of Mdm4, resulting in the stabilization of p53 in a $p14^{ARF}$-independent manner. E1A failed to stabilize p53 in the absence of Mdm4, showing that Mdm4 was required for p53 stabilization by E1A. Moreover, E1A-mediated stabilization of p53 occurred in nucleus. Although it had no effect on the p53-Mdm2 interaction, E1A facilitated Mdm4 binding to p53 and inhibited Mdm2 binding to Mdm4, resulting in decreased nuclear exportation of p53. Thus, our findings highlighted a novel mechanism, whereby E1A stabilized the p53 tumor suppressor through Mdm4.

INTRODUCTION

Adenoviral E1A, a well-known transcription regulator (1, 2), originally was recognized as an oncogene because its expressed proteins immortalize primary rodent cells. However, in the past decade, many studies indicated that E1A was associated with multiple anticancer activities, and E1A gene therapy for cancer has been investigated in clinical trials (3–5). It has been reported that the adenoviral protein E1A increases the half-life of p53 tumor suppressor in rodent and human cells (6), but its mechanism still is not completely defined. Although $p19^{ARF}$ was shown to be involved in E1A-mediated p53 stabilization in rodent cells (7), many studies showed that p53 could be stabilized in cells deficient of $p14^{ARF}$ (the human equivalent of $p19^{ARF}$), especially for the p53 activation in DNA damage response (8). Because E1A is a protein with versatile function, these observations implied that there might be a $p14^{ARF}$-independent mechanism of p53 stabilization by E1A, and other factors may be involved in this mechanism (9).

Mdm4 (also known as MdmX), a protein structurally similar to Mdm2, is one of the factors involved in p53 stabilization mechanism. Like Mdm2, Mdm4 is capable of binding to p53 and repressing p53-dependent transactivation. However, Mdm4 lacks ubiquitin ligase activity and does not target p53 for degradation. Instead, it protects p53 from Mdm2-mediated degradation. Mdm4 also possesses several other properties distinguishing it from Mdm2. Mdm4 does not possess efficient nuclear export capability, and its expression is not regulated by p53 (10–14). It interacts with Mdm2 through their RING finger domains and forms a heterotrimeric complex with p53 (15). Mdm4 also stabilizes Mdm2, whereas Mdm2 promotes the ubiquitination and degradation of Mmd4 (16–18). Genetic evidence also suggests that Mdm4 and Mdm2 are nonredundant critical regulators of p53 (19). Mdm4 is ubiquitously expressed, and the loss of p53 completely rescues the Mdm4−/− embryonic lethality, showing that Mdm4 is a major regulator of p53 *in vivo* (8, 10).

In an effort to identify novel E1A-binding proteins that may contribute to the tumor suppressor activities of E1A, we found that Mdm4 was a novel target of adenoviral E1A by screening yeast two-hybrid library. In this study, we showed the importance of Mdm4 for E1A-induced p53 stabilization. E1A bound to Mdm4 *in vitro* and *in vivo* and targeted Mdm4 to stabilize p53 in a $p14^{ARF}$-independent manner. We also found that E1A prevented p53 from degradation by forming a tricomplex with p53 and Mdm4. Our data also suggested that the tricomplex enhanced nuclear retention of p53, suppressing Mdm2-mediated nuclear export and degradation of p53. These findings shed light to define a general mechanism whereby E1A stabilized the p53 tumor suppressor.

MATERIALS AND METHODS

Cell Culture. Human cell lines MCF-7 (breast cancer, wild-type p53), U2OS (osteosarcoma, wild-type p53), and 293T (expressing wild-type p53 and adenoviral E1A/E1B) were obtained from the American Type Culture Collection (Manassas, VA). p53-null, Mdm2/p53 double null, and Mdm4/p53 double null mouse embryo fibroblast (MEF) cells were generated from knockout mice as described previously (19). All of the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. MCF/E1A, a MCF-7 stable cell line expressing adenoviral E1A, was established by transfecting adenoviral E1A DNA (20) into MCF-7 cells and subsequently G418 selecting. MCF/E1A cells were maintained in DMEM containing 10% fetal bovine serum and 500 ng/mL G418.

Plasmid Constructs and Antibodies. To construct pBTM-E1A, 12S E1A sequence was amplified from the E1A coding region (21) by PCR with primers 5′-GGCTGGAATTCATGAGACATATTATCTGCC-3′ and 5′-CGACGGATCCCTTATGGCCTGGGGCGTT-3′. The generated fragment was inserted into *Eco*RI/*Bam*HI sites of the yeast expression vector pBTM116 as described previously (22), and the subcloned sequence was confirmed by DNA sequencing. To produce the glutathione *S*-transferase (GST) fusion protein, GST-E1A was constructed by digesting BTM-E1A with *Eco*RI/*Sal*I and then inserting the 12S E1A fragment into corresponding sites of the pGEX6p1 vector (Amersham Biosciences, Piscataway, NJ). GST-E1AΔC was made by *Xba*I/*Xho*I digestion of GST-E1A and self-ligation. Other E1A mutants were produced by inserting PCR fragments into *Eco*RI/*Xho*I sites of pGEX6p1. The sequences of forward primers for GST-E1AΔN, GST-E1A (80–243), and GST-E1A (120–243) are 5′-AGCTGAATTCATGCCACCTACCCTTCACGAACTGTATG-3′, 5′-AGCTGAATTCATGCTCACTTTTCCGCCGGCGCCCGG-3′, and 5′-AGCTGAATTCATGGATCTTACCTGCCACGAGGCTGG-3′, respectively, and the reverse primer was 5′-ACTGCCTCGAGTTATGGCCTGGGGCGTTTAC-3′. GST-E1A1108 was derived from dl1108 (23) using the same upstream primer as GST-E1A.

To construct human Mdm4 or its deletion mutant expression vectors, the desired sequences were amplified from Mdm4 coding region by PCR and cloned into *Hind*III/*Bam*HI sites in pCDNA3 vector (Invitrogen Corp., Carlsbad, CA). The forward and reverse primers for full-length Mdm4 are 5′-AA-

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AAGCTTATGACATCATTTTCCACC-3′ and 5′AAGGATCCTTATGCTATAAAAACCTT; for Mdm4 (1 to 107) mutant: 5′-AAAAGCTTATGACATCATTTTCCACC and 5′AAGGATCCGTAGCAGTGGCTAAAGTG; for Mdm4 (107 to 490) mutant: 5′-AAAAGCTTCACTTTAGCCACTGCTAC and 5′-AAGGATCCTTATGCTATAAAAACCTT; and for Mdm4 (107 to 431): 5′-AAAAGCTTCACTTTAGCCACTGCTAC and 5′-AAGGATCCCTGGCAATCCTCCATGTT, respectively. T7-p53 was obtained by subcloning coding sequence of p53 from its expression plasmid LTRXA (22) to pCDNA3 vector, which contains T7 promoter. Dr. Donna L. George provided Mdm4-myc (18). GST-Mdm4 was a gift from Dr. Arthur L. Haas (24). Dr. Yanping Zhang provided CMV-p14^{ARF}.

The mouse monoclonal antibody against p53 (Ab-3, IgG_{2a}/κ) was obtained from NeoMarkers (Fremont, CA), and rabbit polyclonal antibody against p53 was obtained from Cell Signaling (Beverly, MA). Goat antibody against Mdm4 and mouse antibody against histone-H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against E1A and Mdm2 were obtained from BD Biosciences (San Diego, CA). Anti-myc tag antibody was obtained from Roche (Indianapolis, IN; monoclonal) or from Cell Signaling (polyclonal). Rabbit polyclonal antibody against actin was purchased from Sigma (St. Louis, MO).

Yeast Two-Hybrid Screening. The pBTM-E1A (12S) construct was used as bait to screen the yeast two-hybrid library that was arranged as the prey cDNA array. The L40 yeast strain was transformed with pBTM-E1A. The mating partner, AMR70, previously was transformed with each specific prey gene in the pACT2 vector, and each transformant was inoculated in selective medium in 96-well plates. The pBTM-E1A transformant of the L40 strain was allowed to mate with each AMR70 transformant, and subsequently mated yeast clones were selected on a medium lacking histidine, leucine, and tryptophan. The yeast colonies were further duplicated on plates containing X-gal to test β-galactosidase. The E1A-interacting candidate genes in the pACT2 vector were cotransformed with pBTM-E1A or pBTM-lamin (negative control) into the L40 strain to test the binding specificity.

***In vitro* Binding Assays.** GST-E1A or GST-Mdm4 was transformed into *Escherichia coli* BL21. The bacteria were grown at 30°C in LB medium, and GST-fusion protein synthesis was induced with 0.5 to 1.0 mmol/L of isopropyl-l-thio-β-D-galactopyranoside. Cells were harvested after 3 to 4 hours. The cell pellet was resuspended in cold sodium-Tris-EDTA lysis buffer [10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 150 mmol/L NaCl] supplemented with lysozyme (1 mg/mL; Sigma) and incubated on ice for 15 minutes. Just before sonication, 1 mmol/L DTT, 10 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 1% Sarkosyl (Sigma) were added to the cell lysate and mixed thoroughly. The cell lysate then was sonicated at maximum intensity for 20 seconds. Triton X-100 (2%) was added, and the cell lysate was mixed gently for 30 minutes to help the fusion protein dissolve. After centrifugation, the GST-fusion protein was purified by Glutathione Sepharose 4B (Amersham Biosciences) and eluted with buffer [50 mmol/L Tris (pH 8.0)] containing 10 mmol/L glutathione (reduced form; Sigma). To purify E1A protein without GST moiety, the GST-E1A fusion protein was digested with PreScission Protease (Amersham Biosciences), and the GST moiety was removed by glutathione affinity chromatography.

Mdm4 or its deletion mutants and p53 expression constructs were individually transcribed and translated in the presence of [^{35}S]-methionine using the T7-coupled reticulocyte lysate system (TNT; Promega, Madison, WI). The same amounts of GST and GST-fusion proteins were coupled to Glutathione Sepharose 4B beads and mixed with 20 μL of TNT reaction in the pulldown assays. Each reaction was performed in the binding buffer [10 mmol/L HEPES (pH 7.5), 10% glycerol, 300 mmol/L NaCl, 0.5 mmol/L EDTA, 0.1% NP40, 10 μmol/L NaF, 10 μmol/L Na_3VO_4, 1 mmol/L PMSF, and 1% aprotinin] and incubated for 1 hour at 4°C. The beads were washed four times with binding buffer and boiled in 20 μL of SDS loading buffer. Complexes were separated by 8% SDS-PAGE and visualized by exposure to X-ray film for autoradiography.

Immunoprecipitation and Western Blot Analysis. For immunoprecipitation assays, cell lysates (500 μg of protein) were incubated with 1 μg of the appropriate antibody and lysis buffer (final volume, 300 μL) overnight at 4°C with mixing. After the addition of 40 μL of protein A or G agarose beads, the reactions were incubated for 3 hours at 4°C with mixing. The beads were washed four times in immunoprecipitation assay buffer [1% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 10% glycerol, 10 μmol/L NaF, 10 μmol/L Na_3VO_4, 1 mmol/L PMSF, and 1% aprotinin]. The immunoprecipitates were separated by SDS-PAGE and transferred to PROTRAN nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). For immunoblot experiments, 20 to 50 μg of lysates in SDS loading buffer were separated by SDS-PAGE. Western blot analysis was performed using a chemiluminescence system (Amersham Biosciences).

Cell Fractionation. Cells were washed with cold PBS twice and collected in lysis buffer [20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.5% NP40, 100 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 1% aprotinin]. Cell lysates were homogenized for 30 strokes and centrifuged at 4,000 rpm for 5 minutes. After centrifugation at 12,000 rpm for 20 minutes, the supernatant was collected as a nonnuclear fraction. The nuclear pellet was washed with lysis buffer four times and resuspended in nuclear extract Tris-NP40 buffer [150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris (pH 8.0), 0.5% NP40, 25 mmol/L NaF, 2 mmol/L Na_3VO_4, 5 mmol/L PMSF, and 1% aprotinin]. The samples were sonicated for 10 seconds and centrifuged at maximum speed for 20 minutes. The supernatant was saved as a nuclear fraction.

RESULTS AND DISCUSSION

E1A Binds Mdm4 *In vitro* and *In vivo*. To search for novel E1A-binding proteins, we used 12S E1A as bait to screen yeast two-hybrid library and identified that Mdm4, a p53-binding protein, was a positive candidate (Fig. 1*A*). To validate whether E1A-Mdm4 interaction also occurs in human cells, myc-tagged Mdm4 was transfected into E1A-expressing 293T cells. E1A was coimmunoprecipitated with Mdm4 but not with the IgG control, indicating that Mdm4 associated with E1A specifically in human cells (Fig. 1*B*). To further explore whether E1A can directly bind to Mdm4, the GST pulldown assay was performed using purified GST-E1A protein and ^{35}S-labeled *in vitro* translated Mdm4 protein. GST-E1A, not the GST control, was capable of interacting with Mdm4, indicating that E1A is capable of binding to Mdm4 directly (Fig. 1*C*). Because of its structural similarity to Mdm4, we also performed the GST pulldown assay for Mdm2 and E1A under the same conditions. The E1A binding to Mdm2 was barely detectable even after longer exposure of the film (Fig. 1*C, right*), suggesting that E1A preferentially bound to Mdm4. To define the protein domains of E1A required for binding to Mdm4, a series of GST-linked mutants of Mdm4 E1A mutants were generated (Fig. 1*D* and *E*), and their interaction was evaluated by *in vitro* binding assays. Mdm4 (107–431), a mutant lacking the NH_2-terminal p53-binding domain and COOH-terminal region, failed to interact with E1A. The interaction between E1A and Mdm4 (107 to 490) that lacks p53-binding domain also was undetectable. Conversely, Mdm4 (1 to 107), the mutant containing only p53-binding domain, can interact with E1A (Fig. 1*D*). These results showed that the NH_2-terminal region, including the p53-binding domain of Mdm4, was required for its binding to E1A. Conversely, like wild-type E1A-12S, the E1AΔC, E1A-1108, and E1AΔN mutants were capable of binding to Mdm4. However, E1A (80 to 243) and E1A (120 to 243), both which lack CR1 domain, failed to interact with Mdm4, indicating that CR1 domain is required for E1A-Mdm4 interaction (Fig. 1*E*).

E1A Stabilization of p53 through Mdm4 Is Independent of p14^{ARF}. Several studies have shown that E1A can stabilize p53 protein (6, 7), and genetic evidence showed that Mdm4 has been shown to be an essential regulator of p53 (19). Because we found that Mdm4 directly interacted with E1A, we hypothesized that Mdm4 was involved in the E1A-mediated stabilization of p53 by binding to E1A. To evaluate the effects of E1A and Mdm4 expression on p53 protein level, we examine the protein expression in p53-positive MCF-7 cell line and its stable E1A transfectant line MCF/E1A. In agreement with other studies showing that E1A can stabilize p53 protein, the p53 protein level was increased in MCF/E1A cells compared with that in

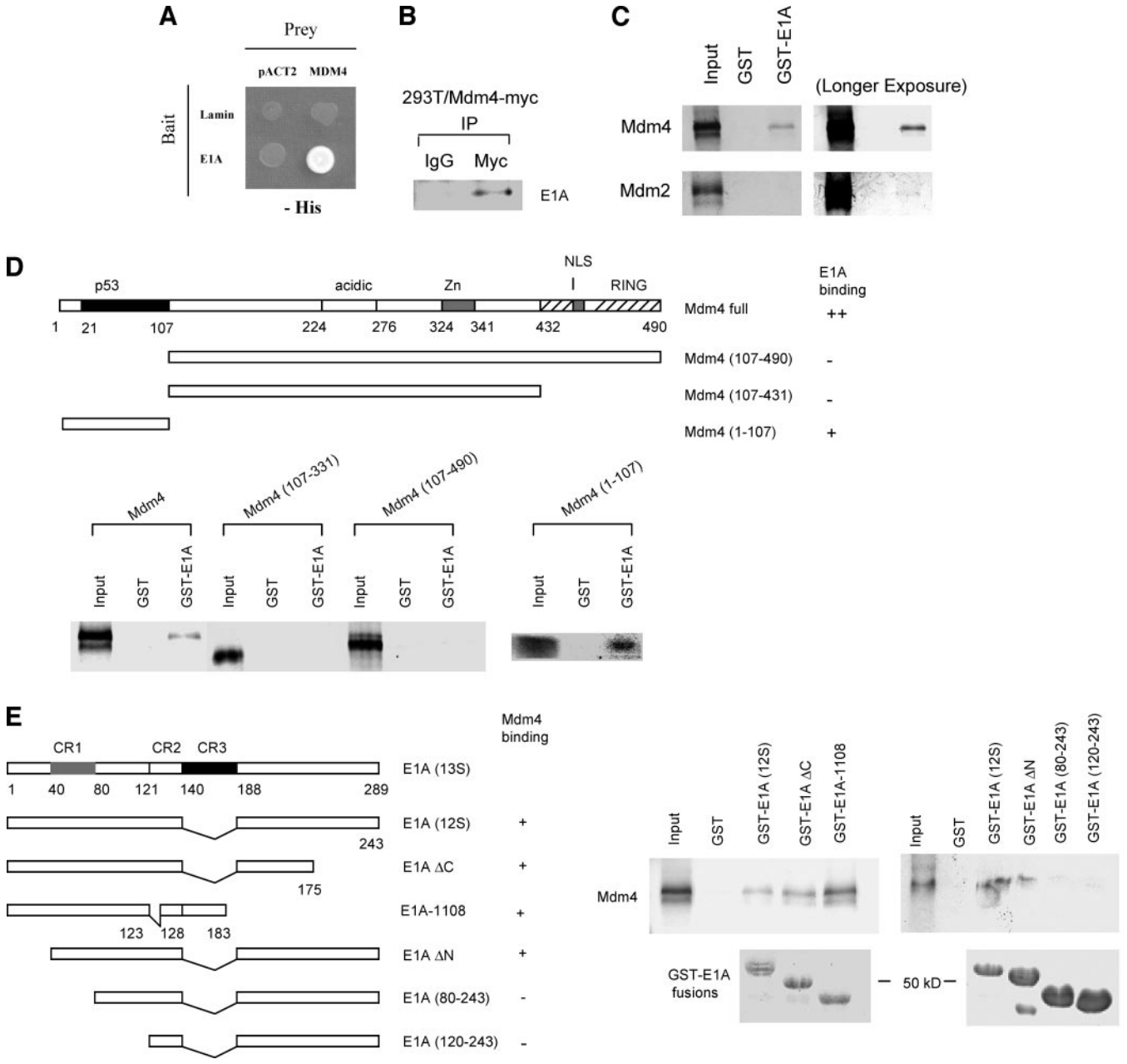

Fig. 1. Interaction between E1A and Mdm4 *in vitro* and *in vivo*. *A,* Mdm4 interacted with 12S E1A in the yeast two-hybrid system. Yeast strain L40 was cotransformed with a bait (12S E1A or the control protein lamin fused to the LexA protein) and a prey protein (MDM4 or control vector pACT2). The interaction was monitored by the yeast clone growth in restriction medium as described in Materials and Methods. *B,* Mdm4 interacted with E1A in human cells. The Mdm4-myc construct was transfected into 293T cells. The Mdm4-E1A complexes were immunoprecipitated from extracts using myc antibody and analyzed by Western blot analysis using the E1A antibody. *C,* Mdm4 bound to E1A directly. The GST pulldown assay was performed using GST-E1A (12S) proteins and ^{35}S-labeled *in vitro* transcribed and translated Mdm4 proteins. The binding of Mdm2 and E1A also was measured under the same conditions. *D.* The NH_2-terminal of Mdm4 was required for Mdm4 binding to E1A. Wild-type Mdm4 and mutant Mdm4 constructs were *in vitro* transcribed and translated individually in the presence of [^{35}S]methionine. The GST pulldown assay was performed. *E,* The CR1 domain of E1A was required for E1A binding to Mdm4. GST-E1A wild-type and mutant proteins were purified and used for an *in vitro* binding assay in the presence of ^{35}S-labeled *in vitro* transcribed and translated Mdm4 protein.

MCF7 (Fig. 2*A*). Exogenous expression of Mdm4 also increased the p53 protein level in MCF7 and MCF/E1A cells (Fig. 2*B*), even though the basal p53 level in the latter is higher than the former. These data supported that the effect of E1A or Mdm4 on p53 stabilization coexisted in MCF-7 cells, and E1A was capable of stabilizing p53 without increasing Mdm4 expression (Fig. 2*A*). It was reported that E1A-induced p53 stabilization depends on $p19^{ARF}$ in rodent cells (7). However, previous studies showed that $p14^{ARF}$, the human equivalent of $p19^{ARF}$, was not expressed in MCF-7 cells (8, 25). Using immunoblot analysis, we confirmed the previous observation that $p14^{ARF}$ was undetectable in MCF-7 cells (data not shown), indicating that $p14^{ARF}$ was not involved in this p53 stabilization mechanism. Our results suggested a novel mechanism that E1A induced stabilization of p53 through Mdm4 in a $p14^{ARF}$-independent manner.

On the basis of the aforementioned results, we then asked whether Mdm4 was required for E1A-induced p53 stabilization. E1A and p53 cotransfected into p53 knockout, Mdm4/p53 double knockout, or Mdm2/p53 double knockout MEF cells. Although E1A could stabilize p53 in p53-null and Mdm2/p53 double knockout MEF cells, it did not change p53 level in Mdm4/p53 double null MEF cells (Fig. 2*C*; the p53 levels were normalized with actin levels, and the p53 increase folds of *Lane 3* versus *Lane 2*, *Lane 6* versus *Lane 5*, and *Lane 9*

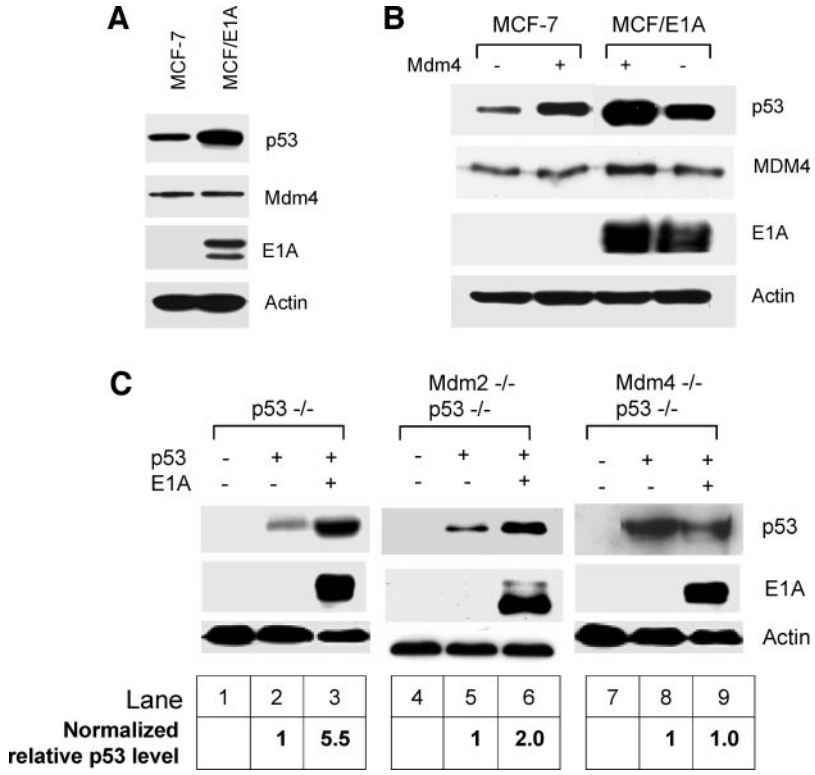

Fig. 2. E1A stabilizes p53 through Mdm4 independently of $p14^{ARF}$. *A,* E1A stabilized p53 in MCF-7 cells. The expressions of Mdm4 and p53 were examined by Western blot analysis of extracts from MCF-7 and MCF/E1A cells. *B.* The Mdm4 expression construct was transfected into MCF-7 and MCF/E1A cells. After transfection for 20 hours, p53 proteins were measured by Western blot analysis. *C.* E1A failed to stabilize p53 in the absence of Mdm4. Equal amounts of p53 cDNA (0.5 μg) were cotransfected with or without E1A (1.0 μg) into p53−/− MEF, Mdm2−/− plus p53−/− double knockout MEF, and Mdm4−/− plus p53−/− double knockout MEF cells, respectively. Cells were harvested before p53 or E1A-induced apoptotic effect was significant. Cell lysates were prepared after 10 hours, and p53 expression was measured by Western blot analysis. The p53 levels were normalized with actin levels, and the p53 increase folds of *Lane 3* versus *Lane 2*, *Lane 6* versus *Lane 5*, and *Lane 9* versus *Lane 8* are shown at the bottom.

versus *Lane 8* are 5.5, 2.0, and 1.0, respectively), indicating the required involvement of Mdm4, but not Mdm2, in E1A-mediated p53 stabilization. Collectively, these results suggest that E1A up-regulates p53 protein level through Mdm4 in a $p14^{ARF}$-independent manner.

E1A Forms a Complex with p53 in the Presence of Mdm4, Facilitating Mmd4-p53 Binding while Inhibiting Mdm4-Mdm2 Binding. Because E1A mediates p53 stabilization not through up-regulation of Mdm4 (Fig. 2*A*), we asked whether E1A could influence p53 binding activity of Mdm4. The effect of E1A on the interaction between Mdm4 and p53 was examined by GST pulldown assay using *in vitro* translated GST-Mdm4 and E1A proteins. As expected, Mdm4 can bind to p53 without other factors (Fig. 3*A, Lane 2*). However, the presence of E1A protein greatly facilitates the direct interaction between Mdm4 and p53 (Fig. 3*A, Lane 3*). To explore how E1A is involved in such binding, the interaction between E1A and p53 was examined with a similar assay. E1A cannot interact with p53 directly (Fig. 3*B, Lane 1*). However, in the presence of Mdm4, E1A can form a complex with p53 (Fig. 3*B, Lane 2*). Interestingly, the p53-binding domain of Mdm4 was required for binding to E1A (Fig. 1*D*), but E1A did not compete with p53 in binding to Mdm4. In contrast, data here implied that they form a tricomplex.

Mdm2 played a critical role in the nuclear export and degradation of p53 (8–13). Because Mdm4 can interact with Mdm2 and p53 (11, 26), we examined whether Mdm4 binding activity of E1A could disrupt the interaction between Mdm4 and Mdm2 by cotransfection and immunoprecipitation (Fig. 3*C*). Mdm4 associated with Mdm2 in MCF7 cells (Fig. 3*C, Lane 4*). However, the expression of E1A decreased Mdm2 binding to Mdm4 *in vivo* (Fig. 3*C, Lane 3*). Collectively, these results suggest that E1A enhances the interaction between p53 and Mdm4 by forming a tricomplex with them, which blocks the binding of Mdm4 to Mdm2.

E1A-Induced p53 Stabilization Accompanies the Accumulation of Ubiquitinated p53. One of the essential mechanisms to regulate p53 protein level is the Mdm2-mediated ubiquitination and degradation of p53. Therefore, we asked whether E1A stabilizes p53 by altering ubiquitinated status of p53 and Mdm2-p53 interaction. Ubiquitin was cotransfected with or without E1A into MCF-7 cells, and the p53 ubiquitination then was measured by immunoprecipitation and immunoblot of p53. Surprisingly, E1A expression enhanced the accumulation of ubiquitinated p53 (Fig. 4*A, Lane 3*) compared with control (Fig. 4*A, Lane 2*). In the presence of the proteasome inhibitor MG132, p53 is highly stabilized and ubiquitinated no matter whether E1A is present (Fig. 4*A, Lanes 4* and *5*), implying the enhanced intensity of ubiquitination may result from accumulation of nondegraded p53. These results suggest that E1A does not inhibit the ubiquitination of p53 but simply decreases the degradation of ubiquitinated p53. In accordance with these results, we also found that

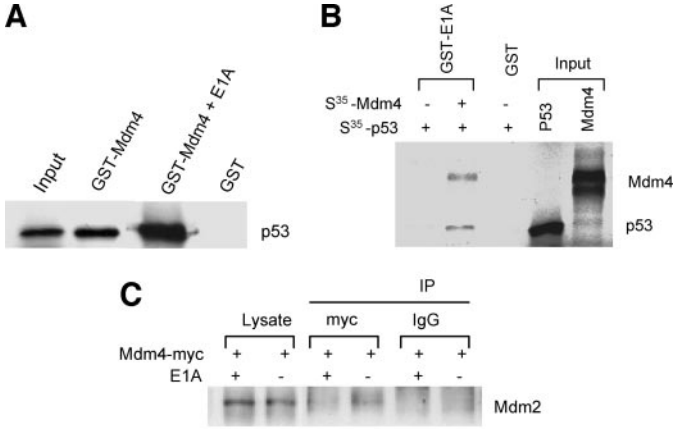

Fig. 3. E1A forms complex with p53 in the presence of Mdm4, facilitating Mmd4-p53 binding while inhibiting Mdm4-Mdm2 binding. *A,* GST pulldown assay was performed using GST-E1A and S^{35}-labeled *in vitro* transcribed and translated Mdm4 and p53. *B,* The binding of Mdm4 to p53 in the presence of purified E1A protein *in vitro* was measured by GST pulldown assay. *C,* The Mdm4-myc construct was transfected into MCF-7 and MCF/E1A cells. The cells were treated with MG132 (20 μmol/L) for 2 hours before the cell lysate was prepared. Mdm4 was immunoprecipitated (*IP*) by the myc antibody, and the Mdm4-Mdm2 interaction then was examined by immunoblot with Mdm2 antibody.

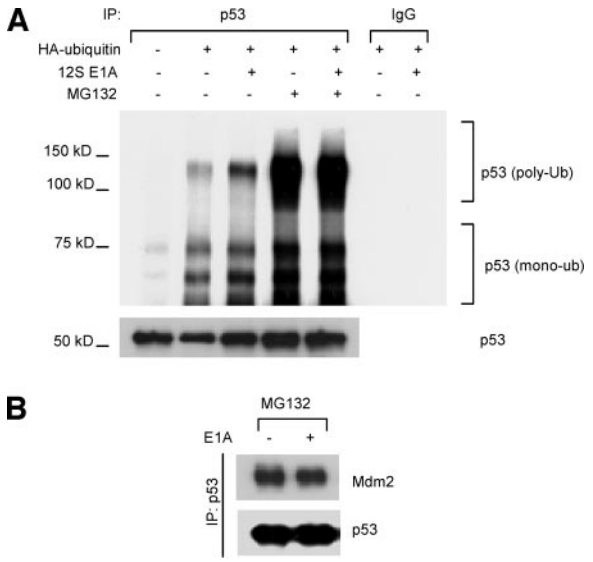

Fig. 4. E1A-induced p53 stabilization accompanies accumulation of ubiquitinated p53. *A.* HA-ubiquitin was cotransfected with 12S E1A into MCF-7 cells. After transfection for 18 hours, cells were treated with or without MG132 (20 μmol/L) for 6 hours, and p53 proteins were immunoprecipitated (*IP*) from the extracts. The ubiquitination (*Ub*) of p53 was shown. *B.* MCF-7 and MCF/E1A cells were treated with MG132 (20 μmol/L) for 6 hours, and cell lysates then were prepared. The Mdm2-p53 complexes were examined by immunoprecipitation and Western blot analysis.

E1A could not inhibit p53 binding to Mdm2 (Fig. 4*B*), which is an E3 ubiquitin ligase of p53. It also was consistent with our results that E1A interacted poorly with Mdm2 (Fig. 1*C*). E1A-induced accumulation of ubiquitinated p53 also has been reported in other study. Interestingly, the authors observed that E1A stabilizes p53 without interfering p53 ubiquitination by Mdm2 (27). Collectively, we concluded that E1A does not change ubiquitination and Mdm2 binding activity of p53 to stabilize it.

E1A Enhances the Nuclear Retention of p53. Our unexpected finding that E1A stabilized p53 without decreasing the ubiquitination level of p53 suggested that other mechanisms might exist to account for E1A/Mdm4-mediated p53 stabilization. To investigate other potential mechanisms, MCF-7 and MCF/E1A cells were untreated or treated with the proteasome inhibitor MG132. MG132 treatment greatly stabilized p53 in MCF7 cells (5.2-fold increase in *Lane 3* compared with *Lane 1*; Fig. 5*A*). Without MG132, E1A expression increased p53 level as expected (3.3-fold increase in *Lane 2* compared with *Lane 1*; Fig. 5*A*). However, in the presence of MG132, E1A expression had little effect on p53 level (1.2-fold increase in *Lane 4* compared with *Lane 3*; Fig. 5*A*). This result suggests that E1A increases p53 level by preventing it from degradation, not by enhancing ubiquitination. It recently has been shown that nucleocytoplasmic shuttling of p53 is essential for Mdm2-mediated cytoplasmic degradation but not ubiquitination (26, 28), suggesting that the ubiquitination of p53 and degradation of p53 are two distinguished activities. To investigate whether E1A exhibits any effect on the p53 shuttling between the cytoplasm and nucleus, the nuclear and cytoplasmic fractions of MCF-7 and MCF/E1A cell lysates were analyzed by immunoblot analysis. In the absence of a proteasome inhibitor, the p53 stabilized by E1A was exclusively localized in the nucleus (Fig. 5*B, Lanes 1* to *4*). In addition, Mdm4 and p53 were mostly localized in the nucleus of MCF-7 and MCF/E1A cells in the absence of MG132 (Fig. 5*B, Lanes 3* and *4*), which also was supported by confocal microscopy observation (data not shown). Interestingly, in

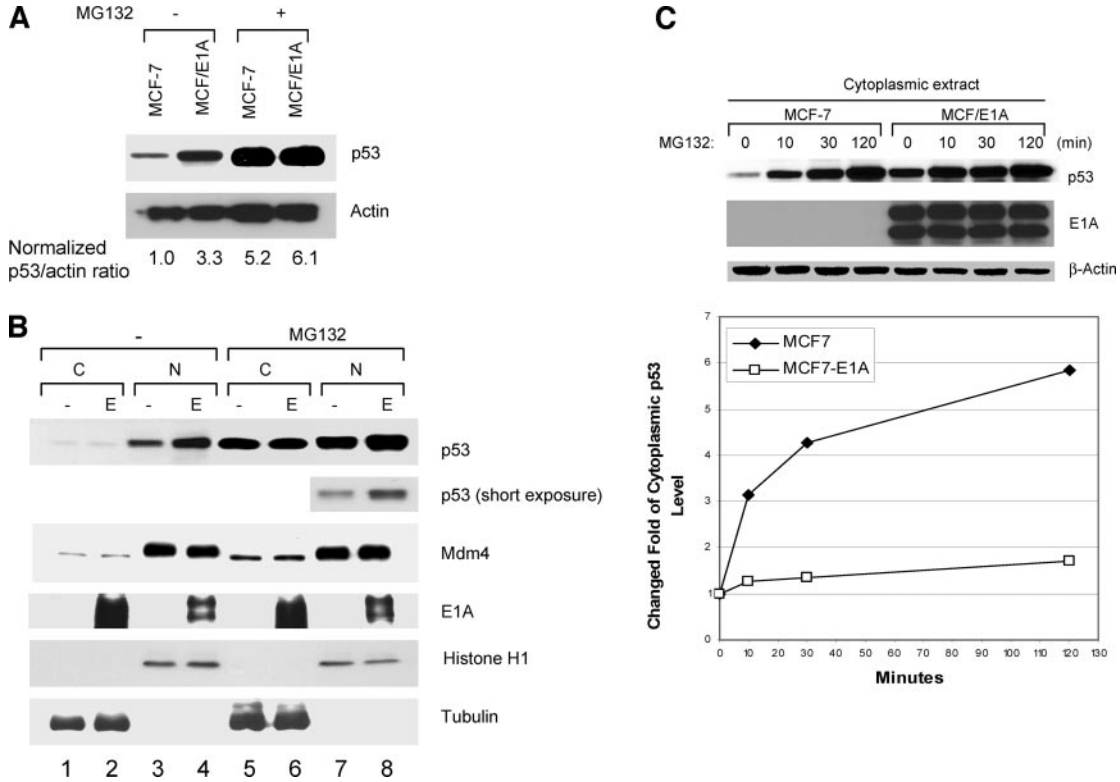

Fig. 5. E1A enhances the nuclear retention of p53. *A.* MCF-7 and MCF/E1A cells were treated with or without MG132 (20 μmol/L) for 6 hours. The levels of p53 proteins were analyzed by Western blot analysis and normalized with actin levels. *B.* Nuclear and cytoplasmic extracts were prepared from MCF-7 and MCF/E1A cells treated with or without MG132 (20 μmol/L) for 6 hours. The nuclear distribution of p53 or Mdm4 was analyzed by Western blot analysis. *C,* p53 (*top*) time course change of the cytoplasmic p53 level in MCF and MCF/E1A cells after inhibition of protein degradation. The cytoplasmic fraction of MCF-7 and MCF/E1A cell lysates was prepared after the treatment of MG132 (20 μmol/L) for different times; the p53, E1A, and β-actin proteins were detected by Western blot analysis. *Bottom,* the changed fold of cytoplasmic p53 level in *top*. The intensity of bands in the immunoblot is quantitated using image analysis software Quantity One 4.3.0 (Bio-Rad, Hercules, CA), and the ratio of p53 level at each time point to that at time 0 is shown.

the presence of the proteasome inhibitor MG132, the nuclear level of the p53 protein in MCF/E1A cells was significantly higher than that in MCF-7 cells (Fig. 5*B, Lanes 7* and *8*; shorter exposure). In contrast, E1A did not alter Mdm4 cellular localization (Fig. 5*B, Lanes 5* and *6*). The results suggested that E1A inhibited nuclear exportation of p53. To further confirm this observation, the cytoplasmic extracts of MCF-7 and MCF/E1A cells were prepared after the treatment of MG132 at different time points. Within 30 minutes after MG132 treatment, the cytoplasmic p53 protein level in MCF/E1A cells did not significantly change (Fig. 5*C, top*; MCF/E1A). Contrarily, under the same condition, the cytoplasmic p53 protein level in MCF-7 cells significantly increased (Fig. 5*C, top*; MCF). The quantitated result of p53 intensity in the immunoblot (Fig. 5*C, bottom*) also showed that the accumulating rate of cytoplasmic p53 level in MCF7 cells was much higher than that in MCF7/E1A cells. These results showed that E1A was capable of decreasing the nuclear exportation of p53 proteins.

Collectively, we showed that E1A increased Mdm4-p53 interaction through the formation of a tricomplex *in vitro* (Fig. 3) and that Mdm4 was required for E1A-induced p53 stabilization (Fig. 2). These results suggest that E1A enhances nuclear retention of p53 by forming a tricomplex with Mdm4 and p53, preventing it from nuclear exportation and degradation in cytoplasm. Interestingly, an alternative splicing form of Lyn tyrosine kinase, which was located in the nucleus, increased the nuclear levels of ubiquitinated p53 by inhibiting the export of p53 to the cytoplasm (29). In an attempt to examine whether Lyn might participate in the E1A-mediated nuclear retention, the coimmunoprecipitation experiment did not show E1A could enhance interaction between p53 and Lyn (data not shown). It requires additional investigation whether Lyn may have a role in the E1A-mediated nuclear exportation of p53. In summary, we identified a novel mechanism for E1A-induced p53 stabilization through Mdm4 that is independent of $p14^{ARF}$. E1A has been tested in multiple clinical trials (3–5). The current study unravels another mechanism for E1A-mediated p53 stabilization, which may be important to modulate anticancer activity of E1A.

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