The Targeting of the Proteasomal Regulatory Subunit S2 by Adenovirus E1A Causes Inhibition of Proteasomal Activity and Increased p53 Expression*

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Although adenovirus early region 1A (AdE1A) can modulate protein expression through its interaction with transcriptional regulators it can also influence the ability of the cell to degrade proteins by binding to components of the 26 S proteasome. We demonstrate here that AdE1A interacts with the S2 subunit of the 19 S regulatory complex in addition to the ATPase subunits S4 and S8 previously identified. S2 forms complexes with both the 13 and 12 S AdE1A proteins both in vivo and in vitro. Mutational analysis has shown direct binding through a short sequence toward the N terminus of conserved region 2 of AdE1A, which encompasses the LXCXE motif, involved in interaction with the pRb family of proteins. In vivo, additional contacts are made between AdE1A and proteasomal components, as well as within the proteasome, such that deletion of the N-terminal region of E1A as well as part of conserved region 2 is required to completely disrupt S2 binding. Mutation of AdE1A, which disrupts complex formation with S2, results in the loss of its ability to stabilize the p53 protein. Similarly down-regulation of S2 expression using small interfering RNAs leads to the inhibition of p53 degradation. These effects were observed in normally growing cells and those subjected to UV irradiation. Furthermore, AdE1A had no effect on the Mdm2-mediated ubiquitination of p53. We suggest therefore that interaction of AdE1A with S2, as well as with the ATPases S4 and S8, directly causes inhibition of proteasomal activity and consequent increase in the protein levels of p53.

Adenovirus early region 1A (AdE1A) is the first protein to be expressed following viral infection and is essential for adenovirus-mediated cell transformation (1–4). Two major E1A components are expressed that are of 289 and 243 amino acids in Ad2S, differing in the presence of a 46-amino acid sequence located toward the C terminus of the larger molecule. These proteins are referred to as 13 and 12 S products, respectively, based on the sedimentation coefficients of their mRNAs. Comparison of the sequences of AdE1As from different virus serotypes has allowed four highly conserved regions (CR) to be identified (5, 6). These conserved regions in Ad2/5E1A are distributed throughout the molecule with CR1 extending from residues 41 to 80 and CR2 from residues 121 to 139. CR3 is located in the region unique to the larger 13 S AdE1A species, and CR4 encompasses the C-terminal region.

AdE1A exerts its effect on the target cell, during both infection and transformation, by binding to and radically altering the activity of a large number of cellular proteins (4, 7). Many of the sites of interaction on AdE1A coincide with the conserved regions. For example, the pRb family of tumor suppressor proteins bind through CR1 and CR2 (8–10), whereas components of the general and specific transcriptional machinery (e.g. TATA-binding protein and activating transcription factor) bind to CR3 (11, 12). Similarly, C-terminal binding protein is targeted by the C terminus of E1A (CR4) (13). It is notable, however, that certain significant partner proteins interact through the less highly conserved N-terminal region. For example, the transcriptional coactivators CBP/p300 (14), TATA-binding protein (15, 16), and the ATPase regulatory components of the 26 S proteasome (17) all bind close to the N terminus.

Although AdE1A is primarily able to modulate the level of expression of cellular proteins through its regulation of transcription, it can also affect the rate of protein degradation by targeting the 26 S proteasome (17, 18). The 26 S proteasome is the major nonlysosomal cellular mechanism of protein proteolysis (19–22). It comprises a 19 S regulatory complex (RC) and a 20 S proteolytic core. The 19 S RC itself is composed of a lid and base subcomponents. Present in the base are six homologous ATPases, at least some of which will bind to AdE1A, both in vitro and in vivo. We have previously shown that S4 and S8 interact with AdE1A mainly through a short sequence located toward the N terminus of the molecule (amino acids 15–19 appear to be essential for interaction) (17). The interaction of AdE1A with S4 and/or S8 results in the inhibition of the activity of 26 S proteasomes directed against p53 in in vitro assays. Moreover, deletion mutants of AdE1A, which do not bind the proteasomal ATPase components have lost their ability to stabilize p53 protein in vivo. However, because AdE1A tends to target multiple components in many pathways, we have inves-
Mammalian or bacterial expression plasmids encoding Ad5 E1A proteins are shown. All of the mutants were derived from Ad5 13S. Ad5 wt 12SE1A does not express 13S E1A. Viruses used for infection are listed. All of the mutant viruses were derived from Ad5 dl520, which does not express 12SE1A or E3 proteins. Ad5 dl1520 does not express E1B 55K protein.

| Mutants   | Mutation positions |
|-----------|--------------------|
| Ad5 plasmids |                     |
| 13S/e/E1A  | 13 S only          |
| dl1101     | Δ4–25              |
| R02        | Point mutation 2   |
| Exxon1     | Δ188–289           |
| dl1108     | Δ124–127           |
| RG2/d1108  | R02, Δ124–127      |
| d0108      | Δ4–25, Δ124–127    |
| L122P      | Point mutation 122 |
| T123N      | Point mutation 123 |
| C124R      | Point mutation 124 |
| H125D      | Point mutation 125 |
| E126Q      | Point mutation 126 |
| 12S/e/E1A  | 12 S only          |
| Ad5 viruses |                     |
| wt         |                     |
| dl520      | no 13 S or E3      |
| dl1101     | Δ4–25              |
| dl1104     | Δ48–60             |
| dl1114     | Δ61–69             |
| dl1106     | Δ96–105            |
| dl1107     | Δ111–123           |
| dl1108     | Δ124–127           |
| dl1109     | Δ4–25, Δ49–105     |
| dl1107     | Δ4–25, Δ111–123    |
| dl1108     | Δ4–25, Δ124–127    |
| dl1143     | Δ38–60             |
| dl2408     | Δ38–60, Δ124–127   |
| dl1520     | no E1B 55K         |

EXPERIMENTAL PROCEDURES

**Cell Lines and Transient Transfection—**Ad5HEK293 cells were derived from the transformation of human embryo kidney cells with sheared Ad5 DNA and express Ad5E1A proteins. H1299 and HCT116 are colon carcinoma cell lines. A549 cells were isolated from a human small cell lung carcinoma and have been used for the viral infection experiments. Both HCT116 and A549 cells express wild type (wt) p53, whereas H1299 does not express p53. The cells were grown in HEPESS-buffered Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum and 2 mM glutamine. For transient transfection experiments, the cells at 60–70% confluency were transfected with plasmids using LipofectAMINE 2000 reagent (Invitrogen) 24 h prior to harvesting or UV treatment. The total amount of DNA added to each dish was kept constant using the corresponding empty vector pcDNA3.

**Viruses and Viral Infection—**The mutant adenoviruses used in the coimmunoprecipitation studies are based on dl520, which expresses Ad512SE1A but not 13SE1A. These viruses have various mutations created in the 12SE1A gene as described in Table I. For viral infection, subconfluent A549 cells were infected with dl520 and mutant viruses in serum-free medium at 50 plate forming units/cell for 2 h at 37 °C with intermittent rocking. Infected cells were then washed once and incubated in fresh complete medium for a further 24 h.

**Plasmids and Fusion Protein Production—**Plasmid constructs encoding human 26 S proteasome subunits were described previously (23). Human Mdm2 and p53 plasmids were kindly provided by Prof. David Lane (University of Dundee). Constructs containing various Ad5 E1A mutants have been described previously (24–26) and are shown in Table I. Intact AdE1A inserts were excised from parental plasmids with EcoRI and XhoI and cloned into the corresponding sites of a GST vector (pGEX-4T1) to generate GST-E1A fusion proteins or into pcDNA3 for in vitro transcription/translation and mammalian expression. Point mutations in the LXCIEX motif were generated by site-directed mutagenesis using a QuikChange™ kit (Stratagene). The sequences of the AdE1A mutants were confirmed by direct DNA sequencing. To induce GST-E1A fusion protein expression, a unique colony for each expression vector transformed with a GST-E1A plasmid was cultured in Luria broth in the presence of isopropyl-β-D-thiogalactopyranoside at 37 °C. To purify the GST fusion proteins, bacterial lysates were mixed with glutathione-agarose beads (Sigma) with rotation for 1 h at 4 °C. The resulting beads were washed, and GST fusion protein was eluted with the reduced form of glutathione (25 mM) for a further 1 h at 4 °C. The GST-E1A protein was carefully collected and dialyzed against 50 mM Tris buffer (pH 7.3).

In Vitro GST Pull-down Assays—Ad5 E1A mutants, human S5b, and FIG. 1. Ad5SE1A binds to the non-ATPase subunit S2 in vivo. A and B, Ad5HEK293 cell lysates were immunoprecipitated with antibodies against S2 and S8 and cyclin B1. Immunoprecipitates were fractionated on 12% polyacrylamide gels in the presence of SDS (A) or 7% urea (B). The proteins were transferred to nitrocellulose filters and coimmunoprecipitated Ad5E1A detected with antibody M58. The lysates were electrophoresed in the first track of each gel. C, lysates from Ad5 HEK293 cells and A549 cells infected with Ad5 wt virus were immunoprecipitated with antibody against AdE1A. The immunoprecipitates were fractionated on 12% polyacrylamide gels in the presence of SDS. After Western blotting, bound S2 was detected using a rabbit polyclonal antibody. IP, immunoprecipitation; Ab, antibody.
Ad5E1A binds to S2 but not S5b in vitro. Bacterially expressed GST or GST-Ad5E1A protein (10 μg) was incubated with 35S-labeled in vitro translated S2 or S5b (10 μg) for 2 h at 4 °C. Glutathione-agarose beads were added, and GST, GST-Ad5E1A, and bound proteins were isolated. After washing, the beads were resuspended in SDS sample buffer, and the proteins fractionated by SDS-PAGE. 35S-labeled S2 or S5b was detected by fluorography and autoradiography.

intact S2 and S2 fragments were expressed by in vitro transcription/translation, either in the absence or presence of L-[35S]methionine (Amersham Biosciences), using the TnT-coupled wheat germ or rabbit reticulocyte lysate systems, according to the manufacturer’s instructions (Promega). Typically, to assay in vitro binding capacity, 5–10 μg of GST conjugates were mixed with 10 μl, or equivalent proportions, of the respective translation mixture for 2 h at 4 °C in 1 ml of immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.4), 0.825 mM NaCl and 1% Nonidet P-40. GST complexes were precipitated using 30 μl of packed, preswollen, glutathione-agarose beads for 1 h at 4 °C. The precipitated complexes were washed four times with 1 ml of 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 5% sucrose (w/v), 1 mM EDTA, and 1% Nonidet P-40. The beads were washed once more with 50 mM Tris-HCl (pH 7.4), 0.125 M NaCl, and 1% Nonidet P-40, prior to being resuspended in SDS-PAGE sample buffer. The precipitated proteins were separated by SDS-PAGE and analyzed by fluorography and autoradiography.

Immunoprecipitation, Western Blotting, and Antibodies—The cells were harvested, after washing in ice-cold saline, by aspiration. For Western blotting studies the cells were lysed in 9 μl urea, 50 mM Tris-HCl (pH 7.3), and 0.15 μl β-mercaptoethanol, and the proteins were fractionated by SDS-PAGE in the presence of 0.1 μl Tris, 0.1 μl Bicine, and 0.1% SDS or in the presence of 7 μl Tris, 93 mM EDTA, and 1% Nonidet P-40. The cells were preincubated with AdE1A prior to the addition of p53 and HPV16 E6. GST complexes were precipitated using 30 μl of packed, preswollen, glutathione-agarose beads for 1 h at 4 °C. The precipitated complexes were washed four times with 1 ml of 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 5% sucrose (w/v), 1 mM EDTA, and 1% Nonidet P-40. The antigens were immunoprecipitated using the appropriate antibodies and protein G-linked agarose (Sigma). Ad5E1A was detected with the mouse monoclonal M58 (Pharmingen) and M73. Human S2 and S8 were detected with rabbit polyclonal antibodies (23), p53 was detected in Western blots with the monoclonal antibody DO1.

In Vitro Protein Degradation Assays—Human p53 and HPV16E6 were in vitro translated using the TnT-coupled wheat reticulocyte system in the presence or absence of L-[35S]methionine. Ad5E1A proteins were generated from GST fusion vectors. HPV16 E6-mediated degradation of p53 was as described by Crook et al. (27). For the TnT-coupled wheat germ lysates and reticulocyte lysates, the cells were lysed in 50 mM Tris-HCl (pH 7.5), 0.225 M NaCl, and 1% Nonidet P-40. The antigens were immunoprecipitated using the appropriate antibodies and protein G-linked agarose (Sigma). Ad5E1A was detected with the mouse monoclonal M58 (Pharmingen) and M73. Human S2 and S8 were detected with rabbit polyclonal antibodies (23), p53 was detected in Western blots with the monoclonal antibody DO1. The cells were preincubated with Ad5E1A or GST-Ad5E1A mutants, rabbit reticulocyte lysates were incubated with GST fusion vectors. HPV16 E6-mediated degradation of p53 was as described by Crook et al. (27) as modified by Turrell et al. (17). In vitro translated p53 and HPV16 E6 (at a ratio of 2:1) were incubated in degradation buffer (25 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 3 mM dithiothreitol) containing additional rabbit reticulocyte lysates to a final concentration of 40%. In incubations containing GST-Ad5E1A or GST-Ad5E1A mutants, rabbit reticulocyte lysates were preincubated with Ad5E1A prior to the addition of p53 and HPV16 E6. After the allotted time the aliquots were withdrawn and mixed with SDS sample buffer prior to SDS-PAGE and autoradiography.

In Vitro Ubiquitination Assays—The assays contained 35S-labeled p53 (1 μl), 100 mM Tris (pH 7.6), GST-Mdm2 (40 ng), ubiquitin activating enzyme E1 (75 ng), ubiquitin conjugating enzyme E2 (UbEHa5a, 75 ng), ubiquitin (2.5 μg), ubiquitin aldehyde (140 ng), 10 μM ATP, and MgCl2. GST-Ad5E1A was added at an equal molar concentration to Mdm2 or in 5-fold molar excess. After incubation at 37 °C for 2 h, the reaction was stopped by the addition of SDS sample buffer. 35S-Labeled p53 was fractionated by SDS-PAGE and detected by autoradiography. GST-Mdm2 was a gift from Prof. David Lane (University of Dundee). E1 and E2 proteins were kindly provided by Prof. Ron Hay (University of St. Andrews).

RNA Interference Assays—All purified, annealed double-stranded siRNAs were purchased from Qiagen. The targeted sequences of proteasome subunits are as follows: 5′-aag cag cac gac gag att tt-3′ (nucleotides 1201–1223) for S2 and 5′-aa aac gag tgt atg gct gtt gtt-3′ (nucleotides 485–507) for S8. Transient transfection of siRNAs into cells was performed using Oligofectamine reagent according to the instructions from the manufacturer (Invitrogen). The concentration of siRNAs used in the study is indicated in the figure legends. A nonsilencing siRNA was used as a negative control. The experiments were repeated two to four times depending on the experiment with duplicate plates, and highly reproducible results were generated.

Quantitative Real Time RT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega). Expression of specific messenger RNAs was determined using the ABI PRISM 7700 Sequence Detection System. RT-PCR was carried out in 25-μl volumes on 96-well plates, in a reaction buffer containing 1× TaqMan Universal PCR Master Mix, 100–200 nmol of TaqMan probe, and 900 nmol of primers.
resuspended in 1 ml of phosphate-buffered saline containing 10 (v/v) ethanol for 1 h. Following fixation, the cells were washed and adherent cells. The cell pellet was then washed and resuspended in 70% from the culture dish with trypsin/EDTA and combined with the non-adherent cells were washed in phosphate-buffered saline and detached with acridine orange dye and examination for apoptotic lation labeled with propidium iodide. Apoptosis was confirmed by stain-

The predesigned probe and primers to detect p53 were purchased from PE Biosystems. S2 primers (5′-GCCAAGGACCCAAACACTT and 3′-CCCCGGCAGCTCTTGTT) and S2 probe (TCTGTTGCGCTTTGGCAGTTA) were designed in-house and purchased from Eurogentec. All of the reactions were multiplexed with a preoptimized control probe for 18 S ribosomal RNA (PE Biosystems), enabling data to be expressed in relation to an internal reference, to allow for differences in RT efficiency. p53 and S2 probes were labeled with FAM dye, and the 18 S primers were used in the S2 binding assay: d11108 and RG2/dl1108. D, radiolabeled S2 bound to various GST-E1A mutant proteins with single amino acid substitutions in the LXCXE motif in CR2.

**RESULTS**

**Interaction of AdE1A with the 26 S Proteasome Subunit S2**—It has previously been shown that AdE1A will bind directly to the S4 and S8 ATPase subunits of the 19 S RC (17, 18). It can be seen from the data presented in Fig. 1 showing that when the non-ATPase subunit S2 is immunoprecipitated from Ad5 HEK293 cells, S2 is coprecipitated (Fig. 1A). When the immunoprecipitates were fractionated on a “urea gel” (i.e. in the absence of SDS) the 12 and 13 S forms of AdE1A were distinguishable, both having interacted with S2 (Fig. 1B). Similarly, when AdE1A was immunoprecipitated from either Ad5 transformed human cells (Ad5 HEK293) or Ad5 wt virus-infected A549 cells, S2 was coprecipitated (Fig. 1C).

Because AdE1A will bind to several proteasomal components, it is possible that the results shown in Fig. 1 could be attributable to coprecipitation of intact proteasomes and binding of AdE1A to the ATPase subunits. To demonstrate direct interaction S2 was expressed using an in vitro transcription/translation kit and labeled with l-α-[35S]methionine. This material was incubated with GST-Ad5E1A (or GST alone), and bound protein was identified by PAGE and autoradiography (Fig. 2). Direct binding of S2 to GST-AdE1A was clearly evident, although no interaction of GST with S2 was observed.
To confirm the specificity of the interaction between S2 and AdE1A, GST-Ad5E1A was also incubated with the L-H9251-[35S]methionine-labeled unrelated proteasome component S5b. AdE1A did not bind to S5b (Fig. 2B).

The Site of Interaction for AdE1A on S2—To determine the binding site for AdE1A, three L-H9251-[35S]methionine-labeled fragments of S2 (encoding amino acids 1–416, 417–701, and 702–908; Fig. 3A) were incubated with GST Ad5E1A, and bound protein was detected by SDS-PAGE and autoradiography. It can be seen (Fig. 3B) that GST-Ad5E1A bound strongly and specifically to only the C-terminal fragment. No interaction with the N-terminal and intermediate polypeptides of S2 was observed, nor did any S2 polypeptide bind to GST alone.

The Site of Interaction for S2 on AdE1A—The S4 and S8 ATPase subunits of the 19 S proteasome bind to the N terminus of AdE1A between amino acids 4 and 25 (17). It was therefore of considerable interest to investigate whether S2 bound through the same region. Initially a series of Ad5E1A mutants (summarized in Table I) were expressed as GST fusion proteins in E. coli and purified. These proteins were incubated with L-[35S]methionine-labeled S2. Bound S2 was detected by PAGE and autoradiography. It can be seen from Fig. 4A that a deletion at the N terminus of AdE1A, which interfered with S4 and S8 interaction (dl1101) had no effect on binding to S2. Similarly the C-terminal fragment of S2 formed a complex with these AdE1A derivatives (Fig. 4B). However, when a second set of mutants with lesions in CR2 were examined in the in vitro assay, it was observed that that loss of amino acids 124–127 (dl1108), toward the N-terminal end of CR2, disabled the binding capacity of the protein (Fig. 4C). Amino acids 124–127 comprise part of the Rb-binding site LXCXE (28). However, mutation of any single residue in this site did not negate binding of S2 (Fig. 4D). It is presumed that several of the amino acids in this short sequence are required for interaction.

Binding of S2 to AdE1A in Vivo—The coimmunoprecipitation studies shown in Fig. 1 established that AdE1A and S2 form a complex in adenovirus-infected and -transformed cells. Mutant AdE1A proteins have been used to establish a binding site for S2 in vitro (Fig. 4). Mutant viruses were used to investigate whether the same and/or additional sites on AdE1A are required for interaction in vivo. Viruses carrying mutations in AdE1A (summarized in Table I) were used to infect A549 cells. After 24 h the cells were harvested, and S2 was immunoprecipitated. Bound AdE1A was detected by Western blotting (Fig. 5A). AdE1A and S2 were expressed at comparable levels in all of the samples (Fig. 5A, two lower panels). Interestingly, when S2 was immunoprecipitated from cells infected with dl1108 (the CR2 deletion that did not bind to S2 in vitro), AdE1A was coprecipitated (Fig. 5A, top panel). However, the deletion of a second region (amino acids 4–25) at the N terminus of AdE1A disrupted interaction between Ad5E1A and S2 (mutant dl0108), although deletion of the N-terminal region alone

Fig. 6. AdE1A-induced stabilization of p53 requires its binding to S2 in vivo. H1299 cells were transfected with plasmids expressing p53 and AdE1A mutants for 24 h. The cells were then treated with the protein synthesis inhibitor anisomycin for the times shown. A, p53 was determined by Western blotting. B, relative p53 level was obtained by densitometric scanning of the Western blots shown in A. Expression of AdE1A mutant proteins is shown in C. H1299 cells were transfected with p53 and various amounts of 12SE1A-expressing plasmid for 24 h prior to quantitative analysis of mRNA by RT-PCR. The levels of p53 mRNA in the presence of AdE1A are shown in D.
Targeting of S2 by AdE1A Causes an Increase in p53 Half-life—The half-life of p53 is normally very short (<20 min) (29). Because 13S wt E1A and its mutants. After 24 h the protein synthesis inhibitor, anisomycin, was added, and the samples harvested at the appropriate time, p53 levels were determined by Western blotting (Fig. 6A). The half-life of p53 was found to be less than 20 min in this system but was increased to more than 1 h in the presence of wt AdE1A and dl1101 (Fig. 6, A and B). The mutant dl0108, which did not bind S2, had no significant effect on p53 half-life (Fig. 6B). Mutant and wt AdE1A were expressed at similar levels in all of these experiments (Fig. 6C). Confirmation that AdE1A did not have an effect at the transcriptional level is provided by the real time PCR data shown in Fig. 6D, demonstrating that increased AdE1A had no effect on the level of p53 mRNA.

S2 Binding by AdE1A Is Required for the Neutralization of the Ability of HPV16E6 to Target p53 for Proteasomal Degradation—p53 is rapidly degraded in the presence of HPV16E6 by proteasomal components present in rabbit reticulocyte lysates (27, 30, 31). We have previously demonstrated that both Ad5 and Ad12E1A can inhibit HPV16E6-mediated p53 degradation (17). When a deleted form of Ad5E1A, such that it was unable to interact with S4 and S8, was included in the assay, it was unable to stabilize p53. To examine the effect of S2 binding in this assay, a series of HPV6-mediated p53 degradation assays were set up in the presence of mutant GST-E1A proteins. It can be seen from the data presented in Fig. 7 that wt AdE1A inhibited proteosomal degradation of p53 (Fig. 7A). However, the inclusion of Ad5d/d1101 (bearing an N-terminal deletion and unable to bind to S4 and S8) or Ad5d/d1108 (with a CR2 deletion and unable to bind to S2) made very little difference to the rate of E6-mediated p53 degradation (Fig. 7B). Furthermore, mutation of AdE1A arginine 2 to glycine gave rise to a protein that stabilized p53 (Fig. 7C) almost to the same extent as wt. All of the mutant and wt AdE1A proteins were expressed at similar levels in the assays (Fig. 7D). These data...

FIG. 7. S2 binding by AdE1A inhibits the ability of HPV E6 to target p53 for proteasomal degradation in vitro. In vitro degradation of L-α-[35S]methionine-labeled p53 was performed as described under “Experimental Procedures.” Aliquots were removed at appropriate times, as indicated, during the assay and fractionated by SDS-PAGE. Residual p53 was detected by autoradiography. A, E6-mediated degradation. The assays contained in vitro translated p53 and added HPV16E6, and AdE1A as indicated. B and C, in vitro degradation of p53 in the presence of Ad5E1A mutants. The assays were set up as described under “Experimental Procedures.” Either wt or mutant Ad5E1A proteins were included in the assays as indicated. Residual p53 was detected by fluorography and autoradiography. GST-E1A proteins were expressed at similar level in the assays as shown in D.
suggest that ablation of the binding of E1A to S2 results in the loss of its ability to stabilize p53.

Mdm2 and AdE1A-mediated p53 Stability in Vivo—Ubiquitination and degradation of p53 are regulated, in vivo, by the E3 ubiquitin ligase Mdm2 (32–36). A series of experiments were undertaken to examine the effect of Mdm2 on the degradation of p53 in the presence of AdE1A. p53 was transfected into the p53-null cell line H1299, together with AdE1A mutants dl0108 and dl1108. dl1108 caused some stabilization of p53, whereas dl0108 had no effect, confirming that dl0108 does not inhibit proteasomal degradation of the protein (Fig. 8A). An additional experiment was undertaken in which Mdm2 was cotransfected into H1299 cells (Fig. 8B). Mdm2 caused a marked reduction in p53 levels (compare lanes 1 and 2). Significantly, the dl0108 mutant had no effect on Mdm2-mediated degradation. In contrast, expression of dl1108 inhibited degradation both in the presence and absence of Mdm2, although protein degradation was more marked in the presence of Mdm2. We have concluded that mutant AdE1A that is unable to bind the proteasome is unable to inhibit Mdm2-mediated degradation.

Fig. 9. Effect of AdE1A on Mdm2-mediated p53 ubiquitination. A, H1299 cells were transfected with p53 and control or 13S wt E1A plasmids for 24 h. p53 and AdE1A were detected by Western blotting. The central panel where the ubiquitinated forms of p53 are indicated is an overexposed version of the top panel. B, A549 cells were either mock infected or infected with Ads dl1520 virus expressing 12 and 13 S AdE1A. The cells were harvested at various time points. The ubiquitinated forms of p53 (Ub-p53) were observed by prolonging exposure time. C, in in vitro ubiquitination assay radiolabeled p53 was incubated with purified ubiquitin, E1, UbcH5a, GST-Mdm2, and GST-E1A or GST. The concentrations of GST-E1A or GST proteins added compared with GST-Mdm2 were at a ratio of 1:1 or 5:1 as indicated. Ub, ubiquitin.
AdE1A S2 Targeting Causes Proteasomal Activity Inhibition

Fig. 10. Down-regulation of S2 stabilizes p53 protein. A549 (A) or HCT116 (B) cells were transfected with nonsilencing or S2 siRNA oligonucleotides (0.2 μM). After the times indicated the cells were harvested and subjected to Western blotting for expression of S2, S8, and p53. C, Ad5 HEK293 cells were transfected with S2 siRNA at various concentrations for 24h. After harvesting, S2 and p53 levels were determined by Western blotting. D, HCT116 cells were transfected with S8 siRNA at various concentrations for 24 h. The levels of S8, S2, and p53 were determined by Western blotting. E, A549 cells were transfected with nonsilencing or S2 siRNA (0.2 μM). After 24 h cells were treated with anisomycin for times indicated and harvested for detection of p53 and S2. F, A549 cells were transfected with nonsilencing or S2 siRNA at various concentrations. After 24 h the cells were harvested, and mRNA was purified from them. The levels of p53 mRNA were determined by real time RT-PCR.

degradation, whereas AdE1A molecules that can still interact with the proteasome will inhibit degradation in vivo. These results contrast with those obtained in vitro when dl1108 was unable to inhibit E6-mediated degradation of p53 (Fig. 7B). However, it must be borne in mind that the two assay systems, in vitro E6-mediated p53 degradation (Fig. 7) and in vivo Mdm2-mediated degradation (Fig. 8), are not directly comparable.

AdE1A Does Not Inhibit Ubiquitination of p53—In the preceding sections it has been established that binding of S2 by AdE1A inhibits proteasomal activity, causing stabilization of p53 and increasing steady state levels. It might be supposed that the same result could be achieved by inhibiting ubiquitination of p53, thus reducing the concentration of protein targeted to the proteasome. To address this point, H1299 cells were transfected with p53 in the presence or absence of AdE1A (Fig. 9A). It can be seen that AdE1A inhibited p53 degradation but did not affect ubiquitination of the protein (seen as a ladder of p53 species of increasing molecular weight). Furthermore, infection of A549 cells with the replication defective virus Ad5 dl1520 caused an increase in p53 expression that was accompanied by the ubiquitinated forms of the protein, seen on the overexposed Western blot shown in Fig. 9B. The effect of AdE1A on the ubiquitination of p53 was also assessed in in...
expression was determined by Western blotting. As shown in were transiently transfected with control (nonsilencing and S2 interference assays. A549, HCT116, and Ad5 HEK293 cells comparable with that shown in Fig. 9, we performed RNA fication of proteasome activity directed against p53 to investigate whether inhibition of S2 can cause modi- tion this aspect of the p53 degradation pathway.

**Down-regulation of S2 Expression Inhibits p53 Degradation**—To investigate whether inhibition of S2 can cause modification of proteasome activity directed against p53 in vivo, comparable with that shown in Fig. 9, we performed RNA interference assays. A549, HCT116, and Ad5 HEK293 cells were transiently transfected with control (nonsilencing) and S2 siRNAs for periods of 24, 48, and 72 h. After harvesting, protein expression was determined by Western blotting. As shown in Fig. 10 (A and B) the protein level of S2 was decreased in the presence of S2 RNAi, whereas p53 levels increased significantly from 24 h post-transfection. This decrease in S2 and the concomitant increase in p53 were apparent at least up to 3 days post-transfection. These changes were not observed following transfection of control nonsilencing oligonucleotides. No change in S8 protein expression was observed in response to S2 siRNAs (Fig. 10, A and B). When Ad5 HEK293 cells were treated with S2 siRNAs for 24 h at various concentrations, a similar decrease in S2 protein level was observed, together with an increase in p53 (Fig. 10C). Similarly, the addition of S8 siRNAs to HCT116 cells caused a reduction in S8 expression and an increase in p53 expression without change in S2 (Fig. 10D). These results suggest that inhibition of proteasome activity by targeting even one regulatory subunit such as S2 or S8 is sufficient to inhibit p53 degradation in vivo. This observation is consistent with the data showing that d1108, which does not bind S2 but still binds S8 and S4, was capable of inhibiting p53 degradation.

To determine the mechanism by which reduction in S2 level resulted in increased p53 expression, the cells were transfected with S2 siRNA and then treated with anisomycin to inhibit nascent protein synthesis. It can be seen from the data presented in Fig. 10 (E and F) that there was an appreciable increase in p53 stability (half-life) in the presence of S2 siRNA. RNAi had no effect on the transcription of p53, with no increase in p53 mRNA even when there was a marked increase in protein (Fig. 10F). The increased level of p53, seen as a result of S2 RNAi administration, is ubiquitinated as would be expected if the effect of S2 RNAi were directly, and only, on the expression of a proteasomal component (Fig. 11).

Increase in p53 by S2 RNAi and AdE1A Correlates with Sensitization of Cells to UV Light-induced Apoptosis—The data presented in Fig. 10 (A–D) clearly demonstrate that the reduction in S2 expression correlates with a reduction in proteasomal activity, leading to an increase in cellular p53 level. We have enlarged these studies by examining the effects of either reduction of S2 or expression of AdE1A in conjunction with UV irradiation (Fig. 12). HCT116 cells, treated with S2 siRNA, were subjected to UV irradiation. The p53 level was elevated in response to increasing doses of UV irradiation in cells treated with either nonsilencing or S2 siRNAs. However, there was an additional increase of p53 in samples treated with S2 siRNA (relative to UV irradiation alone), presumably because of inhibition of proteasome activity (Fig. 12A). FACS analysis of cells treated with S2 siRNA and UV irradiation showed that the increase in p53 level correlated well with an increase in apoptosis, as judged by the increase in sub-G1 population of cells (Fig. 12B). Similarly, an increased level of p53 was observed in cells expressing wt AdE1A compared with those expressing d10108 either in the presence or absence of UV irradiation (Fig. 12C). In addition, this effect correlates well with the ability of AdE1A to sensitize cells to UV light-induced killing (Fig. 12D). FACS analysis shows that the level of UV light-induced apo- ptosis was increased in HCT116 cells following transfection of wt AdE1A but was unaffected by d10108, which expresses mut- ant AdE1A that does not bind to proteasome proteins.

**DISCUSSION**

Many of the cellular targets for AdE1A are involved in the regulation of transcription (2, 4). By means of these interactions, the virus is able to influence the level of expression of a number of viral and cellular proteins. However, we have re- cently demonstrated that AdE1A is also able to control the level of certain proteins through direct action on the proteasome (17, 18). Specifically, it has been shown that AdE1A interacts with the S4 and S8 ATPase components of the proteasomal 19 S regulatory complex. This has the effect of inhibiting the activity of S4 ATPase (17). AdE1A is able to inhibit the ability of the proteasome to degrade p53 in in vitro assays by overcoming the activity of HPV16E6. It is also able to stabilize p53 in HeLa cells that express HPV18E6. Deletion of the S4- and S8-binding sites at the N terminus of AdE1A markedly reduced the ability of the viral protein to stabilize p53 (17).

In our original analysis of the relationship between AdE1A and the proteasome, it was observed that AdE1A could bind to several components of the 19 S RC (18). Most of these had molecular weights of 40,000–65,000, although one protein had a molecular weight of about 100,000. We have now shown that AdE1A will form a complex with the S2 subunit present in the 19 S RC (molecular weight, 100,000). Using communoprecipitation protocols complex formation between S2 and Ad5E1A12S and 13 S products has been confirmed in vivo (Fig. 1). In addition, direct binding has been demonstrated using GST pull-down assays (Fig. 2).

These data show that AdE1A targets not only the ATPase components of the 19 S RC but also at least one other 19 S RC component: S2. Because S2 has no sequence homology to the ATPases, it seemed probable that a novel binding site on AdE1A would be involved, quite distinct from the N-terminal region that is required for interactions with S4 and S8. Using a series of deletion mutants it has been shown in in vitro assays that a region in CR2 of Ad5E1A binds to the C-terminal domain of S2 (Fig. 4). This site on AdE1A has been mapped to the LXCXE motif, which is essential for interaction with the Rb family of proteins. Deletion of single amino acids across the region did not noticeably interfere with the interactions. It has been concluded, therefore, that the binding site encompasses much of the LXCXE sequence. Because mutation of single
amino acids within this motif will negate Rb binding, it is
apparent that S2 does not interact via Rb but binds directly. It
is interesting to note that no obvious amino acid sequence
homology between pRb and S2 could be found.

When AdE1A was immunoprecipitated from cells following
infection with mutant viruses, it was observed that deletion of
the AdE1A CR2 region did not impair binding to S2 (dl0108).
After 24 h, the cells were irradiated with UV light (30 J/m²). The cells were cultured for a further 24 h prior to harvesting. p53 level was determined by Western blotting and densitometry. The percentage of apoptotic cells in sub-G₁ phase is shown. C. HCT116 cells were transfected with a nonsilencing or S2 siRNA as described previously. After 24 h, the cells were irradiated with UV light at different doses as indicated. The cells were cultured for a further 24 h prior to harvesting. The p53 level was determined by Western blotting and densitometry. The level of S2 is shown. D. FACS analysis was carried out on samples treated with S2 siRNA followed by UV irradiation (30 J/m²). The percentage of apoptotic cells in sub-G₁ phase is shown.

AdE1A S2 Targeting Causes Proteasomal Activity Inhibition

FIG. 12. Inhibition of the proteasome by AdE1A or S2 RNAi enhances UV light-induced apoptosis through increasing p53 levels. A, HCT116 cells were transfected with a control or Ad5 13SE1A plasmid that expresses wt or mutant protein incapable of binding to S2 (dl0108). After 24 h, the cells were irradiated with UV light (30 J/m²). The cells were cultured for a further 24 h prior to harvesting. p53 level was determined by Western blotting and densitometry. B, FACS analysis was carried out on samples treated with wt or dl0108 followed by UV irradiation. The percentage of apoptotic cells in sub-G₁ phase is shown. C, HCT116 cells were transfected with a nonsilencing or S2 siRNA as described previously. After 24 h, the cells were irradiated with UV light at different doses as indicated. The cells were cultured for a further 24 h prior to harvesting. The p53 level was determined by Western blotting and densitometry. The level of S2 is shown. D, FACS analysis was carried out on samples treated with S2 siRNA followed by UV irradiation (30 J/m²). The percentage of apoptotic cells in sub-G₁ phase is shown.

AdE1A is able to stabilize p53 and increases its steady state
level through an increase in protein half-life. In vivo the CR2 mutant dl1108, which is unable to interact with S2 directly but
is still capable of binding the proteasomal ATPases, can increase the half-
life, whereas the AdE1A mutant (dl108), which does not in-
teract with the 19 S RC, fails to increase the p53 level (Fig. 6).
As with binding to S4 and S8, it was shown that E1A interac-
tion with S2 inhibited p53 degradation in in vitro assays (Fig.
7), although the CR2 mutant (dl1108) was somewhat less im-
paired than the N-terminal mutant (dl1101). Loss of binding to
CBP/p300 (the RG2 mutation; Fig. 7C) did not significantly alter the ability of E1A to stabilize p53, indicating that binding to proteasomal proteins and not CBP is the major determining factor for E1A (38).

These studies were extended to consider the effects of the mutant AdE1A on the proteasome in vitro and to assess the ability of Mdm2 to target p53 for degradation in the presence of AdE1A both in vivo and in vitro. In vivo the CR2 mutant dl1108 is able to stabilize p53 regardless of the fact that it is unable to bind to S2. However, the double mutant (dl1018) lacking the N-terminal S4/S8-binding site as well as the S2-binding site in CR2 had no effect on p53 levels (Fig. 8A). In the presence of additional Mdm2, dl1108 was also able to reduce proteasome-mediated degradation of p53, whereas dl1018 was not. Thus, mutant AdE1A (dl1018), which does not bind the proteasome, is unable to reduce degradation of p53 in the presence or absence of exogenous Mdm2 (Fig. 8B). The correlation between AdE1A interaction with the proteasome and its stabilization of p53 clearly suggests that AdE1A stabilizes p53 by targeting the proteasome.

This relationship of AdE1A to Mdm2 has been considered in rather more detail because it might be suggested that AdE1A could increase p53 levels by inhibition of the ubiquitin ligase activity of Mdm2 as well as inhibiting the proteasome directly. It has been shown here (Fig. 9), however, that p53 is ubiquitinated in vivo in response to transfected AdE1A or AdE1A expressed during viral infection. Therefore, it seems unlikely that AdE1A has any marked effect on ubiquitination by Mdm2 as has been previously suggested. Furthermore, the in vitro assays presented in Fig. 9C also confirm that there is no difference in the ubiquitinated forms of p53, because of the action of Mdm2, in the presence of AdE1A even when the latter protein is present in 5-fold molar excess (Fig. 9C).

A recent report has suggested that although Mdm2 is responsible for the monoubiquitination of p53, polyubiquitination requires the additional activity of the E4 ubiquitin ligase p300 (39). Binding of AdE1A to p300 inhibited its ability to ubiquitinate p53, and it was suggested that by this interaction AdE1A could up-regulate p53 expression (39). However, it is important to note in the study presented here that the AdE1A RG2 mutant, which is known not to bind p300 or CBP had a similar effect to wt AdE1A in in vitro assays, markedly inhibiting p53 degradation (Fig. 6C). Furthermore, other studies have confirmed that polyubiquitination of p53 by Mdm2 can occur both in vitro and in vivo (40, 41).

In an effort to determine whether targeting of specific proteasomal regulatory proteins by AdE1A is responsible for increased p53 levels, we have compared its effects with those obtained following knock-down of proteasomal components using RNA interference. Thus, the addition of appropriate siRNAs for S2 or S8 increased p53 expression because of increased protein half-life (Fig. 10). This observation is consistent with data recently published by Wojcik and DeMartino (42), who demonstrated that reduced expression of a number of proteasome subunits resulted in a reduction of proteasome activity.

A further study presented here showed that both AdE1A and down-regulation of S2 were able to potentiate UV light-induced apoptosis. Thus, an additional increase in p53 expression in response to UV irradiation was seen in cells treated with S2 siRNA, and this was able to induce apoptosis (Fig. 12, A and B). Similarly wt AdE1A potentiated UV light-induced apoptosis, whereas the mutant dl1018, which is unable to target the proteasome, had no effect (Fig. 12C).

Exactly why it is necessary for AdE1A to target multiple proteasomal subunits is not clear at present. In the assays described here and previously, the effects of interaction of AdE1A with S2, S4, and S8 seem to be broadly similar. We presume that, in vivo, E1A binding to the different proteasomal proteins produces subtly different effects that are necessary for the ability of AdE1A to modulate the levels of cellular protein precisely. Thus, it is possible that subpopulations of proteasomes could be targeted at certain times by AdE1A; for example nuclear proteasomes could be inhibited, whereas those in the cytoplasm are unaffected.

Unique roles for components of the regulatory complex are now becoming apparent. Within the base subcomplex the ATPase subunits form a hexameric ring with S1, S2, and S5a non-ATPases closely associated. These proteins are responsible for mediating most of the interactions between the base and lid. The base itself appears to be essential for unfolding proteins entering the proteasome and translocation of substrates into the proteolytic core (43). S5a plays an essential role in the recognition of ubiquitinated substrates (44), whereas S4 is involved in opening of the 20 S proteolytic core (45). S5a is a polyubiquitin receptor protein with a substrate-specific function and a role in 19 S stability (46, 47). As noted above, S2 forms a tetrameric complex with S4, S7, and S6b. Our studies do not provide any direct evidence as to whether AdE1A will disrupt these interactions nor, indeed, any of the other multiple binding reactions that have been observed. However, it seems reasonable to suppose that interaction of AdE1A with a number of these proteasomal proteins will subtly affect the dynamics of binding within the 19 S RC as a whole and therefore its ability to degrade cellular substrates. Down-regulation of S2 expression with siRNAs appears to duplicate the effect of AdE1A on the activity of the 26 S proteasome. Although there is no general increase in protein expression, we have observed a marked increase in level of certain normally short-lived proteins such as p53 (Fig. 10).

Interestingly, proteasomes have recently been shown to associate with a number of novel proteins that appear to be previously unidentified components (48). A deubiquitinating enzyme Ubp6 (Usp14 in mammals) binds to S2, which locates it close to the substrate translocation channel (48). The observation that proteasome binding activates Ubp6 over 300-fold suggests that it plays an important role in protein degradation. By removing ubiquitin from proteasomal substrates it has been suggested that Ubp6 contributes appreciably to protein degradation. It is possible that binding of AdE1A to S2 could modulate (inhibit) Ubp6 activity, thus affecting the overall rate of proteolysis and therefore increasing the cellular concentration of potential substrates. Furthermore, S2/Rpn1 binds to ubiquitin-like protein domains (49), and so it is possible that S2 could be involved in the recognition of ubiquitinated p53 by the proteasome. Any or all of these interactions could be a target for AdE1A.

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The Targeting of the Proteasomal Regulatory Subunit S2 by Adenovirus E1A Causes Inhibition of Proteasomal Activity and Increased p53 Expression
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