Adenovirus E1A targets p400 to induce the cellular oncoprotein Myc


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Adenovirus E1A drives oncogenesis by targeting key regulatory pathways that are critical for cellular growth control. The interaction of E1A with p400 is essential for many E1A activities, but the downstream target of this interaction is unknown. Here, we present evidence that the oncoprotein transcription factor Myc is the target of this interaction. We show that E1A stabilizes Myc protein via p400 and promotes the coassociation of Myc and p400 at Myc target genes, leading to their transcriptional induction. We also show that E1A requires Myc for its ability to activate Myc-dependent gene expression and induce apoptosis, and that forced expression of Myc is sufficient to rescue the activity of an E1A-mutant defective in p400 binding. Together, these findings establish that Myc, via p400, is an essential downstream target of E1A.

Results and Discussion

To examine the effects of E1A on Myc protein stability, human U2OS cells were infected with the Ad5 adenovirus Δ520, which expresses WT 12S E1A, and endogenous Myc protein levels were assessed by Western blot (WB; Fig. 1A). Six to 8 h after infection, at the point at which E1A expression was first detected, we observed an increase in steady-state Myc levels, which gradually subsided over a 12- to 24-h period. Consistent with previous reports (5), the increase in Myc protein levels at 6 h was accompanied by a decrease in the levels of Myc mRNA (Fig. 1B). Although Baluchamy et al. (7) have reported that adenovirus activates Myc gene expression, these experiments were done in quiescent cells; our assays, and those of Lohr et al. (5), were performed in cycling cells, suggesting that regulation of Myc transcription by adenovirus is influenced by the growth status of the cells.

The transient increase in Myc levels we observed, together with the decline in Myc mRNA, suggested that Myc is stabilized during the course of adenovirus infection. This notion was confirmed by treating infected cells with cycloheximide (CHX), and monitoring Myc levels by WB (Fig. 1C). Under these conditions, adenovirus stabilized Myc considerably, and in a manner that depended on E1A; a virus that expresses LacZ (β-gal) instead of E1A did not induce Myc stability (Fig. 1C). Importantly, expression of E1A alone was sufficient to stabilize Myc; retroviral expression of 12S E1A in U2OS cells resulted in a potent stabilization of Myc (Fig. 1D) and a commensurate decrease in Myc mRNA levels (Fig. 1E). Thus, confirming earlier work (5), E1A promotes Myc stability.


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E1A has been shown to interact with multiple subunits of the 19S proteasome, and this interaction has been proposed to inhibit proteasomal proteolysis (6). Because Myc proteolysis requires proteasome function (8), global inhibition of proteasome activity by E1A could account for its ability to stabilize Myc. It is important to note, however, that E1A has not been shown to elicit widespread proteasome inhibition in vivo. We therefore asked whether adenoviral infection stabilizes the synthetic protein U-GFP (9), a substrate that is widely used as an in vivo reporter of proteasome activity (Fig. 1F). Under conditions where adenovirus stabilized Myc, there was little, if any, change in the rate of destruction of U-GFP, demonstrating that proteasome function is not generally attenuated in adenovirus-infected cells. This notion was supported by comparing the effects of adenovirus infection and proteasome inhibition on Myc localization [supporting information (SI) Fig. S1]. Whereas proteasome inhibition results in the redistribution of Myc from the nucleoplasm to the nucleolus (10), infection of cells with adenovirus expressing E1A did not significantly alter the nuclear distribution of the Myc protein. Thus the consequences of stabilization of Myc by E1A are different from those of proteasome inhibition. Taken together, these data support a model in which the ability of E1A to stabilize Myc does not involve general inhibition of proteasome function.

**E1A Stabilizes Myc via p400.** We next probed for the regions of E1A that are required to stabilize Myc. We analyzed a panel of adenovirus E1A deletion mutants, depicted in Fig. 2A, for their ability to stabilize Myc at 6 h after adenovirus infection (Fig. 2B). This experiment revealed that, of the 11 mutant viruses tested, all but three were able to stabilize Myc efficiently. The three viruses that failed to stabilize Myc (dl1101, dl1102, and dl1103) encode E1A proteins with deletions that span residues 4–49. This region of E1A is important for its interaction with p400, CBP/p300, TBP, and Rb, but the smallest deletion that disrupts Myc stabilization, Δ26–35 (dl1102), interacts with all of these proteins, with the exception of p400 (4). Although the Δ26–35 mutation also disrupts interaction of E1A with the coactivator TRRAP (11), interaction with TRRAP additionally requires CR1; disruption of CR1 (as in the dl1104 and dl1105 viruses) did not block the ability of E1A to stabilize Myc (Fig. 2B). Importantly, we also observed that WT E1A, but not the Δ26–35 mutant, stabilized Myc in Rat1 and IMR90 cells (Fig. S2), demonstrating that the ability of E1A to interact with p400 correlates with Myc stabilization in a variety of cell types. Together, these data indicate that adenoviral-mediated stabilization of Myc requires interaction of E1A with p400.

To determine whether p400 plays a role in E1A-mediated stabilization of Myc, we knocked down expression of p400 in U2OS cells by using short-hairpin-mediated gene silencing and examined the effects of E1A on Myc turnover. This analysis (Fig. 2C) showed that knockdown of p400 attenuates the ability of E1A to stabilize Myc. Moreover, we found that knockdown of p400, in the absence of E1A expression, increased the rate of Myc proteolysis and decreased steady-state Myc protein levels (Fig. 2D), indicating that p400 normally acts to promote Myc stability. Consistent with this idea, overexpression of p400 promoted accumulation of Myc protein (Fig. 2E) and specifically reduced the formation of high-molecular-weight Myc–Ub conjugates (Fig. 2F); p53–Ub conjugates, which we assayed as a control, were unaffected by p400 expression. Based on these results, we conclude that p400 acts to stabilize Myc by reducing the extent of Myc ubiquitylation and that E1A targets this process.

**E1A Promotes Formation of a p400–Myc Complex.** Given the ability of p400 to stabilize Myc, a simple model to explain our observations is that E1A promotes the association of Myc and p400. To test this model, U2OS cells expressing HA-tagged Myc and FLAG-tagged p400 were infected with various adenoviruses, and Myc–p400 complexes were detected by coimmunoprecipitation analysis (Fig. 2F). By recovering either Myc or p400 immune complexes, we found that WT E1A, but not the Δ26–35 mutant, promoted coassociation of both proteins. Importantly, WT E1A could also stimulate the association of Myc and p400 on promoter DNA, as assayed by ChIP. Because commercially available anti-p400 antibodies did not function for ChIP (data not shown), we expressed FLAG-tagged p400 in cells infected with various adenoviral vectors for these analysis. We first performed ChIP by using an antibody against Myc and found that expression of WT E1A (encoded by dl520), but not the Δ26–35 mutant (dl1101), promoted the association of Myc with the nucleophosmin (B23) promoter (Fig. 3B). We then recovered Myc–DNA complexes from the ChIP reaction and performed a second round of immunoprecipitation with anti-FLAG antibody to recover chromatin that was bound by both Myc and
Fig. 2. p400 is required for E1A to stabilize Myc. (A) 12S E1A is depicted, showing conserved regions 1 (CR1) and 2 (CR2). The positions of adenovirus E1A deletion mutants dl1004 and dl1101–dl110 are shown above, and residues required for interaction with p400 are shown below. (B) U2OS cells were infected with adenovirus deletion mutants, or dl520, and incubated for 6 h, and CHX chase was performed. WB was used to monitor levels of E1A and endogenous Myc. (C) U2OS cells were transduced with retroviral expression constructs encoding (i) a short hairpin directed against p400 (sh p400), or vector control, and (ii) WT 12S E1A, or vector control (-). CHX chase followed by WB analysis was used to determine levels and stability of Myc and E1A. (D) WB analysis was used to determine levels of Myc and p400 in control (−) and p400 hairpin (+)-expressing cells. (E) U2OS cells were transiently transfected with HA-tagged Myc, in the absence (−) or presence of an expression construct encoding p400. Levels of Myc and p400 were determined by immunoblotting. (F) U2OS cells were transfected with expression vectors encoding either Myc or p53, together with FLAG-tagged p400 (or vector control), and a plasmid expressing His-tagged Ub (20). Ub conjugates were recovered by Ni-NTA chromatography, and ubiquitylated Myc and p53 proteins were detected by WB.

and p400. Consistent with the ability of E1A to promote association of Myc and p400 in solution, expression of E1A also promoted an enrichment of Myc–p400 cocomplexes at the B23 promoter (Fig. 3C); as expected, this enrichment was not observed with the Δ26–35 E1A mutant. The ability of E1A to promote both the association of Myc with a target gene, and, beyond this, to promote corecruitment of Myc and a coactivator, supports a model in which E1A functionally targets Myc via its interactions with p400.

Functional Interaction Between Myc and E1A. E1A and Myc share a number of functional similarities, including the ability to promote ectopic S-phase entry, sensitize cells to apoptosis, and collaborate with oncogenes like Ras to transform rat fibroblasts (12). Given our demonstration that E1A can induce both Myc levels and the Myc–p400 interaction, we speculated that the overlapping activities of these proteins may result, in part, from the ability of E1A to function through Myc. To probe this idea, we first asked whether E1A can stimulate Myc’s transcriptional activity. We examined expression of two Myc target genes (Rcl1 and Cad) in diploid IMR90 cells transduced to express either WT E1A or the Δ26–35 E1A mutant (Fig. 4A). Compared with vector control, we found that both genes were induced by expression of WT E1A, to a level comparable to that observed upon overexpression of Myc. The Δ26–35 E1A mutant, in contrast, failed to activate either gene. Importantly, activation of Rcl1 (Fig. 4B) and AHCY (Fig. 4C) by E1A depended on Myc, as siRNA-mediated knockdown of Myc in these cells attenuated the ability of E1A to activate these genes. Similar behavior was observed at the B23 and PCNA genes (Fig. S3). Thus E1A can activate a Myc target gene in a Myc-dependent manner, supporting the notion that it functionally stimulates the Myc protein.

The E1A–p400 interaction has recently been found to be important for the ability of E1A to promote apoptosis (4). If, as our model predicts, the E1A–p400 interaction functions through Myc, we would expect that Myc will also be required for induction of apoptosis by E1A. We therefore asked whether E1A
can induce apoptosis in Rat1 fibroblasts in which both copies of the c-myc gene were disrupted by homologous recombination (13). In Rat1 cells, E1A was a potent inducer of apoptosis triggered by adriamycin (Fig. 4D; see Fig. S4A for expression data on the E1A and Myc proteins). In congenic Myc-null cells, however, E1A was unable to induce apoptosis. This deficit was caused by a loss of Myc and not a secondary mutation, because reintroduction of Myc into Myc-null cells restored the ability of E1A to induce apoptosis. Importantly, this deficit was not caused by a general defect in E1A activity in Myc-null cells. Our previous studies have shown that binding of E1A to Rb is important for inducing the expression of several caspases, and that this induction potentiates cell death in E1A-expressing cells (14). When we examined caspase-7 levels in our system (Fig. 4E), we found that E1A was capable of inducing caspase-7, and that this induction was not diminished in Myc-null cells. This result demonstrates clearly that the ability of E1A to function via the Rb pathway does not depend on Myc expression and reveals that only a subset of E1A activities require Myc.

A key prediction of our model is that overexpression of Myc should rescue defects in E1A that are associated with loss of the p400 interaction. To challenge this prediction, we asked whether overexpression of Myc can restore the ability of the E1A Δ26–35 mutant to sensitize cells to apoptosis (4). As reported (15), expression of E1A in IMR90 fibroblasts sensitizes them to apoptosis in the presence of adriamycin (Fig. 4F Left) and results in the induction of both ARF and p53 (Fig. S4B). Under these conditions, E1A is a more potent inducer of apoptosis than Myc, and its proapoptotic...
activity (Fig. 4F), and ability to induce ARF and p53, is disrupted by the Δ26–35 mutation. As predicted from the model, overexpression of Myc in the presence of the Δ26–35 E1A mutant rescued both E1A’s ability to trigger apoptosis (Fig. 4F) and induce ARF and p53 (Fig. S4B). The rescue of mutant E1A function was specific, because overexpression of Myc did not rescue the apoptotic defects of an E1A mutant (ΔCR2) that still interacts with p400 but fails to interact with Rb (Fig. 4F). Thus, increased expression of Myc can specifically rescue apoptotic defects associated with loss of the E1A–p400 interaction.

Finally, we asked whether the same phenomenon applied to the ability of E1A to drive human cell transformation. Our previous studies have shown that expression of E1A and activated Ha-RasV12 in early passage normal human foreskin fibroblasts (designated BJ) allows the formation of colonies in soft agar (Fig. 4G and ref. 16). Blocking the ability of E1A to interact with p400 also attenuates its ability to collaborate with Ras to drive human cell transformation in this assay (Fig. 4G). Importantly, this activity can be restored by overexpression of Myc. Thus, overexpression of Myc can rescue both the transformation and apoptotic defects that result when the ability of E1A to interact with p400 is blocked. These data are consistent with the idea that the critical function of the E1A–p400 interaction in transformation and apoptosis is to induce Myc.

**Conclusions**

Together, our data support a model in which the binding of E1A to p400 promotes the formation of a Myc–p400 complex at Myc-target gene promoters. The increase in interaction of Myc and p400 is associated with stabilization of the Myc protein and an induction of Myc target genes. These functions of E1A are required for its ability to induce the ARF/p53 pathway, promote apoptosis, and drive cellular transformation, revealing that the downstream arm of the E1A–p400 interaction is mediated via Myc. Although it has long been known that E2F functions as the downstream target of the E1A–Rb interaction, the molecular processes downstream of the E1A–p400 connection have remained obscure. Our data indicate that Myc is the ultimate target of this connection. This finding not only provides an explanation for the overlapping biological functions of Myc and E1A, but also reveals an interesting viral strategy that Myc is the ultimate target of this connection. To induce apoptotic cell death, however, cells must also be treated with adriamycin for 24 h. Adherent and nonadherent cells were then pooled and analyzed for viability by trypsin blue exclusion. At least 200 cells were counted for each data point. To assess the ability of E1A to induce apoptosis in Myc−/− cells, HO.15.19, and parental Rat1 cells, TGR-1 (13) were transduced with retroviral expression constructs for E1A, E1A Δ26–35, or Myc, in the indicated combinations. Relative apoptosis was determined by comparing cell death 24 h after treatment with increasing doses of adriamycin. Data presented are the average of three independent experiments.

**Cell Viability.** To assay the ability of Myc to rescue the apoptotic defect of the E1A Δ26–35 mutant, IMR90 cells were stably transduced with pLPC E1A (21), pLPC E1A Δ26–35 (4), pLPC ΔCR2 (4), or pBabe Hygro HAM Myc (22) by retroviral infection. The resulting cell lines were plated into 12-well dishes at a density of 1 × 10^5 cells per well. Twenty-four hours later, cells were treated with adriamycin for 24 h. Adherent and nonadherent cells were then pooled and analyzed for viability by trypan blue exclusion. At least 200 cells were counted for each data point. To assess the ability of E1A to induce apoptosis in Myc−/− cells, HO.15.19, and parental Rat1 cells, TGR-1 (13) were transduced with retroviral expression constructs for E1A, E1A Δ26–35, or Myc, in the indicated combinations. Relative apoptosis was determined by comparing cell death 24 h after treatment with increasing doses of adriamycin. Data presented are the average of three independent experiments.

**Anchorage-Independent Growth.** BJ normal human primary foreskin fibroblasts were stably transduced with pBABE-Puro Ha-RasV12, pW2LNeo E1A, or Hygro-MarkII-Myc in the indicated combinations by retroviral infection and analyzed for anchorage-independent growth in semisolid media as described (16).

**ChiP and Re-ChIP Analysis.** ChiP analyses were performed in U2OS cells that had been transduced with either control (pUC119) or pCMV-FLAG-p400 construct by using Fugene 6 (Roche). After 48 h, cells were infected with control, d520, and d11T22 adenosine for 8 h. Primary immunoprecipitation was performed by using anti-Myc (N262) antibody; for re-ChIP, a secondary immunoprecipitation using anti-FLAG (M2) antibody was performed. Comparison of ChIP and Re-ChIP DNAs after each round were assayed by quantitative PCR using either the B23.C (specific) and B23.M (nonspecific control) amplicons (23). Re-ChIP DNA signals for FLAG-p400 were further normalized to those from cells transduced with the vector control.

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