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Biochemistry

Induction of transforming growth factor β_1 resistance by the *E1A* oncogene requires binding to a specific set of cellular proteins

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ABSTRACT **Transforming growth factors β (TGF-βs) are potent inhibitors of epithelial cell growth in culture and might play a similar role *in vivo*. Several studies have suggested that acquisition of TGF-β resistance is an important step in epithelial tumor development. Here, we show that resistance to TGF-β_1 growth inhibition can be induced by transformation of keratinocytes with the *E1A*, but not the *ras*, oncogene. Mutational analysis revealed that these effects closely correlate with the ability of *E1A* proteins to bind to the retinoblastoma gene product (p105) as well as to three other cellular proteins (p60, p107, and p300). Only partial resistance to TGF-β_1 growth inhibition was elicited by E1A mutants that bind to a subset of proteins, whereas complete resistance was induced by E1A mutants that bind to all four proteins together. Total protection against TGF-β growth inhibition was also induced by concomitant introduction into cells of an E1A mutant binding to the p60/p105/p107 proteins and one binding to p300. In parallel with these effects, epidermal transglutaminase, a marker of keratinocyte differentiation, was induced by TGF-β in control but not in *E1A*-transformed cells. TGF-β_1 receptor levels were only partially down-modulated by an intact *E1A* gene and not significantly affected by the various truncated mutants. Thus, the ability of *E1A* to induce TGF-β resistance depends on its ability to bind, and presumably inactivate, several cellular proteins that may be involved in transmission of the TGF-β signal and seem to act downstream from its receptor(s).**

Several genetic events involving both gain and loss of specific functions are probably required for tumor development (1). The ability of certain viral oncogenes, such as the adenovirus *E1A* or human papilloma virus *E7*, to cooperate with *ras* in establishing transformation (2, 3) might directly connect with their ability to bind—and presumably inactivate—a small subset of cellular proteins, including the *Rb* tumor-suppressor gene product (4–6).

A separate line of work has suggested that the ability to escape the inhibitory influences of a normal cellular environment is an important step in tumorigenesis (1, 7). One mechanism for control of tumor formation by surrounding normal cells is release of diffusible growth inhibitory factors, of which the transforming growth factor β (TGF-β) family is one of the best examples (8, 9). These bifunctional factors can either stimulate or inhibit cellular growth, depending on cell type and culture conditions. The particularly strong inhibitory effects that TGF-βs have on growth of epithelial cells can be associated with induction of differentiation (10, 11). The possible involvement of these factors in homeostasis of normal epithelial tissues has also been suggested by a number of *in vivo* studies (9), including our recent demonstration that a TGF-β-like factor plays an important role in skin tumor control (C.M., S. Ramon y Cajal, and G.P.D., unpublished work). These results, together with previous *in vitro* work (8, 12, 13), indicate that acquisition of TGF-β resistance could be an important determinant of epithelial tumor development.

Very little is known about the molecular events involved in TGF-β signal transduction. Specific cell-surface receptors have been identified that are thought to mediate TGF-β action and that are lost in a number of TGF-β-resistant cells (14, 15). The second-cell-messenger system activated by TGF-β has not yet been identified. Recent work has shown that the 105-kDa retinoblastoma gene product (p105-Rb) may play a role in this process and suggested that binding to this protein by certain viral oncoproteins—such as the adenovirus 5 *E1A*, simian virus 40 large tumor antigen, and human papillomavirus type 16 *E7*—may explain their ability to induce TGF-β resistance (16, 17). However, transformation of keratinocytes with human papillomavirus type 16 viruses—expressing the *E7* oncogene—is not by itself sufficient to induce TGF-β resistance, and additional changes are probably required (18, 19). Here, we show that induction of full TGF-β resistance by the *E1A* oncogene requires binding not only to the p105-Rb protein, but also to other *E1A*-associated proteins, of which p300 seems the most important one.

MATERIALS AND METHODS

Cells. Mouse Pam212 cells (20) were grown in Dulbecco's modified essential medium (DMEM)/10% calf serum (GIBCO). Approximately 20% confluent cultures were infected with Harvey sarcoma virus (carrying the v-*ras* oncogene) (7), or a retrovirus carrying the G418-resistance gene alone [pC6M$^-$ neo (21)], or a virus transducing an E1A 12S cDNA (22) together with a G418-resistance gene (MD-E1a12S). Construction of this latter virus was identical to that described for an equivalent E1A-13S retrovirus (23). G418 selection (0.5 mg/ml; GIBCO) was subsequently applied to ensure a homogeneously infected population except for Harvey sarcoma virus, where the presence of helper ensured infection of most, if not all, cells in the dish (as verified by high *ras*-p21 expression levels). The E1A mutants used in this study have been described (5, 24). The various *E1A* sequences (carrying their own promoter) were transferred from the original plasmids into SV2neo (25) (*Bam*HI site) to link them with a G418-resistance gene. The resulting E1A/SV2neo plasmids were transfected into Pam212 cells by lipofection (BRL). Approximately 500 independent G418-resistant colonies were recovered from each transfected DNA and pooled together. Experiments were done with this set of cells, but biological effects of the various mutants were also confirmed by use of a second independent set of transfected cells. A similar approach was used to test complementation in trans of two different E1A mutants. The NTd1598 gene was inserted into SV2hygro (26) (between *Cla* I and *Hin*dIII sites) to link it to a hygromycin-resistance gene and transfected into cells already harboring the d1922/947 gene. Hygromycin-resistant colonies were pooled together

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Abbreviations: TGF-β, transforming growth factor β; PMA, phorbol 12-myristate 13-acetate.

before testing for their TGF-β response. As before, experiments were confirmed by use of a second, independent set of transfected cells.

E1A Coimmunoprecipitation Experiments. Near-confluent dishes of the various transfectants were switched to methionine-free DMEM/2% calf serum and labeled with [^{35}S]methionine at 500 μCi/ml (13.3 mCi/ml; 1112 Ci/mmol; 1 Ci = 37 GBq; ICN) for 4 hr. Conditions for cell extract preparation and immunoprecipitation were as described by Whyte *et al.* (5). The E1A-specific monoclonal antibodies M73 (27) and the control anti-simian virus 40 large tumor antigen Pab 416 monoclonal antibodies (28) were from E. Harlow (Cold Spring Harbor Laboratory). Products of the immunoprecipitation reaction were analyzed by 6% PAGE. The gel was fluorographed before autoradiographic exposure.

Mitogenicity Assays. Control and variously transformed Pam212 keratinocytes were plated onto 24-well trays (0.5–1 × 10^5 cells per well) and tested 1 day later. At the time of the experiment, cultures were switched to serum-free DMEM/epidermal growth factor at 10 ng/ml (Collaborative Research)/insulin at 5 μg/ml/transferrin at 5 μg/ml/selenium at 5 ng per ml (ITS, Collaborative Research) plus or minus TGF-β_1 at 10 ng/ml (R & D Systems, Minneapolis). After 20-hr incubation, DNA synthesis was measured by a 1-hr pulse with [*methyl*-^{3}H]thymidine (3.3 μCi/ml; 40–60 Ci/mmol; ICN). Trichloroacetic acid precipitation was done directly on the dish, and labeled DNA was recovered by extraction with NaOH (0.2 N), essentially as described by Reiss and Sartorelli (11).

Transglutaminase Assays. Epidermal transglutaminase activity was measured by incorporation of [2,3-^{3}H]putrescine into casein at pH 9.5, as described (29). Prior to test, cells were plated onto 24-well trays (2 × 10^4) and grown for 5 days to near confluence in low-calcium (0.05 mM) minimal essential medium (MEM)/4% Chelex-treated fetal calf serum (Flow Laboratories)/epidermal growth factor at 10 ng/ml. We found that, relative to cells grown in normal DMEM, Pam212 cells grown under these conditions had lower basal levels of transglutaminase activity, which allowed better detection of enzymatic induction.

^{125}I-Labeled TGF-β_1-Crosslinking Experiments. The assay was done essentially as described by Massague (30). Briefly, subconfluent cultures were washed with binding buffer A (DMEM/0.1% bovine serum albumin/25 mM Hepes, pH 7.4) and then incubated in the same buffer for 1 hr at 37°C; a second wash was performed with binding buffer B (128 mM NaCl/5 mM KCl/5 mM $MgSO_4$/1.2 mM CaCl/50 mM Hepes, pH 7.5, containing bovine serum albumin at 2 mg/ml) for 30 min at 4°C to completely remove bound ligand. ^{125}I-Labeled TGF-β_1 in binding buffer B (150 pM) was added to the monolayers for 4 hr at 4°C on an oscillating platform. Unbound radioactivity was removed by washing with binding buffer B lacking bovine serum albumin. The cross-linking agent, disuccinimidyl suberate (Pierce), was added at a concentration of 0.135 mM, and plates were agitated for 15 min at 4°C. Cells were scraped off in detachment buffer (0.25 M sucrose/10 mM Tris/1 mM EDTA, pH 7.0/0.3 mM phenylmethylsulfonyl fluoride) and sedimented by centrifugation. Cell pellets were solubilized in 70 μl of solubilization buffer (125 mM NaCl/10 mM Tris/1 mM EDTA, pH 7.0/1% Triton X-100/0.3 mM phenylmethylsulfonyl fluoride/leupeptin at 0.01 mg/ml/pepstatin at 0.01 mg/ml/trypsin inhibitor at 0.1 mg/ml) for 40 min at 4°C with end-over-end mixing. Soluble material was subjected to SDS/7.5% PAGE under reducing conditions. Gels were fluorographed before autoradiography.

RESULTS

Induction of Resistance to TGF-β Growth Inhibition Requires Concomitant Binding of E1A to a Specific Set of Cellular Proteins. Acquisition of TGF-β resistance in epithelial cells involves poorly understood genetic events. We attempted to reproduce these events by introducing into keratinocytes activated *ras* or *E1A* oncogenes. For our previous studies, primary keratinocyte cultures were used because established cells are likely to have significantly altered growth-control mechanisms. Primary mouse keratinocytes, however, are not easy to passage and cannot be efficiently transfected with foreign DNA. Therefore, for the present study, a spontaneously derived keratinocyte line, Pam212 (20), was used. In spite of several other changes, this cell line was still highly responsive to TGF-β_1 growth inhibition (C.M. *et al.*, unpublished work; Fig. 1).

Introduction of an *E1A* oncogene into Pam212 cells by use of a retroviral vector (MD-E1a12S) induced total resistance to TGF-β_1 growth inhibition, as assessed by an *in vitro* [^{3}H]thymidine incorporation assay (Fig. 1*A*). In contrast, cells transformed by the v-*ras* oncogene (by Harvey sarcoma virus infection) or carrying only a G418-resistance gene (by infection with a control retrovirus) retained full sensitivity to the growth inhibitor (Fig. 1*A*).

Mutational analysis has previously suggested that the transforming and *ras*-cooperating activity of *E1A* closely correlates with its ability to bind to three distinct cellular proteins, p105 [the product of the *Rb* tumor-suppressor gene (4, 31)], p107, and p300 (5). In addition, binding to a fourth, cell-cycle-related protein, p60, could be important for *E1A* transformation (32, 33). Association with these proteins may also be required for induction of a TGF-β-resistant phenotype. Accordingly, several E1A mutants that differ in their ability to bind to the *E1A*-associated proteins (5, 24) were tested for their effects on keratinocytes. The various mutated *E1A* genes were linked to a G418-resistance gene and introduced into Pam212 cells by transfection. For each gene, >300 independent transfectants were selected and pooled together, so as to minimize individual clone variations. Differential association of the various mutated E1A proteins with specific cellular proteins was verified by coimmunoprecipitation experiments with M73 anti-E1A monoclonal antibodies (Fig. 2).

E1A mutants d1787N and d1799N contain internal deletions that leave intact the two discrete regions required for cell transformation and binding to the p105, p107, and p300 (5) as well as p60 proteins (Fig. 2). Introduction of these mutants into Pam212 cells induced complete resistance to TGF-β growth inhibition, similar to that observed after infection with the MD-E1a12S virus (Fig. 1*B*; Fig. 3).

The NTd1814 mutant contains an amino-terminal deletion of 83 amino acids that destroys most of the transforming region of E1A and retains only the binding region for p107 (5). Cells transfected with this mutant were sensitive to TGF-β growth inhibition but consistently less (15%) than control cells carrying no *E1A* sequences (Fig. 1*B*; Fig. 3). This fact suggests that association with p107 can by itself confer a low level of TGF-β resistance.

NTd1598 and NTd1646 correspond to deletions of the first 13 and 29 amino acids of *E1A*, respectively, and prevent binding of the p300 protein without preventing that of p105 and p107 (5). We found that, unlike NTd1814, these mutations do not affect binding to p60 (Fig. 2). Cells transformed by these mutants exhibited an intermediate phenotype, their growth being inhibited by TGF-β, but to a 30–40% lesser extent than the controls (Fig. 1*B*; Fig. 3).

Mutant d1922/947 has retained the ability to bind to p300 but has lost that for p105 and p107 (5). Interestingly, binding to p60 is also prevented by this mutation (Fig. 2). A consistently higher level of TGF-β resistance (60%–70%) was observed after transformation of Pam212 cells with this mutant relative to the previous ones (Fig. 1*B*; Fig. 3).

If induction of full TGF-β resistance by *E1A* requires concomitant association with the p60/p105/p107 and p300

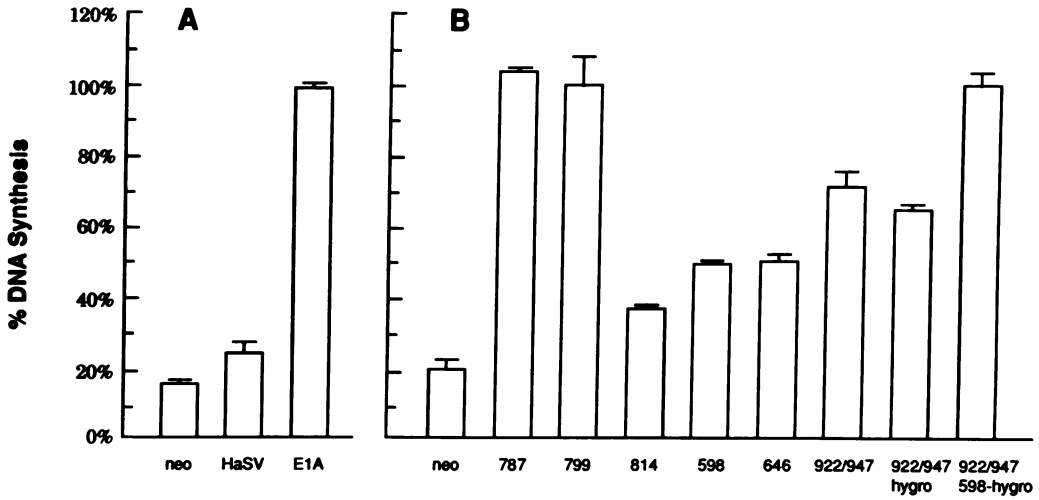


FIG. 1. Different sensitivity of oncogene-transformed keratinocytes to TGF-β growth inhibition. Values are expressed as % [^{3}H]thymidine incorporation of TGF-β-treated cells versus untreated controls (100%). In each case, duplicate wells were tested, and SDs were as indicated by bars. A TGF-β_1 concentration of 10 ng/ml was used, but comparable results were also obtained in other experiments with concentrations of 3 and 20 ng/ml. (*A*) Pam212 cells infected with a retrovirus carrying the G418 resistance gene alone (neo) or the v-*ras* oncogene (HaSV) or an E1A 12S cDNA (E1A). (*B*) Cells carrying the SV2neo plasmid control, the various E1A mutants, and the double transfectants with d1922/947/neo plus SV2hygro or NTd1598/hygro.

proteins, E1A mutants that bind separately to these two groups of proteins might be able to complement each other in trans. This possibility was tested by linking the NTd1598 gene to an hygromycin-resistance gene and introducing it into keratinocytes already transformed with d1922/947. Control d1922/947 cells that were transfected with the hygromycin-resistance gene alone exhibited partial resistance to TGF-β growth inhibition, similar to that seen with their parent cells. In contrast, the double NTd1598-d1922/947 transfectants showed complete TGF-β resistance, as observed with intact *E1A* genes (Fig. 1*B*).

Thus, association of *E1A* to either the p107, p60/p105/p107, or p300 proteins can induce partial TGF-β_1 resistance, but binding to all four proteins is required for complete resistance.

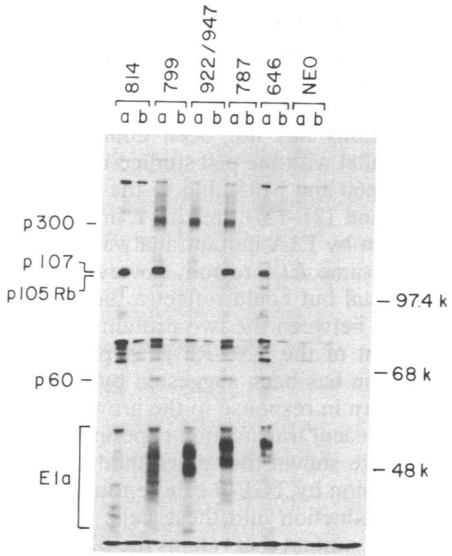
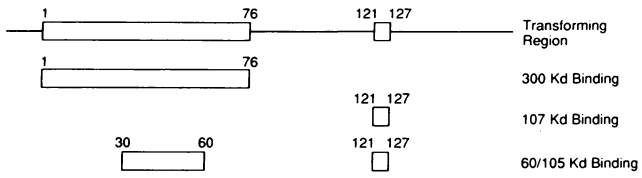


FIG. 2. Different association of various E1A mutants with specific cellular proteins in stable Pam212 transfectants. Extracts from [^{35}S]methionine-labeled Pam212 cells stably transfected with the various E1A mutants were incubated with either anti-E1A M73 (27) (lanes a) or anti-simian virus 40 large tumor antigen Pab416 (28) (lanes b) monoclonal antibodies. k, kDa.

E1A Transformation Blocks the Differentiating Effects of TGF-β. Epidermal transglutaminase is an enzymatic marker of differentiation that can be induced by exposure of keratinocytes to calcium or phorbol 12-myristate 13-acetate (PMA) (29). Consistent with their resistance to calcium (20, 29), Pam212 cells showed only a slight transglutaminase induction in response to this agent (data not shown). In contrast, significant increase of enzymatic activity was seen after exposure of these cells to PMA (Fig. 4) in agreement with their responsiveness to this substance (20, 29). Similar, if not stronger, induction was also seen after treatment with TGF-β_1 (Fig. 4), indicating that this factor can act as a differentiating agent of Pam212 cells, as reported for certain keratinocytes (11) even if not others (40). This response was not affected by *ras* transformation (Fig. 4). In contrast, cells harboring an *E1A* gene with an intact transforming region



Mutants	Aminoacids deleted	Binding p60	p105	p107	p300	TGF-β resistance
NTdl814	2-85	–	–	+	–	15%
NTdl598	2-13	+	+	+	–	37%
NTdl646	2-29	+	+	+	–	35%
dl922/947	122-129	–	–	–	+	61%
dl787N	77-85	+	+	+	+	93%
dl799N	81-85	+	+	+	+	100%
SV2neo	/	–	–	–	–	0%

FIG. 3. Protein-binding domains and biological effects of the various E1A mutants. Different association of the mutated *E1A* products to the p60, p105, p107, and p300 cellular proteins is as described (5, 24, 32, 35) and as assessed by the E1A coimmunoprecipitation experiment of Fig. 2. Resistance to TGF-β_1 growth inhibition was calculated from an average of five independent experiments, including the one shown in Fig. 1, as % of DNA synthesis relative to similarly treated control cells carrying SV2neo only. Kd, kDa.

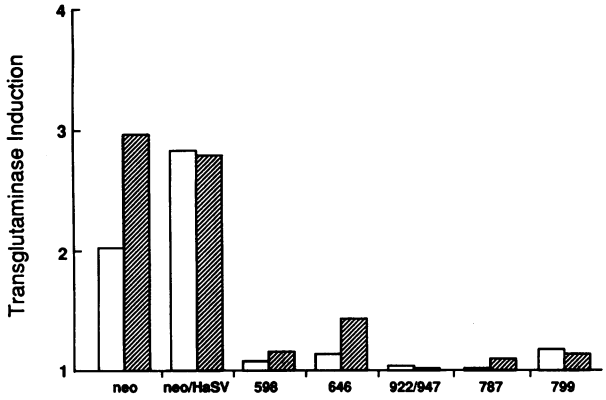


FIG. 4. Induction of transglutaminase activity as a marker of keratinocyte differentiation. Transglutaminase activity was assayed by measuring incorporation of [2,3-^{3}H]putrescine into casein at pH 9.5, as described. Cells harboring the various E1A mutant plasmids or SV2neo or SV2neo plus Harvey sarcoma virus (HaSV) were either untreated or treated with PMA (100 ng/ml; white bars) or TGF-β (10 ng/ml; striped bars) for 24 hr before testing. Values are expressed as -fold induction of treated versus untreated controls. All assays were done in duplicate and normalized for protein content. Background radioactivity from controls (containing no cell lysate) was subtracted in all cases.

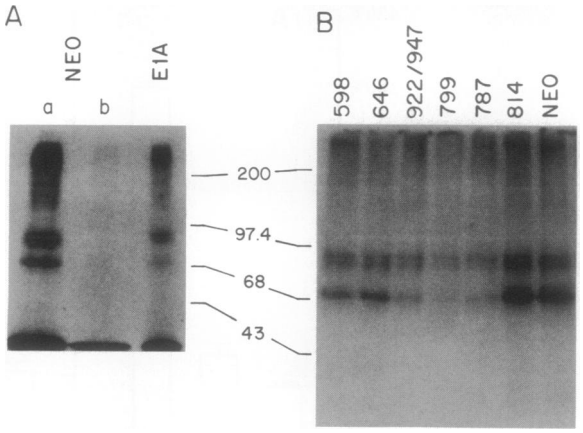


FIG. 5. TGF-β_1 cell-surface receptors in variously transformed cells as detected by affinity labeling with ^{125}I-labeled TGF-β_1. Subconfluent cultures of cells were affinity labeled with ^{125}I-labeled TGF-β followed by chemical crosslinking with disuccinimidyl suberate as described. In all cases, samples were normalized for equal protein content before analysis by gel electrophoresis and autoradiography. (*A*) Cells infected with a control retrovirus (Neo) or a virus carrying an E1A 12S cDNA (E1A). Specificity of binding was demonstrated by incubating control cells with ^{125}I-labeled TGF-β_1 alone (lane a) or with a vast excess (20 nM) of unlabeled TGF-β_1 (lane b). (*B*) Cells carrying the various E1A mutant plasmids or SV2neo. Relative to the other cells, the particularly strong signal detected with cells harboring the NTd1814 mutant and SV2neo in this particular experiment was not seen in a second independent experiment. Signal intensity seemed also to depend on state of confluence of various cultures.

(mutants d1787N and d1799N) showed little or no transglutaminase induction after TGF-β exposure (Fig. 4). A similar lack of response was also seen with all other tested E1A mutants (Fig. 4). Interestingly, a similar block of transglutaminase induction was observed in the various E1A transformants in response to PMA (Fig. 4). This result is consistent with the possibility that at least some PMA effects on keratinocytes be mediated by inducing TGF-β production (34).

TGF-β_1 Receptors Are Only Marginally Affected by E1A Transformation. Previous work has shown that one mechanism for acquisition of TGF-β resistance involves loss of TGF-β cell-surface receptors (14, 15). This possibility was evaluated by direct cross-linking of iodinated TGF-β_1 to the various transformed keratinocytes. Of the three surface glycoproteins that bind TGF-β_1 with high affinity [≈53, 73–95, and 300 kDa proteins, defined as type I, II, and III receptors, respectively (14)], there was a partial down-modulation of type I receptor (to ≈20–30% of control levels; quantification obtained by densitometric scanning of autoradiographs in two independent experiments) in cells transformed by *E1A* genes with an intact transforming region (Fig. 5). In the same cells, a weaker and less consistent down-modulation of the other two receptor types was also seen. No significant reductions in receptor number were detected in cells carrying E1A mutants with a truncated transforming region relative to control or *ras*-transformed keratinocytes.

DISCUSSION

In the present communication, we have shown that transformation of keratinocytes with the *E1A* oncogene is sufficient to prevent the growth inhibitory and differentiating effects that TGF-β_1 has on these cells. Association of *E1A* with a number of distinct cellular proteins seems required for full resistance to TGF-β growth inhibition. E1A mutants that bind to these proteins separately induced only partial TGF-β resistance when tested on their own but induced full resistance when they were concomitantly introduced into cells.

The cellular response to TGF-β might be affected by *E1A* at different levels. The partial down-modulation of TGF-β receptors seen in *E1A*-transformed keratinocytes, is an unlikely explanation for the TGF-β resistance of these cells because only 10–20% receptor occupancy is required for full TGF-β biological activity (8). Moreover, transformation with the various truncated E1A mutants resulted in partial TGF-β resistance without affecting TGF-β receptor levels. Thus, *E1A* is likely to act mainly on intracellular pathways downstream of the TGF-β receptor. Very little is known about these pathways, but *E1A*-associated proteins might be involved.

One of these proteins, p105, has been identified as the product of the retinoblastoma tumor-suppressor gene (4, 31), while another, p60, is a cdc2-associated cell-cycle regulatory protein (32, 33). Both of these proteins might play a role in TGF-β signal transduction and be inactivated or otherwise functionally modified by their association with *E1A*. The identity of the p60 protein that we have detected in our immunoprecipitations has not been conclusively proven. However, in parallel with the p60 studied by others (35), we have found that p60 and p105 bind to the same *E1A* region (residues 30–60 and 121–127), making it impossible to separate their function by E1A mutant analysis. Binding of p60 and p105 to the same *E1A* region, however, might not be merely coincidental but could reflect a functional or structural relationship between the two proteins.

An involvement of the p105-Rb gene product in TGF-β signal transduction has been suggested by its altered phosphorylation pattern in response to the growth inhibitor (16). Furthermore, transient transfection experiments with mouse keratinocytes have shown that down-modulation of exogenous *myc* expression by TGF-β can be totally prevented by concomitant introduction into these cells of an intact *E1A* gene or of an E1A mutant that retains the p105/p107, but not the p300, binding region (17). The significance of *myc* down-regulation for induction of keratinocyte-growth arrest is not completely clear because transformation with a constitutively and highly expressed *myc* gene is not sufficient to induce TGF-β resistance (36; our unpublished observation). Furthermore, although block of *myc* down-regulation might

be from binding to p105/p107 (17), association with p60 might be involved as well. In any case, we have shown here that E1A mutants binding to p60–p105/p107 induce only partial resistance to TGF-β growth inhibition, indicating that additional events besides interference with p60/p105 function are required for induction of complete TGF-β resistance.

Two other proteins, p107 and p300, were found on the basis of their association with *E1A*. An E1A mutant binding only to p107 could induce partial TGF-β resistance, but at rather low levels. On the other hand, a p300-binding mutant induced TGF-β resistance at elevated levels, consistently higher than those seen with the p60–p105/p107-binding mutants. The particularly strong effects that were exerted by the p300-binding mutant in this system contrast with its lack of biological activity in conventional transformation assays (5, 24). Nothing is known about the p300 protein, except that it may be involved in control of DNA synthesis (37). Our results suggest that p300 might have an especially important role in transmission of negative growth signals of the kind elicited by TGF-β.

In addition to its growth-inhibitory effects, TGF-β_1 was able to act as an inducer of keratinocyte differentiation, as revealed by epidermal transglutaminase induction and recent *in vivo* results (C.M. *et al.*, unpublished work). This effect was blocked by E1A mutants with an intact transforming region or which retained only the p60/105–107- or p300-binding domains. This fact suggests that the various *E1A*-associated proteins are involved in transmission of the TGF-β differentiation signal and that interference with either p60/p105–p107 or p300 function is sufficient to totally block it.

In conclusion, the adenovirus *E1A* oncogene encodes for a multifunctional protein with the ability to interfere with normal cell metabolism at a variety of levels (38, 39). It was shown here that another property of this gene is to block the normal response of cells to TGF-β. We recently found that *E1A* transformation *in vivo* releases *ras*-transformed keratinocytes from control of a TGF-β-like inhibitory factor (C.M. *et al.*, unpublished work). Thus, the ability of *E1A* to cooperate with other oncogenes might be associated with its ability to allow cells to escape from environmental control of tumorigenesis, at least as mediated by TGF-β factors.

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