The adenoviral E1A oncoproteins interfere with the growth-inhibiting effect of the cdk-inhibitor p21\(^{CIP1/WAF1}\)

Petra Keblusek, Josephine C. Dorsman, Amina F. A. S. Teunisse, Hans Teunissen, Alex J. van der Eb and Alt Zantema

Laboratory for Molecular Carcinogenesis, Leiden University Medical Center, PO Box 9503, 2300 RA Leiden, The Netherlands

The cdk-inhibitor p21\(^{CIP1/WAF1}\) inhibits the activities of cyclin-dependent kinases and proliferating cell nuclear antigen, thereby repressing cell-cycle progression and DNA replication. Transforming oncogenes, such as E1A of human adenovirus 5 (Ad5), may interfere with such growth-inhibitory proteins. In this study, we show that in various Ad5E1-transformed cells, p21\(^{CIP1/WAF1}\) is expressed and that, in general, expression is not downregulated. In addition, colony-formation assays show that in Ad5E1-transformed cells highly overexpressed p21\(^{CIP1/WAF1}\) can still cause growth inhibition. FACS experiments indicate, however, that a G1 arrest induced by moderate overexpression of p21\(^{CIP1/WAF1}\) can be overcome by E1A. The E1A proteins may interfere with the function of p21\(^{CIP1/WAF1}\) by binding. Indeed, p21\(^{CIP1/WAF1}\) binds with its cyclin/cdk-binding N terminus to the transforming N-terminal and CR1 region of the E1A proteins. Together, these results lend support to the model that E1A can interfere directly with p21\(^{CIP1/WAF1}\) function and thereby stimulates cell growth.

Introduction

Expression of the adenoviral (Ad) 12S and 13S E1A proteins can force quiescent cells to re-enter the cell cycle and can result in the transformation of susceptible mammalian cells. The non-conserved N-terminal domain and the conserved regions CR1 and CR2, which are present in both the 12S and 13S E1A proteins, are important for transformation. Binding of E1A to a number of cellular proteins contributes to this growth-stimulatory effect by altering and, in most instances, inactivating the function of these proteins (reviewed in Moran & Mathews, 1987; Peep & Zantema, 1993; Moran, 1994). Cellular proteins bound to the transforming regions of E1A include tumour-suppressor protein pRb (Whyte et al., 1988) and related proteins p107 (Ewen et al., 1991) and p130 (Hannon et al., 1993; Li et al., 1993), transcriptional co-activators p300 and CBP (CREB-binding protein) (Arany et al., 1994, 1995; Eckner et al., 1994; Lundblad et al., 1995), cyclin-dependent kinase cdk2 and cyclin A/E (Faha et al., 1993). The binding of E1A to cellular proteins may be accompanied by changes in gene expression that favour cell-cycle progression. For example, binding of E1A to pRb prevents the formation of repressive pRb/E2F transcription-factor complexes, thereby activating E2F-responsive genes needed for the onset of S-phase (reviewed in Nevins, 1992; Moran, 1994).

Recently, a new class of growth-inhibitory proteins was discovered, the so-called cdk inhibitors. In normal cells, regulation of the activity of cyclin-dependent kinases is crucial for an orderly progression through the cell cycle. Cdk inhibitors can bind to and inhibit the function of cyclin-dependent kinases and thereby negatively affect cell growth (reviewed in Sherr, 1994; Hunter & Pines, 1994; Nasmyth & Hunt, 1993; Peters, 1994; Elledge et al., 1996; Sherr, 1996). They can be sub-divided into the CIP/KIP family which inhibit several cyclin-dependent kinases with varying efficiencies: p21\(^{CIP1/WAF1}\) (Harper et al., 1993; El-Deiry et al., 1993), p27\(^{KIP1}\) (Polyak et al., 1994) and p57\(^{KIP2}\) (Lee et al., 1995), and the INK4-family which inhibit only the activity of the cyclin D-type-specific cyclin-dependent kinases: p15\(^{INK4B}\) (Hannon & Beach, 1994), p16\(^{INK4A}\) (Serrano et al., 1993), p18\(^{INK4C}\) (Guan et al., 1994) and p19\(^{INK4D}\) (Chan et al., 1995).

The first cdk inhibitor discovered was p21\(^{CIP1/WAF1}\). Various groups independently have cloned the same gene, either as an inhibitor of cdk2 activity (Cip1/CAP20) (Gu et al.,
1993; Harper et al., 1993), as a transcripational target of the p53 tumour-suppressor protein (WAF1) (El-Deiry et al., 1993) or as a gene that is induced at senescence (Sdi) (Noda et al., 1994). Besides binding to cyclin-dependent kinases, p21\(^{CIP/WAF1}\) can also bind to and inactivate stress-activated kinases (Shim et al., 1996) and casein kinase II (Götz et al., 1996). This cdk inhibitor can also fulfill other functions, such as inhibition of DNA replication by binding to proliferating cell nuclear antigen (PCNA) (Flores-Rozas et al., 1994; Waga et al., 1994; Luo et al., 1995). Evidently, p21\(^{CIP/WAF1}\) plays a central and diverse role in cell-cycle regulation and thus is an obvious target for viral-transforming proteins, such as the E1A proteins.

It has already been shown that E1A indeed can interfere with induced expression of the p21\(^{CIP/WAF1}\) gene. The induction of expression of this gene which is observed upon differentiation of keratinocytes can be abrogated by the E1A protein (Missiro et al., 1995). Likewise, no induction of p21\(^{CIP/WAF1}\)-gene expression was observed after DNA damage or TGF-β treatment in E1A-expressing human cells in contrast to the induction detected in control cells (Datto et al., 1997; Steegenga et al., 1996; Somasundaram & El-Deiry, 1997). Furthermore, E1A binds to the p21\(^{CIP/WAF1}\)-related inhibitor p27\(^{KIP}\) and inactivates its function upon treatment with TGF-β (Mal et al., 1996). Apparently, E1A can counteract the induction of active p21\(^{CIP/WAF1}\) and p27\(^{KIP}\) by various mechanisms. The inhibitory protein p21\(^{CIP/WAF1}\), however, is also a likely target for the E1A proteins upon transformation of susceptible mammalian cells.

In this study, we investigated whether in cells expressing the Ad transforming proteins the levels and/or activities of p21\(^{CIP/WAF1}\) are influenced in comparison to untransformed cells. Colony-formation assays were performed to determine whether the Ad transforming proteins can counteract the growth-inhibitory functions of p21\(^{CIP/WAF1}\). In addition, FACS experiments were performed to determine whether E1A can counteract the function of this inhibitor. We also investigated whether E1A exerts its effect on p21\(^{CIP/WAF1}\) by binding to it.

**Methods**

**Cell lines, tissue culture, transfections and colony-formation assays.** U2OS cells (a human osteosarcoma cell line), normal rat kidney 49F (NRK) cells, primary human embryonic retina (HER) cells, two independent NRK cell lines transformed with the E1 region of Ad5 (NRK-Xho c2.1 and c2.4) and two independent HER cell lines and one baby rat kidney (BRK) cell line transformed with the same region of Ad5 (HER-Xho c1, HER-Xho c2 and BRK-Xho c22) were used. These or comparable cell lines have been described previously (Schrier et al., 1979; Zantema et al., 1985; Dorsman et al., 1995) and all cell lines used express high levels of Ad-E1 proteins. All cells, except for the BRK cells, were grown in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco) supplemented with 8% foetal calf serum and antibiotics. The BRK-Xho c22 cells were maintained in Minimal Essential Medium (MEM, Gibco) supplemented with 8% newborn calf serum and antibiotics. Insect cells (Spodoptera frugiperda, Sf9) were obtained from the ATCC (CRL 1711) and were grown in TC-100 medium (Gibco) supplemented with 10% foetal calf serum and antibiotics.

All transfections were performed with the calcium phosphate technique, essentially as described before (van der Eb & Graham, 1980). For the colony-formation assays, NRK, HER-Xho c1 and BRK-Xho c22 cells were transfected with either 4 μg pCMX-p21 or pCMV-p16 in the presence of 1 μg pCMV-neo. As a control, 5 μg pCMV-neo was transfected alone. After 3 weeks of maintaining the cells in G418-containing medium, G418-resistant colonies were fixed and stained with Coomassie Brilliant Blue according to standard procedures.

**Plasmid constructs.** The full-length p21\(^{CIP/WAF1}\) cDNA fragment was isolated from pCEP4-Waf1 (a kind gift from W.S. El-Deiry, University of Pennsylvania School of Medicine, PA, USA) (El-Deiry et al., 1993) and cloned into a CMV-promotor-containing vector (pCMX). Plasmid pCMX-p27 was kindly provided by T. Hunter (Salk Institute, La Jolla, CA, USA) (Yoshiosea & Hunter, 1994) and plasmid pCMV-p16 by D. Beach (Cold Spring Harbor Laboratory, NY, USA) (Serrano et al., 1993). Plasmid pCMV-CD20 was generously provided by R. Bernards (Netherlands Cancer Institute, Amsterdam, The Netherlands). Plasmids pRSV-neo and pRSV-5E1A have been described previously (Offringa et al., 1990).

**Antibodies, preparation of extracts and Western blotting.** The mouse monoclonal antibody M73 against Ad5-E1A exon 2 was a kind gift of E. Harlow (Massachusetts General Hospital Cancer Centre, Charlestown, MA, USA) (Harlow et al., 1985). The polyclonal rabbit antibody against p21\(^{CIP/WAF1}\) (C19), the rabbit polyclonal antibody against p16\(^{CDK4}\) (H-156), the rabbit polyclonal antibody against cyclin A (H-432) and the mouse monoclonal antibody against PCNA (PC10) were purchased from Santa Cruz. Anti-cdk2 (rabbit polyclonal antiseraum) was described before (Kranenburg et al., 1995).

For the determination of protein levels, extracts were prepared as follows: exponentially growing cells were washed twice with PBS and incubated for 30 min at 4 °C in a buffer consisting of 50 mM HEPES pH 7.4, 250 mM NaCl, 0.5% NP-40, 50 mM NaF and 10 mM Na\(^3\)-VO\(_4\) supplemented with PMSF, trypsin inhibitor, leupeptin, pepstatin and aproginin as protease inhibitors. Subsequently, the lysate was centrifuged for 10 min at 4 °C and the supernatants were used for further analysis. For the analysis of protein–protein interactions, extracts were prepared as described above, with the exception that a different buffer was used (binding buffer: 50 mM Tris pH 8, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM NaF, 10 mM Na\(^3\)-VO\(_4\) supplemented with protease inhibitors). Protein concentrations were measured with the Bio-Rad protein assay kit. Western blotting was essentially performed as recommended by Santa Cruz, on Immobilon P membranes (Millipore). For detection, ECL reagent was used (Amersham).

**In vitro translation.** The p21\(^{CIP/WAF1}\) protein was synthesized using two different methods. Firstly, for the experiment shown in Fig. 4 (B), a PCR reaction was performed on plasmid pCEP4-Waf1 with an oligonucleotide containing the T7 promoter sequence followed by a Kozak sequence as the forward primer (Roest et al., 1993). The p21\(^{CIP/WAF1}\) protein was subsequently synthesized in vitro with the Tnt-coupled transcription–translation system as recommended by the manufacturer (Promega), with the PCR fragment as a template. Secondly, for the experiment shown in Fig. 4 (C), pCMX-p21 and pCMX-p27 were in vitro transcribed and translated with the same Tnt-coupled transcription–translation system.

**GST ‘pull-down’ experiments.** The following GST-E1A fusion proteins were used: GST-E1A, GST-E1A-N-CR1, GST-E1A-exon 1 (N-CR1-CR2), GST-E1A-exon 2 (J.C. Dorsman, unpublished). Ex-
expression plasmids encoding GST-p21(1–164), GST-p21N(1–103) and GST-p21C(74–164) were a generous gift of R. Fotedar (Institut de Biologie Structurale, Grenoble, France) (Fotedar et al., 1996).

For the GST-E1A ‘pull-down’ experiments, equal amounts of GST alone and GST-E1A fusion proteins were bound to glutathione-Sepharose beads which were subsequently incubated with in vitro translated, [35S]methionine-labelled p21(1–164)/WAF1 or p27/KIP1 in 400 µl binding buffer. After 3–4 h of incubation, the beads were centrifuged and washed, and GST or GST fusion proteins with associating proteins were resolved on a 12% SDS–polyacrylamide gel. Associated p21(1–164)/WAF1 and p27/KIP1 were visualized by autoradiography after treatment of the gels with PPO–DMSO. As input 15% of the total amount that was used in the GST ‘pull-down’ experiments, was loaded.

For the GST-p21 ‘pull-down’ experiments essentially the same procedure as described above was used with the following exceptions. The GST proteins were mixed with 900 µg whole cell extract prepared from E1A-expressing NRK-Xho cells. Bound E1A was visualized in a Western procedure with monoclonal antibody M73 against E1A as first antibody. As input 10 µg whole cell extract was loaded. In the experiment shown in Fig. 4(b) smaller amounts of GST-p21 than of the other GST fusion proteins were used since it was more difficult to obtain high expression levels of GST-p21 in bacteria.

The possibility of a direct interaction between E1A and p21(1–164)/WAF1 was analysed in ‘pull-down’ experiments with purified GST-p21 and purified baculovirus-derived His-E1A. Purified His-E1A was obtained as follows: a recombinant baculovirus expressing Ad5-12S E1A with six histidine residues linked to the N terminus (His-E1A) was constructed according to a previously described procedure (Peeper et al., 1992). Sf9 cells were infected with virus and after 42 h cell extracts were prepared and His-E1A was purified on an Ni2+-NTA–agarose column (Qiagen) according to the manufacturer’s recommended protocol. As input 10% of the total amount that was used in the GST ‘pull-down’ experiments was loaded.

Fluorescence-activated cell sorting (FACS) analysis. U2OS cells were plated on a 9 cm dish and transfected with 10 µg pCMX, 1 µg pCMX-p21 and 9 µg pCMX, 3 µg pCMX-p21 and 7 µg pCMX, or 10 µg pCMX-p21, in the presence of either 30 µg pRSV-5E1A or 30 µg pRSV-neo and in the presence of 3 µg pCMV-CD20. In parallel, the same experiments were performed with pCMV-p16. After 24 h the precipitate was removed, medium supplemented with 50 ng/ml nocodazole was added and the cells were incubated for 16 h. The cells were subsequently washed with PBS, removed from the dish by treatment with trypsin, and washed with PBS supplemented with 20 mM HEPES pH 7.6 and 0.1% BSA. After centrifugation, the cells were incubated in a 20 ml CD20–FITC solution (Pharmingen) for 1 h. Cells were subsequently washed with PBS and fixed in 3% ethanol in PBS for at least 30 min. After removal of the ethanol, cells were resuspended in PBS containing 0.2 mg/ml RNaseA and 0.01 mg/ml propidium iodide. The samples were analysed with a Becton-Dickinson FACS scan with LYSIS II software. In parallel, cells in 6 cm dishes were transfected with the same precipitate; nocodazole was again added after 24 h, and 16 h later lysates were prepared in Laemmli sample buffer (Harlow & Lane, 1988). These lysates were analysed for expression of the transfected genes in Western blot experiments.

**Results**

**p21\(^{1+1}\) is expressed in Ad5E1-transformed cells and still functions as a growth inhibitor**

The cdk-inhibitor p21\(^{1+1}\) is a negative regulator of progression through the cell cycle. Since this regulation in Ad5E1-transformed cells is severely disturbed, we examined whether the levels of expression and/or functions of this inhibitor are influenced by the Ad transforming proteins. Firstly, various human and rat Ad5E1-transformed cell lines were tested for p21\(^{1+1}\) expression levels (see Fig. 1). In two independent Ad5E1-transformed human cell lines, HER-Xho c1 and c2, the levels of p21\(^{1+1}\) were higher than in the primary cells (see Fig. 1, lanes 2 and 3 vs lane 1), whereas in two transformed rat cell lines, NRK-Xho c2.1 and c2.4, expression was slightly lower than in the untransformed NRK cells (see Fig. 1, lanes 5 and 6 vs lane 4). Apparently, p21\(^{1+1}\) is still expressed in Ad5E1-transformed cells and expression is, in general, not downregulated.

To determine whether the growth-inhibitory pathway of p21\(^{1+1}\) is still intact in Ad5E1-transformed cells, we tested whether overexpression of p21\(^{1+1}\) is able to inhibit the growth of these cells. To this end, we performed a colony-formation assay in which Ad5E1-transformed BRK-Xho c22 cells, Ad5E1-transformed HER-Xho c1 cells or untransformed NRK cells were transfected with equal amounts of pCMV-p21, pCMV-p16 or a control vector (pCMV-neo). The cell lines chosen for this study can all be transfected with good efficiencies with the calcium phosphate method. After 3 weeks of selection with G418-containing medium, neomycin-resistant colonies were counted and relative percentages of colony formation were calculated. Fig. 2 shows that, as expected, overexpression of either p21\(^{1+1}\) or p16\(^{INK4A}\) in untransformed NRK cells suppresses colony formation. In Ad5E1-transformed cells, overexpression of p21\(^{1+1}\) still negatively affects the outgrowth of colonies, although slightly less efficient than in untransformed cells (see Fig. 2). These results indicate that, in principle, p21\(^{1+1}\) and its downstream targets can still function in these cells. In contrast, no inhibition of colony formation is seen in Ad5E1-transformed cells, when p16\(^{INK4A}\) is overexpressed (see also Fig. 2). These results are as expected, since previous studies showed that p16\(^{INK4A}\) functions as a growth inhibitor only in cells with
active pRb (Lukas et al., 1995; Medema et al., 1995) and in Ad5E1-transformed cells pRb is inactivated by binding to E1A (Nevins, 1992). In the BRK-Xho cells, there was even an increase in the number of colonies, when p16INK4A is overexpressed.

**Ad5-E1A can partially overcome a p21<sup>CIP1/WAF1</sup>-induced G1 arrest**

Since the colony-formation assays suggest that the Ad5E1 region may be able to partially relieve growth repression mediated by p21<sup>CIP1/WAF1</sup>, we investigated whether a p21<sup>CIP1/WAF1</sup>-induced G1 arrest could be (partially) relieved by E1A. To this end, we performed FACS analysis of U2OS cells transfected with increasing amounts of either pCMV-p21 or pCMV-p16 together with either pRSV-5E1A or a control vector. All cells were co-transfected with a CD20-expressing plasmid to analyse the transfected cells afterwards. Cells were also stained with propidium iodide to determine the DNA content. For an accurate determination of the number of cells arrested in the G1 phase we added nocodazole, which arrests cells in the G2/M phase (Zieve et al., 1980). Accordingly, cells that have not already been arrested in the G1 phase by the overexpression of the inhibitory proteins, will arrest in the G2/M phase. In Fig. 3(B), the percentages of cells in the G1 phase are shown compared with cells transfected with the control vectors. Increasing amounts of pCMV-p21 or pCMV-p16 induce a G1 arrest which is reflected by increasing percentages of cells in G1 phase (Fig. 3). In this experiment, lower amounts of pCMV-p16 than of pCMV-p21 already gave rise to a maximal G1 arrest. As expected for cells in which pRb is inactivated, p16<sup>INK4A</sup> does not function as a growth inhibitor when co-expressed with E1A (Fig. 3 and Lukas et al., 1995; Medema et al., 1995). However, increasing amounts of pCMV-p21 still give rise to an increase in G1-phase cells in the presence of pRSV-5E1A. This result is in agreement with the colony-formation assay (see Fig. 2) in which overexpressed p21<sup>CIP1/WAF1</sup> retained the capacity to function as a growth inhibitor in the presence of E1A. However, E1A can overcome the growth-inhibitory properties of moderately overexpressed levels of p21<sup>CIP1/WAF1</sup> and partially those of higher levels of p21<sup>CIP1/WAF1</sup>, as can be seen in Fig. 3: the increase in G1-phase cells observed with increasing pCMV-p21 concentrations is decreased in the presence of pRSV-5E1A. Essentially the same results were obtained with E1A and E1B together or without addition of nocodazole (data not shown). As a control, expression levels of the transfected proteins were tested by Western blot analysis. The levels of overexpressed p21<sup>CIP1/WAF1</sup> and p16<sup>INK4A</sup> are not affected by co-expression of E1A (data not shown). Together, these results suggest that E1A can partially abrogate the p21<sup>CIP1/WAF1</sup>-induced G1 arrest.
E1A overcomes p21-induced growth arrest

Fig. 3. Effect of E1A on a p16INK4A- or p21CIP1/WAF1-induced G1 arrest. (A) Cell-cycle distribution determined by FACS analysis of CD20-positive U2OS cells. Dishes (9 cm) of U2OS cells were transfected with 3 µg pCD20 and cotransfected with increasing and indicated amounts of pCMV-p16 or pCMV-p21 plasmid in the presence or absence of 30 µg pRSV-5E1A. Nocodazole (50 ng/ml) was added to the medium for the duration of 16 h after a 24 h transfection period. The average values of two independent experiments are depicted. (B) Graph showing the absolute increase in G1 phase in the same experiments as in (A).

The transforming domains of the E1A proteins can bind to p21CIP1/WAF1

Since E1A can partially overcome a p21CIP1/WAF1-induced G1 arrest, we examined whether the observed inactivation of p21CIP1/WAF1 by E1A may be caused by binding. To this end, we performed GST 'pull-down' experiments with various GST-SE1A fusion proteins and in vitro translated, [35S]methionine-labelled p21CIP1/WAF1. The composition of the GST fusion proteins is shown in Fig. 4(A). It is evident from Fig. 4(B) that GST-SE1A binds to p21CIP1/WAF1 and that this association is specific since GST alone does not display binding to p21CIP1/WAF1 (Fig. 4B, lane 2). In addition, the same figure shows that the N-terminal domain and the CR1 region of E1A are sufficient for binding to p21CIP1/WAF1, whereas no binding to the C-terminal domain of E1A encoded by exon 2 can be observed (see Fig. 4B, lanes 3 and 5). When GST 'pull-down' experiments were performed with extracts prepared from NRK cells as a source for p21CIP1/WAF1, essentially the same results were obtained (data not shown). Subsequently, we compared the efficiency of binding of p21CIP1/WAF1 to E1A with that of an already identified E1A-binding protein, the highly related cdk-inhibitor p27KIP1 (Mal et al., 1996; Nomura et al., 1998). Fig. 4(C) shows that the binding of p21CIP1/WAF1 to E1A is at least as efficient as the binding of p27KIP1 to E1A. Furthermore, binding experiments with purified GST-p21 and purified baculovirus-derived His-E1A suggest that the interaction is direct (Fig. 4D). All forms of E1A can bind equally well to the p21CIP1/WAF1 protein, as was visible on longer exposures (results not shown). Together the results indicate that p21CIP1/WAF1 has the capacity to bind directly to regions of E1A that are important for transformation (Moran & Mathews, 1987).

The binding region of E1A on the p21CIP1/WAF1 protein was also determined. The p21CIP1/WAF1 cdk-inhibitor contains two separate domains, an N-terminal domain binding to cyclins and cyclin-dependent kinases and a C-terminal domain binding to PCNA (Luo et al., 1995). To map the binding to the N- or C-terminal domain, GST-p21(1–164) encompassing the full-length protein, GST-p21N(1–103) encompassing the N-terminal domain and GST-p21C(74–164) encompassing the C-terminal domain were incubated with extracts prepared from Ad5E1-transformed NRK cells. GST fusion proteins and associating proteins were isolated and Western blot analysis was performed with anti-E1A antibodies (see Fig. 4E). The results suggest that E1A binds primarily to the N terminus of p21CIP1/WAF1 (see Fig. 4E, lane 4), while almost no binding to the C-terminal half of p21CIP1/WAF1 can be observed (see Fig. 4E, lane 3). As a control, the same Western blot was also probed with antibodies against the already identified p21CIP1/WAF1-binding proteins cdk2 and PCNA (Luo et al., 1995). In the case of cdk2, only GST-p21(1–164) and GST-p21N(1–103) containing the N terminus of p21CIP1/WAF1 showed association, as expected, whereas in the case of PCNA only fusion proteins containing the C terminus of p21CIP1/WAF1 exhibited binding (data not shown).
Fig. 4. Ad5-E1A binds to p21<sub>CP1/WAF1</sub> in vitro. (A) Composition of GST fusion proteins used in ‘pull-down’ experiments. (B) GST (lane 2), GST-5E1A N-CR1 (lane 3), GST-5E1A exon 1 (lane 4), GST-5E1A exon 2 (lane 5), GST-5E1A (lane 6), immobilized on glutathione–Sepharose beads, were mixed with in vitro translated, [<sup>35</sup>S]methionine-labelled p21<sub>CP1/WAF1</sub> (lane 1, input). Bound p21<sub>CP1/WAF1</sub> was visualized by autoradiography. (C) GST (lanes 2 and 6), GST-5E1A exon 1 (lanes 3 and 7) and GST-5E1A exon 2 (lanes 4 and 8) were mixed with in vitro translated, [<sup>35</sup>S]methionine-labelled p21<sub>CP1/WAF1</sub> (lanes 1–4) or p27<sub>KIP1</sub> (lanes 5–8). Bound p21<sub>CP1/WAF1</sub> and p27<sub>KIP1</sub> were visualized by autoradiography. (D) GST (lane 2), glutathione–Sepharose beads alone (lane 3) or GST-p21 (lane 4) were mixed with purified baculovirus-derived His-E1A (lane
Discussion

In Ad5E1-transformed cells the normal regulation of progression through the cell cycle is disturbed due to interference with the functions of important cellular regulatory proteins. The E1A oncoproteins affect the function of several cell-cycle-regulatory proteins via direct associations which can lead to the altered expression of other cellular genes (reviewed in Peepér & Zantema, 1993; Moran, 1994). Since the cdk-inhibitor p21\(^{CIP/WAF1}\), like its family members p27\(^{KIP1}\) and p57\(^{KIP2}\), is a potent growth inhibitor, it seems likely that inhibition by E1A of its level and/or function may stimulate cell growth and transformation. In the present study, we therefore have analysed the interplay between E1A and this inhibitor.

The expression levels of p21\(^{CIP/WAF1}\) were rather high and, in general, not downregulated in the Ad5E1-transformed cells used in this study. In two independent human HER-Xho cell lines higher levels and in two independent rat NRK-Xho cell lines slightly lower levels of p21\(^{CIP/WAF1}\) were detected compared to untransformed cells. It had been reported previously that in transformed cells p21\(^{CIP/WAF1}\) is released from cyclin A/cdk2 complexes (Xiong et al., 1993). In the Ad5E1-transformed cells used in this study, however, complexes consisting of cyclin A/cdk2/p21\(^{CIP/WAF1}\) could still be detected. Significantly, no p21\(^{CIP/WAF1}\)-associated kinase activity was detected whereas antibodies raised against cyclin A can efficiently precipitate kinase activities (data not shown). These results indicate that p21\(^{CIP/WAF1}\) is in principle still capable of functioning as a cdk inhibitor in Ad-transformed cells. In agreement with this observation, overexpressed p21\(^{CIP/WAF1}\) can still negatively affect cell growth in Ad5E1-transformed cells. Firstly, overexpression of p21\(^{CIP/WAF1}\) in Ad5E1-transformed cells leads to a reduction in the number of colonies in a colony-formation assay. Secondly, FACS analysis showed that increased amounts of pCMV-p21 can still give rise to an increase in G1-phase cells in the presence of pRSV-E1A. These results indicate that p21\(^{CIP/WAF1}\) and its downstream targets are, in principle, still able to function in Ad5E1-transformed cells.

However, in the presence of E1A, the growth inhibition induced by p21\(^{CIP/WAF1}\) is less pronounced, as was observed in both the colony-formation assays and, more clearly, in the FACS experiments. Consequently, E1A can counteract the growth-inhibitory effect of p21\(^{CIP/WAF1}\) at moderate concentrations of p21\(^{CIP/WAF1}\). A possible cause for this counteractive effect of E1A could be an increase in the levels of cyclin A, which is a growth-stimulatory protein (Buchou et al., 1993; Zerfass et al., 1996). However, Western blots of cell extracts of the same transfected cells as used in the FACS analysis showed no significant increase in cyclin A levels (data not shown). Together, these data suggest that E1A is able to partially overcome the concentration-dependent p21\(^{CIP/WAF1}\)-induced G1 arrest by acting directly on p21\(^{CIP/WAF1}\) or dose-dependently on a downstream target of p21\(^{CIP/WAF1}\). Similar results were obtained for p21\(^{CIP/WAF1}\) and SV40 large T antigen, which can also act in a mutually antagonistic manner (Harper et al., 1993).

In contrast to p21\(^{CIP/WAF1}\), the cdk-inhibitor p16\(^{INK4A}\) no longer shows a growth-inhibitory effect in Ad5E1-transformed cells at any concentration. These results are in agreement with the facts that cell-cycle inhibition by p16\(^{INK4A}\) is pRb-dependent (Lukas et al., 1995; Medema et al., 1995) and that pRb is inactivated in Ad5E1-transformed cells by binding to E1A (Nevins, 1992). In the Ad5E1-transformed BRK cell line, we even observed an increase in the number of colonies when p16\(^{INK4A}\) is overexpressed. However, this phenomenon is not observed in the HER-Xho cell line.

The best known mechanism by which E1A interferes directly with the function of cell-cycle-control proteins is by association. Therefore, we tested whether the partial relief by E1A of a p21\(^{CIP/WAF1}\)-induced G1 arrest, which we observed in the FACS experiments, could be caused by the inactivation of p21\(^{CIP/WAF1}\) via association to the E1A proteins. In vitro binding studies indeed showed specific binding of p21\(^{CIP/WAF1}\) to E1A and that this binding is direct. Confirming previous studies (Mal et al., 1996; Nomura et al., 1998) we also detected binding of E1A to the related cdk-inhibitor p27\(^{KIP1}\), but the observed interaction was slightly less efficient as that observed for p21\(^{CIP/WAF1}\). The regions of E1A that bind to p21\(^{CIP/WAF1}\) are not conserved and are the non-conserved N-terminal region and the CR1 region. Both these E1A domains are required for transformation and can bind to and inactivate a number of regulatory proteins (Peepér & Zantema, 1993; Moran, 1994). Hence, the observed binding of p21\(^{CIP/WAF1}\) to this region of E1A could be functionally relevant and important for the transformation process.

The binding site of E1A on p21\(^{CIP/WAF1}\) was also determined. We found that E1A predominantly binds to the N-terminal half of p21\(^{CIP/WAF1}\), in which the cyclin/cdk-binding domain is located (Luo et al., 1995). This is the region of p21\(^{CIP/WAF1}\) which displays a clear homology with the N-terminal region of the cdk-inhibitor p27\(^{KIP1}\) (Toyoshima & Hunter, 1994). The mapping of the domain in p27\(^{KIP1}\) involved in binding to E1A, however, led to different conclusions on the importance of the N-terminal region (Mal et al.).
al., 1996; Nomura et al., 1998). The fact that in those studies different fragments representing the N-terminal regions have been used may be relevant for this discrepancy. Nomura et al. (1998) used constructs which are comparable to our constructs and, consistent with our results, binding to the conserved N-terminal domain of p27 \( \text{KIP1} \) was observed. Together these data suggest that E1A can interfere with functions of p21 \( \text{CIP1/WAF1} \) and p27 \( \text{KIP1} \) by similar mechanisms and thereby prevent p21 \( \text{CIP1/WAF1} \) and p27 \( \text{KIP1} \) from inducing a cell-cycle arrest.

Recently, various groups have reported on binding studies between p21 \( \text{CIP1/WAF1} \) and an oncoprotein which displays similarities to E1A, the papillomavirus E7 protein. In differentiated keratinocytes or after DNA damage, an interaction of p21 \( \text{CIP1/WAF1} \) and E7 could be observed (Jones et al., 1997; Funk et al., 1997). In contrast to E1A, which primarily binds the N terminus of p21 \( \text{CIP1/WAF1} \), E7 can bind the C terminus of this inhibitory protein (Funk et al., 1997). However, cases have also been reported in which no binding of p21 \( \text{CIP1/WAF1} \) to E7 could be detected (Ruesch & Laimins, 1997; Morozov et al., 1997; Jones & Münger, 1997). In fact, we were unable to detect a significant amount of binding of p21 \( \text{CIP1/WAF1} \) to E1A in vivo in normally growing Ad5E1-transformed human HER-Xho cells, although in some experiments a weak interaction could be observed (data not shown). Possibly, in exponentially growing cells only a small portion of the total amount of p21 \( \text{CIP1/WAF1} \) is bound to E1A or, alternatively, the binding is rather weak and therefore difficult to detect. It remains possible, however, that binding has an effect on p21 \( \text{CIP1/WAF1} \) function. Since p21 \( \text{CIP1/WAF1} \), and also p27 \( \text{KIP1} \), are thought to maintain a certain threshold in the cell needed to be overcome by cyclin-dependent kinases before progression through the cell cycle can take place (reviewed by Nasmyth & Hunt, 1993; Peters, 1994), a decrease of this critical level for cell-cycle progression through inactivation of at least a part of the total amount of functional p21 \( \text{CIP1/WAF1} \) and possibly p27 \( \text{KIP1} \) might provide a growth advantage. This may be one of the mechanisms by which E1A promotes cell growth. Moreover, as also holds for the related E7 protein, binding of E1A to p21 \( \text{CIP1/WAF1} \) may be more prominent in certain cell situations than under other cell conditions.

In summary, it is evident that viral proteins such as E1A, E7 and SV40 large T may use various strategies to promote cellular growth by influencing the expression levels and/or functions of important cell-cycle-control proteins (reviewed in Jansen-Dürr, 1996). The finding that E1A, E7 and SV40 large T all interfere with p21 \( \text{CIP1/WAF1} \) function suggests that inhibition of this cdk inhibitor may play an important role in transformation and oncogenesis, possibly by diminishing the threshold for cell-cycle progression.

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