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Transcriptional Down-regulation of Poly(ADP-ribose) Polymerase Gene Expression by E1A Binding to pRb Proteins Protects Murine Keratinocytes from Radiation-induced Apoptosis*

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Adenovirus E1A confers enhanced cell sensitivity to radiation and drug-induced DNA damage by a mechanism involving the binding to cellular proteins. Mutant analysis in E1A-transfected murine keratinocytes demonstrates that increased sensitivity to DNA damage requires at least E1A binding to the p300/CREB-binding protein (CBP) transcriptional coactivators and to pRb family members, indicating that this biological activity of E1A is the result of the concomitant perturbation of different cell pathways. Here we show that in the same cells E1A binding to members of the retinoblastoma protein family induces transcriptional down-regulation of the poly(ADP-ribose) polymerase (PARP) gene, coding for a NAD-dependent enzyme stimulated by DNA breaks. Inhibition of PARP expression is accompanied by a decrement of γ -irradiation-induced apoptosis, which is overridden by reconstitution of wild type levels of PARP. Hence, E1A effects on PARP transcription are central determinant of the apoptotic sensitivity of E1A-expressing keratinocytes. Conversely, E1A binding to only p300/CBP results in an increase in PARP enzyme activity and consequently in cell death susceptibility to irradiation, which is effectively counteracted by the PARP chemical inhibitor 3-aminobenzamide. Therefore, our results identify in the E1A-mediated effects on PARP expression and activity a key molecular event involved in E1A-induced cell sensitization to genotoxic stress.

Adenovirus E1A proteins stimulate entry into S-phase of the infected cells and, in combination with E1B proteins, induce transformation. This effect is probably the consequence of perturbation of a variety of cellular basal programs, like proliferation, differentiation, and programmed cell death (1). A well characterized effect of transfected E1A alone is a marked increase in cell sensitivity to DNA damaging agents (ionizing radiation and DNA-targeted chemotherapeutic compounds such as doxorubicin and 5-fluorouracil) in epithelial tumor (2, 3) and immortalized cells (4, 5), as well as in primary embryo fibroblasts (6, 7). At the doses employed in the cited studies, the morphology of cell death induced by these agents is that of apoptosis, which under conditions of serum deprivation and

high cell density could be elicited *per se* by E1A transfection, without any additional stimulus (8). Therefore, it is likely that the basis of E1A-induced enhanced sensitivity to DNA damage relies at least partially in the lowering of the apoptotic threshold, by acting at a distal step in the programmed cell death pathway. E1A encodes two mRNA species, named 13S and 12S, which give respectively a 289- and a 243-amino acid protein, identical apart from an internal stretch of 46 amino acids. E1A proteins present two conserved regions (CR1 and CR2), which are known from mutational studies to be responsible for the induction of transformation and to be the site of interaction with several identified cellular proteins (reviewed in Ref. 9). These proteins are p107, p130, and p105^{Rb}, belonging to the pRb tumor suppressor gene family and which interact primarily with CR2 with a weak cooperativity of CR1, and p300 and CREB¹-binding protein (CBP) of the p300/CBP family of transcriptional coactivators, which require a portion of CR1 and the N terminus for binding (10). Since these proteins have key regulatory roles in different cell pathways, the perturbations induced by their association with E1A are thought to be responsible for the complex biological activity of E1A (11). Nevertheless, the mechanism leading to E1A-induced cell sensitization and the exact role in this process of the bound cellular proteins are almost totally unclear.

This study was designed with the aim of finding downstream substrates for the action of E1A that could be involved in its sensitization to ionizing radiation-induced apoptosis. We used an established *in vitro* model of spontaneously immortalized murine keratinocytes, stably transfected with full-length E1A 13S or with E1A 13S deletion mutants unable to bind either p300 (mutant NTdl598) or p105^{Rb} (mutant NTdl922/947) family members (5). In these cells, we demonstrate that E1A down-regulates the expression of PARP (EC 2.4.2.30), an abundant nuclear enzyme endowed with DNA damage-triggered ADP-ribosylating activity and involved in the late apoptotic cascade as a substrate/effector protein (12). By testing the behavior of the two mutant E1A-expressing cell lines, we also show that this effect is mediated by E1A binding to pRb proteins and is accompanied by apoptosis resistance to γ -rays, while E1A binding to the transcriptional integrators p300/CBP enhances PARP activity and increases irradiation-induced cell death.

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¹ The abbreviations used are: CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; 3-AB, 3-aminobenzamide; FL E1A, full-length E1A; PARP, poly(ADP-ribose) polymerase; DMEM, Dulbecco's modified Eagle's medium; Gy, gray; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); CAT, chloramphenicol acetyltransferase.

EXPERIMENTAL PROCEDURES

Cells, Cell Cultures, and Treatments—Mouse Pam212 cells are a spontaneously immortalized keratinocyte line (13), while Pam212-derived transgenic cell lines carrying full-length E1A, and the NTdl598 and NTdl922/947 E1A mutants were obtained by lipofection with retroviral constructs (MD-E1a 13S, MD-E1a NTdl598, MD-E1a NTdl922/947, respectively) carrying the G418 resistance gene (14). The E1A mutants used in this study have been previously described (15, 16). Mutant NTdl598 (amino acids 2–13 deleted) binds only to members of the pRb family, p105 and p107 at least, while mutant NTdl922/947 (amino acids 122–129 deleted) binds only to p300. All transgenic cell lines expressed similar levels of E1A, as previously assessed by Western blot and immunoprecipitation (14). All cells were grown in DMEM supplemented with 10% FCS (Life Technologies, Inc., Paisley, United Kingdom) 200 units/ml penicillin, 100 μ g/ml streptomycin (Sigma, Milan, Italy), 1 mg/ml G418 (Life Technologies, Inc.) for the transgenic lines, and incubated at 37 °C in a 5% CO₂ atmosphere. Cells were γ -irradiated with a ⁶⁰Co source at 57-cm distance and at a dose rate of 1.55 Gy/min. In all experiments cells were plated in 90-mm Petri dishes 24 h before irradiation, and the time of irradiation was taken as the zero time. For irradiation in the presence of the PARP inhibitor 3-AB, cells were incubated with 5 mM 3-AB for 2 days, then irradiated and subjected to time-lapse videomicroscopy scoring of cell death.

Cell Viability and Long Term Survival—Cellular viability was assessed by the crystal violet method. Briefly, cells were plated in 24-well plates (2 × 10⁴/well), fixed at the indicated time with 1% glutaraldehyde for 10 min, washed twice with PBS, and stained by 1.5 ml of 0.1% crystal violet solution for 30 min. Wells were rinsed with distilled water until the dye was washed off and the absorbance was read at 590 nm after taking the dye up in 10% acetic acid. The long term survival was determined by a clonogenic survival assay as described previously (17).

Cell Extracts and Western Blotting—Cells were plated 24 h before treatment, and the determination of the cell number was done by classical trypsinization procedure; for protein extraction, cells were scraped in cold PBS (pH 7.4), spun 10 min at 1000 rpm at 4 °C, and suspended at 2 × 10⁵ cells/ml in 1 × sample buffer (62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% 2-mercaptoethanol). Cells were then lysed by sonication on ice for 20 s (microtips at limit, 40% duty cycle, Sonicator Vibracell, Sonics and Materials, Dunbury, CT) and incubated 15 min at 65 °C before loading. Samples were electrophoresed on a SDS-polyacrylamide minigel (8% W/v) for 1 h and 15 min at 100 V. Proteins were transferred onto nitrocellulose membrane (Hybond C from Amersham Pharmacia Biotech) at 4 °C under stirring at 100 V for 1 h. The membrane was blocked in PBSBT (PBS 1 × (pH 7.4), 5% BSA, 0.1% Tween 20) for 30 min, incubated with the polyclonal anti-PARP antibody C2–10 (18), provided by Dr. G. Poirier, at a final dilution of 1:10,000 for 2 h in blocking solution. Bands were visualized with horseradish peroxidase-conjugated antimouse immunoglobulin (1:2500 in blocking solution), enhanced chemiluminescence reagent (ECL, Amersham Pharmacia Biotech), and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham Pharmacia Biotech).

Activity Gel—The PARP automodification activity was detected on the gel using the incorporation of [³²P]NAD⁺ into [³²P]poly(ADP-ribose) as described (19). The extracts prepared from control and treated cells were electrophoresed in a 7.5% polyacrylamide gel. Thereafter the gel was soaked at room temperature with shaking for 30 min in renaturation buffer (50 mM Tris-HCl (pH 8.0), 3 mM 2-mercaptoethanol), for 30 min in renaturation buffer containing 6 M guanidine hydrochloride, and finally for 30 min in renaturation buffer. The enzymatic reaction was carried out for 1 h at 37 °C in 3 ml of 100 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 μ M NAD, 1 mM dithiothreitol, and 5 μ Ci/ml [³²P]NAD (800 Ci/mmol, NEN Life Science Products). Nonincorporated NAD was removed by washes with cold 10% trichloroacetic acid followed by washes with cold 1 M HCl. The gel was dried and subjected to autoradiography using Kodak X-Omat AR5 films. Alternatively radioactive bands were detected and quantified with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA).

Time-lapse Videomicroscopy—Cells were plated in 25-cm² flasks and incubated in a 5% CO₂ atmosphere under a time-lapse videomicroscopy apparatus (20). Briefly, randomly chosen fields of 100 cells were monitored at a rate of one frame every 3 min for 72 h. Cells were considered to undergo apoptotic cell death when exhibiting membrane blebbing, shrinkage, and fragmentation in apoptotic bodies, followed in some cases by membrane disruption and a morphology of secondary necrosis. The scoring was carried out manually marking the dying cells on a transparency attached to the time-lapse monitor. Results are expressed

as either the cumulative number of apoptotic events at the final time point or plotted against time with scoring every 5 h. For experiments involving transient expression of the pCDNA3-mPARP vector cells were plated in 24-well plates, transfected as reported, and incubated in a 5% CO₂ chamber under the time-lapse apparatus.

Semiquantitative RT-PCR—For the quantification of PARP mRNA levels in Pam212 cells, total RNA was extracted using the RNeasy Midi kit (Quiagen, Hilden, Germany) and treated with RQ1 RNase-free Dnase (Promega, Madison, WI) before reverse transcription, to exclude any possible amplification of contaminating genomic DNA. After ethanol precipitation RNA was dissolved in 10 μ l of RNasin (1 unit/ μ l), quantitated and analyzed on agarose gel. The reverse transcription mixture (100 μ l) contained 10 μ l (about 10 μ g) of total RNA, 100 units of RNasin, 25 μ g of BSA, 1 mM dNTPs, 12 μ M random hexamers (Genosys, The Woodlands, TX), 50 mM Tris-HCl (pH 8.3), 75 μ M KCl, 3 mM MgCl₂, and 10% glycerol. After heating at 80 °C for 5' and rapid cooling, 1000 units of Moloney murine leukemia virus RT (Promega) were added, the mixture was incubated at 37 °C for 60 min, and the reaction was stopped by heating at 95 °C for 15 min. The cDNA was then purified by phenol-chloroform, ethanol-precipitated, and resuspended in water. Semiquantitative determination of PARP mRNA levels was done by an internal standard-based PCR assay with serial dilution of cDNA and using β -actin as reference gene, as described previously (21). A 279-bp segment of the murine PARP cDNA sequence (GenBankTM accession number X14206) was targeted with upstream primer 5'-AGGCCCTAAAGGCTCAGAAT-3', bases 791–810, and downstream primer 5'-CTAGGTTTCTGTGTCTTGAC-3', bases 1051–1070, while a 326-bp segment of the murine β -actin sequence was amplified with upstream primer 5'-GCGGGAAATCGTGCCTGACATT-3', bases 2106–2127, and downstream primer 5'-GATGGAGTTGAAGGTAGTTTCGTG-3', bases 2409–2432. Fifty μ l of the PCR reaction mixture were as follows: 0–5 μ l of cDNA solution, 1 μ M primers, 200 μ M dNTPs, 15 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), sterile water to 45 μ l, 2.5 units of *Taq* Gold polymerase (Perkin-Elmer, Norwalk, CT). The PCR profile was 5 min at 94 °C and then 30 min at 94 °C, 30 min at 54 °C (PARP), or at 60 °C (β -actin) and 30 min at 72 °C for 25 cycles. Amplification products were run on a 2% agarose gel and the ethidium bromide-stained bands quantitated by densitometric analysis. Within the linear range of amplification at least three values of PARP amplification products were normalized to the starting cDNA volumes and referred to the corresponding β -actin values.

Plasmid Constructs—Murine genomic DNA extracted from Pam212 cells by the DNeasy Tissue Kit (Quiagen) was PCR-amplified following standard procedures with the primers 5'-GTGGAAATTATTTGGGC-GAAC-3' (upstream, from 309 to 328 of GenBankTM accession number D14553) and 5'-AATACTCTCGTGCATTCT-3' (downstream, from 741 to 760), which give a 451-bp product spanning a portion of the murine PARP 5' regulatory region highly homologous to the previously characterized rat PARP promoter (from –237 to +13, see Ref. 39). The resulting amplification product was cloned in the pCRII vector, sequenced, digested with *Sac*I and *Xho*I, and subcloned into the pCAT3-Basic vector (Promega, Madison, WI), a CAT expression plasmid optimized for the analysis of enhancer and promoter sequences, after linearization with *Sac*I and *Xho*I. The pCAT3-B-mmpPARP vector was amplified in the bacterial strain JM109. For production of the murine PARP expressing vector an aliquot of the murine cDNA obtained from Pam keratinocytes was PCR-amplified with the primers 5'-GGGCT-GCGGCAGCACGAGAA-3' (upstream, from 63 to 82) and 5'-GACTCG-GCTACCCCTCGCAC-3' (downstream, from 3127 to 3146) to obtain the full PARP open reading frame (3083 bp). After cloning in pCRII and partial sequencing for confirmation, the insert was digested with *Hind*III and *Xho*I and subcloned into pCDNA3 (Invitrogen, Carlsbad, CA) after linearization with the same restriction enzymes to obtain the pCDNA3-mPARP vector, in which the expression of murine PARP is under the control of the cytomegalovirus promoter.

Transient Transfection and CAT Assay—For CAT assay in the wild type and E1A Pam212 keratinocytes the plasmids pCAT3-B-mmpPARP and pCAT3-Control were used, the latter containing SV40 promoter and enhancer sequences and being a standard for comparison of CAT activities in different cell lines. Transient transfection of these CAT reporter plasmids and of the pCDNA3-mPARP expression vector was done by a lipofection procedure. The day before transfection, cells were plated in 24-well plates at 8 × 10⁴ cells/well, being 70% confluent after 24 h. For each well of cells 0.4 μ g of plasmid DNA and 2 μ l of LipofectAMINE 2000 Reagent (Life Technologies, Inc.) were diluted separately in 50 μ l of cell culture medium and incubated at room temperature for 10 min. The diluted DNA and cationic lipid were combined and incubated further for 20 min. The growth medium was removed from

cells and 0.5 ml of DMEM added, then the plasmid-lipid complexes (100 μ l) were directly added to each well. Cells were incubated for 5 h, the DMEM replaced with complete medium and further incubated for additional 24 h. CAT activity was then monitored by liquid scintillation counting of CAT reaction products. 48 h post-transfection cell extracts obtained with 100 μ l of xylene were incubated in a reaction mix containing 3 H-labeled chloramphenicol and *n*-butyryl coenzyme A. The *n*-butyryl chloramphenicol partitions mainly into the xylene phase, while unmodified chloramphenicol remains predominantly in the aqueous phase (22). The xylene phase was mixed with scintillant and counted in a scintillation counter. The ratio of the counts obtained for each cell line with pCAT3-B-mPARP and pCAT3-Control, normalized to the protein content of the sample determined by the BCA Protein assay (Pierce), gave the level of activity of the PARP murine promoter. For normalization of the transfection efficiency of the pCDNA3-mPARP vector, an aliquot of the transfected cells was subjected to activity gel determination of PARP as described previously, and only those samples giving a PARP activity comparable with the wild type Pam212 keratinocytes (80%) were subjected to the time-lapse analysis.

RESULTS

Short and Long Term Survival of Wild Type and E1A-transformed Keratinocytes Exposed to γ -Irradiation—We assessed the response to 5 Gy of γ -irradiation of a panel of murine Pam212 keratinocytes stably expressing full-length (FL) and deleted E1A 13S constructs. The effect of γ -rays was evaluated by measuring cell viability with the crystal violet method and proliferative potential with the clonogenic survival assay. As shown in Fig. 1A, at 72 h after 1, 3 and 5 Gy of irradiation only the FL E1A Pam212 keratinocytes exhibited a significant decrease in viability with respect to the unirradiated control, which was dose-dependent and reached 65% at 5 Gy. Wild type Pam212 keratinocytes and Pam cells stably expressing two E1A mutants, NTd1598 and NTd1922/944, that are incapable of binding respectively to p300/CBP and to p105^{Rb} family members (16), showed no statistically significant difference with respect to the untreated cells. Similarly, a clonogenic survival assay carried out after 7 days from irradiation (Fig. 1B) indicates that only the FL E1A-transformed keratinocytes are profoundly impaired in their proliferative potential, as demonstrated by the marked reduction in the number of cells capable of colony formation with respect to the slight survival decrease observed for the wild type and the mutant E1A-expressing cells. In line with a previous characterization of this model of stably transfected keratinocytes (5) and with other results (24), we conclude that E1A-induced enhancement of radiosensitivity depends on E1A binding to both p300/CBP and pRb proteins.

Effects of E1A Binding to p300 and pRb Family Members on PARP Expression and Activity—E1A binding to its cellular targets provokes perturbation of unknown pathways that could be implicated in the cell sensitivity to genotoxic stress. Since one of the determinants of cell radiosensitivity is the PARP nuclear enzyme, we wanted to verify if the activity of this protein could be implicated in the modulation of cell response to γ -ray by E1A, due to E1A binding to the p300/CBP transcriptional coactivator, to pRb proteins, or both. Fig. 2 reports the level of PARP activity in basal conditions and after 5 Gy of γ -irradiation (assessed by the activity gel method) and the level of PARP expression (assessed by Western blotting and semi-quantitative RT-PCR) for the wild type and the E1A-expressing Pam212 keratinocytes. The binding of E1A to only pRb proteins (NTd1598 mutant) induced a reduction of PARP enzymatic activity paralleled by a decrease in PARP protein and mRNA content to values under the basal physiologic values for these cells. On the contrary, the mutant NTd1922/947, able to bind only p300/CBP, did not affect PARP expression but induced a marked increment of PARP activity, quantified as about three times the basal wild type levels (data not shown). The binding of E1A to both sets of proteins (FL E1A) resulted in a decrease of PARP expression comparable with that induced by the

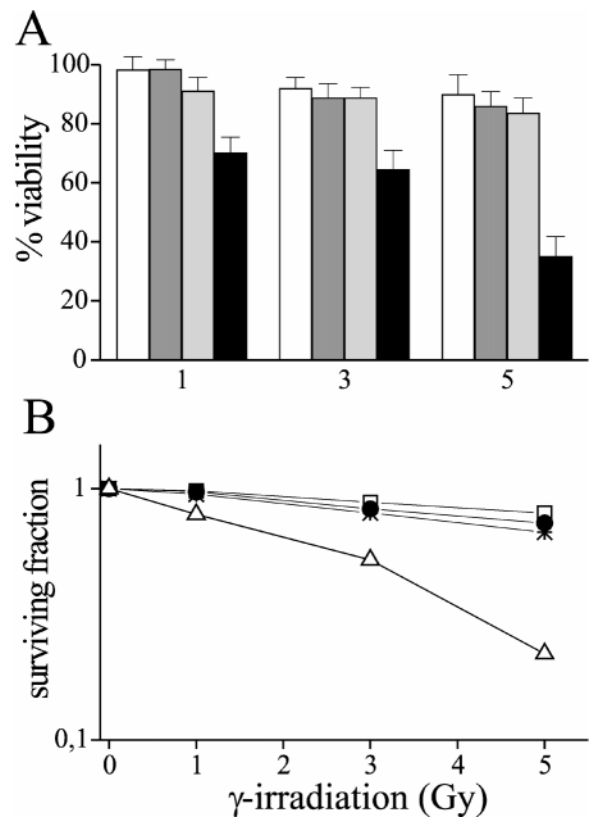


FIG. 1. Increased sensitivity to γ -irradiation in E1A-expressing murine keratinocytes is due to E1A binding to both p300/CBP and to pRb family members. A, at 24 h from plating, cells were irradiated (5 Gy) at the indicated doses and viability was assessed by crystal violet method after 72 h from irradiation, as described under "Experimental Procedures." Viability is expressed as percentage of unirradiated cells for each cell line. Symbols: white columns, wild type Pam212 keratinocytes; dark gray columns, NTd1598; Pam 212 cells stably expressing an E1A deletion mutant that binds only pRb family members; light gray columns, NTd1922/947; Pam212 cells stably expressing an E1A deletion mutant which binds only p300/CBP; black columns, FL E1A cells. Each point represents the mean \pm S.E. of four independent experiments. B, surviving fraction was evaluated 7 days post-irradiation at the indicated doses and is expressed as the ratio between the number of colonies scored in the irradiated and the unirradiated plates. Each point represents the mean of six independent experiments. Symbols: □, wild type; ●, NTd1598; ×, NTd1922/947; Δ, FL E1A.

NTd1598 mutant, but the level of enzyme activity was the same as in untransfected keratinocytes. The pattern of PARP activity in the cell panel obtained after γ -irradiation was substantially analogous to that of the untreated cells, showing only, as expected, a generalized slight increment of signal attributable to DNA damage-induced PARP overactivation, since PARP protein levels remained unchanged (not shown). Therefore, E1A affects PARP activity differently by binding pRb proteins or p300/CBP, the first effect being a down-regulation of PARP expression at the mRNA and protein level and the second an opposite action of increment of PARP activity without any change in gene expression. The overall effect of FL E1A on PARP appears to be the combinative result of the effects induced by the two mutants, that is a down-regulation functionally compensated by an increased enzymatic activity.

Transcriptional Activity of the Mouse PARP Promoter in Wild Type and E1A-transformed Keratinocytes—The down-regulation of PARP mRNA steady state levels in cells expressing E1A proteins able to bind pRb (NTd1508 and FL E1A, Fig. 2C) prompted us to investigate if this effect could be due to lowering of the PARP transcriptional activity. Since the regulatory sequences in the rat PARP proximal promoter have been identi-

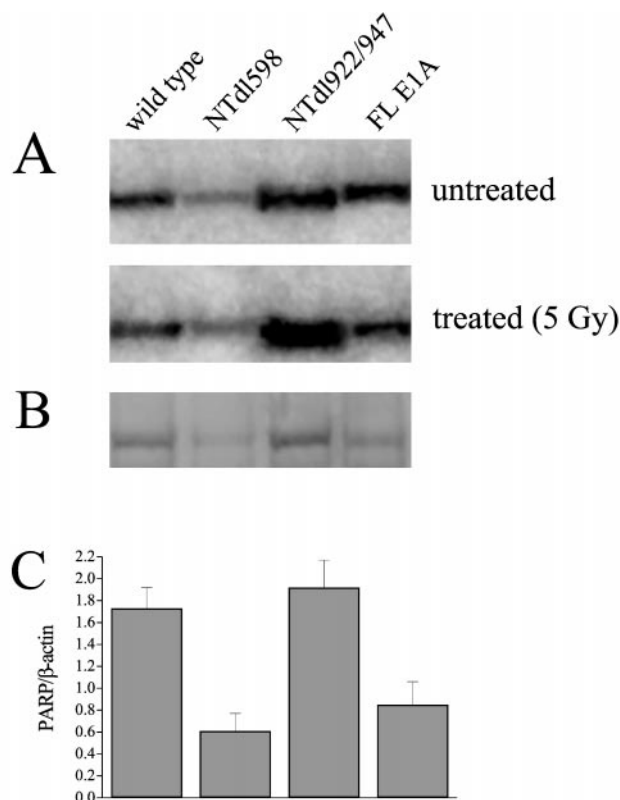


FIG. 2. E1A affects PARP expression and activity differently by binding pRb proteins or p300/CBP. Equal amounts of extracts from each cell line were subjected to activity gel with (*upper panel*) and without (*lower panel*) a previous exposure to 5 Gy of γ -irradiation (the γ -ray-treated extracts were prepared 4 h from irradiation) (A) and to Western blot analysis (B), to evaluate PARP activity and expression levels. Both Western blotting and activity gel analysis were carried out in cell extracts obtained as described under "Experimental Procedures." A representative experiment is reported. (C), graphic representation of PARP mRNA levels evaluated by an internal standard-based (β -actin) semiquantitative RT-PCR technique (see "Experimental Procedures" for details). Amplification products were run on an agarose gel and the ethidium bromide-stained bands were quantitated by densitometric analysis. Each histogram represents the mean \pm S.E. of three to four values of PARP amplification products normalized to the starting cDNA volumes and referred to the corresponding β -actin values.

fied (25), we cloned the mouse corresponding DNA region upstream to a CAT reporter plasmid, and we transiently transfected our panel of E1A-expressing keratinocytes. CAT activities reported in Table I demonstrate that E1A binding to pRb (NTdl508 and FL E1A) results in a dramatic down-regulation of the PARP promoter, while no significant effect is seen after E1A binding to p300 (NTdl922/947). Taken together, the data presented in Fig. 2 and Table I strongly suggest that the pRb-sequestering activity of E1A is responsible for transcriptional down-regulation of the PARP gene.

E1A Sensitization to γ -Irradiation-induced Apoptosis in Murine Keratinocytes Is the Result of Two Contrasting Actions due to Binding to pRb Proteins and to p300/CBP—From the data presented in Fig. 1 we know that both sequestering activities of cellular proteins are necessary for E1A-induced radiosensitivity in murine keratinocytes. We know that cell sensitivity to γ -rays could depend both on perturbation of the cell cycle and on direct cell killing. Since recently PARP has been positively implicated in the pathway of DNA damage-induced cell death, we reasoned that the down-regulation of PARP expression exerted by the pRb-sequestering activity of E1A should counteract the overall cytolethality of E1A. To segregate the cytolethal from the cytostatic effect of γ -rays, we used time-lapse video-

TABLE I

Down-regulation of PARP expression by E1A binding to pRb proteins is due to transcriptional inhibition

Cells were plated at 8×10^4 cells/well in 24-well plates and after 24 h transiently transfected (see "Experimental Procedures" for details) with the CAT expression plasmids pCAT3-mpPARP and pCAT3-Control. Cells were harvested, then levels of CAT activity were determined by scintillation counting and normalized to the cell protein content. Values are reported as mean CAT activity \pm S.D. ($n = 12$) of the ratio between the level directed by pCAT3-mpPARP and pCAT3-Control in the same cell line, and as percentage of the wild type Pam212 cells.

	Wild type Pam212	NTdl598	NTdl922/947	FL E1A
% CAT activity	100 \pm 18	15 \pm 3	107 \pm 15	19 \pm 8

microscopy scoring of radiation-induced cumulative cell death events in the panel of E1A-transfected cells. We counted the number of cell deaths that occurred over a 72-h observation interval after irradiation in a population of 100 cells. As reported in Fig. 3A, the administration of 5 Gy of γ -irradiation to wild type keratinocytes produced cell death figures (presenting invariably the morphology of apoptosis, not shown) that were the same number of the spontaneous cell deaths observed in a 100-cell field in 72 h, about 10 apoptotic figures. This demonstrates that the administered radiation dose, at least in the time window of observation, is unable to change the apoptotic threshold of Pam212 keratinocytes. The number of deaths was instead almost tripled in the clone stably expressing full-length E1A, indicating that E1A-induced radiosensitization is accompanied by a substantial decrease of the apoptotic threshold.

When we tested the cell lines expressing the two mutant E1A constructs, we saw that the NTdl598 mutant, able to bind to pRb proteins but not to p300/CBP, completely lost the ability of E1A to lower the apoptotic threshold to γ -rays of these cells. Moreover, the binding of E1A to only p300/CBP (mutant NTdl922/947) oversensitized cells to apoptosis with respect to the wild type E1A protein. This cell death profile of the E1A mutants is in agreement with two opposite effects exerted by E1A on the apoptotic susceptibility of Pam212 cells, one negative due to binding to pRb and one positive due to binding to p300/CBP. To better characterize the possible antiapoptotic effect of E1A binding to pRb we exposed the Pam212 wild type and NTdl598 keratinocytes to increasing doses of γ -rays, obtaining the dose-response curve reported in Fig. 3B, which clearly indicates, particularly at the higher radiation doses, the antiapoptotic activity of E1A sequestering of pRb proteins.

To reconcile the increased apoptotic susceptibility with the absence of change in radiosensitivity of the NTdl922/947 keratinocytes (reported in Fig. 1 as viability and clonogenic potential), we performed a scoring of the mitotic events in this mutant, which demonstrates a marked increment of the proliferation rate.² This increased mitotic activity could compensate for the enhanced cell death observed and is indicative of the complexity of effects exerted by E1A on the pathways leading to cell proliferation and death.

PARP Expression and Activity Levels Are Determinant of Apoptotic Sensitivity in Murine Keratinocytes Stably Expressing E1A Binding Mutants—The unexpected finding of the combined proapoptotic-antiapoptotic activity of E1A was suggested by the down-regulation of PARP expression in the NTdl598-expressing keratinocytes. To directly test if this single molecular event could be a determinant of apoptotic sensitivity for the different E1A mutants, we transiently transfected the NTdl598 keratinocytes with a pcDNA3-PARP expression vector, bearing human PARP cDNA under the control of the cytomegalovirus promoter. Activity gel determination of PARP

² A. Pacini and P. Nassi, unpublished data.

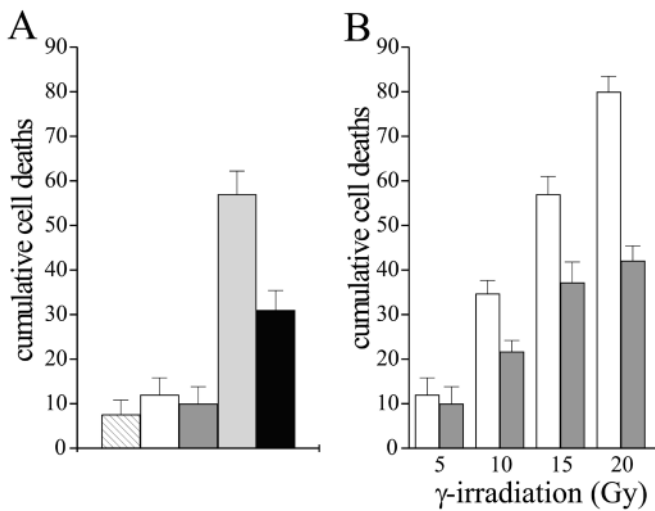


FIG. 3. E1A binding to pRb proteins counteracts sensitization to radiation-induced apoptosis due to E1A binding to p300/CBP. A, cell lines were plated in 25 cm² flasks (3×10^5 cells/flask) and after 24 h exposed or not to 5 Gy of γ -irradiation, then a field of 100 cells was monitored by time-lapse videomicroscopy for the subsequent 72 h. During the time-lapse recording, cells were maintained at 37 °C in a 5% CO₂ atmosphere. The cell death scoring was as described under "Experimental Procedures." The cumulative number of cells at the time end point is reported as mean \pm S.E. of four independent experiments. Symbols: hatched column, unirradiated wild type Pam212 cells; white column, wild type Pam212 cells; dark gray column, NTd1598 cells; light gray column, NTd1922/947 cells; black column, FL E1A cells. B, time-lapse dose-response curve of cumulative cell death events obtained after exposure of wild type and NTd1598-expressing keratinocytes to increasing doses (from 5 to 20 Gy) of γ -rays. Symbols: white columns, wild type Pam212 cells; gray columns, NTd1598 cells.

showed that plasmid transfection reproducibly reconstituted PARP enzymatic activity at levels comparable with those of the wild type cells or higher (not shown). Fig. 4A reports that the apoptotic sensitivity to 15 Gy of γ -irradiation is restored after 24 h from transfection of NTd1598 Pam212 cells with the PARP expression vector, becoming even higher than in the wild type cells. Conversely, the administration of 3-AB, a specific inhibitor of PARP enzymatic activity, lowered the level of cell death present in the NTd1922/947 mutant after 5 Gy of γ -irradiation. The dose of 5 mM of 3-AB administered 48 h before the exposure to γ -irradiation was chosen because it was able to decrease PARP activity by 70% in wild type Pam212 cells (not shown). As reported in Fig. 4B, 3-AB decreased the apoptotic sensitivity of cells expressing the NTd1922/947 mutation to levels corresponding to that of cells expressing the full-length E1A, demonstrating that inhibition of PARP activity acts on the apoptotic threshold of this cell model in the same manner of E1A sequestering of pRb proteins.

DISCUSSION

Despite the substantial amount of data describing the details of the interaction of E1A with cellular proteins, limited information is available on how this interaction brings to the pleiotropic effects of E1A, including cell sensitization to genotoxic agents. Since these agents are known to elicit a complex pathway starting with the change in activity of DNA damage sensor proteins (26), an obvious hypothesis is that E1A could directly or indirectly affect the expression and activity of these proteins to produce its profound perturbations in the downstream signals. A first confirmation of this assumption has come with the recent demonstration that p300/CBP coactivators are essential for the transactivation function of the p53 oncosuppressor protein (27) and that E1A sequestration of p300/CBP disrupts functions mediated by p53 (28, 29). Since p53 is known to be

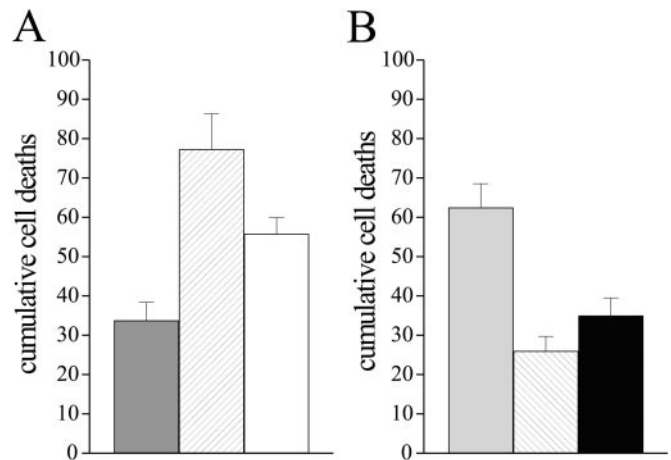


FIG. 4. Apoptotic sensitivity in cells expressing E1A mutants correlates with PARP expression and activity. A, NTd1598 keratinocytes were transiently transfected with a pcDNA3-PARP expression vector as reported under "Experimental Procedures." Cells were plated in 24-well plates at 8×10^4 cells per well, transfected, and after 24 h checked for PARP enzymatic activity. Those samples that gave an activity comparable with that of wild type Pam212 cells were further processed. Cells were γ -irradiated (15 Gy) 24 h after transfection, then they were analyzed by time-lapse videomicroscopy for 72 h as described. Each point represents the mean \pm S.E. of six independent transfection and irradiation experiments. Symbol: gray column, untransfected NTd1598 cells; hatched column, transfected NTd1598 cells; white column, wild type Pam212 cells. B, 48 h before irradiation, NTd1922/947 cells were plated in 25-cm² flasks and treated with 5 mM 3-AB. After administration of 5 Gy of γ -irradiation cells underwent to time-lapse videomicroscopy as described. Each point is the mean \pm S.E. of four independent experiments. Symbols: gray column, NTd1922/947 cells; hatched column, NTd1922/947 cells + 3-AB; black column, FL E1A.

activated by its direct association with DNA strand breaks (30, 31), we wanted to study the effects of E1A on another well known DNA lesion-scanning protein, the PARP enzyme.

Similarly to p53, PARP is activated by binding to breaks in DNA, binding that triggers its enzymatic domain to consume NAD for the synthesis of chains of poly(ADP-ribose) on target proteins, including PARP itself (32). We focused on an established model of spontaneously immortalized and stably E1A-transfected cells, the murine Pam212 keratinocytes, to look for a role of PARP in modulation by E1A of the response to γ -irradiation. We found that full-length E1A induced a clear down-regulation of expression of PARP both at the protein and the mRNA level and that this effect is due to transcriptional repression of the PARP promoter. Moreover, mutant analysis clearly indicated that this E1A activity is dependent on its association with pRb-related proteins. A well described mechanism of *in vivo* stimulation of transcription by pRb is that mediated by binding to the transcriptional activator Sp1 (33–37). Since in the rat PARP promoter five consensus sites for Sp1 are present to whom Sp1 has been demonstrated to bind strongly stimulating transcription in reporter assays (38–40), the pRb-Sp1 activator complex could be responsible for positive regulation of the PARP promoter, destroyed by E1A-induced sequestration of pRb. This activity of E1A is also associated with a reduction of apoptosis elicited by γ -ray, and the role of PARP down-regulation in this process, among the other events induced by E1A binding to pRb proteins, is sustained by the fact that PARP expression in the same mutant cell line under the control of a viral promoter restores cell sensitivity to γ -ray. An antiapoptotic activity of E1A mediated by PARP down-regulation is in agreement with the demonstration of profound protection from experimental cerebral ischemic injury in PARP null mice (41) and with the partial protection exerted in wild type mice by PARP chemical inhibitors (42). Cerebral ischemia

is supposed to elicit a pathway starting with the excessive release of glutamate by overexcited neurons and resulting in the production of DNA damaging species (43), which in turn overactivate PARP with the consequent depletion of NAD and a sudden ATP fall induced by the cell effort to resynthesize NAD, ultimately responsible for death (23).

Besides our demonstration of the role of PARP activity in E1A cell sensitization to radiation-induced apoptosis, the complex effects of E1A on the expression and activation of PARP could represent a future tool for dissecting the regulation of this gene, as happened in the past for other nuclear proteins directly or indirectly affected by E1A.

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