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Tissue Transglutaminase-Dependent Posttranslational Modification of the Retinoblastoma Gene Product in Promonocytic Cells Undergoing Apoptosis

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The retinoblastoma gene product (pRB) plays an important role in controlling both cell release from the G_1 phase and apoptosis. We show here that in the early phases of apoptosis, pRB is posttranslationally modified by a tissue transglutaminase (tTG)-catalyzed reaction. In fact, by employing a novel haptenized lysis synthetic substrate which allows the isolation of glutaminyl-tTG substrates in vivo, we identified pRB as a potential tTG substrate in U937 cells undergoing apoptosis. In keeping with this finding, we showed that apoptosis of U937 cells is characterized by the rapid disappearance of the 105,000- to 110,000-molecular-weight pRB forms concomitantly with the appearance of a smear of immunoreactive products with a molecular weight of greater than 250,000. The shift in pRB molecular weight was reproduced by adding exogenous purified tTG to extracts obtained from viable U937 cells and was prevented by dansylcadaverine, a potent enzyme inhibitor. The effect of the pRB posttranslational modification during apoptosis was investigated by determining the E2F-1 levels and by isolating and characterizing pRB-null clones from U937 cells. Notably, the lack of pRB in these U937-derived clones renders these p53-null cells highly resistant to apoptosis induced by serum withdrawal, calphostin C, and ceramide. Taken together, these data suggest that tTG, acting on the pRB protein, might play an important role in the cell progression through the death program.

Transglutaminases belong to a family of Ca^{2+}-dependent enzymes that catalyze cross-linking of polypeptide chains in which the γ-carboxamide groups of peptide-bound glutamine residues serve as acyl donors and primary amino groups of several compounds function as acceptor substrates (13–15). The reaction results in the posttranslational modification of proteins by establishing ε(γ-glutamyl)lysine cross-linkages and/or covalent incorporation of biogenic amines (di- and polyamines and histamine) into proteins (13, 37). The transglutaminase-dependent formation of stable cross-links induces protein polymerization which confers high resistance to mechanical and chemical attack to the polypeptides involved in the linkage (11, 13); in fact, these polymers can be destroyed only by proteolytic degradation of the protein chains (12, 13).

Tissue transglutaminase (tTG) binds guanine nucleotides and hydrolyzes GTP (15). Nakaoka et al. (34) have demonstrated that the 74-kDa α subunit ($G_{\alpha h}$) associated with the 50-kDa β subunit of the GTP-binding protein, G_h, is tTG. This dimer acts in association with the rat liver α_1-adrenergic receptor in a ternary complex. Thus, $G_{\alpha h}$ (tTG) is a multifunctional protein which not only acts as a transglutaminase but, by binding GTP (in a $G_{\alpha h}$-GTP complex), activates phospholipase C after receptor stimulation (34).

Recently, much emphasis has been given to the genetic control exerted by various regulatory elements during the early phases of the death program, which results in the synthesis and/or activation of effector killer genes (8, 17). The tTG gene is one of the few genes induced during apoptosis (17, 38, 39). In fact, tTG protein is undetectable in the majority of cells, and its mRNA is transcribed as a consequence of the induction of apoptosis (38, 39). However, the tTG gene is constitutively expressed in some cell types, including endothelial cells, smooth muscle cells, and mesangial cells in vivo (for a review, see reference 39). It has been shown that tTG not only is associated with apoptosis but in some cell types may also play an important role in the killing process (14, 30, 41). Overexpression of tTG in human neuroblastoma SK-N-BE(2) cells, L929 cells, and NIH 3T3 fibroblasts enhances their susceptibility to undergo apoptosis (14, 30, 41). Human neuroblastoma cells overexpressing tTG showed a large reduction in their growth capacity, not only in vitro but also when xenografted into SCID mice (30, 40). By contrast, transfection of the same neuroblastoma cells with an expression vector containing a segment of the human tTG cDNA in the antisense orientation resulted in a decrease of both spontaneous and retinoic acid (RA)-induced apoptosis (30). It is difficult at the moment to establish the precise position of tTG in the cascade of events leading to apoptosis. However, a large body of evidence indicates that tTG induction parallels or slightly precedes *bcl-2* downregulation and is not susceptible to the inhibitory effect of Bcl-2 protein (30).

The present study was undertaken to investigate whether in the early stages of apoptosis, the controlled tTG-mediated posttranslational modification of a specific protein substrate(s) might have a role in cell commitment to apoptosis.

MATERIALS AND METHODS

Chemicals. [1,4-(*n*)-^{3}H]putrescine dihydrochloride (26.3 Ci/mmol) was from Amersham (Bucks, United Kingdom). Optifluor was from Packard (Zurich, Switzerland). *N*,*N*′-Dimethylcasein, bovine serum albumin, and putrescine hydrochloride were from Fluka (Bucks, United Kingdom). Cell culture media and plastic were from Flow (Irving, United Kingdom). All-*trans*-RA, bovine serum albumin, guinea pig liver tTG, dansylcadaverine, and propidium iodide were

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purchased from Sigma Chemical Co. (St. Louis, Mo.). Calphostin C was from Calbiochem Novachem Co. (La Jolla, Calif.). RPMI 1640 medium supplemented with L-glutamine, fetal calf serum (FCS), penicillin, and streptomycin was from Seralab Ltd. (Crawley Down, United Kingdom). Streptavidin-biotin immunoperoxidase staining systems were from BioGenex (San Ramon, Calif.). Anti-human retinoblastoma protein (pRB) antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, N.Y.). The anti-human E2F-1 antibody was obtained from Santa Cruz Biotechnologies.

Cell culture. The parental U937 cell line was grown at 37°C, under a humidified atmosphere containing 5% CO_2 in air, in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. Where indicated, prior to treatment with calphostin C, cells (5×10^5/ml) were shifted overnight to a growth medium containing 0.5% serum. Drug administration and the initial 20-min incubation were carried out in the dark; cells were then exposed to light for a further 20 min, and incubation was allowed to proceed in an incubator with occasional cell exposure to microscope light; control samples were treated with the proper amounts of carrier dimethyl sulfoxide (DMSO) and run in parallel. In those experiments employing dansylcadaverine (1 mM final concentration), the tTG inhibitor was added 1 h before calphostin C administration.

For the RA studies, cells were plated at 50×10^3 cells/cm^2 in growth medium containing 1 μM RA (from a 5 mM stock solution dissolved in 70% ethanol) and 1.25% DMSO. Medium was replaced daily. Control cultures were treated with 0.07% ethanol. Cell numbers and viability were evaluated in a Thoma hemocytometer chamber.

For the isolation of DALP {3-[*N*-α,*N*ε-(2′,4′-dinitrophenyl)-amino-*n*-hexanoyl-L-lysylamido]-propane-1-ol}-labeled protein, lysates obtained from U937 cells treated for 72 h with RA in the presence of 1.25% DMSO were precipitated with cold acetone and then treated with 100% trifluoroacetic acid for 30 min on ice to hydrolyze carbamate esters, lyophilized, and redissolved in 2% sodium dodecyl sulfate (SDS)–125 mM Tris-HCl (pH 6.8)–0.01% bromophenol blue. The sample was applied to a PrepCell 491 (Bio-Rad Laboratories, Hercules, Calif.) filled with 3% gel. Isolated proteins were applied to a column (0.5 by 5 cm) filled with rabbit anti-dinitrophenyl immunoglobulins coupled with hydrazide-linked beads prepared with an Affi-gel Hz immunoaffinity kit (Bio-Rad Laboratories). Affinity-purified proteins were released from the beads by boiling in 2% SDS–125 mM Tris-HCl and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

tTG assay. Cells were washed with phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) and sonicated at 4°C for 20 s. tTG activity was measured by detecting the incorporation of [^{3}H]putrescine into *N*,*N*′-dimethylcasein as previously reported (38). Determination of tTG protein in both RA-treated and untreated U937 cells was carried out by Western blot analysis. Aliquots (50 to 300 μg) of total protein were suspended in 0.1 M Tris-HCl buffer (pH 7.0) containing 1% SDS, 0.05% β-mercaptoethanol, 2.5% glycerol, and 0.001% bromophenol blue and boiled for 3 min. The extract was subjected to SDS–10.5% PAGE (30).

Apoptosis detection. Apoptosis was determined by both morphological and flow cytometric analyses (24). For flow cytometric analysis, aliquot of 5×10^5 cells were centrifuged at $300 \times g$ for 5 min; pellets were washed with PBS, placed on ice, and overlaid with 0.5 ml of a solution containing 200 μg of propidium iodide per ml, 1.0% Triton, and 0.9% NaCl. After gentle resuspension in this solution, cells were left at 4°C for 30 min, in the absence of light, before analysis. Propidium iodide-stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Bedford, Mass.); fluorescence was measured between 565 and 605 nm. The data were acquired and analyzed with the Lysis II program (Becton Dickinson).

Western blotting. Western blotting was carried out as previously described (30). In brief, aliquots of total protein extracts (100 μg) from cells after different treatments were suspended in 0.1 M Tris-HCl buffer (pH 7.0) containing 1% SDS, 0.05% β-mercaptoethanol, 2.5% glycerol, and 0.001% bromophenol blue, boiled for 3 min, and subsequently size fractionated by SDS–7.5% PAGE. The gel was electroblotted overnight onto nitrocellulose paper at 40 mA, and the bands were revealed with horseradish peroxidase-conjugated goat anti-mouse or anti-human immunoglobulin G (IgG) (Bio-Rad). The reaction was developed with the ECL detection system (Amersham). Molecular weights (MWs) were evaluated by plotting R_f against log MW with the Rainbow protein high-MW markers (Amersham) as a standard.

Immunohistochemistry. Immunohistochemical staining of human U937 cells was performed with, as the primary antibody, an affinity-purified monospecific IgG raised in rabbits against human erythrocyte-soluble tTG (1:100) (9); the anti-human Bcl-2 was from Dako (Glostrup, Denmark). Incubations with the primary antibody were carried out in a wet chamber overnight at 4°C. A biotinylated goat anti-rabbit IgG was used as the second antibody, followed by a preformed streptavidin-horseradish peroxidase complex (BioGenex). The reaction was developed by using aminoethylcarbazole (CRL, Richmond, Va.) as a chromogen substrate and 0.01% H_2O_2. Cells were counterstained in Mayer's hemalum.

RESULTS

Identification of tTG substrate proteins in U937 cells undergoing apoptosis. We used DALP, a novel synthetic substrate of tTG that is able to permeate cells, to isolate and identify glutaminyl-tTG substrate proteins in cells undergoing apoptosis (35). The substrate properties of DALP were tested by detecting its tTG-dependent incorporation into dimethylated casein and identification of γ-glutamyl-DALP (35). The K_m of DALP (3 μM) is about 50-fold lower than that of the best available amine substrate of tTG (35). The carbamate methyl ester derivative of DALP is nontoxic and easily penetrates into the U937 cells, where is hydrolyzed by cellular esterases and, in the presence of tTG, covalently incorporates into cellular proteins (35). Proteins bearing the haptenic group were isolated from U937 cells undergoing apoptosis upon treatment with RA, a potent inducer of tTG and apoptosis (Fig. 1; see reference 39 for a review), by immunoaffinity chromatography with antibodies against the dinitrophenyl haptenic group of DALP (Fig. 2). Analysis of the isolated proteins revealed that the major DALP-labeled proteins in U937 undergoing apoptosis had molecular masses of 43, 90, and 110 kDa; in addition, we isolated protein aggregates which could not enter the gel (Fig. 2A). It is interesting that no substrate proteins were isolated from viable U937 cells, thus suggesting that they act as a tTG substrate only in U937 cells undergoing apoptosis (data not shown). By immunostaining the blots obtained from the affinity-purified proteins with the C15 anti-human pRB monoclonal antibody (MAb), we found that the band showing an MW of 110,000 was specifically recognized by the anti-pRB MAb (Fig. 2A); the same results were obtained by using an anti-pRB MAb corresponding to amino acids 619 to 928 (not shown). The SDS-insoluble high-MW protein complex was also stained, suggesting that pRB might be incorporated into this large protein polymer as a conseque of a tTG-dependent reaction. In a separate set of experiments, the 43-kDa tTG protein substrate isolated with an immunoaffinity column and not stained by the two anti-pRB MAbs was identified as actin (35).

To investigate whether pRB may indeed act as a substrate for tTG in U937 cells, we incubated crude U937 cell extracts in the presence of purified tTG for 60 min (Fig. 2B). After incubation, the cell lysates were analyzed by SDS–7.5% PAGE and immunoblotted with the previously reported anti-human pRB MAb (Fig. 2B). In control cells, this antibody evidenced a broad band in the 105- to 110-kDa area, corresponding to pRB in different phosphorylation states, and a smaller 68-kDa product recently identified in U937 cells as one of the cleavage products of pRB by caspase (5, 22). Treatment of cells with tTG brought about a dramatic modification of this pattern, consistent with the rapid disappearance of the pRB 105- to 110-kDa immunopositive band(s) and the appearance of a smear of products with molecular masses higher than 250 kDa (Fig. 2B).

pRB acts as a tTG substrate in U937 cells undergoing apoptosis. Calphostin C, a potent and selective protein kinase C inhibitor (6), has been demonstrated to induce apoptosis in various cell systems (23, 28) and to sensitize cells to apoptosis induced by other agents (31). In U937 cells maintained in 0.5% serum and treated with 250 nM calphostin C, the onset of apoptosis was observed 1 h after drug administration, and by 3 h about 70 to 80% of cells had died (Fig. 3). Figure 3b shows that calphostin C-induced apoptosis, at its onset, was consistent with a selective loss of cells in the G_1 phase of the cycle. It has been established that multiple phosphorylation of pRB is a key event in the control of G_1-to-S cell cycle phase transition and apoptosis (22, 43). We therefore investigated the effect of

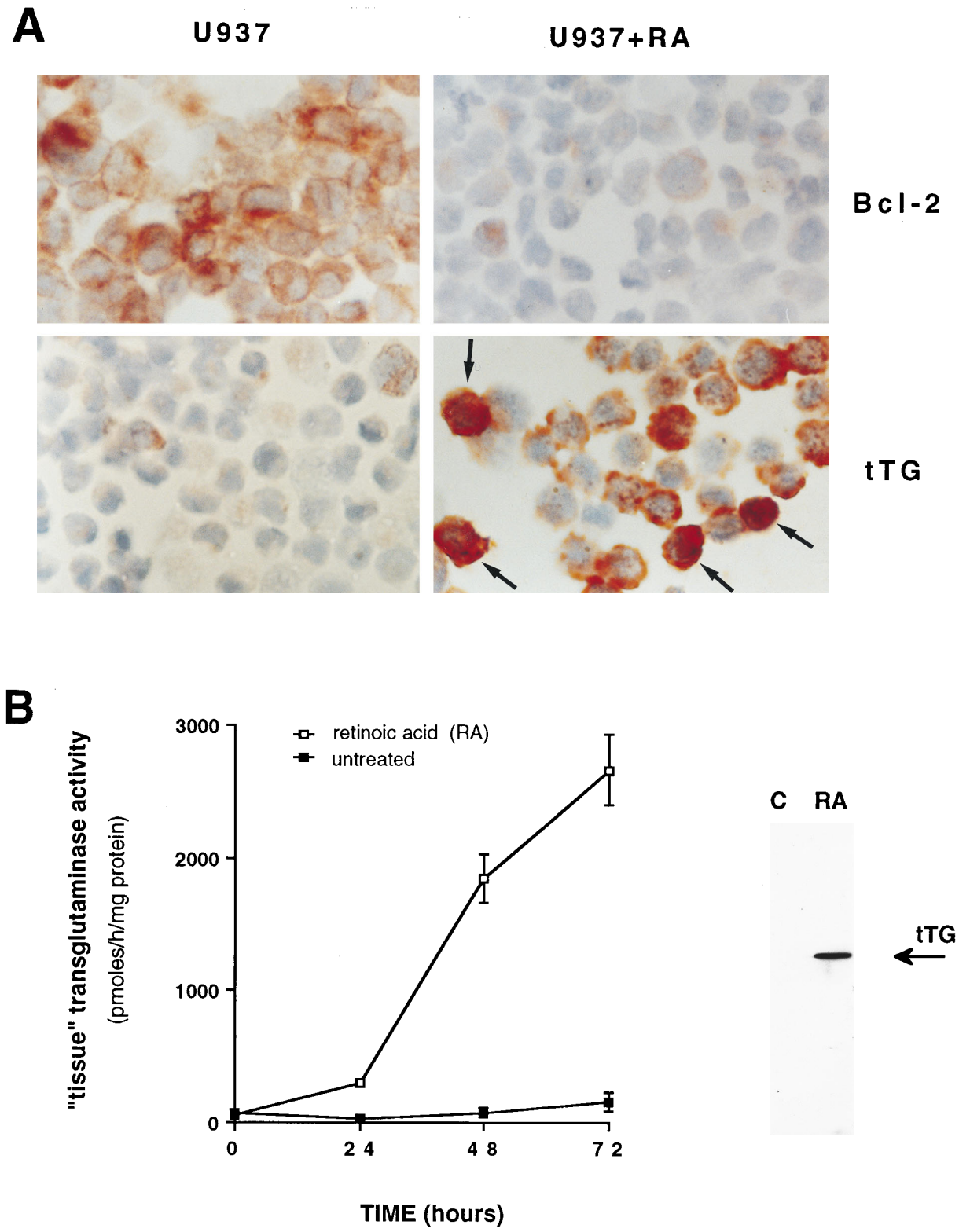

FIG. 1. Effect of RA on tTG and Bcl-2 levels in U937 cells. (A) Effect of RA on Bcl-2 and tTG immunostaining in U937 cells. Cells were grown on slides in the presence (left) or absence (right) of RA (1 μM) and 1.25% DMSO for 2 days (cell viability was reduced to 50%). After treatment, cells were fixed in 2.5% paraformaldehyde, and after immunostaining, they were counterstained with hematoxylin. Note the marked *bcl-2* downregulation highlighted by the pale Bcl-2 staining in RA-treated U937 cells. By contrast, note the intense tTG immunoreaction in U937 cells treated with RA, which, in the typical apoptotic bodies, is localized in the entire cell, including the nucleus (arrows). Magnification, ×2,200. (B) Left panel, U937 cells grown in 10% FCS were either left untreated or treated with 1 μM RA for 24, 48, or 72 h. Cells cultured as described above were extensively washed in PBS, and tTG activity was measured in vitro as picomoles of [^{3}H]putrescine incorporated into protein per hour per milligram of protein (see Materials and Methods). Data are the means ± standard errors of the means for triplicate determinations in three different experiments. Right panel, U937 cells grown in 10% FCS, either left untreated (lane C) or treated with 1 μM RA for 48 h (lane RA), were extensively washed in PBS, and Western blot analysis was carried out as described in Materials and Methods.

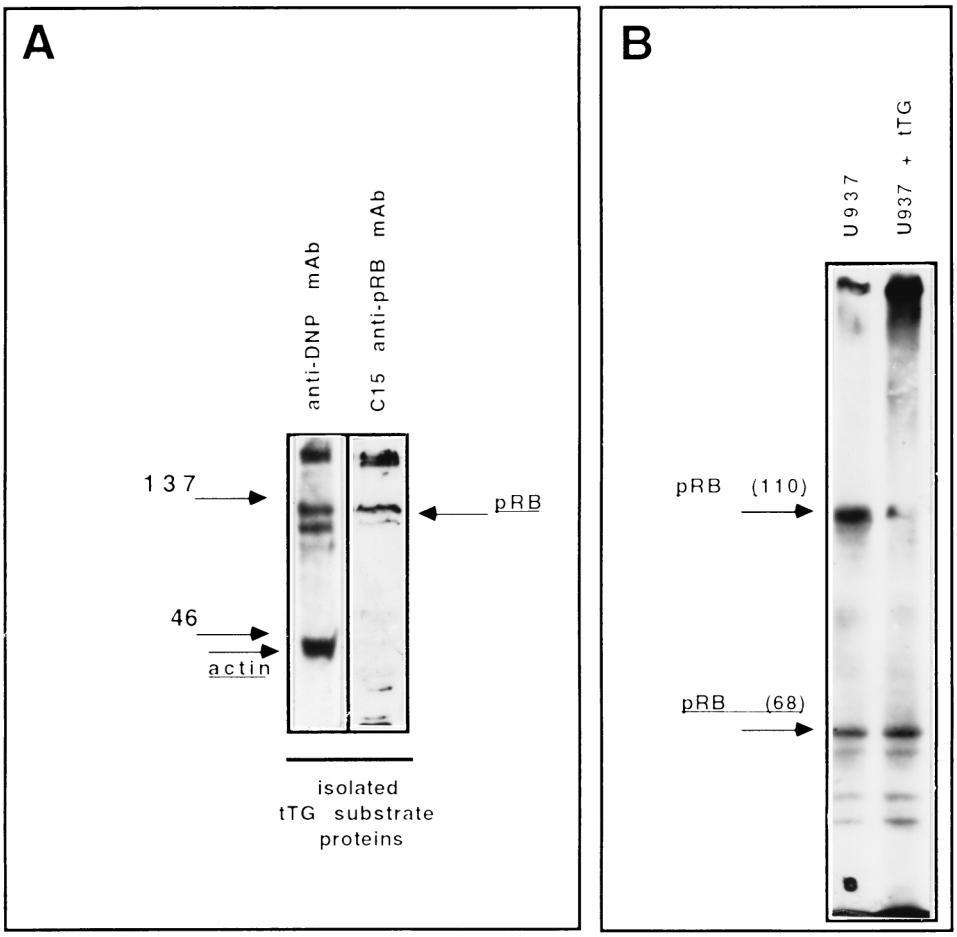

FIG. 2. Characterization of tTG protein substrates in U937 cells. (A) Western blot analysis of the affinity-purified endogenous tTG substrate proteins from U937 cells undergoing apoptosis. U937 cells were grown in 10% FCS and treated for 3 days with 25 mM DALP-methyl carbamate–1 μM RA–1.25% DMSO. After immunopurification, aliquots (5 μg of total protein) of the isolated tTG substrate proteins were analyzed by Western blotting with the anti-dinitrophenyl and the C15 anti-pRB MAbs as described in Materials and Methods. (B) Cell lysates obtained from U937 cells grown in 10% FCS were incubated in the presence of either bovine serum albumin (U937) (10 μg/ml) or purified guinea pig liver tTG (U937 + tTG) (1 μg/ml) for 1 h. After incubation, aliquots of 100 μg of total protein were used for Western blot analysis carried out as described in Materials and Methods, with the C15 anti-pRB MAb used for staining. Numbers on the left in each panel are molecular weights in thousands.

calphostin C-induced apoptosis on pRB. As shown for RA-induced apoptosis (Fig. 1), calphostin C-induced apoptosis also occurs in the presence of increased tTG activity (45 ± 10 pmol/h/mg of protein in untreated U937 cells versus 220 ± 35 pmol/h/mg of protein in 250 nM calphostin C-treated U937 cells). Lysates of U937 cells treated with calphostin C for 30 or 60 min were analyzed by SDS–7.5% PAGE and immunoblotted with the C15 anti-human pRB MAb. In untreated cells (maintained for 18 h in 0.5% FCS) this antibody evidenced the broad band in the 105,000- to 110,000-MW area corresponding to pRB in different phosphorylation states together with the other band showing an MW of 68,000 (Fig. 4A). Treatment of cells with calphostin C brought about a drastic modification of this pattern, consistent with the rapid disappearance of all of the pRB-immunopositive bands (Fig. 4A); in addition, in keeping with the in vitro experiments reported in Fig. 2B, the smear of products with MWs higher than 250,000 was also detected. To confirm that the effect of calphostin C on pRB was dependent on tTG action, we blocked tTG activity (before the addition of calphostin C) by treating U937 cells with 1 mM dansylcadaverine, a well-known tTG inhibitor (15). As shown in Fig. 4A, the disappearance of the pRB 105,000- to 110,000-MW and 68,000-MW bands observed in calphostin C-treated cells was largely reduced in the presence of dansylcadaverine, thus confirming the hypothesis that pRB acts as a substrate for tTG in cells undergoing apoptosis.

It is interesting that in some experiments, calphostin C-induced disappearance of the pRB 105,000- to 110,000-MW bands was not quantitative; in this case, residual pRB (10 to 20% of the total pRB immunostaining) was found in the hypophosphorylated form (Fig. 4B). Although this phenomenon remains to be better investigated in future studies, at present one could entertain the possibility that the hyperphosphorylated pRB might be a better substrate for the tTG than the hypophosphorylated counterpart. The phosphorylation, for instance, could modify pRB folding, making the lysine and glutamine residues better exposed on the surface to the action of tTG.

Isolation and characterization of pRB-null U937 cell lines. To investigate the functional relationship between pRB and tTG in apoptosis, we attempted to isolate U937 cells which had a null pRB expression. By using a limiting-dilution approach, we were able to isolated several clones which showed a specific reduction in the levels of pRB (Fig. 5A). In fact, no significant changes in the expression of other genes, such as *bcl-2* and the

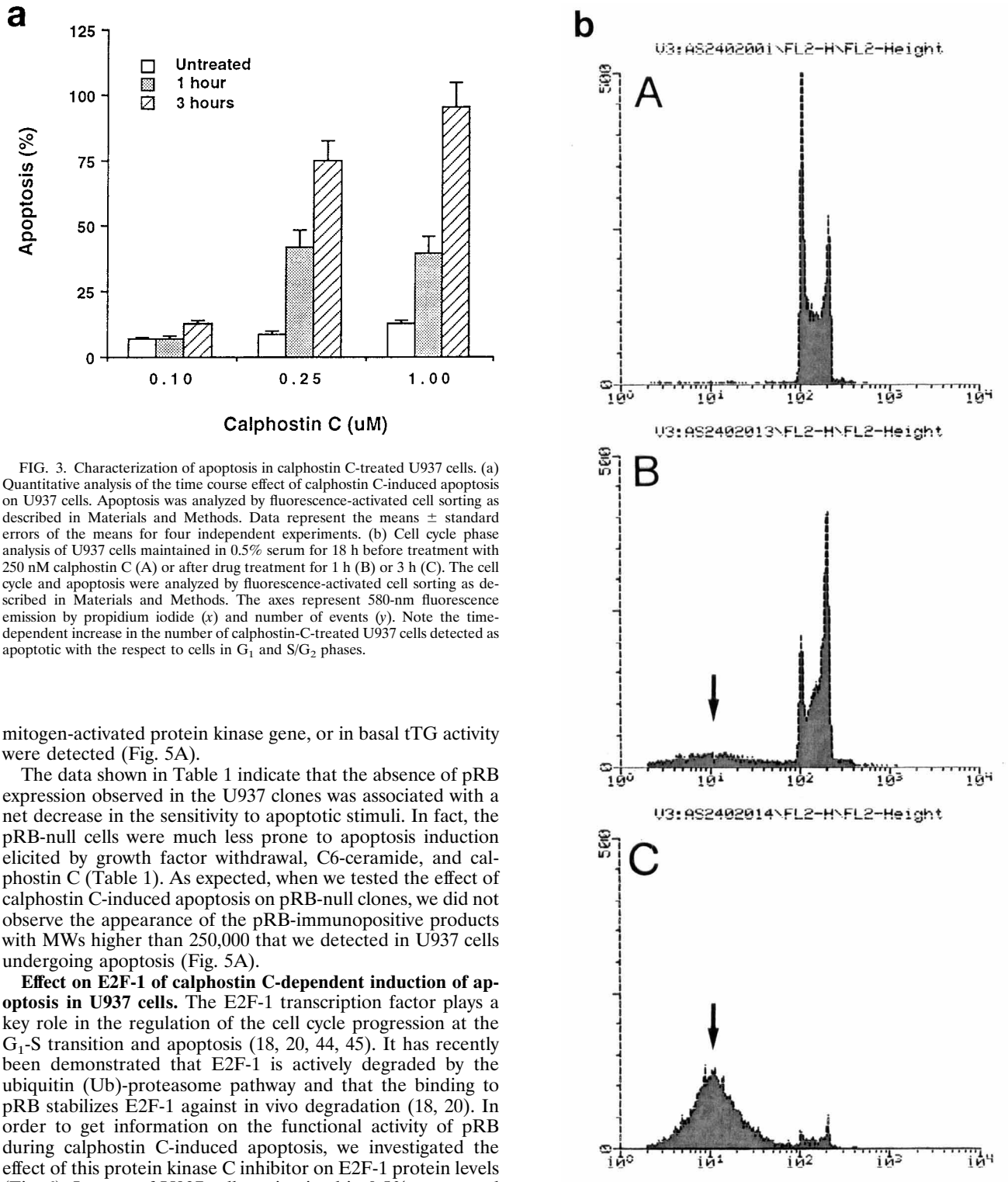

FIG. 3. Characterization of apoptosis in calphostin C-treated U937 cells. (a) Quantitative analysis of the time course effect of calphostin C-induced apoptosis on U937 cells. Apoptosis was analyzed by fluorescence-activated cell sorting as described in Materials and Methods. Data represent the means ± standard errors of the means for four independent experiments. (b) Cell cycle phase analysis of U937 cells maintained in 0.5% serum for 18 h before treatment with 250 nM calphostin C (A) or after drug treatment for 1 h (B) or 3 h (C). The cell cycle and apoptosis were analyzed by fluorescence-activated cell sorting as described in Materials and Methods. The axes represent 580-nm fluorescence emission by propidium iodide (*x*) and number of events (*y*). Note the time-dependent increase in the number of calphostin-C-treated U937 cells detected as apoptotic with the respect to cells in G_1 and S/G_2 phases.

mitogen-activated protein kinase gene, or in basal tTG activity were detected (Fig. 5A).

The data shown in Table 1 indicate that the absence of pRB expression observed in the U937 clones was associated with a net decrease in the sensitivity to apoptotic stimuli. In fact, the pRB-null cells were much less prone to apoptosis induction elicited by growth factor withdrawal, C6-ceramide, and calphostin C (Table 1). As expected, when we tested the effect of calphostin C-induced apoptosis on pRB-null clones, we did not observe the appearance of the pRB-immunopositive products with MWs higher than 250,000 that we detected in U937 cells undergoing apoptosis (Fig. 5A).

Effect on E2F-1 of calphostin C-dependent induction of apoptosis in U937 cells. The E2F-1 transcription factor plays a key role in the regulation of the cell cycle progression at the G_1-S transition and apoptosis (18, 20, 44, 45). It has recently been demonstrated that E2F-1 is actively degraded by the ubiquitin (Ub)-proteasome pathway and that the binding to pRB stabilizes E2F-1 against in vivo degradation (18, 20). In order to get information on the functional activity of pRB during calphostin C-induced apoptosis, we investigated the effect of this protein kinase C inhibitor on E2F-1 protein levels (Fig. 6). Lysates of U937 cells maintained in 0.5% serum and treated with calphostin C for 30 or 60 min were analyzed by SDS-PAGE and immunoblotted with anti-human E2F-1 (epitope corresponding to amino acids 418 to 437). In control cells, this antibody evidenced a band showing an MW of about 60,000 (Fig. 6). Treatment of cells with calphostin C brought about a rapid modification of this pattern, leading to the disappearance of the E2F-1-immunopositive band (Fig. 6), thus suggesting a rapid degradation by the Ub-proteasome pathway.

DISCUSSION

The tTG gene is induced in dying cells under physiological and pathological conditions in vivo as well as in several in vitro contexts (2, 10, 25, 30, 39). A definitive role for tTG in apoptosis has not yet been firmly established; however, tTG ex-

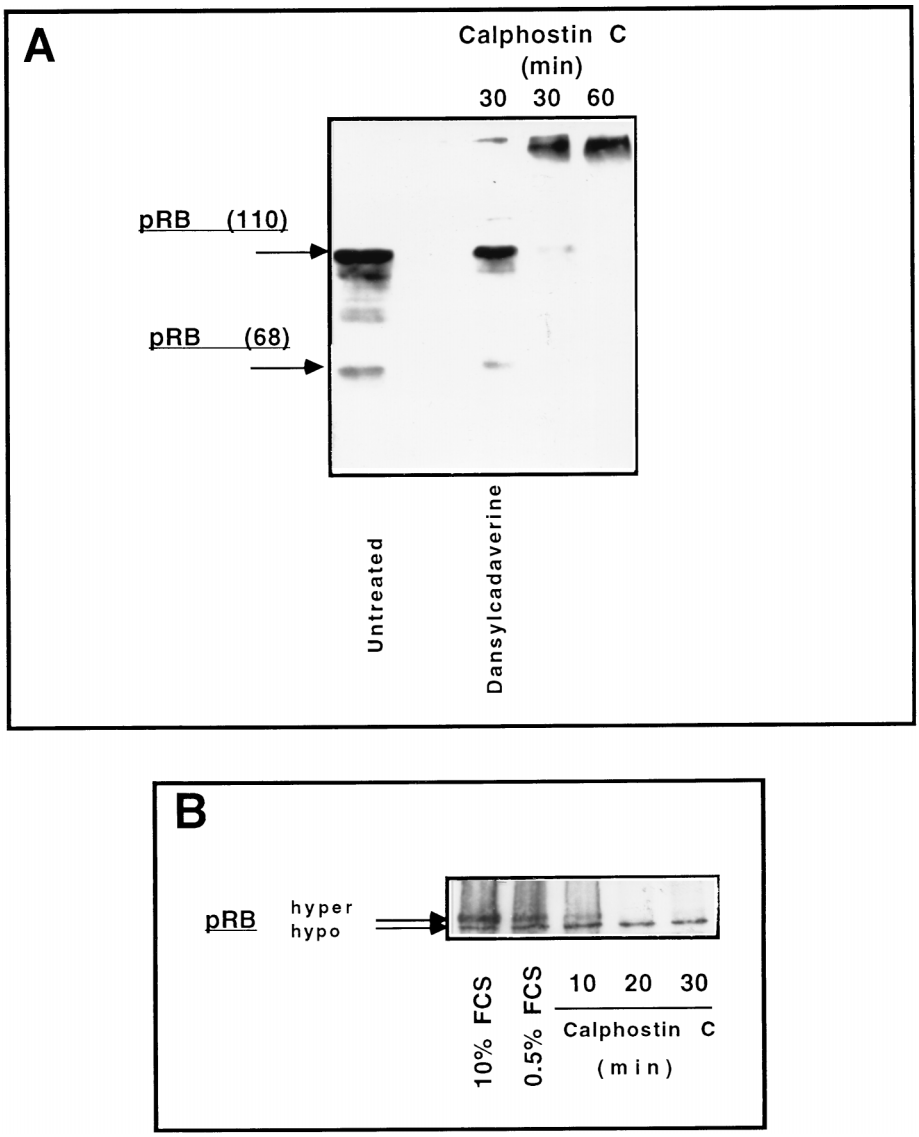

FIG. 4. Effect of calphostin C on pRB in U937 cells. (A) Western blot analysis of cell lysates obtained from U937 cells maintained for 18 h in 0.5% FCS and either left untreated or treated for 30 min (in the presence or in the absence of 1 mM dansylcadaverine) or 60 min with 250 nM calphostin C. Numbers on the left are molecular weights in thousands. (B) Cells grown in 10% FCS were shifted to 0.5% FCS for 18 h and either left untreated or treated for the indicated times with 250 nM calphostin C. Western blot analysis was carried out by using SDS–12.5% PAGE for protein separation and the anti-pRB MAbs against amino acids 619 to 928 for staining the immunoblot.

pression is not a late epiphenomenon, since antisense tTG transfection did not affect disposal and degradation of (poorly cross-linked) apoptotic bodies but actually reduced apoptosis (30).

By using various experimental approaches, we have provided evidence that in U937 cells the polymerization of pRB, which precedes apoptosis, is mediated by its posttranslational modification catalyzed by tTG. Furthermore, we have shown that in these p53-null cells (26), the lack of pRB largely increases the resistance to apoptosis induced by different stimuli.

Previous studies suggested that an extensive tTG activation leads to the assembly of intracellular cross-linked protein polymers, which irreversibly modifies the cell organization, contributing to the widespread, ultrastructural changes occurring in cells undergoing apoptosis (10, 11, 25). This extensive tTG-dependent protein polymerization stabilizes apoptotic cells before their clearance by phagocytosis, thus contributing to the prevention of inflammation in the surrounding tissues (3, 11, 17, 38, 41). The question arises as to whether in the early stages of the death pathway, a regulated tTG-mediated posttranslational modification of a specific protein substrate(s) might have a role in the commitment to apoptosis. In keeping with this hypothesis, we showed that during the early phases of apoptosis, tTG posttranslationally modifies pRB, thus suggesting that the activation of this cross-linking enzyme might determine an irreversible commitment to death. In fact, pRB polymerization was already detectable after 10 to 20 min in calphostin C-treated U937 cells, when the level of apoptosis was still unchanged (Fig. 4).

pRB plays a key role in cell cycle control (7, 43). It is generally accepted that pRB, in the hypophosphorylated state, binds to and impairs the activities of several proteins, including

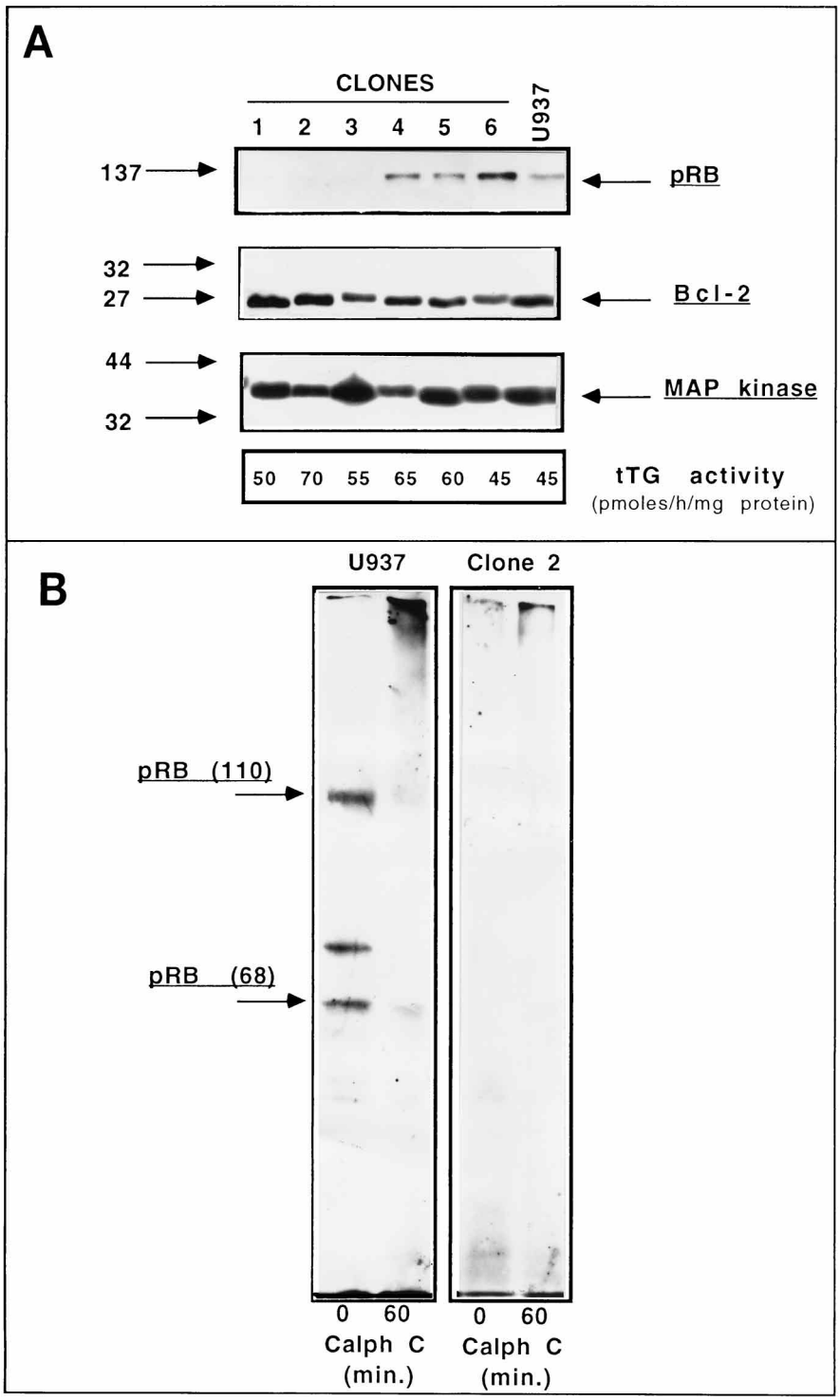

FIG. 5. Effect of calphostin C on U937-derived pRB-null clones. (A) U937 cells and derived pRB-null clones were grown in 10% FCS. After extensive washings in PBS, Western blot analysis of cell lysates was carried out with anti-pRB, -Bcl-2, and -mitogen-activated protein kinase MAbs on 100 μg of total protein. tTG activity of the U937 cell clones was measured as described in Materials and Methods. (B) Western blot analysis of cell lysates obtained from U937 cells and the derived pRB-null clones. Cells maintained for 18 h in 0.5% FCS were either left untreated (0 min) or treated for 60 min with 250 nM calphostin. Numbers on the left in each panel are molecular weights in thousands.

the transcription factors of the E2F family (7, 33). The range of pRB functions has recently been discovered to be more complex, since it has been shown that the absence of functional pRB may result in apoptosis rather than in uncontrolled cell proliferation (1, 32, 42) and that overexpression of functional pRB may induce apoptosis or rescue cells from death, depending on the system (16, 29, 46). Homozygous pRB-null mice die during gestation, showing massive induction of apoptosis dur-

TABLE 1. Effect of serum, calphostin C, and C6-ceramide on apoptosis in pRB-null U937-derived cell clones[a]

Treatment	% Apoptosis (mean ± SEM [n = 3]) in:		
	U937	Clone 2	Clone 3
FCS (10.0%)	7.7 ± 1.5	4.2 ± 2.0	5.4 ± 1.1
FCS (0.5%)	24.3 ± 4.0	13.2 ± 3.0	12.7 ± 3.5
Calphostin C (250 nM)	75.3 ± 14.0	25.2 ± 3.0	27.7 ± 3.5
C6-Ceramide (30 μM)	54.3 ± 9.0	23.0 ± 3.0	29.7 ± 6.5

[a] Apoptosis in cells growing in 10% FCS as well as after a shift to 0.5% FCS for 18 h in the absence of any treatment or after a 3-h exposure to calphostin C or C6-ceramide at the incated concentration was evaluated. Cells were washed extensively in PBS, and the relative percentage of apoptotic cells was evaluated by fluorescence-activated cell sorter analysis as described in Materials and Methods.

ing liver erythropoiesis and neuronal development (43). The question arises as to the functional implications of the tTG-dependent polymerization of pRB occurring during the early phases of apoptosis. In keeping with the possibility that pRB polymerization might lead to its functional inactivation, we showed that the tTG-dependent polymerization of pRB is paralleled by the rapid disappearance of E2F-1, which occurs when the transcription factor is not protected by pRB binding from entering the Ub-proteasome pathway (18, 20). U937 cells provide a p53-null line (reference 26 and our unpublished observations), leading to the question of whether loss of pRB function is sufficient for the occurrence of apoptosis. Indeed, the simultaneous absence of pRB and p53 is not incompatible with life. In fact, we have showed here that the absence of pRB leads to a an increased resistance to several apoptotic stimuli, such as growth factor deprivation and calphostin C and C6-ceramide treatment. Taken together, these findings suggest that the polymerization of pRB observed in U937 cells might represent a key signal for the initiation of apoptosis. By contrast, the lack of pRB, in the absence of p53, may have dramatic consequence for cycling cells. In fact, as we observed for the U937-derived pRB-null cell clones, this condition results in uncontrolled proliferation associated with a high resistance to apoptosis (44).

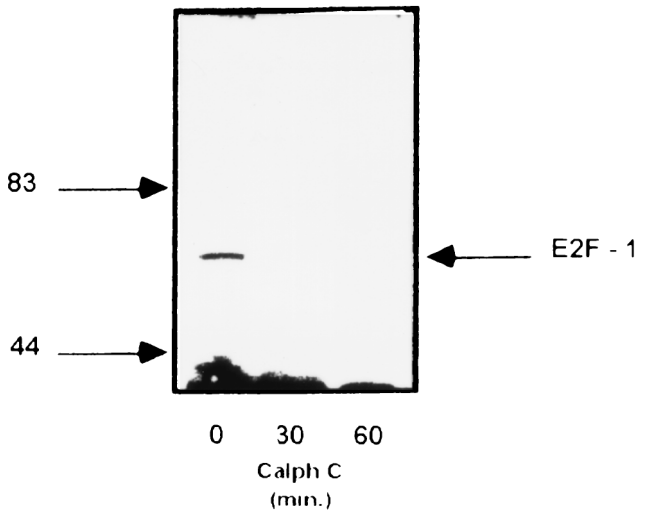

FIG. 6. Effect of calphostin C on E2F-1 levels in U937 cells. Western blot analysis, with the anti-human E2F-1 MAb, of lysates obtained from U937 cells maintained for 18 h in 0.5% FCS and either left untreated (0 min) or treated for 30 or 60 min with 250 nM calphostin C is shown. Numbers on the left are molecular weights in thousands.

It is now becoming clear that in cells undergoing apoptosis, a set of specific proteins are degraded mainly by the caspase family of proteases (27). It has recently been demonstrated that pRB also is a substrate for interleukin-converting enzyme-like proteases in the context of apoptosis induced by certain antitumor drugs (5, 22). It is interesting that several substrates for the caspases (27) are also substrates for tTG in vivo. In fact, in addition to pRB, actin (25, 35) and histones (4) are post-translationally modified in cells undergoing apoptosis induced by both tTG and caspases. The functional significance and the reciprocal influence of these two events in the commitment to and execution of apoptosis are interesting matters for future studies.

In conclusion, the data reported in this paper seem to indicate that tTG might have more than one function within the cascade of events leading to the establishment of the apoptotic phenotype: (i) an early regulatory function which, through the polymerization of pRB, may influence the decision to undergo apoptosis or to survive, and (ii) a late effect leading to the stabilization of the dying cells by the formation of intracellular cross-linked protein polymers (41).

Recent experiments have suggested that cells may undergo a process morphologically similar to apoptosis without a nucleus and in the absence of de novo protein synthesis by simply activating the downstream elements (17, 21), thus suggesting that all machinery is already in place before the apoptotic signal and that, at least in some circumstances, the final events of apoptosis may be controlled at the posttranslational level. The plasticity provided by a multifunctional enzyme like tTG represents an interesting example of how a complex phenomenon such as apoptosis may be regulated at the posttranslational level. The modulation of the tTG activity or $G_{\alpha h}$ protein functions by the intracellular environment (such as the levels of GTP, Ca^{2+}, and free di- and polyamines) may have a relevant role in the decision of cells to survive or die.

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