

Intracellular Clusterin Induces G₂-M Phase Arrest and Cell Death in PC-3 Prostate Cancer Cells¹

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ABSTRACT

Enhanced clusterin gene expression has been related frequently to organ remodeling, tissue involution, and cell death. Whether clusterin represents a leading cause or a consequence of apoptosis induction is still a matter of debate. Clusterin is known as an extracellular secreted glycoprotein in the mature form. However, truncated isoforms of the protein and nuclear localization of clusterin have been described recently in association to cell death. Here, we show the biological effects triggered in PC-3 androgen-independent prostate cancer cells by overexpression of an intracellular, not secreted form of clusterin (intracellular-clusterin). Transient transfection of PC-3 cells with intracellular-clusterin resulted in nuclear localization signal-independent massive nuclear localization of the protein leading to G₂-M phase blockade followed by caspase-dependent apoptosis. Constitutive expression of intracellular-clusterin (pFLAG-intracellular-clusterin) in recombinant PC-3 cells caused clonogenic toxicity. The rare pFLAG-intracellular clusterin surviving clones showed inhibition of the proliferation rate and altered phenotype with impaired mitosis and endoreduplication. In these cells, caspase-independent cell death was induced. Impaired cell cycle progression in pFLAG-intracellular-clusterin clones was associated to arrest at the G₂-M checkpoint by down-regulation of the mitotic complex cyclin B1/cyclin-dependent kinase 1. Intriguingly, intracellular-clusterin was localized exclusively in the cytoplasm in stably transfected cells, suggesting a negative correlation between nuclear clusterin accumulation and cell survival. These findings may possibly explain the conflicting results obtained in different laboratories, suggesting that clusterin might be a proapoptotic or a survival gene, also opening new perspectives for the characterization of androgen-independent and apoptosis-resistant prostate cancer cells.

INTRODUCTION

Prostate cancer is the most common malignancy, and it is the second-leading cause of cancer-related deaths in North America (1). Whereas in the case of other neoplastic diseases molecular research has progressed substantially (such in breast and colon cancer), the identification of molecular mechanisms leading to onset and progression is still a major challenge in the field of prostate cancer and requires urgent attention by research laboratories (2). Elucidation of molecular mechanisms leading to androgen-independence and resistance to apoptosis in prostate cancer cells is urgently needed. At present, androgen-independent metastatic human prostate cancer PC-3 cells are a useful model to address this topic. Nevertheless, the specific involvement of the apoptotic machinery as well as the modulation of pathways taking part in cell transformation are far from being understood completely in this very common kind of cancer (3).

Clusterin (also known as ApoJ, TRPM-2, and SGP-2) is a secreted glycoprotein that is induced highly during rat prostate involution (4–8) as well as in many experimental models of tissue regression or

cellular stress (9–13). Although clusterin is an apoptosis-associated gene, a precise relationship between this gene activity and programmed cell death has not been elucidated clearly as yet. In prostate cancer, clusterin has been reported both down- (14, 15) and up-regulated (16, 17), whereas its role in cell survival is still a matter of debate. This ambiguity between possible pro- and anti-apoptotic activities of clusterin has been addressed recently and independently in two manuscripts in which nuclear isoforms of clusterin have been found to be associated with cell death (18, 19). The ability of the clusterin gene to code for different/alternative protein isoforms was reported previously (5, 20–23), and the hypothesis that the secreted form might play a cytoprotective role, although the intracellular form could be cytotoxic, now seems to reconcile most of the authors in the field (19, 24, 25). The intracellular isoform of the protein has been proposed to induce cell death through its nuclear localization. According to the experimental model used, different pathways have been suggested to explain intracellular clusterin retention and nuclear migration. Treatment with tumor necrosis factor α has been described to change the activity of Golgi-resident enzymes leading to the formation of nonglycosylated clusterin accumulating in the nucleus. This happened in coincidence with DNA fragmentation (19). Transforming growth factor β was reported to induce the synthesis of a truncated form of the protein, starting from a second in-frame ATG. This clusterin isoform lacks the secretion signal sequence, does not enter endoplasmic reticulum, and is retained in the cell (20). Leskov *et al.* (18) have reported for the first time a possible alternative splicing of clusterin mRNA in MCF-7 cells treated with X-rays. This splice clusterin variant, which starts from the second ATG codon mentioned above and, therefore, omitting the endoplasmic reticulum-targeting signal, was found to enter the nucleus and initiate apoptosis. Taken together, these findings support the hypothesis of a link between intracellular clusterin accumulation and cell death.

Little is known about the subcellular localization of clusterin in prostate cells. No evidences of proapoptotic roles possibly played by truncated or nuclear isoforms of this protein are available in the same model. Thus, we designed an expression vector capable of driving the expression of a truncated isoform of clusterin (intracellular-clusterin), starting from the second in-frame ATG on full-length human clusterin cDNA. Then, we have studied its intracellular localization and the biological effects induced by transient and stable overexpression of intracellular-clusterin in PC-3 cells.

MATERIALS AND METHODS

Antibodies and Reagents. Mouse antihuman cyclin-dependent kinase 1 (CDK1) and antihuman cyclin B1 primary antibodies were purchased from BD Bioscience (Erembodegem, Belgium) and used at dilutions 1:400 for Western blotting and 1:40 for immunocytochemistry. Mouse antihuman clusterin (clone 41D, Upstate Biotechnology, Lake Placid, NY) was used at 1:1,000 for Western blotting and 1:100 for immunocytochemistry. Rabbit anti-FLAG (Sigma, Alcobendas, Madrid, Spain) was used at dilutions 1:3,000 for Western blotting and 1:300 for immunocytochemistry. Immunoblotting detection was done with antimouse and antirabbit secondary horseradish-peroxidase-conjugated antibodies from Dako (Glostrup, Denmark) diluted at 1:2,000. Immunofluorescence staining was evidenced with antimouse and antirabbit Alexa green secondary antibodies (Molecular Probes, Inc., Eugene, OR) diluted at

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1:200. Etoposide (Sigma) was dissolved in DMSO and added at final concentrations of 100 μM in cell culture medium. The *z*-Val-Ala-Asp-fluoromethyl ketone (*z*-VAD-fmk; Clontech Laboratories, Inc., Palo Alto, CA) was administered at final concentrations of 5 μM in cell culture medium.

Expression Vector Constructs. Full-length human clusterin (GenBank accession no. M64722) cDNA was generated by reverse transcription-PCR of total RNA extracted from normal human fibroblast in primary culture using the following primers: 5'-GAC TCC AGA ATT GGA GGC ATG-3' (forward); and 5'-ATC TCA CTC CTC CCG GTG CT-3' (reverse). Full-length clusterin cDNA was then first cloned in pGEM T-easy (Promega, Southampton, United Kingdom). Using 5'-GGC GGC CGC GGG GAT CCG AT-3' as forward primer and 5'-GAC CTG CAG GCG GCC GCG AAT-3' as reverse primer, the same sequence was then subcloned in pIRES-hyg1 (Clontech, Oxford, United Kingdom) at the *Bam*HI and *Nor*I sites to obtain a full-length clusterin expression vector. A truncated clusterin cDNA fragment was then amplified from the full-length expression vector using the primers 5'-GTC TCA GAC AAT GGG ATC CAG GA-3' (forward) and 5'-GAC CTG CAG GCG GCC GCG AAT-3' (reverse) and inserted in pIRES-hyg1 at the same *Bam*HI and *Nor*I sites to generate a second expression vector for driving intracellular clusterin expression. Flagged intracellular clusterin was derived from full-length clusterin by amplification of the clusterin fragment [primers: 5'-CAG GAA ATG TCC AAG CTT GGA AGT A-3' (forward); and 5'-GCG GGA TCC TCT AGT GCG G-3' (reverse)] and subcloning at the *Hind*III and *Bam*HI sites of pFLAG-CMV-4 (Sigma). Nuclear localization signal-mutant vectors were produced using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), starting from pFLAG-intracellular-clusterin. Three sets of two oligonucleotide primers, each complementary to opposite strands of the vector and containing the desired mutations, were designed to create three different constructs carrying mutations in one (N-mut), two (N-mut + 324-mut), or all three nuclear localization signal (N-mut + 324-mut + C-mut). These oligonucleotides are as follows: 5'-GAA GAA GCC AAG GAG GAG AAA GAG GAT GC-3' (forward) and 5'-GCA TCC TCT TTC TCC TCC TTG GCT TCT TC-3' (reverse) for N-mut; 5'-CGA ATC CCC CCA GGC CGC TGA GGG GTT GAC CAG G-3' (forward) and 5'-CCT GGT CAA CCC CTC AGC GGC CTG GGG GGA TTC G-3' (reverse) for 324-mut; and 5'-GAA TAC CGC AAA GAG CAC GGG GAG GAG TG-3' (forward) and 5'-CAC TCC TCC CCG TGC TCT TTG CGG TAT TC-3' (reverse) for C-Mut. Empty pIRES-hyg1 and pFLAG-CMV-4 were used to generate mock controls. All of the constructs have been sequenced by ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Foster City, CA) before carrying out expression experiments.

Cell Cultures and Transfections. PC-3 is an androgen-independent cell line isolated from bone metastasis of human prostate carcinoma (26). PC-3 cells were purchased from the American Tissue Culture Collection (Manassas, VA) and maintained at 37°C in 5% CO₂, in DMEM/Ham F12 1:1 supplemented with 2 mM L-glutamine, and in 7% FCS (Life Technologies, Inc., Grand Island, NY). Transfections were done with the nonliposomal reagent FuGENE 6 (Roche, Mannheim, Germany), according to the manufacturer's protocol. The 5 × 10⁴ and 10⁵ cells were plated, respectively, in 24-well and 35-mm dishes and transiently transfected with 0.5 μg /well and 2 μg /dish of plasmid DNA. Transfected cells were then used for immunoblotting and fluorescence microscopy. Efficiency of transient transfection experiments was assessed routinely by green fluorescence protein expression using the pEGFP-N1 vector (Clontech Laboratories, Inc.), and it was higher than 50% of total PC-3 cells. Stable transfections were conducted on 5 × 10⁵ cells plated in 60-mm dishes with 6 μg of plasmid DNA/plate. For selection of stably transfected cells, geneticin (G418, Life Technologies, Inc.) was added to the culture medium 48 h after transfection at a concentration of 600 μg /ml.

Colony Formation, Proliferation Assay, FACS Analysis, and Caspase-3 Activity. Individual colonies from stable clones were fixed in paraformaldehyde 4% and stained with crystal violet (Sigma) 14 days after the beginning of selection in G418. Time course of cell proliferation was measured by cell count using trypan blue exclusion test in triplicate. Cell cycle analysis was evaluated by fluorescence-activated cell sorter (Beckman Coulter Epics XL) after fixation of the cells with 70% ethanol at -20°C overnight, followed by treatment with RNase A, 20 μg /ml, and staining with propidium iodide. Caspase-3 activity was measured by Caspase-3 Colorimetric Assay Kit (Eppendorf, Hamburg, Germany), according to the manufacturer's instructions.

Immunocytochemistry. Cells were seeded on 12-mm diameter coverslips (Marienfeld, Lauda-Koenigshofen, Germany), fixed with 4% paraformaldehyde for 15 min, and permeabilized with 70% ethanol for 15 min. Then, cells were washed in 50 mM Tris-HCl (pH 7.5–150 mM) NaCl (TBS), and blocking was done with 1% BSA plus 0.1% Tween 20 in TBS. Immunostaining was carried out by incubation at room temperature for 1 h with the appropriate primary antibody in BSA-0.1% Tween 20 in TBS. Cell-coated coverslips were washed with TBS, incubated for 1 h at room temperature with secondary antibody, washed in TBS, and mounted on slides with 70% glycerol. Slides were visualized by TCS-NT argon/krypton confocal laser microscope (Leica, Wetzlar, Germany).

Immunoblotting. Whole cell extracts or culture medium were added to buffer containing 1% SDS, 1% DTT, and 10% glycerol, heated to 100°C for 5 min, resolved by 10% SDS-PAGE, and blotted to polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA). Membranes were blocked in 5% nonfat dried milk-0.1% Tween 20 in TBS for 1 h prior to incubation at 4°C overnight with the appropriate primary antibody. Detection was done using horseradish-peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Pharmacia, Buckinghamshire, United Kingdom). For protein staining, Ponceau S solution (Sigma) was used.

Real-Time Reverse Transcription-PCR. One microgram of total RNA from each experimental condition was primed with 0.75 μg of random primers (Life Technologies, Inc.) and incubated at 42°C for 60 min in a 30 μl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM deoxynucleoside triphosphates, and 300 units of Superscript II Reverse Transcriptase (Life Technologies, Inc.). One microliter of each cDNA preparation was PCR-amplified using the following primers: 5'-CAC TTC CTT CGG AGA GCA TC-3' (forward) and 5'-GAG GAA AGT GCA CCA TGT CA-3' (reverse) for cyclin B1; 5'-AGT GTG GCC AGA AGT GGA AT-3' (forward) and 5'-TTT CGA GAG CAA ATC CAA GC-3' (reverse) for CDK1; and 5'-CAG CCT CAA GAT CAT CAG CA-3' (forward) and 5'-TGT GGT CAT GAG TCC TTC CA-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase. PCR conditions were as follows: 96°C for 30 s; 55°C for 30 s; and 72°C for 30 s. Real time-PCR was done by DNA Engine Opticon (MJ Research, Waltham, MA) using the ×2 SYBR Green PCR core reagents mix (Applied Biosystem).

RESULTS

Synthesis of Intracellular Clusterin. Translation from the second in-frame ATG codon at position 152 of human clusterin coding sequence (GenBank accession no. M64722) located just downstream of the endoplasmic reticulum-targeting signal was suggested to yield a nuclear proapoptotic form of the protein (18, 20). To test this hypothesis also in the case of prostate cancer cells, we have transfected PC-3 cells with an expression vector (pIRES-hyg1) loaded with a shortened clusterin cDNA (intracellular-clusterin) that starts from the nucleotide 152 and, therefore, lacks the secretion signal sequence (Fig. 1A). As a positive control, cells were transfected with the full-length clusterin cloned in the same expression vector. As expected, the ATG₁₅₂ was able to initiate the translation of a very stable protein form of about *M_r* 50,000 that was not secreted but retained into the cells (Fig. 1B). The molecular weight of the clusterin isoform determined by electrophoresis was compatible with the predicted size of *M_r* 48,800, as determined by ExPASy Molecular Biology Server,³ thus, suggesting that this clusterin isoform is a nonglycosylated single-chain protein. The molecular weight difference between intracellular-clusterin and full-length clusterin was nearly *M_r* 20,000 by immunoblotting detection (Fig. 1B, cell extracts), although full-length clusterin sequence is only 11 amino acids longer after the loss of the secretion peptide (Fig. 1A). Consistently, although culture medium derived from full-length clusterin-transfected cells was rich with extracellular secreted clusterin, intracellular-clusterin transfected cells

³ Web address: <http://us.expasy.org>.

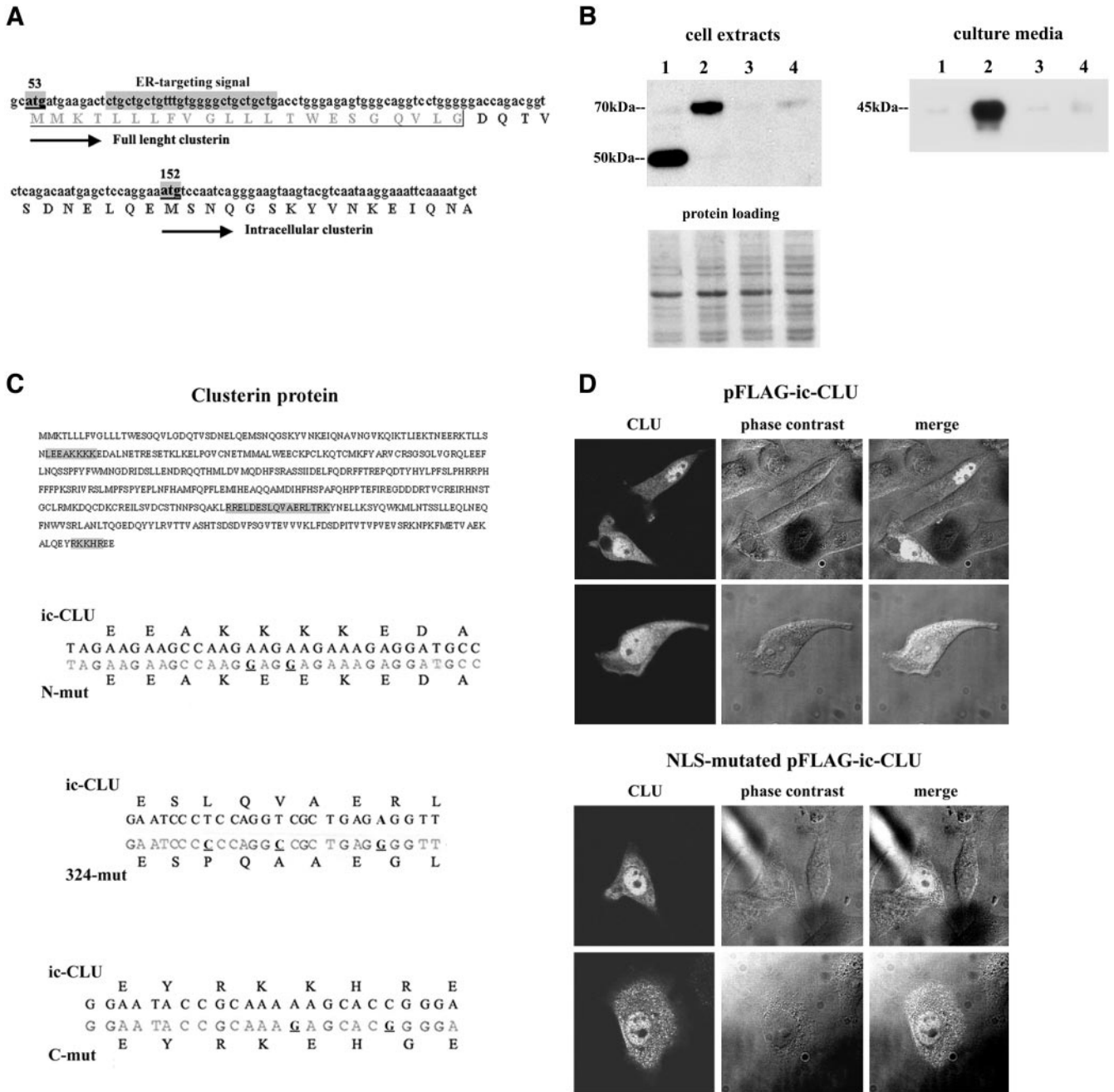


Fig. 1. A, schematic representation of the open reading frames coding for full-length clusterin and intracellular-clusterin (truncated, intracellular clusterin). Full-length clusterin starts from ATG at position 53, whereas intracellular-clusterin originates from ATG at position 152 of the same sequence (GenBank accession no. M64722). Both start codons and endoplasmic reticulum-targeting signals are highlighted in gray. Start codons are also underlined. After the removal of the first 22 amino acid residues (the secretion signal peptide, gray), full-length clusterin will be processed for secretion as a heterodimer and will contain 11 amino acids (DQTVSDNELQE) that are not present in intracellular-clusterin. The intracellular-clusterin cDNA has been cloned in both pIRES-hyg1 (intracellular-clusterin) and pFLAG-CMV-4 (pFLAG-intracellular-clusterin) expression vectors for transfection experiments (see Materials and Methods). The intracellular-clusterin cloned in pFLAG-CMV-4 has a short additional FLAG sequence just before ATG₁₅₂ that permits the specific detection of exogenous intracellular-clusterin instead of endogenous clusterin by using rabbit anti-FLAG (Sigma) antibodies. B, Western blots done with mouse antihuman clusterin (clone 41D, Upstate Biotechnology) showing that intracellular-clusterin was neither glycosylated nor secreted when overexpressed in PC-3 cells. Cell extracts were from PC-3 cells transiently overexpressing intracellular-clusterin (1), full-length clusterin (2), mock-transfected cells (3), or wild-type parental cells (4). M_r 70,000 full-length clusterin (2) is identical to the endogenous form (3, 4), whereas M_r 50,000 intracellular-clusterin shows on the gel the predicted size (M_r 48,800) of a polypeptide that is not glycosylated. As expected, only weak endogenous clusterin secretion was detectable in intracellular-clusterin overexpressing cell culture media (1), whereas clusterin secretion is evident in full-length clusterin overexpressing cells. Very faint bands of secreted endogenous clusterin are also present in mock-transfected (3) or wild-type parental cells (4). Ponceau S staining of the blot is provided to show equal loading of the protein samples. The data are representative of four independent experiments. C, human clusterin protein sequence (GenBank accession no. M64722) has three potential nuclear localization signals (highlighted in gray). Each one of the three nuclear localization signals has been mutated. The intracellular-clusterin and mutated intracellular-clusterins sequences are aligned for comparison. N-mut: two adenines have been changed to two guanines (underlined), leading to the change of two lysine residues to two glutamic acid residues; 324-mut: two thymines and one adenine have been changed to two cytosines and one guanine (underlined), leading to the change of one leucine, one valine, and one arginine to one proline and one alanine and one glycine, respectively; and C-mut: one adenine and one cytosine have been changed to two guanines (underlined), leading to the change of one lysine and one arginine to one glutamic acid and one glycine, respectively. D, Nuclear accumulation of intracellular-clusterin is nuclear localization signal-independent in PC-3 cells. Simultaneous mutations of all three nuclear localization signals (bottom panel; nuclear localization signal-mutated pFLAG-intracellular-clusterin) did not change the percentage of clusterin nuclear-positive cells in comparison with not mutated pFLAG-intracellular-clusterin (top panel) when PC-3 cells were transiently transfected with the appropriate constructs. The overall transfection efficiency in these experiments was not <50%, as assessed using pEGFP-N1 vector and detecting green fluorescence protein as reporter gene. The result is representative of four independent experiments in which at least eight different fields containing not <50 cells were analyzed. Not <75% of positively transfected cells exhibited nuclear localization of intracellular-clusterin. The immunocytochemistry experiment was obtained with rabbit anti-FLAG (Sigma) antibodies. Mouse antihuman clusterin (clone 41D, Upstate Biotechnology) gave the same result (see Materials and Methods). Magnification, ×63. (ic-CLU, intracellular-clusterin; CLU, clusterin; ER, endoplasmic reticulum; NLS, nuclear localization signal)

did not show any significant secretion activity of the overexpressed intracellular form of clusterin (Fig. 1B, culture media).

Nuclear Localization of Intracellular-Clusterin Is Nuclear Localization Signal-Independent. Although basal levels of clusterin in wild-type and mock-transfected PC-3 cells are barely detectable (Fig. 1B), we produced a flagged intracellular-clusterin form with the pFLAG-intracellular-clusterin vector to unambiguously discriminate exogenously transfected intracellular-clusterin from endogenous native clusterin. In fact, in our experimental model, flagged intracellular-clusterin is detectable by both anti-Flag and antihuman clusterin commercial antibodies (see Materials and Methods). Similarly to intracellular-clusterin, FLAG-intracellular-clusterin was not secreted, did not enter the endoplasmic reticulum, and was not glycosylated. Fig. 1D shows the intracellular localization of flagged intracellular-clusterin in transiently transfected PC-3 cells by immunocytochemistry analysis using rabbit anti-FLAG-specific antibody. About 75% of the positively transfected cells showed strong nuclear localization of intracellular-clusterin (Fig. 1D, upper plate). The use of mouse anti-human clusterin gave the same result, also showing that only low levels of cytoplasmic endogenous clusterin were present in mock-transfected cells (data not shown). Sequence analyses indicated the presence of three potential nuclear localization signals in human clusterin, starting from the NH₂ terminus to the COOH-terminus of the protein (Fig. 1C). The first nuclear localization signal (NLS_{n-term}) is LEEAKKKK, between the residues 74–81; the second (NLS₃₂₄) is RREDES LQVAERLTRK, between the residues 324–340; and the third (NLS_{c-term}) is RKKHR, between the residues 442–446 (Fig. 1C). To check whether these signal peptides were functional in determining pFLAG-intracellular-clusterin nuclear localization, we produced three pFLAG-intracellular-clusterin constructs carrying mutations in each one of the three nuclear localization signals (Fig. 1C). Then, we overexpressed three different pFLAG-intracellular-clusterin constructs carrying one (N-mut), two (N-mut + 324-mut), or three mutated nuclear localization signals (N-mut + 324-mut + C-mut). In each case, no significant differences were detected in nuclear localization of the protein after overexpression of all three mutated pFLAG-intracellular-clusterin constructs in transiently transfected PC-3 cells in comparison to the original pFLAG-intracellular-clusterin (Fig. 1D). Although we cannot rule out the existence and functionality of a noncanonical nuclear localization sequence, these results suggested a nuclear localization signal-independent mechanism driving nuclear clusterin migration/retention in PC-3 cells.

Transient pFLAG-Intracellular-Clusterin Overexpression Triggered Cell Death. Transient transfection of pFLAG-intracellular-clusterin in PC-3 cells caused a transitory increase of the S and G₂-M phase cell population, followed by progressive cell death induction, when compared with pFLAG mock controls (Fig. 2A). The increase in the rate of apoptosis was already significant 24 h after the beginning of experiments, becoming dramatic at 36 and 60 h (Fig. 2A; Table 1). We also treated PC-3 cells with the topoisomerase II inhibitor etoposide as a positive control of programmed cell death induction for comparison. Etoposide is a well-known chemotherapeutic drug capable of inducing apoptosis by enhancing enzyme-mediated DNA breaking in several cell lines (27–29). As shown in Fig. 2A, 24 h of 100- μ M etoposide treatment were sufficient to induce G₂-M arrest and apoptosis in PC-3 cells. Also, pFLAG-intracellular-clusterin overexpression caused a progressive increase of apoptosis rate, reaching at 60 h values that were very similar to those caused by 24 h etoposide [Fig. 2A and Table 1 compare etoposide (24 h) to pFLAG-intracellular-clusterin (60 h)]. The same experiments were carried out using unflagged intracellular-clusterin (pIRES-hyg1 vector) with equivalent results.

Decreased proliferation and increased cell death was specifically caused by caspase-dependent apoptosis induction when intracellular-

clusterin was transiently overexpressed in PC-3 cells. The administration of 5 μ M of z-VAD-fmk broad-spectrum caspase inhibitor significantly reduced proapoptotic activities of both pFLAG-intracellular-clusterin and etoposide in PC-3 cells (Fig. 2B; Table 1). This result was confirmed by measuring caspase-3 activity in both transfected (60 h) and etoposide-treated (24 h) cells (Fig. 2C). The significant increase of caspase-3 activity in both pFLAG-intracellular clusterin-transfected and etoposide-treated cells compared with pFLAG mock controls was abolished by z-VAD-fmk administration (Fig. 2C; Table 1). Caspase-3 activity of parental PC-3 cells was comparable with pFLAG mock controls (data not shown). As a consequence of z-VAD-fmk-mediated rescue, both etoposide-treated and pFLAG-intracellular clusterin-transfected cells exhibited a significant increase in the number of cells in G₂-M phases of the cell cycle (Fig. 2B; Table 1), suggesting the involvement of G₂-M cell cycle checkpoint induction of caspase-cascade in intracellular-clusterin-mediated toxicity.

Recombinant PC-3 Cell Clones Stably Overexpressing pFLAG-Intracellular-Clusterin Showed Reduced Proliferation and Mitotic Catastrophe. Stable transfection of pFLAG-intracellular-clusterin in PC-3 cells dramatically decreased colony formation ability in comparison with pFLAG-transfected cells. Both number and size of pFLAG-intracellular clusterin-derived cell colonies were reduced drastically in comparison with controls (Fig. 3A). The few pFLAG-intracellular clusterin-resistant clones recovered (pFLAG-intracellular-clusterin #1, pFLAG-intracellular-clusterin #2, and pFLAG-intracellular-clusterin #3) were slow-growing cells (Fig. 3B) displaying variable expression of the M_r 50,000 intracellular-clusterin protein form (Fig. 3C). At the same time, endogenous clusterin form was expressed at low levels as in the case of mock control clones (pFLAG #1, pFLAG #2, and pFLAG #3), in which only the presence of M_r 70,000 clusterin was detectable (Fig. 3C). Cell growth analysis, done on each one of the stable pFLAG-intracellular-clusterin overexpressing clones mentioned above, showed impairment of cell proliferation rate when compared with mock controls (Fig. 3B). This phenomenon was consistent with the finding of a strong increase in abnormal mitosis figures, leading to formation of giant multinucleated cells (Fig. 4A). Not <30% of total pFLAG-intracellular-clusterin recombinant cells showed endoreduplication and several micronuclei. These cells were unable to carry out successfully the cell division. Intriguingly, z-VAD-fmk administration was not effective at significantly inhibiting cell death in these recombinant clones (Fig. 4B; Table 1). This data suggests strongly that the high rate of cell death displayed by these cells is likely because of caspase-independent apoptosis. In the FACS analysis experiments, most of the giant multinucleated cells were recognized as cell doublets and, therefore, excluded by the cell sorter. Intriguingly, intracellular-clusterin localization in stably transfected cells surviving to intracellular-clusterin overexpression was confined exclusively to the cytoplasm (Fig. 4A), suggesting that inhibition of intracellular-clusterin translocation to the nucleus might be involved in escaping apoptotic doom by PC-3 cells.

It is known that the CDK1 interacts with cyclin B1 to form the “mitosis-promoting factor,” the activity of which regulates the cell cycle timing of mitosis (30). The intracellular-clusterin-overexpressing stable clones showed lower levels of both CDK1 and cyclin B1 in comparison with mock control clones (Fig. 5, A and C). This peculiar expression profile of both CDK1 and cyclin B1 was independent from cell confluence and/or synchronization (data not shown). Real-time PCR experiments (Fig. 5B) showed that down-regulation of these two genes in recombinant clones was not because of transcriptional suppression caused by intracellular-clusterin. Furthermore, direct protein-protein interaction between intracellular clusterin and both cyclin B1 and CDK1 were excluded by coimmunoprecipitation analyses (data not shown).

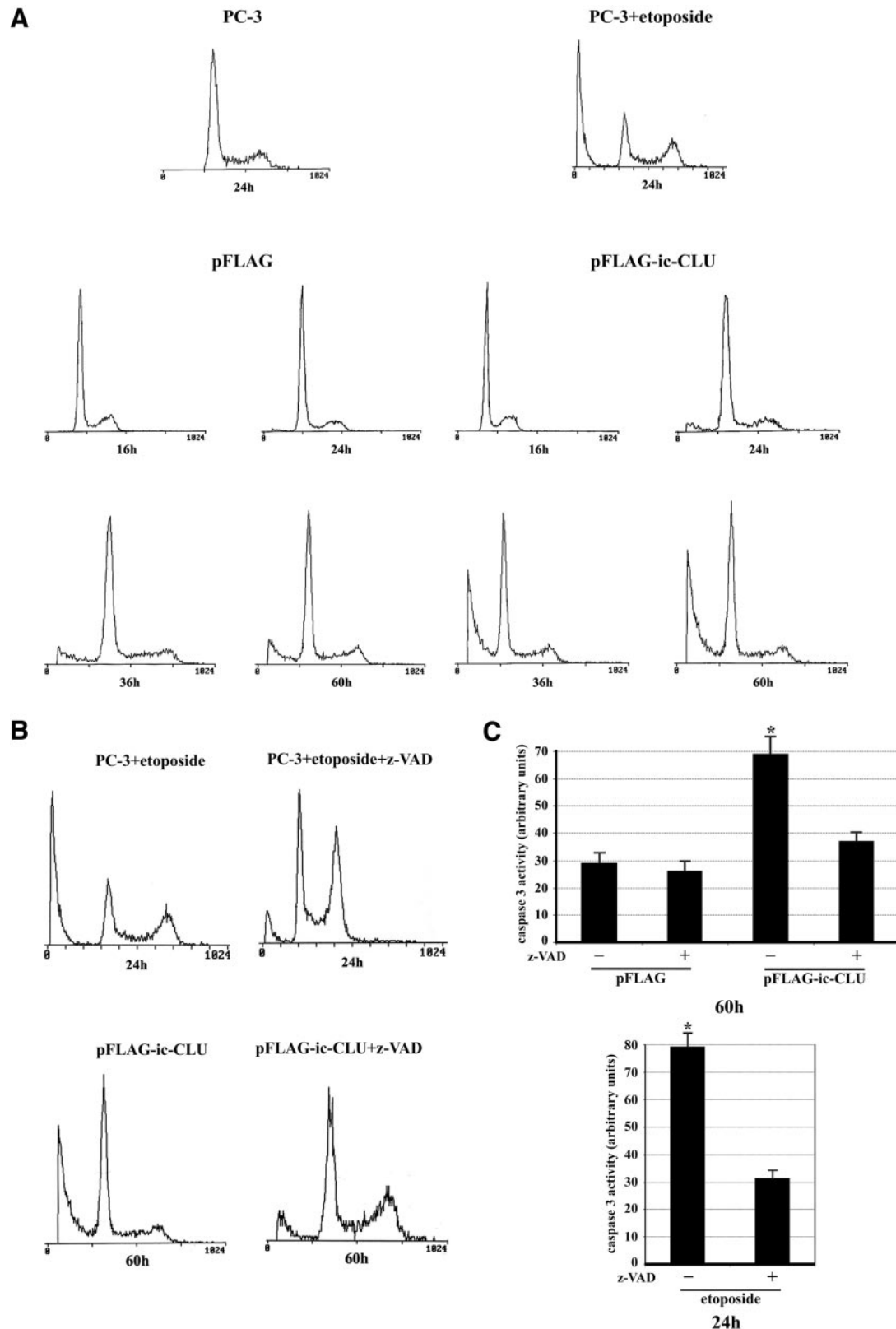


Fig. 2. *A*, Transient transfection of flagged-intracellular-clusterin (pFLAG-intracellular-clusterin) caused time-dependent increase of cell death when compared with mock controls transfected with the empty vector (pFLAG). Quantification and statistical analysis of the data are provided in Table 1. The intracellular-clusterin overexpression progressively increased cell death to levels very close to those obtained by etoposide treatment (see Table 1). The cell debris accumulation was always significantly higher when compared with controls from 24- to 60-h transfection time, whereas S + G₂-M phase increase was significant only at 16 h (see Table 1). Transfection done with pIRES-intracellular-clusterin gave the same result when compared with pIRES-hyg1 mock-transfected controls. The result is representative of four independent experiments. *B*, Both etoposide treatment and pFLAG-intracellular-clusterin overexpression induced caspase-dependent apoptosis. Quantification and statistical analysis of the data are provided in Table 1. Transfection done with pIRES-intracellular-clusterin gave the same result when compared with pIRES-hyg1 mock-transfected controls. The data are representative of four independent experiments. *C*, Both pFLAG-intracellular clusterin-transfected (60 h) and etoposide-treated (24 h) cells showed a significant increase of caspase-3 activity, with respect to parental PC-3 and pFLAG controls. The increase in caspase-3 activity was specifically inhibited by z-VAD-fmk administration. Parental PC-3 cells and pFLAG mock-transfected cells showed comparable levels of caspase-3 activity in this experimental model. The data are representative of three independent experiments. Bars, \pm SD. (*ic-CLU*, intracellular-clusterin) (t test; *, $P < 0.01$).

Table 1 Flow cytometry data analysis of transfected and etoposide treated PC-3 cells

Cell culture conditions	S + G ₂ -M (%)	Cell debris (%)
PC-3 24 h	38.2 ± 2.8	3.8 ± 1.2
PC-3 + etoposide 24 h	31.2 ± 3.5	52.8 ± 5.7*
PC-3 + etoposide 24 + z-VAD-fmk	58.6 ± 5.1†	19.1 ± 2.9†
pFLAG 16 h	40.3 ± 2.4	4.5 ± 1.5
pFLAG-ic-CLU 16 h	55.3 ± 3.1‡	4.5 ± 1.1
pFLAG 24 h	35.6 ± 3.2	8.6 ± 1.8
pFLAG-ic-CLU 24 h	34.9 ± 2.4	17.1 ± 2.6§
pFLAG 36 h	29.8 ± 1.9	18.3 ± 3.2
pFLAG-ic-CLU 36 h	25.4 ± 2.2	38.6 ± 3.7
pFLAG 60 h	28.4 ± 2.1	20.8 ± 2.8
pFLAG-ic-CLU 60 h	21.3 ± 1.3	44.4 ± 4.5¶
pFLAG-ic-CLU 60 h + z-VAD-fmk	43.2 ± 4.4**	20.3 ± 2.8**
pFLAG stable clones	27.4 ± 3.1	17.7 ± 2.8
pFLAG stable clones + z-VAD-fmk	25.6 ± 2.3	14.3 ± 2.2
pFLAG-ic-CLU stable clones	34.7 ± 4.2	37.1 ± 4.6††
pFLAG-ic-CLU stable clones + z-VAD-fmk	37.8 ± 3.9	34.4 ± 4.1††

NOTE: These values were calculated from data collected from four independent experiments.

Abbreviation: ic-CLU, intracellular-clusterin.

* *T* test, *P* < 0.01 versus PC-3 24 h.

† *T* test, *P* < 0.01 versus PC-3 + etoposide 24 h.

‡ *T* test, *P* < 0.01 versus pFLAG 16 h.

§ *T* test, *P* < 0.01 versus pFLAG 24 h.

|| *T* test, *P* < 0.01 versus pFLAG 36 h.

¶ *T* test, *P* < 0.01 versus pFLAG 60 h.

** *T* test, *P* < 0.01 versus pFLAG-ic-CLU 60 h.

†† *T* test, *P* < 0.01 versus pFLAG stable clones.

DISCUSSION

The link between clusterin overexpression and cell stress, tissue degeneration and apoptosis is well established in the literature, but whether clusterin accumulation represents a cause or a consequence of apoptotic death still remains an open question. In the attempt to explain controversial findings and opposite hypothesis, the possibility of a double feature for this gene has been suggested recently. Depending on the cell metabolic contest and stimulus, clusterin would have both pro- and anti-apoptotic properties, probably because of different protein products (clusterin isoforms) coded by the same gene. In this view, a secreted form of clusterin would act as a cytoprotective protein (31–33), also acting as an extracellular chaperone-inducing phagocytosis (34, 35), although a nuclear form, possibly starting from a second in-frame ATG, was suggested to be a cell death protein (18, 20). As a consequence of different pro-apoptotic stimuli (and/or through different mechanisms not elucidated completely yet), the biosynthesis of a not-secreted, truncated form of clusterin would take place in the cell, and its accumulation would commit cells to apoptotic death.

Our earlier works showed that clusterin is subjected to early down-regulation in prostate cancer cells (14, 15, 36) and that clusterin overexpression inhibited cell cycle progression of SV40-immortalized human prostate epithelial cells (37). Thus, we studied the biological effects caused by intracellular-clusterin transient and stable overexpression in PC-3 androgen-independent prostate cancer cells. Our experiments showed that the second in-frame ATG positioned at nucleotide 152 of full-length human clusterin cDNA sequence (accession no. M64722) can be activated by deletion of the 5' flanking sequence containing the first ATG codon. Transient overexpression experiments showed that a shortened intracellular-clusterin is synthesized as a stable product and accumulated in PC-3 cells. The intracellular-clusterin protein had an estimated size of M_r 50,000, consistent with the hypothesis that it is a nonglycosylated protein monomer. In fact, its secretion rate was undetectable and showed the expected

nuclear localization in PC-3 cells (Fig. 1, B and D). At least 75% of the transiently transfected cell population (average transfection efficiency of PC-3 was >50%) showed intracellular-clusterin in the nucleus, regardless of mutations in all three potential nuclear localization signal signals present in the sequence (Fig. 1, C and D). Consistent with the observations of O'Sullivan *et al.* (19), these

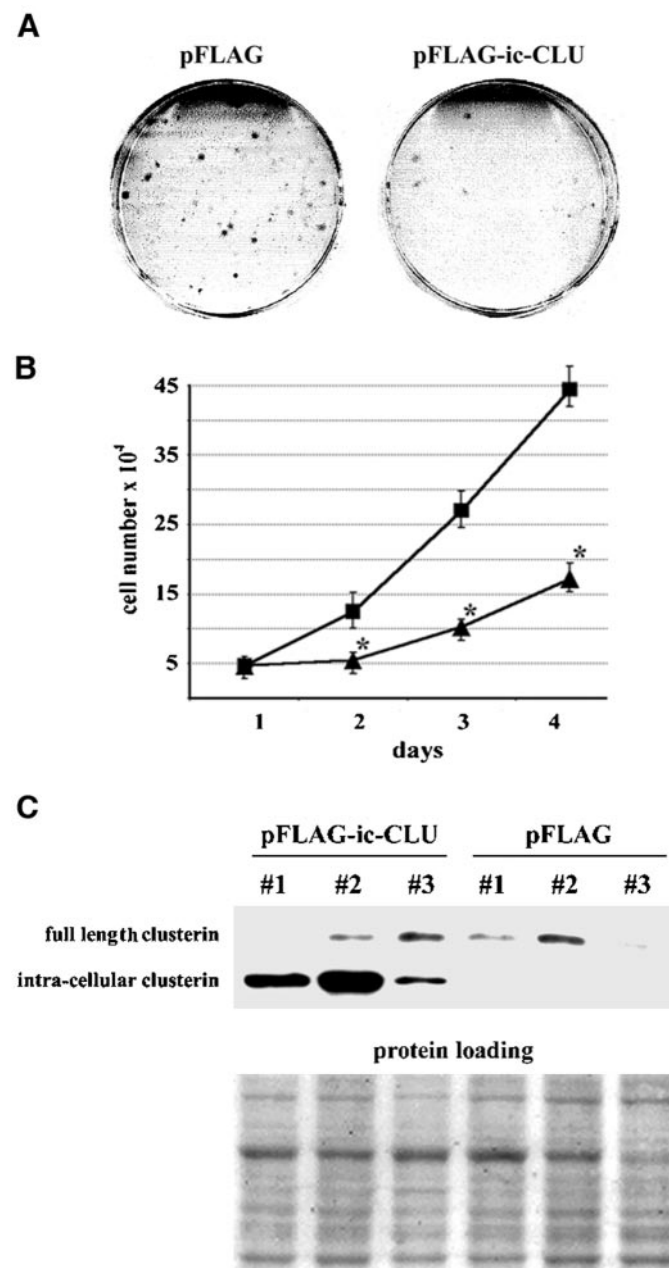


Fig. 3. A, clonogenic toxicity of intracellular-clusterin. Colony formation capability of PC-3 cells during G418 selection of recombinant clones with pFLAG-intracellular-clusterin was ~10-fold lower than pFLAG control cells. The data are representative of four independent attempts to establish recombinant PC-3 cells. B, growth curve of pFLAG (■) and pFLAG-intracellular-clusterin (▲) survivor recombinant clones. The pFLAG-intracellular-clusterin cells showed a doubling time that was increased significantly when compared with pFLAG controls. The data are the mean of three independent cell growth experiments done with each one of the three recombinant clones; bars, ±SD (*n* = 9). Statistically significant differences are indicated (*t* test; *, *P* < 0.01). C, Western blot analysis showing the level of expression of the M_r 50,000 intracellular-clusterin in recombinant, stably transfected PC-3 survivor clones. Data were obtained with mouse antihuman clusterin (clone 41D, Upstate Biotechnology). The pFLAG mock control clones express only the endogenous M_r 70,000 form. Ponceau S staining of the blot is provided to show equal loading of the protein samples. The data shown are representative of four independent experiments. (ic-CLU, intracellular-clusterin)

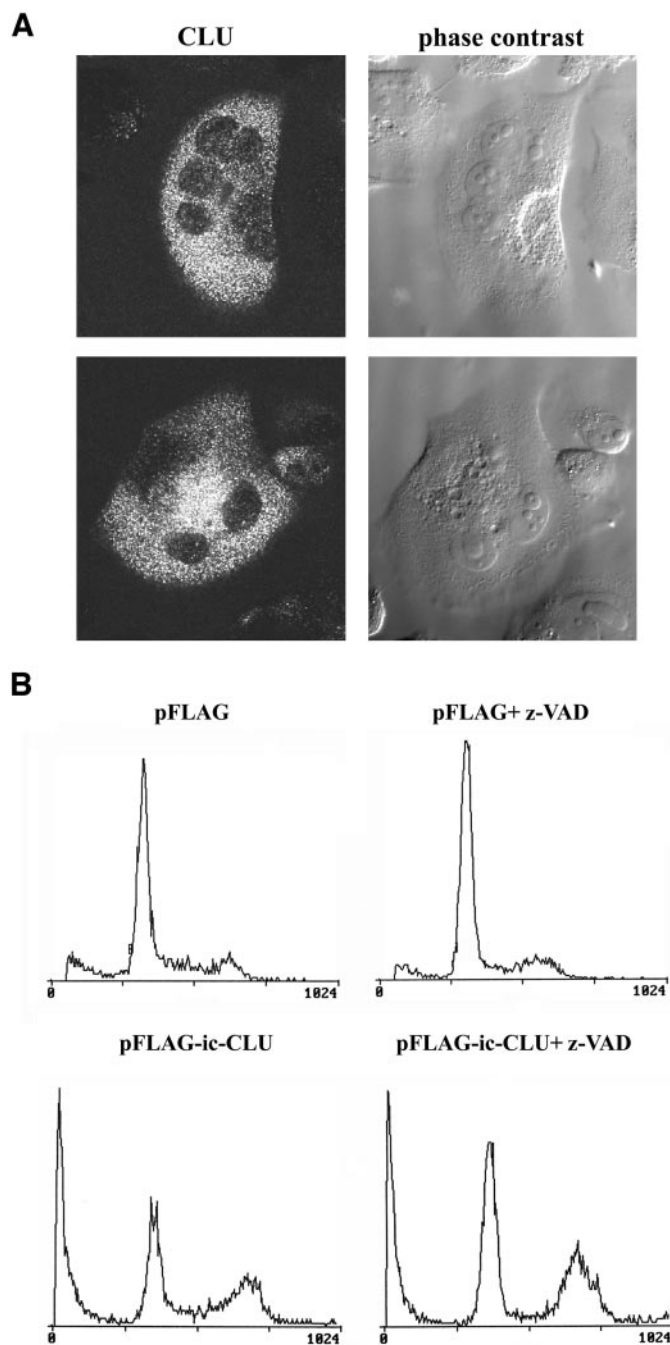


Fig. 4. *A*, Immunocytochemistry experiments showing multinucleation (endoreduplication) of pFLAG-intracellular-clusterin-survivor clones overexpressing intracellular clusterin. In these cells, clusterin was found exclusively in the cytoplasm. Both antihuman clusterin and anti-FLAG antibodies were used and gave the same results (see Materials and Methods). The result is representative of intracellular-clusterin subcellular localization determined in all three intracellular-clusterin recombinant clones considered in Fig. 3C, as determined in at least eight different fields, containing not <50 cells in each case. Magnification, $\times 63$. *B*, Fluorescence-activated cell sorter analyses of pFLAG and pFLAG-intracellular-clusterin clones showing higher levels of apoptosis in surviving, intracellular-clusterin overexpressing PC-3 recombinant cells. Quantification and statistical analysis of the data are provided in Table 1. In this case, treatment with z-VAD-fmk caspases inhibitor was ineffective at inhibiting apoptosis rate (compare to Fig. 2B). The data shown are representative of four independent experiments. (*CLU*, clusterin; *ic-CLU*, intracellular-clusterin)

findings suggest nuclear localization signal-independent mechanisms for intracellular-clusterin nuclear accumulation in PC-3 cells.

Concomitant to transient intracellular-clusterin overexpression, PC-3 cells undergo progressive increase in caspase-dependent apo-

ptosis as a consequence of the early increase of S and G₂-M phase cell population. The ability of etoposide to induce cell cycle arrest at the G₂-M checkpoint followed by apoptosis is well known in many biological models, including prostate cells (38). A comparison between the two systems showed that intracellular-clusterin overexpression and etoposide caused similar changes along cell cycle progression of PC-3 cells. The blocking of caspase-cascade by z-VAD-fmk administration resulted in apoptosis inhibition and G₂-M phase arrest in both etoposide-treated and intracellular-clusterin transiently transfected cells (Fig. 2B). These results suggest that the triggering of G₂-M cell cycle checkpoint-induced apoptosis by intracellular-clusterin nuclear accumulation is caspase-dependent.

The proapoptotic activity of intracellular-clusterin was also confirmed by establishing recombinant PC-3 clones stably overexpressing intracellular-clusterin. After transfection and antibiotic selection, intracellular-clusterin constitutive expression markedly reduced colony formation capability, thus showing clonogenic toxicity (Fig. 3A). Moreover, the few clones surviving intracellular-clusterin overexpression showed a decreased proliferation rate when compared with mock controls (Fig. 3B). Furthermore, intracellular-clusterin clones displayed distinct alterations in cell shape, size, and DNA content. At least 30% of the intracellular-clusterin overexpressing cells seemed unable to carry on cell division, leading to the formation of giant multinucleated cells. The percentage of giant cells did not change on several cell culture passages, suggesting a continuous production of mitotic catastrophes in intracellular-clusterin recombinant clones. We can hypothesize that survivor intracellular-clusterin cells have developed a certain degree of resistance to intracellular-clusterin-mediated apoptosis during G418-growth as a positive selection response. Thus, this altered phenotype might be the result of such adaptation. As a matter of fact, these cells acquired the capability to survive intracellular-clusterin overexpression, but their proliferation rate was much slower than controls. Intriguingly, this feature is believed to be possessed by transformed cells within advanced prostate cancer, a kind of tumor in which inhibition of caspase-cascade dependent cell death is observed frequently in strict association to slow proliferation rate and gross genetic alterations (39, 40). Interestingly, in all of the survivor clones, intracellular-clusterin was found exclusively in the cytoplasm. Thus, a positive selection of only those recombinant cells with no or very low (tolerable?) nuclear intracellular-clusterin localization was very likely to take place. These findings emphasize the importance of nuclear clusterin localization for the balance between cell death and viability in PC-3 cells. The still unknown mechanism through which intracellular intracellular-clusterin was retained in the cytoplasm during clone selection does not necessarily involve mutations on any of the nuclear localization signals present in the human clusterin cDNA.

Silencing of clusterin expression by small interfering RNA was reported recently (41) and found to induce apoptosis and cell sensitization to genotoxic stress in PC-3 cells. Interestingly, small interfering RNA did not exert any effect on the nuclear form of clusterin and had only minor effects on the cytoplasmic form of the protein. Thus, the reported induction of cell death by small interfering RNA was very likely because of specific inhibition of the extracellular protein production. These findings, together with the data presented here, may suggest a possible explanation of conflicting results obtained in different laboratories, suggesting that clusterin might be a proapoptotic or survival gene. In fact, given that clusterin expression and accumulation within the cell are enhanced because of specific stimuli, the kind of clusterin protein isoform prevailing in the cell and its subcellular localization (*i.e.*, in the nucleus or in the cytoplasm) might be the key for interpretation of the biological effects. Thus, this hypothesis would open new perspectives for the characterization of androgen-independent and apoptosis-resistant prostate cancer cells,

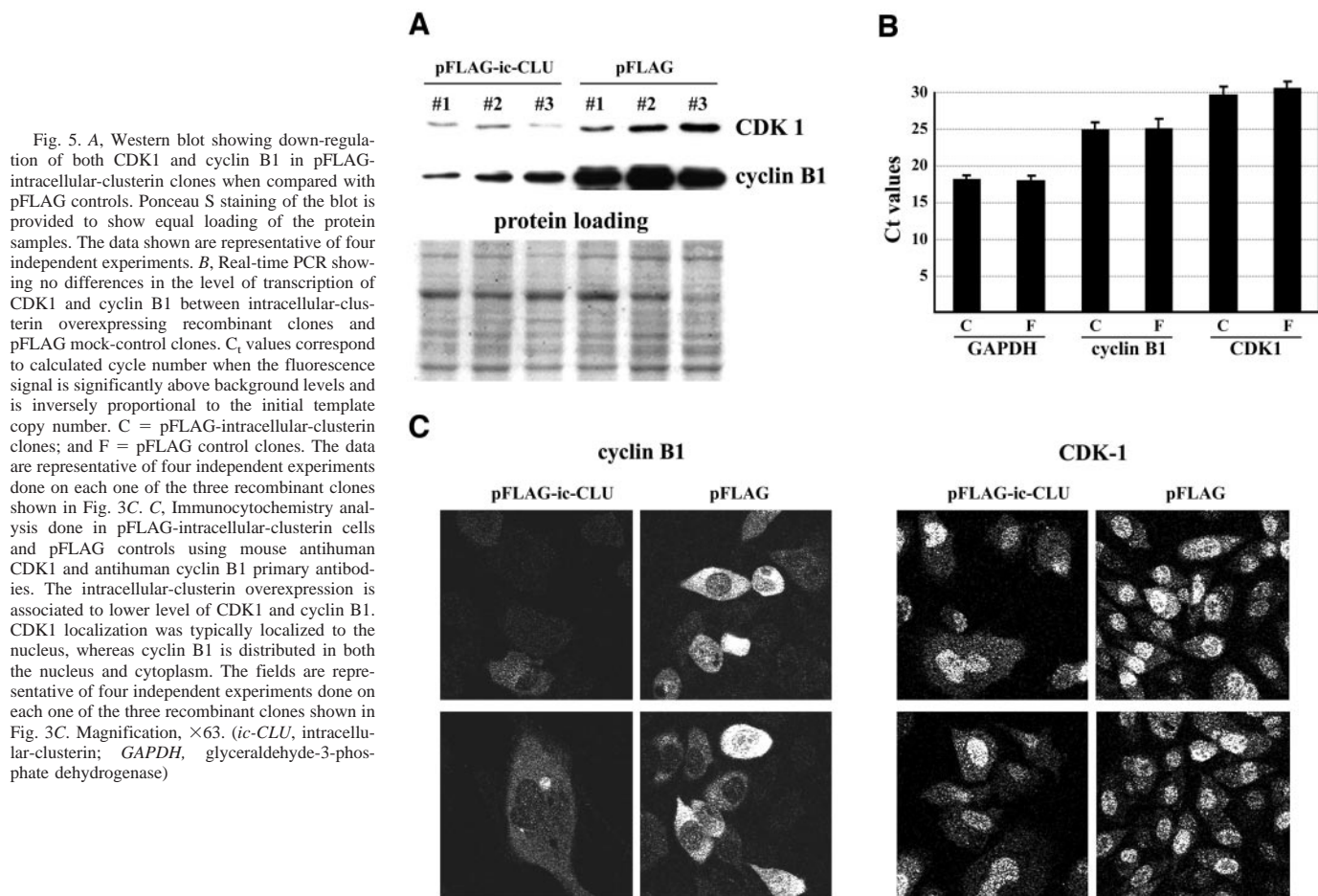


Fig. 5. *A*, Western blot showing down-regulation of both CDK1 and cyclin B1 in pFLAG-intracellular-clusterin clones when compared with pFLAG controls. Ponceau S staining of the blot is provided to show equal loading of the protein samples. The data shown are representative of four independent experiments. *B*, Real-time PCR showing no differences in the level of transcription of CDK1 and cyclin B1 between intracellular-clusterin overexpressing recombinant clones and pFLAG mock-control clones. Ct values correspond to calculated cycle number when the fluorescence signal is significantly above background levels and is inversely proportional to the initial template copy number. C = pFLAG-intracellular-clusterin clones; and F = pFLAG control clones. The data are representative of four independent experiments done on each one of the three recombinant clones shown in Fig. 3C. *C*, Immunocytochemistry analysis done in pFLAG-intracellular-clusterin cells and pFLAG controls using mouse antihuman CDK1 and antihuman cyclin B1 primary antibodies. The intracellular-clusterin overexpression is associated to lower level of CDK1 and cyclin B1. CDK1 localization was typically localized to the nucleus, whereas cyclin B1 is distributed in both the nucleus and cytoplasm. The fields are representative of four independent experiments done on each one of the three recombinant clones shown in Fig. 3C. Magnification, $\times 63$. (*ic-CLU*, intracellular-clusterin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase)

because increased accumulation of clusterin in the cytoplasm would be a molecular event marking the acquisition of resistance to caspase-dependent apoptosis and/or lack of cell cycle control exerted by androgens and other growth factors. As a matter of fact, we have described already this event in rare foci of transformed cells detected in human prostate cancer specimens from androgen-ablated patients (15).

The morphological alterations detected in recombinant intracellular-clusterin clones correlated with down-regulation of both cyclin B1 and CDK1. The cyclin B1/CDK1 complex is required for correct transition from G₂ to M phase of the cell cycle. An inactivation and/or down-regulation of both CDK1 and cyclin B1 is described to cause mitotic catastrophe and induce cell death in several experimental models (42–44). A correlation between down-regulation of the cyclin B1/CDK1 mitotic complex and occurrence of endoreduplication was found previously in maize and fruit flies (43). Thus, we can speculate that overexpression of intracellular-clusterin may provoke G₂-M arrest, leading to apoptosis through cyclin B1/CDK1 complex deregulation in PC-3 cells. At present, we are unable to define a precise pathway that links clusterin to the mitotic complex. However, it seems unlikely that intracellular-clusterin modulation of these factors might be at the transcriptional level, or because of a direct protein-protein interaction between intracellular-clusterin and cyclin B1/CDK1 complex. This suggests that ubiquitin-dependent proteolysis of cyclin B1, leading to cyclin B1/CDK1 down-regulation, might be enhanced after intracellular-clusterin overexpression.

Taken together, our results indicate a proapoptotic role for the truncated form of clusterin (*i.e.*, intracellular-clusterin), which specifically affects cell cycle regulation through the cyclin B1/CDK1 com-

plex in PC-3 prostate cancer cells. We believe that prostate cancer management will benefit from additional studies aimed at discovering new therapeutic approaches inducing clusterin nuclear shuttling in highly malignant prostate cancer cells.

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