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Clusterin Isoforms Differentially Affect Growth and Motility of Prostate Cells: Possible Implications in Prostate Tumorigenesis

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Abstract

Besides a fully processed, secreted form of clusterin (sCLU), an alternative proapoptotic form of the protein targeting the nucleus (nCLU) was recently described. The possible differential roles played by the two clusterin forms in growth and motility of nonmalignant and malignant prostate cells are investigated here. sCLU or nCLU was transiently transfected in both androgen-independent prostate cancer cells (PC3 and DU 145) and immortalized prostate epithelial cells (PNT1A, a nontumoral control). Then, cell growth, motility, and cytoskeleton organization were studied. We found that (*a*) in PNT1A cells, both sCLU and nCLU significantly decreased cell proliferation and motility; (*b*) in PC3 and DU 145 cancer cells, only nCLU inhibited cell growth and migration, with sCLU being ineffective; and (*c*) the antimotility effect of nCLU was accompanied by a dramatic dismantling of the actin cytoskeleton. Moreover, transfection with "full-length" CLU cDNA produced both sCLU and nCLU in nonmalignant PNT1A cells, whereas only sCLU was found in cancer cells. Thus, *CLU* gene expression might play a crucial role in prostate tumorigenesis by exerting differential biological effects on normal versus tumor cells through differential processing of CLU isoforms in the two cell systems. We also found that nCLU binds to α-actinin, a key protein for the regulation of actin cytoskeleton, and that nCLU and α-actinin colocalize in the cytoplasm. Thus, the antimotility activity of nCLU and its ability to cause dismantling of the actin cytoskeleton seem to be mediated by its binding to α-actinin. [Cancer Res 2007; 67(21):10325–33]

Introduction

Clusterin (CLU) is a gene/protein highly conserved and expressed in several human tissues and fluids. Also known as testosterone repressed prostate message 2, apolipoprotein J, or sulfated glycoprotein 2 (1, 2), CLU has many biological functions (1, 2). CLU is considered an enigmatic protein because of the difficulties in the definition of its precise functions; at the moment, an important debate concerns its implication in carcinogenesis, particularly in prostate cancer development. On this issue, the data currently available are controversial because CLU has been suggested as being either a proapoptotic or a prosurvival factor (3–9).

The recent detection of two possibly related protein forms produced by the same gene has depicted a new scenario that might help to unravel its possible role. The *CLU* gene is located on human chromosome 8 (10). *CLU* gene translation produces a protein precursor that is driven by an initial signal peptide to the endoplasmic reticulum, where it is cleaved into two distinct peptides (α and β) held together by five disulfide bonds. The product is then glycosylated, and the mature heterodimeric glycoprotein, named sCLU, is secreted (1, 11, 12). An alternative isoform of CLU has recently been identified and named nuclear CLU (nCLU; refs. 13, 14). nCLU is synthesized from a second in-frame AUG codon located in position 152. AUG_{152} becomes functional by (*a*) alternative splicing of CLU mRNA (13) or (*b*) alternative initiation of translation (14). As a consequence, nCLU lacking the leader peptide would not enter the endoplasmic reticulum, thus skipping α/β cleavage and glycosylation, mainly localizing in the nucleus (1, 6, 9, 14, 15). The existence of sCLU and nCLU might explain the contradictory roles apparently played by *CLU* gene in the control of cell growth processes: sCLU would act as a survival factor whereas nCLU would be responsible for cell death induction (5–7, 9, 13, 15–18).

Prostate cancer is the second leading cause of male cancer deaths in Western countries (19). The disease is initially androgen dependent, and androgen-deprivation therapy represents the first-line treatment for prostate cancer patients (20). After an initial remission, prostate carcinoma progresses toward a condition of hormone resistance, characterized by high proliferation rate, strong metastatic behavior, and refractoriness to classic chemotherapy (21). CLU level of expression has been found to be either down-regulated (22–24) or up-regulated (25) in prostate cancer specimens. Antisense oligonucleotides and small interfering RNAs (siRNA) targeting the full-length CLU have been reported to induce apoptosis and chemosensitivity *in vitro* in prostate cancer xenografts (26–29) and in prostate cancer patients (30, 31). On the other hand, nCLU accumulation has been observed in dying prostate cells challenged with proapoptotic stimuli (4, 5, 17). Overexpression of nCLU has been reported to induce G_2-M phase arrest and caspase-dependent apoptosis in prostate cancer cells (6). Thus, although the role played by *CLU* gene in prostate cancer development has not been fully clarified, the data currently available suggest that the specific protein forms that are prevalently expressed within the cell and their subcellular localization might be crucial for understanding the function(s) of this enigmatic gene. As a confirmation of this, increase of nCLU expression during antisense oligonucleotide or siRNA targeting has been considered as a possible explanation for chemosensitization of breast cancer cells (32). These observations suggest that sCLU is a prosurvival factor, whereas nCLU is proapoptotic. The relative balance between sCLU and nCLU might drive cell fate and play an important role in cell transformation.

The aim of this work was to clarify whether, and through which mechanisms, *CLU* gene plays a role in the control of growth and metastatic behavior of prostate cancer cells. Thus, sCLU and nCLU have been transiently overexpressed in prostate cancer cells (PC3 and DU 145) and in SV40-immortalized PNT1A prostate cells, a nontumorigenic cell model. The biological effect of such maneuver has been studied by evaluating cell growth, cell motility, and cytoskeleton organization. The results obtained have been interpreted in relation to differential processing and intracellular localization of clusterin in normal versus cancer cells.

In addition, we used the two-hybrid approach and coimmunoprecipitation and colocalization analyses in the search for nCLU binding partners and molecular interactions that might explain the effects of this protein form on the migratory behavior of prostate cells.

Materials and Methods

Materials

Antibodies used were mouse anti-human clusterin antibody (clone 41D, Upstate Biotechnology); mouse anti-human actin antibody (clone Ab-1, Oncogene); mouse anti-human α-actinin antibody (Chemicon International); mouse anti-human pan-cytokeratin antibody (clone PCK-26, Sigma Chemical Co.); polyclonal goat anti-human α-actinin antibody (C20, Santa Cruz Biotechnology); and FITC-conjugated goat anti-mouse (Alexa Fluor 488), TRITC-conjugated goat anti-mouse (Alexa Fluor 594), and FITC-conjugated rabbit anti-goat (Alexa Fluor 488) secondary antibodies (Molecular Probes, Inc.). FITC-phalloidin, secondary horseradish peroxidase–conjugated rabbit anti-mouse antibody, and laminin were from Sigma Chemical. Fibronectin was from Biochrom.

Cell Cultures

PNT1A cell line was established by immortalization and cloning of normal prostate epithelial cells with SV40 large T antigen (33, 34). PC3 and DU 145 androgen-independent prostate cancer cells were purchased from the American Tissue Culture Collection. PNT1A and PC3 cell lines were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glutamine (1 mmol/L), and antibiotics (100 IU/mL penicillin G sodium and 100 μg/mL streptomycin sulfate). DU 145 cells were grown in the same medium supplemented with 5% FBS. Cells were incubated at 37°C in 5% CO_2.

Expression Vectors and Transfections

Full-length human clusterin cDNA was generated as described (15). Briefly, reverse transcription-PCR of normal human fibroblast total RNA was done using the primers 5′-GACTCCAGAATTGGAGGCATG-3′ (forward) and 5′-ATCTCACTCCTCCCGGTGCT-3′ (reverse). The cDNA was cloned into pGEM-T Easy (Promega). CLU full-length cDNA was then subcloned into the bicistronic expression vector pIRES-hyg (Clontech) to produce the pIRES-sCLU vector. A truncated CLU cDNA fragment was then amplified from the full-length expression vector using the primers 5′-GTCTCAGA-CAATGGGATCCAGGA-3′ (forward) and 5′-GACCTGCAGGCGGCCGCGA-AT-3′ (reverse). Truncated cDNA was inserted in pIRES-hyg to generate the pIRES-nCLU vector (6). Constructs have been sequenced before carrying out expression experiments. Transfections were done with the nonliposomal reagent FuGENE 6 (Roche). Cells (10^5) were plated in 35-mm dishes and transiently transfected with 3 μg/dish of plasmid DNA (pIRES-sCLU or pIRES-nCLU). In each experiment, control cells were transfected with the empty vector pIRES-hyg. Efficiency of transfection was routinely assessed by green fluorescence protein (GFP) expression using the pCMV-GFP-LpA vector (Clontech Laboratories). Transfection efficiency was usually higher than 50% of total cells (6). The size of the pCMV-LpA vector is comparable with that of the vector used for CLU overexpression. The high efficiency of the procedure used is shown by the detection of the protein bands by Western blotting (Fig. 1) and of CLU fluorescence signals by immunocytochemistry (Figs. 3 and 4).

Cell Growth Assays

Transiently transfected PNT1A, PC3, and DU 145 cells, grown in 35-mm dishes, were harvested and counted with a hemocytometer at different time intervals (24–72 h) after transfection. Each experimental condition was replicated six times and each experiment was repeated thrice. The data were analyzed by one-way ANOVA followed by Bonferroni's test.

Western Blotting

Whole-cell extracts. Transfected cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [0.05 mol/L Tris-HCl (pH 7.7), 0.15 mol/L NaCl, 0.8% SDS, 10 mmol/L EDTA, 100 μmol/L NaVO₄, 50 mmol/L NaF, 0.3 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L iodoacetic acid] containing leupeptin (50 μg/mL), aprotinin (5 μl/mL), and pepstatin (50 μg/mL). The extracts were centrifuged to remove insoluble material.

Culture media. Conditioned media obtained from transfected cell cultures were centrifuged at $12,000 \times g$ for 10 min and 30 μL of supernatants were processed for Western blotting. Pan-cytokeratin expression was used as loading control.

Cytoplasm and nuclear extracts. Transfected cells were prepared using the Nuclear Extraction kit (Chemicon International). Protein concentration was determined using the bicinchoninic acid method.

Whole-cell extracts (30 μg), culture media (30 μL), and cytoplasm and nuclear extracts (50 μg) were resuspended in sample buffer [0.5 mol/L Tris-HCl (pH 6.8), 20% glycerol, 10% SDS, 0.2% 2β-mercaptoethanol, 0.05% blue bromophenol] and heated at 95°C for 5 min. After electrophoretic separation by 10% SDS-PAGE, proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 3% bovine serum albumin (BSA) before incubation at room temperature for 2 h with the mouse anti-clusterin primary antibody (1:1,000). Detection was done using a horseradish peroxidase–conjugated anti-mouse secondary antibody and enhanced chemiluminescence reagents (SuperSignal Chemiluminescence Detection System, Pierce Biotechnology, Inc.). Lack of contamination by cytoplasm proteins in nuclear extracts was monitored by Western blotting of cytokeratin expression on membranes after stripping the blots and blocking nonspecific sites. Mouse monoclonal anti-human pan-cytokeratin was used at a dilution of 1:100,000.

Actin and α-actinin expression was detected in whole-cell extracts from PC3 cells transfected with nCLU. Protein preparations (30 μg) were processed for Western blotting as described above with mouse anti-human actin (1:5,000) or mouse anti-human α-actinin (1:1,000) as primary antibodies, respectively.
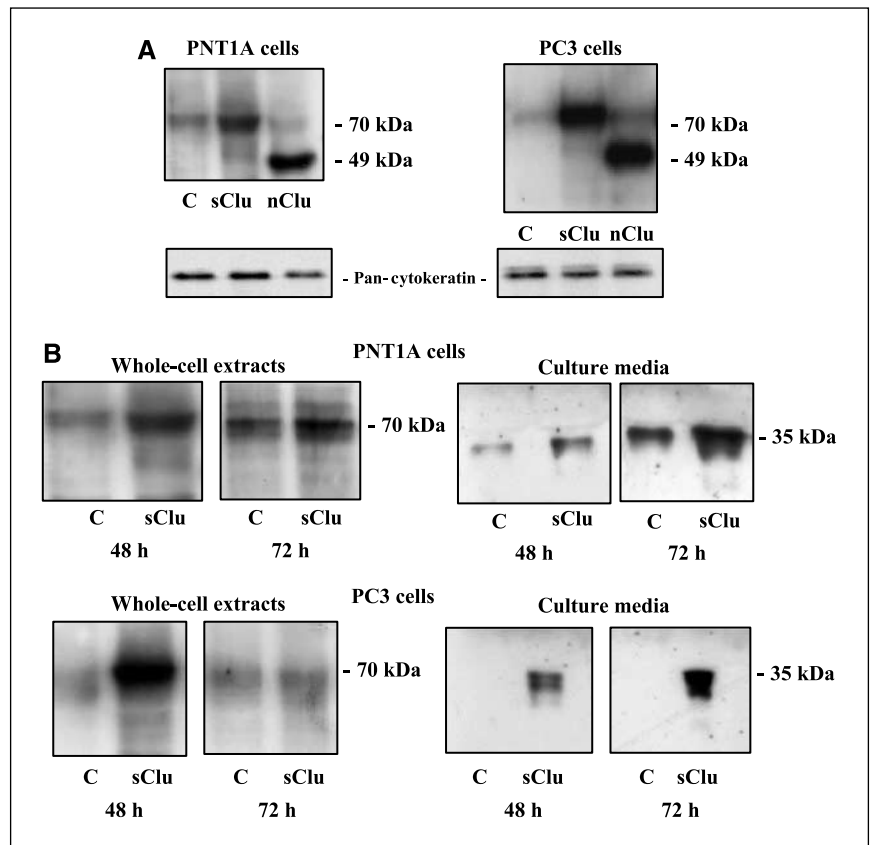
Cell Migration Assays

Haptotactic assays were done using 48-well Boyden chambers (Neuroprobe) in which each pair of wells were separated by polyvinyl pyrrolidone–free polycarbonate porous membrane (8 μm pore size). In preliminary experiments, the most effective stimulus for PNT1A and PC3 cell lines was laminin at 1.5 μg/cm²; the best stimulus for DU 145 cells was fibronectin at 2.5 μg/cm² (data not shown). Thus, laminin was used for PNT1A and PC3 cells and fibronectin was used for DU 145 cells. Transfected (48 h) cells were counted and placed in the open-bottom wells of the upper compartment of a Boyden chamber (10^5 cells/50 μL) in which the lower surface had been precoated with either laminin or fibronectin. The lower compartment of the chambers was filled with serum-free medium. The chambers were kept in the cell culture incubator for 4 h. Cells that migrated through the pores and found adherent to the underside of the membrane were fixed, stained (DiffQuick kit, DADE), and mounted onto glass slides. Six random objective fields of stained cells were counted for each well (eight wells per experimental group) and the mean number of migrating cells per square millimeter was calculated. Assays were repeated thrice, and triplicate wells were analyzed for each time point. Data were analyzed by one-way ANOVA followed by Bonferroni's test. The effect of clusterin isoform overexpression on cell migration is not explained by decreased cell proliferation because the haptotactic assay is too short (4 h) for detecting a possible antiproliferative effect.

Yeast Two-Hybrid Screen

For nuclear clusterin protein-protein interaction studies, we used the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). nCLU cDNA was

Figure 1. *A,* Western blot analysis for determination of sCLU and nCLU in nonmalignant (PNT1A) and malignant (PC3) prostate epithelial cells. Whole-cell extracts from transiently transfected (48 h) PNT1A and PC3 cells were analyzed by SDS-PAGE and Western blotting with a mouse anti-human clusterin antibody. Western blot analysis of cytokeratin expression is provided to show equal loading of the samples. C, mock-transfected controls. Representative of three different experiments done independently, which gave the same results. *B,* Western blot analysis for determination of sCLU in whole-cell extracts and in culture media of nonmalignant (PNT1A) and malignant (PC3) prostate epithelial cells. PNT1A and PC3 cells were transiently transfected with sCLU vector. Whole-cell extracts and culture media were collected at 48 and 72 h after transfection. The full-length cytoplasm sCLU isoform (70 kDa) and the α chain of the secreted heterodimeric form (35 kDa) were detected in cell extracts by SDS-PAGE and Western blotting with a mouse antihuman clusterin antibody. Representative of three different experiments.

cloned in *Nde*I and *Bam*HI sites of pGBKT7 bait vector using the primers 5′-ATGAGCTCCAGCATATGTCCAATC-3′ (forward) and 5′-GCGGGATCCT-CTAGTGCGG-3′ (reverse). The resulting construct was used to screen a human prostate cDNA library made in the pACT2 prey vector. Bait and prey vectors were transformed in AH109 yeast strain and colonies were grown and selected in media lacking adenine, histidine, leucine, and tryptophan.

Coimmunoprecipitation

Transfected (48 h) PC3 cells were lysed in RIPA buffer. Extracts were centrifuged to pellet the cell debris. Protein extracts from supernatants were incubated with anti-clusterin antibody (2 µg) and rabbit anti-mouse immunoglobulin G at room temperature for 2 h. Protein A-Sepharose beads were added and incubated overnight at 4°C with gentle rotation. After centrifugation, immunoprecipitated pellets were washed five times with ice-cold wash RIPA buffer and thrice with water in a refrigerated microcentrifuge. The pellets were dissolved in reducing sample buffer, electrophoresed, and blotted onto nitrocellulose membranes. Membranes were blocked with 3% BSA and incubated for 2 h at room temperature with mouse monoclonal anti–α-actinin antibody (1:1,000). Membranes were then incubated with rabbit anti-mouse secondary antibody for 1 h at room temperature. Signal was detected with enhanced chemiluminescence reagents (SuperSignal Chemiluminescence Detection System, Pierce Biotechnology).

Immunofluorescence Analysis

PNT1A, PC3, or DU 145 cells were seeded on 13-mm-diameter coverslips. After transfection (48 h), cells were fixed with 3% paraformaldehyde in 2% sucrose-PBS for 15 min and permeabilized with 0.5% HEPES/Triton buffer (20 mmol/L HEPES, 300 mmol/L sucrose, 50 mmol/L NaCl, 3 mmol/L MgCl$_2$, 0.5% Triton X-100) for 1 min.

Detection of clusterin. Fixed cells were incubated with the unlabeled monoclonal anti-clusterin primary antibody (1:100) followed by FITC-conjugated goat anti-mouse secondary antibody (Alexa Fluor 488).

Detection of actin. Cells were seeded on coverslips coated with laminin (1.5 µg/cm^2) and stained with FITC-phalloidin (1:2,000). In transfected PC3 cells, α-actinin was detected in fixed cells incubated with the unlabeled monoclonal antibody (1:100) followed by TRITC-conjugated goat anti-mouse secondary antibody (Alexa Fluor 594).

Analysis of nCLU/α-actinin colocalization. nCLU-transfected PC3 cells were fixed and incubated with the unlabeled monoclonal anti-clusterin primary antibody (1:100) followed by TRITC-conjugated goat anti-mouse secondary antibody (Alexa Fluor 594). Cells were then incubated with the goat anti-human α-actinin primary antibody (1:100), followed by FITC-conjugated rabbit anti-goat secondary antibody (Alexa Fluor 488). In each experiment, labeled cells were examined under a Zeiss Axiovert 200 microscope with a 63×/1.4 objective lens linked to a Coolsnap Es charge-coupled device camera (Roper Scientific-Crisel Instruments).

Results

sCLU and nCLU overexpression in PNT1A and PC3 cells. Cells were transiently transfected with either full-length CLU cDNA (sCLU vector) or truncated clusterin cDNA (nCLU vector; ref. 6). Protein expression was evaluated in whole-cell extracts from both PNT1A and PC3 cells by Western blotting. The expression profile of CLU in mock controls was identical to that in untransfected cells for both cell lines (data not shown). Figure 1*A* shows that, in both cell lines, transfection of the full-length cDNA (sCLU) leads, 48 h later, to overexpression of a protein band of ∼70 kDa, corresponding to the predicted size of an intracellular sCLU precursor. As expected, the shorter ATG$_{152}$ cDNA generated a smaller protein form (nCLU), whose molecular weight was 49 kDa, which is compatible with the predicted size of uncleaved, unglycosylated nCLU (9). To further confirm correct processing and secretion of sCLU, whole-cell extracts and conditioned culture medium preparations from both cell lines at 48 and 72 h after transfection

were analyzed by Western blotting. Figure 1*B* shows that sCLU is overexpressed in cell extracts of both cell lines at 48 h after transfection. In PNT1A cells, but not in PC3 cells, sCLU levels were still elevated at 72 h. In culture media from both cell lines, a protein band of ~35 kDa, corresponding to the α chain of the mature heterodimeric form of CLU, was particularly evident at 72 h after transfection (Fig. 1*B, Culture media*). From Fig. 1*B*, it also seems that sCLU is present in the culture media of control PNT1A cells, but it is undetectable in the culture media of control PC3 cancer cells. This observation might suggest that the "prosurvival" isoform of clusterin is preferentially retained in the cytoplasm of cancer cells, possibly to facilitate at this level their malignant potential.

As previously shown (6), the basal levels of the secreted form of clusterin (corresponding to the endogenous secretion) were not modified in the culture media of PNT1A and PC3 cells after transfection with nCLU vector (data not shown), confirming that nCLU was retained inside the cells instead of being secreted.

Effects of sCLU and nCLU on PNT1A, PC3, and DU 145 cell growth. We checked whether the two clusterin isoforms might differentially affect the growth of nonmalignant and malignant cells. Figure 2*A* shows results obtained at 48 h after transfection because, at this time interval, we observed the most significant effects in all cell lines. Transfection of sCLU or nCLU vector caused a significant decrease in PNT1A cell growth when compared with mock controls (Fig. 2*A*). In PC3 and DU 145 cells, overexpression of nCLU elicited a significant antiproliferative effect, whereas sCLU did not affect cell proliferation (Fig. 2*A*). Thus, nCLU is antiproliferative in both nonmalignant and malignant prostate cells, whereas sCLU is antimitogenic in normal, but not in malignant, prostate cells.

Effects of sCLU and nCLU on PNT1A, PC3, and DU 145 cell motility. PNT1A, PC3, and DU 145 cells transfected (48 h) with sCLU or nCLU vector were subjected to haptotactic assay. The results show that overexpression of both sCLU and nCLU significantly decreases the migration of PNT1A cells when compared with mock controls (Fig. 2*B*). In PC3 and DU 145 cells, cell motility was significantly reduced only by nCLU, with sCLU being ineffective (Fig. 2*B*).

Effects of sCLU and nCLU on actin cytoskeleton organization in PNT1A, PC3, and DU 145 cells. Immunofluorescence staining for F-actin was done in transfected (48 h) PNT1A, PC3, and DU 145 cells. Figure 2*C* shows that mock-transfected PNT1A cells (control) display a diffuse actin cytoskeleton with an intense actin staining at the level of stress fibers and under the cell cortex. sCLU and nCLU overexpression induced a dramatic change in cell morphology and a decrease in intracellular actin staining, indicating a complete disorganization of the actin cytoskeleton (Fig. 2*C*). Figure 2*C* also shows the effects of sCLU or nCLU overexpression on immunofluorescence staining of actin in PC3 and DU 145 cells. In mock-transfected PC3 and DU 145 cells (control), F-actin is widely diffused in the cytoplasm and localizes at the cell periphery and at the level of filopodia. Transfection of PC3 and DU 145 cells with sCLU vector did not change actin organization; on the contrary, nCLU dramatically changed cell morphology and completely disrupted the actin cytoskeletal organization (Fig. 2*C*).
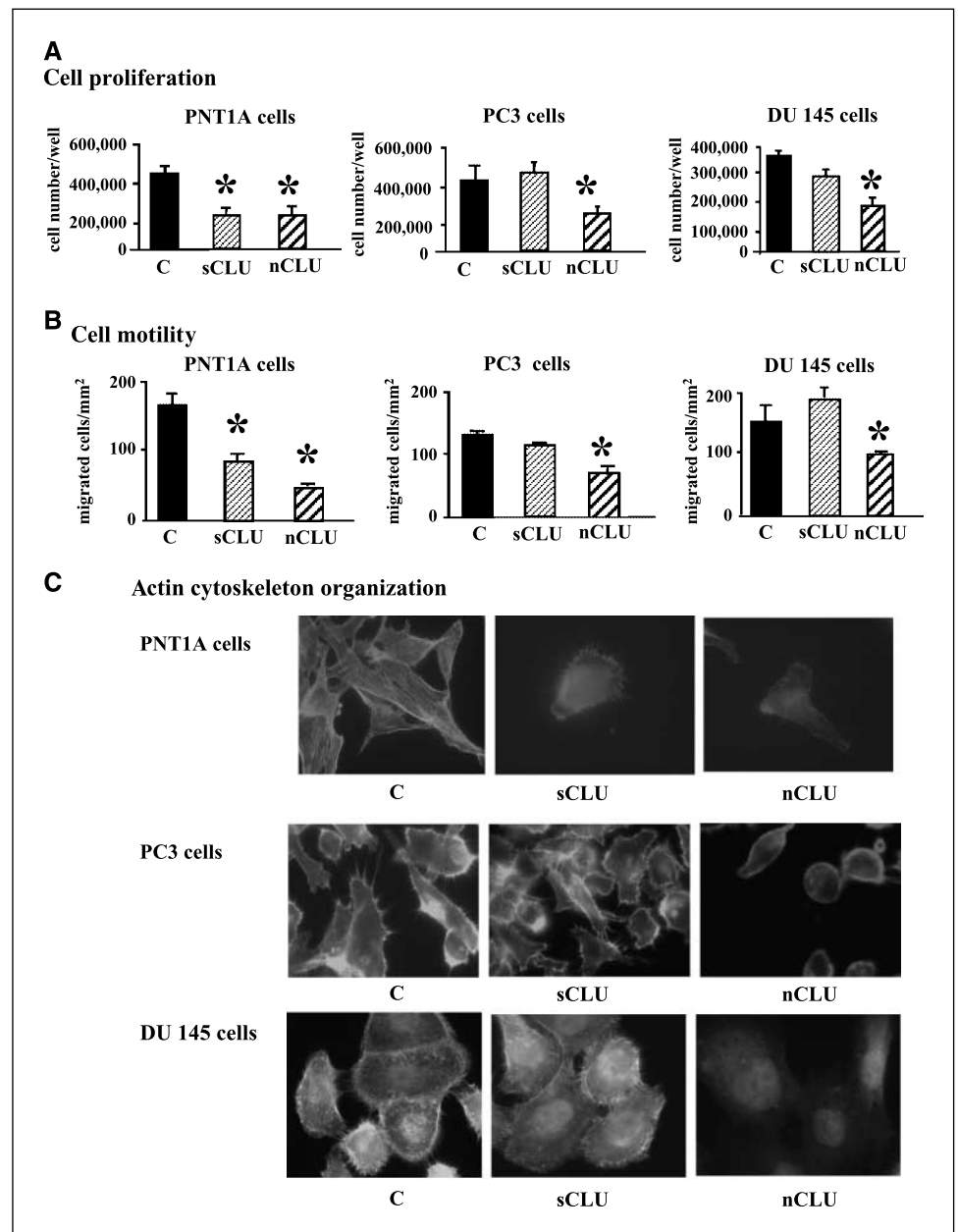
Intracellular localization of CLU isoforms in PNT1A, PC3, and DU 145 cells. The differential effects of sCLU and nCLU on growth and motility of normal and malignant prostate cells suggested that full-length CLU cDNA might drive the synthesis of both sCLU and nCLU in nonmalignant cells, whereas only sCLU

would be produced in cancer cells. To verify this hypothesis, the intracellular localization of protein forms was analyzed in PNT1A and PC3 cells by immunofluorescence and Western blotting after transfection (48 h) with either sCLU or nCLU. In PNT1A cells, transfection with sCLU originated a specific staining for CLU in the cytoplasm (Fig. 3*A, top, lane 1*). However, a nuclear signal was also observed in most of the cells (Fig. 3*A, top, lanes 2* and *3*). Interestingly, when the protein staining was mainly present in the cytoplasm, cells maintained their normal flattened shape (Fig. 3*A, top, lane 1*). On the contrary, cells with clusterin also present in the nucleus (Fig. 3*A, top, lanes 2* and *3*) showed an altered round shape morphology, suggesting that these cells might undergo apoptosis by anoikis as previously showed (3–5). Western blotting experiments done on cytoplasm and nuclear extracts from PNT1A cells overexpressing sCLU confirmed that these cells produced a protein band of ~70 kDa in the cytosolic fraction (Fig. 3*A, bottom*). However, PNT1A cells also produced a protein band of ~49 kDa, corresponding to the molecular weight of nCLU. This band was also present in the nuclear fractions (Fig. 3*A, bottom*). Thus, in normal prostate cells, transfection with full-length cDNA originates the cytoplasm "full-length" sCLU form and also a certain amount of the shorter nuclear form of the protein. On the contrary, overexpression of full-length cDNA in PC3 cancer cells only produced a protein form of ~70 kDa, exclusively localized in the cytoplasm, as shown by both immunofluorescence staining (Fig. 3*B, top, lanes 1* and *2*) and Western blotting of cytosol and nuclear extracts (Fig. 3*B, bottom*). Thus, the nCLU isoform is produced by full-length cDNA in the cytoplasm and then translocated into the nucleus only in normal prostate cells. This does not happen in prostate cancer cells. In contrast, transient transfection with nCLU cDNA was followed by the immunofluorescence staining in the cytoplasm and in the nucleus of both PNT1A and PC3 cells (Fig. 3*C* and *D, top, lanes 1* and *2*). Western blot analysis further confirmed that a protein band of ~49 kDa, corresponding to nCLU, is detectable in the cytoplasm as well as in the nuclear fraction in both cell lines (Fig. 3*C* and *D, bottom*). The same pictures show that, in mock control cells, the endogenous 70-kDa band is detectable in PNT1A cells and is known to express higher basal levels of CLU. In PC3 cells, the level of expression of endogenous CLU is much lower instead. These data indicate that in both normal and cancer prostate cells, nCLU cDNA gives rise only to the nuclear protein isoform, which appears in the cytoplasm before being translocated into the nucleus as previously suggested (9). In Western blotting experiments, the lack of contamination of nuclear extracts by cytosol proteins was verified by cytokeratin analysis.

The intracellular localization of CLU isoforms in DU 145 cells is reported in Fig. 4. In DU 145 cells, sCLU was found to be exclusively localized in the cytoplasm (Fig. 4, *top, lanes 1* and *2*), whereas nCLU could be detected both in the cytoplasm and nucleus (Fig. 4, *bottom, lanes 1* and *2*), in line with the results obtained in PC3 cells. These results confirm that, in prostate cancer cells, the full-length CLU isoform fails to give rise to the nuclear form of the protein (as shown in normal prostate cells).

nCLU binds to and colocalizes with α-actinin. The possibility that the nuclear isoform of clusterin might interact with α-actinin, a protein involved in the organization of actin (35), has been investigated in nCLU-transfected PC3 cells (in which only nCLU, and not sCLU, was effective in disrupting actin cytoskeleton). We investigated whether nCLU overexpression might affect the expression of α-actinin by immunofluorescence analysis and Western blotting. Figure 5*A* confirms that transfection of nCLU

Figure 2. Effects of sCLU and nCLU overexpression on proliferation (*A*), cell motility (*B*), and cell morphology and actin cytoskeleton organization (*C*) of nonmalignant (PNT1A) and malignant (PC3 and DU 145) prostate epithelial cells. *A,* PNT1A, PC3, and DU 145 cells were transiently transfected with sCLU or nCLU vector and the cell number per well was determined at 48 h after transfection. Each experimental condition was replicated six times and each experiment was repeated thrice. *Columns,* mean number of cells per well; *bars,* SE. *C,* mock-transfected cells. *B,* PNT1A, PC3, and DU 145 cells were transiently transfected (48 h) with sCLU or nCLU vector and seeded in the upper compartment of a Boyden chamber for the haptotactic assay. Each experimental condition was replicated six times and each experiment was repeated thrice. *Columns,* mean number of migrated cells per square millimeter; *bars,* SE. *, $P < 0.05$, versus mock-transfected cells. *C,* immunofluorescence staining of F-actin in mock-transfected (*C*), sCLU-transfected (*sCLU*), and nCLU-transfected (*nCLU*) PNT1A, PC3, and DU 145 cells 48 h after transfection. In nonmalignant PNT1A cells, both sCLU and nCLU induce a dramatic change in the architecture and morphology of the cells with a complete dismantling of the actin cytoskeleton. In malignant PC3 and DU 145 cells, at variance with nonmalignant prostate epithelial cells, sCLU does not change cell morphology and F-actin organization. On the other hand, as observed in nonmalignant prostate epithelial cells, nCLU induces a dramatic change in the morphology of PC3 and DU 145 cells with a complete disassembly of the actin cytoskeleton. Representative pictures from three experiments done independently, which gave the same results.

dramatically changes cell morphology and disrupts the actin cytoskeletal organization (F-actin). Figure 5*A* also shows that nCLU does not affect α-actinin staining, which remains diffusely distributed into the cytoplasm; nCLU-transfected cells (merge) do not show α-actinin/actin colocalization. Both actin and α-actinin protein levels were not affected by nCLU (Fig. 5*B*). These data indicate that nCLU does not exert its effects on actin cytoskeleton by reducing the expression of α-actinin.

We further analyzed whether nCLU might act by binding to α-actinin; in fact, the "chaperone"-like activity of clusterin has already been shown in different experimental models (1). Results from two-hybrid experiments indicated that α-actinin could be one of the possible intracellular protein partners of nCLU (Fig. 6*A*). Other potential protein partners of nCLU have also been detected (Katanin p60, ZW10 interactor, and TRAF4-associated 1; further experiments are ongoing to explore such molecular interactions).

To validate this observation, coimmunoprecipitation analysis was done. Figure 6*B* (*lane 2*) shows that, in PC3 cells transfected with nCLU, α-actinin specifically binds to nCLU. No coimmunoprecipitation bands could be detected in negative controls (Fig. 6*B, lane 1,* protein extracts from mock-transfected cells; *lane 3,* no cell extracts with anti-clusterin antibody; *lane 4,* protein extracts without anti-clusterin antibody). To further confirm these observations, colocalization analysis has been done by immunofluorescence. Figure 6*C* (*lanes 1* and *2*) shows that, after transfection, nCLU is present in the cytoplasm before entering the nucleus (nCLU); in both cells, nCLU substantially colocalizes with α-actinin, mainly in the perinuclear region (Fig. 6*C, lanes 1* and *2, merge*). Figure 6*C* (*lane 3*) shows a transfected PC3 cell in which most of nCLU has already entered the nucleus. In this cell, no α-actinin/ nCLU colocalization can be detected at the cytosolic level (Fig. 6*C, lane 3, merge*).
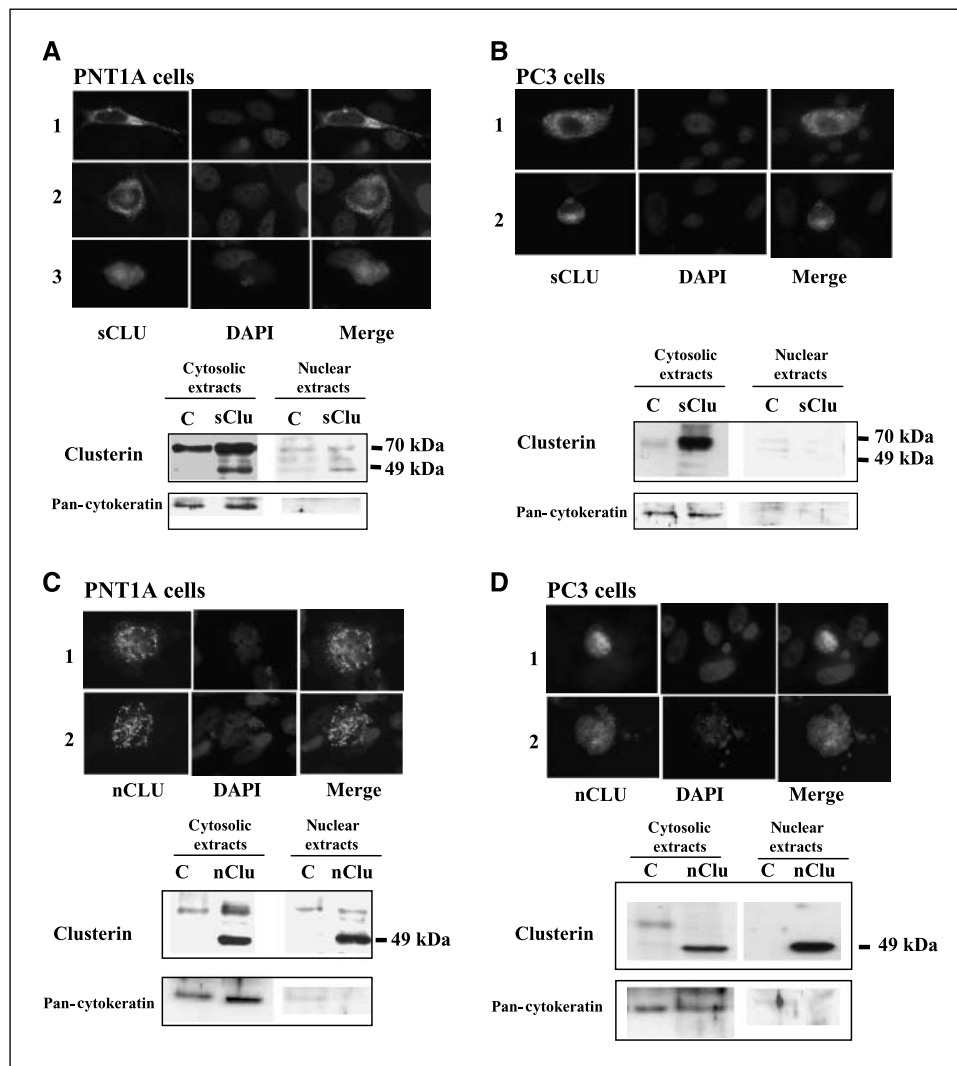
Figure 3. *A* and *B,* intracellular localization of sCLU after transient transfection in nonmalignant (PNT1A; *A*) and malignant (PC3; *B*) prostate epithelial cells. *A, top,* immunofluorescence detection of clusterin staining is reported for three different PNT1A cells (*lanes 1–3*) 48 h after transfection. *Lane 1,* clusterin staining is mainly localized in the cytoplasm; the cell shows a normal flattened shape. *Lanes 2* and *3,* clusterin staining is detected not only in the cytoplasm but also in the nucleus. These cells show a round shape morphology, typical of cells undergoing apoptosis and detaching from plate (*lane 2*) with condensed nucleus (*lane 3*). *Bottom,* cytosol and nuclear extracts were prepared from PNT1A cells transfected (48 h) with sCLU and submitted to Western blot analysis. As expected, a protein band of 70 kDa accumulated in the cytosolic fraction of transfected PNT1A (*sCLU*) when compared with mock-transfected controls (*C*). Interestingly, a band of ~49 kDa, corresponding to the molecular weight of the nuclear form of the protein, is also present in both the cytosol and the nuclear fraction of the cells. Representative pictures from three experiments done independently, which gave the same results. *B, top,* immunofluorescence detection of clusterin staining is reported for two different PC3 cells (*lanes 1* and *2*) 48 h after transfection. In both cells, clusterin staining is exclusively localized in the cytoplasm. *Bottom,* cytosol and nuclear extracts were prepared from PC3 cells transfected (48 h) with sCLU and submitted to Western blot analysis. As expected, transfection leads to accumulation of a protein band of 70 kDa exclusively in the cytosolic fraction of transfected PC3 cells as compared with mock-transfected controls. This band is absent in nuclear extracts from the same cells. No band of 49 kDa can be detected in the cytosolic or nuclear fraction of PC3 cells. Representative pictures from three experiments done independently, which gave the same results. *C* and *D,* intracellular localization of nCLU after transient transfection in nonmalignant (PNT1A; *C*) and malignant (PC3; *D*) prostate epithelial cells. *C, top,* immunofluorescence detection of clusterin staining is reported for two different PNT1A cells (1, 2) 48 h after transfection. In both cells, clusterin localizes both in the cytoplasm and in the nucleus. *Bottom,* cytosol and nuclear extracts were prepared from PNT1A cells transfected (48 h) with nCLU and submitted to Western blot analysis. A protein band of 49 kDa clearly accumulates in both the cytosolic and nuclear fractions of transfected PNT1A cells (*nCLU*). This band is completely absent in mock-transfected controls. Representative pictures from three experiments done independently, which gave the same results. *D, top,* immunofluorescence clusterin staining is reported for two different PC3 cells (*lanes 1* and *2*) at 48 h after transfection. As previously observed in nonmalignant cells, the two PC3 cells show positive clusterin staining, detectable both in the cytoplasm and nucleus. *Bottom,* cytosol and nuclear extracts were prepared from PC3 cells transfected (48 h) with nCLU and submitted to Western blot analysis. Following transfection, a protein band of 49 kDa clearly accumulates both in the cytosol and nuclear fractions of PC3 cells when compared with mock-transfected controls. Representative pictures from three experiments done independently, which gave the same results. Western blot analysis of cytokeratin expression is provided to show absence of cross-contamination of nuclear extracts.

Discussion

We have studied how the two clusterin isoforms sCLU and nCLU affect growth and motility of normal (PNT1A) and tumoral (PC3 and DU 145) human prostate epithelial cells. We found that nCLU significantly decreased the growth of both normal and prostate-cancer cells, confirming the peculiar antiproliferative/proapoptotic

functions previously hypothesized (7, 9, 13) and then described in breast (12) and prostate cancer (6, 15) cells, also following different proapoptotic stimuli (4, 5, 8, 17) or in other experimental models (36–39). Transfection with sCLU vector differentially affected the two cell models because accumulation of sCLU did not change the proliferation rate of prostate cancer cells while eliciting a

significant decrease in cell growth in nonmalignant prostate cells, as previously reported (3). The lack of effect of sCLU on the growth of cancer cells is consistent with previous observations indicating a prosurvival, antiapoptotic effect of this protein form. Transfection of sCLU in cancer cells is associated with increased survival in the presence of cytotoxic drugs (40–42), whereas down-regulation of sCLU by means of antisense oligonucleotides decreases drug resistance in cancer models (27, 28, 32, 42–47).

Then, we investigated whether or not the two protein isoforms modulate cell motility and cytoskeletal organization. We found that nCLU caused a significant decrease in cell motility in both non-malignant and malignant prostate cells. This effect was accompanied by a dramatic alteration of cell morphology and complete dismantling of actin cytoskeleton. In contrast, the sCLU form exerted an antimigratory effect only in normal, and not in tumor, prostate cells. It is important to take into account that the modality of induction of cell death by nCLU has previously been characterized. In previous works (9, 10, 14), it has been shown that nCLU induces anoikis, a cell detachment–induced cell death that takes from 24 h to a few days to be fully appreciated in prostate cells. Only when Ca^{2+} is fully depleted by BAPTA-AM is the phenomenon much faster (17). This important information is given here to exclude the possibility that the antimigratory effect
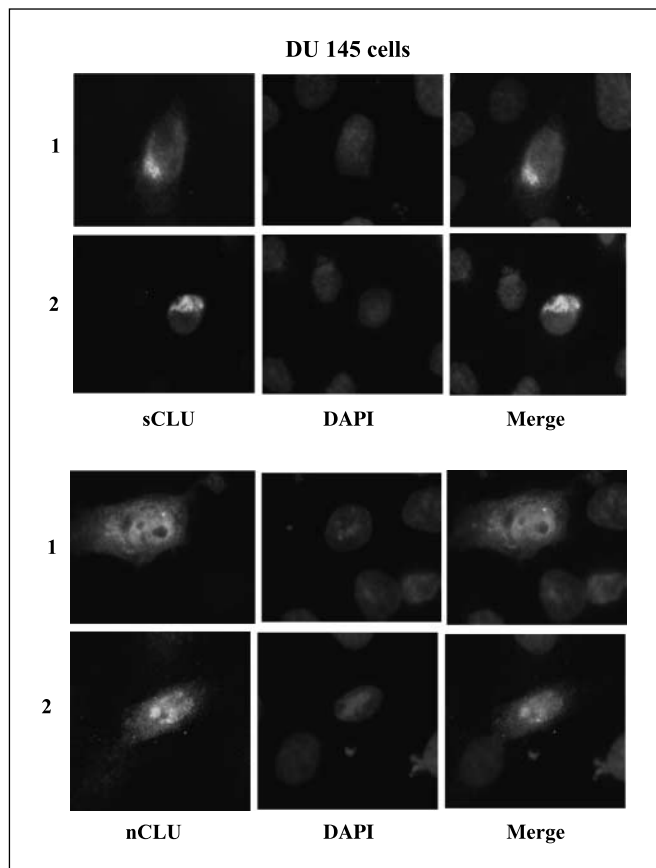

Figure 5. Effects of nCLU overexpression on α-actinin expression in malignant PC3 prostate epithelial cells. *A,* immunofluorescence staining of F-actin and α-actinin in mock-transfected (*C*) and nCLU-transfected (*nCLU*) PC3 cells 48 h after transfection. The dramatic effect of nCLU on cell morphology and actin cytoskeleton organization is not accompanied by alteration of α-actinin staining; however, nCLU-transfected cells (*Merge*) do not show α-actinin/actin colocalization. *B,* Western blot analysis of actin and α-actinin expression in PC3 cells transfected with nCLU. The expression of both proteins is not affected by nCLU overexpression. Representative pictures from three experiments done independently, which gave the same results.

detected in our experiments (which lasted 4 h) is biased by a specific loss of CLU-overexpressing cells. The experimental data thus far available on the possible role played by the secreted form of the protein in the control of cell motility are scarce and rather controversial. Overexpression of sCLU has been reported either to inhibit (48) or to stimulate (16, 49) the metastatic behavior of cancer cells. The reasons for this discrepancy are not clear; however, the different experimental conditions used need to be carefully considered in the final interpretation of the data.

The relative lack of production of the nuclear form of clusterin in prostate cancer cells suggests that full-length clusterin cDNA and/or protein precursor might be differentially processed in normal versus transformed cells. We observed that transfection of full-length CLU cDNA only resulted in production of a protein band of ~70 kDa (corresponding to the full-length isoform) in prostate cancer cells, which was exclusively localized in the cytoplasm and then converted into the fully processed and secreted clusterin (sCLU) form of the protein. On the contrary, normal PNT1A cells produced not only the expected cytoplasm precursor and the secreted form but also a protein product of 49 kDa corresponding to the nuclear form of clusterin. This 49-kDa protein band was found both in the cytoplasm and in the nuclear extracts, confirming that nCLU is synthesized as a precursor cytoplasmic protein, which is then translocated (on activation?) into the nucleus (9). A similar intracellular distribution of clusterin (i.e., cytoplasmic and nuclear localization) was observed after transfection of the nCLU cDNA both in normal and cancer cells. Because we have shown that nCLU reduced cell motility by acting on the cytoskeleton organization, we hypothesize that this protein isoform might exert these effects when still present in the cytoplasm.

In normal prostate cells, the full-length *CLU* gene originates both the secreted (and probably cytoprotective) and the truncated antimitogenic/antimigratory form of the protein, which is known to localize into the nucleus to exert its potent effects. Thus, a shift in the production of CLU isoforms may play a crucial role in

Figure 4. Intracellular localization of sCLU and nCLU after transient transfection (48 h) in DU 145 prostate cancer cells. *Top,* immunofluorescence detection of clusterin staining is reported for two different DU 145 cells (*lanes 1* and *2*) transiently transfected with sCLU. In both cells, clusterin staining is exclusively localized in the cytoplasm. *Bottom,* immunofluorescence detection of clusterin staining is reported for two different DU 145 cells (*lanes 1* and *2*) transiently transfected with nCLU. The two DU 145 cells show positive clusterin staining, detectable both in the cytoplasm and in the nucleus.
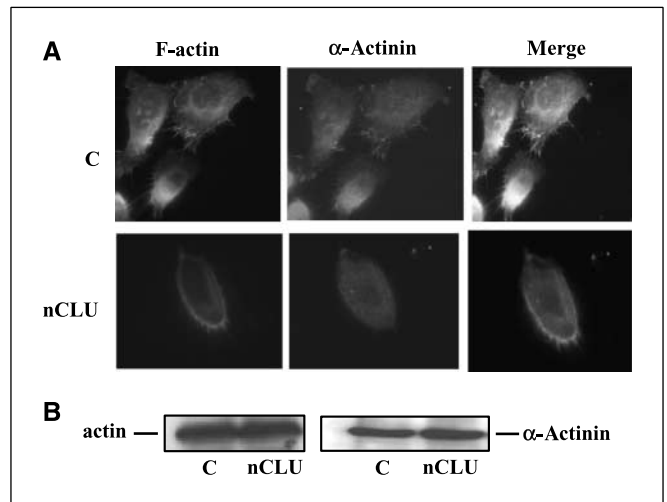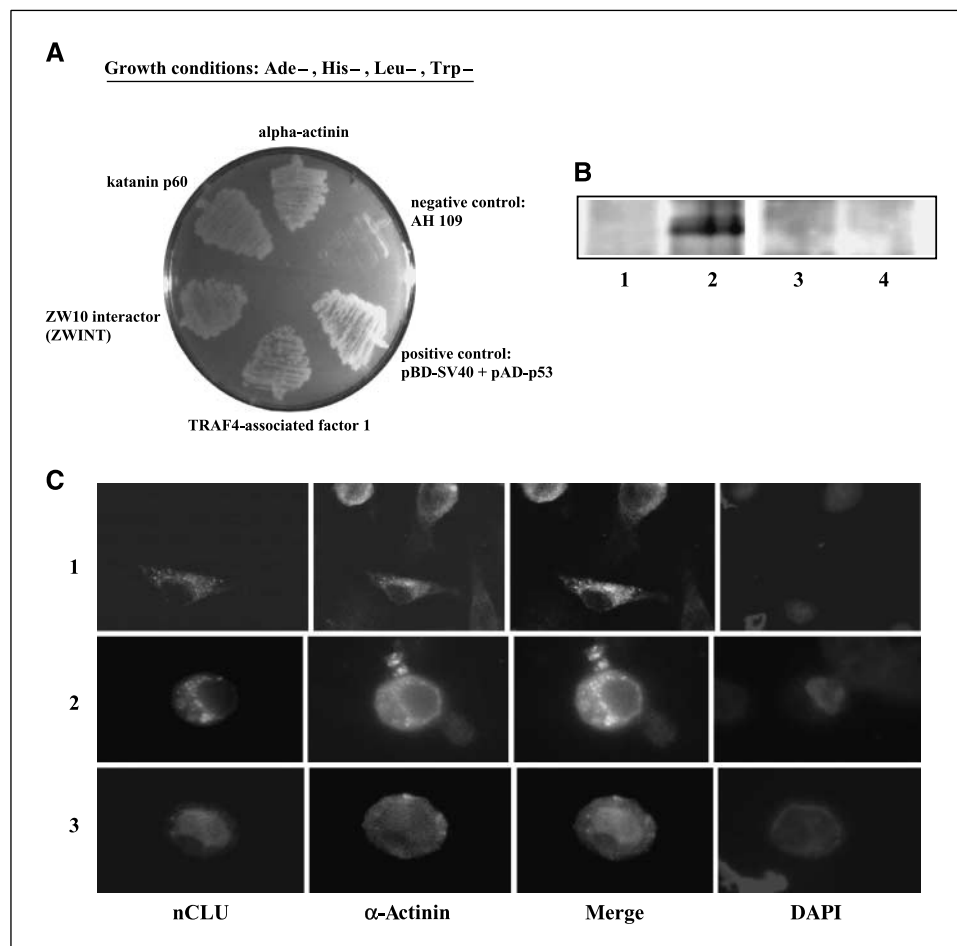
Figure 6. Binding of overexpressed nCLU to α-actinin in malignant PC3 prostate epithelial cells. *A,* yeast two-hybrid analysis of nCLU interactions with α-actinin. AH 109 yeast strain was selected for growth on SD/−Leu, −Trp, −His, −Ade with 10 mmol/L 3-amino-1,2,4-triazole. Positive control was done by cotransfection of pBD-SV40 as bait vector with pAD-p53 as prey. The negative control consisted of nCLU (bait) with empty pACT2 vector (prey). *B,* coimmunoprecipitation experiments on PC3 cells transiently transfected (48 h) with nCLU. Protein extracts from transfected PC3 cells were immunoprecipitated with anti-clusterin antibody, resolved by SDS-PAGE, and processed for immunoblotting with anti–α-actinin antibody (*lane 2*). No coimmunoprecipitation bands can be detected in negative controls: *lane 1,* protein extracts from mock-transfected PC3 cells; *lane 3,* no cell extracts with anti-clusterin antibody; *lane 4,* protein extracts from nCLU-transfected cells without anti-clusterin antibody. Representative pictures from three experiments done independently, which gave the same results. *C,* immunofluorescence staining of nCLU and α-actinin in nCLU-transfected malignant PC3 prostate epithelial cells. *Lanes 1* and *2,* in these cells, nCLU is still localized in the cytoplasm before entering the nucleus (*nCLU*); nCLU and α-actinin substantially colocalize at the cytoplasmic level (*Merge*), mainly in the perinuclear region. *Lane 3,* in this cell, nCLU is already localized in the nucleus; no colocalization with α-actinin (*Merge*) at the cytoplasmic level. Representative pictures from three experiments done independently, which gave the same results.

keeping a balance between cell proliferation and cell death, deeply affecting the cell fate. In this view, neoplastic transformation of prostate cells might require early escape from this mechanism, with the prosurvival sCLU action prevailing over the proapoptotic/antimotility properties of nCLU. Under these conditions, nCLU might not be efficiently produced and/or translocated into the nucleus. In line with our observations, it has been reported that healthy colonic mucosal cells display a strong nuclear localization of clusterin, consistent with its role in apoptosis induction; progression toward high-grade colon carcinomas is characterized by appearance of the cytoplasm form of clusterin, accompanied by a complete loss of the nuclear isoform (50). The molecular mechanisms responsible for the shift in clusterin isoform synthesis are still unclear. Leskov et al. (13) have suggested that nCLU might originate from the full-length *CLU* gene by alternative splicing of CLU mRNA. The experimental conditions adopted in the present article (transfection with the full-length CLU cDNA) strongly support an alternative initiation of translation. This point is crucial for cell transformation and deserves further studies.

Because nCLU was found to be associated with decreased motility and a dramatic disassembly of the actin cytoskeleton, we investigated the possible mechanism of this activity. We found that nCLU specifically binds to and colocalizes with α-actinin, an actin-cross-linking protein, which plays crucial roles in regulating plasticity of the actin cytoskeleton (35). We hypothesize that binding of nCLU to α-actinin would reduce its ability to cross-link actin filaments, thus promoting cytoskeletal disassembly and decreased motility. Because the binding of nCLU to α-actinin is specifically localized in the cytoplasm, we suggest that nCLU might exert its effects on the cytoskeletal organization when still in the cytoplasm and before entering the nucleus. These results show for the first time that nCLU is an α-actinin binding protein causing disassembly of the cytoskeleton. The finding that nCLU reduces the motility of the cells provides a rationale for anoikis-death induction previously described when nCLU was induced (4, 5, 8, 17, 37).

Because of the controversy about the possible proapoptotic versus antiapoptotic role for this protein, and the down-regulation versus up-regulation of *CLU* gene in prostate cancer, unraveling the role of CLU in prostate tumorigenesis is critical. In this light, it is important to consider that clinical trials aimed at inhibiting CLU expression with antisense oligonucleotides are ongoing in prostate cancer patients (30). Chi and coworkers showed that it is possible to achieve a significant down-regulation of CLU and increased apoptotic rate in prostate cancer cells without major adverse effects for patients. Unfortunately, the relative percentages of sCLU and nCLU affected by this down-regulation are not known. Our results suggest that prostate cancer patients would benefit from an ideal scenario where sCLU is knocked down while nCLU is up-regulated. Lowering the sCLU/nCLU ratio might be the right strategy to control prostate cancer cell outgrowth and metastatic diffusion.

In conclusion, the data reported here represent the first demonstration that the proapoptotic nuclear isoform of clusterin is endowed with a potent antimigratory activity through binding to α-actinin. Moreover, the pattern of clusterin isoform production

is different in normal versus cancer prostate cells; escaping the mechanisms leading to nCLU production from the full-length *CLU* gene might play a crucial role in prostate tumorigenesis.

Acknowledgments

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