

Increased Expression and Activity of Nuclear Cathepsin L in Cancer Cells Suggests a Novel Mechanism of Cell Transformation

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Abstract

It is generally accepted that the role of cathepsin L in cancer involves its activities outside the cells once it has been secreted. However, cathepsin L isoforms that are devoid of a signal peptide were recently shown to be present in the nucleus where they proteolytically process the CCAAT-displacement protein/cut homeobox (CDP/Cux) transcription factor. A role for nuclear cathepsin L in cell proliferation could be inferred from the observation that the CDP/Cux processed isoform can accelerate entry into S phase. Here, we report that in many transformed cells the proteolytic processing of CDP/Cux is augmented and correlates with increased cysteine protease expression and activity in the nucleus. Taking advantage of an antibody that recognizes the prodomain of human cathepsin L, we showed that human cells express short cathepsin L species that do not contain a signal peptide, do not transit through the endoplasmic reticulum, are not glycosylated, and localize to the nucleus. We also showed that transformation by the ras oncogene causes rapid increases both in the production of short nuclear cathepsin L isoforms and in the processing of CDP/Cux. Using a cell-based assay, we showed that a cell-permeable inhibitor of cysteine proteases is able to delay the progression into S phase and the proliferation in soft agar of ras-transformed cells, whereas the non-cell-permeable inhibitor had no effect. Taken together, these results

suggest that the role of cathepsin L in cancer might not be limited to its extracellular activities but may also involve its processing function in the nucleus. (Mol Cancer Res 2007;5(9):899–907)

Introduction

Proteolytic processing has emerged as an important post-translational mechanism that modulates the subcellular localization and/or biochemical activities of proteins with functions as diverse as receptor, ligand, signaling molecules, and transcription factors (1). In the case of transcription factors, the consequence of proteolytic processing is often to redirect the localization of proteins from a cytoplasmic site to the nucleus (reviewed in ref. 2). However, in some instances, the peptides generated by processing exhibit specific half-life, biochemical properties, and biological functions, which are distinct from that of their precursors (reviewed in ref. 3).

CCAAT-displacement protein/cut homeobox (CDP/Cux) belongs to a family of transcription factors involved in the control of proliferation and differentiation (reviewed in ref. 4). Genetic studies in *Drosophila* and the mouse showed that the CDP/Cux/Cut gene plays an important role in the development and homeostasis of several tissues (5-7). The CDP/Cux transcription factor was reported to acquire novel DNA binding and transcriptional properties as the result of a proteolytic processing event that takes place at the end of the G₁ phase (8, 9). At the cellular level, the p110 processed isoform was shown to stimulate cell proliferation and to accelerate S phase entry in various cell types (10). These results extended early observations linking CDP/Cux to the control of gene expression in S phase (11, 12). In transgenic mice, two short CDP/Cux isoforms, p75 and p110, were shown to cause malignancies in several organs and cell types (13).

A nuclear isoform of cathepsin L was found to be responsible for the proteolytic processing of CDP/Cux (14). Cathepsin L is a ubiquitously expressed cysteine protease that localizes primarily to the lysosomes and can be secreted (15-17). However, translation initiation within the murine cathepsin L mRNA was shown to take place with low efficiency at two internal start sites, thereby generating cathepsin L species devoid of the NH₂-terminal signal peptide (14). Moreover, immunofluorescence imaging revealed the presence of cathepsin L in the nucleus after the G₁ phase of the cell

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cycle. These results uncovered a novel role for cathepsin L in the processing of a nuclear protein and, by extension, in the regulation of gene expression.

Expression of cysteine cathepsins was reported to be elevated in many types of cancers (reviewed in refs. 18, 19). In particular, tumor promoters, such as phorbol ester, and certain oncogenes, such as ras, v-src, SV40 large T, and raf, were shown to induce cathepsin L expression and secretion (20-25). Abundant literature has extended this observation to human tumors originating from various tissues. Notably, human colorectal carcinomas containing K-ras mutations showed greater increases in cathepsin L activity (26-29). Moreover, the metastatic potential of transformed cell lines was shown to correlate with cathepsin L expression levels (22). More recently, a role in cell proliferation and tumor growth was suggested from studies of multistage carcinogenesis in a mouse model (30, 31).

Short CDP/Cux isoforms were reported to be expressed at higher level in human uterine leiomyomas and in breast tumors (32, 33). In the present study, we have compared CDP/Cux expression in normal and transformed cell lines. We report that proteolytic processing of CDP/Cux is activated in many transformed cell lines and correlates with increased cathepsin L expression and activity in the nucleus. These results suggest that the role of cathepsin L in cancer may also involve its nuclear functions.

Results

ras-3T3 Cells Express Higher Level of Short CDP/Cux Proteins

We observed that the ratio of full-length and truncated CDP/Cux isoforms was different in ras-3T3 cells compared with the parental 3T3 cells (Fig. 1A). DNA binding by p110 CDP/Cux was correspondingly increased in ras-3T3 cells (Fig. 1B, lanes 1 and 2). In contrast, DNA binding to an Oct consensus binding site was similar in both cells (Fig. 1B, lanes 3 and 4). The two retarded complexes observed with the CDP/Cux consensus binding site in Fig. 1B (lane 2) correspond to the p110 (1) and the p200 (2) isoforms, as shown from supershift analysis with various antibodies and competition with cold oligonucleotides (Fig. 1B, lanes 5-9 and 10-13). The NFY-ATCG oligonucleotides, which contain an optimal binding site for p200 but a low affinity binding site for p110, competed away the #2 retarded complex at low concentration and the #1 retarded complex only at higher concentration (Fig. 1B, compare lane 5 with lanes 14-17; ref. 34). To confirm the link between transformation by ras and the increase in CDP/Cux processing, 3T3 cells were infected *de novo* with a retrovirus expressing ras-V12 or with an empty vector and CDP/Cux expression was analyzed after 5 days. The ras-expressing cells displayed higher levels of the endogenous p110 CDP/Cux isoform (Fig. 1C).

Processing of CDP/Cux Is Increased in ras-Transformed 3T3 Cells

To begin to assess the mechanism responsible for the higher expression of p110 CDP/Cux, we transfected 3T3 and ras-transformed 3T3 cells with a vector that expresses the full-length CDP/Cux protein with the influenza virus hemagglutinin (HA) epitope tag at its COOH terminus (diagram in Fig. 1F). As

seen in Fig. 1D, a higher level of the processed p110 isoform was observed in ras-3T3 cells. Yet, the half-life of a recombinant protein similar to p110 was not significantly different in the two cell lines (Fig. 1E). Altogether, these results indicated that processing of CDP/Cux is increased in ras-transformed cells.

p110 CDP/Cux Levels Are Increased in Many Tumor Cell Lines

We next asked whether the observation made in ras-transformed cells could be extended to tumor-derived cell lines. Western blot analysis of a panel of human breast tumor cell lines indicated that the p110 CDP/Cux steady-state level was also elevated in many but not all transformed cells (Fig. 2A, lanes 2, and 6). Consistent with this findings, SkBr3, Hs578T, and MDA231 cells displayed higher levels of short CDP/Cux isoforms following transfection of the CDP-HA vector (Fig. 2B, lanes 1, 2, and 5). Indeed, an activating ras mutation is present in MDA231 and Hs578T cells, and ras activity was reported to be elevated in SkBr3 cells, whereas it was low in MCF7 and TD47D (35). Overall, these results suggest that the proteolytic processing of CDP/Cux is more efficient in some human tumor cell lines.

Expression of p110 CDP/Cux Is Not Regulated in a Cell Cycle-Dependent Manner in Transformed Cells

Proteolytic processing of CDP/Cux was previously shown to augment as cells approach S phase (8, 14). To verify whether expression of p110 CDP/Cux remained cell cycle dependent in transformed cells, fractions of cells in different phases of the cell cycle were obtained by counterflow centrifugal elutriation. In NIH3T3 cells, little or no p110 was detected in fractions 1 and 2, which are enriched in G₁; expression of p110 became apparent in fraction 3 and increased steadily up to fractions 6 and 7 (Fig. 2C, NIH3T3). In contrast, in ras-NIH3T3 cells, expression of both p200 and p110 was relatively constant throughout the cell cycle (Fig. 2C). We conclude that expression of p110 CDP/Cux is constitutive in ras-transformed NIH3T3 cells.

Human Breast Tumor Cells Express Short Nuclear Cathepsin L Isoforms

We have established thus far that ras-transformed cells as well as some human cancer-derived cell lines express higher levels of the p110 CDP/Cux isoform through a mechanism that does not involve protein stabilization but, more likely, increased processing. CDP/Cux was previously shown to be proteolytically processed by cathepsin L in the nucleus (14). We therefore hypothesized that the increased processing of CDP/Cux might be associated with an elevation in the level of cathepsin L in the nucleus of transformed cells. Targeting of cathepsin L to the endoplasmic reticulum depends on the presence of an NH₂-terminal signal peptide, and subsequent activation of cathepsin L in the endosomes involves cleavage of the prodomain (36-39). In a previous study, the presence of cathepsin L in the nucleus of mouse cells was associated with the synthesis of cathepsin L species that are devoid of the NH₂-terminal signal peptide as a result of translation initiation taking place at

internal AUG sites within the cathepsin L mRNA (14). We speculated that shorter cathepsin L prodomain would not bind as tightly to the active groove, thereby allowing catalytic activity (14).

To verify whether shorter cathepsin L species devoid of a signal peptide could be detected in human cells, we first confirmed that translation of human cathepsin L mRNA could start at internal AUG sites. In humans, there are four alternative cathepsin L mRNAs that differ at their 5' end and a switch in favor of the short mRNAs was found to occur in cancer cells (40). Human cathepsin L AI and AIII mRNAs were tested in an *in vitro* transcription-translation system. As previously described, translation was more efficient with the AIII cathepsin L mRNA, which contains a shorter 5' untranslated region (Fig. 3A, lanes 1 and 2). Importantly, the presence of several

bands suggested that translation could initiate at more than one site. Indeed, replacement of the first AUG codon with a UUG codon eliminated the slowest migrating species without preventing expression of the shorter species (Fig. 3A, lane 3).

Human cathepsin L isoforms were analyzed by immunoblotting following cell fractionation. Absence of contamination from the endoplasmic reticulum in the nuclear fraction was confirmed by immunoblotting with an anticalexin antibody (Fig. 3B, bottom, lanes 5 and 6). In addition, glycosylated species (which have transited through the endoplasmic reticulum) were identified by their lower molecular weight following treatment with *N*-glycanase (Fig. 3B, compare lane 7 with lane 8). Samples were migrated in parallel with the products of *in vitro* transcription-translation system to help identify the full-length and short cathepsin L species (Fig. 3B,

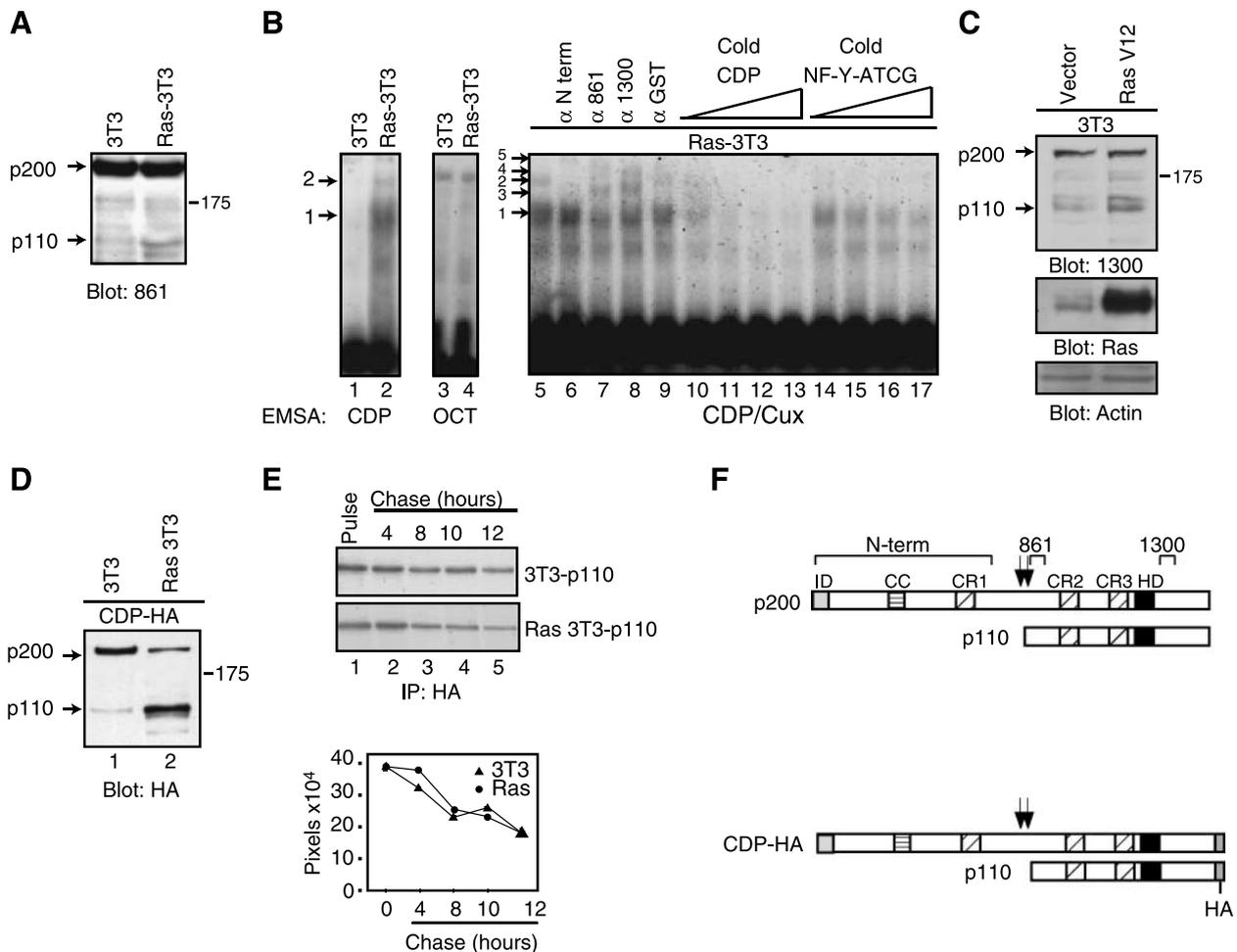


FIGURE 1. Increased DNA binding activity and constitutive processing of CDP/Cux in transformed cells. **A** and **B**. Nuclear extracts were prepared from 3T3 and ras-transformed 3T3 cells (*Ras-3T3*) and analyzed by immunoblotting with anti-CDP/Cux α 861 antibody (**A**) and electrophoretic mobility shift assay (EMSA) with a CDP/Cux consensus binding site (**B**). The CDP/Cux retarded complexes are indicated by the numbered arrows: 1, p110; 2, p200; 3, supershift of p110 with α 861 or α 1300; 4, supershift of p200 with α 861 or α 1300; 5, supershift of p200 with α N-term. **C**. Immortalized mouse fibroblasts were infected with a retrovirus expressing ras-V12 or with an empty vector. Top, 5 d later, nuclear extracts were prepared from populations of puromycin-resistant cells and analyzed for CDP/Cux processing using the anti-CDP/Cux α 1300 antibody; middle, cytoplasmic fraction (20 μ g) was used to analyze ras expression; bottom, equal loading was confirmed by reblotting the membrane with an anti-actin antibody. **D**. 3T3 and ras-3T3 cells were transfected with a vector encoding CDP-HA. Nuclear extracts were analyzed by immunoblotting with an anti-HA antibody. **E**. A vector coding for a HA-tagged p110 CDP/Cux protein was introduced into 3T3 and ras-3T3 cells and pulse-chase analysis was done. The pixel decay for both cell lines is shown on the graph. **F**. Schematic representation of the full-length (p200) and proteolytically processed (p110) CDP/Cux isoforms and of the recombinant proteins. The regions recognized by each antibody are indicated.

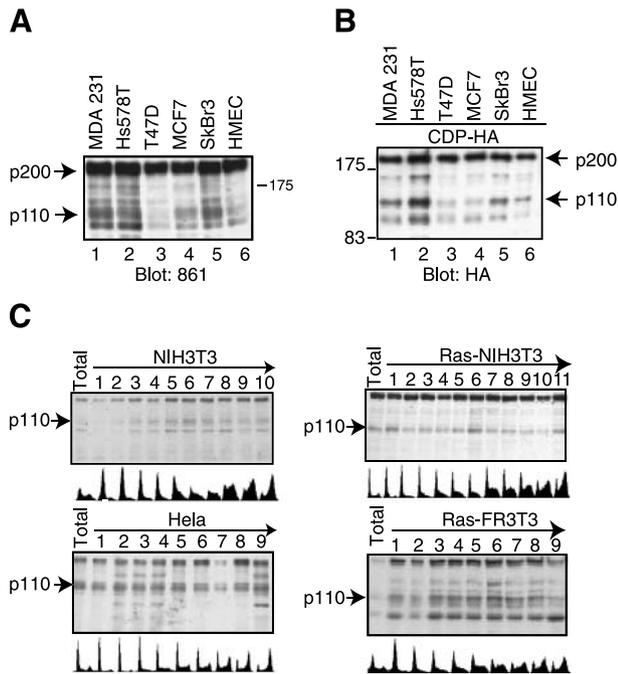


FIGURE 2. Increased levels of the p110 CDP/Cux isoform in transformed cells from fibroblastic and epithelial origin. **A.** Nuclear extracts were prepared from various breast tumor cell lines and primary human mammary epithelial cells (*HMEC*) and submitted to immunoblotting using the anti-CDP/Cux 861 antibody. **B.** The indicated cell lines were transfected with the vector encoding CDP-HA and nuclear extracts were analyzed by immunoblotting using anti-HA antibodies. **C.** Cells were subjected to counterflow centrifugal elutriation. For each fraction, nuclear extracts were analyzed by immunoblotting with the anti-CDP/Cux α 861 antibody, and the cell cycle profile was monitored by fluorescence-activated cell sorting analysis after DNA staining with propidium iodide.

lanes 1 and 2). We also took advantage of the existence of antibodies that can recognize epitopes in different regions of human cathepsin L (41). The 33-1 antibody, which recognizes an epitope present in all cathepsin L species, revealed three major bands in the cytoplasmic fraction. These bands correspond to the full-length, single chain, and heavy chain species and are sensitive to *N*-glycanase (Fig. 3B, lanes 7 and 8). The 2D4 antibody, which was raised against an epitope present in the prodomain, as expected did not recognize the single and heavy chains but detected the full-length cathepsin L and several faster migrating species (Fig. 3B, lanes 3-8). Two of these faster migrating bands were detected by both the 2D4 and 33-1 antibodies, they were present in the nuclear fraction, they comigrated with the short cathepsin L species produced in the *in vitro* transcription-translation system, and their migration was not affected by the *N*-glycanase treatment (Fig. 3B, see identical bands labeled "S" in lanes 5 and 6 and compare with lane 2; also note that the band labeled with an asterisk corresponds to *N*-glycanase itself).

Similar cathepsin L species were observed in MCF10A mammary epithelial cells and in Wi-38 human diploid fibroblasts (Fig. 3C and D, lanes 3 and 4). That these bands indeed correspond to cathepsin L species was confirmed by the fact that their intensity was decreased when cells were transfected with RNA interference against cathepsin L, whereas a random

siRNA duplex had no effect (Fig. 3C, compare lane 3 with lane 4). Altogether, these results show that human cells express short cathepsin L species that do not contain a signal peptide, do not transit through the endoplasmic reticulum, are not glycosylated, and localize to the nucleus.

Oncogenic ras Increases Both Expression of Nuclear Cathepsin L and Processing of CDP/Cux in Human Diploid Fibroblasts

We then verified whether oncogenic transformation would affect the expression of short nuclear cathepsin L isoforms and, as a consequence, the processing of CDP/Cux. Wi-38 human diploid fibroblasts were infected with a retrovirus expressing ras-V12, and cathepsin L expression was analyzed 48 h later. Immunoblotting with both the 33-1 and 2D4 antibodies showed that cathepsin L expression was augmented in cells expressing ras-V12 (Fig. 3D, lanes 8 and 9). These results agree with previous reports (20-25). Moreover, analysis of nuclear fractions with the 2D4 antibodies also revealed that higher amounts of short nuclear cathepsin L species were present in cells that were infected with the ras-V12-expressing vector compared with cells infected with an empty vector (Fig. 3D, lanes 3 and 4). In accordance with this result, coexpression of ras-V12 with a recombinant CDP/Cux protein in Wi-38 cells led to an increase in processing (Fig. 3E). These results show that activated ras causes an increase in the expression and activity of cathepsin L in the nucleus.

Inhibition of Intracellular Cysteine Protease Activity Delays S-Phase Entry and Hinders Proliferation

We used cysteine protease inhibitors that are cell permeable or not to investigate the role of intracellular cysteine proteases in cell proliferation. The permeability and efficiency of the inhibitors was assessed by adding to the inhibitor-treated cells the fluorescent small activity-based probe GB111 (42). Following a 1-h treatment with either inhibitor, the activity-based probe was added for another hour before cell fixation. A strong fluorescent signal was observed in cells that had received the vehicle or the non-cell-permeable inhibitor JPM-565 (Fig. 4A). In contrast, a much lighter signal was detected in cells that received the cell-permeable inhibitor JPM-OEt, indicating that most papain family of cysteine proteases was inhibited (Fig. 4A). Inhibition of papain family cysteine proteases in this context was further confirmed by subjecting whole-cell lysates to SDS-PAGE followed by analysis of the GB111 fluorescence using a Typhoon scanner. As shown previously, only the cell-permeable inhibitor JPM-OEt efficiently prevented labeling of active proteases by GB111 (Fig. 4B; ref. 42). We then tested whether those inhibitors could affect on cell proliferation. Whereas the non-cell-permeable inhibitor JPM-565 had no effect on cell cycle progression or growth in soft agar, the cell-permeable inhibitor JPM-OEt delayed entry into S phase following serum starvation and restimulation (Fig. 4C) and hindered the growth of ras-3T3 cells in soft agar (Fig. 4D). Not only was there a significant reduction in the number of colonies, but the size of colonies also was considerably reduced (Fig. 4D). These findings strongly suggest that the intracellular activity of cysteine proteases is required for rapid cell cycle progression and maximal cell proliferation.

Discussion

Results from the present study revealed two novel findings. We showed that proteolytic processing of the CDP/Cux transcription factor was activated in many cancer cells. In parallel, we showed that cathepsin L expression and activity were also increased in the nucleus of transformed cells. Below, we discuss the significance of these findings and we argue that the processing activity of cathepsin L in the nucleus can contribute to the development of cancer.

Microarray analyses revealed that CDP/Cux mRNA expression was elevated in several cancers originating from

various tissues (43–47). In a survey of human breast cancers, CDP/Cux protein expression was found to be significantly increased in high-grade carcinomas and was inversely correlated with survival (48). Collectively, these data suggested that increased expression of CDP/Cux is a contributing factor in tumor development and/or progression. In the present study, we uncovered not only a quantitative but also a qualitative difference in the expression of CDP/Cux, as relatively more of the processed isoform was expressed in many transformed cells. Importantly, increased processing of CDP/Cux is not a phenomenon that is limited to transformed cells in tissue culture

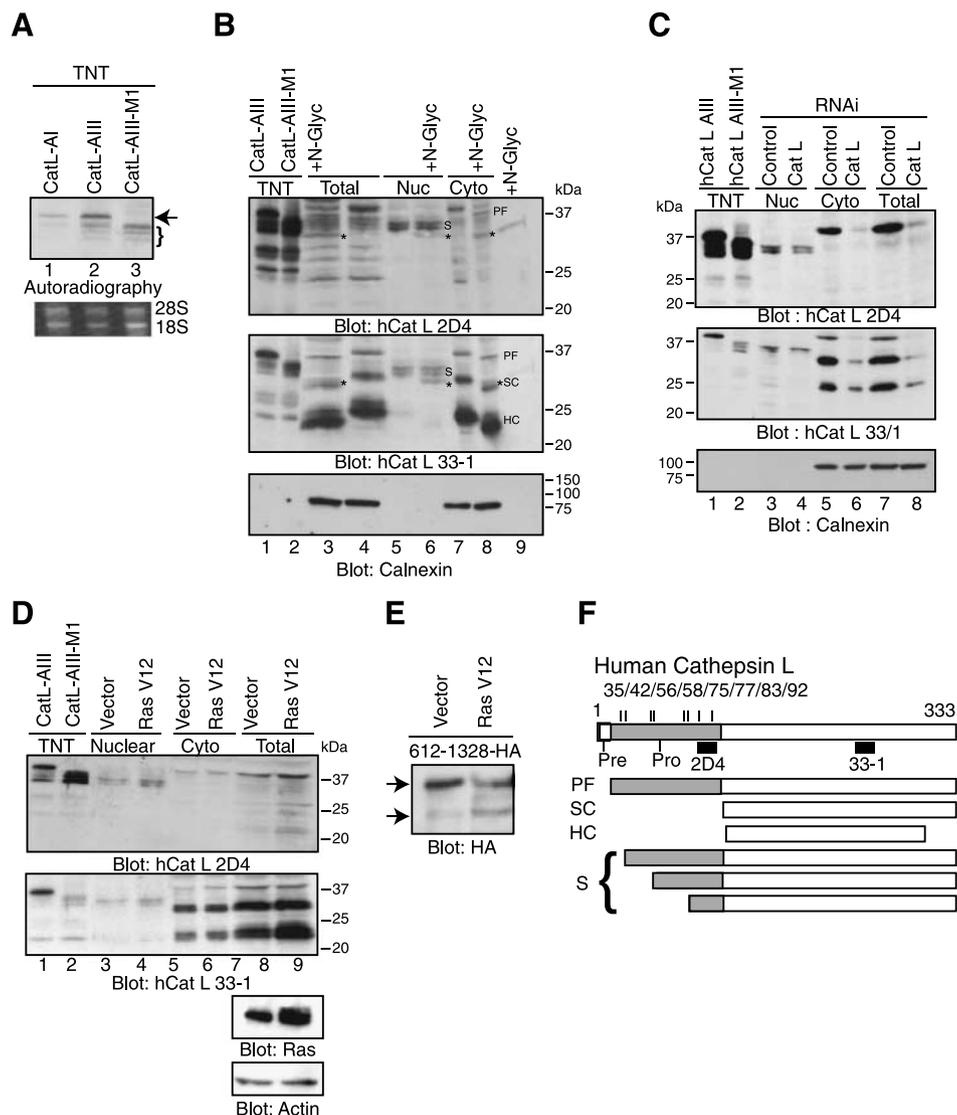


FIGURE 3. Human diploid fibroblast infected with pBabe-ras and Hs578T cells display higher levels of nuclear short cathepsin L isoforms. **A.** Wild-type or mutated human cathepsin L (*CatL*) vectors were expressed *in vitro* using the Promega transcription-translation system (TNT). AI and AIII refer to the two alternatively spliced human cathepsin L isoforms (lanes 1 and 2), and M1 refers to the methionine codon that was mutated (AUG to UUC). Arrow, full-length cathepsin L species; bracket, short cathepsin L species. **B.** Nuclear and cytoplasmic fractions were isolated from Hs578T cells and analyzed, along with total extracts, by immunoblotting using the indicated antibodies. Where indicated, the extracts were pretreated with *N*-glycanase F (*N*-Glyc). Asterisk, *N*-glycanase enzyme; PF, human cathepsin L (*hCat L*) proform; SC, single chain; HC, heavy chain; S, short isoforms. **C.** MCF10A cells were transfected with a random or human cathepsin L–specific siRNA. Extracts and immunoblots were done as described in **B**. **D.** Human diploid fibroblasts Wi-38 were infected with a retrovirus expressing ras-V12 for 48 h. Total extracts and cellular fractions were prepared as in **B** and blotted with the indicated antibodies. **E.** Wi-38 cells were infected with a retroviral vector coding for ras-V12 or with an empty vector and then transfected with a plasmid expressing the recombinant CDP/Cux protein 612-1328/HA. Nuclear extracts were analyzed by immunoblotting with anti-HA antibodies. **F.** Schematic representation of the human cathepsin L isoforms. The AUGs are indicated above, and the epitopes recognized by the two antibodies 2D4 and 33-1 are shown below.

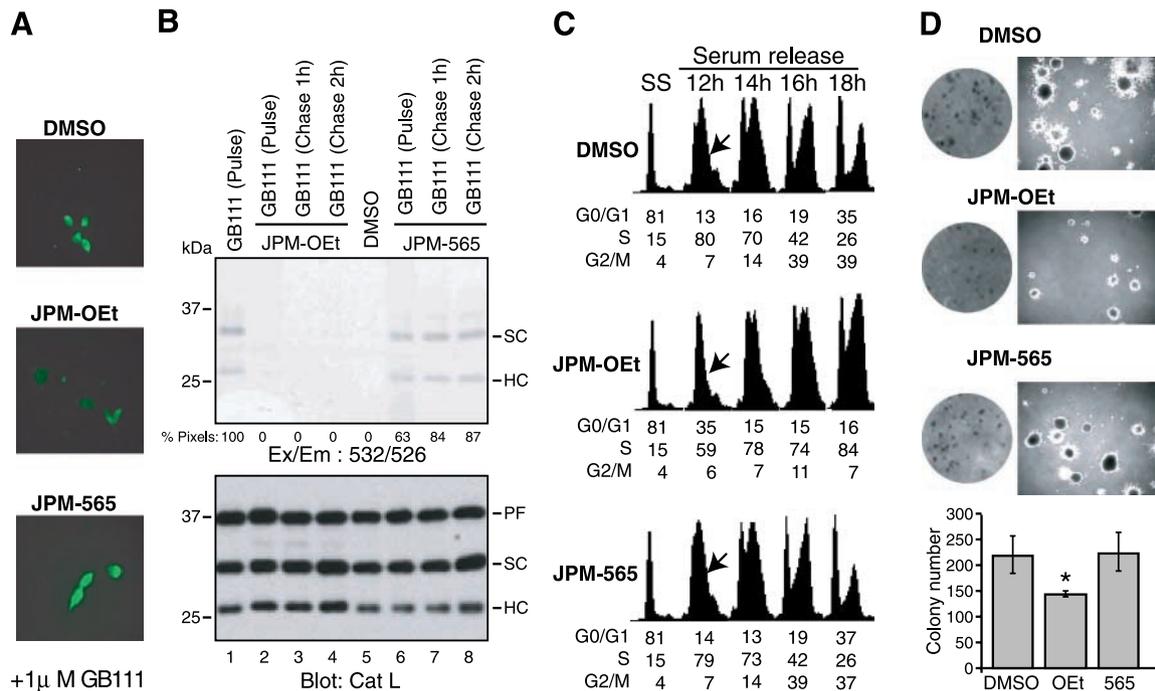


FIGURE 4. Inhibition of intracellular cysteine protease activity delays cell cycle progression and the anchorage-independent proliferation of ras-3T3 cells. **A.** ras-3T3 cells were treated with either DMSO (vehicle) or 100 $\mu\text{mol/L}$ of the cell-permeable (JPM-OEt) or non-cell-permeable (JPM-565) cysteine protease inhibitor before labeling with 1 $\mu\text{mol/L}$ of the GB111 activity-based probe. Cells were fixed and visualized by fluorescence. **B.** ras-3T3 cells were treated with the protease inhibitors and GB111 as in **A**. Whole-cell lysates were subjected to SDS-PAGE and labeled active proteases were visualized by scanning the gel using a Typhoon scanner (top). The fluorescence in each lane was measured by counting pixels from the single and heavy chain bands. The relative percentages of pixels are indicated below the panel and are expressed relative to the untreated sample (100%; lane 1) and the unlabeled sample (0%; lane 5). Bottom, equal loading was confirmed by Western blot using a cathepsin L antibody. **C.** ras-3T3 cells were serum starved for 72 h and restimulated with fresh medium supplemented with 10% serum in the presence of either DMSO or 100 $\mu\text{mol/L}$ of JPM-OEt or JPM-565. At the indicated times, cell cycle distribution was monitored by fluorescence-activated cell sorting analysis. **D.** ras-3T3 cells were plated in soft agar in the presence of DMSO, JPM-OEt, or JPM-565. Shown is one plate from a triplicate for each condition and a $\times 10$ magnification of a representative field is also depicted (bottom). The variation in colony numbers is presented in the histogram below. Asterisk, statistically significant difference ($P < 0.05$, Student's two-tailed t test).

because in human uterine leiomyomas p110 CDP/Cux was found to be frequently expressed at higher level in the tumor than in the adjacent myometrium (32). Interestingly, more than one mechanism seems to be used in cancer cells to favor the expression of short CDP/Cux isoforms. Transcription initiation at a downstream site within intron 20 was also found to contribute to the expression of another short CDP/Cux isoform called p75 (33). In invasive breast tumors, a significant association was established between higher intron 20-mRNA expression and a diffuse infiltrative growth pattern (33). The functional significance for the overexpression of short CDP/Cux isoforms was established in mouse models. Transgenic mice engineered to express the p75 CDP/Cux isoform displayed heightened susceptibility to a disease defined as a myeloproliferative disease-like myeloid leukemia (49). Interestingly, the p110 CDP/Cux transgenic mice did not display a higher incidence of myeloproliferative diseases but developed malignancies in other tissues and cell types.⁸ Thus, globally, results from several studies indicate that higher expression of CDP/Cux short isoforms is associated with, and contribute to, the development of cancer.

⁸ Cadieux et al., in preparation.

The higher expression of cathepsin L in many transformed cells and primary human material has been abundantly reported (26, 29, 50, 51). A causal link between ras activation and the higher expression of cathepsin L has also been documented in numerous studies (16, 20, 22-25). Whereas previous studies reported the overall increase in cathepsin L expression as well as its secretion, our results revealed that cathepsin L expression and activity were also increased in the nucleus of transformed cells (Fig. 3). It is generally accepted that cathepsin L plays a role in metastasis through its extracellular activities once it has been secreted (22, 26, 27, 30, 52). Our results lead us to propose that cathepsin L also plays a role within cells and notably within the nucleus where it can change the properties of some transcriptional regulators. We acknowledge that the catalytic activity of short cathepsin L isoforms remains to be shown in an *in vitro* system. Despite our efforts with many expression systems, cathepsin L isoforms that include a partial prodomain did not fold properly in our hands and did not exhibit catalytic activity (data not shown). These negative results could mean that short cathepsin L species simply have no enzymatic activity or, alternatively, that our *in vitro* systems do not adequately recapitulate the *in vivo* situation. We favor the latter hypothesis because our results established a clear link between ras transformation, the level of cathepsin L in the nucleus, and the processing of CDP/Cux (Figs. 1A, C, D, and E

and 3D and E). These findings agree with previous results showing a correlation in normal cells between the increase in CDP/Cux processing in S phase and the increase in cathepsin L signal in the nucleus in indirect immunofluorescence (14). A role for cathepsin L inside the cells was also supported by the difference in the effects of protease inhibitors that are permeable or not. A cell-permeable cysteine protease inhibitor was able to delay entry into S phase and hinder the formation of foci in soft agar, whereas the inhibitor that was not permeable had no effect on these processes (Fig. 4C and D). This result strongly suggests that intracellular cysteine proteases play a role in cell proliferation. As the processed CDP/Cux isoform was shown to stimulate cell proliferation by accelerating S-phase entry, we speculate that the effect of cysteine proteases on proliferation is mediated, at least in part, through the proteolytic processing of CDP/Cux (10, 14). Whether cathepsin L is the sole cysteine protease that accounts for the effect on cell proliferation could not be verified using cathepsin L RNA interference because its expression, in particular of its nuclear isoforms, could not be reduced to a sufficiently low level (Fig. 3C, *top*, compare *lanes 3* and *lanes 4-6* and *lanes 7* and *8*).

The findings reported in the present study add to the increasing evidence linking the aberrant activity of certain proteases with the altered localization or change in biochemical properties of proteins in cancer cells (53-57). As site proteolysis has the potential to generate truncated protein isoforms with novel biochemical properties, the aberrant expression or activity of intracellular proteases may lead to the activation of some proto-oncogenes and inactivation of some tumor suppressors. In the case of cathepsin L, thus far, only one substrate has been identified in the nucleus but there is no doubt that other proteins must be targeted particularly in cancer cells. Future studies should attempt to identify the full repertoire of targets in the nucleus and define the functional consequences of their cleavage. Notably, nuclear cathepsin L has the potential to influence the transcriptional program of cells by changing the properties of transcriptional factors. In this manner, higher nuclear cathepsin L activity could potentially affect many stages of tumorigenesis, including hyperplasia, angiogenesis, cell movement, and invasion.

Materials and Methods

Cell Culture

NIH3T3, Ha-ras-V12 transformed 3T3, and SkBr3 cells were cultured in DMEM supplemented with 10% fetal bovine serum; MCF7, MDA231, T47D, and Hs578T cells were cultured in DMEM with 5% fetal bovine serum. Human mammary epithelial cells were purchased from Clonetics and cultured using the manufacturer's instructions. All transfections were done using ExGene500 (Fermentas) or Gene Juice (Novagen) according to the manufacturer's instructions. Cell synchronization was done as before (14).

Counterflow Centrifugal Elutriation

Cells (2×10^8) in 10 mL of cold PBS + 1% fetal bovine serum were submitted to counterflow centrifugal elutriation using a JE-5.0 rotor at constant speed (2,460 rpm) in a

Beckman Coulter Avanti J-20 centrifuge. Fluorescence-activated cell sorting analysis was done as before (58).

Retroviral Infections

Cells were infected by the addition of virus-containing supernatant from 293VSV producer cells in the presence of polybrene (8 $\mu\text{g}/\text{mL}$; ref. 59). Forty-eight hours later, cells were either analyzed or trypsinized and selected for 5 days with the appropriate drug.

Soft Agar Assays

ras-3T3 cells (10,000) previously mixed with 0.3% agar-DMEM supplemented with 10% fetal bovine serum and either DMSO or 100 $\mu\text{mol}/\text{L}$ JPM-OEt or JPM-565 were seeded on a 4% agar cushion. Cells were fed every 3 days with 0.3% agar-DMEM-10% fetal bovine serum with the appropriate inhibitor or DMSO. After 3 weeks, the colonies were fixed with PBS-formalin and stained with Giemsa.

Cell Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts and electrophoretic mobility shift assay were obtained as described in ref. 8. Alternatively, nuclei were prepared using the Nuclei EZ Prep (Sigma) kit according to the manufacturer's protocol. Total extracts were made in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS]. Buffers were supplemented with protease inhibitor mix tablets (Roche), 1 mmol/L phenylmethylsulfonyl fluoride, 100 $\mu\text{mol}/\text{L}$ E-64, and 1 mmol/L DTT. Where indicated, extracts were treated with *N*-glycanase F for 1 h at 37°C (Biolabs).

Pulse-Chase Labeling

Two days after transfection, cells were incubated 1 h with methionine/cysteine-free medium followed by a 4-h pulse with medium containing 0.1 mCi/mL [^{35}S]methionine. Cells were then chased with cold medium. At different time points, cells were harvested and lysed. Immunoprecipitation was done using HA antibody (Covance) and protein G-agarose (Amersham Pharmacia Biotech).

Plasmid Description

All plasmids have been described previously (8, 14).

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