Carboxyl-terminal Proteolytic Processing of CUX1 by a Caspase Enables Transcriptional Activation in Proliferating Cells*

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Proteolytic processing at the end of the G1 phase generates a CUX1 isoform, p110, which functions either as a transcriptional activator or repressor and can accelerate entry into S phase. Here we describe a second proteolytic event that generates an isoform lacking two active repression domains in the COOH terminus. This processing event was inhibited by treatment of cells with synthetic and natural caspase inhibitors. In vitro, several caspases generated a processed isoform that co-migrated with the in vivo generated product. In cells, recombinant CUX1 proteins in which the region of cleavage was deleted or in which Asp residues were mutated to Ala, were not proteolytically processed. Importantly, this processing event was not associated with apoptosis, as assayed by terminal dUTP nick end labeling assay, cytochrome c localization, poly(ADP-ribose) polymerase cleavage, and fluorescence-activated cell sorting. Moreover, processing was observed in S phase but not in early G1, suggesting that it is regulated through the cell cycle. The functional importance of this processing event was revealed in reporter and cell cycle assays. A recombinant, processed, CUX1 protein was a more potent transcriptional activator of several cell cycle-related genes and was able to accelerate entry into S phase, whereas mutants that could not be processed were inactive in either assay. Conversely, cells treated with the quinoline-Val-Asp-2,6-difluorophenoxymethylketone caspase inhibitor proliferated more slowly and exhibited delayed S phase entry following exit from quiescence. Together, our results identify a substrate of caspases in proliferating cells and suggest a mechanism by which caspases can accelerate cell cycle progression.

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The roles of caspases in cytokine maturation and apoptosis have been thoroughly documented (for a detailed review, see Refs. 1 and 2). Caspases are expressed as inactive zymogens, and their activation proceeds by one of two mechanisms. Caspase-2, -8, -9, and -10, the initiator caspases, are activated by dimerization. They contain CARD (caspase recruitment domain) or DED (death effector domain) domains and are brought together via homotypic interactions with upstream molecules. Caspase-3, -6, and -7, the effectors, are present as inactive dimers that are activated by proteolysis (3). Caspase substrates have aspartate in the P1 position, and the specificity of recognition by individual caspases is determined by the amino acids in the P2, P3, and P4 positions.

In addition to their functions in cell death, caspases participate in nonapoptotic processes, such as differentiation and proliferation. Caspase activity has been documented during the differentiation of diverse cell types, including erythrocytes, keratinocytes, lens fiber, and sperm cells, and was often accompanied by some of the morphological changes associated with apoptosis (e.g. chromatin condensation, enucleation, and remodeling of the cytoplasm) (4–6). In contrast, differentiation of monocyte into macrophages did not involve morphological signs of apoptosis (7).

Evidence for a role of caspases in proliferation comes mostly from the study of the immune system. A number of studies revealed that caspases were activated following T cell stimulation with phytohemagglutinin, IL-2, or other mitogens (8–11). However, no evidence of cell death was detected. Moreover, a number of caspase inhibitors were able to block T cell proliferation (8, 9). In agreement with these findings, an inherited mutation in humans, causing defects in the activation of T, B, and NK (natural killer) cells, was mapped to the caspase-8 gene (12). In mice, T cell-restricted ablation of the caspase-8 gene resulted in a decrease in the number of peripheral T cells and impaired activation-induced T cell proliferation (13). Indirect evidence for the involvement of caspases in T cell proliferation was also suggested from studies on Fas-associated via death domain, FADD. In mice that lack FADD function, either due to gene ablation or expression of a dominant negative, T cells were defective in activation-induced proliferation (14, 15). In accordance with these findings, T cells expressing dominant negative FADD mutant arrested in G0/G1 (15). Mice expressing a FADD mutant in which serine 191 was mutated to aspartate were smaller and anemic and presented splenomegaly. No defect in apoptosis was noticed, but an impairment in the cell cycle progression of T cell was evident (16). Interestingly, the
equivalent serine in human FADD is differentially phosphorylated throughout the cell cycle, further suggesting that post-translational modifications could modulate FADD function (16).

Much less is known about the mechanism(s) by which caspases participate in cell proliferation. One study reported a correlation between cell proliferation in the BJAB B-lymphoid cell line and a caspase-like activity that caused the truncation of the p27Kip1 Cdk inhibitor (17). Following T cell receptor activation, caspase-8 was shown to induce the nuclear translocation of NF-kB in a manner dependent on its catalytic activity (18, 19). Interestingly, the long isoform of FLICE-like inhibitory protein, cFLIPL, was found to function both as an activator and substrate of caspase-8 (18, 20). cFLIPL was shown to activate caspase-8 by forming a heterodimer with it. In turn, the cleavage of cFLIPL into p43FLIP enhanced its ability to recruit adaptor proteins that promote activation of NF-kB. Apart from cFLIPL, no other caspase substrate has been conclusively identified in this system. Thus, although a number of substrates have been described in apoptotic conditions, substrates that are associated with proliferation still remain to be identified.

Limited proteolysis regulates the activities of many transcription factors. Cleavage of Notch, SREBP, and others results in their translocation to the nucleus (21, 22). Alternatively, limited proteolysis of IRF2, Stat3, -5, and -6, and CCAAT/enhancer-binding protein removes their activation domains (23). CUX1 is a transcription factor that has variously been named CDP, CUTL1, CUX, or CDP/Cux. CUX1 has recently been identified as a substrate for the caspase-8-like enzyme caspase-9, which regulates the nuclear translocation of NF-kB. The product of this proteolysis, p110, lacks the amino-terminal inhibitory domain and the cut repeat 1 (CR1) and binds more stably to DNA (24, 25). Although the full-length CUX1 protein was characterized as a transcriptional repressor, the processed p110 isoform was found to function both as a repressor and as an activator (reviewed in Ref. 27; see Refs. 25, 28, and 29). The molecular basis for the regulatory effect of p110 CUX1 remains to be defined.

In the present study, we show that another proteolytic processing event results in the removal of the COOH-terminal region of CUX1, which contains two active repression domains. Treatment of cells with a panel of protease inhibitors suggested that a caspase was responsible. Using site-directed mutagenesis, we mapped the region of cleavage to one or more aspartate residues downstream of the Cut homeodomain. This cleavage was observed in proliferating cells and was confirmed to occur in the absence of apoptosis. In vitro processing assays suggested that CUX1 can be a substrate for multiple caspases. Interestingly, a truncated recombinant protein was a more potent activator in reporter assays and was also able to accelerate entry into S phase. These results identify a novel caspase substrate that plays a role in cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**

Sequences and/or maps will be provided upon request for CUX1-(831–1505) Δ1320–1351, Myc-CUX1-(878–1505)-HA D1320,36,39A, Myc-CUX1-(878–1336), and pTriEx/his/Myc1062–1505/CBP/protA constructs. All other CUX1 constructs have been described in our previous studies, although they were called CDP/Cux (25, 30).

**Expression and Purification of CUX1 Fusion Proteins**

The pTriEx/his/Myc1062–1505/CBP/protA expression plasmid was introduced into the BL21 (DE3) strain of *Escherichia coli* and induced with 1 mM isopropyl l-thio-β-d-galactopyranoside for 1.5 h. Cleared lysates were resuspended in IPP-100 buffer (10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM imidazole, 1 mM magnesium acetate, 4.5 mM CaCl₂, 10 mM β-mercaptoethanol, 10% glycerol, 0.1% Nonidet P-40) and incubated with calmodulin affinity resin (Stratagene). EGTA was used to elute the purified CUX1-containing complexes from the calmodulin column.

**Recombinant Caspase Expression, Purification, and Titration**

Recombinant caspases were expressed in *E. coli* as COOH-terminal His-tagged fusion proteins using the pET expression system (Novagen, Madison, WI). Proteins were expressed in the BL21 (DE3) *E. coli* strain (Novagen) and purified by Ni²⁺ affinity chromatography as previously described (31).

**Cell Culture, Transfection, and Synchronization**

Hs578T cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS). NIH3T3 and MCF-7 cells were grown in DMEM supplemented with 10% FBS. Kit225 T cells stably expressing CUX1 proteins were generated as in Ref. 32. Cells were grown in RPMI supplemented with 10% FBS and 75 ng/ml human IL-2. Transient transfections were performed with Genejuice (Novagen) according to the manufacturer’s instructions.

**Cell Cycle Synchronization**

Synchronization of NIH3T3 cells was performed by two methods.

- **Serum Starvation/Stimulation**—Post-transfection, cells were maintained in DMEM for 72 h and then in DMEM plus 10% FBS for the indicated times.
- **Thymidine Block**—Post-transfection, cells were cultured overnight in DMEM plus 10% FBS supplemented with 2 mM thymidine and harvested. Stably infected Kit225 cells were deprived of IL-2 for 48 h, followed by IL-2 addition for the indicated times. Cell cycle distribution was monitored by fluorescence-activated cell sorting following ethanol fixation and propidium iodide staining (32).

**Carboxyfluorescein Diacetate Succinimidyl Ester Staining**

Cells were stained using the CellTrace™ carboxyfluorescein diacetate succinimidyl ester staining kit and were analyzed by flow cytometry with 488-nm excitation and emission filters appropriate for fluorescein, according to the

**3 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; IL, interleukin; Z, benzoxycarbonyl; fmk, fluoromethyl ketone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid; HA, hemagglutinin; Q-VD-OPh, quinoline-Val Asp-2,6-difluorophenoxymethylketone; DEVD-CHO, Asp-Glu-Val-Asp-aldehyde.


**CUX1, a Caspase Substrate in Proliferating Cells**

A

B

C

D

E

FIGURE 1. CUX1 is proteolytically processed downstream of the homeodomain in proliferating cells. A, Hs578T cells were transfected with vectors expressing recombinant CUX1 proteins with Myc epitope tag at their NH2 terminus. Nuclear extracts were prepared and analyzed by Western blot with a Myc antibody. The arrows indicate cleaved isoforms. B, NIH3T3 cells were transfected with a vector expressing Myc-CUX1-(878–1505)-HA and synchronized by serum starvation and restimulation or thymidine block. Cells were harvested, and cell cycle distribution was monitored by fluorescence-activated cell sorting analysis after staining of the DNA with propidium iodide. Nuclear extracts were analyzed by Western blot with the Myc antibody. The indicated antibodies, run on SDS-PAGE, and exposed to film overnight. C–20, NIH3T3 cells were transfected with a vector expressing CUX1-(878–1505)-HA and synchronized by serum starvation and restoration of thymidine block. Cells were harvested, and cell cycle distribution was monitored by fluorescence-activated cell sorting analysis after staining of the DNA with propidium iodide. Nuclear extracts were analyzed by Western blot with the Myc antibody. The indicated antibodies, run on SDS-PAGE, and exposed to film overnight.

**Luciferase Assay**

Luciferase assays were performed as previously described (25). Because the internal control plasmid is itself often repressed by CUX1, as a control for transfection efficiency the purified β-galactosidase protein (Sigma) was included in the transfection mix, as previously described (33). The luciferase activity was then normalized based on β-galactosidase activity.

**CUX1 Antibodies**

Antibodies 510, 861, 1061, and 1300 have been described previously (25, 34). Antibody c-20 is a goat polyclonal antibody raised against the last 20 amino acids of CUX1 (sc-6327; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Preparation of Total and Nuclear Extracts and Western Blot Analysis**

Nuclear extracts were prepared according to the procedure of Lee et al. (35), except that nuclei were obtained by submitting cells to one freeze/thaw cycle in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol). Nuclei were then resuspended in Buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA) and incubated at 4 °C for 30 min. After 15 min of centrifugation, the supernatant was collected. Buffers A and C were supplemented with protease inhibitor mix tablet purchased from Roche Applied Science and 1 mM phenylmethylsulfonyl fluoride. Total extracts were prepared by applying modified radioimmune precipitation buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, protease inhibitors as above) to a monolayer plate. After a 10-min incubation on ice, the resulting slurry was centrifuged for 15 min at 4 °C, and the supernatant was collected. Proteins were blotted onto polyvinylidene difluoride membrane and probed with the antibodies indicated. An actin antibody was used to monitor equal loading (sc-1615). Horseradish peroxidase-conjugated α-rabbit (1:4000) (sc-2313), α-mouse (1:10,000) (Jackson Immunoresearch), or anti-goat (1:4000) (sc-2020) secondary antibodies were used. Immunoreactive proteins were visualized by chemiluminescence with an ECL Western blotting detection kit (Amersham Biosciences).

a Myc antibody. The arrows indicate cleaved isoforms. B, NIH3T3 cells were transfected with a vector expressing Myc-CUX1-(878–1505)-HA and synchronized by serum starvation and restimulation or thymidine block. Cells were harvested, and cell cycle distribution was monitored by fluorescence-activated cell sorting analysis after staining of the DNA with propidium iodide. Nuclear extracts were analyzed by Western blot with the Myc antibody. The CUX1 bands were scanned using the Li-Cor Odyssey Infrared Imaging System, and the ratio of the full-length over the processed species is indicated below each lane. C, Hs578T cells were grown overnight in medium containing 35S-labeled Met and Cys. Whole cell extracts were immunoprecipitated (IP) with the indicated antibodies, run on SDS-PAGE, and exposed to film overnight. D, nuclear extracts from Hs578T cells were immunoprecipitated with the antibodies indicated and analyzed by Western blot with the 1300 antibody. E, a schematic representation of recombinant CUX1 proteins used in this study. Note that there are several potential NH2 termini for the endogenous p110 CUX1 isoform, since cathepsin L was shown to cleave at amino acids 643, 747, and 755 (24).
35S Labeling and Immunoprecipitation

Cells were grown for 16 h in medium containing [35S]Met and [35S]Cys. Total extracts were prepared as above (modified radioimmune precipitation buffer), and precleared with protein A-agarose beads for 1 h. After overnight incubation at 4 °C with antibody, beads were added for 1 h. Complexes were washed two times with wash buffer I (20 mM Tris-HCl, pH 8, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.2% SDS), two washes with wash buffer II (20 mM Tris-HCl, pH 9, 2 mM EDTA, 2 mM EGTA, 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS), and one wash with 1× phosphate-buffered saline. Samples were boiled in sample buffer and run on SDS-PAGE.

Protease Inhibitors

Protease inhibitors were purchased from Calbiochem and used at the following working concentrations: 20 μM E-64d, 40 μM MG132, 100 μM Z-VAD-fmk, 100 μM DEVD-CHO, 100 μM pepstatin A. Q-VD-OPh was purchased from Enzyme Systems Products; a 100 μM working concentration was used.

Electrophoretic Mobility Shift Assay

An electrophoretic mobility shift assay was performed with the indicated quantity of purified protein. Samples were incubated at room temperature for 20 min in a final volume of 30 μl of 25 mM NaCl, 10 mM Tris, pH 7.5, 1 mM MgCl₂, 5 mM EDTA, pH 8.0, 5% glycerol, 1 mM dithiothreitol, 3 μg of bovine serum albumin with 0.2 pmol of radiolabeled oligonucleotides. Samples were loaded on a 5% polyacrylamide (29:1), 0.5 TBE gel and separated by electrophoresis at 8 V/cm in 0.5 TBE. Gels were dried and visualized by autoradiography.

In Vitro Proteolytic Processing Assay

In vitro cleavage reactions were performed using 50 mM Hepes, pH 7.2, 50 mM NaCl, 0.015% CHAPS, 1 mM sodium citrate, 10 mM dithiothreitol (for caspase-2, -3, -8, -9, and -10) or 20 mM PIPES, pH 7.2, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose (for caspase-6 and -7). Caspases were activated at 37 °C for 10 min prior to the addition of 65 nM purified CUX1 protein. Reactions were carried out at 37 °C for 20 min, after which loading buffer was added and samples were boiled. The second order rate constant was calculated as described in Ref. 31. Briefly, \( k_{cat}/K_m = k = \ln(2)/t \times E \), where \( E \) is the active caspase concentration that cleaved 50% of the CUX1 substrate in the incubation period \( t \).

Terminal dUTP Nick End Labeling Assay

A terminal dUTP nick end labeling assay for free DNA 3'-OH ends was performed as per the manufacturer's directions with the ApopTag kit S7110 (Serologicals).

Immunofluorescence

NIH3T3 cells were plated on coverslips and transfected or not (see figure legends). Cells were fixed with 2% paraformaldehyde and then solubilized (5% FBS + 0.5% Triton X-100 in PBS) and incubated with primary antibodies α-Myc9e10, falloidin, α-cytochrome c, or α-Tom-20. Secondary antibody (anti-mouse Alexa-488 (1:1000) or anti-rabbit Alexa-594) was added for 30 min. Cells were visualized using a Zeiss AxioVert 135 microscope with a ×63 objective or using a Zeiss LSM 510 confocal microscope.
RESULTS

CUX1 Is Proteolytically Processed Downstream of the Homeodomain in Proliferating Cells—Previous studies, in which immunoblotting was performed with an NH2-terminal antibody, have revealed a species that was shorter than the full-length CUX1 protein (27, 28). These results raised the possibility that CUX1 was proteolytically processed at its COOH terminus. To verify this hypothesis, a panel of CUX1 proteins starting at amino acid 1, 659, or 878 were expressed in Hs578T cells and analyzed by immunoblotting with an antibody against the NH2-terminal Myc epitope tag. In addition to the full-length protein, in each case we could detect a species that was shorter than the parental molecule by 20–30 kDa (Fig. 1A). We next asked whether this proteolytic event was cell cycle-regulated. NIH3T3 cells were transfected with a vector expressing Myc-CUX1-(878–1505)-HA and were then synchronized in early G1, mid-G1, and S phases by serum deprivation and restimulation or thymidine block (see “Experimental Procedures”). The COOH-terminally cleaved protein was barely visible in early G1 and mid-G1 but was strongly expressed in unsynchronized cells and in S phase using either method of synchronization (Fig. 1B). Importantly, there was no sub-2N peak, which indicates the presence of apoptotic cells, at the time points where processing was observed (Fig. 1B; also see Fig. 3B). These results suggested that CUX1 is COOH-terminally truncated in proliferating cells.

Evidence for the Existence of an Amino- and Carboxyl-terminally Truncated CUX1 Isoform—We then verified whether an endogenous CUX1 protein lacking both the NH2 and COOH terminus could be detected. Hs578T cells were labeled overnight with [35S]Met and [35S]Cys. Whole cell extracts were immunoprecipitated with 861 and 1300 antibodies or, as a control, an HA antibody (Fig. 1C). Following autoradiography, the p200 and p110 CUX1 isoforms were detected, as well as an 80-kDa isoform that could be the product of processing events at the NH2 and COOH termini. In order to address this possibility and to attempt to map the epitopes present in the 80-kDa isoform, nuclear extracts were immunoprecipitated with a series of CUX1 antibodies, followed by Western blotting with the 1300 antibody (Fig. 1D; see Fig. 1E for a diagram of the protein and antibodies). As expected, p200 was immunoprecipitated by all of the antibodies (Fig. 1D, lanes 1–5). In contrast, p110 was detected following immunoprecipitation with transfected CUX1 (Myc) and F-actin (phalloidin). B, Hs578T cells were transfected with Myc-CUX1-(878–1505)-HA. The following day, cells were fixed and stained with propidium iodide, and the DNA content was analyzed. The bar indicates where cells with sub-2N content would be found. C, extracts from Fig. 2B were analyzed by Western blot with an anti-poly(ADP-ribose) polymerase antibody. D, NIH3T3 cells were plated on coverslips, and half were transfected with a construct expressing Myc-CUX1-(878–1505). The following day, the untransfected sample was treated with tumor necrosis factor-α (DAP) (blue), Myc (red), or free 3′-OH (green). E, NIH3T3 cells were plated on coverslips and transfected or not with a construct expressing Myc-CUX1-(878–1505). The following day, the untransfected sample was treated with tumor necrosis factor-α and cycloheximide for 5 h. Cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue), Tom20 (red), or for cytochrome c (green). In parallel, coverslips with transfected cells were stained with a Myc antibody (data not shown). In the transfected, untreated sample, 3 of 155 cells (1.9%) had no mitochondrial staining.

FIGURE 3. CTD processing occurs in nonapoptotic conditions. A, NIH3T3 cells were transfected with a construct expressing Myc-CUX1-(878–1505). Immunofluorescence was performed with antibodies that recognize trans-
antibodies 861, 1061, and 1300 but not with 510 and only weakly with C-20 (Fig. 1D, lanes 1–5). The 80-kDa species was immunoprecipitated with antibodies 861, 1061, and 1300 but not with 510 or C-20 (Fig. 1D, lanes 1–5). These results are consistent with the notion that an endogenous 80-kDa CUX1 species is truncated at both its COOH and NH2 termini. Hereafter, this species will be called p80.

A Caspase Cleaves CUX1 in the COOH-terminal Region—To determine which protease(s) cleaves the CUX1 COOH terminus, NIH3T3 cells were transfected with a vector expressing Myc-CUX1-(878–1505)-HA, and, following treatment with a series of protease inhibitors, nuclear extracts were analyzed by Western blot with the Myc antibody (Figs. 2A and 7A). Proteolytic processing was partially inhibited by treatment with Z-VAD-fmk (Fig. 2A) and Q-VD-OPh (Fig. 7A), both broad spectrum caspase inhibitors, but not by any of the other inhibitors tested (Fig. 2A, compare lane 5 with the other lanes). Inhibition by Z-VAD-fmk was also observed in transfected MCF-7 and Kit225 T cells stably expressing Myc-CUX1 747–1505-HA (Fig. 2A, compare lanes 9 and 13 with lanes 10–12 and 14). Inhibition of processing by Z-VAD-fmk and the absence of inhibition by E-64d and MG132 implicated a cysteine protease and discriminated against cathepsins B and H. Indeed, co-transfection of a vector expressing a viral caspase inhibitor variant with broad range caspase inhibition, CrmA<sub>QMD</sub>, inhibited COOH-terminal processing (Fig. 2B). Expression of a recombinant CUX1-(831–1505) protein generated a processed species that migrated close to the recombinant CUX1-(831–1336) protein (Fig. 2C, lanes 1 and 2). Examination of the amino acid sequence in this region revealed the presence of four Asp residues, Asp-1320, Asp-1336, Asp-1339, and Asp-1351, all of which represent potential caspase cleavage sites (Fig. 1E). Deletion of amino acids 1320–1351 prevented proteolytic processing (Fig. 2C, compare lanes 2 and 3). Moreover, replacement of 1, 2, or 3 Asp residues with alanine reduced or eliminated proteolytic processing (Fig. 2D). Altogether, the inhibition of cleavage in the presence of Z-VAD-fmk, Q-VD-OPh, or CrmA<sub>QMD</sub> or following the deletion or replacement of Asp residues within CUX1 implicated a caspase-like activity in the COOH-terminal processing of CUX1.

CUX1 Is Processed in Nonapoptotic Conditions—To ensure that processing was not occurring post-lysis (36), Z-VAD-fmk was added to the cells 5 min prior to lysis and was included in all buffers used in subsequent steps. The processed isoform was still observed in Western blot analysis (data not shown). We next asked whether there was any evidence of apoptosis in cells in which processing was observed. No evidence of membrane blebbing or abnormal cellular morphology was observed upon actin staining (Fig. 34; data not shown). No apoptotic population was observed above background in flow cytometry profiles of cells stained with propidium iodide (Fig. 3B). Using the terminal dUTP nick end labeling assay, we examined 250 Myc-positive cells and found that none of these transfected cells was terminal deoxynucleotidyltransferase-positive (Fig. 3D). In contrast, terminal deoxynucleotidyltransferase-positive cells were easily detected following 5 h of treatment of NIH3T3 cells with tumor necrosis factor-α and cycloheximide (Fig. 3D). Protein extracts from transfected cells (see Fig. 2B) were immunoblotted for the caspase substrate, poly(ADP-ribose) polymerase, but cleavage products were not detected, again ruling out postlysis artifacts (Fig. 3C). Another indicator of apoptosis, the release of cytochrome c from the mitochondria, was only observed upon induction of apoptosis with tumor necrosis factor-α and cycloheximide (Fig. 3E). Altogether, these results confirmed that proteolytic processing of CUX1 was occurring in nonapoptotic cells.

CUX1 Is a Substrate for Caspases in Vitro—A COOH-terminally epitope-tagged CUX1 protein was produced in bacteria and affinity-purified on calmodulin beads. The approximate concentration of substrate was determined by comparing
Coomassie staining to bovine serum albumin standards (data not shown). 65 nM substrate, which is below the range of $K_m$ for natural substrates (31) and therefore allows the observation of first order kinetics, was incubated with increasing amounts of a panel of activated titrated recombinant caspases for 30 min at 37 °C. Western blot analysis with a Myc antibody revealed that CUX1 was a substrate for caspase-2, -3, -7, -8, -9, and -10 but not for caspase-6 (Fig. 4). A recombinant protein truncated at 1336 co-migrated with the caspase cleavage product (Fig. 4), confirming that cleavage happens at or near Asp-1336, as it does in vivo (Figs. 2, C and D, and 4). COOH-terminal processing was observed in MCF-7 cells, which lack caspase-3, and caspase-8-null Jurkat cells (data not shown). These findings indicate that other caspases must cleave CUX1 in these cells, but the involvement of caspase-3 and -8 in some cells cannot be excluded. Altogether, these results confirm that CUX1 can be a substrate for one or more caspase(s).

A CTD-truncated CUX1 Isoform Stimulates the DNA Polymerase α Gene Promoter—Functional assays were performed to investigate the consequence of COOH-terminal processing. The Myc-CUX1-(878–1505)-HA construct was introduced into Hs578T cells, and nuclear extracts were prepared from cells treated or not with Z-VAD. As predicted, this treatment inhibited the production of the processed isoform (Fig. 5A). Electrophoretic mobility shift assays with a consensus CUX1 binding site demonstrated that the processed isoform was able to bind to DNA (Fig. 5B, lane 3, p80). Note that although the p110-retarded complex was supershifted by both the Myc and HA antibodies, the p80 complex was shifted by the Myc antibodies but not by the COOH-terminal HA antibodies (Fig. 5B, lanes 1 and 2). In previous studies, the p110 isoform was shown to function as a transcriptional activator of the DNA polymerase α gene promoter (25, 26, 28, 29). However, in retrospect, we realized that the p110 isoform was always expressed together with its COOH-terminally processed isoform (e.g. see Fig. 3B in Ref. 28). The question arose, therefore, as to which of the two isoforms was able to transactivate this promoter, given that proteolytic processing in the COOH-terminal region results in the removal of two active repression domains (37, 38). The availability of mutants that are defective in this processing...
event enabled us to compare the transactivation potential of both the processed and unprocessed p110 isoforms. Whereas both the recombinant CUX1 (878–1505) and (878–1336) proteins strongly stimulated the DNA polymerase α reporter, the two mutants that are not COOH-terminally processed, 878–1505D1320–1351 and 878–1505D1336,1339A, were unable to transactivate this reporter (Fig. 5C, 878–1505D1320–1351, Fig. 5E, 878–1505D1336,1339A). Importantly, the mutant 878–1505D1320–1351 was able to repress the p21WAF1/CKI1 gene reporter, a finding that confirms that the protein was able to bind to DNA and carry transcriptional regulation (Fig. 5C). The failure of processing mutants to transactivate was observed with other reporters as well; although 878–1336 strongly stimulated the cyclin A2, CAD, DHFR, and B-myb reporters, the 878–1505D1336,1339A had little effect, if any (Fig. 5G). We have recently shown that CUX1 cooperates with E2F transcription factors in the transcriptional activation of cell cycle-regulated genes.4 878–1336 cooperated with E2F1 in the transactivation of the DNA polymerase α reporter. However, no cooperation was observed between 878–1505A1320–1351 and E2F1 (Fig. 5H), suggesting that it is p80 and not p110 that forms a complex with E2F1 on the DNA polymerase α promoter and mediates transcriptional activation. Altogether, these results demonstrate that only the COOH-terminally processed isofrom is able to transactivate the DNA polymerase α reporter.

Overexpression of a Truncated CUX1 Protein Accelerates Entry into S Phase—In cell-based assays, the p110 isofrom was previously shown to stimulate cell cycle progression by accelerating entry into S phase (32). Again, the availability of mutants that are less efficiently processed enabled us to investigate the cell cycle activities of the distinct isoforms. We used the IL-2-dependent Kit 225 T cell line and generated populations of cells stably carrying a retroviral vector expressing either nothing or the CUX1 747–1505, 747–1336, or 747–1505D1320,1336,1339A proteins. Cell cycle progression assays were performed three times with similar results. A representative example is shown in Fig. 6A. Following IL-2 starvation and restimulation, the control Kit 225 cells started to enter into S phase after 18 h (Fig. 6A). Expression of either CUX1 (747–1505) or (747–1336) stimulated cell cycle progression, since a greater proportion of cells were in S phase at 18 h (Fig. 6A). These results are consistent with those from a previous study (32). In contrast, cells expressing the 747–1505D1320,1336,1339A protein progressed just like the control cells (Fig. 6A). These results suggest that COOH-terminal processing of CUX1 is required for the stimulation of S phase entry. However, we cannot exclude the possibility that a stronger effect is mediated in the presence of both the processed and nonprocessed isoforms.

*Treatment of Cells with Q-VD-OPh Decreases the Proliferation Rate and Delays S Phase Entry—The* fact that only the cleavable form of CUX1 was able to accelerate S phase entry suggested that caspase activity might be required for cell cycle progression and cell proliferation. To investigate the role of caspases in cell proliferation, we monitored the effect of a broad spectrum caspase inhibitor, Q-VD-OPh, on the proliferation and cell cycle progression of two types of cells, NIH3T3 and Kit 225 T cells. The treatment of NIH3T3 cells with 100 μM Q-VD-OPh significantly reduced the steady-state level of the processed CUX1 isofrom (Fig. 7A). Cells treated with the same amount of Q-VD-OPh proliferated more slowly, with a doubling time of 42 h, compared with 37 h for the Me2SO control (Fig. 7B). Following serum starvation and restimulation, the addition of Q-VD-OPh delayed entry into S phase by ~2 h. Although some of the Me2SO (vehicle)-treated cells began to enter S phase at 18 h and had already moved into G2 at 22 h, the Q-VD-OPh-treated cells started to enter S phase at 20 h and were still behind control cells at 22 h (Fig. 7C). Similarly, the treatment of Kit 225 cells with Q-VD-OPh inhibited cell proliferation as measured in two assays, cell counting and CSFE staining (Fig. 7, D and E). Following IL-2 starvation and restimulation, Q-VD-OPh delayed entry into S phase of Kit 225 cells, albeit to a lesser extent than with the NIH3T3 cells (Fig. 7, C and F). In agreement with our findings, another group showed inhibition of cell cycle entry following cytokine-

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induced quiescence in the presence of 50 μM Q-VD-OPh (39). Although we cannot exclude the possibility that pro- 

these results are consistent with the notion that caspase activity is required for proliferation and S phase entry.

**DISCUSSION**

In this study, we demonstrated a proteolytic processing event that results in the removal of the carbox- 
yl-terminal region of the CUX1 transcription factor. A number of results implicated a caspase-like activity in the COOH-terminal processing of CUX1. Proteolytic processing was inhibited in cells that were maintained in the presence of Z-VAD-fmk or Q-VD-OPh as well as in cells that overexpressed the CrmA/DQMD caspase inhibitor (Figs. 2, A and B, 5A, and 7A). Moreover, the replacement of aspartate with alanine residues within the CUX1 COOH-terminal region reduced or eliminated proteolytic processing (Figs. 2D and 5F). In vitro, several caspases cleaved CUX1 in the COOH-terminal region to produce a peptide with similar electrophoretic mobility as that of the in vivo generated peptide (Fig. 4). Importantly, CUX1 was cleaved with relative efficiency by caspase-2, -3, -7, -8, -9, and -10. Indeed, the $k_{cat}/K_m$ ratio was estimated to be in the $10^{-8}$–$10^{-7}$ M$^{-1}$ s$^{-1}$ range, which is close to that of known caspase substrates, such as poly(ADP-ribose) polymerase, whose $k_{cat}/K_m$ ratio is in the $10^{-6}$ M$^{-1}$ s$^{-1}$ range (Fig. 4; data not shown) (31).

Interestingly, the proteolytic processing of CUX1 by a caspase-like activity was not associated with signs of apoptosis. Immunoblotting analysis of the same cell extracts did not reveal the cleavage product of CUX1 COOH-terminal region to produce a peptide with similar electrophoretic mobility as that of the in vivo generated peptide (Fig. 4). Importantly, CUX1 was cleaved with relative efficiency by caspase-2, -3, -7, -8, -9, and -10. Indeed, the $k_{cat}/K_m$ ratio was estimated to be in the $10^{-8}$–$10^{-7}$ M$^{-1}$ s$^{-1}$ range, which is close to that of known caspase substrates, such as poly(ADP-ribose) polymerase, whose $k_{cat}/K_m$ ratio is in the $10^{-6}$ M$^{-1}$ s$^{-1}$ range (Fig. 4; data not shown) (31).

Interestingly, the proteolytic processing of CUX1 by a caspase-like activity was not associated with signs of apoptosis. Immunoblotting analysis of the same cell extracts did not reveal the cleavage product of poly(ADP-ribose) polymerase, a known caspase substrate in apoptotic cells (Fig. 3C). Cells displayed a normal morphology without any membrane blebbing, nuclear fragmentation, or evidence of cytochrome C release (Fig. 3). Apoptotic cells were not detected in the termi-
judged from the ratio of the full-length over the processed species, was higher in populations of cells enriched in S phase, whether by serum starvation/restimulation or thymidine block, than in populations enriched in G0/G1 or in G1 (Fig. 1B). In accordance with the notion that the cleavage of CUX1 was not associated with cell death but, instead, with proliferation, functional assays indicated that the COOH-terminally processed isoform of CUX1 was a more potent transactivator in reporter assays and was able to accelerate entry into S phase (Figs. 5, E and G, and 6). In contrast, mutants of CUX1 that were not processed as efficiently were less efficient in the transactivation assay, and were unable to accelerate S phase entry (Figs. 5, C, E, and G, and 6). Altogether, the absence of features associated with apoptosis, the cell cycle profile of cells, and the heightened functional properties of the processed CUX1 isoform all concur that the COOH-terminal processing of CUX1 is linked to cellular proliferation.

The COOH-terminal processing of CUX1 was mapped to the region immediately downstream of the Cut homeodomain. This region does not show much sequence conservation, apart from the relatively high concentration of alanine and proline residues, a distinctive feature of some transcriptional repression domains (40). Indeed, two active repression domains were mapped within the COOH-terminal region of CUX1 and were shown to be able to repress transcription when bound to DNA at a distance from the transcription start site (37). The mechanism of active repression appears to involve the recruitment of both the histone deacetylase, HDAC1, and of the histone lysine methyltransferase, G9a (38, 41). Deletion or replacement mutations that hindered the COOH-terminal processing of CUX1 also impaired the transactivation activity of the protein in the reporter assay (Fig. 5, C, E, and G). One interpretation of these results is that the active repression domains must be removed in order to convert the CUX1 repressor into an activator. Although this model is attractive and certainly fits with much of the data, we are not convinced that this is the sole mechanism that explains how CUX1 can function as a repressor or an activator. In particular, we are struck by the fact that, independently of the isoform tested, CUX1 functions only as a repressor on the p21 promoter and only as an activator on the DNA polymerase α promoter (26, 28–30, 41, 42). Moreover, we have recently identified a target, the N-myc gene, on which COOH-terminal processing was not an obligate prerequisite for CUX1 to function as an activator. It appears, therefore, that the promoter context has a major impact on the regulatory effect of CUX1. This can be best explained by assuming that CUX1 associates with specific partners on distinct promoters and that the direction of the regulation is determined by the type of protein complex that forms on a specific promoter. In this context, we could envisage that a CUX1 isoform that carries its repression domain might still be able, on some promoters, to engage in a complex that ultimately mediates activation, whereas on other promoters the removal of the COOH-terminal region would facilitate the formation of an activation complex. Moreover, we can envisage that other post-translational modifications, besides proteolytic processing, can modulate the interactions between CUX1 and various co-regulators.

Previous studies have identified many substrates of caspases that play a role in cell cycle progression, such as cyclin E (43), Cdc2 (44), Cdc27, Wee1 (45), pRb (46), and MDM2 (47). Cleavage of these substrates invariably caused their inactivation and was associated with apoptotic conditions. So far, only a few caspase substrates have been linked to proliferation. One study reported a correlation between cell proliferation in the BJAB B-lymphoid cell line and a caspase-like activity that caused the truncation of the p27Kip1 Cdk inhibitor (17). Inactivation of p27Kip1 would be assumed to enable higher cyclin-dependent kinase activity; however, this was not directly tested. The cleavage of cFLIP into p43cFLIP is believed to promote the assembly of a protein complex that ultimately triggers the activation of the NF-κB transcription factor (reviewed in Ref. 48). The SATB1 transcription factor was shown to be inactivated as a result of cleavage by caspase-6 upon activation of quiescent human tonsillar B cells (49). The activation of NF-κB and inactivation of SATB1 are believed to play an important role in the overall change in transcriptional program that takes place upon reentry into the cell cycle. In the case of CUX1, caspase-mediated cleavage led to an increase in the transactivation potential of this factor on a number of its cell cycle targets, including the DNA polymerase α, cyclin A2, CAD, DHFR, and B-myb genes. The fact that a truncated CUX1 mutant was a better activator and was able to accelerate entry into S phase indicates that the caspase-mediated cleavage of CUX1 indeed has an impact on cell cycle progression.

In this study, the treatment of cells with the broad spectrum caspase inhibitor, Q-VD-OPh, delayed S phase entry and decreased the rate of proliferation in fibroblasts and in T cells (Fig. 7). These results are in agreement with previous reports showing that various caspase inhibitors were able to block the proliferation of B and T cells (49, 50). The identification of CUX1 as a substrate of caspase activity provides one mechanism by which caspases may play a role in cell cycle progression, but there is little doubt that caspases target additional substrates in proliferating cells. We envisage that caspase-mediated cleavage will both inactivate proteins that block proliferation and activate proteins that promote proliferation. Future studies should also decipher the regulatory mechanisms that determine whether caspases accelerate cell proliferation or promote cell death.

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REFERENCES

CUX1, a Caspase Substrate in Proliferating Cells