

Development of an *in Vitro* Assay for the Proteolytic Processing of the CDP/Cux Transcription Factor

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The CDP/Cux transcription factor was previously shown to be proteolytically processed at the G1/S transition. In view of characterizing and eventually identifying the protease responsible for CDP/Cux processing, we have established an *in vitro* proteolytic processing assay. CDP/Cux recombinant proteins expressed in mammalian or bacterial cells were efficiently processed *in vitro* using as a source of protease either whole cell extracts, the nuclear or the cytoplasmic fraction. Processing was found to take place optimally at a lower pH, to be insensitive to variations in salt concentration, and to be inhibited by the protease inhibitors MG132 and E64D. Interestingly, the bacterially-produced substrate was more efficiently processed than the substrate purified from mammalian cells. Moreover, processing *in vitro* was more efficient when CDP/Cux substrates were purified from populations of cells enriched in the S phase than in the G1 phase of the cell cycle. Altogether, these results suggest that post-translational modifications of CDP/Cux in mammalian cells inhibits processing and contributes to the cell cycle-dependent regulation of processing. The *in vitro* processing assay described in this study will provide a useful tool for the purification and identification of the protease responsible for the processing of CDP/Cux.

Keywords: CDP/Cux, Proteolytic processing, *In vitro* assay, Cell cycle

Introduction

CDP/Cux (CCAAT-displacement protein/cut homeobox) belongs to a family of transcription factors present in all

metazoans and involved in the control of proliferation and differentiation (see review by Nepveu, 2001). In *Drosophila melanogaster*, a large number of phenotypes were found to be caused by the insertion at various positions of a transposable element that disrupted the expression of the *cut* gene in a tissue-specific manner (Modolell *et al.*, 1983; Bodmer *et al.*, 1987; Blochlinger *et al.*, 1990; Blochlinger *et al.*, 1991; Jack *et al.*, 1991; Liu *et al.*, 1991; Liu and Jack, 1992; Dorsett, 1993; Jack and DeLotto, 1995; Cai and Levine, 1997). Cloning of the *cut* cDNA revealed that the gene coded for a homeodomain protein (Blochlinger *et al.*, 1988). In higher, vertebrates, there are two CDP/Cux genes called CDP-1 and CDP-2 in human, and Cux-1 and Cux-2 in mouse and chicken (Neufeld *et al.*, 1992; Valarche *et al.*, 1993; Quaggin *et al.*, 1996). The *cux-1* knockout mice displayed phenotypes in various organs including curly whiskers, growth retardation, delayed differentiation of lung epithelia, altered hair follicle morphogenesis, male infertility, a deficit in T and B cells, and a surplus in myeloid cells (Tufarelli *et al.*, 1998; Ellis *et al.*, 2001; Sinclair *et al.*, 2001; Luong *et al.*, 2002). In contrast to the small size of the *cux-1* knock-out mice, transgenic mice expressing Cux-1 under the control of the cytomegalovirus virus (CMV) enhancer/promoter displayed multi-organ hyperplasia and organomegaly (Ledford *et al.*, 2002). Thus, from genetic studies both in *Drosophila* and the mouse, it is clear that the CDP/Cux/Cut genes play an important role in the development and homeostasis of several tissues.

A number of studies demonstrated that CDP/Cux is regulated in a cell cycle-dependent manner and may have a specific function in S phase. The histone nuclear factor D (HiNF-D), which was found later to include CDP/Cux as its DNA binding partner, was shown to be up-regulated in S phase in normal cells (van Wijnen *et al.*, 1989; Holthuis *et al.*, 1990; van Wijnen *et al.*, 1991; Wright *et al.*, 1992; van Wijnen *et al.*, 1996). The up-regulation of stable DNA binding at the G1/S transition was shown to involve at least two post-translational modifications: dephosphorylation of the Cut homeodomain by the Cdc25A phosphatase, and proteolytic cleavage of CDP/Cux p200

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between CR1 and CR2 to generate CDP/Cux p110 (Coqueret *et al.*, 1998a; Moon *et al.*, 2001). Later in the cell cycle, DNA binding was found to decrease in G2 following phosphorylation by cyclin A/Cdk1 of two serines residues in the region of the Cut homeodomain, S1237 and S1270 (Santaguida *et al.*, 2001).

At the molecular level, CDP/Cux is a complex protein containing four evolutionarily conserved DNA binding domains: three Cut repeats (CR1, CR2, and CR3) and a Cut homeodomain (HD) (Neufeld *et al.*, 1992; Andres *et al.*, 1994; Aufiero *et al.*, 1994a; Harada *et al.*, 1994). *In vitro* DNA binding studies revealed that various combinations of the Cut repeats and the Cut homeodomain can cooperate to bind DNA with distinct specificity and kinetics (Andres *et al.*, 1994; Aufiero *et al.*, 1994b; Harada *et al.*, 1994; Catt, Harada *et al.*, 1995; Luo and Skalniak, 1999; Moon *et al.*, 2000). In particular, CR1CR2 can make a rapid but transient interaction with DNA, whereas CR2CR3HD and CR3HD bind more slowly, but stably to DNA (Moon *et al.*, 2000). Somewhat surprisingly, *in vitro* a purified full length CDP/Cux protein, which we refer to as CDP/Cux p200 behaved like CR1CR2 in that it made an unstable interaction with DNA (Moon *et al.*, 2000; Moon *et al.*, 2001). However, CDP/Cux p200, was found to be proteolytically processed at the G1/S transition of the cell cycle, thereby generating the CDP/Cux p110 isoform that contains three DNA binding domains, CR2, CR3, and HD (Moon *et al.*, 2001). Predictably, CDP/Cux p110 exhibited DNA binding properties similar to that of CR2CR3HD and CR3HD, and made a stable interaction with DNA. Moreover, in a transient transfection assay the processed isoform, but not the full length protein, was able to stimulate expression of a reporter containing the promoter from the DNA polymerase alpha gene (Moon *et al.*, 2001). Thus, proteolytic processing of CDP/Cux at the G1/S transition serves to generate an isoform with distinct DNA binding and transcriptional properties.

Interestingly, an increase in CDP/Cux processed isoforms was observed in uterine leiomyomas suggesting that deregulation of this cell cycle event may occur in cancer cells (Moon *et al.*, 2002; Lee and Park, 2003).

In the present study, we have begun to establish an *in vitro* processing assay that would recapitulate the cleavage of CDP/Cux. This assay is aimed to serve two purposes. It could be used as a functional assay to help in the purification and identification of the protease responsible for the processing of CDP/Cux (Kim *et al.*, 2002). Secondly, it could provide useful information on the cell cycle-dependent regulation of CDP/Cux processing. Results presented in this study indicated that the CDP/Cux substrate was processed with varying efficiency depending on whether it was obtained from bacteria or mammalian cells in G1 or in S phase. We conclude that post-translational modifications of the substrate are involved in the cell cycle-dependant regulation of CDP/Cux processing.

Materials and Methods

Cell lines and tissue culture NIH3T3 cells were maintained in

Dulbecco's modified Eagle's medium (DMEM) containing 1% (v/v) glutamine, 1% (v/v) penicillin/streptomycin, and 10% (v/v) fetal bovine serum (FBS). HS578T cells were maintained as above but with 5% (v/v) FBS. Cells were grown at 37°C, 5% CO₂.

Modified pTriEx2 vector preparation pTriEx2 vectors (Novagen, Madison, USA) were modified by the addition of an influenza virus hemagglutinin (HA) epitope and nuclear localization sequence (NLS), and the removal of the 3' His-tag as follows. The pTriEx2 vector was digested with *PmlI* and treated with phosphatase. Following purification, the vector was ligated to kinased and annealed oligos containing an NLS (5'-GT GCA CTT AAA AAG AAG CGC AAG GTT C-3') with T4 DNA ligase. The pTriEx2/NLS vector was prepared for the addition of an HA epitope through digestion with *XhoI* and phosphatase treatment. Oligos containing the appropriate sequence (5'-C TAT CCA TAC GAC GTA CCT GAC TAC GCA C-3') and the required overhangs were annealed and kinased and then ligated to the purified vector. pTriEx2/NLS/HA was further modified through the removal of the 3' His-tag by digestion with *XhoI* and *DraIII*, T4 DNA polymerase treatment, and re-ligation to produce the pTriEx2/NLS/HA/ Δ 3' His vector.

Plasmids The complete sequence of the plasmids will be provided upon request. All constructs were prepared using the human CDP/Cux sequence (GenBank accession M74099). The pXJ42 vector was obtained from Dr. A. G. Wildeman. The pXJ/MCH plasmid derives from pXJ42 and contains HSCDP nt 44-4558 with a myc epitope tag at the 5' end and an influenza virus hemagglutinin (HA) tag at the 3' end. The pTriEx2 vector (Novagen, Madison, USA) was modified by the addition of an HA epitope tag and nuclear localization sequence (NLS), and the removal of the 3' His-tag. pTriEx2/HSCDP aa 612-1328/HA/ Δ 3' His was constructed through digestion of pTriEx2/HA/ Δ 3' His with *SmaI* and *PmlI* to produce the vector and digestion of pKS/GinMe1A1 with *XmnI* and *EagI* as the insert.

Transfections Transfections were performed with ExGen 500 (MBI Fermentas) according to the manufacturer's instructions. Briefly, NIH3T3 cells were transfected at approximately 80% confluency with 5 μ g of DNA/100 mm plate under serum-free conditions. Two h later medium was added to produce a final concentration of 10% FBS. Drug treatments, if indicated, were performed the following day for four hours. MG132 was used at a final concentration of 20 μ M and ethanol treatments were performed using an equivalent volume as a negative control for MG132 treatment. E64d (Sigma, St. Louis, USA) was used at a final concentration of 25 μ M.

Synchronization and FACS analysis NIH3T3 cells were plated at 3.2×10^5 cells per 150 mm plate. The following day transfections were performed with ExGen 500 as described above. Two h later transfection medium was added to create a final concentration of 0.4% FBS. After 48 h of serum starvation, cells were restimulated with 10% FBS for 4 or 14 h for G1-phase and S-phase synchronizations, respectively, or maintained in 0.4% FBS for G0 synchronization. Drug treatment was performed with MG132 or ethanol for 2 h and the proteins were purified as described below. Untransfected cells were treated in the same manner to produce

synchronized whole cell extracts. Cells were fixed in 70% ethanol and stained with propidium iodide prior to fluorescence activated cell sorting (FACS) analysis, as previously described (Coqueret *et al.*, 1998a).

Cellular extracts Nuclear extracts were prepared as previously described (Santaguida *et al.*, 2001). Cytoplasmic extracts were prepared by collecting supernatant following incubation in Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) and centrifugation. Whole extracts were prepared using Buffer X (0.4 M KCl; 4 mM NaF; 4 mM Na₃VO₄; 0.2 M EDTA; 0.2 M EGTA; 0.1% (v/v) NP-40; 10% (v/v) glycerol), as previously described (Santaguida *et al.*, 2001). Buffer X was prepared at pHs of 7.9 and 7.0 using Hepes, pH 6.5 using carbonate, and pH 5.75 using Mes. Unless otherwise specified, Buffer X was used at pH 5.75.

Protein purification The 612-1328 recombinant protein, His/CDP/Cux 6121328/HA, was isolated from transformed *Escherichia coli* BL21(DE3) or NIH3T3 cells transfected as described above. Bacteria were disrupted by freezing-thawing (Benov and Al-Ibraheem, 2002). Purification was performed with Ni-NTA agarose according to the manufacturers' instructions (Qiagen, Hilden, Germany), and recovered in Solution 3 (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl; 250 mM imidazole), with the exception that mammalian purification required the use of nuclear extracts diluted with Solution 1 (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl; 10 mM imidazole; 1 protease inhibitor tablet/10 mL).

In vitro processing assay *In vitro* assays were performed with 10 g of nuclear extracts or 0.5 µg of purified protein as substrate in a volume of 2 µL of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) or solution 3 (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl; 250 mM imidazole), respectively. Specified amounts of NIH3T3 or HS578T whole cell extracts were added as a source of protease. Buffer X was used to ensure that the volume of all reactions was equal. Average sample volumes were from 10–15 µL. Results were analyzed by SDS-PAGE and Western blotting.

Western blotting Western blotting was performed as previously described using a 1/1000 dilution of mouse-derived antibodies against the HA isotope (Covance, Princeton, USA) and a 1/4000 dilution of anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (Coqueret *et al.*, 1998a).

Results

Proteolytic processing of CDP/Cux is inhibited by MG132 and can take place *in vitro* using a whole cell extract as a source of protease. The CDP/Cux transcription factor was previously shown to be proteolytically processed at the G1/S transition of the cell cycle (Moon *et al.*, 2001). With the aim of developing a functional assay which would help in the purification and characterization of the protease responsible for CDP/Cux processing, we set out to verify whether CDP/Cux could be processed *in vitro*. As a first step, we tested a

crude system comprised of a nuclear extract from cells expressing a CDP/Cux substrate, and a whole cell extract as a source of protease. Previous studies revealed that proteolytic processing of CDP/Cux is a highly regulated event. Not only was there little or no processing in the G1 phase of the cell cycle, but even in S phase only a fraction of CDP/Cux was cleaved (Moon *et al.*, 2001). Regulation may involve the protease, the CDP/Cux substrate, or both. The protease may be regulated by limiting its expression, appropriate localization, or activation to S phase alone. On the other hand, post-translational modifications of CDP/Cux may control whether it is processed or not. If correct, the latter hypothesis would predict that those CDP/Cux proteins that become appropriately modified are readily cleaved in the cells. In contrast, the rest of the CDP/Cux proteins may not be recognized as substrates for processing. With this hypothesis in mind, we decided to use nuclear extracts from cells in which CDP/Cux processing had been temporarily inhibited as a source of substrate. Fig. 1 shows the effect on processing of a 4-h treatment with MG132. NIH3T3 cells were transfected with a vector expressing CDP/Cux with an influenza virus hemagglutinin (HA) tag at its carboxy-terminus. The following day, cells were treated for 4 h with either ethanol or MG132. Nuclear extracts were prepared and submitted to Western blotting analysis using an anti-HA antibody. In the sample treated with MG132, the cleaved product was clearly reduced in intensity, but not completely eliminated (Fig. 1, compare lanes 1 and 2). Although nuclear extracts from cells treated with MG132 contain an appreciable amount of cleaved products, we decided to try using these extracts as a source substrate for *in vitro* processing assays. As a source of protease for the *in vitro* processing assay, we prepared whole cell extracts from the breast tumor cell line, HS578T. These cells were found to express a relatively high level of the CDP/Cux processed isoforms, and thus were deemed to represent a good source of protease. Whole cell extracts from HS578T cells were incubated with nuclear extracts (2 µg) from CDP-HA-transfected and MG132-treated NIH3T3 cells at 37°C for 30 min (Fig. 1, lane 3). Comparison of lanes 2 and 3 showed that incubation *in vitro* generated two cleaved products which co-migrated with CDP/Cux peptides produced *in vivo*. The identical electrophoretic mobility of the CDP/Cux peptides generated *in vivo* and *in vitro* lead us to conclude that proteolytic processing of CDP/Cux can be reproduced *in vitro*.

Proteolytic processing of CDP/Cux *in vitro* is more efficient at low pH, is observed with nuclear and cytoplasmic extracts and is inhibited by MG132 and E64. To verify whether proteolytic processing could be affected by the pH, whole cell extracts were prepared in various buffers as described in Materials & Methods. Proteolytic processing of CDP-HA was clearly stimulated when the pH of the whole cell extract was reduced to 5.75 (Fig. 2A, compare lane 3 with lanes 4–6). In contrast, no change was observed when the salt concentration was varied from 0.008 M to 0.4 M KCl (data

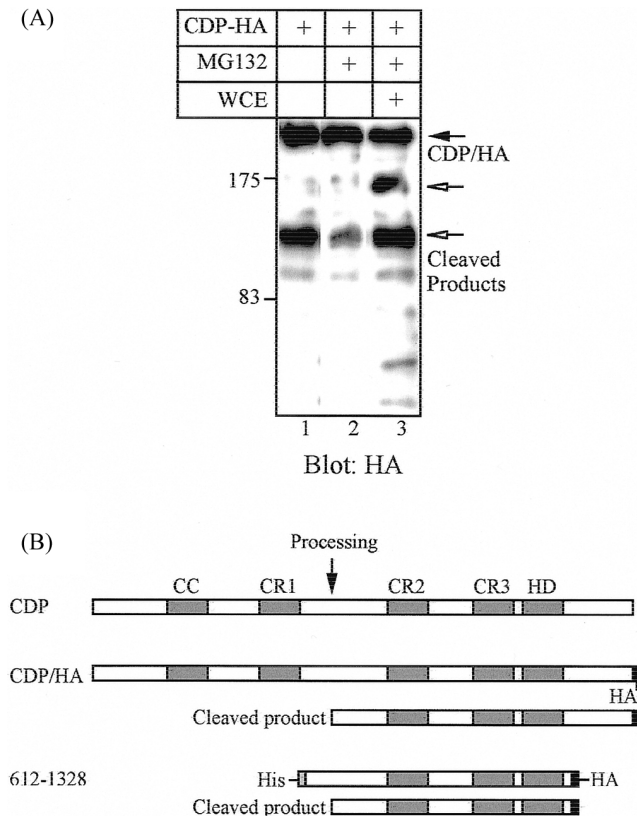


Fig. 1. Proteolytic processing of CDP/Cux is inhibited by MG132 and can take place *in vitro* using a whole cell extract as a source of protease. (A) Lanes 1 and 2, proteolytic processing of CDP/Cux *in vivo*. NIH3T3 cells were transfected with a vector, CDP-HA, expressing the full length CDP protein with an influenza virus hemagglutinin (HA) tag at its carboxy-terminus. The following day, cells were treated with either ethanol (lane 1) or MG132 (lane 2) for 4 h and nuclear extracts were prepared. Lane 3, proteolytic processing of CDP/Cux *in vitro*. Nuclear extracts (2 μ g) from CDP-HA-transfected and MG132-treated NIH3T3 cells were incubated at 37°C for 30 min in the presence of 15 μ g of whole cell extract (WCE) prepared from HS578T cells. Samples were analyzed in Western blot with an anti-HA antibody. The black arrow indicates the position of CDP/H A; the white arrow, the processed isoform. (B) Diagrammatical representation of CDP and the proteins encoded by the vectors used in this study. The boxes represent the evolutionarily conserved domains of CDP: CC, coiled-coil; CR1, CR2 and CR3: Cut repeats 1, 2 and 3; HD, homeodomain.

not shown).

To verify whether post-translational modifications of CDP/Cux may affect proteolytic processing, we wanted to be able to purify a CDP/Cux substrate from both bacterial and mammalian cells. To this end, we used the vector pTriEx2 to express a recombinant protein containing amino acids 612 to 1328 of CDP with histidine (His) and HA tags at its N- and C-termini, respectively. We reasoned that this protein would be small enough to be expressed efficiently in bacteria, yet it would likely contain all important regulatory elements,

notably the Cut homeodomain and the region where processing occur. The histidine tag would serve to purify a full-length substrate, whereas the HA-tag would help visualize processing in Western blot analysis. When the 612-1328 protein was expressed in NIH3T3, we observed two cleaved products whose appearance was partially inhibited in the presence of MG132 (Fig. 2B, lanes 1 and 2). To verify the *in vitro* processing of this protein, nuclear extracts from MG132 treated cells were incubated with whole cell extracts from NIH3T3. Two cleaved products of identical electrophoretic mobility were produced *in vitro* (Fig. 2B, compare lanes 3 and 4). We conclude that the 612-1328 recombinant protein is proteolytically processed, *in vivo* and *in vitro*, in a manner similar to that of the full-length CDP/Cux protein. The *in vitro* processing reaction was also carried out using either the nuclear or the cytoplasmic fraction as a source of protease. In both cases, the same two cleaved products were generated *in vitro* (Fig. 2B, lanes 4-6). These results suggest either that the protease is present both in the cytoplasm and the nucleus, or that it can diffuse during fractionation.

The 612-1328 protein was expressed in bacteria, purified by affinity chromatography on nickel beads and tested in the *in vitro* processing assay using whole cell extract from NIH3T3 cells as a source of protease. The same two cleaved products were generated *in vitro* as what was observed when the protein was expressed in NIH3T3 cells (Fig. 2C, compare lanes 3 and 4 with 1 and 2). Incubation *in vitro* in the presence of E64 or MG132 inhibited to some extent the production of the two cleaved products whereas the ethanol solvent had no effect (Fig. 2C, compare lanes 5, 6 and 7). Thus, proteolytic processing of CDP/Cux can be reproduced *in vitro* using a purified protein from bacteria. These results indicate that the CDP/Cux substrate does not need to be post-translationally modified in order to be recognized as a substrate. Importantly, when the CDP/Cux substrate was purified by affinity chromatography, we did not observe any cleaved product in the control lane (compare lanes 3 and 2, substrate from bacteria and mammalian cells, respectively). Therefore, purifying the substrate represents an improvement in the method.

Proteolytic processing *in vitro* is more efficient with a CDP/Cux substrate purified from bacteria than from mammalian cells. The 612-1328 protein was expressed in NIH3T3 or bacteria, purified by affinity chromatography on a nickel column and then incubated *in vitro* in the presence of whole cell extract from NIH3T3 cells. No cleaved product was observed in the control lanes (Fig. 3, lanes 2 and 6). Incubation of either the bacterial or mammalian protein with whole cell extract *in vitro* generated two cleaved products that co-migrated with the cleaved products generated *in vivo* (Fig. 3, compare lanes 3 and 4 with lane 1). Interestingly, a larger amount of cleaved product was produced when the bacterially expressed protein was used as a substrate (Fig. 3, compare lane 1 with lanes 3 and 4). These results suggest that the

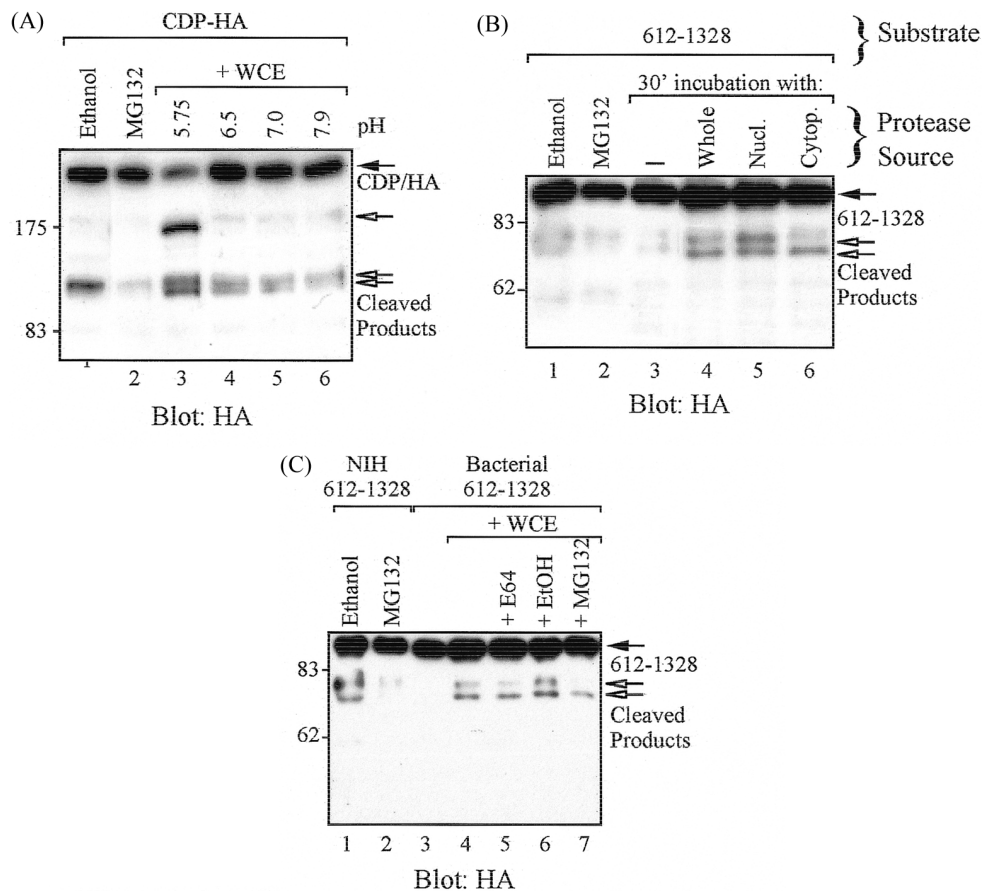


Fig. 2. Proteolytic processing of CDP/Cux *in vitro* is more efficient at low pH, is observed with nuclear and cytoplasmic extracts and is inhibited by MG132 and E64. (A) Proteolytic processing *in vitro* was performed as in Fig. 1, except that whole cell extracts were prepared at the various pHs indicated. Samples were analyzed in Western blot with an anti-HA antibody. The black arrow indicates the position of CDP/HA; the white arrows, the processed isoforms. In lane 1, nuclear extracts from ethanol-treated NIH3T3 cells served as a control for proteolytic processing *in vivo*. (B) The vector pTriEx2-His-CDP/Cux 612-1328-HA was designed to allow the expression, in bacterial and mammalian cells, of a recombinant protein, 612-1328, which contains amino acids 612 to 1328 of CDP with histidine (His) and HA tags at its N- and C-termini, respectively (see Fig. 1B). The vector was introduced into NIH3T3 cells and nuclear extracts were prepared two days later following a 4-h treatment with ethanol (lane 1) or MG132 (lane 2). Nuclear extracts were incubated with itself or with 15 μ g of whole, nuclear, or cytoplasmic extracts prepared from NIH3T3 cells. Samples were analyzed in Western blot with an anti-HA antibody as in (A). (C) The bacterially expressed 612-1328 protein was purified by affinity chromatography on a nickel column (lane 3). 500 ng of the purified 612-1328 protein was incubated with 9 μ g of whole cell extract from NIH3T3 cells in the presence or absence of protease inhibitors as indicated (lanes 4 to 7). E64 and MG132 were used at concentrations of 25 and 20 μ M respectively. Samples were analyzed in Western blot with an anti-HA antibody as in (A).

protein expressed in mammalian cells is modified in a manner that renders it resistant to cleavage.

Proteolytic processing *in vitro* is more efficient with a CDP/Cux substrate purified from cells in the S than in the G1 phase of the cell cycle. Proteolytic processing of CDP/Cux was previously shown to be up-regulated at the G1/S transition of the cell cycle (Moon *et al.*, 2001). To begin to understand the regulatory mechanisms at play, we compared processing *in vitro* using a CDP/Cux substrate purified from cells enriched for either the G1 or the S phase of the cell cycle. In parallel, as a source of protease we prepared whole cell extracts from either unsynchronized cells or from cells

enriched for the G1 or the S phase of the cell cycle. Whereas no cleaved product was observed in the control lane for the G1-phase substrate, a number of shorter products were present, albeit at weak intensity, in the control lane for the S-phase substrate (Fig. 4A, compare lanes 3 and 8). This result was reproducible over several attempts. Although great care was taken in the purification of the CDP/Cux substrate from S-phase cells, we consistently observed shorter products in these samples. We consider the possibility that a small amount of the protease was brought along with the substrate when the latter was purified from S-phase cells. Interestingly, upon incubation *in vitro* with extracts from various sources, the substrate obtained from cells in S phase was more efficiently

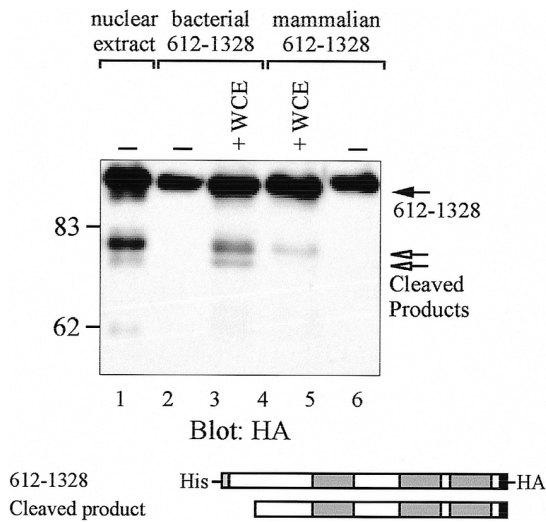


Fig. 3. Proteolytic processing *in vitro* is more efficient with a CDP/Cux substrate purified from bacteria than from mammalian cells. The 612-1328 protein was expressed in NIH3T3 cells and in bacteria. The protein from either source was purified by affinity chromatography on a nickel column, and was incubated at 37°C for 30 min in the presence or absence of 12 µg of whole cell extract from NIH3T3 cells, as indicated. Samples were analyzed in Western blot with an anti-HA antibody. Nuclear extract from transfected NIH3T3 are included as a control for processing *in vivo* (lane 1). The black arrow indicates the position of 612-1328; the white arrows, the processed isoforms.

processed than the substrate from cells in G1 (Fig. 4, compare lanes 4 and 5). In contrast, processing *in vitro* was not significantly different when the whole cell extracts originated from populations of cells enriched for the G1 or the S phase of the cell cycle (Fig. 4, compare lanes 6 and 7). We conclude that regulation of CDP/Cux processing during the cell cycle is likely to involve the post-translational modification(s) of CDP/Cux itself. We cannot, however, exclude that the protease is also modulated in some way during cell cycle progression.

Discussion

We have presented evidence demonstrating that the proteolytic processing of CDP/Cux can be reproduced *in vitro*. Using various recombinant CDP/Cux proteins as substrates, we were able *in vitro* to produce cleaved proteins that exhibited identical electrophoretic mobility to that of the processed proteins generated in cells. Moreover, proteolytic processing was inhibited, *in vitro* and *in vivo*, in the presence of the protease inhibitors, E64D and MG132. Although we cannot exclude that proteolytic cleavage *in vitro* occurs at a slightly different position than *in vivo*, the comparable sensitivity of the reaction to protease inhibitors and the similar apparent molecular weight of the processed products strongly suggest that cleavage of CDP/Cux substrates involved the same

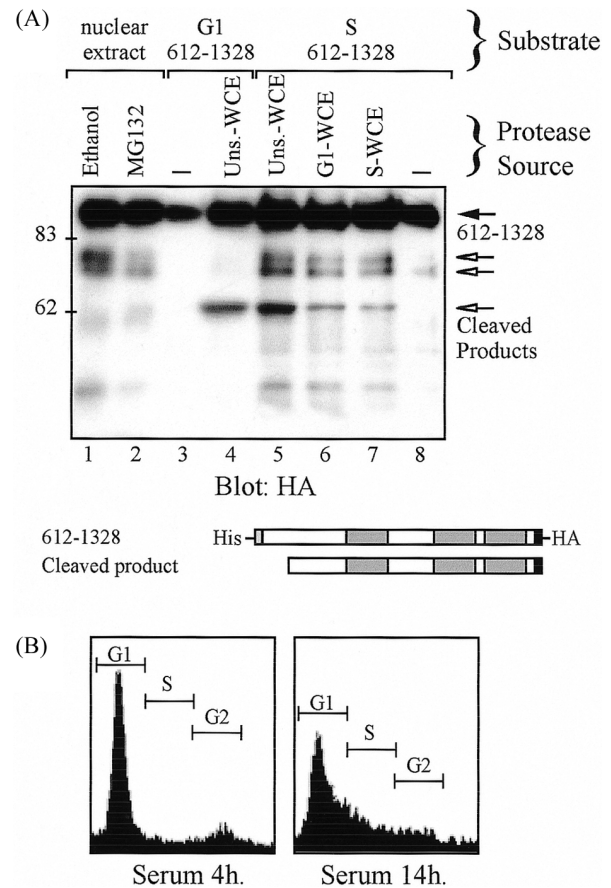


Fig. 4. Proteolytic processing *in vitro* is more efficient with a CDP/Cux substrate purified from cells in the S than in the G1 phase of the cell cycle. (A) NIH3T3 cells were transfected with a vector expressing the 612-1328 protein. Following a 48-h incubation in medium with 0.4% serum, cells were stimulated for 4 or 14 h in medium with 10% serum in order to obtain populations or cells enriched in G1 or in S phase. Nuclear extracts were prepared and the protein was purified by affinity chromatography on a nickel column. In parallel, whole cell extracts were prepared from untransfected NIH3T3 cells that were growing asynchronously or synchronized in G1 or in S phase. The purified protein from either source was incubated at 37°C for 30 min either alone or in the presence of whole cell extracts from the indicated source. Samples were analyzed in Western blot with an anti-HA antibody. The black arrow indicates the position of 612-1328; the white arrows, the processed isoforms. (B) Cell cycle distribution of transfected NIH3T3 cells was monitored by fluorescence-activated cell sorting (FACS) analysis after staining of the DNA with propidium iodide.

protease *in vitro* and *in vivo*.

This *in vitro* processing assay could be used as a functional assay to follow the protease through a purification scheme. Currently, it takes approximately one day to carry the assay, run an SDS-PAGE and perform the Western blot analysis. In practice, this represents a long time, however, we have been able to freeze and thaw the whole cell extracts, nuclear or

cytoplasmic fractions without any loss in activity. Whether it will be possible to freeze and thaw fractions that are more highly purified will have to be determined. If this turns out to be impossible, we could design a fluorogenic substrate in order to speed up the assay.

The *in vitro* processing assay has been used to carry an initial characterization of the proteolytic processing of CDP/Cux. We found that processing *in vitro* was more efficient at a lower pH, insensitive to variations in salt concentration, and inhibited by the protease inhibitors MG132 and E64D. Altogether these properties would point towards a cysteine protease. Using cytoplasmic or nuclear fractions, proteolytic processing of CDP/Cux *in vitro* was not significantly different. This result may mean that the protease is present in both compartments or that it is able to leak into either fraction during the fractionation procedure.

Interestingly, a CDP/Cux substrate that was produced in bacteria and purified by affinity chromatography was efficiently cleaved *in vitro*. This result suggests that CDP/Cux does not need to be post-translationally modified in order to be recognized and cleaved by the protease. In fact, the bacterially expressed CDP/Cux substrate was more efficiently cleaved *in vitro* than the substrate isolated from mammalian cells. We take these results to indicate that proteolytic processing is inhibited by some post-translational modification(s) of CDP/Cux taking place in mammalian cells. This finding would help explain, at least in part, why only a fraction of CDP/Cux is processed in S phase, and perhaps why little or no CDP/Cux is processed in G1 (Moon *et al.*, 2001). Indeed, cleavage *in vitro* was more efficient with a CDP/Cux substrate purified from a population of cells enriched for S phase than for G1. Several post-translational modifications of CDP/Cux have previously been reported. The Cut repeats were shown to be phosphorylated by protein kinase C (PKC) and casein kinase II (CKII), whereas the Cut homeodomain was found to be acetylated by PCAF, phosphorylated by an unknown kinase in G1, and dephosphorylated by the Cdc25A phosphatase in S phase (Coqueret *et al.*, 1996; Coqueret *et al.*, 1998a,b; Li *et al.*, 2000). Of those modifications, only the phosphorylation and dephosphorylation of the Cut homeodomain was seen to vary in a cell cycle dependent manner as it was more highly phosphorylated in G1 than in S phase and the Cdc25A phosphatase is known to reach its peak of activity in S phase (Hoffmann *et al.*, 1994; Jinno *et al.*, 1994; Coqueret *et al.*, 1998a). Whether the phosphorylation state of the Cut homeodomain can affect the efficiency of cleavage between the Cut repeats 1 and 2 will need to be investigated. It is possible as well that processing is modulated by an as yet to be defined modification of CDP/Cux.

In contrast, we did not observe a significant difference in processing efficiency using as a source of protease whole cell extracts from cells in S or G1 phase. This result could mean that the protease is not regulated in a cell cycle dependent manner. However, we cannot exclude that the localization of

the protease varies during the cell cycle and contributes to the regulation of CDP/Cux processing. In this regard, it is noteworthy to mention that CDP/Cux has never been detected outside of the nucleus. Thus, it is likely that processing takes place in the nucleus. Considering that no difference in processing was detected using nuclear or cytoplasmic fractions as a source of protease, we think that our *in vitro* processing assay would not be able to reveal a difference in the localization of the protease between the G1 and S phases. The issue of whether the protease is regulated during the cell cycle will probably have to await its identification.

In summary, we have established an *in vitro* processing assay for CDP/Cux that will be useful in the identification of the protease. Moreover, initial characterization of processing using this assay revealed that post-translational modification(s) of CDP/Cux inhibits processing and probably contributes to the cell cycle regulation of CDP/Cux processing.

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