Regulation of Cyclin D1 by Calpain Protease*

Yung Hyun Choi, Su Jae Lee, Phuong Mai Nguyen, Joungh Soon Jang, Jeen Lee, Ming-Lei Wu, Emiko Takan0, Masatoshi Maki, Pierre A. Henkarti, and Jane B. Trepel**

From the Medicine Branch, Division of Clinical Sciences and the §Experimental Immunology Branch, Division of Basic Sciences, NCI, National Institutes of Health, Bethesda, Maryland 20892, and the ¶National Kyoto Hospital, Kyoto 612, and ¶Nagoya University, Nagoya 464-01, Japan

Cyclin D1, a critical positive regulator of G1 progression, has been implicated in the pathogenesis of certain cancers. Regulation of cyclin D1 occurs at the transcriptional and posttranscriptional level. Here we present evidence that cyclin D1 levels are regulated at the posttranscriptional level by the Ca2+-activated protease calpain. Serum starvation of NIH 3T3 cells resulted in rapid loss of cyclin D1 protein that was completely reversible by calpain inhibitors. Actinomycin D and lovastatin induced rapid loss of cyclin D1 in prostate and breast cancer cells that was reversible by calpain inhibitors and not by phenylmethylsulfonyl fluoride, caspase inhibitors, or lactacystin, a specific inhibitor of the 26 S proteasome. Treatment of intact NIH 3T3, prostate, and breast cancer cells with a calpain inhibitor dramatically increased the half-life of cyclin D1 protein. Addition of purified calpain to PC-3-M lysates resulted in Ca2+-dependent cyclin D1 degradation. Transient expression of the calpain inhibitor calpastatin increased cyclin D1 protein in serum-starved NIH 3T3 cells. Cyclins A, E, and B1 have been reported to be regulated by proteasome-associated proteolysis. The data presented here implicate calpain in cyclin D1 posttranslational regulation.

Cell cycle progression in eukaryotic cells is governed by a family of cyclins and their cognate cyclin-dependent kinases (Cdks). In mammalian cells at least 12 different cyclin genes and 8 cdk genes have been identified (1–9). Numerous abnormalities in cyclin and cdk genes have been identified in human tumors (5). Among the cyclins and CdkS, the gene most clearly identified as a protooncogene is cyclin D1, the prototype member of the G1-regulatory D-type cyclins (5, 10–12). The cyclin D1 gene is amplified and its mRNA overexpressed in several human tumor types, including breast cancer, squamous cell carcinoma of the head and neck, and centrocytic lymphoma (11–14).

Cyclin D1 is required and rate-limiting for G1 progression. Overexpression of cyclin D reduces the serum requirement for cell cycle entry (15–18). Cyclin D1 levels increase in response to mitogenic stimulation and rapidly decline when growth factors or mitogens are withdrawn (15, 19). The principal target of the cyclin D1-Cdk4 complex that has been identified is the retinoblastoma susceptibility gene product, pRB (3, 4, 6, 9, 16). Phosphorylation of pRB by G1 cyclin-Cdk complexes releases the transcription factor heterodimer E2F-DP from inhibition imposed by binding to the pRB large pocket domain. E2F-DP complexes are thought to play a critical role in S phase entry by binding to and activating the promoters of genes required for DNA synthesis and cell cycle progression (9, 14).

Cyclin proteins are characterized by cell cycle-dependent accumulation and destruction. The molecular mechanisms underlying cyclin periodicity involve both transcriptional and posttranscriptional regulation. Many cyclin mRNAs exhibit cell cycle-dependent fluctuation, and in some cases, specific transcription factors have been implicated in controlling this periodicity (20–22). Another major determinant of cyclin periodicity is protein stability. Cyclin B1, cyclin A, and cyclin E have been shown to be degraded by ubiquitin-dependent proteolysis, catalyzed by the 26 S proteasome (23–28). In this report we present evidence that cyclin D1 protein is regulated by the calcium-activated neutral cysteine endopeptidase calpain (EC 3.4.22.17).

EXPERIMENTAL PROCEDURES

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Radiation Medicine, Georgetown University Medical Center, 3970 Reservoir Rd. NW, Washington, D. C. 20007-2197.

‡ To whom correspondence should be addressed: Medicine Branch, NCI, Bldg. 10, Rm. 12N226, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-1547; Fax: 301-402-0172; E-mail: trepel@helix.nih.gov.

The abbreviations used are: Cdk, cyclin-dependent kinase; LLaL, leucyl-leucyl-norleucinal; LLM, leucyl-leucyl-methioninal; PMSF, phenylmethylsulfonyl fluoride; ZVAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl (p-O-methyl) fluoromethyl ketone; ZFA-FMK, carbobenzoxy-phenylalanyl-alanyl fluoromethyl ketone; BD-FMK, Boc-Asp fluoromethyl ketone; PBS, phosphate-buffered saline; GFP, green fluorescent protein.
rpm for 15 min to remove cellular debris. Protein concentration was determined with the BCA reagent (Pierce). Equal amounts of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting. The blots were blocked, incubated with polyclonal anti-cyclin D1 antibody, washed with 10 mM Tris, pH 7.5, 50 mM NaCl and 2.5 mM EDTA, and incubated with horseradish peroxidase-conjugated secondary antibody, and cyclin protein was detected using an enhanced chemiluminescence detection system (Amersham Corp.). The membrane used for anti-ubiquitin Western blot was first autoclaved in water for 20 min to ensure complete denaturation of ubiquitinated proteins prior to probing with anti-ubiquitin antibody (30). To determine cyclin D1 half-life, 60–70% confluent monolayers of cells were treated with cycloheximide (100 µg/ml). At each time point indicated cells were washed with cold PBS, lysed in TNN lysis buffer, and cyclin D1 levels determined by Western blot analysis.

In Vitro Degradation of Cyclin D1 by Purified Calpain—Cells were lysed in 40 mM Tris-HCl, pH 7.5, 120 mM NaCl. Purified calpain (m-calpain, the 80-kDa subunit of Ca²⁺-activated neutral protease from rabbit skeletal muscle, 1 unit per 33 µg of protein) was incubated with 40 µg of cell lysate for 1 h at 30 °C in a total volume of 60 µl. After incubation SDS sample buffer was added, the samples were boiled and centrifuged, and the supernatant was run on a 10% SDS-polyacrylamide gel. Cyclin D1 protein was detected by Western blot analysis using polyclonal anti-cyclin D1 antibody.

Transient Transfection with Calpastatin—NIH 3T3 cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions, and 1.5 µg/ml calpastatin plasmid in pcDNA3 vector, or with pcDNA3 vector alone (31). Vector control and calpastatin-transfected cells were co-transfected with a GFP expression vector (pEGFP-N1, 1.5 µg/ml; CLONTECH, Palo Alto, CA).

Cyclin D1 Indirect Immunofluorescence—Cells were grown on sterile coverslips, fixed with 3.7% formaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 for 10 min at room temperature (32). Nonspecific binding sites were blocked by incubating the cells with 1% bovine serum albumin in PBS for 1 h at 4 °C. Rabbit polyclonal anti-cyclin D1 antibody was added to the coverslip and incubated for 1 h at 4 °C. The coverslip was washed two times for 2 min with PBS and incubated at 4 °C for 1 h with Texas Red-conjugated goat anti-rabbit immunoglobulin (Molecular Probes, Eugene, OR). After incubation, the coverslips were washed with PBS, rinsed quickly with water, air-dried, mounted using SlowFade (Molecular Probes), and imaged using a Zeiss Axioskop microscope interfaced with a CCD camera (Optronics Engineering, Goleta, CA).

RESULTS

Reversal of Cyclin D1 Degradation in Serum-starved Cells—The half-life of the D-type cyclins is very short and the withdrawal of growth factors during the G₁ phase of the cell cycle results in a rapid decrease of D-type cyclin protein (15, 19). When NIH 3T3 cells were incubated in Dulbecco’s modified Eagle’s medium lacking serum, steady-state levels of cyclin D1 protein were markedly decreased between 6 and 12 h (data not shown), and by 24 h the cyclin D1 protein level was undetectable (Fig. 1 lane 6). To determine whether protease inhibitors could prevent the degradation of cyclin D1 we investigated the effect of the peptid aldehyde calpain inhibitors LLnL and LLM, the serine protease inhibitor PMSF, and the proteasome inhibitor lactacystin on cyclin D1 levels in log phase control and serum-starved NIH 3T3 fibroblasts. The protease concentrations and incubation times chosen resulted in cell viability greater than 70% (data not shown). LLnL, LLM, and lactacystin all increased the level of cyclin D1 protein in control NIH 3T3 cells (lanes 1–5). PMSF had no effect (lane 6). In serum-starved cells LLnL completely reversed cyclin D1 loss, LLM partially reversed cyclin D1 loss, and lactacystin had no effect (lanes 6–12). LLnL and LLM are calpain and cathepsin inhibitors. LLnL also has activity as a proteasome inhibitor (24, 33, 35). This raised the possibility that, like cyclins B1, A, and E, cyclin D1 was being degraded by the proteasome. However, the complete absence of reversal of cyclin D1 degradation by lactacystin, the most specific proteasome inhibitor available (36, 37), suggests that cyclin D1 is being degraded by a calpain-type enzyme.

Reversal of Cyclin D1 Degradation in Actinomycin D-treated Cells—We investigated whether these findings could be extended to human tumor cell lines. PC-3-M prostate carcinoma cells and MDA-MB-231 breast carcinoma cells can continue to proliferate even in the absence of serum. To induce loss of cyclin D1 in these cells we treated the cells with the RNA synthesis inhibitor actinomycin D. An initial concentration dependence experiment showed that treatment with 500 ng/ml for 24 h was not toxic to the cells (viability greater than 80%) and that RNA synthesis was blocked by greater than 90% (data not shown). Log phase control or actinomycin D-treated cells were cultured with or without LLnL, LLM, or PMSF for 24 h, and cyclin D1 protein levels were examined by Western blot analysis. As shown in Fig. 2, LLnL prevented the loss of cyclin D1 protein in both PC-3-M and MDA-MB-231 cells (lane 6). LLnL and PMSF had no effect (lanes 7 and 8).

Lack of Effect of Caspase Inhibitors on Cyclin D1 Levels—Recent studies have shown the existence of a family of cysteine proteases, related to the Caenorhabditis elegans death gene ced-3, which are activated during induction of apoptosis (38–40). This caspase family of proteases can be selectively inhibited by synthetic cleavage site-directed fluoromethyl ketone peptide inhibitors (41). We investigated the effect of the active inhibitors ZVAD-FMK and BD-FMK, and the inactive peptidyl analogue ZFA-FMK on cyclin D1 expression in the human prostate carcinoma PC-3-M cells (Fig. 3). Cells were incubated with or without actinomycin D for 24 h, and, where indicated, peptidyl analogues were added to the culture dishes for the last 12 h of incubation. Total cell lysates were prepared and Western blot analyses were performed using anti-cyclin D1 antibody and enhanced chemiluminescence detection. Cyclin D1 protein levels of PC-3-M cells treated with the caspase inhibitors was similar to control cells (lanes 1 and 3–5). Furthermore, these protease inhibitors did not block the ac-
tinomycin D-induced loss of cyclin D1 protein in PC-3-M cells (lanes 6–8). These data further indicate that the proteolysis of cyclin D1 in these cell cultures is specifically calpain-associated.

**Lactacystin Blocks the Degradation of Cyclin B1**—Ubiquitination is known to target cellular proteins for degradation by the proteasome pathway (27, 42, 43). Lactacystin, a Streptomyces metabolite, is a specific proteasome inhibitor that has been used as a tool to investigate intracellular protein degradation pathways (36, 37). Cyclin B1 was one of the first proteins shown to be regulated by ubiquitin-mediated proteasomal degradation (23). Cyclins A and E are also inactivated by ubiquitin-dependent proteolysis (24, 25, 28). We next examined culture conditions in which both cyclin B1 and cyclin D1 protein become undetectable to compare the effect of lactacystin to the effect of LLnL. PC-3-M cells were treated with actinomycin D for 24 h in the presence or absence of lactacystin or LLnL for the last 12 h. Treatment of PC-3-M cells with actinomycin D induced loss of cyclin B1 and cyclin D1 (Fig. 4). LLnL, an inhibitor of both calpain and the proteasome, blocked degradation of cyclin B1 and cyclin D1. In contrast, lactacystin reversed loss of cyclin B1 and not cyclin D1. These data are consistent with previous reports showing that cyclin B1 degradation is through the proteasome and further support a calpain-mediated, non-proteasomal degradation mechanism for regulation of cyclin D1 under these experimental conditions.

**LLnL Blocks Lovastatin-induced Cyclin D1 Loss**—Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.88), an enzyme required for the conversion of HMG-CoA to mevalonic acid in the pathway leading to cholesterol biosynthesis (44). In a number of cell types lovastatin also inhibits cell proliferation by inducing a block in the G1 phase of the cell cycle (45, 46). Hunter and colleagues (46) have shown that lovastatin lowers the level of cyclin D1 protein in NIH 3T3 cells. As shown in Fig. 5A, the steady-state level of cyclin D1 protein was markedly decreased in both PC-3-M and MDA-MB-231 cells exposed to 10 μM lovastatin for 36 h (Fig. 5A). To examine the role of proteases in cyclin D1 regulation in lovastatin-treated cells, log phase control and lovastatin-treated PC-3-M and MDA-MB-231 cells were incubated in the presence or absence of LLnL or lactacystin, and the levels of cyclin B1 and cyclin D1 were examined by Western blot analysis. LLnL reversed the loss of both cyclin B1 and cyclin D1 protein. In contrast, consistent with the data in Fig. 4, lactacystin prevented the loss of cyclin B1 and not cyclin D1 protein. To confirm that lactacystin was active in increasing the level of ubiquitinated proteins in PC-3-M cells, a control experiment was performed in which total cellular levels of ubiquitinated proteins were assessed by Western blot analysis using anti-ubiquitin antibody in untreated cells and in cells incubated for 12 h with lactacystin or LLnL. Lactacystin caused an increase in total cellular ubiquitinated protein (Fig. 5B). LLnL also caused an increase in total ubiquitinated proteins, as would be predicted by the reported ability of LLnL to block proteasome-associated protease activity (33–35, 46).

**Cyclin D1 Half-life Increases in Response to LLnL**—The half-life of cyclin D1 is among the shortest in eukaryotic cells, typically in the range of 30 to 60 min (19). If cyclin D1 is degraded by calpain in vivo, then inhibition of calpain in cells would be expected to stabilize cyclin D1 protein. To test this we determined the half-life of cyclin D1 protein in the presence or absence of LLnL in the NIH 3T3, PC-3-M, and MDA-MB-231 cell lines. Log phase control cells or cells treated with LLnL for 12 h were treated with cycloheximide and harvested at the times indicated. In the absence of calpain inhibitor, the half-life of cyclin D1 was less than 2 h in the three cell types (Fig. 6A). In contrast, in the presence of LLnL the half-life of cyclin D1 was greater than 12 h in each of the cell lines (Fig. 6B). Thus, the cyclin D1 protein half-life increased by at least 6-fold in each cell type in response to calpain inhibitor.

**Degradation of Cyclin D1 by Purified Calpain**—Calpain is an intracellular nonlysosomal cysteine protease that requires calcium ions for activity (33–35, 47). The results presented above
indicated that calpain may act as an endogenous regulator of cyclin D1. These data prompted us to determine if cyclin D1 acts as a substrate for purified calpain in vitro and to examine the Ca\textsuperscript{2+} dependence of cyclin D1 degradation. Cell lysates from control PC-3-M and MDA-MB-231 cells were incubated with purified calpain under varying conditions, and cyclin D1 levels were determined by Western blot analysis. In the absence of added Ca\textsuperscript{2+} there was no evidence of calpain activity toward cyclin D1 (Fig. 7, lane 2). Boiling of calpain before addition also resulted in no detectable loss of cyclin D1 (lanes 4 and 5). In the presence of 6.0 mM CaCl\textsubscript{2}, however, calpain caused a complete loss of detectable cyclin D1 (lanes 2 and 3). We have subsequently determined that the EC\textsubscript{50} for Ca\textsuperscript{2+} in stimulation of calpain-mediated cyclin D1 proteolysis in the PC-3-M cell lysates is between 100 and 500 \mu M (data not shown). Consistent with the Ca\textsuperscript{2+} requirement for calpain-mediated cyclin D1 proteolysis, simultaneous incubation of cell lysates with calpain and the calcium chelator EGTA mediated cyclin D1 proteolysis, simultaneous incubation of cell lysates with calpain and the calcium chelator EGTA resulted in a complete block of calpain activity toward cyclin D1 (lanes 6 and 7).

**Calpastatin Increases the Half-life of Cyclin D1**—Calpastatin is a highly specific endogenous protein inhibitor of intracellular calpain (35, 48). To assess the specificity of calpain in cyclin D1 regulation in vivo, we studied the effect of overexpressing calpastatin on cyclin D1 levels. Cyclin D1 levels were determined by immunocytochemistry in logarithmic phase NIH 3T3 cells growing in 10% serum, greater than 90% of the cells that were successfully transfected, as determined by GFP positivity, were also positive for cyclin D1 expression. Serum starvation of NIH 3T3 cells resulted in less than 30% of the cells being positive for cyclin D1 protein. In contrast, serum starvation of NIH 3T3 cells co-transfected with GFP and the calpastatin expression vector resulted in greater than 80% of the cells co-expressing GFP and cyclin D1. Thus, expression of calpastatin substantially prevented the loss of cyclin D1 protein induced by serum withdrawal. This result is consistent with earlier data showing that at 24 h the loss of cyclin D1 in serum-starved NIH 3T3 could be blocked by LLaNL (Fig. 1) and further implicate calpain in the in vivo regulation of cyclin D1.

---

**FIG. 7. In vitro degradation of cyclin D1 by purified calpain in PC-3-M and MDA-MB-231 cells.** Cell lysates were incubated for 1 h at 30 °C with purified calpain (lanes 2, 3, 6, and 7) or boiled calpain (lanes 4 and 5) in the presence (lanes 3, 5, and 7) or absence (lanes 2, 4, and 6) of CaCl\textsubscript{2} (6 mM) or EGTA (10 mM) (lanes 6 and 7).

**FIG. 8. Effect of calpastatin on loss of cyclin D1 protein in serum-starved NIH 3T3 cells.** The level of cyclin D1 protein was analyzed by immunocytochemistry in NIH 3T3 cells grown in serum-containing medium (A and B) and compared with serum-starved cells (C and D), and serum-starved cells transfected with a calpastatin expression vector (E and F). The transfected cells were co-transfected with GFP: A, C, and E, cyclin D1 protein; B, D, and F, GFP.

**TABLE I**

| Experiment | Serum-starved | Calpastatin |
|------------|---------------|-------------|
| Control    | 12            | 12          |
| GFP\textsuperscript{+} cells | 114 161 | 94 23 |
| Cyclin D1\textsuperscript{+} cells | 114 161 | 94 23 |
| Cyclin D1/GFP (%) | 96 95 | 27 25 | 87 90 |
Calpain-mediated Cyclin D1 Degradation

It is now recognized that proteolysis plays a critical role in the regulation of cell cycle progression (49). Thus far most studies have focused on the role of ubiquitin-dependent proteolysis catalyzed in the 26 S proteasome (27, 42). Initiation of DNA replication requires the ubiquitin-dependent degradation of a Cdk inhibitor. The initiation of chromosome segregation and exit from mitosis requires a distinct ubiquitin-dependent pathway to coordinate degradation of anaphase inhibitors and mitotic cyclins. Ubiquitin-targeted proteolysis plays an important role in the degradation of many cell growth-regulatory proteins, including the Cdk inhibitors p21 and p27, p53, c-Fos, c-Jun, E2F-1, E2F-4, pRB and p107 (23, 26, 27, 50–53). Cyclin B1 was one of the first proteins to be identified as a substrate for ubiquitin-targeted degradation (23). Cyclins A and E have also been shown to be substrates for ubiquitination and proteasome-mediated proteolysis (24, 25, 27, 28, 49).

The regulation of cyclin D1 protein as a function of cell cycle phase has not been clearly defined, potentially due to differences in cell type and other variables, including methods of cell cycle synchronization. In some reports cyclin D1 protein is high in G1, and falls markedly before S phase, whereas other investigators find little variation in cyclin D1 protein if the cells are maintained in a high growth factor milieu (15, 19). It has been reported that cyclin D1 levels fluctuate with the cell cycle in both normal and transformed cells and, in contrast, that transformation specifically eliminates cell cycle-associated cyclin D1 fluctuations (11, 13, 16, 18). Thus far, the data do suggest that unlike mitotic cyclins, which must be degraded for exit from mitosis, cyclin D1 degradation rates are synchronized with the rate of cyclin D1 transcription, and cyclin D1 degradation is not a prerequisite for G1 progression (24).

Structurally, cyclin D1 is smaller than cyclins A, B1, or E (1, 2, 10, 54). Although highly homologous to the other cyclins for most of its coding sequence, cyclin D1 is missing an amino-terminal domain containing the region that has been identified as the cyclin destruction box, a region that is required for ubiquitination and rapid degradation of mitotic cyclins (1, 23, 49). The lack of this domain, characteristic of the three D-type cyclins, suggests that an alternate mechanism may be utilized for posttranslational regulation of the D-type cyclins. As discussed above, the existence of cell cycle-dependent fluctuations is unclear. However, it has been established unambiguously that cyclin D1 is a highly responsive sensor of the growth factor environment of the cell and that cyclin D1 protein is rapidly degraded when mitogenic factors are withdrawn (15, 19).

As shown in Fig. 1, removing serum from NIH 3T3 cells resulted in loss of cyclin D1 protein that was completely reversible by incubation of the intact cells with the calpain inhibitor L-LLnL. L-LLnL is a calpain inhibitor that is also reported to block activity of the 26 S proteasome (33–35, 47). Therefore we tested for ubiquitin- and proteasome-associated proteolysis of cyclin D1. We found that the proteasome-specific inhibitor lactacystin completely reversed loss of cyclin B1, which has been shown to be degraded by the proteasome but had no effect on cyclin D1. We tested the calpain inhibitor LLM, which is generally not considered to inhibit the proteasome, and LLM reversed the loss of cyclin D1, although to a lesser extent than L-LLnL. These data also suggested that degradation of cyclin D1 was through calpain, rather than the proteasome. The endogenous peptide calpastatin is a highly specific inhibitor of calpain (35, 48). Transient transfection experiments in which calpastatin protein was overexpressed demonstrated that calpastatin could markedly reverse the loss of cyclin D1 protein in serum-starved NIH 3T3 cells. Together these data suggest that, at least for the cells and conditions studied here, a calpain-like protease plays an important role in cyclin D1 regulation. Our data do show that incubation of untreated, logarithmically growing cells for 12 h with lactacystin caused a small increase in the level of cyclin D1 protein (compare Fig. 1, lanes 1 and 5; Fig. 4, lanes 1 and 3). This increase, which was not consistently seen (Fig. 5A, lanes 5 and 6), may reflect a role for the proteasome in the constitutive turnover of cyclin D1 in these cells. Thus, the proteolytic pathway for cyclin D1 may differ according to the signals impinging on the cell, i.e. there may be a difference in the proteolytic pathway for cyclin D1 in logarithmic phase cells maintaining a fairly high level of cyclin D1 protein, compared with cells undergoing a metabolic stress, in which cyclin D1 protein is eliminated from the cell through transcriptional and posttranslational mechanisms.

Calpain, a family of cysteine proteases, can be divided into ubiquitous and tissue-specific isozymes (33, 34, 55, 56). The ubiquitous forms are μ calpain or calpain I, which has an in vitro requirement for Ca2+ in the μM range, and μ-calpain or calpain II, which has an in vitro Ca2+ requirement in the mM range (55, 56). Calpain isozymes have the general structure of a chimera between a calmodulin-like calcium binding protein and a papain-like cysteine protease (56). Calpain has been implicated in a wide range of in vivo events including platelet activation, long term potentiation, oocyte maturation, apoptosis, and nonapoptotic postischemic neurodegeneration, as well as in the pathogenesis of Alzheimer’s disease (57). Reported calpain substrates include the cytoskeletal associated proteins filamin, fodrin, and talin, focal adhesion kinase, c-Mos protein kinase C, and the transcription factors c-Fos, c-Jun, and p53 (33, 56–58). Calpain-catalyzed proteolysis is typically limited, resulting in a clipped but substantially intact protein. In contrast, we have not observed fragments of cyclin D1 protein. This discrepancy may be explained by the proposal of Suzuki and colleagues (48) who suggested that limited proteolysis by calpain could render a substrate protein destabilized and susceptible to a variety of cellular proteases. Consistent with a potential role in growth regulation, calpain activity is subjected to many forms of posttranslational control in vivo, including changes in cytosolic free Ca2+ levels, translocation from cytosol to membrane, limited autolysis, and association with the endogenous protein inhibitor calpastatin (35). Rechsteiner and colleagues (59) suggested that one role for calpain may be in initiating proteolysis of the group of rapidly degraded proteins that contain the PEST (proline-glutamic acid-serine-threonine) motif. Although cyclin D1 does not contain a cyclin destruction box, it does contain carboxyl-terminal PEST sequences (2). The yeast G1 cyclins CLN2 and CLN3 contain PEST domains, and it has been shown that removal of the PEST sequences from yeast G1 cyclins stabilizes their half-life (60, 61). Analysis of the protease sensitivity of cyclin D1 protein mutated in the PEST region would facilitate examination of the potential role of the PEST domain in calpain-mediated cyclin D1 proteolysis.

Acknowledgment—We thank Alfred W. Alberts, Merck Sharp & Dohme Research Laboratories, for kindly providing lovastatin.

REFERENCES
1. Xiong, Y., Connolly, T., Futer, B., and Beach, D. (1991) Cell 65, 691–699
2. Xiong, Y., Menninger, J., Beach, D., and Ward, D. C. (1992) Genomics 13, 575–584
3. Sherr, C. J. (1993) Cell 73, 1059–1065
4. Sherr, C. J. (1994) Cell 79, 551–553
5. Hunter, T., and Fines, J. (1994) Cell 79, 573–582
6. Weinberg, R. A. (1995) Cell 81, 323–330
7. Tassan, J.-P., Jaquenoud, M., Leopold, P., Schultz, S. J., and Nigg, E. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8871–8875
8. Bates, S., Rowan, S., and Vousden, K. H. (1996) Oncogene 13, 1103–1109
9. Sidle, A., Palaty, C., Dirks, P., Wiggan, O., Kiesel, M., Gill, R. M., Wong, A. K., and Hamel, P. A. (1996) Crit. Rev. Biochem. Mol. Biol. 31, 257–271
10. Motokura, T., Bloom, T., Kim, H. G., Jappner, H., Ruderman, J. V.,
