p55Cdc is a mammalian protein that shows high homology to the cell cycle proteins Cdc20p of *Saccharomyces cerevisiae* and the product of the *Drosophila* fizzy (*fzy*) gene, both of which contain WD repeats and are thought to be required for the metaphase-anaphase transition. The *fzy* mutants exhibit a metaphase arrest phenotype, which is accompanied by stabilization of cyclins A and B, leading to the hypothesis that *fzy* function is required for cell cycle-regulated ubiquitin-mediated proteolysis. p55Cdc expression was initiated at the G1/S transition and steady state levels of p55Cdc were highest at M and lowest in G1. Inhibition of the 26 S proteasome prevented both mitotic exit and loss of p55Cdc at the M/G1 transition, suggesting that p55Cdc degradation was mediated by the cell cycle-regulated proteolytic pathway. Immune complexes of p55Cdc obtained at different cell cycle stages showed a variety of proteins with dramatic differences observed in the pattern of associated proteins during the transition from G2 to M. Immunolocalization of p55Cdc demonstrated dynamic changes in p55Cdc localization as the cells transit mitosis. p55Cdc appears to act as a regulatory protein interacting with several other proteins, perhaps via its seven WD repeats, at multiple points in the cell cycle.

In eukaryotic cells, different complexes of kinases and their associated activating or inhibitory proteins control progression through discrete steps of the cell cycle. The best understood complexes known to play a central role in cell cycle progression are the cyclins in association with their cyclin-dependent kinases (Cdk's) (reviewed in Refs. 1–3). In addition to regulation by phosphorylation and association with Cdk inhibitors, the cyclin-Cdk complexes are subject to a more irrevocable form of regulation, i.e. the degradation of cyclin via the cell cycle-regulated ubiquitin-mediated proteolytic pathway (4–7). The work of a number of laboratories has recently converged to identify three tetratricopeptide proteins, CDC16, CDC23, and CDC27, as components of the E3 complex that becomes activated during mitosis (8) and catalyzes the mitosis specific conjugation of ubiquitin to B type cyclins in yeast (9), *Xenopus* (10), and humans (11). These three proteins can form a large complex whose function is required for the metaphase to anaphase transition (12). Many of the genes encoding tetratricopeptidase proteins have been reported to functionally interact with members of the WD repeat family (13, 14).

Recent investigations have demonstrated that other cell cycle-regulated proteins undergo proteolysis at defined cell cycle stages, and that proteins other than cyclins must be degraded to allow the metaphase to anaphase transition (6, 7). Cenome protein (CENP)-E and CENP-F are mammalian kinetochore localized proteins whose expression peaks at mitosis, exhibit dynamic changes in their localization at different mitotic stages, and are rapidly degraded after mitosis (15, 16). In *Aspergillus nidulans* mitotic exit apparently requires the destruction of the cell cycle-regulated NIMA protein kinase (17), and in *Drosophila* the separation of sister chromatids has been reported to require the product of the *pimples* gene, a protein that is degraded after the metaphase-anaphase transition (18). Most of these proteins do not have the cyclin destruction box motif, which targets the A and B type cyclins for destruction (4).

We have (19) identified a protein, p55Cdc, which appears to be a mammalian counterpart of products of the *Saccharomyces cerevisiae CDC20* (20), *Drosophila fzy* (21), and the newly described fission yeast slp1 (22) genes, due to the strong homologies within their WD repeats (14). *cdc20* mutants arrest in mitosis at the nonpermissive temperature after the formation of a complete short spindle and nuclear migration to the neck between the mother cell and a large bud (23). It has been suggested that the cdc20p is required for modulation of microtubule structure, either by altering the surface of microtubules or by promoting disassembly (20, 24). The *fzy* mutants have demonstrated that the failure to degrade cyclins A and B, and the failure of sister chromatids to separate, is due to a lack of functional Fizzy (Fzy) protein (21, 25). Dawson *et al.* (21) have postulated that Fzy function is required for cell cycle-regulated ubiquitin-mediated proteolysis. *slp1* mutants are defective in chromosome separation and recovery from DNA damage arrest (22). The high degree of homology between p55Cdc, Fzy, Cdc20p, and slp1 protein, as well as their essential role in cell cycle, has prompted the proposal that they are orthologous members of a gene family within the highly degenerate WD repeat superfamily (21, 22).

Mammalian p55Cdc exhibits several properties that are consistent with its role in the cell cycle. p55Cdc, not expressed in differentiated or quiescent cells, is readily detectable in dividing cells. p55Cdc appears to be essential for cell division, since transfection of antisense p55Cdc cDNA into Chinese hamster ovary cells results in isolation of only those cells that exhibit a compensatory increase in p55Cdc transcripts in the sense orientation (19). Immune complexes of p55Cdc exhibited a protein
kinase activity that was higher in actively proliferating cells than in quiescent cells and fluctuated with the cell cycle. Overexpression of p55Cdc in myeloid cells inhibited granulocytic differentiation and accelerated apoptosis, suggesting that p55Cdc regulation is critical for normal cell cycle control during myeloid cell proliferation and differentiation (26).

This study was undertaken to examine the expression, phosphorylation, degradation, and localization of p55Cdc at different stages of the mammalian cell cycle. The mitotic Cdk, p34\(^{cdk2}\), was analyzed in parallel and served as an additional control in the course of this work. Pulse labeling experiments with \(^{35}\)S or \(^{33}\)P showed that both the biosynthesis and phosphorylation of p55Cdc were low at G1 and peaked at G2 with a significant drop in phosphorylation during the G2 to M transition. Immunocomplexes, obtained from different cell cycle stages and subcellular fractions, showed distinct p55Cdc-associated proteins. A steady accumulation of p55Cdc during the course of the cell cycle culminated in rapid loss at the M to G1 transition. This loss could only be prevented by a 26 S proteasome inhibitor, suggesting that p55Cdc may be another cell cycle protein to be degraded by the cell cycle-regulated ubiquitin-mediated proteolytic pathway. Localization of p55Cdc by indirect immunofluorescence displayed dynamic changes during mitosis, from the centromeres at prometaphase, mitotic spindle in metaphase, to the spindle equator in anaphase. These observations indicate a role for p55Cdc in G2 and/or M.

**MATERIALS AND METHODS**

**Cell Culture, Synchronization, Labeling, and Subcellular Fractionation**—HeLa and IMR-90 cells were grown in 10-cm minimum essential medium or minimum essential medium, respectively, supplemented with 10% fetal bovine serum, glutamine, and nonessential amino acids (Life Technologies, Inc.). HeLa cells were synchronized at the beginning of S (G2/S) by the double thymidine/aphidicolin block (27). S phase cells were harvested 4 h, G1 cells were harvested 8 h, and mitotic cells were obtained by shake off 10–11 h following release from the aphidicolin block. The remaining adherent monolayer was the late G2 population. To obtain a G1 population, the nonadherent pseudo-mitotic (G2/M) cells were harvested 7 h after nocodazole treatment. The washed cells were replated in media without nocodazole. Approximately 60% of the cells had adhered to the plates by 3 h. At this stage, the nonadherent cells were rinsed off. The cells were used for G1 analysis 4–8 h following replating. To obtain cells at the M1G2 transition, pseudo-mitotic cells were harvested 7 h after nocodazole treatment. The washed cells were resuspended in media without drugs (carboplatin, methyl sulfoxide for HeLa) with media containing the following additions: 50 μM N-acetylleucyl-norleucinyl (L-LnL), 50 μM (2S,3S)-trans-epoxysuccinyl-1-leucyl-amido-3-methyl-butanoyl ethyl ester (E64d), 0.1 μM staurosporine, or 5 μM dihydrocyclophasalin (DDBC), (Sigma Chemical Co). Following a 2.5-h incubation, the cells were analyzed for DNA content by flow cytometry (DNA QC particles kit, Becton Dickinson), and for p55Cdc, cyclin B, and p34\(^{cdk2}\) by Western analysis. An identical analysis was performed on cells that had been treated for 15 h with 5 μg/ml aphidicolin to obtain a population of cells accumulated in G1 and S phase. IMR-90 cells were arrested in G0 by growing the cells for 3 days in low serum to the S100 fraction to make its composition closer to the other two fractions. The resuspended P100 and nuclear fractions were spun for 30 min at 20,000 × g to obtain a clarified lysate for immunoprecipitation experiments. Preparation of a total cell lysate has been described previously (19). All lysates were stored at −80 °C until needed.

**Antibody Preparation and Immunoprecipitation**—The preparation of the antibody against a glutathione S-transferase-p55Cdc fusion construct has been described (19). Rabbit antibodies were generated against the carboxyl-terminal end of p55Cdc (CKSSLIHGQGR) conjugated to keyhole limpet hemocyanin antigen. Antisera were affinity-purified by passage through a Sulfolink (Pierce) column to which the peptide had been coupled. The column was washed with 10 column volumes of PBS, and the affinity-purified antibodies were eluted with 0.1 M glycine, pH 2.8, into tubes containing 1 M Tris, pH 8. The pH of the pooled fractions was adjusted to neutral, and they were stored in aliquots at −80 °C. Protein concentrations were estimated with the bicinchoninic acid reagent (Pierce). The protocol for immunoprecipitations has been described (19). The concentration of antibody was increased to 5 μg/100 μg of lysate for the glutathione S-transferase-p55Cdc fusion protein antibody and to 15 μg/100 μg for the carboxyl-terminal peptide antibody. The concentration of the control adsorbed antisera was always 5–10-fold higher than that of the affinity-purified antibodies. Antibodies to p34\(^{cdk2}\) (monoclonal antibody 17), cyclin A (BF683), cyclin E (E-19), and goat polyclonal antibody against the carboxyl-terminal peptide of human lamin B were purchased from Santa Cruz Biotechnology Inc. The monoclonal antibody to human cyclin B1 (GNS-1) was purchased from Pharmingen, and the monoclonal antibody to human lactate dehydrogenase was from Sigma. The band intensity was quantitated by PhosphorImager analysis (Molecular Dynamics).

**Immunoblots and Immunofluorescence**—Total cell lysates were prepared in 1.5 × SDS sample loading buffer (28) at a concentration of 106 cells/ml. The subcellular fractions were prepared as described above, except that the S100 fraction was concentrated so that normalization was by cell number for all three fractions. The proteins were transferred to Hybond nitrocellulose (Amersham Corp.) for 2 h at 80 mA with the Pharmacia Multiphor II apparatus (Pharmacia Biotech Inc.). The transferred proteins were visualized by the ECL detection system (Amersham Corp.). For indirect immunofluorescence experiments, an asynchronous population of HeLa cells was fixed in −20 °C methanol, 2 mM EDTA for 10 min, blocked, and permeabilized for 7 min in PBS containing 5% donkey serum and 0.1% Triton X-100 and rinsed briefly in PBS, 0.1% BSA. The cells were incubated with 10 μg/ml p55Cdc affinity-purified antibody and 1:50 dilution of β-tubulin monoclonal antibody from Amersham Corp. or 50 μg/ml control adsorbed antisera for 75 min. The slides were washed twice in PBS, 0.1% BSA and blocked again with 5% donkey serum in PBS. Incubation with fluorescence-labeled secondary antibodies (1:400 diluted Cy3 conjugated to AffiniPure F(ab’)2 fragment donkey anti rabbit IgG, 1/100 diluted FITC-conjugated AffiniPure F(ab’)2 fragment donkey anti rabbit IgG and 1/100 diluted FITC-conjugated AffiniPure F(ab’)2 fragment donkey anti mouse IgG, Jackson Immunoresearch Labs) was for 1 h. Slides were washed twice in PBS, 0.1% BSA and once in PBS containing 2.5 μg/ml Hoescht 33342 (Molecular Probes). The slides were rinsed in distilled water and mounted in Vectashield (Vector Laboratories).

Results were observed under fluorescence with a Nikon Microphot-FXA equipped with a Plan Apo 50× oil immersion lens (Nikon Inc). Cells at various mitotic stages were photographed under identical conditions with the appropriate filters. All experiments were performed reproducibly at least twice.

**RESULTS**

**p55Cdc Expression Is Initiated at the G1/S Transition**—Previous work demonstrated that p55Cdc expression was readily detectable in a rapidly proliferating, but not quiescent population of Rat1 fibroblasts (19). To identify the stage of the cell cycle at which p55Cdc expression is initiated, a normal diploid untransformed human fibroblast cell line was examined. IMR-90 cells were arrested at G0 by serum starvation and induced to enter the cell cycle by the addition of 20% serum to the plates with a cell scraper. The nuclei were pelleted by a 1000 × g spin in a swinging bucket rotor for 10 min. The supernatant was separated into SI00 (cytosol) and P100 (particulate) fractions by spinning for 1 h at 100,000 × g. The nuclear and P100 pellets were resuspended in radiolabeled precipitation assay buffer, described previously (19). Nonidet P-40 and NaCl were added to the S100 fraction to make its composition closer to the other two fractions. The resuspended P100 and nuclear fractions were spun for 30 min at 20,000 × g to obtain a clarified lysate for immunoprecipitation experiments. Preparation of a total cell lysate has been described previously (19). All lysates were stored at −80 °C until needed.

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kinase(s) activity, which fluctuates with the cell cycle (19) and precipitated. Since p55Cdc is known to be associated with protein times, the cells were solubilized and the lysates immunopre-
tivated. Since p55Cdc is known to be associated with protein biosynthesis of p55Cdc in HeLa cells was detectable in G1 and increased steadily through the cell cycle to peak at G2 (Fig. 3, b and c). The regulation of p55Cdc biosynthesis was similar to that of p34\(^{cd2}\), being very low at G1 and peaking at G2. The p55Cdc immunoprecipitates showed the presence of a 100-kDa protein in the P100 and nuclear fractions through the entire cell cycle. The p34\(^{cd2}\) immunoprecipitates showed a faint band that appeared to comigrate with p55Cdc and could be due to cyclin A and/or B. A 10-fold increase in the rate of p34\(^{cd2}\) synthesis has been observed as HeLa cells progress from G1 to G2 (33). Although the bulk of newly synthesized p34\(^{cd2}\) was found in the S100 fraction, p55Cdc had the highest relative concentration in the P100 fraction (Fig. 3c) and this pattern did not change with the cell cycle. As expected, the nuclear p34\(^{cd2}\) in the G2 samples was primarily the slower mobility (inactive) form, while that in the S100 and P100 fraction of mitotic cells (Fig. 3b) was of the faster mobility (active) form.

The results of labeling HeLa cells with \(^{35}\)S for 1 h at different stages of the cell cycle are shown in Fig. 4. The late G2 cells represent the adherent monolayer after the mitotic cells have been harvested by repeated pipetting. This population also has cells that have already exited mitosis and entered G1, p55Cdc phosphorylation is undetectable in any of the fractions obtained from a G1 population of cells, followed by progressive increase in phosphorylation of p55Cdc as the cells proceed from G1 to G2. The p55Cdc immunoprecipitates from P100 fractions (Fig. 4b, lanes 2 and 3) contain a unidentified band of 35 kDa that co-migrates with the slower mobility form of p34\(^{cd2}\) and is undetectable in the mitotic P100 fraction. The S100 fractions (Fig. 4a, lanes 2 and 3) showed a diffuse band at longer exposures (data not shown), which could represent different phosphorylated forms of p55Cdc. Immunocomplexes obtained from the nuclear fraction with antibody against glutathione S-transferase-p55Cdc fusion protein (Fig. 4c, lane 2) also showed a slower migrating band around 58 kDa that was not detected by the p55Cdc COOH-terminal antibody (Fig. 4c, lane 3). Since the nuclear membrane has disintegrated in the mitotic cells, only the S100 and P100 fractions were obtained. The mitotic S100 fraction showed a unique profile of p55Cdc-associated phosphorylated proteins in the immune complex, including the appearance of proteins of 72 and 140 kDa. The same profile was obtained by two different antibodies to p55Cdc (Fig. 4a, lanes 2 and 3). The rate of p34\(^{cd2}\) phosphorylation in the various subcellular fractions from different stages in the cell cycle (Fig. 4, a, b, and c, lane 4) paralleled that of p55Cdc, although unlike p55Cdc, phosphorylated p34\(^{cd2}\) was detectable during G1. Enhancement in the rate of p34\(^{cd2}\) phosphorylation at G2 has been observed previously (34).

The data obtained in Fig. 4 were subjected to PhosphorImager analysis, and the results are shown in Fig. 5. Except for the late G2 population, both proteins exhibit the highest concentration of phosphorylated protein in the P100 fraction. This is noteworthy since \(^{35}\)S labeling detected most p34\(^{cd2}\) in the cytosol fraction and similar results were obtained for the distribution of p34\(^{cd2}\) in the various subcellular fractions (Fig. 6, lanes 1–3), although longer exposure showed detectable low mobility form of p34\(^{cd2}\) in the membrane fraction (data not shown). Thus, the p34\(^{cd2}\) associated with cell membranes must be in a highly phosphorylated state. This is consistent with the report that myt1, the inhibitory kinase that phosphorylates p34\(^{cd2}\) on both threonine 14 and tyrosine 15, is a membrane-

\(^2\) J. Weinstein, unpublished observations.
associated kinase (35, 36). In this context, it is interesting that human cyclin B2 has been localized primarily to the Golgi apparatus (37). The phosphorylation rate of both p55Cdc (Fig. 5a) and p34\(^{cd2}\) (Fig. 5b) peak at G2 and show a dramatic dephosphorylation at M. However, although p55Cdc showed an overall dephosphorylation in transition from G2 to M, the S100
fraction showed an increase in net phosphorylation of p55Cdc during mitosis.

**Steady State Levels of p55Cdc**—To estimate the steady state levels of p55Cdc at different points in the cell cycle, synchronized HeLa cells were harvested and lysed in SDS-PAGE sample buffer. The amount loaded per well was normalized by cell number and not protein content and gave approximately equal amount of protein in lanes 4–10 (Fig. 6a). To obtain the G1 population, rounded cells were collected following a nocodazole block, plated, and the adherent monolayer was harvested 4 and 8 h later. Western blots were performed with antibodies against p55Cdc, p34\(^{cdc2}\), cyclin E, and cyclin A (Fig. 6a). The results demonstrated that p55Cdc was present at a much lower concentration in G1 compared with the other stages of the cell cycle. The concentration of p55Cdc increased as the cells progressed through the cell cycle and peaked at M. This was followed by a dramatic loss of p55Cdc as the cells started a new cycle at G1. In contrast, cyclin A and E do not show such a dramatic fluctuation. Cyclin A peaked at G1/S and was maintained at a high level through G2. This result is in agreement with that of Dulic et al. (38), but does not completely agree with that of Pines and Hunter (39), who observed a continuous increase in cyclin A levels up to 8 h following release from an aphidicolin block. However, their samples were normalized for protein content and not cell number. The cyclin E levels peaked at G1/S and declined thereafter as had been observed (38). Subcellular fractionation of a G2 population (fractions normalized by cell number) showed that both cyclin A and E are present in the nucleus as had been observed previously by immunofluorescence (40, 41). The same analysis of p55Cdc showed an increase in net phosphorylation of p55Cdc during mitosis.

**Characterization of the affinity-purified p55Cdc antibody used in this study** is shown in Fig. 6b. Increasing amount of total HeLa cell lysate was loaded in lanes 1 and 2 and the blot developed with the p55Cdc fusion protein antibody preparation. A major band of 55 kDa and a weaker band at about 33 kDa were detected. The 33-kDa band could be due to degraded p55Cdc as suggested previously (19). A duplicate blot probed with p55Cdc adsorbed antiserum did not show any reaction (data not shown). The results demonstrate the specificity of the antibody and indicate that the proteins detected in the p55Cdc immune complexes (Figs. 3 and 4) are not due to contaminating antibodies. The quality of the subcellular frac-
Loss of p55Cdc in Transition from M to G1

Fig. 6. Steady state levels of p55Cdc. a, total cell lysates from synchronized or asynchronous (A) HeLa cells were prepared for SDS-PAGE as described under "Materials and Methods." A G1 population was used to prepare subcellular fractions (S100, lane 1; P100, lane 2; nuclear, lane 3). Subcellular fractions obtained from 0.66 × 10⁶ cells and total lysates from 0.33 × 10⁶ cells were loaded for Western blot analysis. The nitrocellulose blots were developed with affinity-purified p55Cdc antibody; p34cdc2 antibody; cyclin A (BF683) antibody and cyclin E (C-19) antibodies. b, characterization of affinity-purified p55Cdc antibody. Total cell lysates from 0.07 and 0.20 × 10⁶ HeLa cells were loaded on lanes 1 and 2, respectively, for immunoblot analysis with affinity-purified p55Cdc antibody. c, characterization of subcellular fractions. Total cell lysate from 0.17 × 10⁶ cells (lane 1) and subcellular fractions (S100, lane 2; P100, lane 3; nuclear, lane 4) from 0.33 × 10⁶ cells were loaded. Appropriate sections of the blot were probed with three different antibodies: antibody to human lactate dehydrogenase as a cytosolic marker, antibody to β-tubulin as a cytosolic and cytoskeletal membrane protein marker, and antibody to lamin B as a nuclear protein marker.

Results obtained by the simple process of hypotonic cell lysis followed by differential centrifugation is shown in Fig. 6c. Total cell lysate was loaded in lane 1 and S100, P100, and nuclear fractions in lanes 2–4, respectively. The glycolytic enzyme lactate dehydrogenase served as a marker for the cytosol fraction (S100), β-tubulin was a marker for both cytosol and cytoskeletal membrane fraction (P100), and the nuclear membrane protein lamin B was a marker for the nuclear fraction. As expected, the lactate dehydrogenase antibody detected the enzyme in the S100 fraction, the β-tubulin antibody detected this protein primarily in the S100 and P100 fractions, and the lamin B antibody demonstrated that this protein is present only in the nuclear fraction. These results verify the purity of the subcellular fractions.

p55Cdc Is Degraded at the M to G1 Transition—This analysis was performed to understand the mechanism of p55Cdc loss that had been observed by Western analysis of HeLa cells at various cell cycle stages. Cells that had been arrested at G2/M or G1 and S were released to continue their cell cycle progression either in the absence or presence of a variety of drugs. The cell-permeable cysteine protease inhibitor LLaLnL has been shown to inhibit cyclin B degradation and induce cell cycle arrest in Chinese hamster ovary and HeLa cells (42) and has been used as a specific 26 S proteasome inhibitor in many studies (43–46). The lipid-soluble cell-permeable calpain 1 and 11 and lysosomal protease inhibitor E64d is a useful control since it does not appear to inhibit the 26 S proteasome and does not show any cell cycle perturbations (44, 47). Staurosporine is a nonspecific protein kinase inhibitor, which can inhibit certain Cdk-cyclin complexes at very low concentrations (48) and has been used to inhibit the p34cdc2 kinase following release from a nocodazole arrest (49). This treatment leads to a reduction in histone phosphorylation, chromosome decondensation, and nuclear membrane reformation, suggesting a premature exit from mitosis and resetting of the cell cycle clock to a G1 state in the absence of cytokinesis (49). Dihydroxytocolazalin B is a drug that interferes with actin assembly and cell cleavage (50). A large proportion of cells treated with DCB following release from a nocodazole arrest failed to complete cleavage yet entered interphase (G1) by the criteria of decondensed chromatin and reformed nuclear envelopes (50). HeLa cells that had been arrested in mitosis by nocodazole (Fig. 7, a and b, 1–6) or in G1 and S by a single aphidicolin treatment (Fig. 7, a and b, 7–12) were allowed to reenter the cell cycle in the presence or absence of these drugs. After 2.5 h, all the cells were harvested and analyzed for the levels of p55Cdc, cyclin B, or p34cdc2 by Western analysis (Fig. 7a) or for DNA content to obtain cell cycle status (Fig. 7b). Although most of the cells treated by Me2SO alone (carrier) had exited into G1 at this time (Fig. 7b, 2), those treated by LLaLnL and staurosporine showed complete arrest at 4 h. The DCB treatment resulted in a partial block and even the E64d treatment showed some inhibition of mitotic exit. Results from the immunoblot revealed that the only drug able to prevent the proteolysis of p55Cdc and cyclin B was the 26 S proteasome inhibitor LLaLnL, although E64d did show some inhibition of cyclin B degradation. Overall, the pattern of p55Cdc loss was very similar to that of cyclin B, which is known to be destroyed by the cell cycle-regulated ubiquitin-mediated 26 S proteasome pathway during anaphase/telophase (4, 7). Neither p55Cdc nor cyclin B showed readily detectable ubiquitinated bands moving with lower mobility on the SDS-PAGE gels in the LLaLnL-treated samples. Other investigators have found the need to overexpress a cyclin (46) or overexpress a cyclin along with an engineered ubiquitin construct to enable the detection of polyubiquitinated species (47). As expected, the levels of p34cdc2 were unaffected by any of the drug treatments and the enzyme stayed in its high mobility dephosphorylated form (Fig. 7a, 1–6). Release from aphidicolin arrest caused a surge of DNA synthesis, as seen by the peak between the 2 and 4 N peaks under all experimental conditions except one (Fig. 7b, 8–10 and 12). The only drug that showed a detectable inhibition of DNA synthesis was staurosporine, probably by virtue of its ability to inhibit Cdk2 the catalytic component of both Cdk2-cyclin E and Cdk2-cyclin A complexes (48). The cells released from aphidicolin arrest showed no detectable p55Cdc accumulation with any of the inhibitors including LLaLnL when compared with cells treated with carrier alone (Fig. 7a, 7–12), demonstrating that the cell cycle-regulated p55Cdc degradation is active at the M to G1 transition and has been turned off by the time the cells are in late G1 and S phase of the cell cycle.

Immunolocalization of p55Cdc in Mitotic HeLa Cells—Indirect immunofluorescence was performed to visualize the cellular location of p55Cdc during mitotic stages. The colocalization of p55Cdc and β-tubulin at progressive mitotic stages is shown in Fig. 8. The immunofluorescence of p55Cdc in mitotic cells was more intense than that in interphase cells and, in early mitotic stages, appeared to interfere with the ability of the β-tubulin monoclonal antibody to stain the mitotic spindle (Fig. 8a).
8a). The intense speckled nuclear staining of prophase (Fig. 8a) was reduced dramatically at prometaphase (Fig. 8a), at which point it appeared to be localized to the centromere region of the chromosome rosette formation where juxtaposed centromeres can appear as a single fluorescent ring (51). The kinetochores of Ptk1 cells show p55Cdc localization during early mitotic stages.3 Chromosomes within the rosette are oriented with their arms projecting outward from the centromere ring, which forms at the rim of the hub of the chromosome rosette (51). At this stage, p55Cdc appears to be localized to the centromere ring and cell cortex. As the cells enter metaphase (Fig. 8b), p55Cdc localized to the mitotic spindle and the cytosol (in both a normal bipolar and an abnormal tripolar spindle) but was now excluded from the region occupied by the condensed chromosomes. At this stage, the interference with β-tubulin staining has lessened sufficiently to allow the detection of microtubules by the β-tubulin antibody. Some spindle pole association could still be detected during early anaphase (Fig. 8c), along with p55Cdc staining as a bisecting strip at the spindle equator, which continued as intracellular bridge staining when the cells were in late anaphase and telophase (Fig. 8, d and e). The intracellular bridge staining did not continue as midbody staining in cytokinesis (Fig. 8f) in contrast to the intense β-tubulin staining in this region, although a small dot of p55Cdc staining persisted at the site of maximum cleavage furrow constriction. p55Cdc fluorescence was at its highest intensity during prophase and metaphase and declined steadily thereafter, so that by cytokinesis (Fig. 8f) it was down to the levels detected in interphase.

The intensity of p55Cdc staining was always higher when no attempt was made to co-localize with β-tubulin. To clearly visualize the polar spindle staining during anaphase (Fig. 9a) and telophase (Fig. 9b), indirect immunofluorescence was performed on HeLa cells with the affinity-purified p55Cdc antibody alone. No immunofluorescence was detectable with the control p55Cdc adsorbed antiserum (data not shown). The results clearly demonstrate the presence of p55Cdc at the spindle poles during these mitotic stages. Although p55Cdc was localized to the spindle poles, p55Cdc staining was never detected exclusively at the centrosome with the staining protocol used in this study.

DISCUSSION

Mammalian p55Cdc is a cell cycle protein that is expressed in an actively cycling population of cells (19) and first appears,
following activation of a quiescent population of cells, during 
late G1 (Fig. 1). This expression pattern is coincidental with 
that of p34\textsuperscript{cdc2}, the synthesis of which is initiated at the G1/S 
transition (30, 31), and the results of Furukawa \textit{et al.} (32) 
suggest that expression of p34\textsuperscript{cdc2} following exposure of T cells
to phytohemagglutinin is coincident with the G1 to S transition 
as part of an orderly sequence of events that occurs at this 
stage. These results imply that the induction of p55Cdc syn-
thESIS at the G1/S transition could be part of such a sequence. 
The potential role of p55Cdc at this transition and in S phase is 
under investigation.

p55Cdc immunoprecipitates are associated with multiple 
protein kinases, with the myelin basic protein phosphorylating 
activity seen predominantly in the S100 fraction and the 
\alpha-casein protein kinase activity segregating with the nucleus (19). 
Therefore, it was decided to examine subcellular fractions 
in an attempt to identify potential protein kinase bands for 
future analysis. p55Cdc has a short half-life of 2 h in an asyn-
chronous population of HeLa cells. Pulse-labeled p55Cdc and 
p34\textsuperscript{cdc2} show dynamic changes in their subcellular locations 
with time. The short-lived p55Cdc rapidly leaves the cytosol to 
accumulate in the membrane and nuclear fractions while the 
stable p34\textsuperscript{cdc2} shows a much slower exit from the cytosol (Fig. 
2, b–d). Fig. 3b shows a 100-kDa protein in association with 
p55Cdc in the P100 and nuclear fractions through the course of 
the cell cycle. It is interesting that this association is no longer 
obscured in a pseudo-mitotic population of cells collected fol-
lowing nocodazole treatment. It is also evident that p55Cdc 
associates with different proteins at different subcellular loca-
tions during the cell cycle, a dramatic change in the pattern of 
associated proteins occurring in the transition from G2 to M 
(Fig. 4). The results of Figs. 3–5 demonstrate that both the 
biosynthesis and phosphorylation of p55Cdc are cell cycle-reg-

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**FIG. 8. Co-localization of p55Cdc and \beta-tubulin in mitotic cells.** An asyn-
chronous HeLa cell population was subjected to indirect immunofluorescence as 
described under “Materials and Methods” and cells representing progressive mitotic 
stages were photographed: a (prophase and prometaphase), b (metaphase), c (an-
aphase), d (late anaphase), e (telophase), and f (cytokinesis). The primary antibod-
ies were affinity-purified p55Cdc antibodies (10 \mu g/ml, a–f), 1/50 diluted \beta-tubulin 
monoclonal antibody (a–f), and p55Cdc adsorbed antiserum (50 \mu g/ml, g).

**FIG. 9. p55Cdc localization at the spindle poles.** An asyn-
chronous HeLa cell population was subjected to indirect immunofluores-
cence as described under “Materials and Methods.” The primary anti-
body was affinity-purified p55Cdc antibody (10 \mu g/ml) and the 
secondary antibody was FITC-conjugated donkey anti mouse IgG. Cells 
representing anaphase (a) and telophase (b) were photographed under 
identical conditions.
ulated, being low at G1 and progressively increasing with the course of the cell cycle to peak at G2. The p55Cdc phosphorylation/dephosphorylation cycle appears to peak prior to the p55Cdc protein accumulation/degradation cycle. Future experiments will determine if this is coincidental or of functional significance.

The identification of the Drosophila fzy gene as a homolog of p55Cdc and CDC20 may provide some clues toward a mechanism for p55Cdc function. p55Cdc and Fzy have 50% overall identity due to the significant homology that extends beyond the WD repeats (21). The observation that the metaphase arrest phenotype caused by fzy mutations is associated with failure to degrade both mitotic cyclins A and B has led to the suggestion that fzy function is required for cell cycle-regulated proteolysis (21). Siggist et al. (25) have also proposed that exit from mitosis is regulated by Fzy-mediated sequential destruction of cyclins A, B, and B3 and that it is involved in sister chromosome separation and segregation. Although there is no direct evidence to implicate the Fzy homologs cdc20p and p55Cdc in a cell cycle-regulated proteolytic pathway, the immunolocalization of p55Cdc during mitosis (Fig. 8) is similar to that reported for Fzy (21) in that the mitotic cytosol is intensely stained by antibodies against both proteins, while these proteins are excluded from the region occupied by the condensed chromosomes. However, Fzy staining was not detected at the centromeres during prometaphase, the spindle during metaphase, or the spindle equator and poles during anaphase and telophase as p55Cdc was (Figs. 8 and 9). These are all locations where a protein involved in microtubule dynamics might be predicted to occur. The reason for this difference in localization of these homologous proteins might be due to species or technical differences.

Ubiquitin-mediated proteolysis is known to be induced by phosphorylation (7, 46, 47, 52), and reversible phosphorylation is implicated in controlling the activity of cyclosome-associated, cyclin-ubiquitin ligase (53). p55Cdc is associated with a protein kinase activity in the p55Cdc immune complex (19), although high levels of p55Cdc are detectable by Western blot (Fig. 6) and indirect immunofluorescence (Fig. 8) during mitosis. Could the release of a protein kinase or modulation of its substrate specificity by the p55Cdc-kinase(s) complex allow it to phosphorylate a specific mitotic substrate hitherto inaccessible? Furthermore, p55Cdc itself is phosphorylated and this phosphorylation is regulated by the cell cycle, being undetectable at G1 and peaking at G2, a pattern that corresponds to that of the phosphorylation of p34cdc2 (Figs. 4 and 5). p55Cdc has five potential phosphorylation sites among amino acid residues 41–109, which conform to the (S/T)P consensus for Cdk phosphorylation (54, 55). The transition from G2 to M is accompanied by a net dephosphorylation of p55Cdc, yet the mitotic cytosol shows an increase in phosphorylated p55Cdc and a unique pattern of p55Cdc-associated phosphorylated proteins. It is intriguing that p55Cdc immune complexes obtained from the mitotic cytosol of 32P-labeled cells showed phosphorylated proteins of 140 and 72 kDa that were undetectable in the same fraction obtained from interphase cells (Fig. 4a). The onset of mitosis is known to coincide with the appearance of many phosphorylated proteins in the cytosol (56). Identification of the proteins associated with p55Cdc may increase our understanding of the cell cycle-regulated, ubiquitin-mediated proteolytic pathway.

The dramatically lower levels of p55Cdc observed at G1 suggested a precise cell cycle-regulated loss of this protein (Fig. 6). A decrease in the half-life of p55Cdc during mitosis from that observed in an asynchronous population (2 h), in conjunction with the very low levels of p55Cdc synthesis observed during G1 (Fig. 3), could account for the observed loss of this protein in transition from M to G1. Steady state levels of mitotic B cyclins peak at M and show a similar precipitous drop at mitosis (40). The degradation of the B type cyclins is triggered at the metaphase-anaphase transition and has been shown recently to continue through G1 in S. cerevisiae and mammalian cells (43, 57). The kinetics of p55Cdc accumulation and degradation also bear an interesting similarity to those observed for CENP-E, a putative kinetochore kinesin-related motor protein (15); CENP-F, a nuclear matrix protein that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis (16); and the mammalian Polo-like kinase Plk1, implicated in mitotic spindle function (58, 59). p55Cdc escapes complete proteolysis through the course of mitosis but is largely lost by cytokinesis (Fig. 8). Drugs that inhibit cytokinesis by a variety of mechanisms were unable to prevent the degradation of p55Cdc or cyclin B (Fig. 7). The only drug capable of inhibiting p55Cdc and cyclin B proteolysis was the 26 S proteasome inhibitor LLnL, suggesting that not only could p55Cdc be an essential regulatory component of this complex (21, 25) perhaps by interacting with the anaphase promoting complex (7), but also be ultimately degraded by it. This significant observation lends further support to the hypothesis that cell cycle-regulated proteolysis plays a major role in regulating mitotic exit and resetting the cell cycle machinery to a G1 state.

The dynamic changes observed in p55Cdc localization at progressive mitotic stages (Fig. 8) imply a role for this protein at more than one mitotic transition. The increase in p55Cdc staining intensity as the cells enter prophase appears to coincide with nuclear envelope breakdown. Recent studies have suggested that the abrupt reorganization of microtubules at nuclear envelope breakdown is the result of a decrease in microtubule polymer level and increase in microtubule dynamics during prophase (60). The microtubule polymer levels recover as the chromosomes attach to the microtubules during prometaphase and metaphase since the captured kinetochore microtubules are protected from the rapid turnover of other microtubules (60, 61). The three-dimensional analysis and ultrastructural design of mitotic spindles from the cdc20 mutant of S. cerevisiae showed that the cdc20p may be involved in cell cycle processes that promote microtubule disassembly (24).

The centromere has been proposed to be the hub of chromosomal activities (62) and is stained by p55Cdc when the chromosomes are transiently arrayed in a chromosome rosette formation (Fig. 8a). During metaphase, p55Cdc staining could be detected at the mitotic spindle and cytosol and at the spindle equator in anaphase. The interference of β-tubulin immunofluorescence by p55Cdc antibodies during these early mitotic stages implies either a direct or indirect association of p55Cdc with β-tubulin or very close spatial proximity of these two antigens during these transitions. Moreover, both the S. cerevisiae and Drosophila homologs of p55Cdc have been implicated in modulating microtubule behavior. The loss-of-function mutations in cdc20 and fzy leads to a mitotic arrest phenotype that is accompanied by an excess accumulation of tubulin in the spindle microtubules (20, 21, 24). These properties would be consistent with a role for p55Cdc as a component of the spindle assembly and metaphase to anaphase transition checkpoints (63, 64).
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