Enhanced Ribosomal Association of p27\textsuperscript{Kip1} mRNA Is a Mechanism Contributing to Accumulation during Growth Arrest\textsuperscript{*}

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\textsuperscript{*}The abbreviations used are: CDK, cyclin-dependent kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction.

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p27\textsuperscript{Kip1} regulates the decision to enter into S-phase or withdraw from the cell cycle by establishing an inhibitory threshold above which G\textsubscript{1} cyclin-dependent kinases accumulate before activation. We have used the HL-60 cell line to study regulation of p27 as cells withdraw from the cell cycle following treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). We found that the amount of p27 is maximal in G\textsubscript{0} cells, lower in G\textsubscript{1} cells, and undetectable in S-phase cells. In contrast to the protein, the amount of p27 mRNA was the same in these populations, suggesting that accumulation of p27 during the cell cycle and as cells withdraw from the cell cycle is controlled by post-transcriptional mechanisms. In S-phase cells, the degradation of p27 appears to predominate as a regulatory mechanism. In G\textsubscript{0} cells, there was an increase in the synthesis rate of p27. Our data demonstrate that, in G\textsubscript{0} cells, accumulation of p27 is due to an increase in the amount of p27 mRNA in polyribosomes.

The activation status of the cyclin-dependent kinases (CDKs)\textsuperscript{3} regulates progression through G\textsubscript{1} (1). The phosphorylation of the Rb protein correlates with transition through the restriction point when cells commit irreversibly to S-phase and subsequent cell division (2). Rb is phosphorylated by cyclin D-CDK complexes early in G\textsubscript{1}, and cyclin E-CDK2 complexes further phosphorylate and maintain the hyperphosphorylated state through the remainder of G\textsubscript{1} (3). A class of proteins that interact with both G\textsubscript{1} CDKs, called Kips (CDK \textsuperscript{i}nhibitory \textsuperscript{p}roteins), might coordinate these two classes of kinases and the start of S-phase. p27\textsuperscript{Kip1} might directly bridge the activation of cyclin E-CDK2 complexes to expression of cyclin D-CDK complexes (4, 5). p21\textsuperscript{Kip1} might regulate cyclin D-CDK2 complexes in a non-inhibitory manner (5). Changing the amount of p27 mRNA can control accumulation of p27 protein; increased accumulation of p27 mRNA contributes to the increase in p27 protein following exposure of U937 cells to vitamin D3 (18). Third, ubiquitin-mediated degradation contributes to p27 accumulation in quiescent IMR-90 and MG-63 cells (19). We now demonstrate a fourth mechanism of p27 regulation that involves translation control, regulating association of p27 mRNA with polyribosomes.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Cell Culture, mRNA, and Protein Analysis—}HL-60 cells were maintained at 2–5 × 10\textsuperscript{5}/ml in RPMI plus 10% fetal calf serum. Under these conditions, all cells incorporated bromodeoxyuridine during a 24-h labeling period (data not shown). To achieve growth arrest, cells were treated with 33 nM TPA. To obtain synchronized cell cycle fractions, 4 liters of cells were grown and elutriated as we described (20). DNA content was determined by propidium iodide staining as described (20). DNA content was determined by propidium iodide staining as described (20). RNA was isolated from cells using RNA-STAT 60 following the recommendations of the manufacturer, and 20 \mu\text{g} of total RNA was used for Northern blotting as described (5). Proteins were isolated by sonicating cells in Tween lysis buffer as described (5). Immunoblot and kinase assays were performed as described previously (5, 20). To quantitatively determine the amount of protein using enhanced chemiluminescence (ECL), we included a standard protein curve in all experiments. This allowed us to determine a suitable range for quantitation based on the linear relationship between amount of protein and the intensity of signal. Signal quantitation was performed by spot densitometry using an IS1000 digital imaging system (Alpha-Innotech). Accumulation of p27 in Cells Exposed to LLnL—Either following elutriation of an asynchronous population into cell cycle phase synchronized fractions or 48 h after TPA-treatment of an asynchronous population of cells, the G\textsubscript{0}, G\textsubscript{1}, or S-phase cells were exposed to either 2 \mu\text{g/ml}
In HL-60 cells, activation of PKC leads to growth arrest and induction of p27. A, flow cytometry. Asynchronous cultures of HL-60 cells were exposed to 33 nM TPA, and aliquots were removed at 6-h intervals as indicated on the right and processed for flow cytometry. B, CDK2 kinase activity is diminished in TPA-treated cells. Cell lysates were prepared, and the ability of CDK2 immunoprecipitates to phosphorylate histone H1 was measured. The lanes are labeled to correspond with panel A. C, p27 protein increases without a change in the amount of p27 mRNA. Protein and RNA were isolated from cell lysates, and the amount of p27 was determined by immunoblot (top) and Northern blotting (bottom). The lanes are labeled to correspond with panel A. p27 protein was detected with an affinity purified p27-specific antibody. Northern blots were probed with either p27 or GAPDH cDNA as indicated on the right.

Cell Cycle-dependent and Growth-dependent Post-transcriptional Regulation of p27 in HL-60 Cells—The decision to either proliferate or withdraw from the cell cycle is made during G1 phase and is affected by the relative amounts of cyclin-CDK complexes and CDK inhibitors (18, 21–25). We used HL-60 cells to study the mechanisms controlling cell cycle withdrawal during differentiation because we could isolate populations of cells in each phase of the cell cycle in sufficient quantities for biochemical analysis. HL-60 cells differentiate into monocytes in the presence of TPA (26). Proliferation in asynchronous cultures of HL-60 cells completely ceased within 36 h following exposure to TPA, and typically, greater than 90% of the treated cells arrested with a G1 content of DNA (Fig. 1A). The significance of the 10% of cells arrested in G2/M is unclear. TPA treatment of enriched G1 or S-phase populations obtained by centrifugal elutriation suggested that cells arrested in a single cell cycle (Table I) at the next G1 phase. The 15% reduction of G1 cells following TPA treatment of elutriated G1 cells is consistent with a TPA restriction point subdividing this phase of the cell cycle.
the cell cycle. Concomitant with accumulation of cells with a G1 content of DNA following exposure to TPA, there was a coordinated decrease in immunoprecipitable CDK2 kinase activity (Fig. 1B) and an induction of p27 protein (Fig. 1C). The amount of p27 mRNA remained the same (Fig. 1C). Similar results were obtained with the phase-enriched populations (data not shown).

Since TPA-treated cells arrest with a 2C DNA content, p27 might accumulate in these cells as an indirect consequence of G1 arrest in a state where p27 protein is stabilized. To address this, we first determined whether p27 expression is cell cycle regulated. We obtained cell cycle phase-specific populations of HL-60 cells by centrifugal elutriation of asynchronous cultures and examined p27 protein expression (Fig. 2). This method does not induce perturbations in cell cycle-regulated protein expression often observed with drug or metabolic-induced cell cycle synchronization. The amount of p27 protein was maximal during the G1 phase of the cell cycle (Fig. 2B). The amount of p27 protein detected was linearly dependent on the amount of cell extract subjected to immunoprecipitation (data not shown; but see Fig. 3A). The amount of p27 mRNA did not change during the cell cycle (data not shown). These data suggest that, during the unperturbed cell cycle, p27 levels are determined by a post-transcriptional mode of regulation. However, the amount of p27 in equal numbers of G0 and G1 cells was quite different (Fig. 3A). G0 cells contained at least 3–4-fold more p27 than G1 cells. We obtained similar results comparing the amount of p27 as a function of total protein rather than cell number (data not shown). The amount of p27 detected was linearly dependent on the amount of cell extract subjected to immunoprecipitation (Fig. 3A, compare lanes 1 and 2). Together, these data suggest that accumulation of p27 reflected post-transcriptional regulation during both the G0/G1 and G1/S transitions.

**Phase-dependent Changes in Synthesis of p27**—The accumulation of p27 represents the sum of protein synthesis and protein degradation. Precedence exists for the regulation of protein half-life as a function of cell cycle phase during both the G1/S transition (27) and at the metaphase/anaphase transition (28). Determination of protein half-life during specific phases of the cell cycle is problematic because traversal of the cell cycle phases during the chase period will obscure the measured value. Consequently, to define the post-transcriptional mechanisms regulating p27, we measured the accumulation of p27 and the actual synthesis of p27 in G0, G1, and S-phase cells treated with LLnL (N-acetyl-leucinyl-leucinyl-norleucinal-H), an inhibitor that binds to the chymotryptic site on the proteasome. To measure accumulation of p27, we titrated each extract against recombinant p27 over a 16-fold range using 2-fold serial dilution. The resulting autoradiograph was analyzed by spot densitometry using an IS-1000 digital imaging system (Alpha Innotech), and we compared only those points that fell within the linear range (established by comparing signal intensity to amount of recombinant protein). Although the amount of p27 increased in all populations treated with LLnL, consistent with detection of newly synthesized p27 (shown below), the magnitude of the increase was cell cycle phase-specific (Fig. 3B). The increase in p27 levels was greatest in S-phase cells (about 160%) and more moderate in G1 and G0 cells (both about 50%).

To measure the amount of p27 synthesis in each population treated with LLnL, we labeled cells with [35S]-methionine and -cysteine for 90 min and immunoprecipitated p27. The short labeling period ensured that synchronized cells did not progress into the next phase of the cell cycle and inclusion of LLnL would prevent protein degradation; however, cells within G1 phase might traverse the restriction point during this period.

After normalizing lysates for total protein synthesis, we isolated p27 with affinity purified p27-specific antibodies. We quantitated the amount of p27 synthesis by scintillation counting of excised bands, normalizing incorporation to 100% in G1 cells. We found that the amount of newly synthesized p27 was greatest in G0 cells and lower in G1 and S-phase cells (Fig. 3C). We were unable to demonstrate if LLnL affected synthesis of p27 in a cell cycle phase-specific manner; however, similar increases in translation of p27 have been reported in other systems where LLnL was not included (29, 30). This suggested that the actual rate of p27 synthesis increased in G0 cells.

**Increased Synthesis of p27 Occurs as a Consequence of an Increase in the Amount of p27 mRNA in Polysomes**—To determine the mechanism responsible for accumulation of p27 in G0 cells, we compared the association of p27 mRNA with polysomes in proliferating and TPA-treated cells. To accomplish this, we fractionated RNA on continuous sucrose gradients and monitored the UV absorbance of the ribonucleoprotein complexes at A260. In this assay, RNA equilibrates within the gradient as a function of the associated proteins; polysomal RNA equilibrates in the densest regions of the gradient. The

### Table I

| Time (h) | S-phase fraction | G1 phase fraction |
|----------|------------------|------------------|
|          | +TPA | −TPA | +TPA | −TPA |
| 0        | 34.3 | 91.0 | 86.8 | 87.5 |
| 6        | 30.7 | 36.2 | 86.8 | 87.5 |
| 12       | 18.8 | 56.5 | 70.7 | 74.6 |
| 18       | 30.7 | 56.5 | 75.0 | 62.0 |
| 24       | 61.4 | 44.4 | 74.2 | 40.3 |
| 30       | 70.8 | 87.2 | 87.2 | 87.2 |
| 36       | 78.4 | 92.0 | 92.0 | 92.0 |

**Fig. 2.** The expression of p27 is regulated during the cell cycle. A. Flow cytometry. Asynchronous cultures of HL-60 cells were elutriated into cell cycle phase-specific fractions. DNA content was determined by propidium iodide. Fractions are numbered on the right. B, accumulation of p27 protein is maximal during G1 phase of the cell cycle. p27 was immunoprecipitated from each fraction of cells indicated at the top of each lane. The lanes are numbered to match panel A. A mock immunoprecipitation with a rabbit anti mouse antibody (RaM) is shown.

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extent of polysomal association is a reflection of the rate of protein synthesis; increased association reflects either an increase in the rate of initiation or an increase in the rate of elongation. To quantitate the p27 mRNA, we dot-blotted each fraction of the gradient and probed the membranes with a p27 cDNA probe. In untreated cells, p27 mRNA distributed in the free mRNA fraction of the gradient and in fractions containing monosomes and small polysomes (Fig. 4B). In TPA-treated cells, p27 mRNA distributes throughout the gradient, with the greatest differences occurring in the densest region of the gradients. Treatment of cells with puromycin will disrupt polysomes and lead to an accumulation of mRNA in monosome and subunit fractions of the gradient. When we exposed TPA-treated cells to puromycin, the p27 mRNA shift was eliminated, confirming polysome association (Fig. 4B). We found approximately 60% more p27 mRNA associated with polysomes in TPA-treated cells (Fig. 4D). This increase appears to occur at the expense of the monosome and small polysome associated mRNA (Fig. 4D), suggesting that p27 synthesis might be regulated at the level of translation elongation. We were unable to detect a significant change in the distribution of p27 mRNA in polyribosomes isolated from G1 and S-phase cells, consistent with our observation that p27 synthesis, in the presence of the proteasome inhibitor LLnL, was approximately equal in these populations (data not shown).

We confirmed the specificity of the dot-blot by isolating RNA from sucrose gradients and performing RT-PCR with p27-specific oligonucleotide primers. In this experiment, we assessed polysomal fractionation by ethidium bromide staining and detection of 28S and 18S rRNA following agarose gel electrophoresis (Fig. 5). Consistent with our dot blot results, we found a shift in the distribution of p27 mRNA with a substantial amount fractionating with heavier polysomes in TPA-treated cells (Fig. 5). We confirmed that the PCR product was p27 by restriction endonuclease mapping (data not shown). Furthermore, addition of a control DNA encoding p27 but missing 100 base pairs of the sequence between the primer binding sites was specifically amplified (data not shown).

To ensure that the change of p27 migration into heavier polysomes did not represent a nonspecific change in bulk translation, we directly probed fractionated RNA with GAPDH. There was little change in the migration of GAPDH mRNA following TPA treatment, either in the RT-PCR analysis (Fig. 5) or dot-blotting procedure (Fig. 4C). This suggests that the changes in polysome distribution of p27 mRNA were specific and did not reflect general changes that cells might undergo during alternative developmental pathways. Together, these experiments demonstrate that increased expression of p27 pro-
tein in cells exposed to TPA could be attributed to an increase in the density of ribosomes associated with p27 mRNA.

**DISCUSSION**

To study how the p27-mediated CDK inhibitory threshold is modulated during G1, we explored the mechanisms regulating p27 as HL-60 cells withdraw from the cell cycle or enter S-phase. We have analyzed synchronous cell populations in G0, G1, or S-phase. We have shown that the translation of p27 changes in a growth-dependent manner, the association of p27 mRNA with polysomes is a determinant of the accumulation of p27 protein. Furthermore, degradation of p27 is a major determinant of accumulation of p27 during the G1/S transition.

We found that an increase in the rate of p27 protein synthesis modulates the amount of p27 as HL-60 cells withdraw from the cell cycle. First, the amount of p27 is higher in growth-arrested cells than in G1 cells although the amount of mRNA remained the same. Second, the p27 synthesis rate is increased in G0 cells compared with either G1 or S-phase cells when proteolysis was prevented by treating cells with LLnL. Third, the polysome distribution of p27 mRNA shifts to heavier fractions in G0 cells, representing a greater density of actively translating ribosomes on each mRNA. This type of polysome distribution might represent more efficient utilization of the p27 mRNA template either by altering the initiation or elongation phases of translation. Translation control is not unique to either TPA or differentiating HL-60 cells. Hengst and Reed.
From the cell cycle. The mechanism regulating association is efficiently with the translation apparatus as cells withdraw be a member of a small group of messages that interact more polysomes occurs as cells re-enter the cell cycle from a non-
following the programmed withdrawal of a cell from the cell cycle and the amount of LLnL used
accumulation during the cell cycle, and the amount of LLnL used in our experiments is sufficient to inhibit that proteasome activity. Confirmation of this hypothesis, however, awaits the ability to measure the half-life of p27 in cell cycle phase-specific extracts capable of degrading p27.

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