Cell Transformation by the E5/E8 Protein of Bovine Papillomavirus Type 4

p27$^{\text{kip1}}$, ELEVATED THROUGH INCREASED PROTEIN SYNTHESIS, IS SEQUESTERED BY CYCLIN D1-CDK4 COMPLEXES

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The E5/E8 hydrophobic protein of BPV-4 is, at only 42 residues, the smallest transforming protein identified to date. Transformation of NIH-3T3 cells by E5/E8 correlates with up-regulation of both cyclin A-associated kinase activity and, unusually, p27$^{\text{kip1}}$ (p27) but does not rely on changes in cyclin E or cyclin E-CDK2 activity. Here we have examined how p27 is prevented from functioning efficiently as a CDK inhibitor, and we investigated the mechanisms used to achieve elevated p27 expression in E5/E8 cells. Our results show that normal subcellular targeting of p27 is not subverted in E5/E8 cells, and p27 retains its ability to inhibit both cyclin E-CDK2 and cyclin A-CDK activities upon release from heat-labile complexes. E5/E8 cells also have elevated levels of cyclins D1 and D3, and high levels of nuclear p27 are tolerated because the inhibitor is sequestered within an elevated pool of cyclin D1-CDK4 complexes, a significant portion of which retain kinase activity. In agreement with this, pRB is constitutively hyperphosphorylated in E5/E8 cells in vivo. The increased steady-state level of p27 is achieved largely through an increased rate of protein synthesis and does not rely on changes in p27 mRNA levels or protein half-life. This is the first report of enhanced p27 synthesis as the main mechanism for increasing protein levels in continuously cycling cells. Our results are consistent with a model in which E5/E8 promotes a coordinated elevation of cyclin D1-CDK4 and p27, as well as cyclin A-associated kinase activity, which act in concert to allow continued proliferation in the absence of mitogens.

The E5/E8 open reading frame of bovine papillomavirus type 4 encodes a small (42-residue) hydrophobic polypeptide that can transform rodent fibroblasts (1). E5/E8 is a member of the E5 family of membrane-localized transforming proteins (2). It has been postulated that BPV-1 E5 transforms cells by at least two distinct but complementary mechanisms as follows: (i) ligand-independent activation of tyrosine kinase growth factor receptors facilitated by E5-induced dimerization (3–6), and (ii) through direct interaction with ductin, a highly conserved component of the vacuolar proton ATPase (7) resulting in the disruption of pH homeostasis in endomembranes and cell transformation by a poorly defined mechanism, which likely involves perturbation of protein/lipid metabolism/trafficking through inhibition of the vacuolar proton ATPase (8).

It remains to be determined if E5/E8 can physically and/or functionally interact with tyrosine kinase growth factor receptors, although it is clear that E5/E8 can interact with ductin, at least in vitro, suggesting that it may have similar transforming mechanisms to other E5 family members (9, 10). Our approach has been to analyze alterations in the expression and function of key cell cycle regulators in NIH-3T3 cells transformed by E5/E8 to gain a better understanding of the signaling pathways that are utilized by E5/E8 and that result in a loss of both adhesion and mitogen requirements for cell proliferation.

Cyclin-CDK2 complexes are candidates to integrate extracellular (mitogen and anti-mitogenic) signals with the cell cycle machinery. A key function of the cyclin-dependent kinases is to phosphorylate the retinoblastoma protein (pRB), resulting in inactivation of this protein as a transcriptional repressor of key genes required for S-phase entry (11, 12). CDK inhibitors (CKIs) antagonize the catalytic activity of cyclin-dependent kinases, and the activity of these inhibitors is also governed by extracellular factors operating through receptor-mediated signaling pathways. The different CKIs may be responsible for promoting and/or maintaining cells in a quiescent state, and depending on the cell type, this may be a prelude to terminal differentiation. Work from a number of laboratories has demonstrated that cyclin D-CDK complexes play a non-catalytic role in facilitating cell cycle entry and $G_1$ progression by sequestering p21 and p27 into higher order complexes, which retain catalytic activity and aid in the nuclear import of cyclin D-CDK complexes. Sequestration functions to relieve cyclin E-CDK2 complexes from the inhibitory activity of CKIs (reviewed in Ref. 13).

A common feature of a number of transformed cells and tumors is a low p27 expression level. Low p27 levels correlate with aggressive tumor growth (reviewed in Ref. 14), and in some tumors, lack of p27 is an independent indicator of poor clinical prognosis (15, 16).
Many dominantly acting oncogenes target p27 expression and/or function, at least in vitro. A number of studies in cell lines have revealed that c-Myc overexpression overcomes a p27-mediated cell cycle arrest by promoting down-regulation of p27 expression. Oncogenic Ras, alone or in concert with c-Myc, may also down-regulate p27 (17–20), whereas c-Myc expression may also interfere with the binding of residual p27 to cyclin E-CDK2 (17). Viral transforming proteins such as E7 from human papillomavirus type 16 (21) and adenovirus E1A also appear to inactivate p27, although the following two reports have highlighted different mechanisms of action for E1A: in one case by direct interaction with p27 to prevent cyclin-CDK inhibition (22), and the other reported a bypass in p27 G1 arrest independent of a physical interaction between E1A and p27 (23).

Given that p27 is a major negative regulator of G1 progression, our initial finding that p27 levels remain high in E5/E8-transformed cells prompted us to investigate further this unusual situation. Here we have tested how E5/E8 prevents p27 functioning efficiently as a CDK2 inhibitor, and we investigated the mechanism used to achieve constitutive, elevated p27 expression in E5/E8 cells. Intriguingly, p27 remains predominantly nuclear and remains functional as a cyclin-dependent kinase inhibitor. Cyclin D1 expression is elevated in E5/E8 cells, and there is an increase in the abundance of cyclin D1-CDK4 complexes, and most of the p27 in E5/E8 cells is associated with these complexes. p27 immunoprecipitates from E5/E8 cells have substantial pRB kinase activity but little or no histone H1 kinase activity, whereas cyclin D1 immunoprecipitates contain elevated levels of pRB kinase activity. Together these results demonstrate that E5/E8 cells contain elevated levels of active p27-D1-CDK4 ternary complexes. In agreement with this, pRB is constitutively hyperphosphorylated in E5/E8 cells.

Both the steady-state level of p27 mRNA and the protein half-life are unaltered in E5/E8 cells, and the increased steady-state level of p27 is achieved largely through increased protein synthesis, most likely increased translation. This is the first report of enhanced p27 synthesis as the main mechanism for increasing protein levels in continuously cycling cells.

Our results are consistent with a model in which E5/E8 promotes a coordinated elevation of p27 and cyclin D1 to provide raised and constitutively active cyclin D1-CDK4 activity which, together with elevated cyclin A-CDK activity, acts to enforce pRB inactivation, allowing continued proliferation in the absence of mitogens.

MATERIALS AND METHODS

Cell Culture and Transfection—NIH-3T3 cells expressing E5/E8 have been described previously (1). Unless otherwise stated, the results presented below were obtained using E5/E8 clone 1 (E5/E8.1). Cells were also transfected with pT24, an expression plasmid for oncogenic ras (c-Ha-RasV12G), together with a selection marker (pZipNeo), using a standard calcium phosphate protocol. Cells were selected for G418 resistance, and clonal populations were derived by ring cloning and expansion in culture medium containing 0.5 mg/ml G418. Ras expression was confirmed by Western blotting. All cells were maintained as described previously (1).

Detection of E5/E8 Using Enhanced Autoradiography—Cells were incubated in medium containing [3H]leucine (400 μCi/ml) for 20 h and then lysed in a modified RIPA buffer (see below). E5/E8 was immunoprecipitated using affinity-purified E5/E8 antisera generated in two different rabbits (r280 and r281) against a keyhole limpet hemocyanin-conjugated synthetic peptide representing the hydrophilic C terminus of E5/E8 (residues 31–42, LISTITRLDGWD). Precipitated proteins were washed, analyzed by SDS-PAGE, and transferred to nitrocellulose membranes and then impregnated with EA Wax according to the manufacturer’s instructions and exposed to photographic film. Fluorographs were developed after 6 weeks at −70°C.

Immunofluorescence Microscopy—Cells were grown in chamber slides, fixed in 3% paraformaldehyde in PBS for 15 min at room temperature, permeabilized for 2 min in 0.1% Triton X-100 in PBS, and then washed in PBS and incubated in block buffer (PBS containing 10% fetal calf serum) for 1 h at room temperature. Primary antibody was incubated with the fixed cells for 1 h at room temperature. Antibodies were visualized using the following antibodies from Santa Cruz Biotechnology: cyclin A (sc-596), cyclin D1 (sc-450), CDK4 (SC-260), and p27 (sc-528). After 3 washes in block buffer, cells were incubated with 1:200 dilution of fluorescein isothiocyanate-conjugated, anti-rabbit or antimouse antibody (Jackson Laboratories) in block buffer for 45 min.Slides were washed, as above, mounted using Vectashield (Vector Laboratories), and then viewed using a Leitz Ortholux fluorescence microscope.

Immunoblotting, Immunoprecipitation, and Protein Kinase Assays—Immunoblotting and immunocomplex kinase assays were performed as described before (1). The anti-α-tubulin antibody was from Sigma (T-9026). For coupled immunoprecipitations/Western blots, 500-μg samples were adjusted to 1 mg/ml protein in lysis buffer (1) and pre-cleared for 1 h at 4°C using normal IgG (1 μg/ml), 10 μl of protein A-agarose, and 25 μl of a 50% (v/v) suspension of Sepharose beads (Sigma, CL-4B-200) in lysis buffer. Immunoprecipitation was performed on pre-cleared lysates using 2 μl (or 5 μl for agarse conjugates) of the following antibodies from Santa Cruz Biotechnology: cyclin D1 (sc-450-AC), cyclin D3 (sc-163), CDK4 (sc-601-AC), and p27 (sc-528). Immunoprecipitates were analyzed by Western blotting using antibodies to CDK4 (sc-260) and p27 (sc-528).

To analyze cyclin D1- and D3-associated pRB kinase activity, immunoprecipitates were performed as described above. Kinase reactions were carried out as described before (1), but only non-radioactive ATP was used. The extent of substrate (0.5 μg per sample of GST-pRB; amino acids 769–921 of pRB of mouse origin) phosphorylation on Ser-780, a site preferentially phosphorylated by cyclin D-CDK complexes, was determined using an antibody against an epitope containing phosphorylated Ser-780 (New England Biolabs). Parallel samples were run on SDS-PAGE gels and stained with Coomassie Blue to ensure equal amounts of substrate were included in each kinase reaction.

For heat treatment experiments, p27 immunoprecipitates or total cellular extracts prepared by sonication in 50 mM HEPES, pH 7.5, 150 mM NaCl and clarified by centrifugation (13,000 × g) at 4°C were heated at 100°C for 3 min, quenched on ice, and then added to cyclin E or cyclin A complexes immunoprecipitated from extracts prepared from control and E5/E8 cells grown under normal conditions. In some cases p27 was immunoprecipitated from extracts prepared by sonication/centrifugation, as described above, and then heat-treated. This fraction should represent the total cellular pool of p27 available to the antibody. All heat-treated extracts/immunoprecipitates were clarified by centrifugation (13,000 × g for 5 min at 4°C) and used for kinase assays as described above.

For immunodepletion experiments, two rounds of immunoprecipitation were performed as described above (each round for 1 h at 4°C). Controls used irrelevant antibodies or normal rabbit IgG fractions for mock depletion.

Northern Blotting—Total RNA was prepared using RNazol following the manufacturer’s instructions. Ten micrograms of RNA were electrophoresed in a 1% agarose-formaldehyde gel and transferred to nitrocellulose membrane (Hybnd N, Amersham Pharmacia Biotech). Membranes were hybridized with [3P]-labeled, random-primed cDNA probes for p27 and glyceraldehyde-3-phosphate dehydrogenase (loading control). The p27 probe was generated by polymerase chain reaction using mouse p27 cDNA as template (pCMV-p27 expression vector, a gift from Dr. R. Bernards) and primers homologous to the N terminus of p27 as follows: 5’-CGAGCCTGGAGCGGATGGAC-3’ and 5’-GGGAGGACCG-TCTGAAA-3’ as forward and reverse primers, respectively. Hybridization was performed for 16–18 h at 42°C in 1% SDS, 2× SSC, 50% formamide, pH 7.4. Blots were washed twice in 1% SDS, 2× SSC, pH 7.4, at 42°C and twice more in 0.1% SDS in 2× SSC, pH 7.4, at room temperature, dried, and exposed to photographic film.

RESULTS

Expression of E5/E8 in Clonal Populations of NIH-3T3 Cells—To detect E5/E8 we used metabolic labeling with [3H]leucine as this amino acid residue constitutes ~25% of the protein. After long term labeling, immunoprecipitation was carried out with antisera raised against a synthetic peptide representing residues 32–44 of E5/E8. Polypeptides (Mr, 14,000–40,000, and above the 46,000 molecular weight...
Elevated Expressions of p27 and Cyclin A Are General Features of E5/E8-mediated Transformation but are not due to Transformation per se. Cell lysates were prepared from control cells (Ctrl.) and from representative clonal populations of E5/E8 and RasV12G-transformed NIH-3T3 cells. Equal amounts of cellular protein were analyzed by Western blotting using antibodies to cyclin A and p27 as described under “Materials and Methods.” Levels of α-tubulin served as a loading control. HA/E5/E8.1, clonal line derived following transfection of the parent NIH-3T3 cells with a cDNA for E5/E8 expressed as a fusion protein with a hemagglutinin epitope (YPYDVPDYA) from the influenza virus hemagglutinin positioned at the N terminus. HA/E5/E8 cells, clone 1 shown, and others expressed the correct mRNA and a similar transformed phenotype to E5/E8 cells (data not shown).

The aberrant migration of these proteins (the predicted size of E5/E8 is ~7 kDa) may represent a low ratio of SDS:peptide binding due to the strong hydrophobic nature of E5/E8 but is also consistent with the formation of dimers (Mr ~14,000) and higher order oligomers resistant to SDS and dithiothreitol and/or formation of SDS-resistant complexes with cellular protein(s) and/or post-translational modification(s) of E5/E8. Further work is required to identify the precise nature of the protein species precipitated by the antisera.

Elevated Expressions of p27 and Cyclin A Are Elevated and Remain Predominantly Nuclear in E5/E8 Cells—Our initial findings demonstrated that p27 is non-functional in E5/E8-transformed cells as supraphysiological levels of p27 do not correlate with inhibition of cyclin E- and cyclin A-associated kinase activities or cell cycle arrest (1). We reasoned that E5/E8 may operate, in part, by impairing the ability of p27 to block CDK activity and cell cycle progression either by causing sequestration of p27 from the nucleus, its normal site of function, or by inhibiting the function of nuclear p27. To distinguish between these two possibilities, we used immunocytochemistry to reveal the subcellular distribution of p27 in E5/E8 cell lines. We found that p27 in E5/E8 cells is located largely in the cell nucleus in asynchronous populations (Fig. 3A). Similar results were obtained for two other clonal lines and in pooled populations of transfec-
tants (data not shown) indicating that increased expression and nuclear localization of p27 is a general feature of E5/E8-transformed cells. Cyclin A (Fig. 3B) is also predominantly nuclear in asynchronous populations of E5/E8 cells. For both p27 and cyclin A, expression is not strictly cell cycle-specific as immunostaining is near-uniform throughout the asynchronous cell population.

p27 in E5/E8 Cells Remains Functional but Is Sequestered by Heat-labile Protein(s)—We reasoned that p27 could be sequestered by nuclear protein(s), may be intrinsically non-functional in E5/E8 cells as a result of a post-translational modification, or cyclin E- and cyclin A-CDK complexes from E5/E8 cells may be intrinsically resistant to p27 inhibition. We next determined if p27 from E5/E8 cells retained its ability to inhibit cyclin-CDK complexes by exploiting the heat stability of the molecule (25, 26). p27 was immunoprecipitated from both control and E5/E8 cells heated to 100 °C for 2 min, centrifuged briefly, and the supernatant retained. This crude p27 fraction recovered from both control and E5/E8 cells is capable of inhibiting, in a dose-dependent manner, cyclin A and cyclin E-CDK complexes from both cell types (Fig. 4A). In addition, p27 immunoprecipitates from both cell types contained only background levels of kinase activity against histone H1 substrate when measured in the same time frame used routinely to determine total levels of cyclin A and cyclin E-CDK activity (results not shown). Immunoprecipitations using either normal rabbit immunoglobulin or an antibody to an irrelevant antigen (α-tubulin) did not contain any heat-stable activity inhibitory
toward cyclin E-CDK2 complexes from control cells (results not shown). This indicates that both cyclin E- and cyclin A-CDK complexes from E5/E8 cells are not intrinsically resistant to p27-mediated inhibition. In support of this conclusion, no obvious differences were detected in the composition of cyclin A complexes immunoprecipitated from both control and E5/E8 cell extracts after long term metabolic labeling using radioactive amino acids (results not shown).

Next, we immunodepleted cell extracts of p27 and then performed cyclin A kinase assays on the depleted extracts. As shown in Fig. 4B, there is little or no loss of cyclin A-associated kinase activity from E5/E8 cells even though p27 immunodepletion is efficient (Fig. 4B, lower panel).

We conclude that p27 in E5/E8 cells remains functional as a cyclin-dependent kinase inhibitor, but a large proportion of the total cellular pool of p27 is sequestered from cyclin A complexes by heat-labile components.

p27 in E5/E8 Cells Is Mainly Found in Ternary Complexes with Cyclin D1-CDK4, and Cyclin D1-CDK Activity Is Elevated in E5/E8 Cells—Cyclin D-CDK complexes have been proposed as candidates to sequester p27 and p21 within the cell nucleus allowing activation of cyclin E-CDK2 complexes (13, 25, 27–29). Both p27 and p21, although originally designated broad spectrum cyclin-CDK inhibitors, appear to function in a positive way to promote cyclin D-CDK complex assembly and nuclear targeting but not as effective inhibitors of cyclin D-CDK complexes when the stoichiometry of inhibitor to complex is at or close to parity (27, 29). Sequestration of p27 and p21 away from nascent and existing cyclin E-CDK2 complexes permit G1 progression (reviewed in Ref. 13). We investigated the possibility that in E5/E8-transformed cells elevated levels of cyclin D-CDK complexes serve as the major or sole sequestering proteins of p27.

In E5/E8 cells there is a dramatic elevation in the steady-state expression levels of cyclins D1 and D3 compared with control cells, and expression of these cyclins is largely independent of serum mitogens, whereas in control cells their expression level is much lower and remains largely mitogen-dependent (Fig. 5A). We could not detect cyclin D2 in parental NIH-3T3 cells or in any of our transfected lines derived from...
these cells (results not shown). Most cyclin D1 (Fig. 5C) and D3 staining (not shown) is nuclear, indicating that they co-localize with p27 and, although CDK4 expression is not noticeably altered (Fig. 5B), most CDK4 is expressed in the nucleus of E5/E8 cells (Fig. 5C). There is a small but significant increase in the expression of CDK6 in E5/E8 cells (Fig. 5B), but we have not been able to demonstrate an association between cyclins D1 or D3 and CDK6.

Significantly, the levels of cyclin D1-CDK4 complexes are higher in E5/E8 cells, and the majority of the p27 in the cell is associated with these complexes (Fig. 5D). Ternary complexes of p27-cyclin D1-CDK4 remain functional as p27 immunoprecipitates from E5/E8 cells contain elevated pRB but negligible histone H1 kinase activity, a profile of substrate specificities that is a hallmark of cyclin D-CDK activity (Fig. 6A, 30). Direct measurement of cyclin D-associated kinase activity in rodent cells is often not possible using standard immune-complex kinase assays (30), and we were not able to measure pRB kinase activity in control or E5/E8 cells using a variety of antibodies directed against cyclins D1 or D3 as well as CDK4 and CDK6. Direct measurement of pRB kinase activity in cyclin D1 and D3 immunoprecipitates was achieved using a non-radioactive assay that relies on the identification of specific phosphorylated residues in pRB by immunoblotting.3 Using this approach we found that there is substantially more pRB kinase activity in cyclin D1 immunoprecipitates from E5/E8 cells but a decrease in the activity of pRB kinase activity associated with cyclin D3 in the same cells (Fig. 6B), at least when measured against phosphorylation of serine 780 of pRB, a site preferentially phosphorylated by cyclin D-CDK complexes (12, 31). The pattern of expression and phosphorylation of pRB and the related proteins, p107 and p130, provided direct evidence for the de-regulation of cyclin D-CDK activity in vivo in E5/E8 cells. In control cells, cell cycle exit was associated with changes in the phosphorylation state and/or abundance of pRB, p107, and p130. Hyperphosphorylated pRB was clearly evident in proliferating cells, whereas only the hypophosphorylated, anti-proliferative form of pRB was present in quiescent cells. In addition, the amount of p107 decreased with cell cycle exit, whereas the abundance of p130 increased (Fig. 6C). Both pRB and p107 are largely hyperphosphorylated in E5/E8 cells (Fig. 6C), whereas p130 levels are significantly diminished and are not elevated upon growth factor withdrawal (Fig. 6C), consistent with the inability of serum withdrawal to promote quiescence in E5/E8 cells (1). An increased abundance of p107 and a decreased abundance of p130, as determined by Western blotting, most likely reflect alterations in protein expression, but we cannot rule out that it is due to changes in the affinity of the antibodies used for the different phosphorylated forms of these proteins. Nevertheless, we conclude that E5/E8 expression activates kinases in vivo that phosphorylate pRB, p107, and p130.

Together, these data demonstrate ternary complexes of cyclin D1-CDK4-p27 are more abundant and provide the sole or major p27 sequestering activity in E5/E8 cells. In addition cyclin D1-associated pRB kinase activity is elevated and de-regulated in E5/E8 cells and correlates with constitutive pRB hyperphosphorylation.

Supraphysiological p27 Expression in E5/E8 Cells Is Achieved Largely through Increased Protein Synthesis—The steady-state level of p27 is significantly elevated in E5/E8 cells when compared with control cells but does not increase further after mitogen withdrawal, demonstrating that E5/E8 interferes with anti-mitogenic signals that allow accumulation of p27 following growth factor withdrawal (Fig. 7A).

To understand how the steady-state level of p27 is increased in E5/E8 cells, we first examined levels of p27 mRNA and found that increased mRNA expression in E5/E8 cells cannot account

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3 D. Riley, personal communication.
Fig. 6. Elevated levels of pRB kinase activity in E5/E8 cells. A, cell lysates from control and E5/E8 cells were pre-cleared with normal rabbit immunoglobulin for 1 h at 4 °C before immunoprecipitation (IP) with anti-p27 antibody. The washed immunoprecipitates were assayed for pRB and histone H1 kinase activity (p27) or pRB kinase activity alone using GST-pRB (Santa Cruz Biotechnology, sc-4112) as substrate and [γ-32P]ATP. B, analysis of cyclin D-associated pRB kinase activity using a phosphospecific anti-pRB antibody. Cyclin D1 and D3 complexes were immunoprecipitated from control and E5/E8 cells and kinase assays were performed as described under “Materials and Methods” using only non-radioactive ATP in the kinase reaction master mix. Samples were analyzed by Western blotting for GST-pRB phosphorylation on Ser-780 (a site preferentially phosphorylated by cyclin D-CDK complexes) using an antibody that specifically detects an epitope containing phosphorylated Ser-780 (New England Biolabs). Parallel samples were run on SDS-PAGE gels and stained with Coomassie Blue as a loading control for the amount of substrate (GST-pRB; Total pRB) in each kinase reaction (lower panel). C, expression of pRB, p107, and p130 in control and E5/E8 cells. Cell lysates were prepared from control and E5/E8 cells under normal growth conditions (+ serum) or 24 h after switching to low serum (0.2%; − serum), and 50 μg (150 μg for pRB) of total cellular protein was analyzed per lane by Western blotting. Total pRB was determined using a monoclonal antibody to human pRB (PharMingen 14001). The position of the hypophosphorylated form of pRB is indicated in the lane for serum-starved control cells (arrow with asterisk), and additionally phosphorylated forms of pRB are indicated by a retarded electrophoretic mobility. Separate blots were probed with polyclonal antibodies to p107 and p130 as indicated.

DISCUSSION

In E5/E8 Cells p27 Is Sequestered by Cyclin D1-CDK4 Complexes—The cyclin-dependent kinase inhibitor p27 is thought to play a pivotal role in coupling environmental cues to cell proliferation. As a result of changes in the synthesis and degradation of p27, as well as sequestration of p27 by cyclin D-CDK complexes, the level of free p27 varies during the cell cycle (reviewed in Ref. 13). For fibroblasts, and probably other cell types, the level of p27 must be lowered to allow cyclin-CDK activation and cell proliferation (25, 32, 33). E5/E8 cells confound this model of p27-regulated cell proliferation as increased expression of this CKI is observed under all growth conditions tested (see Ref. 1 and Figs. 2 and 7A). Normal subcellular targeting of p27 is not subverted in E5/E8 cells; p27 is largely nuclear (Fig. 3A) and the protein is full-length as assessed by Western blotting (see Figs. 2 and 7A). However, p27 function appears to be subverted, as high levels of cyclin E- and cyclin A-CDK activity are found in cells with elevated levels of p27 in the nucleus (1), and most cyclin A-associated kinase activity is not associated with p27 in E5/E8 cells (Fig. 4B). Instead, the majority of p27 is complexed with nuclear proteins that are heat-labile, and p27 retains its ability to inhibit both cyclin A- and cyclin E-CDK complexes upon release by heat treatment (Fig. 4A). These data point to p27 being rendered functionally inactive in E5/E8 cells through sequestration. High levels of nuclear p27 are tolerated because the inhibitor is sequestered within an elevated pool of cyclin D1-CDK4 complexes and a significant portion of these ternary p27-D1-CDK4 complexes retain kinase activity.

Cyclin D3 is also elevated in E5/E8 cells (Fig. 5A) and localized predominantly in the nucleus (data not shown). However, for the differences in protein expression (Fig. 7B).

To investigate p27 protein stability, we blocked nascent protein synthesis using cycloheximide and followed p27 levels by Western blotting over time. The half-life of p27 is not significantly altered in E5/E8 cells (data not shown). In view of the possible secondary and differential effects of cycloheximide treatment on mRNA synthesis and stability, and the possible differences in the sensitivity of the two cell types to cycloheximide treatment, we conducted pulse-chase analyses followed by specific immunoprecipitation of p27. The results were in agreement with the cycloheximide treatment and demonstrate that the half-life is not significantly altered in E5/E8 cells (Fig. 7C). However, during the pulse period (30 min), substantially more p27 is synthesized in E5/E8 cells than in control cells (Fig. 7C, compare control cells to E5/E8 cells at time 0). We measured total cellular pools of [35S]labeled amino acids at the end of the pulse period and found an ∼2-fold difference with E5/E8 cells containing the larger pool of radioactive amino acids (Fig. 7C, lower right panel). This difference in amino acid uptake is not sufficient to explain the large (typically 10-fold) difference between the amount of p27 synthesized in the 30-min pulse period in E5/E8 cells compared with control cells (compare t = 0 points in Fig. 7C, top panel). Normalizing for differences in amino acid incorporation, ∼5-fold more p27 is synthesized in E5/E8 cells than in control cells during the pulse period.

We conclude that E5/E8 acts to elevate p27 expression, largely through increased synthesis in cells grown under normal culture conditions, and that p27 expression is uncoupled from environmental signals that normally control protein levels. This is the first report of increased protein synthesis as the major mechanism of establishing elevated p27 levels in cycling cells.

Overall, our data support a model in which nuclear cyclin D1-CDK4 complexes are the chief, or sole, sequestering activity of p27 in E5/E8 cells. Coordinated up-regulation of cyclin D1-CDK4 and p27 may act in concert with an E5/E8-mediated increase in the expression of cyclin A and cyclin A-associated kinase activity to provide mitogen-independent cyclin-CDK activity allowing pRB inactivation and continued cell proliferation.
Coordinated Elevation of D-type Cyclins and p27 in E5/E8 Cells—Overexpression of both D-type cyclins and their catalytic partners is insufficient to promote cyclin D-CDK activity in quiescent cells. Instead, signals from the mitogen-activated protein kinase pathway are required for both the synthesis and assembly of cyclin D-CDK complexes in quiescent cells stimulated to enter the cell cycle (41, 42), whereas here we are looking at continuously cycling cells transformed by E5/E8.

While this work was in progress two reports (19, 20) demonstrated that c-Myc could subvert a G1 cell cycle arrest imposed by overexpression of p27. It appears that c-Myc allows quiescent cells to enter the cell cycle by promoting D-type cyclin synthesis and cyclin D-CDK complex assembly, which in turn sequester p27/p27 away from cyclin E-CDK2 complexes. c-Myc-transformed cell lines, however, are characterized by elevated levels of cyclin E and cyclin E-CDK2 activity and low steady-state levels of p27 and D-type cyclins (17, 19). Cell lines transformed by other dominant oncogenes such as ras also display high levels of cyclin E-CDK2 activity and low levels of p27. The alterations seen in E5/E8 cells are unusual as, in addition to the changes outlined above, cyclin E expression and cyclin E-CDK activity is not de-regulated (1).

E5/E8 may not provide a full complement of growth factor/ integrin signals and therefore uncouple down-regulation of p27 from other positive effects on cell cycle progression, e.g. increasing the levels of cyclins D1 and A as well as cyclin A-associated kinase activity. In addition, most studies have analyzed the intersection of signaling pathways with the cell cycle machinery in quiescent cells stimulated to enter the cell cycle (41, 42), whereas here we are looking at continuously cycling cells transformed by E5/E8.

The pRB kinase activity associated with cyclin D3 immunoprecipitates is diminished in E5/E8 cells (Fig. 6B). Identification of the protein complexes containing cyclin D3 in E5/E8 cells is currently under investigation.

Coordinated Accumulation of D-type Cyclin and CKIs is ob- 

served in a subset of primary breast cancers and derived cell

the pRB kinase activity associated with cyclin D3 immunoprecipitates is diminished in E5/E8 cells (Fig. 6B). Identification of the protein complexes containing cyclin D3 in E5/E8 cells is currently under investigation.

Coordinated Elevation of D-type Cyclins and p27 in E5/E8 Cells—Overexpression of both D-type cyclins and their catalytic partners is insufficient to promote cyclin D-CDK activity in quiescent cells. Instead, signals from the mitogen-activated protein kinase pathway are required for both the synthesis and assembly of cyclin D-CDK complexes in quiescent cells stimulated to enter the cell cycle (34–37). CKI proteins themselves play a role in the assembly of D-CDRs by shifting the distribution of the pool of CDK4/6 into cyclin D-CDK complexes and away from complexes with cytoplasmic folding chaperones. In addition, CKIs promote nuclear targeting of cyclin D-CDK complexes, as the CKIs, but not cyclin D or CDK4/6, contain nuclear localization signals (reviewed in Ref. 13).

Cyclin D1 synthesis requires sustained ERK activity that is provided by a combination of growth factor- and adhesion-de- 

pendent signals (37–40), and MEK-1 activity is required for cyclin D1-CDK assembly (36). It is possible to speculate that E5/E8-induced de-regulation of the Ras/mitogen-activated protein kinase pathway could underlie the observed increase in abundance of nuclear cyclin D1-CDK4 complexes in E5/E8-transformed cells.

Fig. 7. Supraphysiological p27 expression in E5/E8 cells is achieved largely through increased protein synthesis. A, steady-state levels of p27 are increased in E5/E8 cells and are invariant after growth factor withdrawal. Cell extracts (50 μg of total cellular protein) were analyzed by SDS-PAGE, and the expression of p27 was determined by Western blotting. Levels of α-tubulin served as a loading control. B, levels of mRNA are sim- 
ilar in control and E5/E8-transformed cells. Cells were harvested at the indicated times (pulse) and then "chased" in the presence of a 100-fold excess of unlabeled methionine and cysteine for the indicated times. Cell lysates were normalized for protein concentration, and p27 was recovered by immu- 
noprecipitation, resolved by SDS-PAGE, and visualized by fluorography. Radioactivity incorporated into p27 was quantified using a PhosphorImager. Data are from an experiment performed three times with similar results. To measure the uptake of radioactive amino acids during the pulse period, radioactivity in the soluble fraction of the cell extract, following trichloroacetic acid precipitation of macromolecules, was determined by scintillation counting and normalized on cellular protein content.
lines (43, 44). Here it appears that a defect in the coupling of cyclins and CKIs to the proteosome may underlie this phenomenon. In the context of elevated expression of D-type cyclins, CKIs may act in concert with the D-type cyclins to promote continued cell proliferation even in the presence of low levels of cyclin E-CDK2 activity as observed in E5/E8 cells following mitogen withdrawal (1). E5/E8 cells display elevated levels of cyclin D1 and cyclin D3 (Fig. 5A) that, along with p27, are targets for phosphorylation-dependent recognition by SKP2. Coupling to SKP2 in turn leads to ubiquitination and proteosomal degradation (14). It is possible that E5/E8 may interfere with or combine with other steps implied in the degradation of cyclins.

The Steady-state Level of p27 in E5/E8 Cells Is Elevated Largely through an Increased Rate of Protein Synthesis—The expression of p27 is altered in response to environmental cues by changes in the rate of transcription, translation of the mRNA (26, 46–48), and by alterations in the protein half-life (27, 45, 49). The importance of each of these mechanisms in determining the steady-state level of p27 appears to reflect the position of the cell within the cell division cycle (26, 49) with increased translation accounting for the elevated level of p27 in cells that are quiescent or are exiting the cell division cycle (26, 46–48). The results presented here (Fig. 7) reveal that the increased steady-state level of p27 in E5/E8 cells results from a post-transcriptional mechanism that does not rely on extending the half-life of the p27 protein, i.e. as a result of an increased rate of protein synthesis, most likely through enhanced translation. This is the first report highlighting this mechanism for elevating p27 expression in cycling cells.

A recent report (48) demonstrated that a U-rich element located in the 5′-untranslated region of p27 mRNA is required for efficient translation and that a protein complex binds this element in a cell cycle-specific manner, with increased mRNA translation and increased polysomal association of p27 mRNA, correlating with the occupation of the U-rich site by this protein complex. The authors (48) suggest that this complex may be the target of anti-proliferative signals, and it is possible that in E5/E8 cells these control mechanisms are overridden. Interestingly, we found that transcription of a reporter plasmid for the murine p27 promoter was enhanced 4–5-fold in E5/E8 cells (data not shown), whereas the steady-state levels of mRNA did not vary correspondingly (Fig. 7B). The reporter construct used would produce mRNA that would contain a U-rich 5′-untranslated region sequence comparable to the region encoded in the human p27 mRNA (24, 48). We have not ruled out that the p27 mRNA half-life is not shortened in E5/E8 cells, but it is conceivable that the construct is acting as a translational reporter.

In E5/E8 cells there is no further increase in p27 expression following mitogen withdrawal (Fig. 7A), possibly because the mechanism used to achieve this, i.e. elevated translation, is maximally operative in E5/E8 cells under normal culture conditions and uncoupled from cell cycle position. Further work is required to determine which step(s) in the post-transcriptional processing of p27 mRNA and/or p27 mRNA translation are E5/E8 targets.

E5/E8 then may have multiple effects on signaling pathways that normally lead to p27 down-regulation and may also affect the processes that lead to p27 accumulation following mitogen withdrawal. If how this is connected to the observed deregulation of D-type cyclin expression and associations between p27 and cyclin-dependent kinases requires further investigation.

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