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Cyclin E, a redundant cyclin in breast cancer

(cell cycle/retinoblastoma/p16/redundancy)

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ABSTRACT Cyclin E is an important regulator of cell cycle progression that together with cyclin-dependent kinase (cdk) 2 is crucial for the G_1/S transition during the mammalian cell cycle. Previously, we showed that severe overexpression of cyclin E protein in tumor cells and tissues results in the appearance of lower molecular weight isoforms of cyclin E, which together with cdk2 can form a kinase complex active throughout the cell cycle. In this study, we report that one of the substrates of this constitutively active cyclin E/cdk2 complex is retinoblastoma susceptibility gene product (pRb) in populations of breast cancer cells and tissues that also overexpress p16. In these tumor cells and tissues, we show that the expression of p16 and pRb is not mutually exclusive. Overexpression of p16 in these cells results in sequestering of cdk4 and cdk6, rendering cyclin D1/cdk complexes inactive. However, pRb appears to be phosphorylated throughout the cell cycle following an initial lag, revealing a time course similar to phosphorylation of glutathione *S*-transferase retinoblastoma by cyclin E immunoprecipitates prepared from these synchronized cells. Hence, cyclin E kinase complexes can function redundantly and replace the loss of cyclin D-dependent kinase complexes that functionally inactivate pRb. In addition, the constitutively overexpressed cyclin E is also the predominant cyclin found in p107/E2F complexes throughout the tumor, but not the normal, cell cycle. These observations suggest that overexpression of cyclin E in tumor cells, which also overexpress p16, can bypass the cyclin D/cdk4-cdk6/p16/pRb feedback loop, providing yet another mechanism by which tumors can gain a growth advantage.

Progression through the eukaryotic cell cycle is mediated both positively and negatively by a variety of growth regulatory proteins (1–3). Cyclins and their catalytic cyclin-dependent kinase (cdk) partners act positively to propel a cell through the proliferative cycle (4, 5). Activation of cyclin-cdk complexes results in a cascade of protein phosphorylations that ultimately induce cell cycle progression (1, 4). Although the identity of downstream substrates and effectors of cyclin-cdks remains to be firmly established, it is commonly believed that cdk-mediated phosphorylations manifest cell cycle regulation via inhibition of growth inhibitory signals and activation of proteins necessary for each stage of the cell cycle (6). A putative, well-characterized substrate for the G1 cyclins is retinoblastoma susceptibility gene product (pRb; refs. 7 and 8). This protein is sequentially phosphorylated during the cell cycle presumably through the concerted activity of different cyclin-cdk complexes (9–11). This phosphorylation is required for cell cycle progression, and the hypophosphorylated form of pRb inhibits cell cycle progression by tethering and inactivating transcription factors of the E2F family, which are required for the transactivation of S phase-specific proteins, including dihydrofolate reductase, cyclin A, and thymidylate synthase (12–14). The phosphorylation of pRb results in the release of E2F transcription factors, freeing them to stimulate transcription of growth-promoting target genes.

Inhibition of pRb phosphorylation, therefore, represents a potent form of growth inhibition. Such inhibition has recently been exemplified through the characterization of cyclin-dependent kinase inhibitor proteins (reviewed in refs. 15 and 16). To date, these proteins exist as two functionally and structurally distinct groups typified by p21 and its homologues p27 and p57, as well as p16 and p15 and their related homologues (17, 18). As potential tumor suppressors, the cyclin-dependent kinase inhibitor genes have been studied extensively to evaluate the possible contribution of cyclin-dependent kinase inhibitor-specific genomic mutations to neoplastic transformation (17). In particular, the gene encoding p16, or multitumor suppressor 1, on chromosome 9p21 has been postulated to encode a tumor suppressor and has been demonstrated to be mutated in a wide variety of tumor-derived cell lines (19–22).

A curious finding has ensued from the analysis of p16 in cancer; although both pRb and p16 are often mutated in human cancers, these mutations seem mutually exclusive (23–26). This inverse correlation has been established in various tumor cell types both *in vitro* and *in vivo*. A logical conclusion then is that these proteins, which act similarly to inhibit cell cycle progression, are differentially regulated by a common pathway, perhaps involving a negative feedback loop. In fact, the growth suppression mediated via p16 overexpression has been shown to be definitively correlated with pRb status (27, 28). Thus, p16 inhibition of cell proliferation is evident only in cells expressing wild-type pRb. As an inhibitor of the putative pRb kinases cdk4 and cdk6, p16 is thought to bind, inhibit, and sequester these cdks, thereby rendering cyclin-D orphan with respect to cdk association. Some groups have postulated that p16 expression is regulated by pRb or by a feedback mechanism involving pRb (29), and it has been demonstrated by others that p16 is transcriptionally regulated by pRb (30). Such a mechanism would permit high levels of p16 to be expressed only when pRb is inactivated, by hyperphosphorylation, genomic mutation, or association with transforming viral oncoproteins. Although not without exception, the inverse correlation of these two proteins, particularly in breast epithelial cells, may represent a tightly regulated feedback mechanism.

In this report, we have identified and characterized an exception to the pRb/p16 inverse correlation rule. In the cell line MDA-MB-157, pRb is wild-type and phosphorylated, and p16 is significantly overexpressed and effectively binds cdk4 and cdk6, thus preventing cyclin D1 from binding to these kinases. We also have demonstrated that although cyclin D1-cdk4 and cyclin D1-cdk6 complexes are inactivated by p16, pRb is progressively synthesized and phosphorylated during the cell cycle. Cyclin D1, cdk4, and cdk6 are not overexpressed in this cell line; however, cyclin E is overexpressed, and its

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Abbreviations: cdk, cyclin-dependent kinase; pRb, retinoblastoma susceptibility gene product; GST-Rb, glutathione *S*-transferase retinoblastoma.

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levels and associated kinase activity remain constitutively high during all phases of the cell cycle. In addition, cyclin E-cdk2 complex can phosphorylate glutathione *S*-transferase retinoblastoma (GST-Rb) throughout the cell cycle. We conclude, therefore, that there exists a functional redundancy among the cyclins such that overexpression of cyclin E may compensate for the inactivation of cyclin D complexes by p16 with respect to the pRb phosphorylation and cell cycle progression.

METHODS

Cells Lines, Culture Conditions, and Tissue Samples. The culture conditions for all cell lines used in this study were described previously (31, 32). Snap-frozen surgical specimens from patients diagnosed with breast cancer were obtained from the Quantitative Diagnostic Laboratories (Almhurst, IL). 76N normal mammary epithelial cell strain and MDA-MB-157 tumor cell line were synchronized at the G_1/S boundary by a modification of the double thymidine block procedure (33) as described (32). For each time interval, 10^6 cells were subjected to FACScan analysis as described (32, 34).

Western Blot and Immune Complex Kinase Analysis. Cell lysates and tissue homogenates were prepared and subjected to Western blot analysis as described (31, 35). Primary antibodies used were monoclonal antibodies to cyclins E and D1 (Santa Cruz Biochemicals), cdk4 (Transduction Laboratories, Lexington, KY), pRb (PharMingen), and p16 (J.A.D.); and polyclonal antibodies to cdk6 (Santa Cruz Biochemicals) and cyclin A (a gift from J. W. Harper, Baylor College of Medicine, Houston). Immunoprecipitations and H1 kinase assays were performed as described (32, 36). Briefly, for H1 kinase and GST-Rb kinase assays, 500 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with polyclonal antibody to cyclin E. Immunoprecipitates were then incubated with kinase buffer containing either 5 μg of histone H1 or 1 μg of purified GST-Rb, 60 μM cold ATP, and 5 μCi of [γ-^{32}P]ATP in a final volume of 50 μl at 37°C for 30 min. The products of the reaction were then run on a SDS/13% PAGE gel. The gel was then stained, destained, dried, and exposed to x-ray film.

For immunoprecipitation followed by Western blot analysis, 250 μg of protein (unless otherwise indicated in the figure legend) were used per immunoprecipitation with either monoclonal antibody to p16, polyclonal antibody to cyclin D1 obtained from M. Pagano (Mitotix, Cambridge, MA) (37), or monoclonal antibody to cyclin D1-clone HD33 (a gift from E. Harlow and C. Ngwu, Massachusetts General Hospital Cancer Center, Boston) in lysis buffer as described above. The immunoprecipitates were then electrophoresed on a SDS/13% PAGE, transferred to Immobilon P, blocked, and incubated with either polyclonal antibody to cdk4 obtained from M. Pagano (Mitotix) (37) or cdk6 as described in the figure legend.

Gel Retardation Assays. Whole-cell extracts were prepared as described (31, 35), and 15 μg of protein were used per lane. Binding reactions were performed as described elsewhere (13, 38). The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human dehydrofolate reductase promoter (DHFR WT) (13). For antibody perturbation experiments, 2 μl (200 ng) of rabbit polyclonal antibody to cyclin E (Upstate Biotechnology, Lake Placid, NY) was added.

RESULTS

Overexpression of p16 and Absence of Cyclin D1/cdk4-D1/cdk6 Complexes in a Breast Cancer Cell Line with Functional Rb Protein. A panel of 13 breast cell lines was surveyed for the correlation of p16 and Rb status, as well as association of p16 and cyclin D1 with cdks 4 and 6 (Fig. 1). The cell lines used include three proliferating normal mammary epithelial cell strains obtained from reduction mammoplasties and used at early passages, one near diploid normal-immortalized breast epithelial cell line and nine tumor cell lines with different cyclin E levels, estrogen receptor, and p53 status as outlined in Table 1.

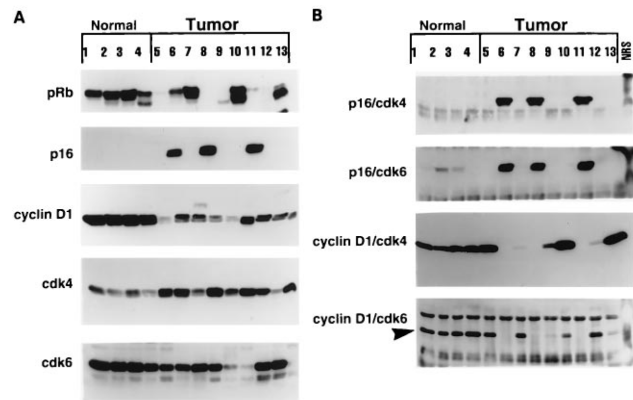

FIG. 1. Expression and complex formation of p16/pRB pathway proteins in normal and tumor-derived breast epithelial cells. (*A*) Western blot analysis: exponentially growing normal and tumor cells were subjected to Western blot analysis using 50 μg of protein for each cell line in each lane of either a 6% (pRb), 13% (cyclin D1, cdk4, and cdk6), or 15% (p16) acrylamide gel and blotted as described. The same blot was reacted with cyclin D1, cdk4, and cdk6 affinity-purified antibodies. The blots were stripped between the three antibodies in 100 mM 2-mercaptoethanol, 62.5 mM Tris·HCl (pH 6.8), and 2% SDS for 30 min at 55°C. (*B*) Immune-complex formation: for immunoprecipitation followed by Western blot analysis, equal amounts of protein (500 μg) from cell lysates prepared from each cell line were immunoprecipitated with either monoclonal antibody to p16 (p16/cdk4 and p16/cdk6), polyclonal antibody to cyclin D1 (cyclin D1/cdk4), or a monoclonal antibody to cyclin D1 (cyclin D1/cdk6), coupled to protein A/G beads, and the immunoprecipitates were washed, boiled for 3 min, separated by SDS/13% PAGE, blotted to Immobilon membranes, and hybridized with either polyclonal antibody to cdk4 (p16/cdk4), polyclonal antibody to cdk6 (p16/cdk6 and cyclin D1/cdk6; arrow pointing to the complexed protein), or monoclonal antibody to cdk4 (cyclin D1/cdk4). The list of normal and tumor cell lines is presented in Table 1 using identical numbers.

We examined the expression of pRb by direct immunoblotting with a monoclonal antibody in which the presence of functional pRb is inferred from the presence of higher molecular weight-hyperphosphorylated forms of the protein. This analysis revealed that besides three tumor cell lines (Fig. 1*A*, lanes 8, 11, and 12; i.e., MDA-MB-436, HBL-100, and Hs-578T) in which pRb is either mutated (42), inactive due to its binding to simian virus 40 large T antigen, or not expressed, pRb is present and functional in all of the other cell lines examined. Furthermore, in all of the pRB-positive cell lines, there are at least two pRb bands present representing different phosphorylation states of pRb. (Due to different levels of pRb expression in each of the cell lines, longer exposures were used to evaluate presence of slower migrating, functional form of pRb, specifically in lanes 1, 2, and 5; data not shown). Next, we correlated the expression of p16 levels with pRb status and found that p16 is overexpressed in three cell lines (Fig. 1*A*, lanes 6, 8, and 11), two of which Rb has been functionally compromised (i.e., MDA-MB-436 and HBL-100). It is curious that in MDA-MB-157, which contains a wild-type pRb, p16 is also markedly overexpressed (Fig. 1*A*, lane 6). Hence, MDA-MB-157, in which cyclin E is severely overexpressed (Table 1; refs. 31, 32), is one exception to the reciprocal p16/Rb correlation rule.

Because overexpression of cdk4, cdk6, or cyclin D1 could counteract the inhibitory effect caused by the overabundance of p16, we also measured the relative levels of these proteins in all 13 cell lines (Fig. 1*A*). Western blot analysis with cyclin D1, cdk4, and cdk6 revealed that these proteins were not overexpressed in MDA-MB-157 cell line relative to the other 12 cell lines examined, suggesting that the overexpressed p16 may adequately sequester cdk4 and cdk6 away from cyclin D1, rendering it inactive. To test this hypothesis, we performed a series of two-step immunoprecipitations followed by Western blot analysis (Fig. 1*B*). When p16 immunoprecipitates were separated on denaturing gels, transferred to poly(vinylidene

Table 1. Characterization of normal and tumor-derived breast epithelial cells

Cell lines	Cell types	Estrogen receptor (31)	p53	Cyclin E (31, 32)	pRb*
1. 70N	N-mortal	−	+ (39)	+	+
2. 81N	N-mortal	−	+ (39)	+	+
3. 76N	N-mortal	−	+ (39)	+	+
4. MCF-10-A	N-immortalized	−	+ (40)	+	+
5. MCF-7	A (pe)	+	+ (40)	+++	+
6. MDA-MB-157	C (pe)	−	− (40)	++++++	+
7. MDA-MB-231	A (pe)	−	− (40)	++++	+
8. MDA-MB-436	A	−	− (41)	+++++	−
9. T47D	DC (pe)	+	− (40)	++	+
10. BT20	C	+	+ (40)	++	+
11. HBL-100	T (bm) SV40 transformed	−	− (40)	+++	−
12. HS-578T	DC	−	− (40)	++++	−
13. ZR75T	IDC	+	+ (40)	+++	+

Cell type, estrogen receptor (ER), p53, and cyclin E status as determined in indicated references. +, wild type; ++(++++), varying degrees of overexpression with MDA-MB-157 showing the highest degree (64-fold, hence 6 + s) of cyclin E overexpression. N, normal breast cells from reduction mammoplasty; A, adenocarcinoma; pe, pleural effusion; C, carcinoma; DC, ductal carcinoma; T(bm), tumor breast milk; SV40, simian virus 40; IDC, infiltrating DC; −, mutant or not expressed.

*pRb status is adopted from Fig. 1, in which + indicates wild type and present in hypo- and hyperphosphorylated forms, and − indicates mutated or virally bound and inactive.

difluoride) membrane, and blotted with antiserum to cdk4 or cdk6, p16 was capable of forming a complex with both cdk4 and cdk6 in the three tumor cell lines in which p16 is overexpressed. Curiously, p16 was also capable of forming a complex with cdk6 in normal breast cell strains in which no overexpression of p16 or cdk6 was noted. However, cyclin D1 immunoprecipitates that were separated and blotted with antibodies to cdk4 or cdk6 revealed that, in the normal cell strains, cyclin D1 formed a complex with cdk4 and cdk6, suggesting that p16 did not completely sequester these kinases from cyclin D1. On the other hand, in tumor cells in which p16 is overexpressed, no complexes were formed between cyclin D1 and cdk4 or cdk6, suggesting that in these three tumor cell lines enough p16 is overexpressed to sufficiently sequester cdk4 and cdk6 away from cyclin D1, preventing it from forming complexes with these kinases (Fig. 1*B*). Collectively, these data provide evidence for the absence of cyclin D1/cdk complexes in a breast cancer cell line with a functional Rb protein.

Cyclin E-Associated Kinase Phosphorylates pRb in the Absence of Cyclin D1/cdk4 or Cyclin D1/cdk6 Complexes in Tumor Cells. To examine the cell cycle regulation of pRb in normal and tumor cells, we synchronized both cell lines by double thymidine block and analyzed the pattern of pRb expression and phosphorylation by Western blot analysis (Fig. 2*A*). Synchrony of both cell types at several times after release from the block was monitored by flow cytometry (Fig. 2*C*). At various times after release from treatment for synchronization, cells were harvested, and extracted proteins were analyzed on Western blots with antibodies to pRb and cyclins E and A (Fig. 2*A*). In normal 76N cells, the pattern of synthesis and phosphorylation of pRb, as well as expression of cyclin E and cyclin A proteins, is consistent with that seen for other normal cell types, with levels rising before S phase and oscillating thereafter in the cell cycle (8, 43, 44). In addition, pRb is present mainly in the hyperphosphorylated form at G_1/S boundary up to G2, where the levels drop to resume again at G_1. Furthermore, there is only one major form (i.e., 50 kDa) of cyclin E protein detected. However, in the tumor cells, pRb and cyclin E proteins do not appear to be cell cycle-regulated. pRb is induced and phosphorylated shortly after release from thymidine block and remains in that phosphorylated state throughout the cell cycle. In addition, multiple isoforms of cyclin E protein are present with similar signal intensities and banding patterns during the time intervals examined. In the same tumor cell extracts, cyclin A protein is cell cycle-regulated with peak levels coinciding with peak S and early M phase. Hence, it appears that in this tumor cell line, pRb and cyclin E are abnormally regulated during the cell cycle.

To decipher whether cyclin E-associated kinase is responsible for the phosphorylation of pRb, cells were immunoprecipitated with cyclin E antibody and used in kinase assays with either histone H1 or a recombinant GST-Rb fusion protein as substrates (Fig. 2*B*). In normal cells, cyclin E-associated kinase is capable of phosphorylating histone H1 and is cell cycle-regulated, coinciding with the levels of cyclin E protein expression (Fig. 2*A*). However, the same cyclin E immunoprecipitates prepared from normal cells were not capable of phosphorylating GST-Rb (Fig. 2*B*). In tumor cells, on the other hand, cyclin E is not cell cycle-regulated and remains in a catalytically active complex throughout the cell cycle, resulting in a constitutive pattern of histone H1 and GST-Rb phosphorylation. Finally, the timing of pRb expression in the tumor cell cycle (Fig. 2*A*) is similar to the timing of phosphorylation of GST-Rb by cyclin E immunoprecipitates (Fig. 2*B*). These observations suggest that overexpression of cyclin E results in an active kinase complex throughout the cell cycle capable of phosphorylating not only histone H1, but also GST-Rb. Hence, in tumor cells that overexpress p16, resulting in the inactivation of cyclin D1/cdk4 or cyclin D1/cdk6 complexes, pRb can still get phosphorylated by cyclin E-associated kinase.

Overexpression of Cyclin E and p16 in Breast Tumor Tissues Is Correlated with Functional pRb. Because the lack of inverse association of pRb and p16 was observed in only one of three breast tumor cell lines overexpressing p16 (Fig. 1*A*), we were interested in deciphering the frequency at which such a phenomenon would occur in breast tissue samples. Therefore, we examined 20 tumor tissue specimens obtained from breast cancer patients. Table 2 lists estrogen and progesterone status, ploidy, and proliferation index expression as measured by immunofluorescence with the respective antibodies followed by image analysis as described (45, 46). We also analyzed the expression of cyclin E, p16, and pRb in these samples by Western blot analysis. The results revealed that cyclin E was severely overexpressed and present in lower molecular weight forms in 18 of 20 tissue samples, which is consistent with the role of cyclin E as a prognosticator for breast cancer (31, 35, 48). The pattern of cyclin E expression observed in these tumor specimens was similar to those used in a previous

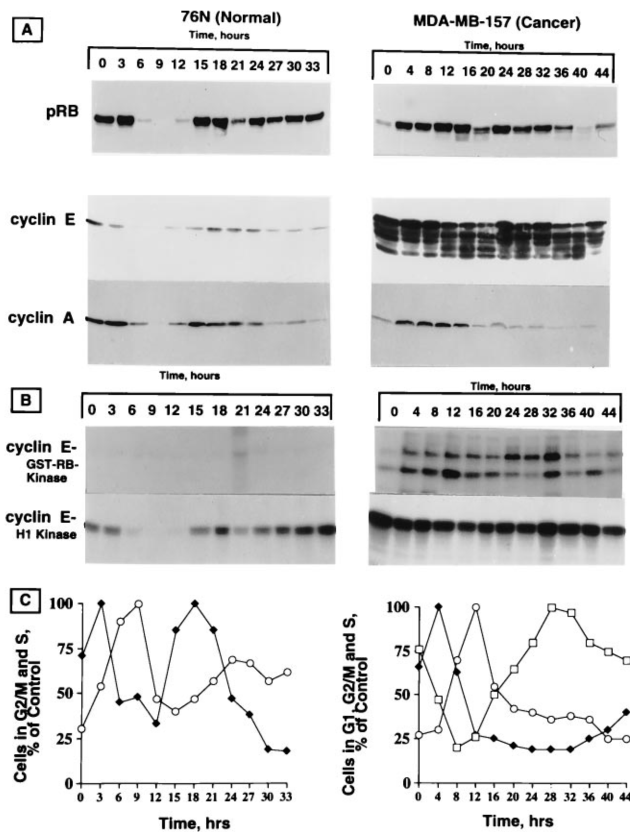

FIG. 2. Phosphorylation of pRb in synchronized population of tumor versus normal cells. Both cell types were synchronized by double thymidine block procedure. At the indicated times after release from double thymidine block, cell lysates were prepared and subjected to Western blot analysis (*A*) and histone H1 or GST-Rb kinase analysis (*B*). Protein (50 μg) for each time point was applied to each lane of either a 6% (pRb) or 10% (cyclins E and A) acrylamide gel and blotted as described. The same blot was reacted with cyclin E monoclonal (HE12) and cyclin A affinity-purified polyclonal antibodies. The blots were stripped between the two assays as described for Fig. 1. For kinase activity, equal amounts of proteins (600 μg) from cell lysates prepared from each cell line at the indicated times were immunoprecipitated with anti-cyclin E (polyclonal) coupled to protein A beads using either histone H1 or purified GST-Rb as substrates. (*C*) The relative percentage of cells in different phases of the cell cycle for each cell line at various times after release from double thymidine block was calculated from flow cytometric measurements of DNA content. ◆, cells in S phase; ○, cells in G_2/M phase; □, cells in G_1 phase.

study (49) showing presence of lower molecular weight forms of cyclin E with increasing stage of the disease. It is interesting that most of the tumor specimens that showed an overexpression of cyclin E also were negative for estrogen and progesterone receptors. A negative steroid receptor status is indicative of poor response to endocrine and cytotoxic chemotherapy characteristics of very aggressive breast tumors (50). Furthermore, p16 was overexpressed in 7 (i.e., KK-005, 086, 147, 173, 190, 369, and 399) of the 20 samples examined. Three of these seven samples had a defect in pRb expression, whereas in the remaining four samples (i.e., KK-005, 147, 173, and 369), pRb was expressed and present in multiple bands, suggesting a functional protein. In addition, cyclin E was severely overexpressed in all four p16/pRb double-positive samples. Hence, these observations suggest that *in vivo*, in breast cancer tissues that overexpress cyclin E, overexpression of p16 is not always accompanied by a defect in pRb, consistent with results obtained with MDA-MB-157 cell line. Cyclin E, which is overexpressed and present in lower molecular weight forms in these tumor tissue samples, may be capable of phosphorylating pRb in the absence of functional cyclin D-containing complexes *in vivo* as well as in cell lines.

Cyclin E Is Present in E2F Complexes Throughout the Cell Cycle of Tumor, but Not Normal, Cells. One of the major targets of growth regulation by pRb is the E2F family of transcription factors. During the G1 phase of the cell cycle, underphosphorylated pRB binds to E2F and represses its transcriptional activity. Phosphorylation of pRb by cyclins during late G_1 and S phase release E2F, which in turn leads to activation of the transcription of genes important for cell cycle progression. Similarly, p107 and p130, two pRb-related proteins, regulate the transcriptional activity of E2F. In addition, both cyclins A and E can bind to p107 and p130 while in complex with E2F. Although the significance of this association is not known, it has been suggested that it regulates the transcriptional activity of E2F.

To determine whether the cyclin E overexpression in the tumor cell lines affected the E2F DNA binding complexes throughout the cell cycle, we performed bandshift assays using an oligonucleotide with an E2F binding site as a probe (Fig. 3). As a control, extracts from a synchronized population of normal cells were prepared. As described (13), normal cells contained several E2F complexes that were present at various times in the cell cycle. The disappearance of E2F complexes at 6, 9, and 12 h after release from the thymidine block occurred when the cells were enriched for G_2/M (Fig. 3*A*; ref. 13). The complex marked with an arrow contained the pRB-related protein p107 and cyclin A, as determined by antibody supershift analysis (data not shown). Addition of cyclin E antibody did not have any effect on the mobility of this complex (Fig. 3*A*), suggesting that cyclin E is not the predominant cyclin in the p107/E2F complex in normal cells. On the other hand, in extracts prepared from tumor cells, E2F complexes were present throughout the cell cycle, and no loss of these complexes was observed during G_2/M. The complex marked with an arrow could be disturbed with anti-p107 and partially with anti-cyclin A antibodies (data not shown). The addition of an anti-cyclin E antibody resulted in a supershift of a large proportion of the complex, suggesting that most of the p107-E2F complex contained cyclin E (Fig. 3*B*). Addition of antibodies to cyclin A and cyclin E to the same extract did not result in the appearance of any different complexes than when both antibodies were added independently (data not shown), suggesting that both cyclins did not form part of the same complex. The association of cyclin E with the E2F complexes in tumor cells paralleled the constitutive expression of cyclin E throughout the cell cycle (Fig. 2*A*, *Right*). Hence, overexpression of cyclin E in tumor cells was capable of forming a major complex with p107 and E2F. This is a second example of how overexpression and constitutive expression of cyclin E could result in a dual role for this cyclin allowing redundancy in function.

DISCUSSION

The interplay between cyclin D1/cdk4-cdk6/p16/pRb has been implicated as a crucial G_1 phase-controlling pathway that becomes frequently deregulated in many types of cancer. Any mutations giving rise to an imbalance in any one of these proteins may therefore result in a cell growth advantage leading to tumorigenesis. In this model, overexpression of p16 would prevent cdk4/cdk6 from phosphorylating pRb, and lead to a G_1 block (27–29). Thus, p16 is thought to negatively regulate the cell cycle (51). In fact, several studies have documented that primary tumors that showed expression of functional pRb protein did not express p16 protein (due to mutations in the gene) and, conversely, cells that expressed p16 protein did not have a detectable pRb protein (23–26). These studies suggest a link between D-type cyclins, cdk4/cdk6, pRb, and p16, such that overexpression of cyclin D1, inactivation of pRb, or loss of p16 may have equivalent consequences for loss of normal growth control. In addition, this model predicts a lack of functional redundancy of this pathway with other cell cycle regulatory proteins.

Even though many studies have corroborated the p16/pRb inverse correlation model, there also has been documentation to the contrary. For example, in their analysis of pRb and p16 expression in lung cancers, Otterson *et al.* (25) reported that 14%

Table 2. Correlation of p16 and pRb status in a series of breast carcinomas

Patient ID no.	ER/PR*	DNA index/ploidy*	Proliferation index (%)*	Cyclin E†	p16†	pRb†
KK005	−/−	1.18/Aneuploid	12.2 (H)	+++	++++	+
KK017	−/−	1.72/Aneuploid	1.5 (L)	++++++	±	+
KK020	−/−	1.73/Aneuploid	14.1 (H)	+++++	−	−
KK036	+/−	1.84/Tetraploid	3.3 (L)	++	±	+
KK061	−/−	ND	ND	++++	±	−
KK070	+/+	ND	ND	+	±	−
KK076	−/−	2.08/Tetraploid	12.5 (H)	+++	±	−
KK086	−/−	1.50/Aneuploid	36.0 (H)	+++++	++	−
KK147	ND	ND	ND	+++++	+++	+
KK173	+/−	1.91/Tetraploid	30.2 (H)	+++++++	++++	+
KK190	−/−	2.09/Tetraploid	31.8 (H)	+++++++	+++	−
KK322	+/−	2.70/Aneuploid	30.0 (H)	+++	−	+
KK369	ND	ND	40.0 (H)	+++++++	++++	+
KK399	−/−	ND	ND	++++	++++	−
KK400	+/−	ND	ND	++++	±	−
KK407	−/−	1.89/Tetraploid	18.0 (H)	++++	−	−
KK428	−/−	1.75/Aneuploid	27.0 (H)	++++	−	−
KK429	−/−	1.71/Aneuploid	28.0 (H)	+++++	−	−
KK457	ND	ND	ND	++++++	−	+
KK458	−/−	1.96/Tetraploid	11.3 (H)	+	−	+

*Quantitation of immunohistochemical staining by image analysis was performed on sections stained with either the monoclonal antibody to estrogen receptor H222 (ER-ICA kit, Abbott), monoclonal antibody to progesterone receptor mPRI (Cell Analysis Systems, Lombard, IL), or monoclonal antibody to Ki67 (Dako) as described (45, 46). Ki67 staining determined growth fraction of the tumor. Values indicate percentage of positive staining: 1.0–7.0% is indicative of low (L) proliferation index, 7.1–11.9 is indicative of moderate (M) proliferation index, and >12.0% is indicative of high (H) proliferation index. For each case, the DNA ploidy was determined by quantitation of the DNA Feulgen stain by computerized microdensitometry as described (47). ND, not determined.

†Cyclin E, p16, and pRb levels were measured using Western blot analysis with HE12 monoclonal antibody to cyclin E (Santa Cruz Biotechnology) as described (31, 32), monoclonal antibodies to p16, and pRb as described in text. Levels of cyclin E in tumor tissue samples were correlated with 76N normal (+) and MDA-MB-157 (++++++) tumor cell lines. For example, cyclin E in MDA-MB-157 cell line is 64-fold (i.e., ++++++) overexpressed compared with 76N cell line (i.e., +) (31). Any tumor tissue overexpressing cyclin E more than MDA-MB-157 received seven +s (i.e., +++++++). p16 levels also were correlated with MDA-MB-157 (++++) cell line. Equal protein loading was monitored by reprobing blots with actin, and all blots were analyzed by densitometry using AGFA scanner and IP Lab Gel software.

of small cell lung cancers and 15% of non-small cell lung cancers examined were p16 and pRb double positives, and Sakaguchi *et al.* (52) reported that 16.4% of non-small cell lung cancers studied immunohistochemically also stained positively for both p16 and Rb protein. In addition, Gerardts *et al.* (53) report that in 43% of all carcinomas examined (breast: 5 of 20; bladder: 7 of 19; colon: 16 of 19; lung: 4 of 17), both pRb and p16 could be detected, suggesting that in common human malignancies, p16 and pRb expression is not mutually exclusive. Furthermore, Musgrove *et al.* (54) report that in 50% of breast cancer cell lines examined, $INK4^{p16}$ mRNA was expressed in the absence of any pRb mutations. Finally, Ueki *et al.* (49) show that 13% of glioblastoma cell lines examined showed neither p16 nor RB alterations, and Wang *et al.* (55) report that regardless of the status of p16 protein, all 15 melanoma cell lines examined showed the presence of pRb protein, ruling out an inverse correlation between the expression of p16 and pRb in these particular cell lines.

One possible explanation for the lack of inverse correlation between p16 and pRb may be due to overexpression of cyclin E, which could act redundantly and replace cyclin D/cdk complexes for phosphorylating pRb. In accordance with this redundancy hypothesis, Hinds *et al.* (56) first demonstrated that overexpression of several different cyclins, including cyclin E, could override the growth arrest properties of pRb in SaOS-2 cells. In addition, we had reported previously that cyclin E is severely overexpressed in all breast cancer cell lines examined (31), and overexpression of cyclin E is accompanied by its constitutive expression and activity throughout the tumor cell cycle (32). Because cyclin E is overexpressed and forms a complex with cdk2 constitutively, the active complex can act upstream of pRb and phosphorylate it even when cyclin D is inactive due to overexpression of p16. To test this model, in this study we used a breast cancer cell line that exemplified an exception to the inverse correlation rule of p16/pRb. In this tumor cell line (MDA-MB-157), cyclin E is markedly overexpressed and present in lower molecular weight isoforms, p16 is also overexpressed, and pRb is not mutated and detectable in both its hypo- and hyperphosphorylated forms. Under these conditions, we show that p16 binds to both cdk4 and cdk6 and inhibits the binding of cyclin D1 to these cdks. We also provide evidence that, in synchronized populations of MDA-MB-157 cells, pRb is phosphorylated throughout the cell cycle following an initial lag, revealing a time course similar to phosphorylation of GST-Rb by cyclin E immunoprecipitates prepared from these synchronized cells. This analysis suggests that cyclin E/cdk2, and not cyclin D/cdk4-cdk6, is a candidate kinase complex capable of phosphorylating pRb throughout the cell cycle of this tumor cell line.

To directly examine the lack of inverse correlation of p16 and pRb *in vivo*, we document in Table 2 that in breast tumor specimen obtained from breast cancer patients in whom cyclin E is markedly overexpressed and p16 also is overexpressed, pRb is detectable in both its hypo- and hyperphosphorylated forms. These studies suggest that phosphorylation of pRb under conditions in which cyclin D/cdk complexes are rendered inactive is not an artifact of the culture conditions and occurs *in vivo*.

Because cyclin E is constitutively expressed in MDA-MB-157 cancer cells and is present during times in the cell cycle when cyclin A is not detected (see Fig. 2), it followed that cyclin E could also replace cyclin A-containing complexes. In fact, as displayed in Fig. 3, cyclin E can function redundantly and replace cyclin A in E2F complexes with cdk2 and p107 in tumor cells. In normal cells, cyclin E was not detected in complex with the pRb-related proteins p107 and p130 and with E2F during the late G_1 and early S phase of the cell cycle. We have found that while this cyclin was a minor component of E2F DNA binding complexes in normal cells, it was a major component of this complex in MDA-MB-157 cells. It is interesting that although normal cells display a down-regulation of E2F DNA binding activity in the G_2/M phases of the cell cycle, MDA-

FIG. 3. Cyclin E is the predominant cyclin in p107/E2F complexes in tumor cells. E2F complexes were analyzed by gel retardation assays using cell lysates (15 μg) prepared from synchronized populations (see Fig. 2) of normal 76N cells (*A*) and tumor MDA-MB-157 cells (*B*). The oligonucleotide used as a labeled DNA probe includes the E2F binding site of the human dehydrofolate reductase promoter. The anti-cyclin E antibody (200 ng) was used to disrupt the E2F complexes.

MB-157 cells show constitutive E2F DNA binding complexes through the cell cycle. This raises the possibility that overexpression of cyclin E perturbed the regulation of E2F activity not only by promoting the hyperphosphorylation of pRb but also by perturbing the cell cycle regulation of E2F by p107.

Based on our observations in breast cancer cell lines and tumor tissue samples, we suggest an alternative order of events along the G_1 phase-controlling pathway culminating in phosphorylation of pRb. In this pathway, cyclin E would act upstream of pRb bypassing cyclin D/cdk4 and giving the tumor cells a selective growth advantage even in the presence of high levels of p16. Hence, abrogation of cyclin D1, cdk4/cdk6, or p16 will not have any effect on the phosphorylation of pRb, which will be accomplished by cyclin E/cdk2 in these cells leading to a deregulated progression through G_1. Our data also demonstrate that cyclin D1 is not required for G_1 progression in tumor cells that exhibit an overexpressed cyclin E and a wild-type pRb. As a result, the function of cyclin D1 is dispensable not only in cell lines in which pRb is inactivated as described (57), but also in cell lines in which cyclin E is overexpressed and constitutively active (ref. 58 and this study). Finally, this study provides evidence for a lack of functional link between p16 and pRb, suggesting that in subpopulations of breast cancers, pRb is not a major substrate for the inhibitory activity of the p16 product. Hence, certain populations of tumor cells can overcome the role of p16 as a tumor suppressor protein by providing a redundant pathway to inactivate pRb and provide a growth advantage to the cells.

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