Non-malignant and Tumor-derived Cells Differ in Their Requirement for p27<sup>Kip1</sup> in Transforming Growth Factor-β-mediated G<sub>1</sub> Arrest*  

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Transforming growth factor β (TGF-β) induces G<sub>1</sub> arrest in susceptible cells by multiple mechanisms that inhibit the G<sub>1</sub> cyclin-dependent kinases (Cdks), including Cdk2, Cdk4, and Cdk6. TGF-β treatment of early passage finite lifespan human mammary epithelial cells (HMECs) led to an accumulation of p27<sup>Kip1</sup> in cyclin E1-Cdk2 complexes and kinase inhibition. The requirement for p27 in the G<sub>1</sub> arrest by TGF-β was assessed by transfection of antisense p27 (ASp27) oligonucleotides into TGF-β-treated HMECs. Despite a reduction in total and cyclin E-Cdk2 bound p27 after ASp27 transfection, HMECs remained arrested in the G<sub>1</sub> phase. Maintenance of the G<sub>1</sub> arrest was accompanied by increased association of the Cdk inhibitor p21<sup>WAF1/Cip-1</sup> and the retinoblastoma family member p130<sup>Rb6</sup> in cyclin E1-Cdk2 complexes along with kinase inhibition. In contrast to the findings in HMECs, p27 was essential for G<sub>1</sub> arrest by TGF-β in two tumor-derived lines. ASp27 transfection into two TGF-β-responsive, cancer-derived lines was not associated with increased compensatory binding of p21 and p130 to cyclin E1-Cdk2, and these cell lines failed to maintain G<sub>1</sub> arrest despite the continued presence of TGF-β. Progressive cell cycle deregulation leading to impaired checkpoint controls during malignant tumor progression may alter the role of p27 from a redundant to an essential inhibitor of G<sub>1</sub>-to-S phase progression.

TGF-β<sub>2</sub> mediates effects on diverse cellular processes such as proliferation, growth, and differentiation via cell surface receptors that in turn regulate the activity of SMAD transcription factors (reviewed in Ref. 1). In many normal cell types, including epithelial and melanocytic cells, TGF-β has a potent antiproliferative effect. In contrast to nontransformed cells, cancer-derived lines show reduced antiproliferative responses to TGF-β or have lost this response altogether (2). In most cases, the loss of TGF-β responsiveness occurs without inactivation of TGF-β receptors or the SMADs. Cell cycle deregulation is believed to contribute to the resistance of malignant cells to G<sub>1</sub> arrest by TGF-β (reviewed in Ref. 3).

TGF-β induces cell cycle arrest in the G<sub>1</sub> phase via a number of pathways that lead ultimately to inhibition of the G<sub>1</sub> cyclin-dependent kinases (Cdks). The Cdks are key mediators of progression through the cell cycle and are regulated by phosphorylation, cyclin binding, and by the binding of Cdk inhibitory proteins (reviewed in Ref. 4). During G<sub>1</sub>-to-S phase progression, the D-type cyclins bind Cdk4 and Cdk6 and the E-type cyclins bind Cdk2, contributing to kinase activation and G<sub>1</sub>-to-S phase progression. The G<sub>1</sub> phosphorytase, Cdc25A, plays an essential role in Cdk activation by the removal of inhibitory phosphates from Cdk2 (5) and possibly also from Cdk4 and Cdk6 (6). Cdc25A may be transcriptionally up-regulated by c-Myc (7). Two families of Cdk inhibitory proteins oppose Cdk activation. p21<sup>WAF1/Cip-1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup> belong to the kinase inhibitory protein (KIP) family and contribute to the inhibition of cyclin E1-Cdk2 complexes in the G<sub>1</sub> phase. p15<sup>INK4B</sup>, p16<sup>INK4A</sup>, p18<sup>INK4C</sup>, and p19<sup>INK4D</sup> belong to the inhibitors of Cdk4 family and act to inhibit Cdk4 and Cdk6 (reviewed in Ref. 4).

In several cell types, including human mammary epithelial cells (HMECs) and mink lung epithelial cells, TGF-β induces and stabilizes the p15 protein, which leads to its binding to and inhibition of Cdk4 and Cdk6 complexes (8–10). TGF-β also causes the accumulation of p27 in cyclin E1-Cdk2 complexes leading to Cdk2 inhibition (11, 12). Changes in several essential cell cycle regulators cooperate to induce TGF-β arrest, including down-regulation of c-Myc (11, 13), Cdc25A (6), and cyclin D1 and, in some cell types, up-regulation of p21 (3). Deregulation of various cell cycle targets including cyclin and Cdk overexpression, Cdk inhibitor inactivation, and Myc or Cdc25A overexpression are believed to contribute to TGF-β resistance in cancer (3). Although mouse embryonic fibroblasts (MEFs) from p27<sup>−/−</sup> mice retain TGF-β sensitivity (14), several studies have indicated an association between altered p27 regulation and the development of TGF-β resistance. Our previous work showed that the acquisition of TGF-β resistance in human mammary epithelial cells was associated with altered phosphorylation, altered Cdk inhibitory activity, and cytoplasmic mislocalization of the p27 protein (15). Although p27 gene mutations are rare in human tumors, increased proteasomal degradation of p27 is observed in a number of cancers, including breast, colon, and prostate, and the reduced p27 levels are associated with poor patient prognosis (reviewed in Refs. 16 and 17). Relatively little is known about the compensatory mechanisms invoked by a nontransformed cell after a reduction in p27 protein levels,
although a few reports support a role for compensation by other Cdk inhibitors to maintain normal cell cycle control. For example, in serum-starved p27−/− mouse embryonic fibroblasts (MEFs), the accumulation of the retinoblastoma family member p130 (20) in cyclin E-Cdk2 complexes compensated for p27 loss and enabled cells to undergo proliferative arrest in the G1 phase (18).

The present study investigated the requirement for p27Kip1 in maintaining G1 arrest by TGF-β in finite lifespan and immortalized HMECs and in cancer-derived lines. Using antisense p27 oligonucleotides to inhibit p27 expression, we show that HMECs, but not the tumor cell lines, maintain G1 arrest after p27 down-regulation via a compensatory accumulation of p21 and p130 in cyclin E-Cdk2 complexes. These data suggest that p27 is required to maintain TGF-β arrest in these malignant lines but has a redundant function in the finite lifespan HMEC that can be compensated for by other Cdk inhibitory mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture—The derivation and culture of normal finite lifespan human mammary epithelial cells from reduction mammaplasty has been described previously (10, 19). The WM35 human melanoma line was derived from a radial growth phase melanoma and was kindly provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (Hyclone Laboratories) (20). MCF-10A cells are spontaneously immortalized human mammary epithelial cells derived from a patient with benign breast disease as described previously (21). MCF-10A cells were kindly provided by Dr. F.-F. Liu (Ontario Cancer Institute) and cultured in MCDB 170 medium with additives as described previously (6). MCF-7 cells (22) were grown in Iscove’s modified essential medium–option Zn2+ supplemented with insulin and 5% fetal calf serum. HMECs and WM35 cells were treated with 10 ng/ml TGF-β; MCF-10A and MCF-7 cells were treated with 100 ng/ml TGF-β purchased from R & D Systems (Minneapolis, MN).

Flow Cytometric Analysis—Cells were pulse-labeled with 10 μM bromodeoxyuridine for 2 h and then fixed, stained with anti-bromodeoxyuridine-conjugated fluorescein isothiocyanate (BD Biosciences) and counterstained with propidium iodide as described previously (23). Cell cycle analysis was carried out on a BD Biosciences FACScan and Cell Quest Software.

Immunoblotting—Cell lysis and immunoblotting were performed as described previously (10). Equal protein loading was verified by blotting for β-actin. To assay cyclin E1 complexes, cyclin E1 was immunoprecipitated from 800 μg of protein lysate with monoclonal anti-cyclin E mAb172. To assay Cdk6 immunoprecipitates, Cdk6 was immunoprecipitated from 300 μg of protein lysate. Immunoprecipitates were resolved, transferred, and blotted with the appropriate antibody for detection of associated proteins. Antibody alone controls were run along side immunoprecipitates. The data presented are representative of at least three repeat assays.

Antibodies—Monoclonal antibodies to p27 and p130 were from BD Transduction Laboratories. Antibodies to p21, Cdk2, Cdk6, Cdc25A, and β-Myc were obtained from Santa Cruz Biotechnology; to cyclin D1 (DCS-6) from Neomarkers; to PSTAIRE from S. Reed (The Scripps Research Institute, La Jolla, CA); to cyclin E (mAbs E12 and E172) and to β-actin from Sigma.

Antisense Oligonucleotide Transfection—184HMEC, MCF-10A, WM35, or MCF-7 cells were treated with TGF-β for 24 h followed by transfection of antisense or missense p27 oligonucleotide transfection using 2.5 μg/ml cofactor G3815 (Gilead Scientific, Foster City, CA) for 6 h as described previously (24) in the presence of TGF-β followed by replacement with fresh media containing TGF-β. Flow cytometry and protein analysis was performed immediately after transfection and at 24 h thereafter. For 184 and MCF-10A cells, neither varying the oligonucleotide concentration from 5 nM to 1, 10, 25, 50, or 120 nM nor varying the transfection time from 6 h to 1, 3, 4, 10, or 24 h abrogated the G1 arrest by TGF-β.

Cyclin-dependent Kinase Assays—Cyclin E1 was immunoprecipitated and reacted with [γ32P]ATP and histone H1 as described previously (12, 25). Radioactivity incorporated in the histone H1 substrate was quantitated using an Amersham Biosciences PhosphoImager and ImageQuant software. Radioactivity incorporated in control nonspecific mouse polyclonal IgG immunoprecipitates was subtracted from test kinase values.

RESULTS

TGF-β Effects on Cell Cycle Profiles—We compared the TGF-β responsiveness of human mammary epithelial cells (184 HMEC, passage 11), WM35, MCF-10A, and MCF-7 cells (Fig. 1A). 184 HMEC are a finite lifespan mammary epithelial strain, MCF-10A is a spontaneously immortalized non-malignant breast epithelial cell line, MCF-7 is a malignant breast cancer line, and WM35 is a malignant melanoma cell line. Cells were treated for 48 h in the absence (–) or presence (+) of TGF-β (Fig. 1A). 184 and WM35 cells had similar sensitivity to TGF-β, undergoing G1 arrest with an ~80% reduction in the proportion of cells in S phase after 48 h of TGF-β treatment (10 ng/ml). The MCF-10A and MCF-7 cell lines were less sensitive than the 184 HMEC or WM35, but both underwent partial G1 arrest with 100 ng/ml TGF-β with more than 50% reduction in the proportion of cells in S phase.
**TGF-β Effects on Cyclin and Cdk Inhibitor Levels**—The levels of the relevant G1 cyclins, Cdkks, and Cdk inhibitors were assayed by Western analysis in 184, MCF-7, WM35, and MCF-10A cells in the absence (–) or after a 48-h exposure (+) to TGF-β (Fig. 1B). Cyclin D1 levels were similar in asynchronously proliferating 184, MCF-7, and WM35 cells and slightly lower in MCF-10A cells. Cyclin E1 levels were −2.5-fold greater in the untreated cancer-derived MCF-7 and WM35 cells compared with the 184 and MCF-10A HMEC. Cyclin E1 and cyclin D1 levels showed no consistent alteration by TGF-β in repeat assays in 184, MCF-7, and WM35 cells. In MCF-10A, however, TGF-β decreased cyclin D1 and cyclin E1 levels by up to 4-fold. The levels of p27 were higher in the cancer-derived lines, with asynchronously proliferating MCF-7 and WM35 cells having p27 levels approximately 15- and 3-fold greater, respectively, than asynchronously proliferating 184 HMEC and MCF-10A cells. TGF-β treatment did not alter p27 protein levels in the HMEC, but p27 levels rose by −1.5-fold in the MCF-7 line, 3-fold in WM35, and 5-fold in MCF-10A. Total p21 levels were similar in 184, MCF-7, and WM35, with reduced levels in MCF-10A. p21 levels were unchanged in the 184 and MCF-7 cells after 48 h of TGF-β treatment. The WM35 cells showed a transient increase in p21 levels at 18–30 h of TGF-β treatment followed by a return to similar levels as in the asynchronous population by 48 h. MCF-10A cells showed a modest decrease in p21 levels at the 48-h time point. p15 levels were much higher in the 184 HMEC compared with the MCF-7 line; WM35 and MCF-10A are p15-null (6, 20). TGF-β treatment of 184 HMEC led to a 3-fold increase in p15 levels. In MCF-7 cells, p15 could not be detected in the short exposure times (3–5 min) that were used to detect p15 from the 184 HMEC. However, a longer exposure of the film (1 h) showed that p15 levels increased by 1.5–2-fold in TGF-β-treated MCF-7 cells (Fig. 1C).

Given that the p130Rho protein, like p21WAF1/Cip-1 and p27Kip1, has a Cdk inhibitory domain and can also accumulate in and inhibit Cdk complexes (18), p130 protein levels were assayed in asynchronously proliferating and TGF-β-treated cells by Western analysis. p130 levels were much lower in HMEC compared with the cancer-derived lines (Fig. 1B). TGF-β modestly increased p130 levels (less than 1.5-fold) in 184 HMEC and also increased p130 in MCF-10A. p130 levels were not affected by TGF-β in the tumor-derived lines. Equal loading was verified by β-actin.

**TGF-β Effects on Cyclin-Cdk Composition and Activities**—The levels of p21, p27, p15, and Cdkks in cyclin E1 complexes were assayed after immunoprecipitation of equivalent levels of cyclin E1 from asynchronously proliferating TGF-β-treated 184, MCF-7, WM35, and MCF-10A cells (Fig. 2A). Cyclin E1-bound Cdk2 levels were similar and were not altered by TGF-β in repeat assays in all four cell types. Cyclin E1-bound p21 levels were unaltered in 184 HMEC after TGF-β treatment, but TGF-β treatment of MCF-7, WM35, and MCF-10A cells led to a modest increase (1.5–2-fold) in p21 binding to cyclin E1. p27 increased in cyclin E1 complexes in all four cell types after TGF-β treatment (Fig. 2B). Paradoxically, asynchronously proliferating cancer-derived lines showed a greater amount of cyclin E1-bound p27 than did HMECs. Cyclin E1-bound p27 levels were −8–15 times higher in proliferating MCF-7 and WM35 than in 184 HMEC. Cyclin E1-bound p21 was also 2-fold higher in MCF-7, WM35, and MCF-10A lines than in 184 HMEC. p130 was detected in cyclin E1 complexes in both asynchronously proliferating and TGF-β-treated cells (Fig. 2A).

Although the total p130 levels were much lower in 184 HMEC and MCF-10A cells compared with the cancer-derived lines (see again Fig. 1B), the levels of cyclin E1-bound p130 were −5–10-fold higher in 184 HMEC than in the cancer-derived lines. p130 binding to cyclin E1-Cdk2 was only modestly increased by TGF-β in 184, MCF-7, and WM35. TGF-β treatment of MCF-10A led to a 3-fold increase in cyclin E1-bound p130.

The histone H1 kinase activity of cyclin E1-complexes shown in Fig. 2A was assayed as described under “Experimental Procedures” (Fig. 2B). Although equal amounts of cyclin E1 were precipitated, cyclin E1-associated kinase activities in MCF-7, WM35, and MCF-10A were 4–5 times greater than that in the asynchronously proliferating 184 HMEC. On repeat assays, TGF-β treatment of the 184 HMEC and MCF-10A led to a nearly complete (−95%) inhibition of cyclin E1-associated kinase activity. WM35 cells also showed 90% reduction in kinase activity associated with the G1 arrest. Cyclin E1-associated kinase activity in the MCF-7 cells was reduced by TGF-β by −60%. Representative histone H1 kinase autoradiography is shown in the inset.

**Loss of p15 Up-regulation by TGF-β in MCF-7 and WM35**—We assayed levels of p15 and cyclin D1 present in Cdk6 complexes from asynchronously proliferating and TGF-β-treated cells (Fig. 2C). Despite similar total cyclin D1 and Cdk6 in 184, MCF-7, and WM35 cells, more cyclin D1 was bound to Cdk6 in asynchronously proliferating MCF-7 and WM35 than in 184 HMEC (see Figs. 1B and 2C). Cdk6-bound cyclin D1 levels in MCF-10A were intermediate between that observed in 184 and MCF-7 or WM35. TGF-β caused a 2–3-fold reduction in the levels of Cdk6-bound cyclin D1 in 184 cells, whereas Cdk6-bound cyclin D1 association was not notably reduced by TGF-β in the MCF-7, WM35, and MCF-10A cells. Cdk6-bound p15 was significantly higher in asynchronously proliferating 184 than MCF-7 cells. The level of p15 bound to Cdk6 increased −5-fold after TGF-β arrest of the 184 HMEC. In contrast, p15 levels were significantly reduced and Cdk6-bound p15 levels did not increase after TGF-β treatment of MCF-7, even on longer exposure of the Cdk6-associated p15 blot (Fig. 2D). WM35 and MCF-10A cells lack p15 because of a biallelic loss of the p15 gene (6, 20).

**Increased c-Myc and Cdc25A Levels in Cancer-derived Lines**—The WM35 and MCF-7 cancer-derived lines in our study showed a number of differences in the regulation of p27, cyclin D1, cyclin E1, and p15 compared with the 184 HMEC. The cyclin E1-associated kinase activities were increased despite the presence of increased cyclin E1-Cdk2 bound p27 in these complexes in both asynchronously proliferating and TGF-β-treated MCF-7 and WM35 lines. We observed increased cyclin D1 bound to Cdk6 complexes and a failure to accumulate p15 in Cdk6 complexes after TGF-β treatment of the cancer-derived lines. These observations prompted us to assay the levels of c-Myc, because c-Myc has been shown to interfere with p27 function at many levels and to repress p15 induction (26–29). c-Myc may also transactivate the Cdc25A gene (7), whose product is an important activator of Cdk2 (7) and whose down-regulation plays an important role in G1 arrest by TGF-β (6).

The levels of c-Myc and Cdc25A proteins were assayed in the cancer-derived MCF-7 and WM35 cell lines and in the 184 HMEC (Fig. 2E). c-Myc levels were 5–10-fold greater in the asynchronously proliferating cancer-derived MCF-7 and WM35 lines compared with 184 HMEC; Cdc25A levels were approximately 15–20-fold higher. c-Myc and Cdc25A levels in MCF-10A were intermediate between those in 184 and in the two cancer-derived lines. After TGF-β treatment, Cdc25A levels were reduced −5-fold in 184, MCF-7, and MCF-10A, and by −2-fold in WM35. Although Cdc25A levels were reduced by TGF-β in all four cell types, the residual amount of Cdc25A protein present in TGF-β-treated cells differed importantly. c-Myc and Cdc25A levels remaining in the TGF-β-treated can-
cer lines were 5–10-fold higher than in the TGF-β-treated HMEC.

ASp27 Activates Cyclin E1-Cdk2 and Abrogates TGF-β-induced G1 Arrest in Tumor-derived Lines but Not in Nonmalignant Lines—p27 was discovered as a mediator of cyclin E-Cdk2 inhibition and G1 arrest by TGF-β (11, 12). However, in different cell types, other changes in G1 regulators seem to contribute to TGF-β-mediated arrest (reviewed in Ref. 3). To specifically address the requirement for p27 in TGF-β-mediated G1 arrest, we tested whether the antisense-mediated inhibition of p27 expression would abrogate TGF-β-arrested 184 HMEC, WM35, MCF-7, and MCF-10A cells. Cells were treated with TGF-β for 36 h followed by a 6-h transfection with antisense-p27, mismatch oligonucleotides, or lipid alone as controls. Fresh media containing TGF-β was added back after transfection. Cell cycle and protein analysis were performed immediately after the transfection and at 24 h. p27 protein levels were reduced by 3–5-fold after ASp27 transfection and levels remained low 24 h after transfection (Fig. 3A). p27 levels in the control and mismatch oligonucleotide-transfected groups were similar. The transfection did not alter protein levels of other G1 regulators examined including p21, p130, cyclin E1, cyclin D1, Cdk2, and Cdk6 (data not shown). ASp27 caused TGF-β-arrested MCF-7 and WM35 to re-enter the cell cycle but had no such effect on arrested 184 HMEC or MCF-10A cells. Flow cytometric analysis at 24 h showed that ASp27 transfection led to a decrease in the proportion of cells in G1 and an increase in the proportion in S phase in the tumor-derived WM35 and MCF-7 lines (Fig. 3B). Approximately 25–30% of these cells were in S phase 24 h after transfection compared with only 9–15% for the lipid and mismatch controls. Equal amounts of cyclin E1 were immunoprecipitated from ASp27-treated and from mismatch p27 or lipid controls, and histone H1 kinase activities were assayed. Reactivation of cyclin E1-dependent kinase accompanied cell cycle re-entry after antisense-mediated inhibition of p27 expression in TGF-β-treated MCF-7 and WM35 cells (shown for WM35 in Fig. 3C).

In contrast, the cell cycle profiles of the finite lifespan 184 HMEC and the immortalized MCF-10A line were not altered by ASp27 transfection. Repeat assays showed that the cyclin E1-associated kinase remained inhibited in G1 arrested ASp27-transfected 184 HMEC and MCF-10A, as it did in TGF-β-treated lipid and mismatch controls (Fig. 3C, data shown for 184 HMEC). Thus, p27 is required to maintain G1 arrest by TGF-β in these tumor-derived lines but not in finite lifespan or immortal, nonmalignant mammary epithelial cells.
The failure of antisense p27-transfected 184 HMEC and MCF-10A cells to re-enter the cell cycle was not caused by toxicity, because replacement of the TGF-β-containing media with complete media (no TGF-β) led to cell cycle re-entry (data not shown). Because early passage and late passage HMEC differ in their responsiveness to TGF-β (30), we repeated the ASp27 transfection experiments with mid-passage (passage 15) and late passage (passage 20) 184 HMEC. Regardless of passage, 184 HMEC maintained G1 arrest in the presence of TGF-β. The failure of the malignant tumor-derived lines to maintain G1 arrest after ASp27 was unlikely to have been caused by differences in the intrinsic TGF-β sensitivity of the cell types. The finite lifespan 184 HMEC and malignant WM35 lines had similar TGF-β sensitivity, as did the nonmalignant MCF-10A and malignant MCF-7 cell lines, yet only 184 and MCF-10A cells maintained arrest by TGF-β after p27 down-regulation.

**Increased Association of p21\textsuperscript{WAF-1/Cip1} and p130\textsuperscript{Rb2} Contributed to Cyclin E1-Cdk2 Inhibition in ASp27-treated HMEC but Not in Cancer-derived Lines—**To investigate mechanisms contributing to maintenance of TGF-β arrest in 184 HMEC and immortal MCF-10A, despite the antisense-mediated decrease in p27 expression, we assayed the levels of p21, p27, and p130 bound to cyclin E1 in ASp27-transfected, TGF-β-treated cells. In all cell types, ASp27 treatment significantly reduced the levels of p21 in cyclin E1 complexes (shown for 184 HMEC and WM35 in Fig. 3D). In the 184 HMEC, the reduction in cyclin E1-bound p21 was associated with a ~3-5-fold increase in cyclin E1-bound p21 and a ~5-10-fold increase in the level of p130 in the cyclin E1 complexes (Fig. 3D). TGF-β-treated MCF-10A also showed increased cyclin E1-bound p130 and p21 after antisense-mediated loss of p27 and maintained the G1 arrest (not shown). In the cancer-derived MCF-7 and WM35 lines, there was no increased p130 association with cyclin E1 complexes; cyclin E1-bound p21 decreased in ASp27-treated cells compared with the lipid and mismatch cells and ASp27 led to cell cycle re-entry (shown for WM35, Fig. 3D).

**DISCUSSION**

Loss of sensitivity to the growth inhibitory effect of TGF-β is common in human tumor-derived cell lines and is thought to contribute to malignant tumor progression (31). Although increased p27 proteolysis and TGF-β resistance have both been shown to occur early in tumorigenesis, previous work has not provided a causal link between p27 deregulation and loss of G1 arrest by TGF-β during oncogenic progression. With the exception of the increased size of p27-null mice compared with wild-type mice, the relative absence of alterations in development, differentiation, and cell cycle control in p27-null mice suggests that compensation by other cell cycle regulators may occur in the absence of p27 (11, 14, 32, 33). Indeed, mouse embryonic fibroblasts (MEFs) obtained from p27-null mice retain sensitivity to many growth inhibitory stimuli, including TGF-β (14). The present study demonstrates that p27 is an essential mediator of G1 arrest by TGF-β in two malignant lines, the MCF-7 breast cancer cell line and the WM35 melanoma line. Antisense-mediated inhibition of p27 expression led to cyclin E1-Cdk2 reactivation and cell cycle re-entry. However, p27 was not essential for G1 arrest by TGF-β in two non-tumor-derived cell types, the finite lifespan 184 HMEC and the immortalized MCF-10A line. In these cells, a compensatory increase in binding of p21\textsuperscript{WAF-1/Cip1} and p130\textsuperscript{Rb2} to cyclin E1-Cdk2 complexes seems to contribute to maintenance of the G1 arrest. A number of studies using oncogene-transformed or cancer-derived cell lines support the notion that p27 loss or deregulation is associated with impaired TGF-β arrest response. Overexpression of the Bcr-Abl kinase in human M07 cells and murine Ba/F3 cells led to the proteosomal degradation of p27.
which was associated with TGF-β resistance (34). Oncogenic ras activation led to cytoplasmic mislocalization of p27 and to TGF-β resistance in epithelial cell lines (35). In the WM35 and 184HMEC used in this study, we reported recently that overexpression of activated protein kinase B impairs TGF-β responsiveness, at least in part through protein kinase B-mediated phosphorylation of p27, leading to its cytoplasmic mislocalization (36). Furthermore, E1A overexpression in mink lung epithelial cells caused TGF-β resistance, and these cells failed to accumulate p27 in cyclin E1-Cdk2 complexes in response to TGF-β (37). In the present study, we observed differences in p27 regulation in the cancer-derived lines compared with two human mammary epithelial cell types, 184 HMEC and MCF-10A. Asynchronously proliferating cancer-derived lines, especially MCF-7, had a paradoxically high amount of p27 present in cyclin E1-cdk2 complexes. In addition, cyclin E1-Cdk2 complexes from both cancer-derived lines had higher kinase activities than asynchronous 184 HMEC, despite more cyclin E1-bound p27 and p21. These data suggest that the Cdk inhibitory activity of the KIPs may be impaired in MCF-7 and WM35 cells. In the context of functional KIP deregulation, even a modest loss of p27 via antisense might have a critical effect because compensatory action by p21 may be impaired.

The p130 protein may play an important compensatory role in maintenance of checkpoints after p27 loss in several cell types, including epithelial cells, as we report here, and fibroblasts. In p27−/− MEFs, the accumulation of p130 in cyclin E-Cdk2 complexes compensated for the absence of p27 and contributed to Cdk2 inhibition and G1 arrest after either pharmacologic phosphatidylinositol 3-kinase inhibition or serum starvation (18, 38). Other studies support a role for p130 in the proliferative arrest by TGF-β. Herzinger et al. (39) showed an accumulation of p130 in E2F complexes and repression of E2F regulated genes during TGF-β arrest of human keratinocytes. We detected p130 in cyclin E1-Cdk2 complexes from both asynchronously proliferating and TGF-β-treated cells. In all cells assayed, TGF-β treatment induced a modest yet similar increase in the levels of cyclin E1-bound p130. Surprisingly, despite 15–20-fold higher p130 protein levels in the two malignant tumor-derived lines than in the nonmalignant cells, the levels of p130 bound to cyclin E1-cdk2 were 5–10-fold less in these cancer-derived lines than in the HMEC. Thus, mechanisms that regulate p130 binding to cyclin E1-Cdk2 complexes may differ between the HMEC and cancer-derived lines.

p130 deregulation has been observed, and may have independent prognostic value, in several types of human cancers (40–42). Altered p130 regulation has been reported in the context of altered p27 regulation and may contribute to loss of responses to antiproliferative stimuli. For example, the viral E1A protein can bind and inactivate both p27 and p130, and E1A overexpression leads to TGF-β resistance (37). In addition, p27−/− lymphocytes, which express lower p130 levels than p27−/− MEFs, fail to commit to G1 arrest after serum starvation (18). Thus, deregulation of both p130 and p27 potentially lead to a loss of normal proliferative control during tumor progression. Future studies may elucidate whether p130 deregulation further stratifies for poor patient outcome among patients whose tumors show reduced p27.

Our data support the notion that deregulation of multiple G1 cell cycle regulators may be required before cells lose responsiveness to antiproliferative effects of TGF-β. Deregulation of several G1 regulators may ultimately be required before p27 becomes essential for G1 arrest by TGF-β. p15 has been shown to cooperate with p27 in G1 arrest by TGF-β (8). p15 is not required for G1 arrest by TGF-β because both MCF-10A (6) and WM35 (20) retain TGF-β responsiveness despite a lack of p15 expression. Moreover, p15 loss per se does not make cells dependent on p27 for TGF-β-mediated G1 arrest. Although both MCF-10A and WM35 are p15-deficient, ASp27 abrogated TGF-β arrest in only the WM35 cells and not the MCF-10A cells. Thus, cell cycle inhibitory pathways activated by TGF-β in p15-deficient MCF-10A that compensate for ASp27-mediated loss do not seem to be functional in WM35. One of these may be the compensatory increase in p130 binding to Cdk2. The disruption of both p130 and p15 regulation in cancers may alter the role of p27 from a redundant to essential mediator of G1 arrest by TGF-β.

c-Myc plays an important role in the regulation of many G1 cell cycle proteins, including cyclin E1, p27, p21, p15, and Cdc25A. Moreover, c-Myc overexpression causes TGF-β resistance (26). In MCF-7, the elevated c-Myc levels may contribute to the reduced induction of p15 by TGF-β. Increased c-Myc may also be linked to the impaired anti-proliferative role of p130 (43, 44) and could contribute to the increased expression of Cdc25A (7) in MCF-7 and WM35.

The increased Cdc25A levels in WM35 and MCF-7 may contribute to the increased cyclin E1-Cdk2 activities observed in these lines. Cdc25A down-regulation contributes to G1 arrest by TGF-β (6). Although TGF-β reduced Cdc25A levels in all of the cell types, MCF-7 and WM35 had significantly higher residual Cdc25A levels remaining after 48 h of TGF-β treatment than did 184 HMEC and MCF-10A. The higher residual levels of the Cdk2 activator Cdc25A in the TGF-β arrested cancer cells may make them more susceptible to cyclin E1-Cdk2 activation after antisense-mediated p27 loss. Cangi et al. (45) have shown increased mortality in breast cancer patients whose tumors expressed both elevated Cdc25A and low p27. In addition, there was a positive correlation between Cdk2 activity and Cdc25A expression in the breast cancers studied. Increased Cdc25A expression and activity would oppose the cyclin E1-Cdk2 inhibitory function of p27. In cancers with Cdc25A overexpression, maintenance of p27 expression and function may become critical for continued responsiveness to such antiproliferative stimuli as TGF-β.

In summary, our data support the notion that a reduction in p27 levels may contribute significantly to the loss of normal responsiveness to growth inhibitory stimuli during cancer progression. Importantly, the reduction in p27 levels alone may be insufficient to disrupt cell cycle arrest responses when other cell cycle inhibitory mechanisms are functional. Our antisense experiments suggest that normal mammary epithelial cells maintain their antiproliferative responses at least in part through activation of the cdk inhibitory function of p21 and p130 when p27 levels are reduced. Loss of these and other normal checkpoint controls during malignant progression may make p27 essential for G1 arrest by TGF-β.

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