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Transforming Growth Factor β Stabilizes p15^{INK4B} Protein, Increases p15^{INK4B}-cdk4 Complexes, and Inhibits Cyclin D1-cdk4 Association in Human Mammary Epithelial Cells

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The effects of transforming growth factor β (TGF-β) were studied in closely related human mammary epithelial cells (HMEC), both finite-life-span 184 cells and immortal derivatives, 184A1^{S}, and 184A1L5^{R}, which differ in their cell cycle responses to TGF-β but express type I and type II TGF-β receptors and retain TGF-β induction of extracellular matrix. The arrest-resistant phenotype was not due to loss of cyclin-dependent kinase (cdk) inhibitors. TGF-β was shown to regulate p15^{INK4B} expression at at least two levels: mRNA accumulation and protein stability. In TGF-β-arrested HMEC, there was not only an increase in p15 mRNA but also a major increase in p15^{INK4B} protein stability. As cdk4- and cdk6-associated p15^{INK4B} increased during TGF-β arrest of sensitive cells, there was a loss of cyclin D1, p21^{Cip1}, and p27^{Kip1} from these kinase complexes, and cyclin E-cdk2-associated p27^{Kip1} increased. In HMEC, p15^{INK4B} complexes did not contain detectable cyclin. p15^{INK4B} from both sensitive and resistant cells could displace in vitro cyclin D1, p21^{Cip1}, and p27^{Kip1} from cdk4 isolated from sensitive cells. Cyclin D1 could not be displaced from cdk4 in the resistant 184A1L5^{R} cell lysates. Thus, in TGF-β arrest, p15^{INK4B} may displace already associated cyclin D1 from cdks and prevent new cyclin D1-cdk complexes from forming. Furthermore, p27^{Kip1} binding shifts from cdk4 to cyclin E-cdk2 during TGF-β-mediated arrest. The importance of posttranslational regulation of p15^{INK4B} by TGF-β is underlined by the observation that in TGF-β-resistant 184A1L5^{R}, although the p15 transcript increased, p15^{INK4B} protein was not stabilized and did not accumulate, and cyclin D1-cdk association and kinase activation were not inhibited.

Cell cycle transitions are governed by a family of cyclin-dependent kinases (cdks), whose activity is regulated by association with positive effectors, the cyclins (41, 61), with negative regulators, the cdk inhibitors (62), and by phosphorylation (45, 66). The cdks control a series of biochemical pathways, or checkpoints, which integrate extracellular mitogenic and growth-inhibitory signals, monitor chromosome integrity, and coordinate cell cycle transitions (23, 42). Passage through G_1 into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. D-type cdks play a major role in phosphorylation of the retinoblastoma protein (pRb) (61), which is required for G_1-to-S phase transition.

Two different families of cdk-inhibitory proteins have been identified (for reviews see references 58 and 62). The kinase inhibitor protein (KIP) family is composed of three members, p21 (also identified as Cip1, Waf1, and Sdi1), p27 (Kip1), and p57 (Kip2). p21 (21, 73) is a transcriptional target of p53 (9) and plays a role in senescence (44) and differentiation (17, 49, 64) and in the coordination of DNA damage repair with cell cycle arrest (6, 13, 34, 52, 72). p27^{Kip1} was identified as an inhibitory activity in cells arrested by TGF-β (see below) (31, 55, 56, 65) and was cloned independently in a yeast two-hybrid screen (71). The third member of this family, p57^{Kip2}, has a more tissue-specific distribution. It shares the highly conserved cdk inhibition domain seen in both p21 and p27 (33, 39). Members of the KIP family of cdk inhibitors bind more efficiently to cyclin/cdk complexes than to either cyclin or cdk alone (18, 22, 57, 71) and can inhibit a wide range of cyclin-cdks in vitro.

Members of the INK4 family of cdk inhibitors binds specifically and inhibit cdk4 and cdk6 activities (3, 16, 20, 26, 60). The first of the INK4 family genes to be cloned, the p16^{INK4A} (60) gene, or multiple-tumor suppressor 1 (28), is mutated in many human tumors and cell lines. The p15^{INK4B} gene (also known as multiple-tumor suppressor 2), located adjacent to the p16^{INK4A} gene (20), may also be inactivated in human tumors (46, 47). Other members of the INK4 family include p18, p19, and p20 (3, 16, 26).

TGF-β is a potent growth inhibitor of normal epithelial cells (38) and inhibits mammary duct development in vivo (53, 63). TGF-β mediates G_1 arrest through a number of mechanisms. Inhibition of pRb phosphorylation (32) by TGF-β involves downregulation of cyclin D1 and cdk4 proteins in certain cell types (11, 12, 30) and induction of p15^{INK4B}, which inhibits cdk4 and cdk6 (20). Reduction of cyclin A and cyclin E mRNA levels and of cyclin A protein (15, 65) contributes to inhibition of cyclin E- and A-dependent kinases (31, 56, 65). Furthermore, G_1 cdks are inhibited by a heat-stable cdk inhibitor, p27^{Kip1} (31, 56, 65). p27^{Kip1} activity is increased in TGF-β-arrested cells and in contact-inhibited cells (24, 31, 55, 56, 65). In cells progressing from G_0 to S phase, this inhibitor activity is highest in G_0 and decreases as cells enter the G_1-to-S phase transition (65).

To further examine the effects of TGF-β on cell cycle regulators in human mammary epithelial cells (HMEC) we have used a series of mammary epithelial cell cultures, including

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nite-life-span HMEC from specimen 184 and immortal derivatives of 184, 184A1^S, and 184A1L5^R that differ in their growth response to TGF-β. Both growth-sensitive and growth-resistant HMEC lines express similar amounts of TGF-β receptors I and II, and all of the cells, including the parental 184, respond to TGF-β by strong induction of extracellular matrix proteins (70). Thus, this system allows the study of TGF-β-induced G_1 arrest in closely related cells which differ in their cell cycle response to TGF-β but retain other TGF-β responses such as the production of extracellular matrix.

In epithelial cells, TGF-β causes upregulation of p15^{INK4B} mRNA and increased binding of a 15-kDa protein to cdk6 (20, 59). This is accompanied by a reduction in cdk4-associated p27^{Kip1} and increased association of p27^{Kip1} with cyclin E-cdk2 complexes (59). The present study extends these observations in the HMEC model. We find that in vivo, p15^{INK4B} complexes do not contain detectable cyclin and p15^{INK4B} can displace cyclin D1 and KIP proteins p21^{Cip1} and p27^{Kip1} from cdk4 complexes in vitro. In sensitive HMEC, the TGF-β-mediated increase in p15^{INK4B} protein not only is due to increased p15 mRNA but also results from a major increase in p15^{INK4B} protein stability. As cdk4- and cdk6-associated p15^{INK4B} increased, there was a loss of cyclin D1, p21^{Cip1}, and p27^{Kip1} from these kinase complexes, and cyclin E-cdk2-associated p27^{Kip1} increased. These data are consistent with a shift in the equilibrium of binding of p27^{Kip1} from cdk4 to cyclin E-cdk2 during TGF-β mediated arrest. The functional significance of these effects in TGF-β-mediated G_1 arrest is supported by our comparison of TGF-β-sensitive and -resistant HMEC lines. The importance of posttranslational regulation of p15^{INK4B} by TGF-β is demonstrated by the finding that in the TGF-β-resistant HMEC line, 184A1L5^R, although the p15 transcript increases, p15^{INK4B} protein is not stabilized and does not accumulate and cyclin D1-cdk association and kinase activation are not inhibited. This study shows for the first time that increased p15 stability is associated with TGF-β-mediated arrest.

MATERIALS AND METHODS

Cell culture. Normal finite-life-span HMEC were obtained from reduction mammoplasty tissues and can undergo 45 to 80 population doublings in serum-free MCDB 170 medium (19, 67). Cells in these experiments were derived from specimen 184. Normal epithelial cells from specimen 184 were transformed to immortality following exposure to the chemical carcinogen benzo[*a*]pyrene, resulting in the established cell lines 184A1 and 184B5 (68). All normal HMEC examined are growth inhibited by TGF-β (27). The immortal lines differ from the parental 184 cells in their response to TGF-β. For clarity, superscript S and R are used to indicate sensitive and resistant phenotypes, respectively. 184B5T1^S, a subclone of early-passage 184B5, is extremely sensitive to growth inhibition by TGF-β. Early-passage 184A1^S cells are sensitive to growth inhibition by TGF-β. The 184A1^S cells used in the present experiments were from passages 25 to 28. With continued passage, 184A1 gradually acquires resistance to growth inhibition by TGF-β. Fully TGF-β-resistant cells can be readily isolated from late-passage 184A1 to give stable growth-resistant sublines such as 184A1L5^R (70). The resistant subpopulation in 184A1^S does not represent a distinct genetic variant since all of four separate single cell clones of 184A1^S examined, exhibited, when they were expanded, the presence of a TGF-β-resistant subpopulation. Thus, the switch from sensitivity to resistance occurs at a population frequency that defies explanation on the basis of a single mutation. Hence mutation of the cdks, cyclins, or cdk inhibitors cannot underlie the phenotypic change observed (conversion from TGF-β growth inhibition to growth resistance).

The immortally transformed cell lines 184B5T1^S, 184A1L5^R, and 184A1^S express mammary cytokeratins, do not display anchorage-independent growth, and are not tumorigenic in nude mice (68). HMEC were grown in MCDB 170 medium (Clonetics Corporation, San Diego, Calif.) (67). Human recombinant TGF-β1 was a gift from Genentech Inc. (San Francisco, Calif.).

A rapid, fully reversible G_0-like growth arrest was achieved in these HMEC through blockage of epidermal growth factor (EGF) receptor signal transduction (removal of EGF from the culture medium and the addition of a blocking antibody to the EGF receptor, monoclonal antibody [MAb] 225, as previously described) (69). Reexposure to EGF leads to a highly synchronous entry into the cell cycle (69), with minor variations in the G_0-to-S duration from one experiment to another.

Determination of labeling index. For autoradiographic analysis, cells were exposed to 2-h pulses of 10 μCi of [^{3}H]thymidine per ml in MCDB 170 medium containing 3×10^{-7} M unlabeled thymidine. Cells were then fixed with methanol and processed for autoradiography, and the percent labeled nuclei was determined as described (65).

Flow cytometric analysis. Cells were pulse-labeled with 10 μM bromodeoxyuridine for 2 h in G_0 and at intervals after release from quiescence with or without the addition of TGF-β or after the addition of TGF-β to asynchronously growing cells. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 95°C to expose the labeled DNA. Cells were then stained with anti-bromodeoxyuridine-conjugated fluorescein isothiocyanate (Becton Dickinson) and counterstained with propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan with Lysis II software.

Antibodies. The anti-EGF receptor monoclonal, MAb 225, was provided by Steve Wiley (University of Utah Medical Center, Salt Lake City, Utah). Antibodies to the pRb, cdk2, and cyclins A, D1, and D2 were obtained from Pharmingen or Santa Cruz Biotechnology, and those to cyclin E (MAbs E12 and E172) were from E. Lees and E. Harlow (Massachusetts General Hospital, Boston). Monoclonal PSTAIRE antibody was a gift from S. Reed (The Scripps Research Institute, La Jolla, Calif.), and the cyclin D1 antibody, DCS-11, was a gift from J. Bartek (Danish Cancer Society, Copenhagen, Denmark). Cyclin A MAb E67 was provided by J. Gannon and T. Hunt (Imperial Cancer Research Fund, London, England). p27 serum was provided by H. Toyoshima and T. Hunter (Salk Institute, San Diego, Calif.), and monoclonal p27 antibody was purchased from Transduction Labs. cdk4, cdk6, p21, and p15-16 polyclonal sera were provided by G. Hannon and D. Beach (Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.). A MAb, JC-6, which recognizes the third ankyrin repeat of human p16 (10) and cross-reacts with human p15 was used for immunoblotting and immunoprecipitation of p15 in these studies.

Immunoblotting. Cells were lysed in ice-cold Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.02 mg each of aprotinin, leupeptin, and pepstatin per ml). Lysates were sonicated and clarified by centrifugation. Protein was quantitated by Bradford analysis, and 50 to 100 μg of protein/lane was resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer and blotting were as described previously (7). For detection of cdk4-associated proteins by immunoprecipitation-Western analysis, cdk4 was immunoprecipitated from 200 to 500 μg of protein lysate, complexes were resolved and blotted, and the blot was reacted with cdk4, cyclin D1, p15, p21, or p27 antibodies. To verify the identity of associated proteins, control cyclin D1, p15, p21, and p27 immunoprecipitations were resolved alongside the cdk4 immune complexes. Similar methods were used to detect cdk6- and cyclin E–cdk2-associated proteins.

Determination of p15^{INK4B} half-life. Cells were grown to 60% confluence on p100 dishes and then labeled metabolically for 1 h with 500 μCi of [^{35}S]methionine in 2 ml of alpha-modified minimal essential medium containing growth factors and lacking cold methionine. Cells were either lysed immediately or transferred to complete MCDB 170 medium containing 40 mM cold methionine and grown for the times indicated below. At intervals, cells were lysed on ice, clarified by centrifugation, and precleared with 1 μl of nonimmune serum and protein A-Sepharose beads. Protein was quantitated by Bradford analysis, and the decay of total protein radioactivity was verified by trichloroacetic acid-insoluble counts. Equal amounts of total protein (300 to 450 μg) were immunoprecipitated for 4 h with antibody (either the p15-p16 monoclonal, JC-6 or cdk6 antibody) in 200 μl of lysis buffer containing protease inhibitors. Immune complexes were collected on protein A-Sepharose beads, washed, and eluted into Laemmli buffer. Samples were resolved by SDS-PAGE, gels were dried, p15 was visualized, and the percentage of radioactivity in p15 was quantitated by PhosphorImager.

Kinase assays. Cyclin D-associated cdk4 kinase assays were performed by the method of Matsushime et al. (40), using cyclin D1 antibody DCS11 (36) for immunoprecipitation and recombinant pRb (gift of Canji Corp., San Diego, Calif.) as the substrate. Quantitation of radioactivity was performed with a Molecular Dynamics PhosphorImager and ImageQuant software.

RNA analysis. Total cellular RNA was isolated, as described previously (4), from cells recovered at intervals following release from EGF depletion in the presence or absence of TGF-β. Northern analysis was as described previously (70). The probe used for analysis of p15 mRNA levels was prepared from the Bluescript vector containing a 1.8-kb *Eco*RI-*Xho*I p15 cDNA insert (provided by G. Hannon, Cold Spring Harbor Laboratories). Blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase to verify loading.

Displacement assay. 184 cells or 184A1L5^R cells were treated with TGF-β for 18 h and lysed, and the protein was quantitated. Lysates containing 600 μg of protein were boiled for 7 min. p15 was then immunoprecipitated with MAb JC-6, complexes were recovered on protein A-Sepharose beads, and heat-stable p15 was released by boiling. A control immunoprecipitation with polyclonal mouse immunoglobulin was carried out in parallel. The p15-containing supernatant and the control immunoglobulin supernatant were recovered, and each was mixed with 100 to 200 μg of G_1 phase cell lysate and incubated at 30°C for 30 min in inhibitor buffer as described previously (65). Both 184 and 184A1L5^R were used as sources of cdk4 complexes. cdk4 was immunoprecipitated (immunoprecipita-

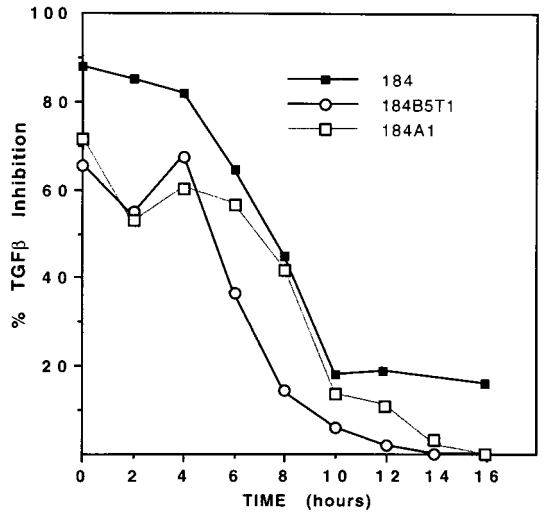

FIG. 1. Period of sensitivity to TGF-β during early G_1. Normal 184 HMEC and TGF-β-sensitive cell lines 184A1^S and 184B5T1^S were released from quiescence at time zero in the presence of 2 μCi of [^{3}H]thymidine per ml. At the indicated times thereafter, inhibitory concentrations of TGF-β were added to the culture dishes, and the percentage of cell nuclei showing incorporation of [^{3}H]thymidine, the labeling index (LI), was determined 18 h after release from G_0. The interval in early G_1 during which the addition of TGF-β could prevent entrance into S phase is demonstrated. The percent inhibition by TGF-β was calculated as follows: 1.00 − [(LI with TGF-β at 18 h)/(LI without TGF-β at 18 h)] × 100.

tion 1) from the lysate to which p15 was added or from the control lysate (no p15 added). To verify that the reduction in cdk4-bound cyclin D1 was not due to degradation but rather that cyclin D1 had been displaced from cdk4 by the added p15, the supernatant remaining after immunodepletion of cdk4 was immunoprecipitated with cyclin D1 antibody (immunoprecipitation 2). cdk4 or cyclin D1 immunoprecipitates were resolved by SDS-PAGE and immunoblotted to detect cdk4, cyclin D1, and associated proteins.

RESULTS

TGF-β sensitivity extends to 8 h from G_0 release. When TGF-β-sensitive cells (184 and 184A1^S) were released from G_0 in the presence of TGF-β, entrance into S phase was inhibited. No inhibition of S-phase entrance was seen in TGF-β-treated 184A1L5^R cells (see the labeling index in Fig. 2 and fluorescence-activated cell sorter [FACS] data in Fig. 6 and 7). TGF-β also mediates G_1 arrest of asynchronously growing sensitive cells (data shown for 184 cells) but not 184A1L5^R cells (for a FACS profile see Fig. 8).

To define the timing of TGF-β-mediated changes in key cell cycle regulators, we first determined the period during the G_0-to-S phase transition in which sensitive HMEC and normal 184, 184A1^S, and 184B5T1^S cells were responsive to growth arrest by TGF-β. Cells released from quiescence typically begin DNA synthesis within 12 h (69) (see Fig. 2). As shown in Fig. 1, TGF-β added during the first 6 to 8 h after release from quiescence inhibited S-phase entrance, whereas by 10 h most cells were committed to S-phase progression. Thus, as observed in other TGF-β-sensitive cells (32, 54), the period during which TGF-β is active in inhibiting HMEC entrance to S phase extends to mid-G_1.

TGF-β inhibits pRb phosphorylation without loss of cyclin D1 or cdk4. Our earlier work with normal HMEC had demonstrated the inhibition of cyclin E- and A-associated kinases by TGF-β (65). The present study was thus directed to the effects of TGF-β on the expression and activity of D-type cyclin-associated kinases. In all HMEC examined (184, 184A1^S, 184A1L5^R, and 184B5T1^S), little or no cyclin D1 was detected in quiescent cells and cyclin D1 levels rose by 4 h after G_0 release. TGF-β did not cause a significant reduction of cyclin D1 in either sensitive or resistant HMEC examined (data for 184, 184A1^S, and 184A1L5^R are shown in Fig. 2). In contrast to the findings of Ewen et al. (11), in HMEC there was no reduction in cdk4 in cells released from G_0 in the presence of TGF-β (Fig. 2). Thus, in this cell system, the inhibition of pRb phosphorylation by TGF-β seen in the sensitive but not in the resistant cells cannot be explained by changes in the levels of cdk4 or cyclin D1. Levels of cyclin D2, cyclin E, and cdk2 proteins were not reduced by TGF-β (as shown earlier for the 184 cells [65]) and did not vary during the G_0-to-S phase transition in either sensitive or resistant HMEC (data not shown). The cyclin D1 and E levels were not different in asynchronously growing sensitive and resistant cells when the different cell types were compared on the same gel. Cyclin D3 was not detected in these cells (negative data not shown).

p15 protein level is increased in TGF-β-arrested HMEC but p21 and p27 levels are not. Inhibitor activity directed against cdk2 is maximal in G_0 and decreases as cells approach the G_1-to-S transition (65). To determine if cell cycle-dependent and TGF-β-mediated changes in inhibitor activity were regulated at the level of protein expression, the cdk inhibitors p15, p16, p21, and p27 were examined by Western analysis in cells released from quiescence in the presence or absence of TGF-β (Fig. 3). In both sensitive and resistant cells, p27 protein was maximal in G_0-arrested cells and decreased in early G_1 (Fig. 3A). In contrast, p21 protein expression was lowest in G_0 and rose within 4 h to a constant level that persisted for up to 24 h following release from quiescence (data not shown). Neither the p21 nor the p27 protein level was affected by TGF-β. Thus, the arrest-resistant phenotype was not due to loss of either of these cdk inhibitors.

p16^{INK4A} mRNA and protein were barely detectable in normal 184 mammary epithelial cells (passage 16) under assay conditions where p16^{INK4A} was readily seen in the fibroblasts derived from the same specimen 184 (data not shown). 184A1^S and the resistant line derived from it, 184A1L5^R, have both lost p16 expression through mutational inactivation (2). Thus, differences in p16 expression do not underlie the different responses of 184A1^S and 184A1L5^R to TGF-β.

p15^{INK4B}, however, was expressed in all HMEC. p15 mRNA levels were highest in G_0 and at 1 h following G_0 release, decreased by 5 h, and remained at a low constant level up to 9 h later (Fig. 3C). In both sensitive and resistant cell lines, release from G_0 in the presence of TGF-β prevented the fall in p15 mRNA levels for up to 9 h. While TGF-β sustained the levels of p15 mRNA in both sensitive and resistant HMEC, the effect of TGF-β on p15 protein levels differed in the two kinds of cell. All HMEC examined expressed a low basal amount of p15 protein in G_0 (data not shown). During G_1-to-S phase progression, p15 protein levels were relatively constant (Fig. 3B). TGF-β treatment of sensitive 184 and 184A1^S cells released from G_0 caused an increase in p15 protein, detectable within 4 h, which was cumulative with time. No increase in p15 protein level was seen in the resistant 184A1L5^R cells (Fig. 3B). Thus, the defect in the p15 response to TGF-β in the resistant 18A1L5^R line is posttranscriptional (see below).

TGF-β increases p15 protein stability. The lack of p15 protein accumulation in the resistant line was not due to loss of TGF-β effects on p15 mRNA. We therefore examined the half-life of p15 by pulse-chase experiments (Fig. 4). In asynchronously growing 184 cells, the p15 half-life was approximately 8.5 h when complexes were immunoprecipitated with cdk6 antibody. Immunoprecipitation with the p15-p16 MAb

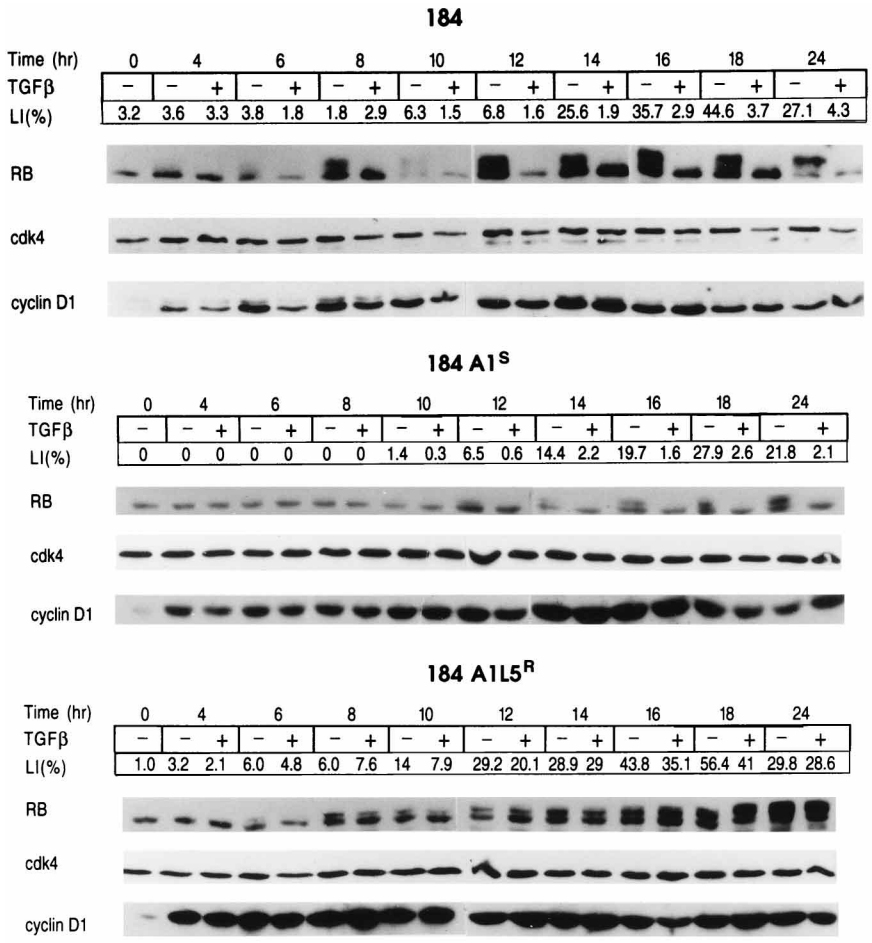

FIG. 2. Effects of TGF-β on the pRb protein, cdk4, and cyclin D1. 184 and immortal 184A1^S and 184A1L5^R cells were synchronized in G_0 by depletion of EGF and exposure to the anti-EGF receptor MAb 225 for 48 h. Cells were released from quiescence by renewal with complete medium, with (+) or without (−) the addition of TGF-β, and recovered at intervals. The percentage of cells entering S phase at each time point was determined by a labeling index (LI). pRb, cdk4, and cyclin D1 proteins were assayed by Western analysis, as described in Materials and Methods. When run on the same gel, lysates from the different HMEC contained similar amounts of cyclin D1 (data not shown).

JC-6 revealed a similar p15 half-life. However, lysates had to be boiled and SDS denatured for appreciable amounts of p15 to be detected with JC-6. Results presented in Fig. 4 are from cdk6 immunoprecipitations. When asynchronous 184 cells were treated with TGF-β for 24 h, the half-life of p15 was dramatically increased to 34 h in one experiment and no decay was detected in a second experiment. It is worth noting that the effect of TGF-β on protein stability did not generalize: cdk6 and cyclin D1 were not stabilized by TGF-β (data not shown). The p15 half-life in asynchronously growing 184A1L5^R cells with and without added TGF-β was measured at 9.7 and 9.15 h, respectively. All pulse-chase experiments (for each cell line, with and without added TGF-β) were confirmed by repeat experiments. Thus, TGF-β causes a change in the posttranslational regulation of p15 that may be more important to the regulation of its target cdks than the effect of TGF-β on p15 mRNA levels.

TGF-β increases p15-cdk4 association and prevents cyclin D1-cdk4 complex formation and activation. The effect of TGF-β treatment on pRb phosphorylation, as seen by mobility of pRb on Western blots (Fig. 2), predicted cyclin D1-associated kinase inhibition by TGF-β. Direct assays of immunoprecipitable cyclin D1-associated kinase activity confirmed that in sensitive but not resistant HMEC, this activity is inhibited by TGF-β (Fig. 5).

The INK protein, p16, can effectively compete with cyclin D1 for binding to cdk4 and cdk6 complexes in vitro (37, 50), and it has been proposed that an excess of p16 could prevent assembly of cyclin D1-cdk in vivo (62). To determine how the increase in p15 protein stability and its accumulation related to TGF-β inhibition of cyclin D1-cdk4, cyclin-cdk complexes were examined. Cells were recovered at intervals following G_0 release with or without exposure to TGF-β at time zero. On exit from quiescence, cyclin D1 protein levels rose (Fig. 2), and there was an incremental association of cyclin D1 and p21 with cdk4 (Fig. 6). In TGF-β-treated sensitive 184 and 184A1^S cells, as p15 protein levels increased, the amount of cdk4-associated p15 increased and the binding of cyclin D1 and p21 to cdk4 was strongly inhibited (Fig. 6). Densitometry of different enhanced chemiluminescence exposures, normalized to the amount of cdk4 immunoprecipitated, revealed that as cells progressed from G_0 into S phase, levels of cdk4-associated cyclin D1 rose by threefold and levels of p21 rose by twofold. There was no increase in cdk4-associated cyclin D1 or p21 levels in TGF-β-treated 184A1^S. In TGF-β-treated sensitive cells, p15 binding to cdk4 increased threefold over that in G_0 cells and, due to the

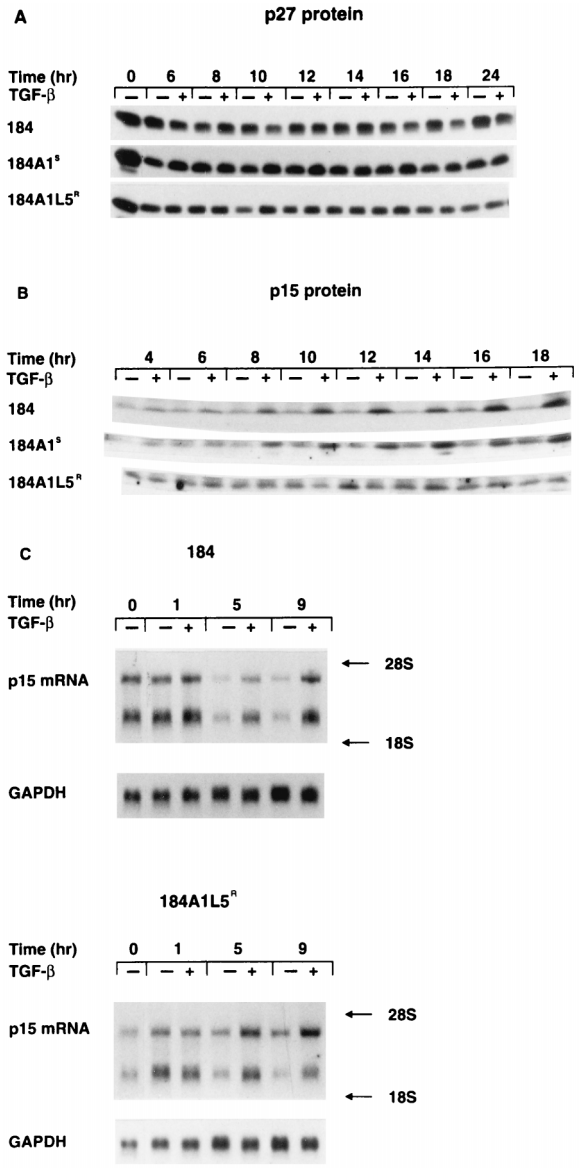

FIG. 3. cdk inhibitor expression in TGF-β-sensitive and -resistant HMEC. The same cells harvested for Fig. 2 were used for p27 (A) and p15 (B) Western blots. The p15 blot from 184A1L5^R is overexposed. The protein levels of p15 in non-TGF-β-treated cells from 184, 184A1^S, and 184A1L5^R were similar when assayed on the same blot (data not shown). (C) Northern blot of p15. 184 (upper gel) and 184A1L5^R (lower gel) were G_0 arrested and released from G_0 with or without the addition of TGF-β at time zero. RNA was extracted at the times indicated, and p15 mRNA levels were assayed by Northern blot analysis as described in Materials and Methods. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

loss of p15 from cdk4 complexes as 184A1^S cells progressed through G_1, TGF-β treated cells had sixfold more cdk4-bound p15 than untreated cells at the G_1-to-S transition. Although very little cyclin D1 was present in quiescent cells, p27 was maximally bound to cdk4 in G_0 arrested HMEC. In TGF-β-treated 184 and in 184A1^S, as the cdk4-associated p15 increased, there was a decline in cdk4-associated p27 of almost twofold at 12 h (Fig. 6A; data shown for 184A1^S). In the resistant 184A1L5^R line, TGF-β did not affect the amount of cdk4-associated p15 or the amounts of cdk4-bound p21 and p27. The binding of p21 to cdk4 roughly paralleled that of cyclin D1 to cdk4 (Fig. 6). As 184A1L5^R progressed into S phase, there was a notable loss of cdk4-associated p21. In the experiment shown in Fig. 6, the G_0-to-S phase progression was slightly faster in 184A1L5^R than in 184A1^S.

TGF-β prevents loss of p27 from cyclin E-cdk2. The abundance of cyclin E-bound cdk2 did not vary as quiescent cells progressed into S phase, nor was it affected by TGF-β treatment in 184, 184A1^S, and 184A1L5^R (data for 184A1^S and 184A1L5^R are shown in Fig. 7). p27 binding to cyclin E-cdk2 was maximal in G_0 and early G_1 and decreased as cells progressed through G_1 in both 184A1^S and 184A1L5^R. In sensitive 184 and 184A1^S, TGF-β inhibited the loss of p27 from cyclin E-cdk2 complexes that occurs as cells approach the G_1-to-S phase transition (data for 184A1^S are shown in Fig. 7A). In 184A1L5^R, TGF-β did not alter the amount of p27 in cyclin E-cdk2 complexes (Fig. 7B). The association of p21 with cyclin E-cdk2 was not affected by TGF-β in either sensitive or resistant cells and remained constant during G_0-to-S phase progression in all HMEC examined (Fig. 7). Thus, while p27 was lost from cdk4 complexes, the association of p27 with cyclin E-cdk2 was maintained in TGF-β-arrested cells.

Increased p15, loss of cdk4- and cdk6-bound cyclin D1, and increased p27-cyclin E-cdk2 accompany the TGF-β arrest of asynchronous cells. To confirm the effects of TGF-β on cyclin-cdk-inhibitor complexes, we examined 184 and 184A1L5^R cells following the addition of TGF-β to asynchronously growing cultures. As can be seen in Fig. 8B, TGF-β caused G_1 arrest in asynchronously growing 184 but did not change the DNA profile in 184A1L5^R. Total cellular p27 levels did not change in either cell line, but in 184 cells there was a progressive increase in p15 protein with increased duration of TGF-β treatment, coincident with the loss of pRb phosphorylation (Fig. 8A). cdk4 and cdk6 levels were not changed by TGF-β. The shifts in cdk-inhibitor complexes that accompanied the arrest of asynchronous cells were essentially the same as those seen during TGF-β arrest following G_0 release. There was a loss of cyclin D1 (3.5-fold by densitometry), p21 (2.7-fold), and p27 (2.7-fold) from cdk4 complexes as the amount of cdk4-associated p15 rose 7-fold. A similar pattern was seen for cdk6 complexes. However, cdk6 complexes from asynchronously growing cells contained more p15 and less cyclin D1 than did cdk4 complexes. The loss of p27 from cdk4 and cdk6 complexes was accompanied by a threefold rise in cyclin E–cdk2-associated p27. The rise in cdk4- and cdk6-associated p15 and in cdk2-associated p27 did not occur in 184A1L5^R.

p15 can displace cyclin D1 and KIPs from cdk4 in vitro. In the experiments described above, control immunoprecipitates showed that while p15 was detectable only in cdk4 and p15 immunoprecipitates, both p21 and p27 were detectable in cyclin E, cyclin D1, cdk2, and cdk4 immunoprecipitates and vice versa. These data are consistent with the observations in vivo and in vitro that KIPs bind most effectively to cyclin-cdk complexes, while in vivo p15, p16, and p19 appear to bind exclusively to their cdk partners (18, 22, 50, 60). Indeed, INK4 protein binding to cdk molecules may preclude stable cyclin D association. While we cannot exclude the possibility that low levels of unstable tripartite complexes of cdk4-cyclin D1-p15 might exist in vivo, we were unable to detect them.

To test whether p15 can displace cyclin D1 from cdk4, as was suggested by the observed effects of TGF-β in vivo (data herein and in reference 59), we used the heat-stable property of the cdk inhibitors to devise an in vitro inhibitor assay. Cell lysates were boiled to release p15 from cdk complexes, and p15 was immunoprecipitated and collected as described in Materials and Methods. When the p15 was added to lysates from 184 cells, it caused dissociation of cyclin D1, p21, and p27 from

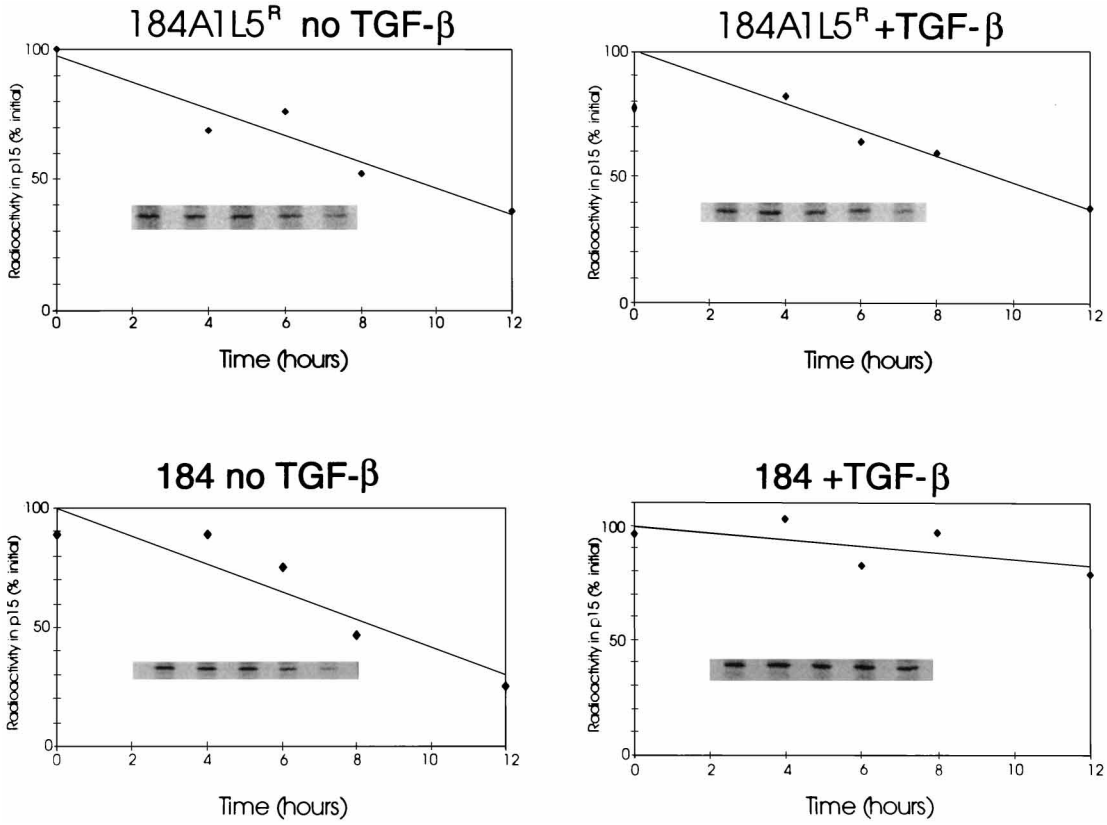

FIG. 4. Determination of the p15 protein half-life in TGF-β-treated and untreated HMEC. Cells (either 184 or 184 A1L5^R) were grown to 60% confluence and then either treated with TGF-β for 24 h or left untreated. Cells were pulsed with [^{35}S]methionine for 1 h and then chased for the indicated times. p15 was immunoprecipitated from equal quantities of protein lysate. The data shown in the insets were quantitated by PhosphorImager and plotted as the percentage of initial radioactivity in p15 versus time. The curves are linear regressions derived from data points plotted.

preformed cdk4-cyclin D1 complexes in these lysates (i.e., less cyclin D1 was seen in cdk4 precipitates after addition of p15) (Fig. 9). That cyclin D1, p21, and p27 were not merely degraded during the process is manifest by their recovery from the supernatant remaining after immunodepletion of cdk4 (data shown for cyclin D1 and p21) (cyclin D1 immunoprecipitation in Fig. 9). With increasing amounts of p15 added, cyclin D1 displacement increased until no cyclin D1 was detected in cdk4 immunoprecipitates. Our in vitro experiments showed that the p15 isolated from both sensitive and resistant cells was competent to displace cyclin D1 and KIPs p21 and p27 from the cdk4 complexes present in sensitive 184 cell lysates. Neither KIPs nor cyclin D1 could be displaced from cdk4 in the resistant 184A1L5^R lysates. Thus, the inability of p15 to displace cyclin from cdk4 in the resistant cells is due not to an intrinsic defect in the p15 protein but rather to altered configurations of the cdk4-cyclin D1-p21 and cdk4-cyclin D1-p27 complexes together with putative protein regulators that have yet to be defined.

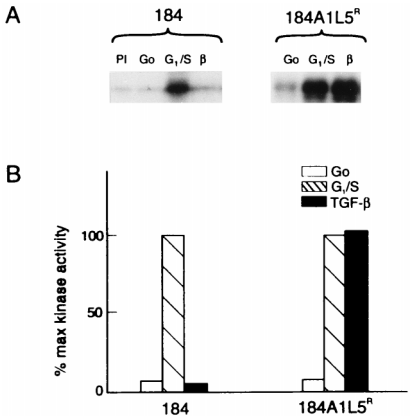

FIG. 5. Cyclin D1-cdk4 kinase assays. 184 cells and 184A1L5^R cells were released from quiescence in the presence or absence of TGF-β. Cells were recovered from G_0 (Go) and at 14 h after G_0 release with or without addition of TGF-β at time zero (β and G_1/S, respectively). Cyclin D1 was immunoprecipitated and kinase activity was assayed with pRb protein as the substrate, as described in the text. Reaction products were resolved by SDS-PAGE, and gels were dried and exposed to film (A). Radioactivity in the pRb substrate was quantitated by PhosphorImager and expressed as a percentage of maximum activity after subtraction of background from control immunoprecipitates (B).

DISCUSSION

Inappropriate proliferation, due to the loss of normal responsiveness to extracellular signals and deregulation of cell cycle checkpoints, is the hallmark of cancer. Loss of sensitivity to the growth-inhibitory effects of TGF-β is common in human tumor-derived cell lines, and this is thought to confer an advantage in tumorigenesis and neoplastic progression (see references 14 and 29 for reviews). In the present study, we examined normal finite-life-span and immortal human mammary epithelial cells that differ in their sensitivity to TGF-β in order to better understand the mechanisms by which TGF-β mediates G_1 arrest and to determine how these regulatory mecha-

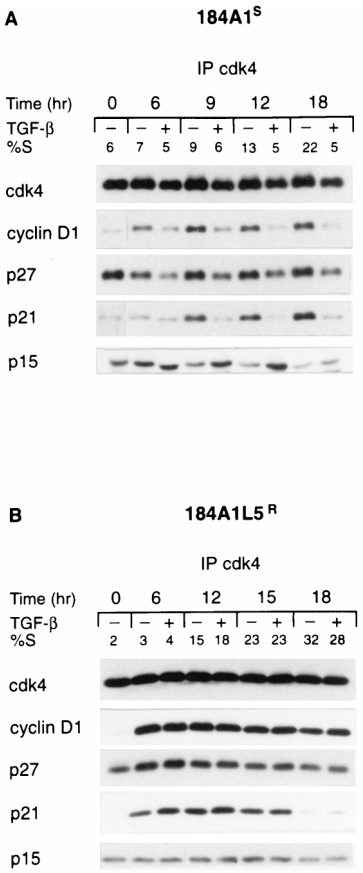

FIG. 6. cdk4 binding to cyclin D1 and cdk inhibitors in HMEC. Cells were recovered at intervals after G_0 release with or without TGF-β. DNA profiles were determined by FACS analysis, and the percentage of cells in S phase is indicated for each time point. Protein lysate (200 to 500 μg) was immunoprecipitated with cdk4 antibody at the indicated times. Complexes were resolved on SDS-PAGE gels and blotted, and blots were reacted with antibodies to detect associated proteins.

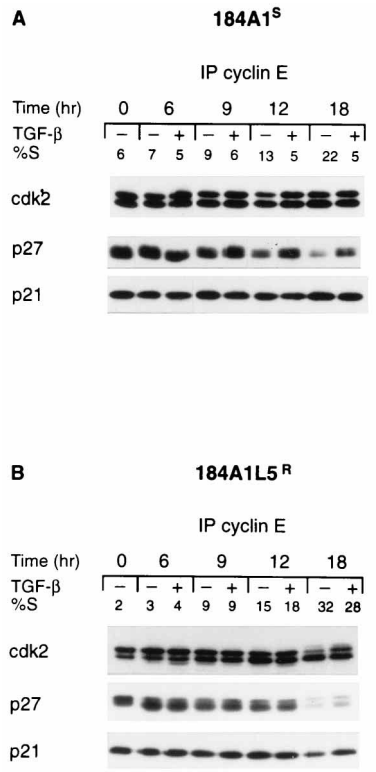

FIG. 7. Cyclin E-cdk2 binding to cdk inhibitors p21 and p27. Cells were recovered for FACS analysis and preparation of protein lysates following G_0 release with or without TGF-β as for Fig. 6. Cyclin E was immunoprecipitated and complexes were resolved and electroblotted as in the experiments for Fig. 6. (The 184A1L5^R 18-h, no-TGF-β lane was underloaded.)

nisms are deranged in cells that are resistant to growth inhibition by TGF-β.

TGF-β has been shown to inhibit pRb phosphorylation in mink lung epithelial cells (31, 32). While TGF-β dramatically inhibited cyclin D1-associated kinase activity and the phosphorylation of pRb in sensitive HMEC, it caused little or no reduction of cyclin D1 or cdk4 protein levels. Thus, in contrast to findings with other cell types (11, 12, 30), in the sensitive TGF-β-treated HMEC the loss of pRb kinase activity cannot be explained by a reduction of either cdk4 or D cyclins.

Of the three cdk inhibitors that we found expressed in HMEC, only p15 levels increased during TGF-β-mediated G_1 arrest. In contrast to what has been reported for other cell types (5, 8, 12, 59), p21 protein was not induced by TGF-β and does not appear to play a role in the arrest of sensitive HMEC. Rather, p21 may be playing a role in the regulation of cyclin-cdk assembly and kinase activation (22, 31a, 35, 74).

In all HMEC lines examined, p27 levels were maximal in G_0 and fell as cells moved into G_1. Although p27 levels did not vary across the cell cycle, as noted for other cell types (25), the association of p27 with cyclin E-cdk2 was periodic, with maximal association in G_0 and early G_1 and loss of p27 from these complexes as cells approach the G_1-to-S phase transition. The TGF-β-sensitive and -resistant HMEC studied expressed similar p27 protein levels, and p27 was not increased in TGF-β-arrested cells. Thus, any differences in the inhibitory activity of p27 in response to TGF-β in these cells must result from altered posttranslational regulation of p27. Modulation of p27 levels by growth-inhibitory stimuli appears to be cell type dependent (12).

The comparison of TGF-β-sensitive and -resistant HMEC demonstrates that TGF-β regulates p15 expression at at least two levels: mRNA accumulation and protein stability. In both sensitive and resistant HMEC, p15 mRNA levels were maximal in G_0 and fell as cells entered G_1 (Fig. 4C). Treatment with TGF-β prevented the fall in p15 mRNA that normally ensues when cells progress into G_1. In sensitive cells, TGF-β caused a marked increase in p15 stability and an accumulation of p15 protein. Although the effect of TGF-β on p15 mRNA levels remained intact in 184A1L5^R, there was no increase in p15 protein in response to TGF-β. Failure to stabilize p15 in response to TGF-β may account for the lack of p15 accumulation in the TGF-β-treated resistant 184A1L5^R line. As was recently demonstrated for p27 (25, 48), posttranslational regulation of p15 may be critical to its effects on target cdks.

It has been postulated that in TGF-β-treated cells, induction of p15 (20) displaces p27 from cyclin D-cdk4 complexes, allowing p27 to bind and inhibit cyclin E-cdk2 (51, 56). In Mv1Lu, TGF-β caused an increase in p15 mRNA and increased the binding of a 15-kDa protein in metabolically labeled cdk6 immunoprecipitates, and there was a progressive loss of p27 from cdk4 complexes, while p27 binding to cdk2 increased (59). Our studies of TGF-β-arrested HMEC confirm and extend these observations. Both in HMEC released from

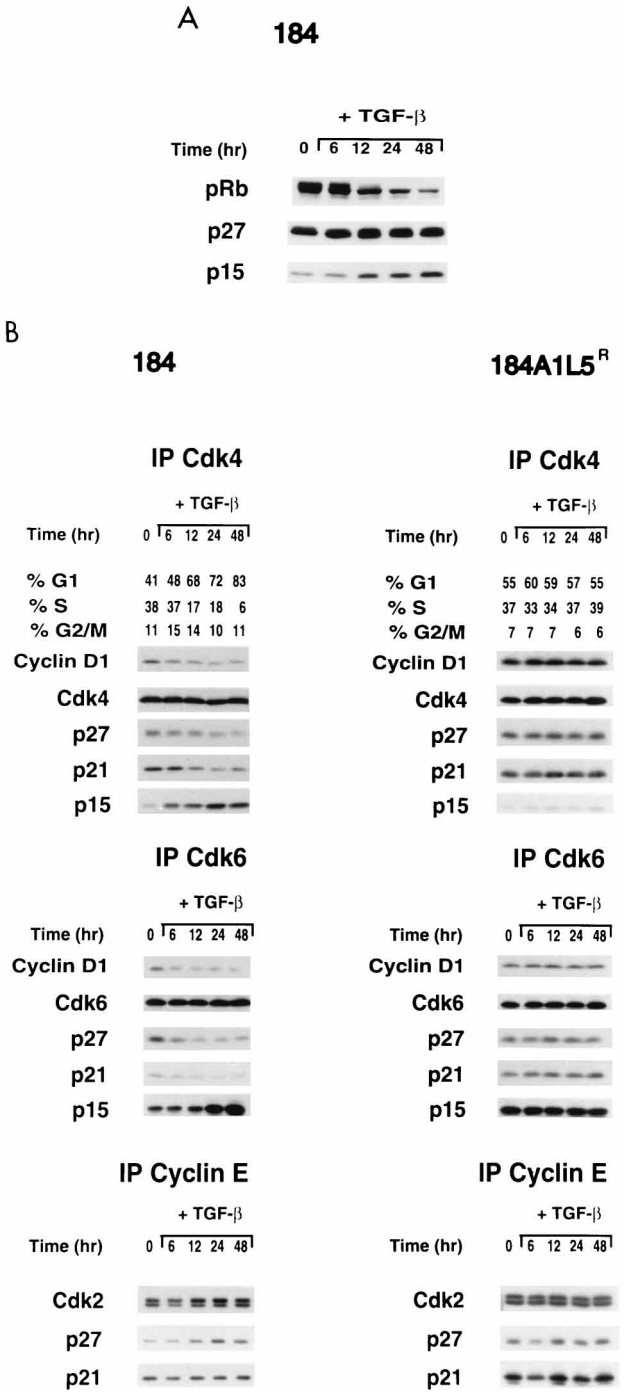

FIG. 8. Cyclin-cdk-cdk inhibitor complexes following treatment of asynchronously growing HMEC with TGF-β. Asynchronous cultures of either 184 or TGF-β-resistant 184A1L5^R were treated with TGF-β for the times shown. At the times indicated, cells were recovered for FACS analysis and for the preparation of protein lysates. (A) Western analysis of pRb, p27, and p15 in TGF-β-treated 184 cells. (B) Cdk4, cdk6, and cyclin E immunoprecipitations (IP) were carried out as described in Materials and Methods. Complexes were resolved by SDS-PAGE, and associated proteins were detected by immunoblotting with the indicated antibodies.

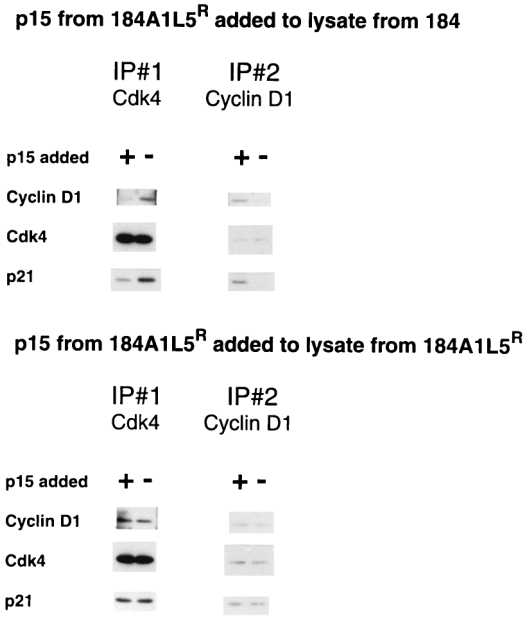

FIG. 9. Displacement of cyclin D1 from cdk4 by p15. p15 was immunoprecipitated from boiled 184A1L5^R lysates and collected on protein A beads. p15 was then mixed with lysates from G_1 cells (either 184 or 184A1L5^R). cdk4 (immunoprecipitation 1 [IP#1]) was then immunoprecipitated from cell lysates to which p15 had been added (+) or not (−). The supernatant from the cdk4 immunoprecipitation was retained, and cyclin D1 was immunoprecipitated (IP#2). Complexes were resolved, and cdk4, cyclin D1, and p21 were visualized by immunoblotting.

G_0 in the presence of TGF-β and in the arrest of asynchronously growing cells by TGF-β, arrest is accompanied by increased p15 binding to cdk4 and cdk6 and loss of cyclin D1, p21, and p27 from these complexes, and there is either stabilization of cyclin E-cdk2-p27 levels or an increase in cyclin E-cdk2-associated p27. Both types of experiment support a shift in the equilibrium of p27-cdk binding from cdk4 and cdk6 into cdk2 complexes. Because cyclins bind to cdks in a 1:1 molar ratio, the loss of cyclin D1-associated kinase activity in TGF-β-arrested HMEC is due at least in part to loss of cyclin D1 from cdk4 complexes.

In TGF-β-sensitive HMEC, high levels of p15 would foster p15-cdk4 association and displace cyclin D1 and might inhibit cyclin D1 reassociation. Disruption of cyclin D1-cdk association leads to loss of cyclin D1 stability (1, 50). p21 and p27 bind their cdk partners with reduced affinity in the absence of associated cyclin (18). Recent in vitro data (31a) and the pattern of p21 and p27 binding to cdk4 in HMEC during the G_0-to-S phase transition support the notion that these KIPs may serve as assembly factors for cyclin D1-cdk4 association. However, TGF-β appears to override this effect, and reassembly of dissociated complexes is inhibited in the presence of high p15 levels. In vitro, premixing of p15 with cdk4 prevented added p27 from binding to cdk4 (59). Dissociation of p27 from cdk4 may facilitate the binding and inhibition of cyclin E-cdk2 by p27. The functional importance of these shifts in cdk-inhibitor binding during TGF-β arrest is underlined by their loss in the TGF-β-resistant 184A1L5^R line.

The study of p15 regulation in 184A1L5^R has yielded two interesting observations. Firstly, loss of the growth-inhibitory effect of TGF-β in this resistant line was associated with failure to stabilize the p15 protein and the loss of p15 accumulation in target cdk complexes in response to TGF-β. Secondly, the p15 protein from these cells was functional in vitro; that is, p15 isolated from 184A1L5^R cells could displace cyclin D1 from preformed cyclin D1-cdk4 complexes present in 184 cell lysates. However, neither p15 from 184 nor p15 from 184A1L5^R

cells could displace cyclin D1 from preformed cdk4 complexes in 184A1L5^R cell lysates. Thus, it would appear that the cyclin D1-cdk4-KIP complexes are altered in the resistant cell line or that p15 is modified in vivo, in 184A1L5^R cells, in a way not reflected in the in vitro displacement assay that we used. In our experiments, mixing the endogenous cellular p15 with 184 cell lysates allowed displacement of cyclin D1 and KIPs from cdk4. In contrast, mixing of recombinant bacterial p19 with preformed baculovirus cyclin D2-cdk4 complexes failed to cause cyclin displacement (26). Purified recombinant proteins may lack posttranslational changes in cyclin-cdk or in p15 itself, which may be required for displacement in vivo.

It is tempting to link the effects of TGF-β on p15 stability and the displacement data in the following hypothesis. In the TGF-β-resistant cell line, although p15-cdk complexes are readily detectable, they do not increase in response to TGF-β. The TGF-β-induced change that allows an incremental association of p15 with cdk4 may also result in stabilization of p15. In the resistant cells the conformation of the cdk4-cyclin D1-KIP complexes may be altered in such a way that it does not permit dissociation of the cyclin from the cdk in vitro. The same conformation change (possibly due to associated protein or posttranslational modification of a component of the cdk complex) may also underlie the failure to stabilize p15 in the TGF-β-treated resistant cell line.

Alternatively, or in addition, TGF-β may increase the affinity of p27 for cdk2 or reduce the affinity of p27 for cdk4. Movement of p27 out of cdk4 complexes could facilitate the association of p15 with cdk4. Our preliminary data suggest that the activity of p27 in quiescent 184A1L5^R is reduced compared to that in quiescent 184A1^S (69a). If this model is correct, a primary defect in the posttranslational activation of p27 by TGF-β could give rise to the observed differences in sensitivity of these HMEC to growth arrest by TGF-β.

Finally, p15 and p27 may act independently and both contribute to TGF-β-mediated inhibition of the cdks in HMEC. Because TGF-β-mediated arrest plays an important role in differentiation and development, a degree of redundancy in the arrest mechanism could have a significant evolutionary benefit. The retention of TGF-β sensitivity in p27 null cells supports this notion (43). The redundancy of TGF-β-mediated arrest mechanisms is also supported by the observation that cells lacking both p15 and p16 expression can undergo TGF-β-mediated arrest (12, 27a).

In this study we present evidence that posttranslational regulation of the INK4 family protein p15 is altered in the TGF-β-resistant human mammary epithelial cell line, 184A1L5^R. Altered regulation of p15 and p27 in this TGF-β-resistant cell line presents a link between the disruption of cyclin D-cdk-pRb cell cycle regulation and the loss of TGF-β sensitivity, both of which are common in human tumors. Altered p15 and p27 regulation may represent important events leading to loss of TGF-β sensitivity during tumor progression in vivo.

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