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Cyclin D1 induction in breast cancer cells shortens G_1 and is sufficient for cells arrested in G_1 to complete the cell cycle

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ABSTRACT **The sequential transcriptional activation of cyclins, the regulatory subunits of cell-cycle-specific kinases, is thought to regulate progress through the cell cycle. Cyclins are therefore potential oncogenes, and cyclin D1 overexpression and/or amplification at its genomic locus, 11q13, are common features of several human cancers. Induction of cyclin D1 is an early response to mitogenic stimulation in several cell types, but the consequences of altered expression of this gene in human cells of epithelial origin remain undefined. We assessed the effects of alterations of cyclin D1 expression in human breast cancer cells by generating T-47D cells expressing human cyclin D1 under the control of a zinc-responsive metallothionein promoter. In cycling cells induction of cyclin D1 after zinc treatment resulted in an increase in the number of cells progressing through G_1 and in the rate of transition from G_1 to S phase, indicating that cyclin D1 is rate-limiting for progress through G_1 phase. In cells arrested in early G_1 phase after growth factor deprivation, zinc induction of cyclin D1 was sufficient for completion of the cell cycle, a process requiring growth factor stimulation in control cells. These data demonstrate a critical role for cyclin D1 in human breast cancer cell-cycle control and suggest that deregulated expression of cyclin D1 is likely to reduce dependence on normal physiological growth stimuli, thereby providing a growth advantage to tumor cells and a potential mechanism of resistance to endocrine therapy.**

Cell-cycle progression in mammalian cells is coordinated at a series of control points, which ensure orderly progress through the complex and tightly regulated processes necessary for cell growth and division. The central mechanism for these control points is thought to be the sequential transcriptional activation of cyclin genes and consequent transient accumulation of different cyclin/cyclin-dependent kinase (CDK) complexes. In synchronized or growth factor-stimulated cells, cyclins C, D1, D2, D3, and E are most abundant during G_1 phase (1–3), suggesting that they function during G_1. The D cyclins have closely related sequences but are differentially expressed: some cells express all three genes [for example, mammary epithelial cells (4)], but many cell types express only one or two (2, 5, 6). Furthermore, these cyclins are not coordinately regulated but, in general, appear sequentially during progress through G_1 phase (2, 6–9), suggesting that they have complementary rather than redundant functions.

Regulation of cyclin D1 gene expression is closely associated with changes in the proliferation rate of breast cancer cells. Increased expression of cyclin D1 occurs within 2 hr of stimulation of T-47D breast cancer cells by peptide mitogens—for example, insulin, insulin-like growth factor I, and basic fibroblast growth factor—and is followed by induction of other G_1 cyclins, cyclins D3 and E, as cells progress through G_1 phase (ref. 8, unpublished data). The proportion of cells that enter S phase is related to the degree of induction of cyclins D1, D3, and E (8). Regulation of cyclin D1 expression also occurs rapidly upon treatment with mitogenic steroids, estrogen, and progestin (ref. 8, unpublished data) and precedes growth inhibition by antiestrogens (8). These data suggest that regulation of cyclin D1 expression could contribute to regulation of breast epithelial cell proliferation by some steroids and steroid antagonists.

It has been proposed that G_1 cyclins are protooncogenes whose inappropriate expression may lead to loss of normal growth control. This hypothesis is supported by several lines of evidence. Aberrant expression of cyclin D1 through chromosomal translocation, gene amplification, and/or overexpression is a common feature of a number of human cancers, including B-cell lymphomas, squamous cell carcinomas of the head and neck, lung, and esophagus, as well as breast and bladder carcinoma (4, 12, 13). Up to 23% of breast carcinomas display amplification of the chromosomal locus of the cyclin D1 gene, 11q13 (14–16), and a greater fraction display increased expression of cyclin D1 mRNA in the presence or absence of gene amplification (4). The functional consequences of cyclin D1 gene regulation or aberrant expression have yet to be investigated in these cells, but the differential expression and distinct functions of the D-type cyclins suggest that their effects may be tissue-specific.

T-47D human breast cancer cells are representative of a well-differentiated, estrogen receptor-positive breast cancer phenotype, in which 11q13 amplification most commonly occurs (13), and are growth-regulated by estrogen, progestins, and their antagonists. No evidence for cyclin overexpression or amplification has been found in these cells (4), and the pattern of cyclin gene expression of T-47D cells after growth factor stimulation is indistinguishable from that of normal mammary epithelial cells (6, 8, 17), in contrast to aberrant cyclin expression in some other breast cancer cell lines (4, 17). Therefore, T-47D cells were used to examine the consequences of increased cyclin D1 expression in human breast cancer. These experiments demonstrated that cyclin D1 is not only cell-cycle regulated in these cells but is cell-cycle regulatory, with effects on both the rate of progress through G_1 phase and the proportion of cells initiating cell-cycle progression.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The T-47D cell line from the E. G. and G. Mason Research Institute (Worcester, MA) was cloned by limiting dilution, and one clonal cell line, T-47D (7-2), was selected for transfection studies. T-47D (7-2) retained the characteristics of the parent line by all the criteria tested—i.e., growth rate in stock culture conditions, sensitivity to growth regulation by steroids and steroid antagonists (progestin, ORG 2058; antiestrogen, ICI 164384; and antiprogestin, RU 486), and abundance of cyclin D1 and c-*myc* mRNA.

RPMI 1640 medium was used throughout. Stock cultures were maintained in medium supplemented with human insu-

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lin at 10 μg/ml (CSL-Novo, North Rocks, NSW, Australia) and 10% fetal calf serum. Serum-free medium was phenol red-free and supplemented with 300 nM human transferrin (Sigma) and, where noted, insulin at 10 μg/ml. Cells were cultured in serum-free medium following a described protocol (18, 19).

Expression Vectors and Transfection Procedures. The inducible expression vector pΔMTcycD1 was constructed by cloning human cyclin D1 coding sequences (5) into the *Sal* I/*Acc* I site of pΔMT (20). Parallel flasks of T-47D (7-2) cells were cotransfected with 50 μg of pΔMTcycD1 or pΔMT and 5 μg of pSV2neo, using standard calcium phosphate precipitation methods, and after 3 weeks colonies resistant to G418 at 600 μg/ml were trypsinized and pooled, to yield the T-47D ΔMTcycD1 and T-47D ΔMT cell lines. Experiments were done within 11 weeks. DNA fingerprinting using a *Pvu* II restriction fragment length polymorphism of the mucin gene confirmed that each cell line was a derivative of T-47D.

Analysis of Cell Cycle Progression. Cell-cycle phase distribution was determined by analytical DNA flow cytometry, as described (21). G_1 exit kinetics were measured using a previously published stathmokinetic technique (22, 23).

RNA Isolation and Northern Blot Analysis. Replicate 150-cm^2 flasks of cells were treated with 50 μM $ZnSO_4$ or vehicle, and total RNA was extracted (using a guanidinium-isothiocyanate-cesium chloride procedure), blotted, and hybridized as described (18). Equivalent RNA loading was verified by hybridization to an oligonucleotide recognizing 18S rRNA sequences (18).

Cell Extraction and Immunoblot Analysis. Cell lysates from replicate 75-cm^2 flasks of cells treated with 10–50 μM $ZnSO_4$ or vehicle were prepared as described (24), except that *p*-nitrophenyl phosphate was omitted from the lysis buffer. Equal amounts of total protein (typically 30 μg per lane) were separated by SDS/10% PAGE. Cyclin D1 protein was detected after incubation (2–4 hr at room temperature) with a 1:1000 dilution of a rabbit polyclonal antibody raised against a cyclin D1 amino-terminal epitope (Santa Cruz Biotechnology) followed by horseradish peroxidase-linked anti-rabbit antibody (1 hr at room temperature) and visualized using the enhanced chemiluminescence detection system (Amersham).

RESULTS

Cyclin D1 Regulates Breast Cancer Cell-Cycle Progression. Alterations in cyclin D1 expression in breast cancer cells were examined by generating T-47D cells expressing cyclin D1 under the control of a metal-inducible truncated human metallothionein IIA promoter lacking steroid-responsive sequences (20); these cells were designated T-47D ΔMTcycD1. Upon zinc treatment the abundance of cyclin D1 mRNA transcripts expressed from the introduced vector increased within 1 hr, reached a maximum at 6 hr, and remained elevated (up to 4-fold) until the last time point examined, 24 hr (Fig. 1*A*). Cyclin D1 protein also increased (≈5-fold) with zinc treatment of T-47D ΔMTcycD1 (Fig. 1*C*). Zinc treatment of cells transfected with vector alone (T-47D ΔMT) had little effect on either cyclin D1 mRNA or protein abundance (Fig. 1 *B* and *D*).

The effects of cyclin D1 induction on cell-cycle progression in cycling cells were determined by using T-47D ΔMTcycD1 and T-47D ΔMT cells cultured in serum-free medium containing insulin as a mitogen. These defined growth conditions result in exponential proliferation at a rate that allows detection of both stimulation and inhibition of proliferation (8, 18). Major changes in cell-cycle-phase distribution were detected after 24-hr zinc treatment of cells expressing exogenous cyclin D1 (Fig. 2*A*). The proportion of S-phase cells increased from ≈22% to 57%, and the G_2 + M peak also significantly increased (from 9% to 16%). In contrast, the

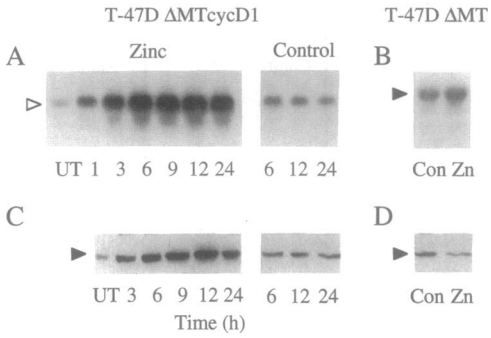

FIG. 1. Zinc induction of cyclin D1 mRNA and protein. T-47D ΔMTcycD1 (*A* and *C*) and T-47D ΔMT (*B* and *D*) cells proliferating in serum-free medium containing insulin were untreated (UT) or treated with 50 μM $ZnSO_4$ or vehicle control (Con). In parallel experiments either total RNA (*A* and *B*) or cell lysates (*C* and *D*) were harvested at intervals (T-47D ΔMTcycD1) or after 6 hr (T-47D ΔMT). The major endogenous cyclin D1 mRNA transcript (4.5 kb) is indicated by a closed arrowhead (*B*), and the exogenous transcript (2.2 kb) by an open arrowhead (*A*). The 35-kDa cyclin D1 protein is indicated by solid arrowheads (*C* and *D*). Experiments done with cells cultured in the same medium supplemented with 5% fetal calf serum gave similar results.

effects of zinc on vector-transfected cells were minor (Fig. 2*A*), confirming that the observed effects on cell-cycle progression resulted from cyclin D1 induction. The temporal changes in cell-cycle-phase distribution of T-47D ΔMTcycD1 cells after zinc induction of cyclin D1 are depicted in more detail in Fig. 2*B*. Commencing after an ≈9-hr treatment there was a rapid decrease in the percentage of G_1-phase cells and a concomitant increase in the percentage of S-phase cells, which continued until maximal effects were evident between 18 and 24 hr. The increase in the S-phase fraction was followed by an increase in G_2/M phase after 18-hr treatment (Fig. 2*B*), as the stimulated cells proceeded through the cycle. The time for cyclin D1-stimulated cells to complete S phase was thus ≈9 hr, an S-phase duration similar to that seen for growth factor-stimulated or mitotically selected T-47D cells (ref. 8, unpublished data). Therefore, although the proportion of cells in S phase increases upon induction of cyclin D1, the level of cyclin D1 has little effect on S-phase duration, suggesting that the major effect of cyclin D1 occurs during G_1 phase. These data imply that cyclin D1 expression governs progress through G_1 phase in breast cancer cells, possibly by regulating the rate of progression through G_1, as observed for G_1 cyclins in other experimental models (10, 25–28), or by controlling the proportion of cycling cells, as suggested by its growth factor regulation.

Cyclin D1 Is Rate-Limiting for G_1 Progression. To directly test the hypothesis that the level of cyclin D1 expression is rate-limiting for G_1 progression in breast cancer cells, G_1-exit kinetics were measured in the presence and absence of cyclin D1 induction. An inhibitor of cytokinesis, ICRF 159 (Razoxane, ICI), was added to zinc-treated and control T-47D ΔMTcycD1 cells to inhibit cells reentering G_1 at the end of the cell cycle (22, 23). Under these conditions the progression of cells into S phase leads to a decrease in the proportion of cells in G_1 phase, and thus the rate of G_1 exit can be determined in cycling cells (Fig. 3). Lines of best fit were calculated by linear-regression analysis and the slope used to derive the average residence time in G_1. Control cells left G_1 phase with a half-time of 9.8 hr (95% confidence interval: 9.1–10.4 hr), whereas the average duration of G_1 phase was reduced by >50%, to 4.5 hr (95% confidence interval: 3.9–5.3 hr) after cyclin D1 induction. These values differed significantly upon two-way analysis of variance (P = 0.0001). Thus, cyclin D1 is rate-limiting for G_1 progression in T-47D breast cancer cells.

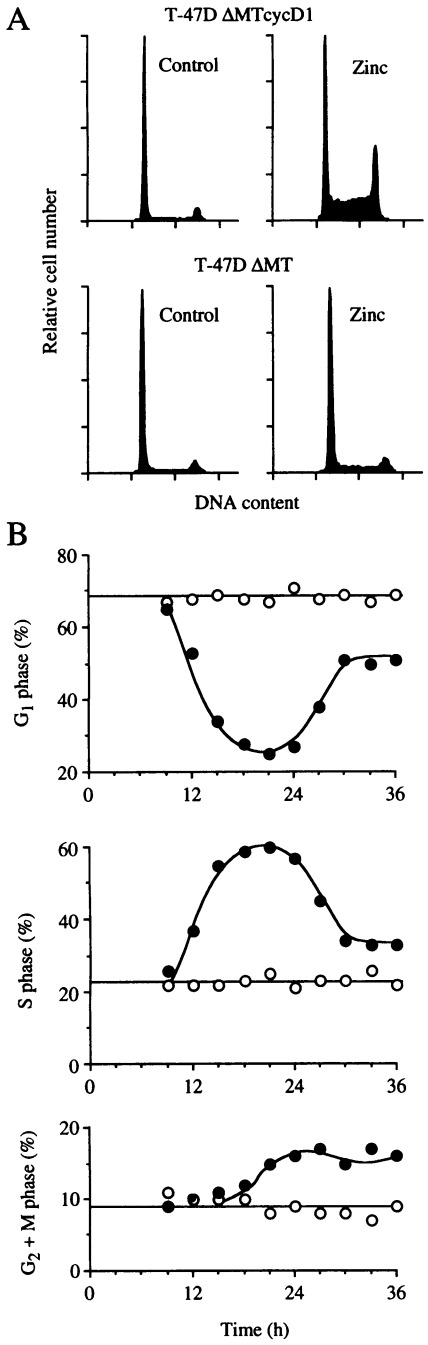

FIG. 2. Effect of cyclin D1 on cell-cycle progression in cycling cells. Replicate flasks of T-47D ΔMTcycD1 or T-47D ΔMT cells proliferating exponentially in serum-free medium containing insulin were treated with 50 μM $ZnSO_4$ or vehicle and harvested at intervals for analytical DNA flow cytometry. (*A*) DNA histograms of T-47D ΔMTcycD1 or T-47D ΔMT cells after 24 hr of $ZnSO_4$ or vehicle (control) treatment. (*B*) Cell-cycle-phase distribution of T-47D ΔMTcycD1 cells treated with $ZnSO_4$ (●) or vehicle (○).

Cyclin D1 Levels Correlate with the Proportion of Cells Entering S Phase. That cyclin D1 might control the proportion of cycling cells was examined in further experiments, using a clonal cell line displaying a large relative induction of cyclin D1 on zinc treatment (T-47D ΔMTcycD1-3). Treatment of these cells with zinc at 10–50 μM resulted in a concentration-dependent increase in relative cyclin D1 protein levels, ranging from <2-fold to a maximum of >7-fold (Fig. 4*A*). As expected, cyclin D1 abundance affected the rate of exit from G_1 phase, which increased with increased zinc concentration (Fig. 4*B*). However, by 21–24 hr the percentage of cells remaining in G_1 phase reached a minimum that was also concentration-dependent (Fig. 4*B*). These data suggest that not only the rate of progression, but also the proportion of the cell population that initiates progression through G_1, depends on cyclin D1 abundance. The cyclin D1 protein abundance 6 hr after zinc treatment correlated with the proportion of cells reaching S phase by 21 hr (Fig. 4*C*), supporting this conclusion.

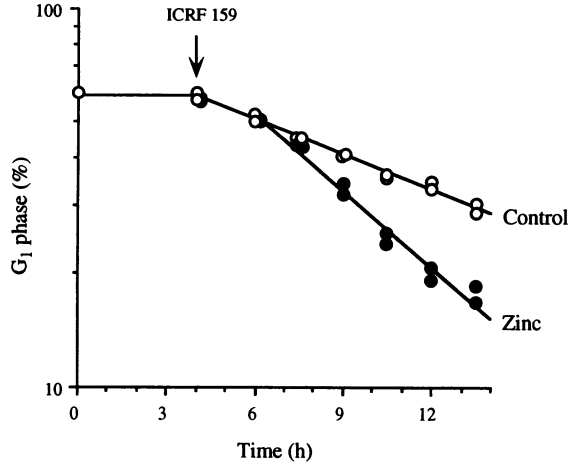

FIG. 3. Effect of induction of cyclin D1 on rate of exit from G_1 phase. T-47D ΔMTcycD1 cells proliferating in medium/5% fetal calf serum were treated with 50 μM $ZnSO_4$ (●) or vehicle (○), and 4 hr later an inhibitor of cytokinesis, ICRF 159 (100 μg/ml), was added. Duplicate flasks were harvested at intervals thereafter for determination of cell-cycle-phase distribution by flow cytometry. Lines of best fit to data from 4–13.5 hr (control) or 6–13.5 hr (zinc-treated) were calculated by linear-regression analysis.

Cyclin D1 Induction Is Sufficient for G_1 Transit in Breast Cancer Cells. In serum-free medium lacking exogenous mitogens, T-47D cells are growth-arrested at the beginning of G_1 phase (19). Upon addition of a single growth factor—e.g., insulin, insulin-like growth factor I, or basic fibroblast growth factor—a cohort of cells reinitiates cell-cycle progression, entering S phase after a delay of ≈12 hr, corresponding to the G_1 duration in cycling cells (refs. 8 and 19, see also Fig. 5). The effects of these growth factors include rapid cyclin D1 induction (8), and the data presented above (Fig. 4) suggest that this induction might lead to reentry into the cell cycle. Therefore, the possibility that cyclin D1 induction was sufficient to allow growth-arrested cells to progress through G_1 and into S phase was examined. Serum-deprived T-47D ΔMTcycD1 cells displayed a higher S-phase fraction than parental or vector-transfected cells under the same conditions (Fig. 5*A*) (19), presumably as a result of constitutive expression from the metallothionein promoter (Figs. 1*A* and 5*A*). However, insulin stimulation resulted in a similar degree of induction of endogenous cyclin D1 and a similar increase in the proportion of cells entering S phase in T-47D ΔMTcycD1 and T-47D ΔMT cells (Fig. 5). Despite the absence of exogenous growth factors, zinc induction of cyclin D1 in T-47D ΔMTcycD1 cells arrested in early G_1 phase resulted in a response similar to that observed in cycling cells—i.e., semisynchronous entry into S phase after ≈12 hr (Fig. 5). Thus, induction of cyclin D1 was sufficient for completion of G_1 phase and subsequent DNA synthesis. The rate of exit from G_1 was slower and the proportion of cells reaching S phase was smaller in the presence of insulin than in the presence of zinc, as expected from the smaller relative induction of cyclin D1 by insulin (Fig. 5). Only minor changes in either cyclin D1 expression or cell-cycle progression were observed after zinc treatment of T-47D ΔMT cells when compared with the effects of zinc on T-47D ΔMTcycD1 (Fig. 5).

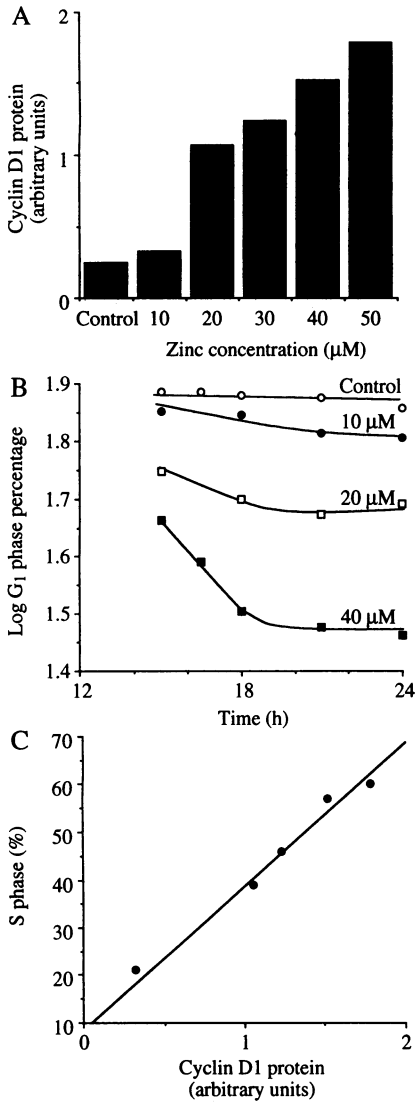

FIG. 4. Relationship between cyclin D1 protein and T-47D cell-cycle progression. In parallel experiments flasks of T-47D ΔMTcycD1-3 cells proliferating exponentially in serum-free medium containing insulin were treated with 10–50 μM $ZnSO_4$ or vehicle, and either cell lysates were prepared (after 6-hr treatment) or cell-cycle-phase distribution was determined. (*A*) Relative levels of cyclin D1 protein were determined by immunoblotting followed by densitometric analysis. (*B*) Percentage of G_1-phase cells after treatment with vehicle (control) or the indicated concentrations of $ZnSO_4$. (*C*) Relationship between percentage of S-phase cells after 21-hr $ZnSO_4$ treatment and cyclin D1 protein abundance after 6-hr $ZnSO_4$ treatment. The line of best fit is shown ($r^2 = 0.984$).

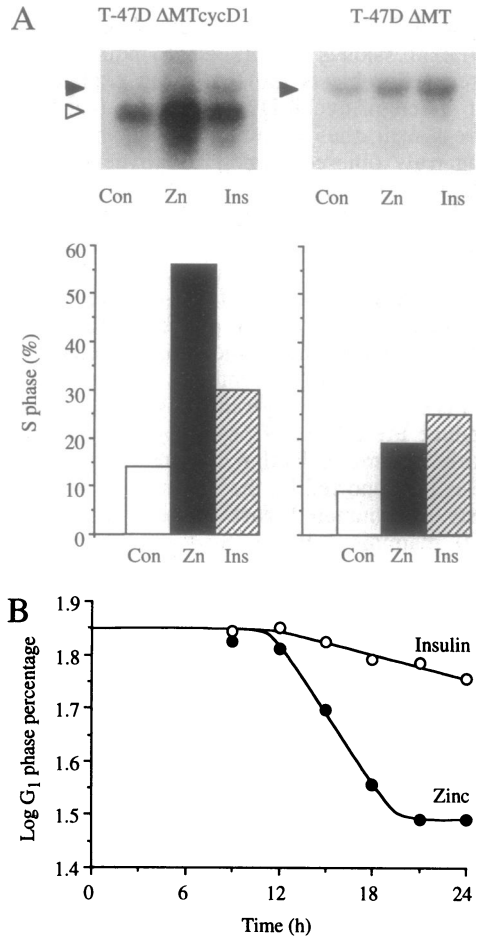

FIG. 5. Effect of cyclin D1 induction in growth-arrested cells. T-47D ΔMTcycD1 and T-47D ΔMT cells were growth-arrested in unsupplemented serum-free medium and then treated with either vehicle (Con), 50 μM $ZnSO_4$ (Zn), or insulin (Ins) at 10 μg/ml 3 or 4 days later. (*A*) Northern blot analysis of cyclin D1 mRNA in cells treated for 6 hr and percentage of cells in S phase after 24-hr treatment. The major endogenous cyclin D1 mRNA species is indicated by closed arrowheads, and the exogenous transcript by an open arrowhead. (*B*) Percentage of G_1-phase cells after treatment of T-47D ΔMTcycD1 with insulin (○) or $ZnSO_4$ (●).

DISCUSSION

In cells that express cyclin D1, regulation of cyclin D1 gene expression is a common response to either stimulation or inhibition of cell-cycle progression (2, 6, 8, 17, 29, 30). Several lines of evidence now support a key role for cyclin D1 in G_1 progression, suggesting that this regulation has functional consequences. (*i*) Inhibition of cyclin D1 expression or function (e.g., by the use of antibodies or antisense techniques) inhibits entry into S phase (28, 32), showing that cyclin D1 is necessary for completion of G_1. Inhibition of cyclin D1 expression has been demonstrated for a variety of agents that inhibit cell proliferation, including tumor necrosis factor α, interferon γ, 8-Br-cAMP, and antiestrogens (8, 29), suggesting that mechanisms for the regulation of proliferation often converge on this gene. (*ii*) Cyclin D1 is rate-limiting for progress through G_1 in cells of both fibroblast (10, 28) and epithelial origin (this study). Only two other mammalian genes have been shown to share this property: c-*myc* (33) and cyclin E (27). Cyclin E appears to regulate progress through the restriction point just before the G_1–S phase boundary (34–36). Antibodies against cyclin D1 inhibit cell-cycle progression when microinjected during mid-G_1 phase but are ineffective near the G_1–S boundary (28, 32), showing that cyclin D1 acts earlier in G_1 than cyclin E. This result is consistent with the idea that the sequential activation of different cyclins reflects functions at consecutive control points during cell-cycle progression.

The proportion of breast cancer cells initiating progress through the cell cycle after induction of ectopic cyclin D1 expression depended on the cyclin D1 level. Similarly, in fibroblasts restimulated from quiescence by low serum concentrations, a much greater proportion of the population reentered the cell cycle in cell lines overexpressing cyclin D1 than in control cells (28). These observations are consistent with data showing a relationship between endogenous cyclin D1 levels and the proportion of cells subsequently entering S phase after mitogen stimulation (2, 8, 30). Furthermore, because induction of cyclin D1 stimulated T-47D cells arrested in early G_1 phase to resume cell-cycle progression and subsequently initiate DNA synthesis, cyclin D1 is sufficient for

completion of G_1 in breast cancer cells. In contrast, increased cyclin D1 expression is not sufficient to induce DNA synthesis in quiescent fibroblasts (30). A likely explanation for the different effects of cyclin D1 induction is that breast cancer cells arrest near the beginning of G_1 phase, rather than becoming truly quiescent upon serum deprivation (19). In serum-deprived normal fibroblasts and mammary epithelium, cyclin D1 induction after mitogen stimulation is not apparent for 4–6 hr (6, 30). This is a delayed response compared with the induction of cyclin D1 in serum-deprived breast cancer cells or colony-stimulating factor 1-deprived macrophages, both of which arrest in early G_1 phase (2, 8) and suggests that cyclin D1 does not act during the initial stage of the transition from quiescence into DNA synthesis. Nevertheless, because cyclin D1 is sufficient for G_1 transit, cyclin D1 overexpression might reduce the effects of physiological growth restraints, particularly in tumor cells that are already transformed. This hypothesis is supported by the observation that both rodent fibroblasts and human breast cancer cells overexpressing cyclin D1 maintain a higher S-phase fraction upon serum deprivation than control cells (ref. 28; this study).

A region of chromosome 11 encompassing the cyclin D1 gene is one of the most frequently amplified regions in human carcinomas (12, 13). Amplification of 11q13 is associated with poor prognosis in breast cancer (37–39), suggesting the presence of a gene contributing to tumor progression within the amplicon. Furthermore, cyclin D1 is the favored candidate oncogene activated by the t(11;14)(q13;q32) translocation in a subset of B-cell lymphomas (11, 31). Fibroblasts overexpressing cyclin D1 do not display features characteristic of transformation (28). However, overexpression of cyclin D1 may contribute to the autonomous growth of a population of tumor cells, particularly in breast cancer, where the estrogen receptor-positive, hormone-responsive phenotype retains regulation of cell proliferation by steroids and growth factors (8, 18). Furthermore, aberrant expression of cyclin D1 may result in resistance to some forms of therapy—e.g., endocrine therapy—because decreased expression of cyclin D1 is a likely mechanism contributing to growth inhibition by diverse agents, including antiestrogens (8).

In summary, this study shows that regulation of cyclin D1 can change the rate of cell-cycle progression, thereby establishing a causal role for cyclin D1 in regulating proliferation in human epithelial cells and providing evidence that deregulation of this gene could contribute to loss of growth control in a range of human carcinomas, including breast cancer.

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