Cell Biology

Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 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 G1 and in the rate of transition from G2 to S phase, indicating that cyclin D1 is rate-limiting for progress through G1 phase. In cells arrested in early G1 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 of these control points is thought to be the sequential transcriptional activation of cyclin genes and consequent transient accumulation of different cyclin-dependent kinase (CDK) complexes. In synchronized or growth factor-stimulated cells, cyclins C, D1, D2, D3, and E are most abundant during G1 phase (1–3), suggesting that they function during G1. 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 G1 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 G1 cyclins, cyclins D3 and E, as cells progress through G1 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 G1 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 G1 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-
lin at 10 \( \mu g/ml \) (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 \( \mu g/ml \). Cells were cultured in serum-free medium following a described protocol (18, 19).

Expression Vectors and Transfection Procedures. The inducible expression vector pAMTcycD1 was constructed by cloning human cyclin D1 coding sequences (5) into the \( Sal I/Acc I \) site of pAMT (20). Parallel flasks of T-47D (7-2) cells were cotransfected with 50 \( \mu g \) of pAMTcycD1 or pAMT and 5 \( \mu g \) of pSV2neo, using standard calcium phosphate precipitation methods, and after 3 weeks colonies resistant to G418 at 600 \( \mu g/ml \) were trypsinized and pooled, to yield the T-47D \( \Delta MTcycD1 \) and T-47D \( \Delta MT \) 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 stathmin kinetic technique (22, 23).

RNA Isolation and Northern Blot Analysis. Replicate 150-cm² flasks of cells were treated with 50 \( \mu M \) ZnSO₄ or vehicle, and total RNA was extracted (using a guanidinium-iodoacetamide-water-isopropyl alcohol 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-cm² flasks of cells treated with 10–50 \( \mu M \) ZnSO₄ 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 \( \mu g \) 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 \( \Delta MTcycD1 \). 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. 1A). Cyclin D1 protein also increased (≈5-fold) with zinc treatment of T-47D \( \Delta MTcycD1 \) (Fig. 1C). Zinc treatment of cells transfected with vector alone (T-47D \( \Delta MT \)) had little effect on either cyclin D1 mRNA or protein abundance (Fig. 1B and D).

The effects of cyclin D1 induction on cell-cycle progression in cycling cells were determined by using T-47D \( \Delta MTcycD1 \) and T-47D \( \Delta MT \) 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. 2A). 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

![Zinc induction of cyclin D1 mRNA and protein. T-47D \( \Delta MTcycD1 \) and T-47D \( \Delta MT \) cells proliferating in serum-free medium containing insulin were untreated (UT) or treated with 50 \( \mu M \) ZnSO₄ 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 \( \Delta MTcycD1 \)) or after 6 hr (T-47D \( \Delta MT \)). 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 were done with cells cultured in the same medium supplemented with 5% fetal calf serum gave similar results.](https://www.pnas.org/content/91/12/8023.full.pdf)
phase reached a minimum that was also concentration-dependent (Fig. 4B). These data suggest that not only the rate of progression, but also the proportion of the cell population that initiates progression through G₁, 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. 4C), supporting this conclusion.

**Cyclin D₁ Induction Is Sufﬁcient for G₁ Transit in Breast Cancer Cells.** In serum-free medium lacking exogenous mitogens, T-47D cells are growth-arrested at the beginning of G₁ 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 G₁ 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 G₁ and into S phase was examined. Serum-deprived T-47D ΔMT cycD1 cells displayed a higher S-phase fraction than parental or vector-transfected cells under the same conditions (Fig. 5A) (19), presumably as a result of constitutive expression from the metallothionein promoter (Figs. 1A and 5A). 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 ΔMT cycD1 and T-47D ΔMT cells (Fig. 5). Despite the absence of exogenous growth factors, zinc induction of cyclin D₁ in T-47D ΔMT cycD1 cells arrested in early G₁ 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 G₁ phase and subsequent DNA synthesis. The rate of exit from G₁ 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 ΔMT cycD1 (Fig. 5).
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 G1 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 G1. 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 G1 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 G1–S phase boundary (34–36). Antibodies against cyclin D1 inhibit cell-cycle progression when microinjected during mid-G1 phase but are ineffective near the G1–S boundary (28, 32), showing that cyclin D1 acts earlier in G1 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 G1 phase to resume cell-cycle progression and subsequently initiate DNA synthesis, cyclin D1 is sufficient for
completion of G1 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 (and perhaps melanoma cells) are already in G1 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 G1 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 G1 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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