Cyclin D3 Is Rate-limiting for the G1/S Phase Transition in Fibroblasts*

(Received for publication, March 24, 1998)

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D-type cyclins are induced in response to mitogens and are believed to control progression through the G1 phase of the cell cycle by activating their corresponding kinase partners (cyclin-dependent kinases). To investigate the function of individual D-type cyclins we have constructed rat fibroblast lines that allow controllable overexpression of a human cyclin D3 cDNA. Overexpression of cyclin D3 led to accelerated passage through G1 in actively proliferating cells with no effect on the overall population doubling time. In cells re-entering the division cycle from a quiescent state, cyclin D3 caused an even more dramatic advancement of S phase entry. Accelerated progression through G0/G1-to-S correlated with premature phosphorylation of the pRb tumor suppressor protein and its relatives, p107 and p130. We conclude that cyclin D3 can act as a rate-limiting G1 cyclin and that this effect involves, in part, the premature phosphorylation of critical substrates.

The eukaryotic cell division cycle is regulated by the successive activation and deactivation of a family of serine/threonine kinases composed of a positive regulatory subunit, termed cyclin, and a catalytic subunit, known as a cyclin-dependent kinase (cdk).¹ The D-type cyclins (cyclins D1, D2, and D3) and their corresponding kinases, cdks 4 and 6, are believed to be involved in the regulation of the transition from the G0/G1 phase to the S phase of the cell cycle, particularly in the coupling of cell cycle progression and mitogenic signals. The high homology between the three D-type cyclins has suggested functional redundancy. On the other hand, tissue-specific expression patterns, different affinity to cdks, and the fact that sequence comparison for individual members reveals a higher degree of conservation between species than between different D-type cyclins within the same species argue for a more specific role of each D-type cyclin (1, 2). Homozygous disruption of the cyclin D1 or D2 genes, however, has a surprisingly limited effect on development of the mouse embryo and subsequent viability. Cyclin D1 knock-out mice are slightly smaller than their wild type littermates, exhibit a retinopathy, and fail to lactate because of abnormal development of the mammary gland during pregnancy (3, 4). Cyclin D2-nullizygous female mice are sterile because of the inability of ovarian granulosa cells to respond to follicle-stimulating hormone (5). Most tissues seem to develop normally in these animals, indicating the possibility that other D-type cyclins might be able to take over the function of cyclin D1 or D2. Paradoxically, however, abrogation of cyclin D1 by antibodies or antisense oligonucleotides prevents cultured fibroblasts from entering S phase (6, 7). This difference between animal and cell culture studies remains to be resolved. On the other hand, overexpression of cyclins D1 or D2 accelerates progression through G1 and reduces the dependence of fibroblasts on growth factors (7–9), suggesting that all D-type cyclins can be rate-limiting effectors of S phase entry.

The human and mouse cyclin D3 genes were originally identified by cross-hybridizing cyclin D1 probes with a mouse phage genomic library or a human cDNA library, respectively (10, 11). However, compared with cyclins D1 or D2, surprisingly little is known about the function of cyclin D3. Successful disruption of the cyclin D3 gene in mice has not been reported. The cell cycle effects of cyclin D3 overexpression have so far only been studied in hematopoietic cells where cyclin D3, like cyclin D2, leads to shortening of G1 and failure to differentiate in response to granulocyte colony-stimulating factor (12). For unknown reasons, a previous attempt to construct stable fibroblast lines constitutively overexpressing cyclin D3 failed (7). In this present study we sought to investigate the effects of cyclin D3 overexpression on the cell cycle in stable fibroblast lines. To avoid potential toxic effects of constitutive cyclin overexpression we employed an inducible expression system.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines—A vector harboring an inducible cyclin D3 (tet-cyclin D3) was constructed by cloning the 2-ki1 base EcoRI fragment of pA7R (11) into pUHD10–3 (13). tet-cyclin D3 was co-transfected with a thymidine kinase promoter-driven hygromycin plasmid into R12 cells, a rat fibroblast line harboring the tet-transactivator (9), and clones inducibly expressing cyclin D3 were selected in medium containing 350 μg/ml G418, 150 μg/ml hygromycin, and 2 μg/ml tetracycline, as described (9).

Cell Cycle Analysis—Cells were labeled with 20 μM bromodeoxyuridine (BrdUrd, Sigma) for 30 min prior to disaggregation with trypsin/EDTA and fixation in 70% ethanol. Cells were stained with a fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (Becton Dickinson) and propidium iodide (Calbiochem) as described (14), and cell cycle phase distribution was analyzed using fluorescent activated cell scanning (FACSsort, Becton Dickinson). For synchronization/re-stimulation experiments, cells were seeded in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 2 μg/ml tetracycline. After 24 h, cells were washed twice with phosphate-buffered saline and re-fed with medium containing 0.1% serum and tetracycline. After another 48 h, cells were washed twice with phosphate-buffered saline and re-fed with medium containing no serum with or without tetracycline. 24 h later, cells were re-stimulated with medium with 10% serum with or without tetracycline.

Immunobots and Kinase Assays—100 μg of cell lysate were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore). Immunoblot analysis was performed using the following antibodies: C-15 α-pRb, C-18 α-p107, C-20

* This work was supported by Public Health Service Grant GM40006 (to S. I. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Research Training Fellowship of the Deutsche Forschungsgemeinschaft.

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¹ The abbreviations used are: cdk, cyclin-dependent kinase; BrdUrd, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis; tet, tetracycline.
Acceleration of G₁ Passage by Cyclin D3

**RESULTS**

Construction of Fibroblast Lines for Inducible Overexpression of Cyclin D3—For the construction of cell lines expressing human cyclin D3 we used the tet-transactivator system developed by Gossen and Bujard (13). By stably transfecting a tet-transactivator-regulated cyclin D3 expression plasmid into R12 (9) fibroblasts we obtained three clones that were characterized by tight suppression of transgene expression in the presence of tetracycline and a high level of induction upon removal of tetracycline from the culture medium (Fig. 1A). Induction of the transgene in cells emerging from quiescence led to high levels of cyclin D3-dependent kinase activity, suggesting that the exogenous human cyclin D3 forms active kinase complexes in rat fibroblasts (Fig. 1B).

Effect of Cyclin D3 Overexpression on Cycling Cells—To determine whether cyclin D3 overexpression increases the rate of cell growth, cells were seeded at low density and grown for 5 days in either the presence or the absence of tetracycline. Fresh culture medium was provided every 48 h. Cells were harvested daily, and the cell number was determined using a hemacytometer. The average population doubling time derived from growth curves ranged between 10.5 and 15 h for the clones analyzed (Fig. 2A). We further investigated whether overexpression of cyclin D3 changes the distribution of cell cycle phases. To this end, cells were analyzed by fluorescence-activated cell sorter analysis after having grown for 3 days in the presence or the absence of tetracycline. In all three clones analyzed, overexpression of cyclin D3 led to diminution of the G₁ subpopulation as compared with noninduced control cells by 22–31% (Fig. 2B). This corresponded to a shortening of the G₁ phase by about 1.2–1.8 h (Table II).

**Overexpression of Cyclin D3 in Cells Emerging from Quiescence—**In the absence of growth factors most cells cease proliferation and enter a quiescent state termed G₀. Upon stimulation with growth factors, quiescent cells re-enter the cell cycle and progress through G₁ into S phase. D-type cyclins are induced as part of the delayed early response to growth factor stimulation. To determine whether cyclin D3 is rate-limiting for G₁ progression in cells emerging from quiescence, we synchronized cells in G₀ by withdrawal of serum for 72 h. Cells were then re-stimulated with 10% serum in either the absence or the presence of tetracycline and harvested in 3-h intervals to monitor changes in cell cycle distribution. Induction of cyclin D3 accelerated entry into S phase by 4.7–5.6 h in all three clones analyzed (Fig. 3 and Table II). Thus, cyclin D3 can act as a rate-limiting factor for the G₁/S transition in rat fibroblasts.

**Phosphorylation of Pocket Proteins—**The retinoblastoma protein pRb and its relatives p107 and p130 are believed to be critical substrates for G₁ cyclins. The phosphorylation status of these proteins can be monitored by immunoblot because of the

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**Table I**

| Clone  | Decrease in length of G₁ | In cycling cells G₁ to S | In cells emerging from quiescence G₀/G₁ to S |
|--------|--------------------------|--------------------------|--------------------------------------------|
| R12D3–1 | 1.19 ± 0.09              | 5.56 ± 1.43              |
| R12D3–2 | 1.55 ± 0.38              | 4.74 ± 0.20              |
| R12D3–3 | 1.79 ± 2.09              | 4.92 ± 0.82              |

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α-p130, and C-16 α-cyclin D3 (Santa Cruz). Cyclin D3-kinase activity was assayed in 200-μg samples of total cellular protein essentially as described (Matsushime), using the DCS28 α-cyclin D3 monoclonal antibody (kindly provided by Jiri Bartek) for immunoprecipitation of cyclin D3-kinase complexes and recombinant full-length retinoblastoma protein (QED Bioscience Inc.) as substrate.
slower migration of more highly phosphorylated species in SDS-PAGE. We investigated the effect of premature cyclin D3 expression on the phosphorylation status of pRb and its relatives. Cells were synchronized in G0 and subsequently stimulated with 10% serum. This resulted in an accelerated passage through G1 phase in actively growing fibroblasts as well as in fibroblasts emerging synchronously from G0 phase. We report here that overexpression of cyclin D3 accelerates progression through G1 phase in actively growing fibroblasts as well as in fibroblasts emerging synchronously from quiescence. Accelerated progression through G1 phase was accompanied by premature phosphorylation of pocket proteins, key substrates of G1 cyclin-dependent kinases. The overall growth rate of the cell cycle was not affected by cyclin D3 overexpression, suggesting that the accelerated passage through G1 phase is compensated for by expanding subsequent cell cycle phases.

Overexpression of cyclin D1 or cyclin E using the same experimental system was previously shown to cause a similar alteration of the cell cycle profile in asynchronous cells. Likewise, constitutive overexpression of cyclin D2 or D3 in 32Dc13 myeloid cells was shown to cause contraction of G1 phase without the concomitant increase in the population doubling time (15). Taken together, these observations suggest functional overlap among D-type cyclins and possibly even cyclin E. The phenotypic similarity of the effects of overexpression of various G1 cyclins does not, however, lead to the conclusion that all operate through identical mechanisms. If one assumes that progression through G1 is controlled by more than one rate-limiting event, a shortening of the overall passage time can be achieved through different mechanisms, possibly operating at different times within the G1 interval.

Indeed, a functional difference between cyclin D3 and cyclin E is suggested from experiments involving cells emerging synchronously from G0 phase. Whereas overexpression of cyclin E shortened G1 by about the same time interval in cycling cells versus re-entering the cycle from G0, cyclins D1 and D3 had a much more pronounced effect if overexpressed during emergence from G0. The time that quiescent cells require to enter S phase can be considered as the sum of the time needed to re-enter the cell cycle (G1→G2) and the time needed for passage through G1. In our experiments, noninduced quiescent R12 fibroblasts began to enter S phase between 12 and 15 h following re-stimulation with serum (Fig. 3), whereas the duration of G1 calculated for cycling cells ranged from 4.4 to 6.2 h. A characteristic feature of D-type cyclins that distinguishes them from other cyclins is the regulation of their accumulation by the action of growth factors. Therefore it is reasonable to
propose that cyclin D3 overexpression would have a strong impact on effectors normally targeted by mitogenic signals early during the G0/G1 transition. Cyclin E, on the other hand, appears to be equally effective in cells emerging from quiescence as in actively cycling cells, suggesting a role specific for a later interval of G1, downstream of the specific effects of mitogens. These observations, however, do not preclude that cyclin E-associated kinase may be a target of regulation during emergence from G0 (16). Nevertheless, these data, as well as evidence for distinct roles with regard to phosphorylation of the retinoblastoma protein, pRb, suggest that D-type cyclins and cyclin E stimulate passage through G1 via different mechanisms (17).

Although the effects of cyclin D3 overexpression provide further evidence for a specific cell cycle function of D-type cyclins as opposed to cyclin E, the issue of functional specificity of individual D-type cyclins still requires clarification. In the cell types and experimental systems utilized, there was no obvious difference between cyclin D1 and D3, suggesting functional redundancy. Proliferation in only a very limited number of cell types or tissues may in fact be controlled exclusively by a single D-type cyclin, an assumption that is indeed corroborated by the narrowly circumscribed defects found in cyclin D1 or D2 knockout mice (3–5). On the other hand, different D-type cyclins might moderate specific functions in other cellular processes not directly related to cell cycle control. Interestingly, overexpression of D-type cyclins in 32De3 myeloid cells showed that only cyclin D2 and D3 but not cyclin D1 can abrogate myeloid differentiation in response to granulocyte colony-stimulating factor, whereas all three conferred similar cell cycle effects in the absence of granulocyte colony-stimulating factor (12). Recently, it has been shown that cyclin D1 can activate estrogen receptor-dependent transcription independently of its kinase regulatory function, although conflicting evidence has been presented concerning the specificity of this function for any particular D-type cyclin (18, 19).

Hypophosphorylated pRb binds the transcription factor E2F and inhibits its transactivation potential. Phosphorylation of pRb in late G1, close to the restriction point, disrupts the pRb-E2F complex, thereby mobilizing the trans-activating potential of E2F (20, 21). Like pRb, the related protein p130 can be found in E2F-DNA complexes during early G1 and is the most prominent E2F-binding pocket protein in quiescent fibroblasts (22, 23). However, because pRb is the preferred substrate for D-type cyclin-dependent kinases in vitro (24), and the G1 arrest caused by abrogation of cyclin D/cdk activity by neutralizing antibodies or the cdk inhibitor p16 depends on the presence of functional pRb (25–27), it is assumed that pRb is the principal functional substrate of D-type cdks in vitro. Our observation that cyclin D3 overexpression triggers the rapid phosphorylation of pRb supports this evidence. Phosphorylation of pRb preceded S phase entry in cyclin D3-overexpressing cells by many hours and is therefore unlikely to reflect a positional effect secondary to the general acceleration of G1 progression. p130, which exhibited a similar acceleration of phosphorylation in these experiments, may well be one of the substrates that cells need to phosphorylate specifically during emergence from G0 into G1. Efficient and rapid phosphorylation of p130 may explain why cells emerging from quiescence benefit even more from overexpression of cyclin D3 than do cycling cells.

Acknowledgments—We thank A. Arnold and J. Bartek for providing plasmids and antibodies as well as members of the Reed lab for helpful discussion and technical support.

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