Overexpression of Cyclin D1 Contributes to Malignancy by Up-Regulation of Fibroblast Growth Factor Receptor 1 via the pRB/E2F Pathway

Etsu Tashiro, Hiroko Maruki, Yusuke Minato, Yuichiro Doki, I. Bernard Weinstein, and Masaya Imoto

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Yokohama 223-8522, Japan [E. T., H. M., Y. M., M. I.]; Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511, Japan [Y. D.]; and Herbert Irving Comprehensive Cancer Center and Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, New York 10032 [I. B. W.]

ABSTRACT

Overexpression of cyclin D1 due to gene rearrangement, gene amplification, or simply increased transcription occurs frequently in several types of human cancers. However, overexpression of cyclin D1 in cell culture system is insufficient, by itself, to cause malignant transformation. In the present study, we found that when rodent fibroblasts that overexpress cyclin D1, but not normal fibroblasts, were treated with basic fibroblast growth factor (bFGF), there was enhanced cell cycle progression, extracellular signal-regulated kinase 2 activation, induction of anchorage-independent growth, and enhanced invasion of a Matrigel barrier. These enhanced responses to bFGF appear to be due to increased expression of fibroblast growth factor receptor 1, at both the mRNA and protein levels, in the cyclin D1-overexpressing cells. We obtained evidence that this increase in fibroblast growth factor receptor 1 expression is mediated through cyclin D1 activation of the pRB/E2F pathway. Taken together, these results suggest that in vivo cyclin D1 overexpression can enhance tumor progression, at least in part, by potentiating the stimulatory efforts of bFGF, which is often produced by stromal cells, and the growth of adjacent tumor cells.

INTRODUCTION

The cyclin D1 gene encodes a regulatory subunit of the cdk4 and cdk6 holoenzyme complex, which phosphorylates and inactivates the tumor suppressor protein pRB as well as pRB-related proteins p107 and p130. The phosphorylation of pRB in mid/late G1 results in its inactivation and the release of E2Fs and other transcription factors that have been sequestered by the unphosphorylated (active) form of pRB. Once liberated by pRB inactivation, E2Fs then proceed to activate genes essential for advance into late G1 and S phase (2). Consistent with its growth-promoting role, cyclin D1 can act as an oncogene. Indeed, rearrangement, amplification, and/or increased expression of the cyclin D1 gene and overexpression of its mRNA have been reported in several types of human cancer, including human parathyroid adenomas; B-cell lymphomas; breast, colon, lung, bladder, and liver cancers; and squamous carcinomas of the esophagus and the head and neck (3–8). The expression of cyclin D1 mRNA and protein peaks during mid-G1, when growth factor-deprived cells are restimulated to enter the cell cycle (9–11). Inhibition of cyclin D1 expression by either antisense methodology or antibody microinjection lengthens the duration of the G1 phase and causes a reduction in proliferation and a loss of tumorigenicity in nude mice (12–16). Overexpression of cyclin D1 leads to a shortened duration of the G1 phase, decreased cell size, and reduced serum dependency in rodent fibroblasts (10, 17–19). Although the cyclin D1-overexpressing rat fibroblasts formed colonies in soft agar, these colonies were much smaller in size than the macroscopic colonies seen with c-Ha-ras-transformed rat fibroblast cells (10). In addition, efficient tumor induction in nude mice with the cyclin D1-overexpressing rat fibroblast cells required the injection of a larger number of cells and a longer latent period than in the case of c-Ha-ras-transformed rat fibroblast cells (10). Therefore, the manner in which cyclin D1 contributes to tumor development and malignant transformation is still obscure.

RTKs play an important role in cell proliferation, differentiation, and embryogenesis. RTKs activate many signaling proteins, such as the Ras/Raf/mitogen-activated protein kinase pathway, via the SH2 domain. RTKs and many of the proteins involved in RTK-dependent signal transduction can also function as oncogenes. Indeed, many RTKs were identified as proto-oncogenes to morphologically transform the mouse fibroblast cell line NIH3T3. Human tumors express high levels of growth factors and their receptors, and many types of malignant cells appear to exhibit autocrine- or paracrine-stimulated growth. The EGF receptor is frequently overexpressed in many types of tumors, and its closely related ErbB2 is overexpressed in breast, ovarian, and stomach cancers (20). The hepatoocyte growth factor receptor is also overexpressed up to 100-fold in thyroid papillary carcinoma (21, 22) when comparing normal epithelial cells with epithelial tumor cells. FGFR overexpression has been shown in brain, breast, prostate, thyroid, melanoma, and salivary gland (23) tumor samples in comparison with normal tissue by immunohistochemistry. In these FGFR-overexpressing tumor cases, one or more FGFRs are often expressed, creating the possibility for autocrine FGF signaling. Because FGFRs play important roles not only in embryonic development and cell proliferation but also in angiogenesis and wound healing (23), they might enhance malignant properties of such cells by an autocrine mechanism. However, causes for most such RTK overexpression are largely uncharacterized.

In this study, we show that cyclin D1-overexpressing fibroblasts acquire enhanced cell cycle progression, Erk2 activation, induction of anchorage-independent growth, and enhanced invasion of a Matrigel barrier in response to bFGF. These enhanced responses to bFGF appear to be due to transcriptionally increased expression of FGFR-1. We obtained evidence that this increase in FGFR-1 expression is mediated through the activation of the pRB/E2F pathway by overexpression of cyclin D1. This is the first report that the cyclin D1/pRB/E2F pathway regulates the expression of growth factor receptor.

MATERIALS AND METHODS

Cells. The mouse fibroblast cell line NIH3T3 and the rat fibroblast cell line Rat4 were maintained in DMEM with 5% FBS and in DMEM with 10% calf serum, respectively. We developed cyclin D1-overexpressing NIH3T3 cells by transfection with a mixture of the LipofectAMINE reagent (Life Technologies,
Inc.) and either the plasmid pcDNA3-human cyclin D1 or the empty vector plasmid. Transfected cells were selected by supplementing the medium with 600 μg/ml G418 (Sigma, St. Louis, MO). G418 resistant cells were isolated, and cyclin D1 expression was evaluated by Western blot analysis using an anti-cyclin D1 antibody (M-20; Santa Cruz Biotechnology, Santa Cruz, CA). The origin of Rat6 and R6ccnD1#4 cells has been described previously (10).

For cell synchronization, the cells were seeded at a density of 1.5 × 10⁶ cells in a 100-mm dish and cultured for 24 h in DMEM supplemented with 5% FBS. The cells were then cultured for an additional 48 h in 0.2% calf serum-containing medium.

Western Blot Analysis. The cells were lysed in lysis buffer [25 mm HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 m NaCl, 5 mm EDTA, 50 mm NaF, 0.1 mm sodium vanadate, 1 mm phenylmethylsulfonyl fluoride, and 0.1 mg/ml leupeptin (pH 7.8)] at 4°C with sonication. The lysates were electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech) and immunoblotted with appropriate antibodies. Detection was performed with the enhanced chemiluminescence reagent (NE5 Life Science Products, Boston, MA). The antibodies used for immunoblotting and their manufacturers are as follows: anti-cyclin D1 (M-20), anti-FGFR-1 (C-15), and anti-EFG receptor (1005) were purchased from Santa Cruz Biotechnology; anti-cyclin E (06-134) and anti-Erk2 (05-157) were purchased from Upstate Biotechnology (Lake Placid, NY); anti-prB (G3-245) was purchased from PharMingen International; and anti-tubulin was purchased from Sigma.

Northern Blot Analysis. Cellular RNA was isolated using Trizol (Life Technologies, Inc.). Twenty μg of total RNA were separated by 1% agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with [32P]dCTP-labeled FGFR-1 cDNA probe. The blots were then stripped and rehybridized with a [32P]dCTP-labeled glyceraldehyde-3-phosphate dehydrogenase probe.

Plasmid. The recovered DNA was analyzed by PCR with primers flanking the putative E2F-1 site: 5'-TTGCCCCAAATGCGTGAATCAC-3', which corresponds to −220 to −197; and 5'-TTCGGCGGGGTCCTCTGGGACAG-3', which

RESULTS

Cyclin D1-overexpressing Cells Are Sensitized to bFGF. To examine the possibility that cyclin D1 overexpression alters growth factor signaling, serum-starved Rat6 fibroblasts that overexpress cyclin D1 (R6ccnD1#4) or vector control (R6pl) cells were stimulated with several growth factors, and G₁–S-phase transitions were monitored by DNA synthesis as measured by [3H]thymidine incorporation. Serum-starved R6pl cells traversed the G₁ phase and initiated DNA synthesis at 20 h after EGF stimulation, whereas DNA synthesis was not induced by bFGF, insulin-like growth factor I, or platelet-derived growth factor, when each was tested at 1–100 ng/ml. In contrast, bFGF and EGF induced G₁ progression in serum-starved cyclin D1-overexpressing R6ccnD1#4 cells (Fig. 1A). Serum-starved cyclin D1-overexpressing NIH3T3 clones also entered S phase at 18 h in response to bFGF, as estimated by DNA synthesis (data not shown). Transition from quiescence to S phase by bFGF in R6ccnD1#4 cells was confirmed by flow cytometric analysis (Fig. 1B). Cell cycle distribution of exponentially growing R6pl cells was similar to that of R6ccnD1#4 cells (Fig. 1B), and 89.1% of R6pl cells and 82.1% of R6ccnD1#4 cells were arrested in G₁-G₂ phase after serum starvation. G₁-S-phase transitions were induced with EGF in R6ccnD1#4 cells and R6pl cells; however, only R6ccnD1#4 cells began to progress through the G₁ phase and entered S phase after bFGF stimulation. Furthermore, although EGF but not platelet-derived growth factor, insulin-like growth factor I, or bFGF induced the activation of Erk2 in control R6pl cells, as determined by the presence of a slower migratory phosphorylated form of the Erk2 protein, both EGF and bFGF induced the activation of Erk2 in R6ccnD1#4 cells (Fig. 1C). Activation of Erk2 by bFGF was also observed in the two cyclin D1-overexpressing NIH3T3 clones (clone D1#5 and D1#11), but not in vector control NIH3T3 clones (clone M3; Fig. 1D). Thus, overexpression of cyclin D1 rendered serum-starved fibroblasts responsive to bFGF.

Up-Regulation of FGFR-1 Expression by Overexpression of Cyclin D1. To explore the mechanism by which cyclin D1 overexpressing cells are sensitized to bFGF, we examined the levels of expression of the FGFR in cyclin D1-overexpressing fibroblasts by Western blot analysis. As shown in Fig. 2A, an increased level of the M₃ 115,000 FGFR-1 was observed in the three cyclin D1-overexpressing NIH3T3 clones (clones D1#5, D1#11, and D1#13) and in the cyclin D1-overexpressing R6ccnD1#4 cells when compared with the two vector control NIH3T3 clones (clones M2# and M3#) and the R6pl control cells, respectively. Furthermore, an elevated level of the FGFR-1 mRNA was detected by Northern blot analysis in the three cyclin D1-overexpressing NIH3T3 clones and in the cyclin D1-overexpressing R6ccnD1#4 cells (Fig. 2B), suggesting that FGFR-1 expression was increased at the level of transcription in the cyclin D1-overexpressing fibroblasts. The FGFR-1 gene has been reported to have several alternatively spliced variants (25). Because the molecular weight of the FGFR-1 β isoform is reported to be M₃ 115,000 (26), the FGFR-1 that is increased in cyclin D1-overexpressing fibroblasts appears to be the β isoform alternative spliced variant. Indeed, reverse transcription-PCR assays indicated that the 614-bp product corresponding to the β isoform of the FGFR-1 was detected in the cyclin D1-overexpressing NIH3T3 clones but not in the control NIH3T3 cells (data not shown). Levels of the FGFR-2 protein were also slightly increased in the cyclin D1-overexpressing NIH3T3 clones, but not in the R6ccnD1#4 cells (data not shown). FGFR-3 and FGFR-4 were not expressed at detectable levels in either cyclin

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D1-overexpressing NIH3T3 or Rat6 clones as judged by reverse transcription-PCR analysis (data not shown). In addition, there was no significant difference in the levels of expression of the EGF receptor between the cyclin D1-overexpressing clones and the vector control clones (Fig. 2). Taken together, these results indicate that overexpression of cyclin D1 induced up-regulation of FGFR-1 expression, thereby sensitizing the cells to growth stimulation by bFGF.

Activation of FGFR-1 Promoter by E2F-1. Cyclin D1 binds to and activates cdk4 and cdk6, which then phosphorylate and inactivate the tumor suppressor protein pRB (3), thereby reversing the function of the suppressive effect of pRB and the E2F-DP transcription factor complex. To delineate the mechanism of increased expression of the FGFR-1 in the cyclin D1-overexpressing fibroblasts, we examined whether FGFR-1 promoter constructs were responsive to E2F. Because the sequence and transcriptional start site of mFGFR-1 were reported previously (27), we cloned the mFGFR-1 promoter and inserted it into a luciferase reporter construct. The luciferase activity of a reporter plasmid containing 793 nucleotides upstream of the mFGFR-1 transcriptional start site (designated \( \text{mHGFR-1} \); Fig. 3A) was stimulated approximately 25-fold by coexpression with wild-type E2F-1 in NIH3T3 cells (Fig. 3B) and also in SAOS-2 human osteosarcoma cells (data not shown). Neither a DNA-binding defective E2F-1 mutant [E2F-1(1-132E); Ref. 28] nor a transcription activity-defective E2F-1 mutant [E2F-1(1–368); Ref. 29] stimulated the activity of this FGFR-1 promoter-luciferase construct (Fig. 3B). Furthermore, E2F-1-stimulated promoter activity was completely inhibited by coexpression with E2F-1(1–368), indicating that E2F-1(1–368) acts as a dominant negative inhibitor (Fig. 3C). As shown in Fig. 3A, the mFGFR-1 promoter sequence contains three putative E2F-responsive-like sequences located at positions \( 323/316 \), \( 28/35 \), and \( 54/+61 \). Truncation of this promoter did not reduce reporter activity because the shortest 5′ deletion mutant of FGFR-1 (−220/mFGFR-1) still remained fully responsive to E2F-1 (Fig. 3D), indicating that −323/316 was not an essential responsive sequence for E2F-1. To identify the E2F-1-responsive sequence in the mFGFR-1 promoter, a point mutation was introduced into the other putative E2F-responsive-like sequence, either at position +33 (C to T)
located in the +28/+35 site or at position +56 (G to T) in the +54/+61 site (designated C33T/mFGFR-1 and G56T/mFGFR-1, respectively). The C33T/mFGFR-1 construct showed a reduced response to E2F-1 (Fig. 3D), but the G56T/mFGFR-1 construct still responded to E2F-1, indicating that the responsiveness of the mFGFR-1 promoter to E2F-1 is dependent on the E2F-responsive sequence located at +28/+35. These results were confirmed by EMSAs. As shown in Fig. 3E, the E2F-1-DP1 complex bound to the putative E2F-responsive sequence located at +28/+35. E2F-1 also associated very weakly with the +54/+61 site, but it failed to bind to the −323/−316 site. We next investigated whether the mFGFR-1 promoter is activated by other members of the E2F family of transcription factors. Both E2F-2 and E2F-3 also increased the activity of the −793/mFGFR-1 promoter construct by 20–25-fold; in contrast, E2F-4 and E2F-5 failed to stimulate the activity of the −793/mFGFR-1 promoter construct (Fig. 3F). We also determined whether the human FGFR-1 promoter is also directly regulated by E2F-1. Because the sequence for the human FGFR-1 promoter was not available, we searched the HTGs database for the human FGFR-1 genomic sequence that overlaps the 5′ end of the published human FGFR-1 cDNA sequence (30), and we identified AC19307 as the human FGFR-1 genomic sequences. We then isolated a 1245-bp genomic fragment (designated human FGFR-1) from human esophageal carcinoma EC17 cells by PCR and found that the FGFR-1 promoter sequence showed 70.8% homology between mouse and human.

Fig. 3. Regulation of the mFGFR-1 promoter by E2F-1. A, schematic illustration of the mFGFR-1 promoter, its 5′ truncation, and E2F-1 binding site mutants. Putative E2F-1 binding sites are indicated. All of these mutants were confirmed by sequencing. B, activation of the FGFR-1 promoter by E2F-1. NIH3T3 cells were transfected with a mFGFR-1 promoter construct (1 μg) and HA-tagged E2F-1 plasmid or HA-tagged mutant E2F-1 plasmids (triangle represents 0, 0.01, and 0.1 μg). C, inhibition of E2F-1-induced FGFR-1 promoter activity by a transcription activity-defective E2F-1 mutant. NIH3T3 cells were transfected with the mFGFR-1 promoter construct (1 μg), HA-tagged E2F-1 plasmid (0.01 μg), and HA-tagged E2F-1(1–368) plasmid (0.1, 0.3 μg). D, response of mutant promoters to E2F-1. NIH3T3 cells were cotransfected with the progressively deleted site-directed mutated mFGFR-1 luciferase reporter plasmids (1 μg) along with HA-tagged E2F-1 plasmids (triangle represents 0, 0.01, and 0.1 μg). E, EMSA assays. Nuclear extracts from 293T cells that were cotransfected with HA-tagged E2F-1 and HA-tagged DP1 plasmids incubated with a 32P-labeled DNA probe corresponding to −323 to −316, +28 to +35, or +54 to +61 of the mFGFR-1 promoter region in the presence or absence of each competitor. *, E2F-1-DP1 complex; **, supershifted by anti-E2F-1 antibody. F, effect of the E2F family of transcription factors on mouse FGFR promoter activation. NIH3T3 cells were transfected with the mFGFR-1 promoter construct (1 μg) and the indicated HA-tagged E2F plasmids (0.1 μg). G, response of human FGFR-1 promoter to E2F-1. NIH3T3 cells were transfected with the −904/human FGFR-1 construct (1 μg) and the HA-tagged E2F-1 plasmid or the HA-tagged mutant E2F-1 plasmids (triangle represents 0, 0.01, and 0.1 μg).
FGFR-1 does not include the sequence corresponding to the +28/+35 E2F-1 binding site that we identified in the mFGFR-1 promoter, it does include three putative E2F-binding sites. A transient cotransfection experiment revealed that the −904/human FGFR-1 reporter was strongly transcribed in NIH3T3 cells in response to E2F-1 in a dose-dependent manner (Fig. 3G), indicating that E2F-1 activates the human FGFR-1 promoter as well as the mFGFR-1 promoter.

**Regulation of FGFR-1 Expression by the Cyclin D1/pRB/E2F-1 Pathway.** To demonstrate that the increased mFGFR-1 expression seen in cyclin D1-overexpressing cells is regulated by the pRB/E2F-1 pathway, the status of pRB phosphorylation in cyclin D1-overexpressing cells was determined by Western blot analysis. Hyperphosphorylated pRB was much more prominent in two cyclin D1-overexpressing clones of NIH3T3 cells and cyclin D1-overexpressing Rat6 cells than in their respective vector control clones (Fig. 4A; data not shown), suggesting that pRB was inactivated in the cyclin D1-overexpressing clones. Furthermore, among E2F-1 target gene products, cyclin E (31–33) and Cdc25A (34, 35), but not proliferating cell nuclear antigen (36), were expressed at high levels in cyclin D1-overexpressing clones (data not shown). In addition, the −793/mFGFR-1 promoter-luciferase construct was transiently transfected into cyclin D1-overexpressing NIH3T3 clones and vector control clones. Luciferase activity was 3–4-fold higher in the cyclin D1-overexpressing cells than in the control clones (Fig. 4B). The enhanced reporter activity seen in the cyclin D1-overexpressing clone was markedly inhibited by cotransfection with dominant negative E2F-1 mutant E2F-1(1–368), providing evidence that E2F-1 was responsible for mFGFR-1 promoter activation (Fig. 4B). Taken together, these results suggest that the cyclin D1-pRB/E2F-1 pathway is responsible for the elevated levels of mFGFR-1 expression in cyclin D1-overexpressing clones. To substantiate this hypothesis, we sought evidence that E2F-1 can bind to and thereby influence the activity of the endogenous mFGFR-1 promoter. ChIP assays were performed using an antibody against E2F-1, and the precipitated DNA was examined for the presence of mFGFR-1 promoter sequences using PCR and Southern blotting. The data shown in Fig. 4C provide evidence for an interaction between E2F-1 and the relevant domain of the mFGFR-1 promoter in the cyclin D1-overexpressing NIH3T3 clone D1#5 (Fig. 4C).

**Regulation of FGFR-1 Expression by Cyclin D1/pRB/E2F-1 Pathway under Physiological Conditions.** Because our finding indicated that FGFR-1 expression results from activation of cyclin D1/cdk4, inactivation of pRB, and therefore release of transcriptionally active E2F-1 in cyclin D1-overexpressing fibroblasts, we next examined whether this mechanism would operate under physiological conditions. We asked whether FGFR-1 expression is induced in a cell cycle-dependent manner in normal cells. To examine the expression levels of FGFR-1 during G1 phase, extracts of serum-starved and restimulated parental NIH3T3 cells were collected at various time points up to 20 h after release from quiescence and examined by Western blot analysis. As shown in Fig. 5A, there was increased expression of M1, 115,000 FGFR-1 at 16 h after release from serum stimulation. The time point of M1, 115,000 FGFR-1 expression was similar to that of cyclin E, which is known to be regulated by E2F-1 (31–33). Expression of cyclin D1 and the hyperphosphorylated form of pRB occurred earlier than induction of FGFR-1 and cyclin E. Because serum-starved NIH3T3 cells began to enter S phase at about 20 h after serum stimulation, the expression of FGFR-1 was induced at mid-G1 phase. In serum-starved Rat6pl cells, at 16 h after serum addition, increased expression of FGFR-1 was also observed (Fig. 5B). Thus, FGFR-1 was expressed in mid-G1 phase possibly through the cyclin D1/pRB/E2F-1 pathway in normal fibroblasts under more physiological conditions.

**Induction of Anchorage-independent Growth of Cyclin D1-overexpressing Cells by bFGF.** The cyclin D1-overexpressing NIH3T3 clones displayed morphological evidence of a transformed phenotype after stimulation with bFGF, but not after stimulation with EGF (data not shown). Therefore, we examined whether bFGF stimulated anchorage-independent growth of the cyclin D1-overexpressing fibroblasts because this is a hallmark of malignantly transformed...
at a magnification of a Matrigel-coated filter to the lower surface were stained and counted under a microscope with or without bFGF in the lower chamber for 12 h. The cells that invaded through the compartment of the chamber in the presence or absence of bFGF (100 ng/ml) and incubated overexpressing NIH3T3 clones or vector control clones were added to the upper compartment of the chamber in the presence or absence of bFGF (100 ng/ml) and incubated with or without bFGF in the lower chamber for 12 h. The cells that invaded through the Matrigel-coated filter to the lower surface were stained and counted under a microscope at a magnification of ×600.

Three cyclin D1-overexpressing NIH3T3 clones and two control clones were seeded on dishes coated with an antiadhesive polymer, PolyHEMA (37), and grown in 1% FBS medium with or without the indicated growth factors for 4 days. Colonies were then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (B). C, invasion assay. Cyclin D1-overexpressing NIH3T3 clones or vector control clones were added to the upper compartment of the chamber in the presence or absence of bFGF (100 ng/ml) and incubated with or without bFGF in the lower chamber for 12 h. The cells that invaded through the Matrigel-coated filter to the lower surface were stained and counted under a microscope at a magnification of ×600.

Fig. 6. bFGF induces malignant properties in cyclin D1-overexpressing fibroblasts. A and B, anchorage-independent growth. Cyclin D1-overexpressing NIH3T3 clones and vector control clones were seeded on PolyHEMA-coated culture dishes containing 1% FBS medium in the presence or absence of bFGF (100 ng/ml) or EGF (100 ng/ml). After 4 days of culture, colony formation was observed (A) and assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (B). C, invasion assay. Cyclin D1-overexpressing NIH3T3 clones or vector control clones were added to the upper compartment of the chamber in the presence or absence of bFGF (100 ng/ml) and incubated with or without bFGF in the lower chamber for 12 h. The cells that invaded through the Matrigel-coated filter to the lower surface were stained and counted under a microscope at a magnification of ×600.

In the present study, we found that overexpression of cyclin D1 rendered quiescent rodent fibroblasts responsive to bFGF, and there was enhanced cell cycle progression and Erk2 activation in response to bFGF. In addition, expression of FGFR-1 was increased in cyclin D1-overexpressing fibroblasts at both the mRNA and protein levels when compared with normal cells. FGFR-2 was also slightly increased in cyclin D1-overexpressing mouse fibroblasts, but not in cyclin D1-overexpressing rat cells, and FGFR-3 and FGFR-4 were not detected in any of these cells. Therefore, we propose that overexpression of cyclin D1 stimulates the expression of FGFR-1 transcriptionally, thereby sensitizing cells to bFGF.

Although overexpression of cyclin D1 can provoke a perturbed progression of the G1 phase of cell cycle (10, 17, 19), it is insufficient to confer transformed properties on primary or established fibroblasts. Nevertheless, cyclin D1 overexpression might be expected to cooperate with other proto-oncogenes in transformation. This prediction has been confirmed in several systems. Cyclin D1 can cooperate with the Ras oncogene to transform primary baby rat kidney cells (1) and rat embryo fibroblasts (2), and it can also cooperate with c-Myc to induce B-cell lymphomas in transgenic mice (38). Moreover, it was shown that Ras requires cyclin D1 to transform mammary gland epithelial cells (39). Our finding that stimulation of cyclin D1-overexpressing fibroblasts with bFGF induces anchorage-independent growth and enhances cellular invasion demonstrates that overexpression of cyclin D1 can cooperate with bFGF to cause further progression of transformation in established fibroblasts through induction of increased expression of FGFR-1. It is unlikely that the changes we observed in the cyclin D1-overexpressing cells are due to spontaneous clonal variation because we examined both a cyclin D1-overexpressing Rat6 clone and three cyclin D1-overexpressing NIH3T3 clones, together with their respective vector control clones. Cell transformation upon bFGF addition has been described in fibroblasts overexpressing FGFR-1 (40). Moreover, it has recently become apparent that subtle changes in the receptor transcript, resulting from the alternative use of different exons, can dramatically alter the structure and function of growth factor receptors. Among several alternatively spliced variants of FGFR-1, the β isoform of FGFR-1 is increased in cyclin D1-overexpressing cells. Although the mechanism underlying increased expression of the FGFR-1 β isoform is not known, elevated expression of the β isoform, but not the α isoform, has been reported in several malignant tumors (41, 42).

The present studies also demonstrate that transcription of human FGFR-1 or mFGFR-1 is regulated by E2F-1 through an E2F-1 binding site within the promoter region of these two genes. Previous studies indicate that E2F-1-mediated transcription is stimulated by an E2F-1 regulatory loop that amplifies expression of the E2F-1 gene itself (43–45). Overexpression of cyclin D1 induces hyperphosphorylation of pRB, thus resulting in activation of this positive feedback loop of E2F-1-mediated transcription. Because expression levels of cyclin E were increased in cyclin D1-overexpressing clones, and cyclin E/Cdk2 activity was also increased in cyclin D1-overexpressing clones (data not shown), activation of the pRB/E2F-1 pathway mediated by overexpression of cyclin D1 might induce up-regulation of the cyclin E/Cdk2 complex, resulting in full inactivation of pRB and hence robust liberation of E2F-1 leading to activation of FGFR-1 transcription. This mechanism apparently explains our finding that FGFR-1, but not the EGF receptor, is expressed at increased levels in cyclin D1-overexpressing cells. The latter finding, in turn, explains our finding that treatment of cyclin D1-overexpressing cells with bFGF, but not with EGF, markedly enhances their transformed phenotype. Additional experiments are in progress using the human FGFR-1 gene.
and several mutants, including E2F-1 dominant negative mutants, and human cultured cells.

The pRB/E2F pathway has been shown to be deregulated in a large majority of human tumors. The importance of the pRB/E2F pathway in normal growth control is emphasized by the frequent loss of pRB functions or mutation of upstream regulators of pRB (e.g., cyclin D1, cdk4, or p16INK4a) in human tumors. Thus, although mutations in E2F have never been detected in human tumors, E2F might be a key regulator not only for inducing S-phase entry but also in tumor development. Most, if not all, of the E2F-targeted genes are growth/ cell cycle-regulated genes involved in control of the cell cycle (e.g., cdk2, cdk4, cdc25A, and cyclins A, D, and E), DNA synthesis (e.g., proliferating cell nuclear antigen, DNA polymerase α, and ribonucleotide reductase), or nuclear transcription factor (c-myc, N-myc, and B-myb). More recently, p19ARF (46), the p73 homologue of p53 (47, 48), and Apaf-1 (49) were also identified as E2F-1-targeted genes, which indicate p53-dependent or -independent apoptosis. However, growth factor or growth factor receptor genes have not yet been reported as E2F-targeted genes. In this study, we demonstrate that FGF-1 represents a new class of genes targeted by E2F. Deregulated expression of various growth factor receptors is also seen in many types of human tumors, and it frequently correlates with both tumor progression and a poor prognosis. Therefore, our findings may provide new insights into the functional significance of the pRB/E2F pathway in regulating growth factor receptor expression and its coupling to enhanced cell growth and tumorigenesis.

There is increasing evidence that in vivo the malignant state emerges from complex interactions in the tumor-host microenvironment, in which the host participates in the induction, selection, and expansion of neoplastic cells (50). Because bFGF can stimulate growth and stromal invasion by tumor cells (50), it is likely that in vivo the extracellular bFGF produced by stromal cells (51) or the locally activated host microenvironment might also enhance the proliferative and invasive behavior of cyclin D1-overexpressing tumor cells because of their increased expression of FGFR-1, thereby enhancing tumor progression.

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