Induction of Anchorage-independent Growth by Transforming Growth Factor-β Linked to Anchorage-independent Expression of Cyclin D1*

(Received for publication, October 26, 1999, and in revised form, January 11, 2000)

Xiaoyun Zhu†, Eric Scharf‡, and Richard K. Asoian¶

From the Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6087

Transforming growth factor-β (TGF-β) was originally identified, characterized, and named on the basis of its ability to induce anchorage-independent growth (phenotypic transformation). This effect has received little attention in recent years, probably because the induction of anchorage-independent growth by TGF-β has been observed only in a few cell lines, of which NRK fibroblasts are among the best studied. We have previously reported that normal rat kidney cells have lost their normal adhesion requirement for expression of cyclin D1, and we now show that this loss is causal for the induction of anchorage-independent growth by TGF-β. First, we show that TGF-β fails to induce anchorage-independent growth in NIH-3T3 cells and human fibroblasts that have retained their adhesion requirement for expression of cyclin D1. Second, we show that TGF-β complements rather than affects cyclin D-cdk4/6 kinase activity in NRK cells. Third, we show that forced expression of cyclin D1 in suspended 3T3 cells renders them susceptible to transformation by TGF-β. These results may explain why the induction of anchorage-independent growth by TGF-β is a rare event and yet also describe a molecular scenario in which the mesenchymal response to TGF-β could indeed involve the acquisition of an anchorage-independent phenotype.

The rate of cell proliferation is normally controlled by the duration of G1 phase, and two cyclin-dependent kinases (cdks), cyclin D1-cdk4/6 and cyclin E-cdk2, regulate progression through this phase of the cell cycle. The induction of cyclin D1 is the rate-limiting step for activation of cyclin D-cdk4/6 (1). The down-regulation of the Cip/Kip family of cdk inhibitors (p21cip1 and p27kip1 in particular) and sequestration of these inhibitors by cyclin D-cdk4/6 is thought to control the activation of cyclin E-cdk2 (2). Once activated, cyclin D-cdk4/6 and cyclin E-cdk2 phosphorylate the retinoblastoma protein (pRb), allowing for the dissociation of bound E2F and the induction of E2F-regulated genes, among which is cyclin A (1, 3, 4). Cyclin A binds to cdk2, and the formation of active cyclin A-cdk2 is required for entry into the S phase of the cell cycle (5).

In non-transformed cells, G1 phase cyclin-cdk activity is regulated by mitogenic growth factors and cell anchorage to the extracellular matrix. We and others (6–10) have shown that the expression of cyclin D1, the down-regulation of Cip/Kips, the activation of cyclin E-cdk2, the phosphorylation of pRb, and the expression of cyclin A are all jointly regulated by growth factor and extracellular matrix signals in non-transformed cells. Thus, normal fibroblasts and most fibroblastic cell lines cultured in suspension fail to induce cyclin D1 or down-regulate p21cip1 and p27kip1 even when exposed to optimal concentrations of mitogens. In contrast, oncogene-transformed cells typically do not require exogenous growth factors or cell anchorage for proliferation. These phenotypes have been termed mitogen-independent and anchorage-independent growth, respectively. Anchorage-independent growth is a particularly good correlate to tumorigenicity in vivo (11).

Early studies with the cytokine TGF-β indicated that it would induce anchorage-independent growth phenotypically (12, 13), but many subsequent investigations have shown that TGF-β is most often a proliferation inhibitor, especially in epithelial cells (14–18). Indeed, the ability of TGF-β to induce anchorage-independent growth is restricted to a few select fibroblast cell lines; NRK fibroblasts are among the best studied in this regard. The fact that this transforming effect of TGF-β is not generally observable clearly reduced interest in this potential aspect of TGF-β biology.

Anchorage-independent growth reflects the fact that cells have lost their adhesion requirement for activation of the G1 phase cyclin-cdkAs. Because both cyclin D-cdk4/6 and cyclin E-cdk2 activities are anchorage-dependent (19), and because both are required for efficient progression through the G1 phase (see above), cells can lose their adhesion requirement for activation of one of those enzymes and still retain the anchorage-dependent for proliferation. For example, we have previously shown that NRK fibroblasts have lost their adhesion requirement for expression of cyclin D1 and yet retain the anchorage-dependent phenotype for growth (6, 20). We now show that loss of the anchorage requirement for cyclin D1 expression, although non-transforming in itself, renders cells susceptible to transformation by TGF-β. Our results can explain why the induction of anchorage-independent growth by TGF-β is a rare event, and yet they also describe a molecular scenario in which the mesenchymal response to TGF-β could indeed involve acquisition of an anchorage-independent phenotype.

EXPERIMENTAL PROCEDURES

Cell Culture—NRK (clone 49F), NIH-3T3 cells, and early passage cultures of human foreskin fibroblasts were cultured and rendered...
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for experiments, cells were trypsinized and reseeded in monolayer (uncoated) or suspension (agar or agarose-coated) tissue culture dishes using 10³ cells in 10 ml of medium (5–10% FCS–DMEM/100-mm dish or 2–3 × 10⁶ cells in 20 ml of medium/150-mm dish. Recombinant EGF and TGF-β1 (both from Life Technologies, Inc.) were added to the cultures at final concentrations of 2 nM and 100 pM, respectively. Anchorage-independent growth was assessed by incorporation of [²H]thymidine into trichloroacetic acid-insoluble radioactivity and by colony formation in soft agar (21, 22).

NIH-3T3 cells were transfected with human cyclin D1 using the tetracycline-repressible expression system. The transfected cells were cultured in the presence of tetracycline (added daily to a final concentration of 2 μg/ml), and stable transfectants were isolated by selection in G418 (0.5 mg/ml) and hygromycin (0.4 mg/ml). Immunoblotting identified several clones in which the expression of cyclin D1 was strongly regulated by tetracycline. Tet-cyclin D1–3T3 cells were maintained in G418 (0.5 mg/ml) and hygromycin (0.4 mg/ml). Immunoblotting identified several clones in which the expression of cyclin D1 was strongly regulated by tetracycline. Tet-cyclin D1–3T3 cells were maintained at <50% confluence in DMEM, 10% calf serum, and 2 μg/ml tetracycline, added daily. G418 and hygromycin were replaced weekly.

**Extractions and Analyses**—Cells were collected by scraping (monolayer cultures) or low speed centrifugation (suspension cultures), washed 2–3 times in phosphate-buffered saline, and extracted in TNE (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, and 10 mM sodium orthovanadate; ~50 μg/10⁶ cells) in preparation for immunoblotting or in TWEEN-containing buffer (~50 μl–5 × 10⁶ cells) in preparation for cdk4 immunoprecipitations and kinase assays using GST-pRb as substrate (23).

The protein concentration of cell extracts was determined by Coomassie binding (Bio-Rad protein assay), and 100-μg aliquots were fractionated on reducing SDS-gels (typically 7.5% acrylamide) prior to electrophoretic transfer to nitrocellulose and immunoblotting using antibodies specific for pRb (Ciba-Corning), cyclin D1 (Upstate Biotechnology), cdk4 (Santa Cruz Laboratories) and cyclin A (prepared in this laboratory). Enhanced chemiluminescence (Amersham Pharmacia Biotech) was used to visualize the bound antibodies.

**RESULTS AND DISCUSSION**

NRK fibroblasts are anchorage-dependent when cultured in suspension with mitogen (serum and EGF)-containing medium, but they are rendered anchorage-independent by exposure to mitogens and TGF-β (Fig. 1A). Even though they are anchorage-dependent, serum/EGF-stimulated NRK cells suspensions readily expressed cyclin D1 (Fig. 1B). In contrast, NIH-3T3 cells and normal human fibroblasts, which are resistant to the transforming effect of TGF-β and NRK fibroblasts (6), we observed with parental NIH-3T3 cells and normal human fibroblasts (h-fib) incubated on agar-coated 35-mm dishes (suspension culture) with mitogen (5–10% FCS, 2 nM EGF) in the presence and absence of 100 μM TGF-β. After 4 days, [²H]thymidine was added to each culture for 24 h. Cells were collected, and the induction of anchorage-independent growth was measured by incorporation of radiolabel into newly synthesized DNA. Shorter and longer incubations did not change the outcome of this experiment (data not shown). In panel B, quiescent cells were prepared and reseeded in monolayer (Mn) or suspension (Sp) with mitogens (5–10% FCS, 2 mM EGF) for 18–24 h. Extracts were prepared and analyzed by immunoblotting to assess the expression of cyclin D1. Blotting with anti-cdk4 was used as a loading control.

In panel A, serum-starved NRK and NIH-3T3 cells and normal human skin fibroblasts (h-fib) were incubated on agar-coated 35-mm dishes (suspension culture) with mitogen (5–10% FCS, 2 nM EGF) for 18–24 h. Extracts were prepared and analyzed by immunoblotting to assess the expression of cyclin D1. Blotting with anti-cdk4 was used as a loading control.

**Fig. 1.** Induction of anchorage-independent growth by TGF-β is associated with anchorage-independent expression of cyclin D1. In panel A, serum-starved NRK and NIH-3T3 cells and normal human skin fibroblasts (h-fib) were incubated on agar-coated 35-mm dishes (suspension culture) with mitogen (5–10% FCS, 2 nM EGF) for 18–24 h. Extracts were prepared and analyzed by immunoblotting to assess the expression of cyclin D1. Blotting with anti-cdk4 was used as a loading control.

The murine cyclin D1 cDNA was cloned into a tetracycline-regulated expression vector and transfected into NIH-3T3 cells. Stable transfectants were selected in the presence of tetracycline and then cultured in monolayer or suspension with mitogens. In contrast to their adherent counterparts (Fig. 3A, Mono + tetracycline), mitogen-stimulated transfectants cultured in suspension with tetracycline poorly expressed cyclin D1, containing hypophosphorylated pRb, and poorly expressed cyclin A (Fig. 3A, Susp + tetracycline). This is the same phenotype we observed with parental NIH-3T3 cells and normal human fibroblasts (6).

Removal of tetracycline allowed for expression of cyclin D1 in suspended tet-cyclin D1–3T3 cells, and the expression of cyclin D1 was associated with pRb phosphorylation and increased cyclin A expression in short-term (18–24 h) suspension cultures (Fig. 3A, Susp + tetracycline). This result supports previous studies indicating that anchorage-dependent expression of cyclin D1 controls anchorage-independent pRb phosphorylation and pRb-dependent cyclin A expression (6, 8, 9). However, in long-term (>1 day) suspension cultures stimulated with mitogens (serum and EGF), cyclin D1 expression was maintained while pRb phosphorylation and cyclin A expression declined (Fig. 3B). The short- and long-term phenotypes of induced tet-cyclin D1–3T3 cells are the same as those observed in other...
NRK cells (refer to Fig. 2, A and C; cells cultured without TGF-β for 1 and 3 days). Thus, by forcing anchorage-independent expression of cyclin D1, we engineered the NRK phenotype into 3T3 cells.

We assessed anchorage-independent growth of the tet-cyclin D1–3T3 cells by measuring both [3H]thymidine incorporation into newly synthesized DNA (Fig. 4A) and colony formation in soft agar (Fig. 4B). Transfectants cultured with tetracycline were anchorage-dependent when treated with mitogens (serum and EGF) or mitogens and TGF-β. Removal of tetracycline allowed for the expression of cyclin D1 (not shown, but refer to Fig. 3), but cyclin D1 expression poorly stimulated anchorage-independent growth of the mitogen-treated cells. Thus, as with NRK cells, expression of cyclin D1 is not sufficient to induce a strong anchorage-independent phenotype. However, the mitogen-stimulated 3T3 transfectants did become anchorage-independent when also treated with TGF-β (Fig. 4, A and B). In fact, the phenotype of induced tet-cyclin D1–3T3 cells resembles that of NRK cells, whereas the phenotype of uninduced tet-cyclin D1–3T3 cells resembles that of parental 3T3 cells (compare Figs. 4 and 1A). Three independent clones of tet-cyclin D1–3T3 cells have given similar results using both [3H]thymidine-based and colony formation-based assays for anchorage-independent growth (not shown). We conclude that TGF-β fails to induce anchorage-independent growth in normal fibroblasts because they are adhesion-dependent for expression of cyclin D1. An extrapolation of our results is that loss of adhesion-dependent cyclin D1 expression is a prerequisite for anchorage-independent growth in fibroblasts.
cell transformation in response to TGF-β. Several years ago, Massagué and co-workers (13) showed that Balb/c 3T3 cells could be rendered responsive to TGF-β if the cells were exposed to insulin-like growth factor activity as well as serum and EGF. Because we now know that cyclin D1 expression is strongly mitogen-dependent (1), the inclusion of insulin-like growth factor activity may have overcome an adhesion requirement for cyclin D1 expression in suspended Balb/c 3T3 cells.

The results shown here raise the question of how TGF-β complements cyclin D1 expression and cyclin D-cdk4/6 activity to induce anchorage-independent growth. We considered the possibility that TGF-β was stimulating long-term cyclin A expression through a pRb-dependent mechanism by enhancing cyclin E-cdk2 activity (e.g. by affecting expression of the Cip/Kip cdk inhibitors). We have detected an increase in cyclin E-cdk2 kinase activity when mitogen-treated NRK cell suspensions are treated with TGF-β (data not shown). However, the loss of cell cycle synchrony in these necessarily long-term cultures has precluded a satisfactory mechanistic examination of this issue. TGF-β could also be regulating cyclin A expression independently of effects on cyclin-cdks, perhaps by activating the non-pRb/E2F sites in the cyclin A promoter (24–27). Resolution of these issues will require further study.

Our results indicate that anchorage-independent expression of cyclin D1 is a prerequisite for induction of anchorage-independent growth by TGF-β. This effect would probably be insufficient to confer TGF-β sensitivity in epithelial cells where TGF-β has been reported to strongly increase p15\(^{ink4b}\), cdk25A, or p21\(^{cip1/cdc25A}\), or p21\(^{cip1}\) (15–18). Nevertheless, the results shown here provide a proof of principle, explaining the long-standing observation that TGF-β potently induces anchorage-independence in certain cell lines while having no effect on others. If anchorage-independent cyclin D1 expression occurs physiologically (e.g. at sites of injury) or pathologically (e.g. in cells that overexpress cyclin D1), then induction of anchorage-independent growth may be a specialized response to TGF-β.

Acknowledgments—We thank Charles Sherr for NIH-3T3 cells and the cyclin D1 and GST-pRb plasmids.

REFERENCES
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doi: 10.1074/jbc.275.10.6703

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