We have previously shown that the expression of α5β1 integrin on the cell surface is dependent upon cell adhesion to the extracellular matrix, and we report here that transforming growth factor-β (TGF-β) overcomes this requirement in normal rat kidney (NRK) fibroblasts. Thus, suspended NRK cells treated with TGF-β show levels of surface α5β1 integrin that are equivalent to those seen in adherent cells. Moreover, several experiments showed that this effect is necessary for the induction of anchorage-independent growth by TGF-β. First, a kinetic analysis showed that surface expression of α5β1 integrin was restored in TGF-β-treated NRK cells prior to the induction of anchorage-independent growth. Second, NRK cell mutants that have lost their TGF-β requirement for surface expression of α5β1 integrin were anchorage-independent in the absence of TGF-β. Third, an antisense oligonucleotide to the β1 integrin subunit or, fourth, stable expression of an α5 antisense cDNA blocked the ability of TGF-β to stimulate anchorage-independent growth. Thus, TGF-β overcomes the adhesion requirement for surface expression of α5β1 integrin in NRK cells, and this effect is necessary for the induction of anchorage-independent growth.

The proliferation of normal cells is dependent upon cell adhesion to a substratum; this phenotype has been termed anchorage dependence. Cell anchorage to substratum is mediated largely by the interaction of cell-surface integrins with the extracellular matrix, and it now seems clear that the anchorage-dependent phenotype reflects the fact that extracellular matrix/integrin-mediated signaling (in cooperation with growth factor/receptor tyrosine kinase-mediated signaling) is required for proliferation through the G1 phase of the cell cycle (1, 2). In contrast, most transformed cells have lost their adhesion requirement for proliferation. This phenotype is termed anchorage independence and is thought to occur because the signaling events normally stimulated by cell adhesion have become constitutively activated. Anchorage-independent growth is an excellent correlate to tumorigenicity in vivo (3, 4).

In addition to initiating growth stimulatory signal transduction cascades, cell adhesion to the extracellular matrix stabilizes the expression of integrins on the cell surface (5-7). Pre-existing surface integrins are internalized and degraded within lysosomes if cells are detached from their substratum. Attachment to substratum also permits surface expression of newly synthesized integrins. We have suggested that this down-regulation of surface integrins may contribute to the anchorage-dependent phenotype by limiting integrin-dependent signaling in suspended cells (5, 6).

Several studies have shown that the adhesive properties of cells are altered when they are exposed to transforming growth factor-β (TGF-β).1 TGF-β typically decreases the expression of matrix-degrading proteases and increases the expression of matrix proteins, integrins, and inhibitors of matrix-degrading proteases (8). TGF-β is most often a negative regulator of cell proliferation (9-12), but it also stimulates anchorage-independent growth of certain fibroblastic cell lines. In NRK fibroblasts, TGF-β cooperates with mitogens (typically serum and EGF or transforming growth factor-α) to induce vigorous colony formation of NRK cells in soft agar. Several years ago, Ignotz and Massagué (13) reported that RGD peptides (which block the binding of several extracellular matrix proteins to their integrin receptors) block the induction of anchorage-independent growth by TGF-β and that fibronectin could replace TGF-β to induce anchorage-independent growth of NRK cells. Since TGF-β stimulates the synthesis of fibronectin, these authors proposed that TGF-β induced anchorage independence by stimulating the secretion of fibronectin, which, in turn, would bind to and activate α5β1 integrin. However, others found that purified fibronectin would not substitute for TGF-β (14), and then we reported that α5β1 integrin is not expressed on the surface of suspended NRK cells (see above). These results are not compatible with the specifics of the original model, but the inhibitory effect of RGD on NRK cell colony formation remains a compelling result that implicates integrins in the transforming effect of TGF-β.

Grotendorst and co-workers (15) have shown that TGF-β induces synthesis of connective tissue growth factor and that this effect is necessary but not sufficient for induction of NRK

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1 The abbreviations used are: TGF-β, transforming growth factor-β; NRK, normal rat kidney; EGF, epidermal growth factor; FCS, fetal calf serum; EMS, ethylmethane sulfonate.
CELL ANCHORAGE-INDEPENDENT GROWTH BY TGF-β. Connective tissue growth factor also stimulates the expression of fibronectin, collagen, and αβ1 integrin in adherent NRK cells (16), indicating that it is a likely effector of the TGF-β signal in this system. However, these studies do not address the functional significance of the matrix or αβ1 integrin effects on anchorage-independent growth. To resolve the relationship between integrin expression and induction of anchorage-independent growth by TGF-β, we developed a system that allowed us to examine the effects of TGF-β on surface integrin expression and anchorage-independent growth simultaneously and within the same cell. We report here that TGF-β overrides the adhesion requirement for surface expression of αβ1 integrin in NRK fibroblasts and that this effect is necessary for the induction of anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Cell Culture and Assessment of Anchorage-independent Growth—Near confluent asynchronous NRK fibroblasts (clone 49F) were trypsinized, plated at half-confluence, and cultured for 24 h with Dulbecco’s modified Eagle’s medium and 5% FCS. The cells were then re-trypsinized and re-plated in 100-mm tissue culture dishes (monolayer cultures) or 100-mm agar-coated dishes (suspension cultures) using 6×10^6 cells in 10 ml of 5% FCS and 2×10^6 EGF × 100 pm TGF-β1 (5). α5-Antisense and α5-control (sense) NRK transfectants were cultured under the same conditions used for parental NRK cells. Proliferation of suspended cells in preparative suspension culture was assessed by incorporation of [3H]thymidine into DNA (trichloroacetic acid-insoluble radioactivity) essentially as described (17). In some experiments, the cultures labeled with [3H]thymidine contained 0.5% or 1% methylicellulose to inhibit diffusion of daughter cells. Colony formation in soft agar was determined as described (18). Recombinant TGF-β1 and purified EGF were purchased from Life Technologies, Inc.

Surface Radiolabeling and Immunoprecipitation of Integrins—Cells were collected from monolayer or suspension cultures, and cell-surface proteins were radioiodinated as described (5). Labeled cells were extracted in lysis buffer A (0.1 M Tris-HCl, pH 8.5, 0.15 M NaCl, 0.5 mM MgCl2, and 0.5% Nonidet P-40), and 5–10×10^6 cells (for anti-integrin antibodies) were brought to 0.5 ml with lysis buffer A and incubated with 2–4 μl of anti-integrin antibodies. Rabbit anti-α5 antibody was prepared in our laboratory; rabbit anti-α5 antibody was a generous gift from E. Marcantonio; and mouse anti-αβ1 monoclonal antibody was a generous gift from S. Carbone. Immune complexes containing rabbit and murine antibodies were collected with 25–50 μl of Pansorbin (Calbiochem) and anti-mouse IgG-Sepharose (Sigma), respectively. Conditions for the immunoprecipitations have been previously described (5, 6), except for the mouse anti-rabbit, αβ1 monoclonal antibody. In this case, the incubations were performed at 4°C for 2 h (primary antibody) and 1 h (secondary antibody-agarose).

Protein A-antibody complexes for αβ1 integrin were typically washed once with lysis buffer A and 1 M KCl prior to extensive washing in lysis buffer A. The washed immunoprecipitates were solubilized in SDS sample buffer lacking reductant, and the radioiodinated integrin subunits were detected by autoradiography after electrophoresis on SDS-polyacrylamide gels containing 5% acrylamide (19:1 acrylamide/bisacrylamide).

Combined Analysis of TGF-β Effects on Integrin Surface Expression and Anchorage-independent Growth—Quiescent suspended NRK cells were prepared in two steps. First, freshly trypsinized cells were replated and allowed to spread for 8–8 h prior to serum starvation for 3 days in defined medium (19). Second, these cells were trypsinized and preincubated in their conditioned medium overnight. These quiescent suspended cells were collected by centrifugation, washed with fresh defined medium, and added (1×10^6 cells) to agar-coated 100-mm dishes containing 10 ml of Dulbecco’s modified Eagle’s medium, 5% FCS, and 2×10^6 EGF × 100 pm TGF-β1. Cells were collected at 12, 24, and 48 h of culture, and adherent and surface-expressed integrins were analyzed by immunoprecipitation and radiiodination. A duplicate aliquot of the quiescent suspended cells was surface-radiiodinated prior to stimulation with mitogens (time 0). Aliquots of the quiescent serum-starved cells (4×10^6) were also added to agar-coated 35-mm dishes in 2 ml of Dulbecco’s modified Eagle’s medium, 5% FCS, and 2×10^6 EGF × 100 pm TGF-β1. The cultures were labeled for 24 h with [3H]thymidine between days 0 and 1, 1 and 2, or 2 and 3. Cells were collected, and trichloroacetic acid-precipitable radioactivity was isolated and quantified to assess the degree of cell cycling.

In some experiments, the overnight preincubation contained 0–20 μg/ml nonsense (GAAAAGATGAATTTNCAAC; control) or (GAAAAGATGAATTTNCAAC; control) or phosphorothioate-modified oligonucleotide (Oligos Etc.) to the β1 integrin subunit. The oligonucleotide-treated cells were then stimulated with FCS, EGF, and TGF-β as described above, except that cells destined for surface radiiodination were added to agar-coated 35-mm wells (six wells/sample), and those destined for pulse labeling with [3H]thymidine were added to agar-coated 15-mm dishes (7.5×10^5 cells in 0.5 ml/well in triplicate) and pulse labeled with [3H]thymidine for 24 h after 2 days in culture. The surface level of β1 integrin and the degree of anchorage-independent growth were assessed by immunoprecipitation and analysis of trichloroacetic acid-insoluble radioactivity, as described above.

RESULTS

TGF-β Overrides the Adhesion Requirement for Surface Expression of αβ1 Integrin in NRK Fibroblasts—We (5, 6) and others (7) have shown that cell-surface integrins are internalized and degraded when cells are cultured in the absence of substrate. This effect presumably contributes to the anchorage-dependent phenotype by preventing integrin signaling in non-adherent cells. We used NRK fibroblasts to examine the effect of TGF-β on this regulation of cell-surface integrin expression.

Cells were cultured in a preparative suspension system in the presence and absence of TGF-β and then radiiodinated for analysis of integrin surface expression by immunoprecipitation, SDS gel electrophoresis, and autoradiography. We detected αβ1 (a collagen/laminin receptor), αβ2 (a laminin, collagen, and fibronectin receptor), and αβ3 (the classical fibronectin receptor) in NRK cell monolayers (Fig. 1, Mn – TGF-β). As expected from our previous studies (5, 6), the surface expression of each of these integrins was lost when the cells were cultured in suspension (Sp – TGF-β). Lack of suitable antibodies prevented similar analysis for rat αβ1 integrin (a collagen/laminin receptor), but αβ1 is also adhesion-dependent for surface expression in NIH-3T3 cells transfected with the human α1 cDNA (data not shown).

Addition of TGF-β resulted in distinct effects on the surface expression of NRK cell integrins. In adherent cells, TGF-β slightly increased the surface expression of αβ1 and αβ3, and inhibited the surface expression of αβ2 (Fig. 1, compare Mn ± TGF-β). Although TGF-β usually stimulates the biosynthesis of multiple integrins in human fibroblasts (20), it can also have selective stimulatory effects on, and even inhibit, the expression of particular integrin subunits (21). For example, TGF-β inhibits the expression of αβ3 integrin in NRK cells (this report) and MG-63 cells (21).

In contrast to these relatively complex and often modest effects, treatment of suspended cells with TGF-β resulted in a dramatic increase in the surface expression of αβ1 integrin (Fig. 1, compare Sp ± TGF-β), completely restoring expression to the level normally seen in adherent cells (compare Mn – TGF-β with Sp + TGF-β). TGF-β also increased the surface expression of αβ1 (compare Sp ± TGF-β), but the surface expression of this integrin was much less than that of αβ1 (compare signal intensities and the amount of radioactivity immunoprecipitated; see “Experimental Procedures”). Moreover, TGF-β failed to restore αβ1 levels in suspended NRK cells to those normally seen in adherent cells (compare Mn – TGF-β with Sp + TGF-β). Not only did NRK cells express very low levels of αβ1 integrin, and treatment with TGF-β did not significantly increase αβ1 surface expression (data not shown). Thus, the predominant effect of TGF-β on integrins in NRK fibroblasts is to permit surface expression of αβ1 integrin when the cells are cultured in the absence of substrate.

Restored surface expression of αβ1 integrin in TGF-β-
treated cells indicates that TGF-\( \beta \) alters the steady-state equilibrium of this integrin on the cell surface, either by inhibiting internalization/degradation or by increasing synthesis/maturation. To directly assess the effect of TGF-\( \beta \) on integrin internalization and degradation, we prepared NRK cells in which plasma membrane \( \beta_1 \)-associated integrins had been biosynthetically labeled with \( ^{35}S \)methionine (see Ref. 6 for detailed procedures). The cells were cultured in suspension to initiate internalization and degradation, and we asked if exposure to TGF-\( \beta \) would inhibit those events. Cells were collected and divided into two equal portions, which were briefly incubated in the presence or absence of Pronase prior to extraction. The level of \( \beta_1 \) integrin subunit in each extract was determined by immunoprecipitation, and the Pronase digestion allowed us to distinguish cell-surface (Pronase-sensitive) from internalized (Pronase-insensitive) \( \beta_1 \) integrin subunit. As shown in the first two lanes of Fig. 2A (0 \( \pm \) Pronase), the very large majority of \( \beta_1 \) integrin subunit was present on the surface of NRK cells prior to incubation in suspension. After \( \sim \)1 day in suspension, cell-surface \( \beta_1 \) integrin levels were greatly decreased, yet no integrin was detected intracellularly (Sp \( \pm \) Pronase). The absence of intracellular \( \beta_1 \) integrin subunit, together with the large decrease in total cell-associated \( \beta_1 \) integrin levels, indicated that \( \beta_1 \) integrin had been degraded, consistent with our previous results (6). Incubation with TGF-\( \beta \) did not block this process (compare Sp \( \pm \) TGF-\( \beta \)).

We then measured the effect of TGF-\( \beta \) on the biosynthesis of the \( \beta_1 \) integrin subunit (Fig. 2B). Control and TGF-\( \beta \)-treated NRK cell suspensions were incubated with \(^{35}S\)methionine for 16 h prior to immunoprecipitation of cell lysates with an antibody recognizing the \( \beta_1 \) integrin subunit. These experiments showed that (i) synthesis of the mature \( \beta_1 \) subunit was readily detected during the incubation with \(^{35}S\)methionine and (ii) TGF-\( \beta \) increased the amount of mature \( \beta_1 \) subunit as well as the amount of pro-\( \beta_1 \) subunit. Although limits of detection prevented the examination of individual \( \alpha \beta_1 \) heterodimers, the results of Fig. 2 (A and B) show that the restorative effect of TGF-\( \beta \) on \( \beta_1 \) integrin surface expression in suspended NRK cells is associated with increased biosynthesis rather than decreased degradation.

**Kinetic and Genetic Relationships between Restored Surface Expression of \( \alpha_\beta_1 \) Integrin and Anchorage-independent Growth**—Since \( \alpha_\beta_1 \) has been strongly implicated in adhesion-dependent cell cycle progression, we reasoned that restored \( \alpha_\beta_1 \) surface expression might be involved in the induction of...
anchorage-independent growth by TGF-\(\beta\). A kinetic analysis showed that TGF-\(\beta\) restored \(\alpha_5\beta_1\) surface expression (shown as the \(\alpha_5\) subunit) within 12 h (Fig. 3A), whereas its effect on anchorage-independent growth (defined as incorporation of \(^{3}H\)thymidine beyond the background level seen with FCS and EGF) required \(>24\) h (Fig. 3B). Thus, restoration of cell-surface \(\alpha_5\beta_1\) integrin by TGF-\(\beta\) was prior to its stimulatory effect on anchorage-independent growth. This result indicates that restored \(\alpha_5\beta_1\) surface integrin is not a secondary consequence of restored cell cycling. Note that the up-regulation of surface \(\alpha_5\beta_1\) and induction of anchorage independence occurred while \(\alpha_5\beta_1\) expression was down-regulated (Fig. 3A). This result argues against a role for this alternative fibronectin receptor and supports a role for \(\alpha_5\beta_1\) integrin in the induction of anchorage-independent growth by TGF-\(\beta\).

We have previously mutagenized NRK cells with EMS (ethylmethane sulfonate) and identified mutants that have lost their adhesion/TGF-\(\beta\) requirement, but retained their mitogen requirement for proliferation (17). These mutants (called NRK/EMS clones B and F) do not produce elevated levels of TGF-\(\beta\), and they respond to exogenous TGF-\(\beta\). However, they proliferate in suspension and form colonies in soft agar when treated with FCS and EGF alone (17). We cultured these NRK/EMS clones in monolayer and suspension with FCS/EGF and examined their adhesion requirements for surface expression of \(\alpha_5\beta_1\) integrin (Fig. 4). In contrast to parental NRK cells, the surface expression of \(\alpha_5\beta_1\) was similar in both adherent and suspended NRK/EMS clones. Thus, these NRK mutants have lost their TGF-\(\beta\) requirements for both anchorage-independent growth and surface expression of \(\alpha_5\beta_1\) integrin. This result (i) indicates that surface expression of \(\alpha_5\beta_1\) integrin and anchorage-independent growth are coupled in NRK cells and (ii) provides genetic evidence supporting the role of restored surface \(\alpha_5\beta_1\) expression in the induction of anchorage-independent growth by TGF-\(\beta\).

Inhibition of Restored \(\alpha_5\beta_1\) Surface Expression Blocks Induction of Anchorage-independent Growth by TGF-\(\beta\)—To determine if restored integrin surface expression was necessary for induction of anchorage-independent growth by TGF-\(\beta\), quiescent suspended NRK cells were preincubated with an antisense or a control (sense) oligonucleotide complementary to a conserved sequence in \(\beta_1\) integrin. The preincubated cells were stimulated with FCS, EGF, and TGF-\(\beta\). Anchorage-independent growth was assessed by \(^{3}H\)thymidine incorporation (Fig. 5), and duplicate cultures were radioiodinated for analysis of \(\beta_1\) integrin surface expression by immunoprecipitation (inset). Consistent with the results in Fig. 1, addition of TGF-\(\beta\) to suspended NRK cells increased the expression of \(\beta_1\) integrins well above the barely detectable levels normally seen in suspended cells (first and second lanes). This TGF-\(\beta\)-mediated increase in cell-surface \(\beta_1\) integrin was partially blocked (\(50\%\)–\(70\%\)) by the antisense oligonucleotide (second and third lanes), whereas the sense oligonucleotide was completely without effect (second and fourth lanes). Parallel immunoprecipitations showed that surface levels of the \(\alpha_5\) integrin subunit were also specifically inhibited by the antisense oligonucleotide (data not shown). The antisense oligonucleotide also inhibited the ability of TGF-\(\beta\) to induce anchorage-independent growth, and this effect was dose-dependent (Fig. 5). Moreover, the concentration of antisense oligonucleotide used to block restored expression of \(\beta_1\) integrin (20 \(\mu\)g/ml) was also effective in blocking anchorage-independent growth. In contrast, the sense oligonucleotide had only a minor effect on TGF-\(\beta\)-induced anchorage-independent growth, and this effect was not dose-dependent.

To assess specifically the role of \(\alpha_5\beta_1\) in the induction of anchorage-independent growth by TGF-\(\beta\), we used NRK cells that had been stably transfected with a 1.3-kilobase pair \(\alpha_5\)-antisense cDNA fragment.\(^2\) The surface expression of \(\alpha_5\beta_1\) integrin is reduced 4-fold in this antisense cell line as compared with parental NRK cells or NRK cells transfected with \(\alpha_5\) cDNA fragment in the sense orientation (control transfectant). Expression of other \(\beta_1\)-containing integrins is similar in \(\alpha_5\)-antisense, \(\alpha_5\)-control, and parental NRK cells. (Note that the \(\alpha_5\) cDNA we transfected encodes only a small part of the \(\alpha_5\) ectodomain and does not result in expression of bona fide \(\alpha_5\) protein when transfected in the sense orientation.) Fig. 6 shows the low level of surface \(\alpha_5\beta_1\) in the control (sense (S)) transfectants

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\(^2\) G. E. Davey, M. Buzzai, and R. K. Assoian, submitted for publication.
inhibits the effect of TGF-β on restored surface integrin expression and anchorage-independent growth. Duplicate cultures of quiescent suspended NRK cells were preincubated for 18 h with 2–20 μg/ml concentrations of the antisense (A) or sense (S) phosphorothioate oligonucleotide prior to stimulation with 5% FCS and 2 μM EGF, and 100 pM TGF-β. After 2 days, [3H]thymidine was added to the cultures; cells were collected 24 h later, and anchorage-independent growth was quantified by isolating and counting trichloroacetic acid-precipitable DNA. Maximal and background thymidine incorporation (first and second columns, respectively) were determined with FCS/EGF-treated NRK cells cultured in the absence of oligonucleotide with and without TGF-β, respectively. Inset, quiescent suspended NRK cells were pretreated with 0 or 20 μg/ml antisense (AS) or sense (S) oligonucleotide for 18 h. The treated cells were then stimulated with 5% FCS and 2 μg/ml EGF ± 100 pM TGF-β for 2 days prior to collection, surface radioiodination, and analysis of β1 integrin surface expression by immunoprecipitation, SDS gel electrophoresis, and autoradiography.

FIG. 7. TGF-β fails to induce anchorage-independent growth in α5-antisense NRK cells. A, suspended α5-antisense and α5-control (sense) NRK transfectants were cultured in suspension for 3 days with 5% FCS and 2 μM EGF ± 100 μM TGF-β. Anchorage-independent growth was quantified by pulse labeling with [3H]thymidine for the last 24 h of the incubation. B, α5-antisense and α5-control (sense) NRK transfectants were cultured in soft agar in the presence of 5% FCS and 2 μM EGF ± 100 μM TGF-β. Shown are phase-contrast photographs taken at 10× magnification after 10 days in culture.

DISCUSSION
Oncogenically transformed cells express low levels of α5β1 integrin (23–25), presumably because these cells have constituently high levels of α5β1 integrin, and Anchorage-independent Growth

FIG. 6. Effects of TGF-β on restored α5β1 surface expression are inhibited in α5-antisense transfectants. Parental NRK fibroblasts and stable α5-antisense (AS) and α5-control (sense (S)) NRK transfectants were incubated in suspension with 5% FCS and 2 μg/ml EGF ± 100 pM TGF-β for 3 days before collection and analysis. Collected cells were surface-radioiodinated and extracted. Equal amounts of trichloroacetic acid-precipitable radioactivity were incubated with anti-α5 antibody, and surface expression of the α5β1 heterodimer was assessed by SDS gel electrophoresis and autoradiography.

cultured in suspension and that exposure to TGF-β increased α5β1 surface expression significantly (compare S ± TGF-β). This result is identical to that seen with parental NRK cells (compare NRK ± TGF-β). TGF-β also increased the expression of α5β1, in the antisense (AS) transfectants, indicating that they have retained TGF-β responsiveness (compare AS ± TGF-β). However, surface α5β1 was barely detectable in the antisense cells lacking TGF-β, and even after adding TGF-β, the surface expression of α5β1 was no higher than the basal levels seen in the control cells lacking TGF-β. Thus, suspended antisense cells treated with TGF-β have much lower surface α5β1 integrin levels than seen in suspended control transfectants treated with TGF-β. (Note that the autoradiogram in Fig. 6 was deliberately overexposed to allow for comparisons of the basal α5β1 surface levels in parental, control, and antisense cells cultured in suspension.)

TGF-β induced anchorage-independent growth of the control (sense) transfectants, and this effect was strongly inhibited in the antisense transfectant (Fig. 7A). Moreover, TGF-β stimulation of an extensive fibronectin matrix was required for anchorage-independent growth of NRK cells induced by TGF-β. We treated NRK cells with TGF-β and looked for the formation of multieellular aggregates that might indicate conversion of serum or cellular fibronectin into a local, extensive fibrillar matrix. NRK cells were induced to undergo anchorage-independent growth by exposure to mitogens and TGF-β. The cells were cultured in soft agar or in different concentrations of methylcellulose to gradually remove the constraints on diffusion of dividing daughter cells. As expected, NRK cells formed discrete multicellular colonies when cultured in soft agar (Fig. 8A), and a similar pattern was observed in high concentrations of methylcellulose (Fig. 8B). An intermediate concentration of methylcellulose led to the appearance of single cells and a reduced number of multicellular aggregates (Fig. 8C). Almost no multicellular aggregates were seen when the cells were cultured in the absence of methylcellulose (Fig. 8D). Simultaneous assessment of anchorage-independent growth by [3H]thymidine incorporation showed that the extent of cell proliferation was the same under all three preparative suspension conditions (Fig. 8E). We conclude that, if diffusion of daughter cells is permitted, induction of anchorage-independent growth of NRK cells by TGF-β does not involve the formation of multicellular aggregates characteristic of colony formation in soft agar.
Like Fava and McClure (14), we also found that fibronectin is unable to replace TGF-β in stimulating anchorage-independent growth (data not shown). However, the data in this report also explain why fibronectin should not be able to replace TGF-β: surface α5β1 would be absent from suspended NRK cells treated with mitogens and fibronectin, whereas it would be present at normal levels in suspended cells treated with mitogens and TGF-β. This difference notwithstanding, our results do support and extend the original hypothesis (13) that the fibronectin-α5β1 interaction is an important aspect of TGF-β action during induction of NRK cell anchorage-independent growth. Since our studies and most others on the induction of anchorage-independent growth by TGF-β were performed in serum-containing medium, either serum-derived fibronectin or TGF-β-induced synthesis of cellular fibronectin could supply the ligand for α5β1 integrin.

We also investigated the nature of fibronectin ligand in NRK cells undergoing anchorage-independent growth in response to TGF-β. We found that if diffusion of daughter cells was not blocked, nonadherent NRK cells treated with TGF-β would proliferate, at least in large part, as a single cell suspension and certainly without the large multicellular aggregates characteristic of colony formation in soft agar. This result indicates that the growth stimulatory effect of TGF-β is distinguishable from a mechanism involving extensive cell-cell adhesion on a local, TGF-β-stimulated matrix.

Although our studies show that restored expression of α5β1 integrin is associated with induction of anchorage-independent growth, others studies show that transformed fibroblasts have a reduced expression of α5β1 integrin (23–25). Moreover, surface α5β1 integrin levels inversely correlate with anchorage-independent growth of nontransformed cells and the ability of restored surface α5β1 integrin to inhibit anchorage-independent growth of nontransformed cells indicate that inhibition and induction of anchorage-independent growth are mechanistically distinct. Indeed, the fact that surface α5β1 integrin is down-regulated when normal (anchorage-dependent) cells are cultured in suspension (Refs. 5 and 6 and this report) strongly argues that loss of surface α5β1 is not causal for the induction of anchorage-independent growth.

Integrins cooperate with growth factor receptor tyrosine kinases to regulate cell proliferation, and α5β1 integrin, in particular, has been implicated in several G1 phase growth stimulatory signaling pathways (1, 2). The results shown here indicate that TGF-β overrides the normal adhesion requirement for surface expression of α5β1 integrin and that this effect is necessary for induction of anchorage-independent growth in NRK cells. Restored α5β1 integrin presumably binds to fibronectin, but the bound fibronectin is not extensively converted into a fibrillar matrix. In this regard, anchorage-dependent proliferation and anchorage-independent proliferation induced by TGF-β are distinguishable processes.

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FIG. 8. Effect of TGF-β on anchorage-independent growth and morphology of NRK cells. A–D, phase-contrast photographs (magnification × 10) of NRK cells stimulated with 5% FCS, 2 nM EGF, and 100 μM TGF-β and cultured for 7 days in medium containing soft agar (A), 1% methylcellulose (B), 0.5% methylcellulose (C), and 0% methylcellulose (D); E, NRK fibroblasts (2 × 10⁴ cells) cultured in preparative suspension containing 5% FCS, 2 nM EGF, and selected concentrations of TGF-β. The cell layer contained 1% (▪), 0.5% (△), and 0% (○) methylcellulose. Anchorage-independent growth was quantified at days 6–7 by 24 h of incubation with [3H]thymidine and subsequent isolation of trichloroacetic acid-precipitable radioactivity.
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