Ectopic expression of the α5 integrin subunit in cancer cells with little or no endogenous expression of this integrin often results in reduced proliferation as well as reduced malignancy. We now show that inhibition resulting from ectopic expression of α5 integrin is due to induction of autocrine negative transforming growth factor-β (TGF-β) activity. MCF-7 breast cancer cells do not express either α5 integrin or type II TGF-β receptor and hence are unable to generate TGF-β signal transduction. Ectopic expression of α5 integrin resulted in enhanced cell adhesion to fibronectin, reduced proliferation, and increased expression of type II TGF-β receptor mRNA and cell surface protein. Receptor expression was increased to a higher level in α5 transfectants by growth on fibronectin-coated plates. Induction of type II TGF-β receptor expression also resulted in the generation of autocrine negative TGF-β activity because colony formation was increased after TGF-β neutralizing antibody treatment. Transient transfection with a TGF-β promoter response element in tandem with a luciferase cDNA into cells stably transfected with α5 integrin resulted in basal promoter activities 5–10-fold higher than those of control cells. Moreover, when α5 transfectants were treated with a neutralizing antibody to either TGF-β or integrin α5, this increased basal promoter activity was blocked. Autocrine TGF-β activity also induced 3-fold higher endogenous fibronectin expression in α5 transfectants relative to that of control cells. Re-expression of type II receptor by α5 transfection also restored the ability of the cells to respond to exogenous TGF-β and led to reduced tumor growth in athymic nude mice. Taken together, these results show for the first time that TGF-β type II receptor expression can be controlled by α5 integrin ligand and integrin signal transduction. Moreover, TGF-β and integrin signal transduction appear to cooperate in their tumor-suppressive functions.
controlling tumor growth. The first of these was the demonstration that removal of autocrine TGF-β activity by stable transfection of a TGF-β antisense expression vector leads to malignant progression of cancer cells in athymic mice (27, 28). This approach blocked autocrine TGF-β activity because endogenous TGF-β was removed from these cells but did not affect the expression of TGF-β receptors and, therefore, permitted the retention of response to exogenously produced TGF-β in the tumor environment from nonmalignant cells. The occurrence of tumor formation indicated that exogenous TGF-β produced by nonmalignant cells was insufficient to achieve tumor suppression. The second line of evidence involved the re-expression of RII in a cell line that was homozygous for mutational inactivation of the gene. Re-expression of RII regenerated autocrine negative TGF-β but did not regenerate an inhibitory response to exogenous TGF-β (25). However, reversion of tumorigenicity did occur, indicating that autocrine TGF-β was critical. Thus, the available evidence indicates that mechanisms for regeneration of RII expression and autocrine negative TGF-β will be of importance in our understanding of controls of TGF-β signal transduction in malignancy and may lead to novel treatment or prevention approaches for cancer.

Previously, we showed that vitamin D3 was inhibitory to wild type MCF-7 clones that expressed RII and to RII-null clones expressing ectopic RII (29). In contrast, RII-null clones were refractory to inhibition (29). Response to vitamin D3 was associated with induction of higher RII levels and enhanced TGF-β autocrine negative activity, suggesting that other growth modulators may cause inhibition by inducing TGF-β autocrine negative activity. Given the ability of α5 integrin to affect growth in a negative fashion (13, 14), we hypothesized that re-expression of cell surface α5β1 integrin in cancer cells deficient in the expression of the α5 subunit would lead to regeneration of RII expression and autocrine negative TGF-β activity. This hypothesis was tested by stable transfection of a MCF-7 breast cancer clone lacking both RII and α5 integrin expression with an α5 integrin cDNA. Transfection resulted in re-expression of RII and regeneration of autocrine TGF-β activity, as well as response to exogenous TGF-β. RII expression and TGF-β responses were dependent upon α5β1 ligation to endogenous MCF-7 fibronectin (FN) and were enhanced when exogenous FN was used to coat culture plates, indicating that growth inhibition by α5β1 ligation involves induction of autocrine TGF-β activity.

MATERIALS AND METHODS

Cell Culture—MCF-7 cells were originally obtained from American Type Culture Collection and adapted to McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS), pyruvate, vitamins, amino acids, and antibiotics (30). Working cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and routinely checked for mycoplasma contamination as described previously (21). MCF-7 limiting dilution clones were obtained by diluting the parental cells in 96-well tissue culture plates at 0.5 cell/well as described previously (21). The strain of MCF-7 cells utilized in this study is insensitive to TGF-β because it lacks RII (21).

Integrin α5 Stable Transfection—An integrin α5 expression vector for mammalian cells was used for stable transfection as described previously (13). The plasmid was linearized and transfected into a typical MCF-7 limiting dilution clone (designated MCF-7 LDC4) by electroporation with a Bio-Rad Gene Pulser at 250 V and 960 microfarads. Control cells were transfected with a Neo-containing plasmid. The transfected cells were plated in 100-mm culture dishes in 10% FBS medium for 2 days. Selection of stable transfectants was carried out by adding Geneticin (600 µg/ml; Life Technologies, Inc.) into the medium. After three weeks, Geneticin-resistant clones were ring-cloned and designated the MCF-7 LDC4 Neo pool.

RNA Analysis—Total RNA was isolated from cultured cells by the guanidine isothiocyanate method (31). For detection of FN mRNA levels, cells (106) were plated in 100-mm culture dishes coated with poly-L-lysine (10 µg/ml; Sigma) or FN (10 µg/ml; Collaborative Biomedical Products) for 4 days in 10% FBS medium, and total RNA was then isolated. For detection of RII mRNA levels, cells (106) were plated in 100-mm culture dishes coated with poly-L-lysine (10 µg/ml) or FN (10 µg/ml) for 1 and 2 h in McCoy’s 5A medium supplemented with 2% bovine serum albumin (Sigma) and 24 and 96 h in McCoy’s 5A medium supplemented with 10% FBS.

The construction of the integrin α5 subunit and RII antisense probes has been described (21, 32). The FN riboprobe was constructed by subcloning a 232-base pair BamHI-PvuII fragment of the human FN cDNA into a pBSK-1 vector (Stratagene Cloning System). T7 RNA polymerase was used to synthesize the FN antisense probe (13, 32). The RNase protection assay was performed by hybridization of the radioactive ribopropbes with total RNA (20 µg) isolated from the control or α5-transfected cells as described previously (32).

Immunoprecipitation—To determine cell surface integrin α5β1 expression, cell surface proteins were labeled with biotin, immunoprecipitated with an anti-α5 subunit monoclonal antibody (Life Technologies, Inc.), and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (32).

Cell Adhesion and 3-(4,5-Dimethyl Thiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide Assays—A 96-well Corning tissue culture plate was coated overnight at room temperature with FN at concentrations of 0, 1.0, 2.5, 5.0, and 10.0 µg/ml, blocked with 3% bovine serum albumin for 3 h, and then rinsed once with phosphate-buffered saline. Confluent cells were trypsinized, plated at 4 × 104 cells/well in 96-well plates, and cultured in serum-free medium, and incubated for 15 min. Unattached cells were gently washed away with three rinses with serum-free medium. The relative number of attached cells was determined by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described previously (33).

The specificity of cell adhesion to FN was determined using a monoclonal anti-human integrin α5 subunit antibody (Life Technologies, Inc.). The cells were incubated in the absence or presence of the antibody (1:100 dilution) for 30 min at 4°C and then plated at 4 × 104 cells/well in 96-well plates coated with FN (10 µg/ml). Determination of cell adhesion and the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay were performed as described above.

Tumorigenicity—Exponentially growing cells of MCF-7 Neo pool and α5 sense clone 15 were inoculated subcutaneously as described previously (21). Growth curves for xenografts were determined by measuring the volume (V) of tumors. V is expressed as V = (L × W)²/2, where L is the length and W is the width of the xenograft.

Mitogenesis Assay—Inhibition of cell proliferation by exogenous TGF-β1, in Neo and α5-transfected cells was determined by measuring [3H]thymidine incorporation as described previously (21). Briefly, cells were plated in 24-well plates at 1.5 × 10⁴ cells/well in the presence of various concentrations of TGF-β1 (0.2–10 ng/ml). After 4 days of incubation, cells received a 1-h pulse with [3H]thymidine (25 µCi) and were washed with supplemental McCoy’s 5A medium three times, and DNA was precipitated with 10% trichloroacetic acid and then solubilized with 0.2 M sodium hydroxyde. The amount of [3H]thymidine incorporated was analyzed by liquid scintillation counting in a Beckman LS6500 scintillation counter. Growth inhibition by TGF-β1 is represented as the percentage of [3H]thymidine incorporation of TGF-β1-treated cells relative to untreated controls.

Plating Efficiency Assay—The effect of TGF-β1, neutralizing antibody on the clonogenic potential of control and α5-transfected cells was compared to determine autocrine TGF-β activity as described previously (25, 26). Cells were seeded at low density (400 cells/well) in 24-well plates in the presence of control IgG (10 µg/ml; R & D Systems, Inc.) or TGF-β1-neutralizing antibody (10 µg/ml; R & D Systems, Inc.). After 8 days of incubation without medium change, cell colonies were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet, and dissolved in 1% Triton X-100 as described by Westergren-Thorsson et al. (34).

Transient Transfection and Luciferase Assay—The TGF-β-responsive luciferase expression plasmid (pSP-T-Lux) was used for transient transfections, and luciferase assays were performed as described previously (25, 26). MCF-7 cells were transfected with 30 µg of pSP-T-Lux and 10 µg of β-galactosidase plasmid by electroporation with a Bio-Rad Gene Pulser at 250 V and 960 microfarads. The electroporated cells were plated onto 6-well tissue culture plates. After the cells attached, control IgG (10 µg/ml) and TGF-β1, neutralizing antibody (10 µg/ml) were added. The cells were harvested with 200 µl of lysis buffer (luciferase assay system; Promega). Luciferase activity was measured in the first 10 s after substrate
Control of Autocrine TGF-β by α5β1 Integrin

RESULTS

Expression of αβ1 Integrin in MCF-7 Cells—We transfected the α5 subunit into a typical limiting dilution clone, MCF-7 LDC4. The α5-positive transfecants were initially screened for increased expression of α5 mRNA by RNase protection assays (Fig. 1). Two positive clones (designated cl.5 and cl.15) were isolated that expressed higher levels of α5 subunit mRNA than the untransfected MCF-7 LDC4 and Neo-transfected (Neo pool) control cells for α5 mRNA levels by RNase protection assay. Actin mRNA levels were used to normalize loading of the samples. M.W. std., molecular weight standard.

Addition using a luminometer (Berthold Lumat LB 9501) and expressed as arbitrary units after normalization with β-galactosidase activity.

Receptor Cross-linking—Human TGF-β1 was purified and iodinated by the chloramine T method as described previously (36). Equal numbers of the cells (10⁵) were plated in 6-well plates, and after 5 days, binding and cross-linking of 200 nm ¹²⁵I-TGF-β1 to the cell monolayer were performed as described by Segarini et al. (37). Labeled cells were solubilized in 200 μl of 1% Triton X-100. Equal amounts of cell lysate protein were electrophoresed by 4–10% gradient SDS-polyacrylamide gel electrophoresis under reducing conditions and exposed for autoradiography.

Induction of Autocrine Negative TGF-β Activity by αβ1 Transfection—To determine whether autocrine negative TGF-β activity was induced as a result of αβ1 expression, enhanced adhesion to FN (Fig. 3A). Both α5 transfectants showed 7–8-fold increased adhesion to FN-coated plates at FN concentrations ranging from 5 to 10 μg/ml, whereas Neo control cells showed only 3–4-fold enhancement. In addition, both α5 transfectants showed ~5-fold increases of binding at FN concentrations ranging from 0 to 2.5 μg/ml, relative to Neo control cells. The specificity of cell adhesion was shown by blocking the attachment to FN with an anti-α5 subunit antibody (Fig. 3B), thus indicating that enhanced cell attachment to FN was due to increased cell surface α5β1 expression.

Ectopic α5 expression leading to enhanced αβ1 function results in enhanced DNA synthesis, whereas disruption of ligation led to increased DNA synthesis in other model systems (13, 14). If our hypothesis that enhanced αβ1 function leads to induction of autocrine negative TGF-β activity was correct, ectopic expression of α5 should result in reduced cell proliferation. Growth curves of α5-transfected clones showed more than 50% inhibition of proliferation relative to wild type cells (Fig. 3C).

Expression of RII in α5 Transfectants—Previously, we showed that MCF-7 cells were insensitive to TGF-β1, because they expressed nearly undetectable levels of RII (21). Therefore, if α5β1-mediated growth inhibition was associated with autocrine negative TGF-β activity, RII expression must be restored. To test this hypothesis, we examined RII mRNA levels in α5 transfectants by RNase protection assay (Fig. 4). High steady state RII mRNA levels were induced in α5-transfected cells compared with Neo control cells when the cells were cultured on poly-l-lysine. This was probably due to enhanced production of endogenous FN, as described below. Growth on exogenous FN further increased RII mRNA levels to 2.5-fold (as determined by densitometry) in α5-transfected cells, whereas it had no effect in Neo control cells. RII mRNA levels remained the same in α5-transfected and Neo-transfected cells (data not shown). TGF-β receptor cross-linking with ¹²⁵I-TGF-β1 showed little binding to RII of control cells, whereas substantially higher binding of TGF-β1 was observed in the α5 transfectants (Fig. 5). Interestingly, binding of TGF-β1 to RII was fairly prominent in control cells, as was binding to RIII. This has been observed in previous studies of this cell line (21, 22), as well as in other cell lines (38). Transfection of α5 subunit resulted in a substantial increase in RI binding. This is in accordance with the increased RII expression because this receptor is thought to be responsible for RI recruitment to the cell surface (35).

Fig. 1. Integrin α5 mRNA levels in α5 sense-transfected MCF-7 LDC4. Total RNA from two positive clones (cl.5 and cl.15) was isolated and compared with untransfected (MCF-7 LDC4) and Neo-transfected (Neo pool) control cells for α5 mRNA levels by RNase protection assay. Actin mRNA levels were used to normalize loading of the samples. M.W. std., molecular weight standard.

Fig. 2. Cell surface integrin α5 and β1 protein levels in α5 sense-transfected MCF-7 LDC4. Cell surface proteins from MCF-7 LDC4 Neo pool, α5 sense clone 5, and α5 sense clone 15 were labeled with biotin. The biotinylated integrin from equal cell numbers of each cell type was immunoprecipitated with an anti-human α5 subunit antibody and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting.
TGF-β neutralizing antibody blockade of endogenously produced TGF-β1 was employed, utilizing a previously described clonal assay (25, 26). Cells expressing autocrine negative TGF-β activity will show enhanced colony formation and growth as a result of the antibody-mediated neutralization of TGF-β1, whereas those cells that do not express autocrine negative activity will be unaffected by antibody treatment. Cell growth and colony formation are determined by crystal violet staining. Standard curves were performed measuring crystal violet levels with known numbers of MCF-7 cells to ensure that the assay was performed over a linear range of MCF-7 cells. As shown in Fig. 6, TGF-β1 neutralizing antibody stimulated colony formation in clone 5 transfectants, whereas it had no effect on Neo control cells (Fig. 6A). The percentage of stimulation by the antibody was calculated and plotted in Fig. 6B. TGF-β1 neutralizing antibody treatment resulted in 25% stimulation for clone 5 and 55% for clone 15.

To confirm the enhancement of autocrine TGF-β activity after α5 expression, we compared the activity of a TGF-β-responsive promoter in control cells with that in α5-transfected cells. The p3TP-Lux promoter contains a TGF-β response element from the plasminogen activator inhibitor gene inserted upstream of the luciferase reporter gene and has been extensively utilized as a marker for TGF-β responsiveness (23, 35). Therefore, it would be expected that induction of autocrine TGF-β activity would result in enhanced expression of the p3TP-Lux construct in α5 transfectants relative to Neo control cells. Fig. 7 shows that both α5 transfectants expressed 5–10-fold higher levels of luciferase activity than Neo control. If increased luciferase activity of p3TP-Lux construct was due to autocrine TGF-β1 neutralizing antibody treatment would reduce expression of the reporter construct. As shown in Fig. 7A, TGF-β1 neutralizing antibody treatment resulted in a substan-
tial decrease in luciferase reporter activity in both $\alpha_5$-transfected clones, whereas it had no effect on Neo control cells. This experiment was repeated four times, and similar results were obtained. Similarly, an $\alpha_5$ neutralizing antibody was used to show that disruption of $\alpha_5\beta_1$ ligation to FN resulted in approximately a 60% reduction in the enhanced luciferase activity associated with the $\alpha_5$ clone 15 transfectant (Fig. 7B), thus confirming that the enhanced endogenous TGF-$\beta$ activity was dependent on the ectopic $\alpha_5$ expression.

We previously showed that autocrine TGF-$\beta$ controlled steady state levels of FN in both native and RII-transfected cells (25, 32). Consequently, induction of autocrine TGF-$\beta$ should be associated with increased endogenous FN expression by $\alpha_5$ transfecants. The $\alpha_5$-transfected cells showed a 3-fold increase (as determined by densitometry) in FN mRNA levels compared with Neo control cells (Fig. 8A). FN mRNA levels were further increased in $\alpha_5$-transfected cells when the cells were plated on FN, whereas the levels in Neo control cells still remained the same. The enhanced FN expression was due to autocrine TGF-$\beta$ as shown by the ability of TGF-$\beta$ neutralizing antibody treatment to repress FN expression in $\alpha_5$ transfectant cells to a level comparable to that of NEO controls (Fig. 8B).

Growth Inhibitory Effects of TGF-$\beta_1$ on $\alpha_5$ Transfectants—Induction of autocrine TGF-$\beta$ activity suggested that response to exogenous TGF-$\beta$ effects should also result from $\alpha_5$ transfection. The MCF-7 LDC4 Neo pool was insensitive to TGF-$\beta_1$ in the absence or presence of exogenous FN (Fig. 9). The $\alpha_5$ transfectants showed reduced basal proliferation relative to NEO controls as indicated above in Fig. 3C. DNA synthesis in the $\alpha_5$ transfectants was further inhibited by TGF-$\beta_1$ in a dose-dependent manner (Fig. 9). When the two $\alpha_5$ transfectants were plated in 24-well culture plates coated with exogenous FN (10$\mu$g/ml), increased sensitivity to TGF-$\beta_1$ was demonstrated. Increased sensitivity on FN was likely due to the increased RII expression when cells were grown on FN as demonstrated in Fig. 4, above.

Effect of Integrin Expression on Tumorigenicity—To assess the effect of $\alpha_5$ expression on the malignant properties of MCF-7 cells, we inoculated Neo control and $\alpha_5$-transfected clone 15 into ovariectomized, estrogen-supplemented nude mice as described previously (21). The size of xenografts formed was monitored with time (Fig. 10). Initially, MCF-7 LD 4 Neo pool and $\alpha_5$ clone 15 formed similar sized xenografts (< 200...
This result indicates that integrin line provided a good model system for this study in that it measured, as described above.

A, stored integrin expression (21–23). Integrin suppressor effects mediated through autocrine TGF-

B, Cells were plated on poly-L-lysine and treated with IgG (−) or TGF-β neutralizing antibody (Ab) (+), and FN mRNA levels were measured, as described above.

mm3) until day 8 after inoculation. After day 8, growth was delayed in α5 clone 15 compared with Neo control. At day 28, Neo controls formed ~2.2-fold larger xenografts than α5 clone 15. This result indicates that integrin α5β1 expression in MCF-7 cells can partially reverse the malignant properties of the cell line.

DISCUSSION

A number of studies have indicated that loss or reduced expression of integrin receptors results in abnormal cell growth (10, 11, 12). To test the hypothesis that α5β1 ligation has tumor suppressor effects mediated through autocrine TGF-β, we restored integrin α5 expression in MCF-7 cells. The MCF-7 cell line provided a good model system for this study in that it expressed low amounts of α5 integrin and was insensitive to the growth inhibitory effects of TGF-β1 due to repression of RII expression (21–23). Integrin α5 transfectants expressed similar levels of cell surface integrin as Hs578T cells, another breast cancer cell line that was sensitive to growth inhibitory effects of TGF-β1. The α5 transfection resulted in an increase in expression of RII, which was accompanied by increased autocrine TGF-β activity as assessed by 1) enhanced clonality following TGF-β neutralizing antibody treatment; 2) decreased endogenous activity of a TGF-β-sensitive reporter system in response to TGF-β neutralizing antibody treatment with either TGF-β1 or α5 antibodies; and 3) stimulation of FN expression, which was reversed by TGF-β neutralizing antibody treatment. Up-regulation of RII expression was also reflected by increased sensitivity to inhibition by treatment with exogenous TGF-β.

Previously, we showed that blockade of FN/α5β1 ligation by antibodies against FN or integrin α5 subunit stimulated DNA synthesis in cancer cell lines with moderate to high α5β1 cell surface expression (13, 14). These results are consistent with a previously described model that suggests that moderate adhesion to a loosely organized extracellular matrix facilitated both migration and growth, but strong adhesion to a fully organized extracellular matrix suppressed proliferation and contributed to inhibition of growth (4). Thus, the low level of expression of integrin α5β1 on the cell surface of wild type MCF-7 cells could contribute to weak adhesion and hence abnormal growth in MCF-7 cells. Ectopic α5 expression leads to results consistent with a model suggesting that higher α5β1 surface expression allows for greater adhesion due not only to α5, but to greater endogenous FN expression as well. Inhibition of proliferation is either due to or augmented by the generation of autocrine TGF-β activity. Exogenous FN coating allows for even stronger adhesion and further enhancement of autocrine TGF-β activity. Most importantly, the results indicate that α5β1 ligation and autocrine TGF-β interact in a reciprocal manner that is self-sustaining for both autocrine negative activity and cell-extracellular matrix interactions. Moreover, this interaction is tumor-suppressive. This model may well apply to other systems given that autocrine TGF-β signaling and α5β1 ligation have both been individually associated with tumor suppression in various model systems. Autocrine TGF-β has been shown to control steady state α5 expression (32) in model systems that also show tumor-suppressive TGF-β function (28).

Our results suggest that α5β1 may have a negative growth regulatory role in some cancer cells and normal cells through modulation of TGF-β sensitivity. Apparently, signal transduction mechanisms for activating the TGF-β pathway are essentially intact in MCF-7 cells because restoration of α5β1 expression leads to autocrine as well as exogenous TGF-β inhibitory responses. However, other types of cancer cells may be resistant to this mode of regulation despite α5β1 expression because of perturbations of the TGF-β pathway resulting from malignant transformation. For example, HCT116 colon carcinoma cells express high levels of integrins that mediate adhesion to FN, but this cell line still exhibits a highly malignant phenotype due to a mutated RII gene (25). It is also possible that downstream signaling messengers encoded by oncogenes or tumor suppressor genes that participate in either a primary or secondary manner in signal transduction are abnormally modulated in some cell types.

An interesting aspect of this study was the enhancement of RII expression and TGF-β function when α5 transfectants were plated on FN-coated plates. These results, along with the demonstration that α5 antibody treatment blocks autocrine TGF-β activity, show that α5β1 ligation to FN is critical to the generation of RII expression and hence TGF-β-mediated signal transduction. Enhanced basal expression of FN in MCF-7 α5 transfectants that were not grown on FN-coated plates was also observed. We postulate that increased steady state expression of FN after α5 transfection allowed for α5β1 ligation, which was critical to the basal RII expression and TGF-β sensitivity associated with transfectant cells that were not exposed to FN-coated plates.

In a previous study, we reported that disruption of α5β1/FN ligation resulted in stimulation of DNA synthesis (14). DNA synthesis was associated with up-regulated CDK2 activity without alterations of CDK inhibitors. DNA synthesis was also found to be dependent upon extracellular receptor kinase 1 and

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*Image 8.* Fibronectin mRNA levels in MCF-7 LDC4 α5 sense transfectants. A, RNase protection assay was performed to determine FN mRNA levels in Neo and α5,5 clones grown either on poly-L-lysine or FN-coated plates for 2 days. Actin mRNA was used to normalize samples. B, Cells were plated on poly-L-lysine and treated with IgG (−) or TGF-β neutralizing antibody (Ab) (+), and FN mRNA levels were measured, as described above.
2 activation. Thus, this previous study indicated $\alpha_5\beta_1$ ligation to FN had a repressive effect on cell cycle progression through repression of cyclin A and CDK2 expression. Exogenous treatment with TGF-$\beta$ has been shown to down-regulate cyclin A (39). Interestingly, TGF-$\beta$ has also been reported to repress ERK1/2 activation in some types of cells (40). This suggests that DNA synthesis resulting from disruption of $\alpha_5\beta_1$ ligation may be a reflection of the disruption of integrin related autocrine TGF-$\beta$ activity resulting in up-regulation of ERK activation and subsequent promotion of cell cycle transit.

There is evidence that integrins transduce signals cooperatively with other growth factor systems in the regulation of cell proliferation. The proliferative response of murine mammary carcinoma cells to platelet-derived growth factor-BB and basic fibroblast growth factor is dependent on the extracellular matrix environment, indicating that modification of extracellular matrix and/or surface integrin receptors may regulate responsiveness to these growth factors (41). Reciprocal enhancement of $\alpha_5\beta_1$-mediated adhesion by insulin and of insulin-mediated signal transduction by $\alpha_5\beta_1$ have recently been reported (42). However, $\alpha_5\beta_1$ ligation does not appear to modulate expression of insulin receptor in this system. Our results indicate that the TGF-$\beta$ signaling pathway can be rescued by re-expression of integrin in MCF-7 cells. Ligation of integrins with their extracellular matrix ligands has been shown to regulate gene expression in a number of studies (43). However, because studies on the regulation of RII mRNA transcription and stability are limited, it is not yet clear how ligation of $\alpha_5\beta_1$ integrin to FN increases RII mRNA level. Nevertheless, the induction of RII and autocrine TGF-$\beta$ activity by $\alpha_5\beta_1$ ligation suggests that integrin-mediated signal transduction plays a cooperative role with TGF-$\beta$ signal transduction in tumor suppression. Moreover, the results indicating reciprocal positive control of autocrine TGF-$\beta$ and $\alpha_5\beta_1$ ligation suggest that TGF-$\beta$ signal trans-
duction and α5β1 integrin signal transduction participate in a mutually self-sustaining tumor-suppressive autocrine loop.

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