[CANCER RESEARCH 64, 1987–1996, March 15, 2004]

Cyr61 Is Overexpressed in Gliomas and Involved in Integrin-Linked Kinase-Mediated Akt and β-Catenin-TCF/Lef Signaling Pathways

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ABSTRACT

Cyr61 is a member of the CCN family of growth factors; these proteins are secreted and can act as ligands of distinct integrins. We show that Cyr61 can enhance tumorigenicity of glioma cells acting through activated integrin-linked kinase (ILK) to stimulate β-catenin-TCF/Lef and Akt signaling pathways. Overexpression of Cyr61 occurred in highly tumorigenic glioma cell lines and in 68% of the most malignant glioblastoma multiforme brain tumors. Forced expression of Cyr61 in U343 glioma cells accelerated their growth in liquid culture, enhanced their anchorage-independent proliferation in soft agar, and significantly increased their ability to form large, vascularized tumors in nude mice. Overexpression of Cyr61 in the U343 cells led to the up-regulation of distinct integrins, including β1 and αvβ3, which have been shown to interact with Cyr61 and ILK. The activity of ILK was increased dramatically in these cells. Overexpression of Cyr61 also resulted in the phosphorylation of glycogen synthase kinase-3β and accumulation and nuclear translocation of β-catenin, leading to activation of the β-catenin-TCF/Lef-1 signaling pathway. Furthermore, forced expression of Cyr61 in the glioma cells activated phosphatidylinositol 3′-kinase pathway, resulting in prominent phosphorylation of Akt and the antiapoptotic protein Bad. Cyr61 appears to stimulate several signaling pathways in the development of gliomas.

INTRODUCTION

Malignant gliomas are the most common primary brain tumors and are among the deadliest of human cancers (1). They develop as the result of stepwise accumulations of multiple genetic alterations, which result in the activation of oncogenes and/or the inactivation of tumor suppressor genes (2). The differential expression of these critical genes and their downstream effectors enables cells to override growth controls and undergo carcinogenesis. Mutation of p53, RB, and PTEN, deletion of $p16^{INK4A}$, activation of the Ras and Akt pathways, and amplification of *CDK4* and *EGFR* contribute to the development of gliomas (2–5). These genetic abnormalities have been well studied in the formation of the most malignant brain tumor, glioblastoma multiforme (GBM). Nevertheless, recent microarray studies reveal that hundreds of gene transcripts may be expressed at significantly altered levels in gliomas compared with normal brain tissue (6–8).

Cyr61 is a member of a growing family of growth factors termed the CCN (*C*ysteine-rich 61/*C*onnective tissue growth factor/*N*ephroblastoma overexpressed) gene family that is characterized by a high degree of amino acid sequence homology ranging from 50–90%. This family is composed of Cyr61 (cysteine-rich protein), connective tissue growth factor (9, 10), *nov* (nephroblastoma overexpressed gene; Refs. 11, 12), and Wnt-1-induced secreted protein 1 (WISP-1; Ref. 13). All of the members of the CCN gene family possess a secretory signal peptide, indicating that they are secreted proteins. Cyr61 is a cysteine-rich, heparin-binding protein that associates with the cell surface and the extracellular matrix and can interact with various cellular integrins (14–16). Expression of Cry61 is induced by a variety of growth factors, hormones, and drug components, including serum, epidermal growth factor, basic fibroblast growth factor, transforming growth factor β, 17β-estradiol, muscarinic acetylcholine receptors, and vitamin D_3 (17–22). Purified Cyr61 protein has been reported to mediate cell adhesion, stimulate chemotaxis, augment growth factor-induced DNA synthesis, foster cell survival, and enhance angiogenesis *in vivo* (16, 18, 19, 23).

Several lines of evidence support a role for CCN molecules in tumorigenesis. Elevated expression of avian nov mRNA was found consistently in all of the myeloblastosis-associated virus 1- and myeloblastosis-associated virus 2-induced avian nephroblastomas (11). The human homologue of avian nov is overexpressed mainly in tumors of predominantly stromal origin, such as Wilms tumors (43). Consistent with its profibrotic properties, connective tissue growth factor is overexpressed in pancreatic cancers (25), melanomas (26), and mammary tumors (27, 28). WISP-1 is expressed strongly in the fibrovascular stroma of breast tumors developing in Wnt-1 transgenic mice and primary human colon cancers (13). Moreover, forced overexpression of WISP-1 in normal rat kidney fibroblasts (NRK-49F) was sufficient to induce their transformation (29). We and several other groups have shown recently that Cyr61 was overexpressed in breast cancers and might be involved in estrogen-mediated tumor development (22, 28, 30, 31).

In this study, we found that Cyr61 was highly expressed in primary gliomas and in cell lines derived from high-grade gliomas. Stable expression of Cyr61 under the regulation of a constitutive promoter in U343 cells accelerated cell proliferation in culture, enhanced anchorage-independent cell growth in soft agar, and significantly increased tumorigenicity and vascularization in nude mice. Furthermore, characterization of the oncogenic activity of Cyr61 demonstrated that it might contribute to tumorigenesis through activation of integrin-linked kinase (ILK)-mediated β-catenin-TCF/Lef and Akt signaling pathways.

MATERIALS AND METHODS

Cell Culture. U87, U118, U138, U343, U373, and T98G glioma cell lines were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 (Life Technologies, Rockville, MD). Culture media were supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA), 10 units/ml penicillin-G, and 10 mg/ml streptomycin (Gemini Bio-Products). All of the cells were incubated at 37°C in 5% CO_2. In experiments in which the effects of phosphatidylinositol 3′-kinase (PI3k) inhibitors were studied, U343 and U343/Cyr61 cells were treated with either wortmannin (100 nM; Sigma, St. Louis, MO) or LY294002 (50 μM; Sigma) for 4 h at 37°C. For effects of either integrin or Cyr61 antibodies, 10 μg/ml LM609 (Chemicon, Temecula, CA) or Cyr61 antibodies were added to the RPMI media.

RNA Preparation and Northern Analysis. Total RNA was isolated from cell lines and patient tissue by using TRIzol reagent (Life Technologies) according to the standard protocol. Cyr61 cDNA probe was labeled with ^{32}P-dCTP using random primers (Life Technologies). Total cellular RNA was

Received 3/16/03; revised 1/14/04; accepted 1/15/04.

Grant support: NIH, Parker Hughes Trust, and the C. and H. Koeffler Research Fund NCFC303T0690 (D. Xie). K. Black holds the Ruth and Lawrence Harvey Chair in Neurosciences. H. P. Koeffler is a member of the Jonsson Comprehensive Cancer Center and holds the endowed Mark Goodson Chair of Oncology Research at Cedars-Sinai Medical Center/UCLA School of Medicine.

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separated on 1.2% formaldehyde-agarose gels and was immobilized on a hybond-N^+ membrane by standard capillary transfer and UV cross-linking. The membrane was hybridized with the Cyr61 probe by standard protocol and was rehybridized with a ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA or β-actin cDNA to confirm equal loading of the samples.

Cell Transfection and Soft Agar Assays. The Cyr61 expression constructs were transfected into U343 cells using Lipofectamine (Invitrogen, Carlsbad, CA) as described previously, and transfectants were selected for G418 resistance (500 ìg/ml; Ref. 22). The selected clones were confirmed to have prominent expression of Cyr61 by Northern and Western blot analysis. For clonogenic assay, cells were plated into 24-well flat-bottomed plates using a two-layer soft agar system with 1×10^3 cells/well in a volume of 400 μl/well as described previously (22). After 14 days of incubation, the colonies were counted and measured. All of the experiments were done at least three times using triplicate plates per experimental point.

Cell Migration Assays. Cell migration assays were performed as described previously (22). Cells were allowed to migrate to the underside of the top chamber for 4–8 h. The migratory cells attached to the bottom surface of the membrane were stained with 0.1% crystal violet in 0.1 M borate (pH 9.0) and 2% ethanol for 20 min at room temperature. The stained cells were extracted using extraction buffer (Chemicon). The number of migratory cells per membrane was determined by absorbance at 550 nm.

Flow Cytometric Analysis. Fluorescence-activated cell sorting (FACS) analysis was performed using LM609 antibody (1:500; Chemicom). After washing the primary antibody with PBS, the cells were incubated with FITC-conjugated antimouse IgG (5 μg/ml) at 4°C for 30 min. The cells again were washed with PBS. FACS sorting was performed using a FACScan (Becton Dickinson, Mountain View, CA), and analysis was performed using CellQuest 2.0 (Becton Dickinson).

Tumorigenicity Assay. Stably transfected U343/Cyr61 and U343/V cells (1.0×10^5 cells/flank) were injected s.c. into 8-week-old female nude mice. Each animal was injected at two sites in the flanks. The resulting tumors were measured once a week, and tumor volume (mm^3) was calculated using the standard formula: length $\times$ width $\times$ height $\times$ 0.5236. Tumors were harvested 8 weeks after injection and individually weighed before fixation. Data were presented as tumor volume (mean $\pm$ SD) and tumor weight (mean $\pm$ SD). Statistical analysis was performed by computer program software (GraphPad, San Diego, CA) using the Student's *t* test.

Cell Proliferation and Cycle Analysis. For the cell proliferation assay, U343/Cyr61 and U343/V cells were plated into 96-well plates at 2.0×10^3 cells/well and cultured for various durations; cell numbers were measured by MTT assay according to the protocol provided by Roche Molecular Biochemicals (Basel, Switzerland). For cell cycle analysis, cells were plated in 100-mm dishes and trypsinized when they reached 60% confluence. After washing twice with PBS, cells were fixed in 70% ice-cold ethanol overnight. After staining with propidium iodide, samples were analyzed using a FACScan.

Purification of the Cyr61 Protein from Sf9 Cells. The pcDNA61 was cloned into the transfer plasmid pVL1392 (BD Biosciences, Franklin Lakes, NJ), and baculovirus-encoding Cyr61 was generated using the BaculoGold system (BD Biosciences). Sf9 insect cells were maintained in TNM-FH Insect Medium (BD Biosciences). His6-tagged Cyr61 protein was produced in Sf9 cells by infecting these cells with Cyr61-baculovirus according to Baculovirus Expression Vector System (BD Biosciences). The Cyr61 was purified from the condition media using a two-step purification method. First, the Cyr61 was markedly enriched after application of the condition media to HiTrap Heparin HP columns (Amersham Bioscience, Piscataway, NJ), and the eluted fraction then was purified using His-Bind Resin (Novagen, Madison, WI). Finally, the eluted fraction from the His-Bind Resin column was applied to the PD-10 column (Amersham Bioscience) to exchange the eluting buffer, which includes imidazole for isotonic PBS.

Real-Time PCR Assay. Primers and probes for Cyr61 and β-actin genes were designed using PRIMER3 software (http://www.genome.wi.nit.edu/cgi-bin/primer/primer3_www.cgi). Primers were purchased from Life Technologies, and probes were from Perkin-Elmer Applied Biosystems (Boston, MA). Amplification reactions contained 5 μl of cDNA, 12.5 μl of the Universal Taqman 2$\times$ PCR mastermix (Applied Biosystems, Foster City, CA), and 2.5 μl of each of the specific primers and the probe. Primer and TaqMan probe concentrations in the final volume of 25 μl were 500 nM and 100 nM, respectively. All of the reactions were performed in triplicate in an iCycler iQ system (Bio-Rad, Hercules, CA), and the thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

ILK and Akt Kinase Assay. The ILK kinase assays were performed using a rabbit immunoaffinity-purified ILK antibody (Upstate Biotechnology, Lake Placid, NY) and myelin basic protein as a substrate. U343/Cyr61 and U343/V cell lysates were centrifuged at 10,000 $\times$ *g* for 5 min, and protein concentrations were determined using a modified Bradford assay protocol (Bio-Rad). The supernatants (100 μg) were precipitated for 12 h at 4°C with protein A-agarose beads precoated with saturating amounts of the antibody. Immunoprecipitated proteins on beads were washed twice with 1 ml of lysis buffer and twice with kinase buffer [50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 mM DTT]. The beads then were resuspended in 40 μl of kinase buffer containing the protein substrate (2 μg of myelin basic protein), 10 mM ATP, and 5 mCi of [γ-^{32}P]ATP (6,000 Ci/mmol; 1 Ci = 37 GBq; Amersham Pharmacia Biotech). The samples were incubated for 30 min at 30°C with occasional mixing, boiled in polyacrylamide gel sample buffer containing SDS, and separated by electrophoresis. Phosphorylated proteins were quantified after exposure to autoradiographic film. The Akt kinase assay was analyzed using IP/kinase assay following the manufacturer's protocol (Cell Signaling Technology, Beverly, MA). Briefly, U343/Cyr61 and U343/V cell extracts (200 ìl) were incubated 2 h with immobilized Akt 1G1 monoclonal antibody. After extensive washing, the kinase reaction was performed in the presence of 200 μM of cold ATP and glycogen synthase kinase-3β (GSK-3β) substrate. Phosphorylation of GSK-3β was measured by Western blot analysis using phospho-GSK-3β antibody.

Immunohistochemical and Immunofluorescence Staining. Immunohistochemical staining for Cyr61 was performed with polyclonal antiserum from Santa Cruz Biotechnology (Santa Cruz, CA). Heat-induced epitope retrieval was performed with a pressure cooker and Tris buffer (pH 9.0) for 2 min. Localization was performed with Dako Envision (Dako, Carpinteria, CA) conjugated to horseradish peroxidase, followed by the diaminobenzidine reaction. Negative controls consisted of substitution of the primary antiserum with normal rabbit serum at the same dilution. For immunofluorescence staining of β-catenin, cells were cultured on a four-well chamber Lab-Tek slide (Nunc, Naperville, IL). After 8 h, cells were fixed in 3% paraformaldehyde in PBS at room temperature for 8 min, permeabilized with 0.3% NP40 in PBS for another 8 min, washed in PBS, and incubated with rabbit polyclonal β-catenin antibody (1 ìg/ml; Santa Cruz Biotechnology) at 4°C overnight. The immunoreactivity was revealed using Rhodamine Red-X-conjugated rabbit IgG (Molecular Probes, Eugene, OR). The cells were examined under a Nikon fluorescence microscope (Image Systems, Columbia, MD).

RESULTS

Cyr61 Is Expressed at High Levels in Glioma Cell Lines and Primary Glioblastomas. To analyze expression of Cyr61 in brain tumor cell lines, Cyr61 expression was examined in a panel of glioma cell lines. Northern analysis showed that Cyr61 mRNA was expressed prominently in the highly invasive and tumorigenic glioma cell lines U87, U118, U138, U373, and T98G; levels were markedly lower in the less invasive and less tumorigenic tumor cell line U343 and was barely detectable in the normal brain cell line HBMEC (Fig. 1*A*).

To determine the pattern of expression of Cyr61 in primary brain tumors, RNA was isolated from quick-frozen brain samples obtained at initial surgery from 102 patients, including 4 normal, 40 with GBM, 19 with astrocytomas, 7 with oligodendrogliomas, 16 with meningiomas, and 16 with other types of brain tumors. The relative level of Cyr61 expression was quantified by real-time PCR and was expressed as a ratio between Cyr61 and the reference gene β-actin to correct for variation in the amount of mRNA. The relative target gene expression for the four normal brain samples also was normalized to a mean value (value = 1). The expression level of Cyr61 mRNA present in brain tumors varied but was increased (3- to >22-fold) in ~40% of the brain tumors (Fig. 1*B*). The highest levels were found in the GBM, which are the most malignant gliomas. Twenty-seven of 40 (68%) GBMs overexpressed Cyr61 compared with only 4 of 19 (21%)

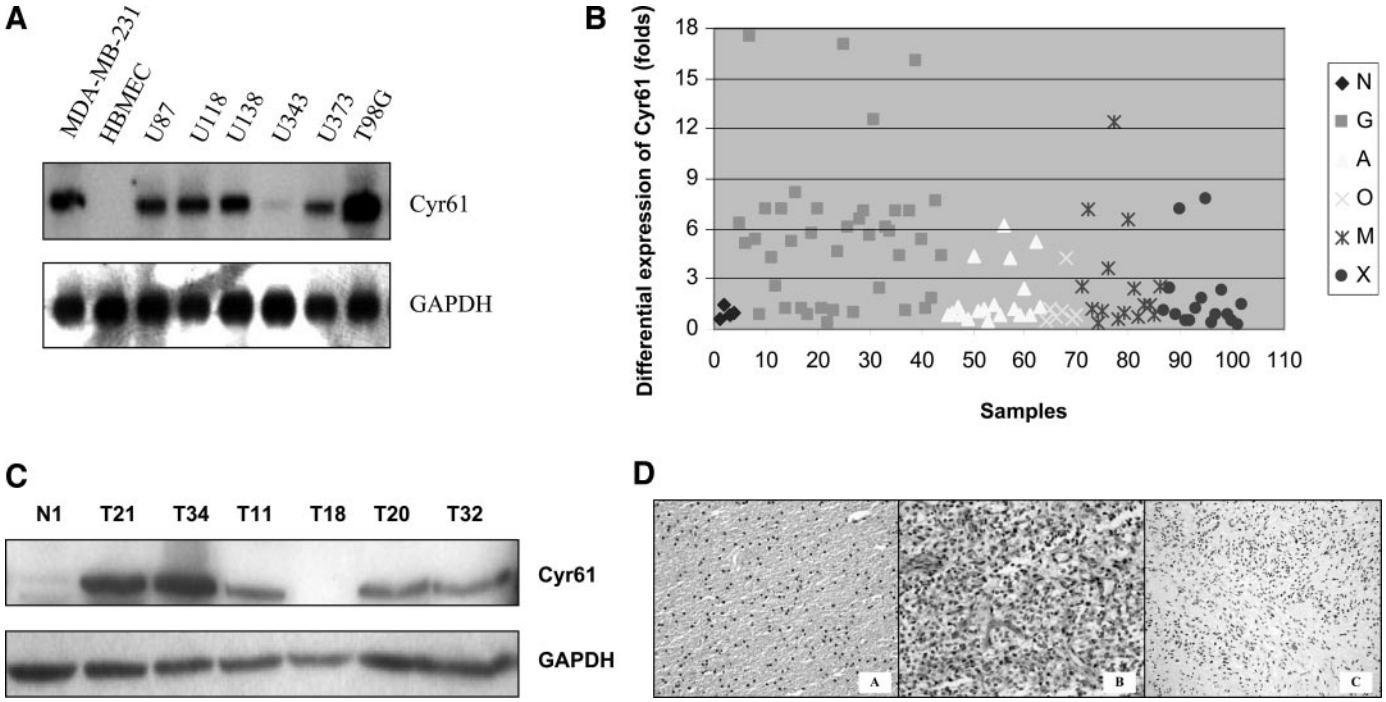

Fig. 1. Expression of Cyr61 in glioma cell lines, human normal brain tissues, and primary brain tumors. For glioma cell lines, total RNA was extracted, subjected to electrophoresis (10 μg RNA/lane), analyzed using Northern blot, and probed with ^{32}P-labeled Cyr61 cDNA. *A*, expression of Cyr61 in glioma cell lines. MDA-MB-231 is a breast cancer cell line that served as a positive control; HBMEC is a normal brain cell line; and U87, U118, U138, U343, U373, and T98G are glioma cell lines. *B*, quantitative expression of Cyr61 in normal brain tissue and primary brain cancer samples. The relative expression levels of Cyr61 in 4 normal brain tissues and 98 primary brain cancer samples were quantified by real-time PCR. The expression levels are displayed as a ratio between the *Cyr61* gene and a reference gene (β-actin) to correct for variation in the amounts of RNA. The relative expression level has been normalized in such a manner that the mean ratio of the four normal brain samples equals a value of 1. *N*, normal; *G*, glioblastoma multiforme (GBM); *A*, astrocytoma; *O*, oligodendroglioma; *M*, meningoma; and *X*, other brain tumors. *C*, expression of Cyr61 protein in primary gliomas as shown by Western blot analysis. The relative value of mRNA expression in tissue samples: N1 = 0.6; T11 = 5.4; T18 = 0.3; T20 = 4.8; T21 = 17.1; T32 = 4.6; and T34 = 16.6. *D*, immunohistochemical staining for Cyr61. *A* is normal brain tissue, which is nearly negative for Cyr61. *B* reveals cytoplasmic staining in a case of GBM; *C* is the negative control for *B*.

astrocytomas and 1 of 7 (14%) oligodendrogliomas. Four of 16 (25%) meningiomas and 2 of 16 (12.5%) other subtypes of brain tumors also had prominent level of Cyr61.

To determine whether overexpression of Cyr61 mRNA was associated with an increased protein level of Cyr61, proteins were extracted for Western blot analysis from normal brain tissue and several human primary glioma samples with different expression levels of Cyr61 mRNA. The results showed that expression of Cyr61 at the protein level paralleled expression level of Cyr61 mRNA measured by real-time PCR in normal brain and human gliomas (Fig. 1*C*).

Immunohistochemical staining was evaluated in three GBM tumors and three normal brain samples. The normal brain tissue was negative for Cyr61 except for sparse cytoplasmic staining in a few glial cells and neurons. In contrast, strong staining for Cyr61 occurred in the neoplastic astrocytoma cells (Fig. 1*D*).

Cyr61 Stimulates Cell Growth in Culture, Colony Formation in Soft Agar, and Cell Migration in Glioma Cells. We first examined the effects of Cyr61 protein on the growth of U343 cells by adding Cyr61 protein into the cultures. The Cyr61 protein stimulated the growth of U343 cells (Fig. 1*A*). Our earlier study showed that forced expression of Cyr61 promoted cell proliferation and anchorage-independent growth in the normal breast cell line MCF-12A and the breast cancer cell line MCF-7 (22). To study whether similar activities occurred in glioma cells, U343 cells, which have low motility and invasiveness, were stably transfected with pcDNA61 containing either full-length Cyr61 or empty vector pcDNA3.1 as a control (32, 33). As expected, Cyr61 was highly expressed in pcDNA61 vector-transfected cells (U343/Cyr61–1 and U343/Cyr61–2) but not in the pcDNA3.1-transfected cells (U343/V) as examined by Northern (data not shown) and Western blot analysis (Fig. 2*B*). Western blot analysis was used to compare expression of Cyr61 in Cyr61-transfected cells (U343/Cyr61) with that observed in the human brain tumor samples. As shown in Fig. 1*C*, the amount of Cyr61 produced in the U343/Cyr61 was comparable with the level of Cyr61 in those human gliomas that highly expressed Cyr61 mRNA. The ability of Cyr61 to enhance growth of cultured cells was investigated by MTT assay. When cells were plated at a density of 1.5×10^5 in RPMI supplemented with 10% fetal bovine serum, the rate of growth was ~2.5-fold greater for the U343/Cyr61 compared with the U343/V control cells. When these cells were grown in RPMI with 0.5% fetal bovine serum, the difference in cell growth rate was even greater (mean, 3.8- $\pm$ 0.8-fold; $P < 0.05$; Fig. 2*D*). Cell cycle analysis showed that U343/Cyr61 cells had a much lower percentage of cells in the G_1 phase (56%) and a higher percentage of cells in the S phase (36%) compared with U343/V cells (82% and 16%, respectively; Fig. 2*E*).

To assess the effect of overexpression of Cyr61 on anchorage-independent growth, the ability of U343/Cyr61 and U343/V cells to form colonies in soft agar was evaluated. U343/Cyr61 cells developed significantly more colonies in soft agar (mean, 2.8- $\pm$ 0.6-fold more colonies; $P < 0.05$; Fig. 2*F*). The colonies formed by U343/Cyr61 also were substantially larger than those formed by U343/V (data not shown).

To determine whether increased expression of Cyr61 enhanced directed cell movement, migration assays of U343/V and U343/Cyr61 were performed in vitronectin-coated Boyden chambers. As shown in Fig. 3, the Cyr61 stably transfected U343/Cyr61 cells had a significantly increased migration compared with the empty vector-transfected U343/V cells ($P < 0.05$). Cyr61 antibody significantly inhibited migration of the U343/Cyr61 cells ($P < 0.05$), suggesting that Cyr61 promoted migration of U343 cells, and this stimulation depended at least in part on Cyr61 being extracellular (Fig. 3).

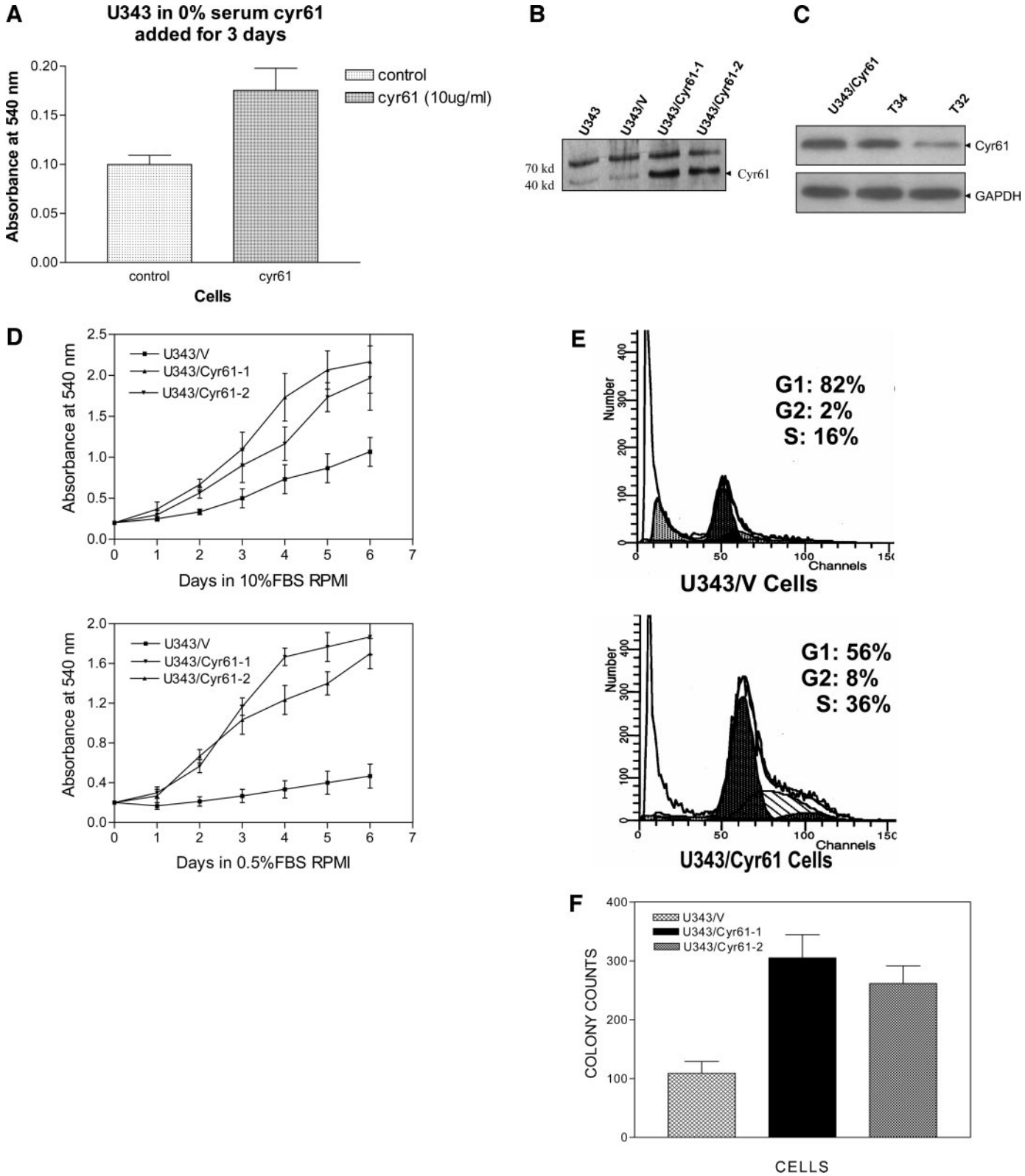

Fig. 2. Effects of Cyr61 on cell cycle, cell growth in liquid culture, and colony formation in soft agar. *A*, effects of Cyr61 protein on growth of glioblastoma multiforme. U343 cells (2×10^3 cells/well) were plated into 96-well plates and cultured for 3 days with 10 μg/ml purified Cyr61 protein. Cell growth was measured by cell proliferation assay (MTT assay). *B*, the U343/Cyr61 and U343/V cell lines were stably transfected with either the empty pcDNA3.1 vector or the Cyr61 expression vector, respectively. U343/Cyr61 clones were selected for high expression of Cyr61. Expression of Cyr61 protein by stably transfected U343/Cyr61 cells is shown by Western blot analysis. Equal loading was ascertained using the internal nonspecific bands at M_r 70,000. *C*, comparison of expression of Cyr61 protein by U343/Cyr61 cells and two primary gliomas as shown by Western blot analysis. *D*, cell growth rates of U343/Cyr61–1 and -2 and U343/V in 10% or 0.5% fetal bovine serum were measured by MTT assay. *E*, cell cycle analysis. Cells were collected with trypsin/EDTA at 60% confluence, fixed with ethanol, and stained with propidium iodide. The cell cycle was analyzed using fluorescence-activated cell sorting. *F*, soft agar assay. A total of 1.0×10^3 cells/well were seeded in soft agar for 2 weeks, and colonies were enumerated. Each experiment was performed in triplicate, and results represent the mean ± SD of three experiments.

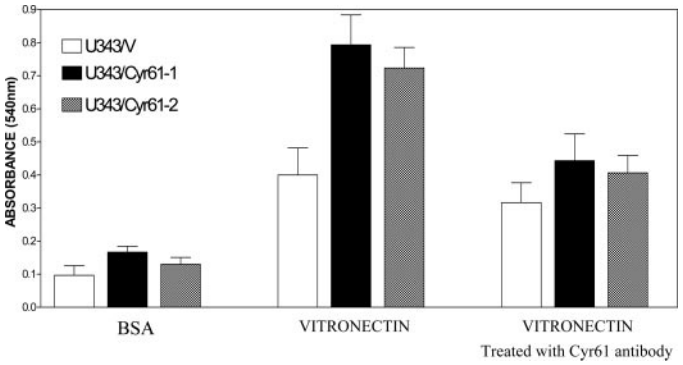

Fig. 3. Cyr61 stimulates cell migration in U343 cells. Cells (5×10^4/well) were seeded in either BSA-coated (control) or vitronectin-coated Boyden chambers. Cells were allowed to migrate for 4–8 h and quantified as described in the QCM-VN protocols (Chemicon). For antibody treatment, Cyr61 antibody (10 ìg/ml) was added to the culture media. The number of cells that migrated through the membrane was determined by absorbance at 550 nm. Each bar represents the mean ± SD of triplicate experiments.

Cyr61 Up-Regulates Expression of Distinct Integrins and Increases the Activity of ILK. Integrins are heterodimeric cell surface receptors that consist of noncovalently associated α and β subunits. These receptors play a role in cell migration, proliferation, and gene transcription and can affect growth and invasion of cancer cells (34–36). Previous studies have shown that CYR61 interacts with various integrins, including $\alpha v\beta 3$, $\alpha 6\beta 3$, $\alpha v\beta 5$, and $\alpha IIb\beta 3$, which can enhance disparate activities, such as cell migration, angiogenesis, and tumorigenesis (14–16, 37). To determine whether overexpression of Cyr61 alters the level of integrin expression in U343 cells, we performed an RNase protection assay to quantify simultaneously 10 mRNA species of distinct integrins from U343/V and U343/Cyr61 cells. Seven of the 10 integrins were expressed more abundantly in the U343/Cyr61 cells compared with U343/V control cells, including integrin αv and $\beta 1$, which were elevated dramatically in the Cyr61 stably transfected cells (Fig. 4*A*). In contrast, glyceraldehyde-3-phosphate dehydrogenase (control) was expressed equivalently in both types. We also used immunofluorescent staining and flow cytometry to examine expression of the integrin $\alpha v\beta 3$, a known receptor of Cyr61 (16). It was expressed at a higher level on the cellular surface of U343/Cyr61 cells compared with U343/V cells (Fig. 4*B*).

Studies have demonstrated that integrins $\beta 1$ and $\beta 3$ could regulate cell migration, cell cycle progression, and anchorage-dependent growth through an ILK (38, 39). ILK can interact directly with the cytoplasmic domain of the $\beta 1$ and $\beta 3$ integrin subunits, and its kinase activity is modulated by interactions with the extracellular matrix. To determine whether overexpression of Cyr61 in U343/Cyr61 cells had an effect on either expression or activation of ILK, we measured the level of ILK by Western blot analysis and ILK activity by ILK kinase assay. As shown in Fig. 4*C*, protein levels of ILK in U343/Cyr61 cells were only slightly higher than those in either the U343 or U343/V cells. In contrast, the ILK activity in both U343/Cyr61 sublines was increased dramatically compared with those in either U343 or U343/V control cells (Fig. 4*D*). These results together showed that overexpression of Cyr61 in U343/Cyr61 cells increased expression of distinct integrins, leading to the activation of the ILK.

Cyr61 Stimulates Nuclear Translocation and Transcriptional Activity of β-Catenin. Previous studies have shown that ILK can activate the β-catenin-TCF/Lef signaling pathway perhaps by phosphorylating GSK-3β, which inhibits its activity, allowing accumulation and translocation of β-catenin into the nucleus (40, 41). Furthermore, ILK can up-regulate cyclin D1, also through phosphorylation and inactivation of GSK-3β (42). To assess whether overexpression of Cyr61, which leads to stimulation of ILK activity, altered the β-catenin signaling pathway, we performed Western blot analysis to evaluate the expression of β-catenin and GSK-3β in the Cyr61 stably transfected cells (U343/Cyr61–1 and U343/Cyr61–2). Protein levels of β-catenin (Fig. 5*A*) were elevated in the U343/Cyr61 cells compared with the U343/V cells. Whereas the level of expression of total GSK-3β was similar in the two cell types, phosphorylated GSK-3β was markedly increased in the U343/Cyr61 cells (Fig. 5*B*). To determine whether the increase of β-catenin can alter its cellular localization, we examined the cells by immunofluorescence. Overexpression of Cyr61 altered dramatically the subcellular localization of β-catenin. β-Catenin remained in the cytoplasm and cell membrane with minimal nuclear localization in the empty vector-transfected U343/V cells; in contrast, the Cyr61 stably transfected U343/Cyr61 cells showed prominent nuclear localization of β-catenin (Fig. 5*C*).

β-Catenin can associate with TCF/Lef transcription factors to activate transcription of target genes that are especially associated with cell proliferation (43–45). Therefore, we examined whether β-catenin was complexed with TCF/Lef in the U343/Cyr61 and U343/V cells by coimmunoprecipitation assay. Cell extracts were isolated from these cells and immunoprecipitated with β-catenin antibody, followed by electrophoresis, Western blot analysis, and probe with an antibody to TCF-4. As shown in Fig. 5*D*, the complex of β-catenin/TCF-4 was detected easily in both sublines of U343/Cyr61 cells but rarely was found in U343/V cells.

Previous studies have shown that the NH_2 terminus of TCF is required for binding to β-catenin and that TCF mutant proteins lacking N-terminal sequences retain DNA binding activity but function in a dominant-negative fashion (46). Hence, we sought to test the effects of such a dominant-negative TCF-4 protein on the Cyr61-overexpressing U343/Cyr61 cells. These cells were transfected with dominant-negative TCF-4 (pcDNA3/ΔN-TCF) and pMACS K^k (codes for cell surface expressed, truncated H-$2K^k$) or with pMACS K^k alone. Cells expressing the truncated H-$2K^k$ were collected using beads coated with an antibody to H-$2K^k$. Expression of the dominant-negative TCF-4 in U343/Cyr61 cells (U343/Cyr61-ΔN-TCF) caused their severe growth arrest compared with control cells transfected with only pMACS K^k(U343/Cyr61-K^k) as measured by MTT (Fig. 5*E*) and colony formation assay (Fig. 5*F*). In the soft agar assay, cells expressing the dominant-negative TCF-4 formed 2.5-fold fewer colonies that also were markedly smaller than the colonies formed by U343/Cyr61 cells (Fig. 5*F*; data for colony size not shown).

We also determined whether the elevated level of endogenous cyclin D1 expression in U343/Cyr61 cells could be inhibited by transient expression of a dominant-negative TCF-4. The dominant-negative TCF-4 reduced dramatically the prominent expression of cyclin D1 induced by overexpression of Cyr61 in U343/Cyr61 cells (Fig. 5*G*). Taken together, these results suggest that Cyr61 stimulates β-catenin-TCF/Lef signaling to enhance expression of proteins associated with cell proliferation. Reduction of levels of cyclin D1 caused by disrupting signaling from β-catenin to TCF probably contributed to the slowing of cell growth because it is one of the rate-limiting factors for progression through the G_1 phase of the cell cycle (47).

Cyr61 Activates Akt through PI3k. Previous studies demonstrated that ILK phosphorylated directly Akt on serine 473 *in vitro* via the binding of PtdIns (3–5) with a PH-like domain of ILK; in contrast, a kinase-deficient form of ILK inhibited severely Akt serine 473 phosphorylation *in vivo* (40). Therefore, we wanted to determine whether overexpression of Cyr61 could regulate phosphorylation and activation of Akt in U343 cells. Because phosphorylation of Akt on serine 473 is required for its activation, we monitored this site using

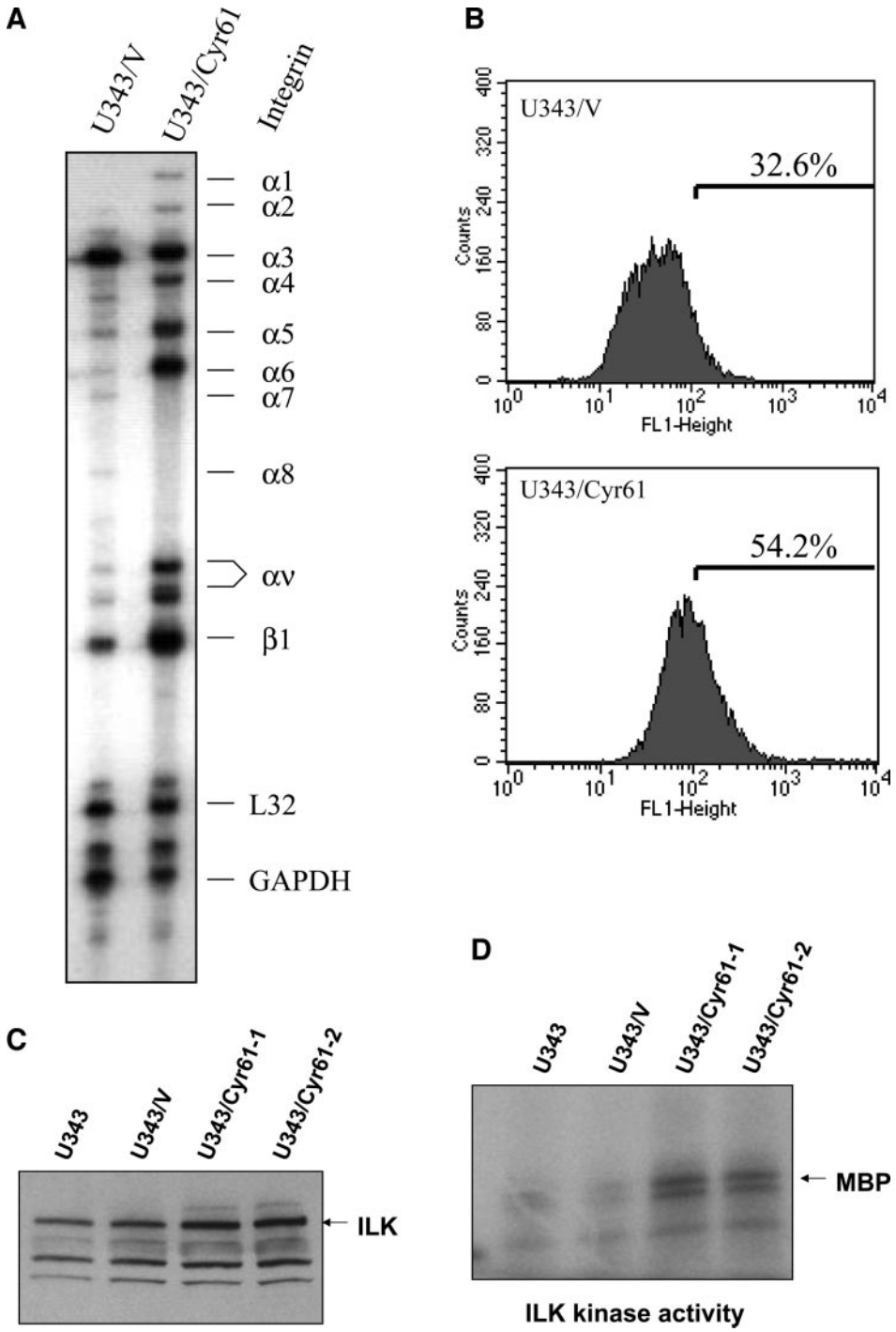

Fig. 4. Cyr61 up-regulates expression of distinct integrins and stimulates the activity of integrin-linked kinase (ILK). *A*, differential expression of integrin genes in Cyr61 stably transfected cells. Expression of integrins in U343/Cyr61 and U343/V cells was detected by the RNase protection assay. A multiprobe template set (PharMingen, San Diego, CA) containing RNA probes of different lengths was hybridized to the sample RNA, and single-stranded RNA was digested with RNase. The remaining RNase-protected probes were run on a gel and visualized by autoradiography. Undigested probes (not shown) were used as markers to aid identification of the RNase-protected bands. *B*, fluorescence-activated cell sorting (FACS) analysis of integrin $\alpha v\beta 3$ expression was performed as described in "Material and Methods" using LM609 (1:500), a monoclonal antibody to integrin $\alpha v\beta 3$. Secondary antimurine IgG was conjugated to FITC before FACS analysis. *C*, expression of ILK in U343/V and U343/Cyr61 cells. *D*, ILK activity assay. ILK was immunoprecipitated from U343/V and U343/Cyr61 cell extracts, and ILK activity was determined by using myelin basic protein as substrate following the protocol as described in "Materials and Methods."

an antibody that reacted specifically with phosphorylated serine 473. Akt was phosphorylated constitutively on serine 473 in Cyr61 stably transfected U343/Cyr61 cells (Fig. 6*A*). Therefore, because we had shown that ILK activity was stimulated constitutively in U343/Cyr61 cells, Cyr61 might regulate the serine 473 of Akt through activation of ILK. Because others have shown that engagement of integrins stimulated PI3k activity leading to activation of Akt (48, 49) and that GSK-3β activity can be regulated by Akt in a PI3k-dependent manner (50), we measured kinase activity of Akt using GSK-3β as a substrate. Akt 1G1 monoclonal antibody was used selectively to immunoprecipitate Akt, and the resulting immunoprecipitate then was incubated with the GSK-3β fusion protein in the presence of ATP. Overexpression of Cyr61 increased kinase activity of Akt and resulted in phosphorylation of GSK-3 (Fig. 6*B*). To test whether these events were linked to PI3k activation, we treated U343/Cyr61 and U343/V cells with two distinct PI3k inhibitors, wortmannin and LY294002. Both PI3k inhibitors blocked Cyr61-induced increase in the Akt activity (Fig. 6*B*), suggesting that activation of Akt by Cyr61 is mediated by a PI3k-dependent mechanism.

Akt is known to phosphorylate and suppress the activity of the proapoptotic protein Bad (51–53). We analyzed the status of Bad on Akt activation in Cyr61-overexpressing cells (U343/Cyr61). Although levels of Bad were similar in U343/V and U343/Cyr61 cells, phosphorylated Bad was detected prominently only in the U343/Cyr61 cells (Fig. 6*C*), suggesting that Cyr61 also may be involved in the Akt-Bad antiapoptotic signaling pathway.

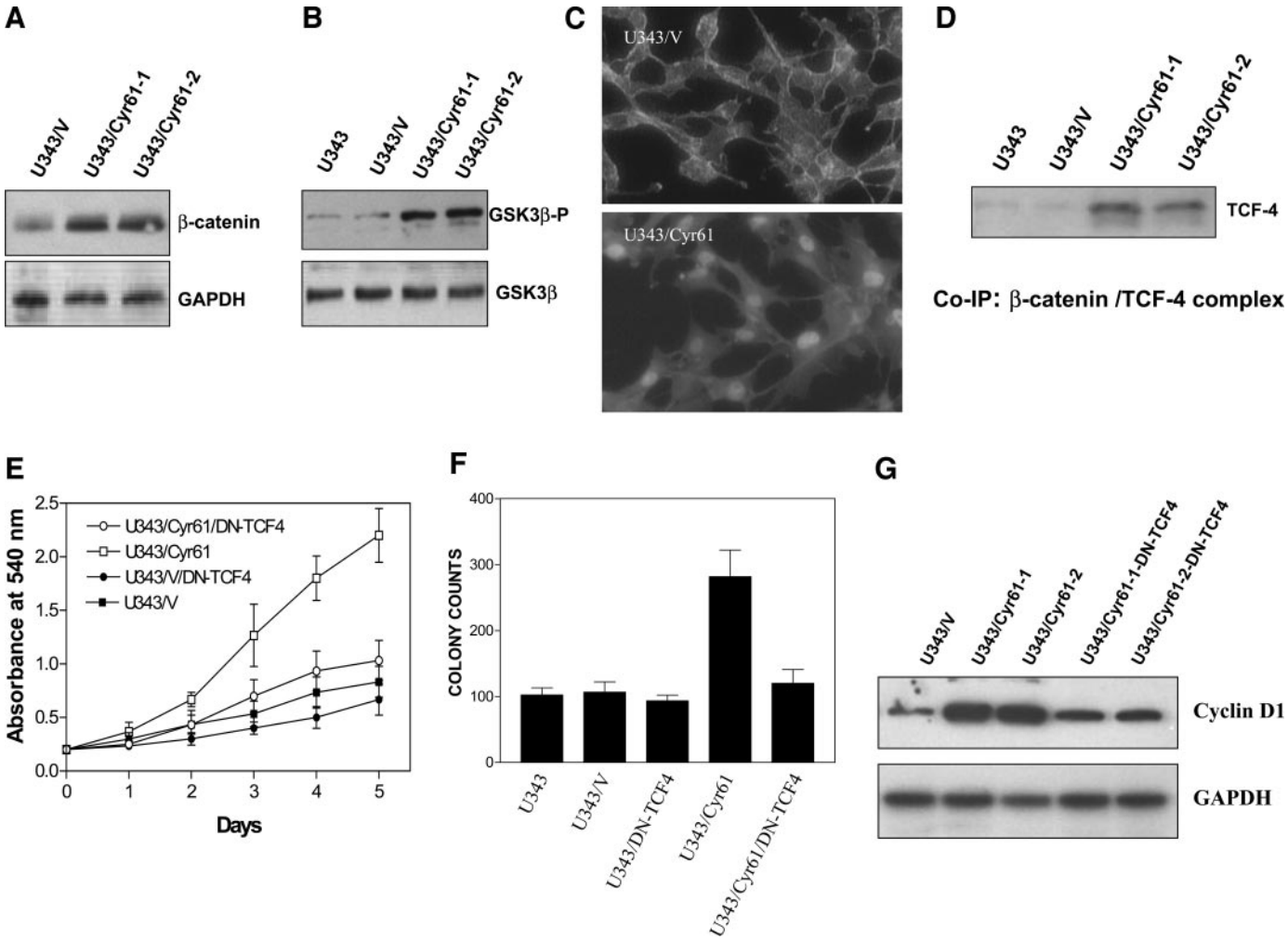

Fig. 5. Cyr61 is involved in β-catenin-TCF/Lef signaling pathway. Forced expression of Cyr61 in U343 cells altered the levels of Wnt-1 signaling regulatory proteins. Lysates from control U343, pcDNA3.1 vector-transfected (U343/V), and Cyr61 stable-transfected (U343/Cyr61) cells were run on a 4–15% polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. Wnt-1 signaling regulatory proteins β-catenin (*A*) and phosphorylated glycogen synthase kinase-3β (*GSK-3β*; *B*) were detected by individual specific antibodies. Either glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or nonphosphorylated GSK-3β served as a loading control. *C*, immunofluorescence staining of β-catenin in U343/V and U343/Cyr61 cells. Cells were stained with a β-catenin antibody, and the immunoreactivity was revealed using Rhodamine Red-X goat antirabbit IgG conjugate. U343/Cyr61 cells demonstrated predominantly nuclear staining of β-catenin. In contrast, most of the U343/V cells exhibited cytoplasmic and membrane β-catenin staining with minimal staining in the nucleus. *D*, coimmunoprecipitation of TCF-4 with β-catenin. Cell extracts (500 μg in NP40 lysis buffer) were immunoprecipitated with 4 μg of rabbit anti-β-catenin antibody and electrophoresed through 4–15% polyacrylamide gel. The gel was Western blotted with goat antibody to TCF-4. *E* and *F*, Cyr61-stimulated cell growth was decreased by dominant-negative TCF-4 (*DN-TCF-4*) in cell culture and soft agar. U343/V and U343/Cyr61 cells were transfected with 10 μg DN-TCF-4 (pcDNA3/ΔN-TCF) and 2 μg pMACS K^k (Miltenyi Biotec, Cologne, Germany) or 2 μg pMACS K^k alone. Cells expressing the truncated H-$2K^k$ were collected by beads coated with an antibody to H-$2K^k$. *G*, DN-TCF-4 was able to partially but significantly block the increased expression of cyclin D1 induced by Cyr61.

Cyr61 Promotes Tumorigenicity and Vascularization in Nude Mice. Our previous studies showed that stable expression of Cyr61 under the regulation of a constitutive promoter in the breast cancer cell MCF-7 significantly increased its tumorigenicity in nude mice. Moreover, overexpression of Cyr61 in normal breast cells (MCF-12A) induced their transformation and formed tumors in nude mice (22). On the basis of our *in vitro* studies indicating that overexpression of Cyr61 promoted anchorage-independent clonogenic proliferation in soft agar and cell migration, we examined whether forced expression of Cyr61 in U343 cells (U343/Cyr61) could enhance their ability to form tumors and stimulate neovascularization in nude mice compared with their controls. These cells were injected s.c. into 8-week-old nude mice, and tumor growth was measured once a week. The U343/Cyr61 cells (sublines 1 and 2), expressing Cyr61 at a high level, developed tumors with a significantly shorter latency (2 weeks after injection; $P < 0.05$) and with a markedly larger size during the 8 weeks of observation compared with the tumors formed by the control U343/V cells (Fig. 7, *A*, *B*, and *C*).

To examine whether the tumors that prominently expressed Cyr61 *in vivo* were associated with angiogenic activity, tumors derived from the U343/Cyr61 and U343/V cells were analyzed histochemically using antibody against CD31. Immunohistochemical analysis demonstrated robustly increased blood vessel density in U343/Cyr61 tumors compared with those from the U343/V controls (Fig. 7*D*).

DISCUSSION

Recent studies have begun to shed light on the tumorigenic role of the growth factor-inducible early response gene *Cyr61* (22, 28, 30, 31). Our early studies demonstrated that Cyr61 is overexpressed in breast cancers and may be involved in the development of estrogen-mediated breast cancer (22). Cyr61 was expressed at high levels in invasive breast cancer cell lines and primary breast cancers, whereas expression of Cyr61 was undetectable in either normal breast tissue or normal breast cell lines. Moreover, expression of Cyr61 mRNA levels increased threefold to fivefold in MCF-7 breast cancer cells after their

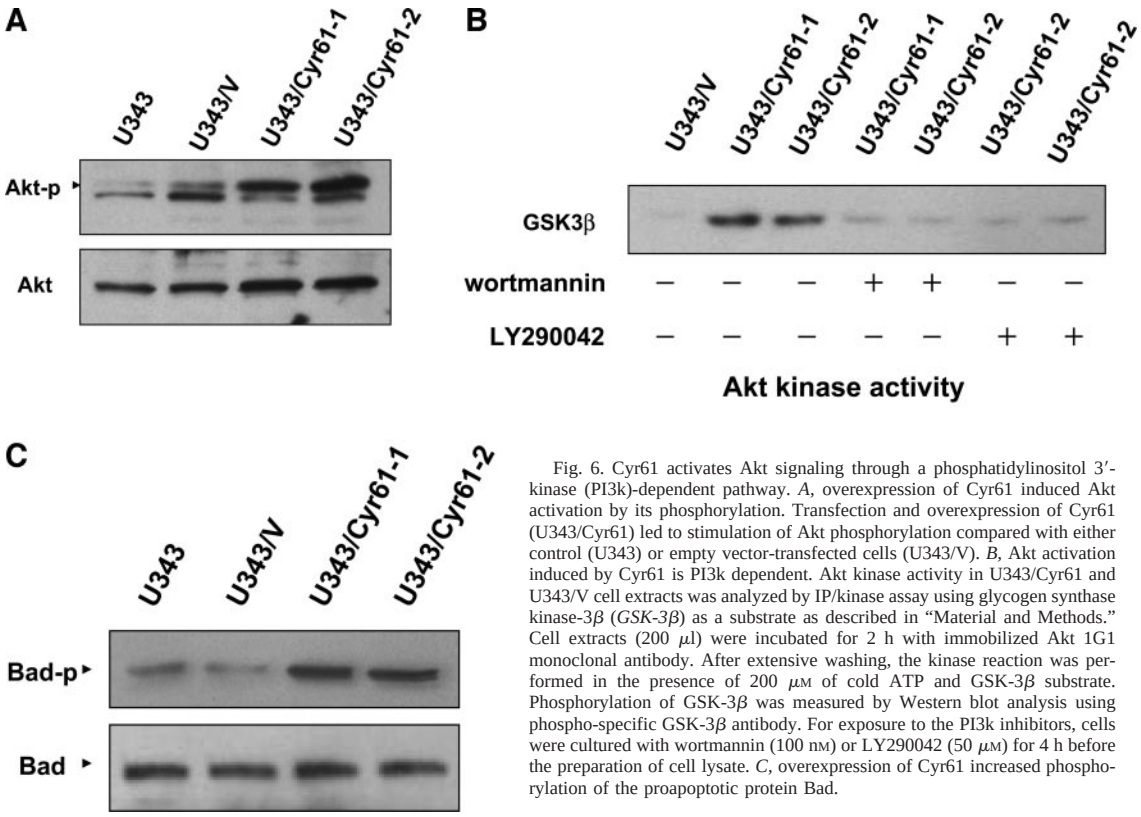

Fig. 6. Cyr61 activates Akt signaling through a phosphatidylinositol 3′-kinase (PI3k)-dependent pathway. *A*, overexpression of Cyr61 induced Akt activation by its phosphorylation. Transfection and overexpression of Cyr61 (U343/Cyr61) led to stimulation of Akt phosphorylation compared with either control (U343) or empty vector-transfected cells (U343/V). *B*, Akt activation induced by Cyr61 is PI3k dependent. Akt kinase activity in U343/Cyr61 and U343/V cell extracts was analyzed by IP/kinase assay using glycogen synthase kinase-3β (*GSK-3β*) as a substrate as described in "Material and Methods." Cell extracts (200 μl) were incubated for 2 h with immobilized Akt 1G1 monoclonal antibody. After extensive washing, the kinase reaction was performed in the presence of 200 μM of cold ATP and GSK-3β substrate. Phosphorylation of GSK-3β was measured by Western blot analysis using phospho-specific GSK-3β antibody. For exposure to the PI3k inhibitors, cells were cultured with wortmannin (100 nM) or LY290042 (50 μM) for 4 h before the preparation of cell lysate. *C*, overexpression of Cyr61 increased phosphorylation of the proapoptotic protein Bad.

exposure to estrogen. This induction was blocked by tamoxifen and ICI182,780, inhibitors of the estrogen receptor. Furthermore, forced expression of Cyr61 in MCF-7 cells enhanced their anchorage-independent cell growth in soft agar and significantly stimulated their ability to form large tumors in nude mice. These findings motivated us to examine the function of Cyr61 protein in other cancers.

In this investigation, we have found that Cyr61 assumed a similar role in gliomas as in breast cancers. It was expressed prominently in the highly tumorigenic astrocytoma cell lines U87, U373, and T98G and expressed very weakly in the less tumorigenic cell U343. Furthermore, overexpression of Cyr61 occurred in 68% of the highly malignant GBM compared with 22% of the astrocytomas and 14% of the oligodendrogliomas, suggesting that Cyr61 might be involved in tumor progression of astrocytomas and oligodendrogliomas into GBM.

The role of Cyr61 in the growth of gliomas was evaluated in several experimental models. Its forced expression in U343 cells (U343/Cyr61) markedly stimulated their proliferation in liquid culture and in an anchorage-independent manner in soft agar and significantly enhanced their tumorigenicity and vascularization *in vivo*. These cells developed larger, more vascularized tumors in nude mice. These cells also migrated more readily. All of these properties are reminiscent of our earlier observations in Cyr61 stably transfected MCF-7 breast cancer cells (22).

An important finding of this study is the characterization of Cyr61 as a tumorigenic enhancer of gliomas through activation of Akt and stabilization of β-catenin and its nuclear translocation, resulting in stimulating β-catenin-TCF/Lef signaling pathway. Overexpression of Cyr61 in the U343 cells resulted in enhanced levels of distinct integrins, including $\alpha v\beta 3$ and $\beta 1$, which are known to be the receptors of Cyr61 (14, 16). We concurrently found that the Cyr61-expressing glioma cells activated the PI3k pathway through ILK. To our knowledge, this is the first report showing that Cyr61 can up-regulate various integrins and activate ILK. Overexpression of Cyr61 in U343 cells also inhibited the activity of GSK-3β by its phosphorylation and induced nuclear translocation of β-catenin. Both of these effects were most likely through the activation of ILK. A previous study showed that activated ILK could inhibit GSK-3β by directly phosphorylating GSK-3β, resulting in translocation of β-catenin into the nucleus in mammary epithelial cells (41). β-Catenin plays a signaling role as a key mediator in the Wnt signaling pathway. The ultimate mediator of this pathway is the nuclear complex of β-catenin acting as a coactivator with TCF/Lef transcription factors to stimulate transcription of a variety of target genes (54). These genes often are associated with stimulating cell proliferation, including cyclin D1, c-*myc*, c-Jun, Fra-1, urokinase-type plasminogen activator receptor, and E-cadherin (55–57). Our studies showed that forced expression of Cyr61 in glioma cells up-regulated transcription of cyclin D1 but not the c-*myc* (data not shown). Whether Cyr61 results in the up-regulation of other TCF/Lef-1 target genes remains to be elucidated.

WISP-1, another CNN family member closely related to Cyr61, was found recently to be a Wnt-1- and β-catenin-responsive oncogene (29). Transfected and overexpressed WISP-1 in a normal rat kidney fibroblast cell line (NRK-49F) induced their morphologic transformation, accelerated their cell growth, enhanced their saturation density *in vitro*, and permitted the cells to form tumors in nude mice. Considering that Cyr61 has four identical structural domains and is related closely to WISP-1, both of these genes may be involved in similar signaling pathways in the development and progression of tumors.

Another interesting finding of this study is that Cyr61 can activate Akt and inhibit the apoptotic effector Bad by its phosphorylation, suggesting that Cyr61 may be involved in more than one signaling pathway. Akt is a key regulator of many intracellular processes implicated in progression of brain tumors (5, 58). Studies have found that Akt-dependent phosphorylation of Bad resulted in its cytosolic sequestration by the ô form of the 14-3-3 proteins and prevented its binding to the survival factor Bcl-X_L at intracellular membrane sites (60). Because Bad exerts its death-promoting effects by heterodimer-

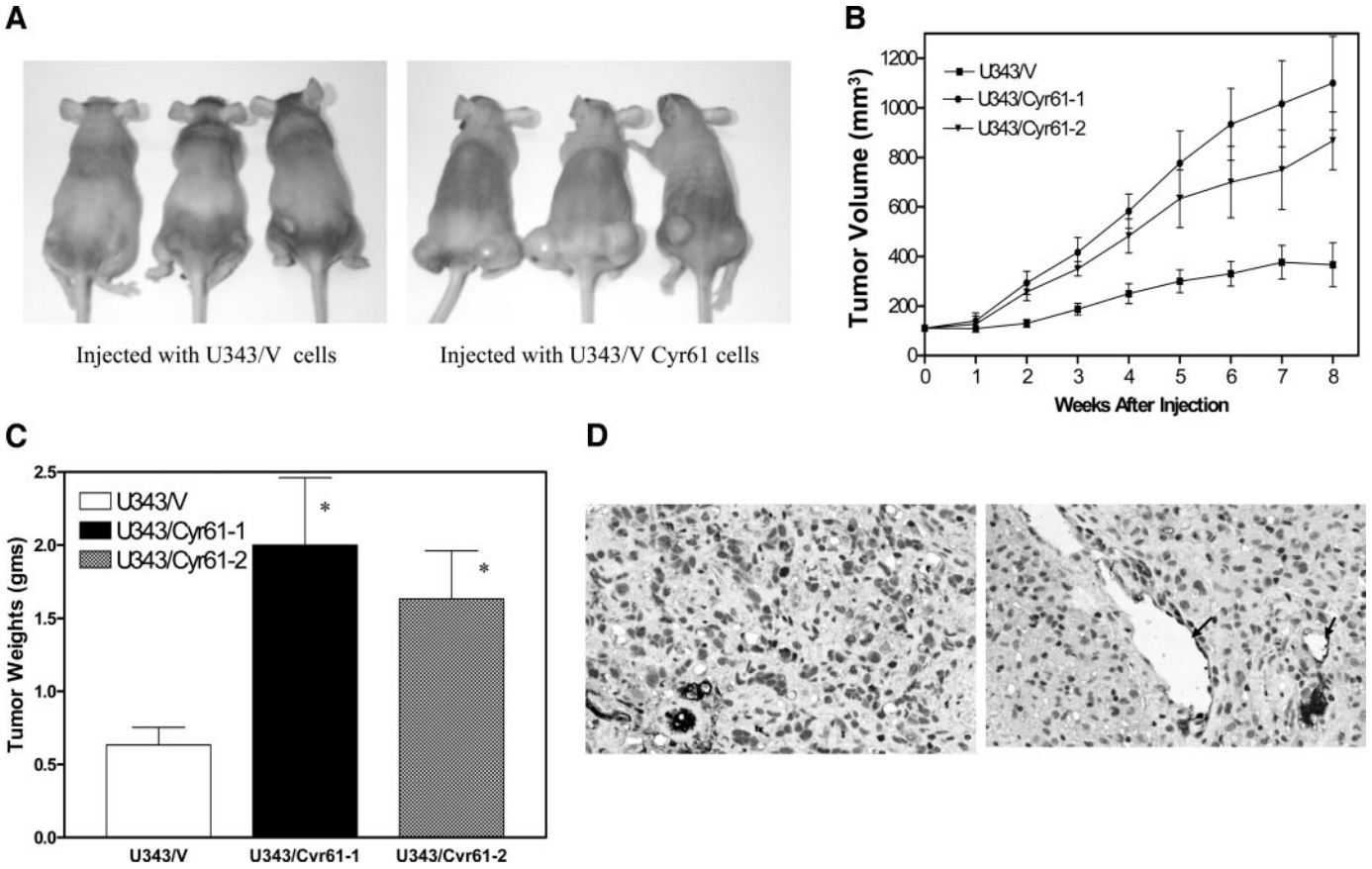

Fig. 7. Effect of forced Cyr61 expression on tumor growth, tumor histology, and neovascularization in nude mice. U343/V cells (vector control) or U343/Cyr61 (Cyr61 expressor) cells were mixed with Matrigel (1:1) and injected s.c. into BNX nude mice (2×10^5 cells/flank). *A*, xenografts growing in nude mice for 8 weeks after injection with either U343/V (*left*) or U343/Cyr61 (*right*). *B*, time course of tumor growth. Tumor volumes were measured every week. Each point represents the mean volume ± SD of eight tumors. *C*, tumor weights at autopsy. At 8 weeks after injection, tumors were removed and weighed. Results are shown as mean ± SD of tumor weights. Statistical significance was determined with a Student's *t* test using the computer program by GraphPad (San Diego, CA); ∗, $P < 0.05$. *D*, immunohistochemical analysis demonstrated robustly increased blood vessel density (*arrows*) in U343/Cyr61 tumors (*right*) compared with those from the U343/V controls (*left*) when immunostained with anti-CD31 antibodies.

izing with and inhibiting the death antagonist Bcl-X_L, phosphorylation of Bad by Akt can preclude its binding to the membrane-anchored Bcl-X_L, leading to increased cell survival (61). Thus, phosphorylation of Bad by Akt is a possible mechanism by which Cyr61 delivers a survival signal, leading to the inhibition of apoptosis. Interestingly, WISP-1 also was shown recently to activate the Akt signaling pathway and up-regulate the antiapoptotic Bcl-X_L protein (62).

We also have demonstrated that Cyr61-dependent activation of Akt can phosphorylate GSK-3β, leading to its inactivation (Fig. 6*B*). Thus, the nuclear accumulation of β-catenin caused by overexpression of Cyr61 may result from inactivation of GSK-3β by Akt. Several studies have found that Akt could decrease GSK-3β activity, but the change was not sufficient to cause translocation of β-catenin into the nucleus in the absence of Wnt signaling (63, 64). In contrast, several other studies have found that the Akt-mediated phosphorylation and inhibition of GSK-3β led to accumulation and nuclear translocation of β-catenin (65–69). ILK has been reported to phosphorylate directly the serine 473 of Akt, resulting in phosphorylation and inhibition of GSK-3β, stimulating nuclear translocation of β-catenin and activation of TCF/Lef transcription factor (40, 42, 59, 70). Furthermore, Fukumoto *et al.* (66) have shown that activated Akt bound to the Axin-GSK-3β complex in the presence of Dishevelled resulted in phosphorylation of GSK-3β and increased free β-catenin, which could be blocked by a dominant-negative Akt.

We showed that overexpression of Cyr61 enhanced tumorigenicity of gliomas cells. Overexpression of Cyr61 results in up-regulation of distinct integrins and activation of ILK mediated by PI3k. Activated ILK inhibits GSK-3β activity by either phosphorylating it directly or first phosphorylating and activating AKT, which then phosphorylates and inactivates GSK-3β. This causes the accumulation of β-catenin in the cytoplasm, resulting in its translocation into nucleus, where it binds to the transcription factors Tcf/Lef, increasing transcriptional activation of cyclin D1 and other target genes. Meanwhile, activated ILK can directly phosphorylate and activate Akt, resulting in inhibition of apoptosis by phosphorylating and suppressing the proapoptotic protein Bad.

In summary, our data indicate that overexpression of Cyr61 may be involved in development of gliomas through activation of the ILK-mediated β-catenin-TCF/Lef and the Akt signaling pathways. This comprehensive elucidation of Cyr61 signaling in brain tumors is an important step to explore the mechanism and function of this protein in the development of gliomas.

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Cancer Res 2004;64:1987-1996.