mTORC2 Activity Is Elevated in Gliomas and Promotes Growth and Cell Motility via Overexpression of Rictor

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Abstract

mTORC2 is a multimeric kinase composed of the mammalian target of rapamycin kinase (mTOR), mLST8, mSin1, and rictor. The complex is insensitive to acute rapamycin exposure and has shown functions in controlling cell growth and actin cytoskeletal assembly. mTORC2 has recently been shown to phosphorylate and activate Akt. Because ~70% of gliomas harbor high levels of activated Akt, we investigated whether mTORC2 activity was elevated in gliomas. In this study, we found that mTORC2 activity was elevated in glioma cell lines as well as in primary tumor cells as compared with normal brain tissue (P < 0.05). Moreover, we found that rictor protein and mRNA levels were also elevated and correlated with increased mTORC2 activity. Overexpression of rictor in cell lines led to increased mTORC2 assembly and activity. These lines exhibited increased anchoragel-dependent growth in soft agar, increased S-phase cell cycle distribution, increased motility, and elevated integrin β1 and β3 expression. In contrast, small interfering RNA-mediated knockdown of rictor inhibited these oncogenic activities. Protein kinase Cα (PKCα) activity was shown to be elevated in rictor-overexpressing lines but reduced in rictor-knockdown clones, consistent with the known regulation of actin organization by mTORC2 via PKCα. Xenograft studies using these cell lines also supported a role for increased mTORC2 activity in tumorigenesis and enhanced tumor growth. In summary, these data suggest that mTORC2 is hyperactivated in gliomas and functions in promoting tumor cell proliferation and invasive potential due to increased complex formation as a result of the overexpression of rictor.

Introduction

Malignant gliomas are the most common form of brain tumors and are highly aggressive, invasive, and typically refractory to current treatments (1, 2). The median survival for patients with glioblastoma multiforme is <1 year (3). Current hypotheses about the genesis of these tumors suggest that they develop due to accumulation of independent genetic mutations, which results in either activation of oncogenic factors or loss-of-function effects on tumor suppressor genes (4). Mutations of p53, Rb, and phosphatase and tensin homologue (PTEN); deletion of p16INK4a, amplification of cyclin-dependent kinase (CDK)-4 and epidermal growth factor receptor (EGFR); and activation of Ras and Akt signaling cascades have been shown to contribute to the development of gliomas (5–7). However, microarray analyses suggest that several hundred transcripts are markedly altered relative to normal brain (8, 9).

mTOR is a serine/threonine kinase belonging to the phosphatidylinositol 3-kinase–related family of kinases and is a common element to two separate multicomponent complexes in the cell. The mTOR–mLST8–rictor complex (TORC1) integrates signals regulating cell size and growth whereas the mTOR–mLST8–Sin1–rictor complex (TORC2) regulates cell cycle–dependent cytoskeleton assembly in addition to cell growth (10). TORC1 is sensitive to rapamycin and regulates cap-dependent mRNA translation and ribosomal component biogenesis (11–13), whereas data show that TORC2 is insensitive to short-term rapamycin exposure (14). In some cell lines, TORC2 activity has been shown to be sensitive to rapamycin on prolonged exposure (15). Activation of the TORC1 branch results in phosphorylation of p70 S6 ribosomal kinase and eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). Phosphorylation of 4E-BP1 results in its liberation from eIF-4E and the formation of functional eIF-4F translation initiation complexes (16, 17). As a result of a gain-of-function effect, rapamycin binds to the small prolyl-isomerase FK506 binding protein 12, forming a complex that binds mTOR and inhibits kinase activity (18). The inhibition of mTOR by these agents typically induces G1 cell cycle arrest but can also result in the activation of apoptosis (19).

Recent data have implicated the TORC2 kinase as the major hydrophobic kinase that phosphorylates Ser473 on Akt (20, 21). Because elevated Akt kinase activity is found in a majority of gliomas (22, 23), we decided to investigate whether mTORC2 activity was elevated in brain tumors. In an initial survey of brain tumor cell lines, we found that the defining component of TORC2, rictor, was expressed at higher levels relative to normal brain tissue. We further show that enhanced rictor expression increased the amount of assembled TORC2 and correlated with elevated activity of the complex. Similarly, an analysis of primary gliomas also showed overexpression of rictor at both the mRNA and protein levels. Ectopic expression of rictor led to increased growth and mobility, whereas RNA interference–mediated inhibition of rictor expression had the reverse effects in vitro and in xenograft experiments. These results support a role for elevated TORC2 activity in glioma growth and invasiveness mediated by the overexpression of rictor.
Materials and Methods

Cell lines, plasmids, transfections, and lentiviral transduction. H4, T98G, U-138MG, LN-229, and U87 cell lines were obtained from American Type Culture Collection. The myc-tagged rictor in pRKS+ (plasmid #11367), the short hairpin RNA (shRNA)/pcDNA.1 targeting rictor (plasmid #1854), scrambled sequence/plK.O1 (plasmid #1864), pCMV-D8.R2.dpr (plasmid #8455), and pCMV-VSVG were obtained from Addgene and previously described (14, 20, 24, 25). pcDNA3.1 was from Invitrogen. The myc-tagged rictor construct was cotransfected with pcDNA3.1 using FuGene 6 (Roche) into the indicated cell lines and transfectants were selected by G418 resistance (500 μg/mL). Stably expressing lines were screened by immunoblotting for the rictor or the myc tag. Lentiviral shRNA production and infection was done as described (20). Cells were infected in media containing 6 μg/mL of protamine sulfate and clones selected for by puromycin resistance. Clones were screened by immunoblotting for endogenous rictor.

RNA analysis. Flash-frozen normal brain and glioma samples were obtained from the Cooperative Human Tissue Network, National Cancer Institute (Western Division, Vanderbilt University Medical Center). Extraction of RNA was done using Trizol (Invitrogen). Total RNA was then quantified and integrity assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). One microgram of total RNA was treated with DNase I (Ambion) before use in quantitative PCR. Northern blot analysis of rictor and 18S RNA was done as previously described (26). 32P-labeled antisense riboprobes for rictor were generated by PCR with primers that incorporated a T7 promoter, was used to generate labeled antisense riboprobe via an in vitro transcription reaction. The primer sequences are as follows: forward primer, 5'-GGAAGATTACTCTCAACATTG-3'; reverse primer, 5'-GCATTATGCTGGATGATCCGGCGTGTTGATGCCCTTAGATG-3'. An antisense riboprobe was used to visualize 18S rRNA levels (27). For quantitative reverse transcription PCR, total RNA was reverse transcribed with random primers using the RETROscript Kit from Ambion. The real-time PCR was done as previously described (27) using a standard curve to determine the relative amounts of rictor mRNA. 18S rRNA was used as an endogenous control and used to normalize transcript levels. The relative expression of rictor mRNA was also normalized to a basal rictor expression value. Primer pairs were designed using Primer Express (version 1.5, PE Applied Biosystems) as described (28). For the rictor gene, the primers used were Rictor-forward 5'-GGAAAGCTTGTGAGGTGTCGACGTCGAT-3' and Rictor-reverse 5'-GCGCTTGCTTTCGAGGAGGAGAGG-3'. Relative rictor mRNA expression level of each sample is shown as the fold difference relative to the basal rictor expression value. Primer pairs were designed using Primer Express (version 1.5, PE Applied Biosystems) as described (28). For the rictor gene, the primers used were Rictor-forward 5'-GGAAAGCTTGTGAGGTGTCGACGTCGAT-3' and Rictor-reverse 5'-GCGCTTGCTTTCGAGGAGGAGG-3'. Relative rictor mRNA expression level of each sample is shown as the fold difference relative to the basal rictor expression value. Primer pairs were designed using Primer Express (version 1.5, PE Applied Biosystems) as described (28).

Protein analysis. In vitro kinase assays and immunohistochemistry. Western blot analysis and TORC2 kinase assays were done as previously described (14, 29). Protein kinase C (PKC)-α kinase assays were done with a PKC assay kit (Upstate Biotechnology) according to the manufacturer's protocol. Sections of paraffin-embedded tumors on slides were processed for immunohistochemistry as previously described (15) with antibodies for either phospho- Ser473 Akt (Cell Signaling Technology) or rictor (Bethyl Laboratories). All other antibodies were obtained from the following sources: raptor, mTOR, and cyclin D1 were from Cell Signaling; mLST8, mLST8/L (G/L) was obtained from Abcam; anti-phospho-Ser407 PCKo and anti-PKCo were obtained from Upstate Biotechnology; anti-myc, actin, CDK2, Rh, integrin β1, and integrin β3 were all from Santa Cruz Biotechnology. Sin-1-specific antibody was prepared by immunizing rabbits with the peptide CKNQWKEKSK0A and sera affinity purified.

Cell proliferation and cell cycle analysis. Cells were plated into 96-well plates at 1,000 per well. After culturing for various time points, cell numbers were measured by 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (Roche) as described by the manufacturer. Cell cycle analysis was done by propidium iodide staining of cells and flow cytometry as previously described (30).

Colony-forming assay and cell migration assay. Clonogenic assays were done by plating a total of 1,000 cells per well in 24-well plates in a total volume of 400 μL using a two-layered soft agar system as previously described (31). Cell migration assays were conducted using precoated modified Boyden chambers from Chemicon as recommended by the manufacturer and previously described (31). For invasion assays through Matrigel, 20,000 cells were loaded in the top well of Boyden chambers that contained growth factor–depleted Matrigel extracellular basement membrane over a polyethylene terephthalate membrane with 8-mm pores (BD Biosciences). Cells were allowed to invade for 24 h before the Matrigel was removed, and invaded cells were fixed and stained. Cells adhering to the bottom surface of the membrane were counted.

 Xenograft studies. H4/rictor cells were cultured and harvested in mid-log phase of growth for tumor implantation into nu/nu mice (Taconic). Cells (6 × 106) were injected s.c. into the flank of 8- to 10-week-old female nu/nu mice. Tumors were measured every 3 to 4 days and tumor volumes calculated using the standard formula length × width × height × 0.5236. Xenografts of U87, U87/mTOR, U87/rictor, LN-229, LN-229/ser shRNA, and LN-229/ΔAHA cells were done in female C.B-17-scid (Taconic) mice as previously described (30). Tumors were harvested for Western blot analysis at autopsy. Statistical analysis was done with Student's t test and ANOVA models using SigmaStat 3.1 (Systat Software).

Results

mTORC2 activity is elevated in gliomas and correlates with high-level rictor expression. To assess whether mTORC2 activity was elevated in glioma, we initially examined the relative level of activity in a panel of glioma and glioblastoma multiforme cell lines as compared with normal brain. Lysates were prepared from five cell lines and one normal brain sample under conditions that allowed the preservation of the rictor-mTOR interaction during extraction (14). mTORCs were immunoprecipitated with mTOR antibody and subjected to in vitro kinase assays using recombinant inactive Akt1 as a substrate. mTORC2-mediated activation of Akt was detected by monitoring Ser173 phosphorylation. As shown in Fig. I,A, high levels of activated mTORC2 are found in these lines relative to the normal brain sample, in which mTORC2 activity is nearly undetectable. We also examined the immunoprecipitated mTORC2 complexes for the relative levels of rictor, raptor, mTOR, mLST8, and mSin1. Interestingly, mTOR complexes from the cell lines contained high levels of rictor relative to raptor, which was consistent with the high levels of Ser173 Akt phosphorylation observed in the kinase assays. Rictor was nearly undetectable in immunoprecipitates from normal brain; however, in the cell lines rictor coimmunoprecipitated at much higher levels with mTOR, suggesting a greater degree of mTORC2 assembly, whereas the ratio of raptor recovered was significantly lower. This was consistent with previous data, which observed an inverse correlation between the relative amounts of rictor and raptor, which independently associated with mTOR (14). Overexpression of rictor was further confirmed in these cell lines by Western blot analysis (Fig. I,B). We also noted that elevated rictor levels did not seem to correlate with the known PTEN status of these cell lines, in that lines expressing either mutated or wild-type PTEN coimmunoprecipitated high levels of rictor with mTOR (see Discussion). These data suggested that mTORC2 activity was elevated in glioma and glioblastoma multiforme cell lines and additionally pointed to high-level
expression of rictor resulting in increased mTORC2 complex formation.

To determine whether mTORC2 activity was elevated in brain tumors, we examined 31 quick-frozen glioma samples and 5 normal samples. Each tumor sample was confirmed histologically, extracts were prepared, and mTORC2 activity was determined as before. We also examined the relative abundance of rictor protein expressed by Western blot analysis and mRNA expression by real-time quantitative reverse transcription-PCR (RT-PCR). As shown in Table 1, the relative level of mTORC2 activity in gliomas was elevated as compared with normal brain ($P < 0.05$). Twenty-two of 31 (70%) tumor samples had markedly higher levels of mTORC2 activity, which we defined as a 2-fold detectable amount of Ser$^{473}$ Akt phosphorylation relative to the mean level observed in normal brain. We also observed that 19 of these 22 (86%) glioma samples had elevated rictor expression (Table 1).

Figure 1C and D shows the expression of rictor in several gliomas relative to normal brain as determined by Western and Northern blot analyses, respectively. We further examined rictor expression levels in sections of glioblastoma multiforme and astrocytoma tumor tissue by immunohistochemistry as shown in Fig. 2. As can be seen, rictor is highly expressed and correlates with elevated phospho-Ser$^{473}$ Akt in these tissues.

**Rictor overexpression results in TORC2 activation, increased proliferation, and enhanced PKCa activity.** To further examine the role of rictor in human brain tumors, a full-length human myc-tagged version of rictor (14) was cotransfected with pcDNA3.1 into the low-grade glioma cell line H4, as well as in U87 cells, and clones that stably overexpressed rictor were identified. As shown in Fig. 3A, basal rictor and mTORC2 activity levels were found to be relatively low in the H4 nontumorigenic malignant glioma line and higher in U87-MG. Two independent clones from each line that overexpressed the transgene (Fig. 3A, top) were selected for further study. These clones possessed elevated mTORC2 activity (Fig. 3B, bottom) as compared with control lines, which were transfected with pcDNA3.1 only (H4/neo and U87/neo). Overexpressing rictor had marked effects on H4 morphology, inducing prominent elongation and the formation of multiple processes (Supplementary Fig. S1D). The growth of H4 cells overexpressing rictor was also significantly increased as compared with control transfectants. As shown in Fig. 3B (top left), rictor overexpression resulted in a 3-fold increase in growth rates as determined by XTT labeling. Moreover, the cell cycle phase distributions of H4 clones overexpressing rictor as compared with H4/neo clones showed a 3-fold higher percentage of S-phase cells (Fig. 3B, bottom left; clone H4/rictor 57 ± 3%; clone H4/neo 17 ± 2%; clone H4/rictor 59 ± 5%; clone H4/neo 14 ± 2%). The effects of rictor overexpression in U87 were more modest in terms of growth. Again, two independent clones were identified that overexpressed rictor and were analyzed. U87 clones overexpressing rictor grew approximately twice as fast as control lines (Fig. 3B, top right). The percentage of U87 cells overexpressing rictor in S phase was also significantly higher than controls (Fig. 3B, bottom right; clone U87/rictor 55 ± 2%; clone U87/neo 50 ± 5%; clone U87/rictor 12 ± 3%). We also determined the expression levels of a number of proteins involved in cell cycle control and cell motility in H4 and U87 clones overexpressing rictor (Fig. 3C). As can be seen, overexpression of rictor resulted in increased levels of cyclin D1 (CCND1), CDK2, integrin $\beta_1$, and integrin $\beta_3$ while decreasing Rb protein abundance relative to control cells. Previous experiments have shown a role for TORC2 in regulating actin cytoskeletal assembly via signaling
Table 1. Relative mTORC2 activity in 31 primary glioma samples

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<th>Relative rictor expression†</th>
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NOTE: Five normal brain and 31 quick-frozen glioma samples were assessed for mTORC2 activity as in Fig. 1 and quantified. The total relative abundance of rictor protein and mRNA was determined by Western blot analysis and real-time quantitative RT-PCR, respectively, and quantified. Twenty-two of 31 tumor samples (70%) has markedly higher levels of mTORC2 activity, which was defined as a ≥2-fold detectable amount of Ser<sup>657</sup> phosphorylation relative to the mean level observed in normal brain.

*Undetectable phospho-Ser<sup>657</sup> Akt.
† Undetectable rictor expression.
‡ mTORC2 activity ≥2-fold above mean of normal brain.
§ Rictor expression ≥2-fold above mean of normal brain.

Rictor promotes clonogenic growth and migration. To determine whether enhanced expression of rictor affected cell migration, we assessed the ability of the rictor-overexpressing H4 and U87 lines to transverse either a vitronectin-coated or fibronectin-coated Boyden chamber as compared with chambers coated with bovine serum albumin (BSA) as a control. The stably transfected clones (H4/rictor and H4/Δric) and U87/rictor and U87/Δric) had significantly increased numbers of cells that migrated toward the vitronectin-coated or fibronectin-coated surfaces relative to the number of cells migrating toward BSA (Supplementary Fig. S1B). We also tested whether these clones showed increased invasive properties as determined by their ability to invade Matrigel. As shown in Supplementary Fig. S1C, both H4 and U87 clones that overexpressed rictor had increased migratory ability as compared with controls. Taken together, these data suggest that increases in rictor protein levels result in enhanced TAC2 activity, which contributes to glioma cell growth and invasiveness.

shRNA-mediated inhibition of rictor expression reduces TAC2 activity, cell growth, and migration. To determine whether reductions in rictor protein levels had effects on any of the oncogenic properties we had previously examined, we generated stable lines that expressed a shRNA via a lentiviral expression system that robustly suppresses rictor expression (20). We infected LN-229, T98G, and U87 cells with virus and obtained cells stably expressing either the shRNA targeting rictor or a control shRNA containing a nontargeting sequence. Cells expressing the shRNA targeting rictor had significantly reduced rictor levels (>50% reduction) as well as reduced phosphorylated Ser<sup>473</sup> Akt levels, whereas in control cells shRNA rictor and phospho-Ser<sup>473</sup> Akt levels were unaffected (Fig. 4A; Supplementary Fig. S3). We then determined whether knockdown of rictor expression affected cell growth in these lines. As shown in Fig. 4B, LN-229 (top left), T98G (top middle), and U87 (top right) clones expressing the shRNA targeting rictor grew at significantly slower rates as compared with controls. Similarly, the percentage of cells in S-phase dropped markedly in LN-229 (Fig. 4B, bottom left), T98G (Fig. 4B, bottom middle), and U87 (Fig. 4B, right) cells with reduced rictor levels as compared with controls (>50% reduction). We then examined the effects of rictor reduction on the ability of these cells to form colonies in soft agar. As shown in Supplementary Fig. S2A, LN-229 (left), T98G (middle), and U87 (right) cells expressing the shRNA targeting rictor had significant reductions in the number of colonies as compared with controls (50% reduction).
colonies as compared with controls. The number of colonies that formed from LN-229/shRNA was ~2-fold less as compared with controls, whereas the ability of T98G/shRNA and U87/shRNA to form colonies was reduced 8- to 9-fold and 2-fold, respectively. The ability of cells expressing the shRNA targeting rictor to migrate was also impaired. Supplementary Fig. S2B shows the relative migration of LN-229/shRNA (left), T98G/shRNA (middle), and U87/shRNA (right) cells relative to controls. The ability of LN-229/shRNA, T98G/shRNA, or U87/shRNA cells to migrate on either vitronectin or fibronectin in Boyden chambers was reduced by ~50% relative to the parental or nontargeting scrambled shRNA–expressing control cells. As shown in Supplementary Fig. S2C, the ability of LN-229 (left), T98G (middle), and U87 (right) clones expressing the shRNA targeting rictor to migrate through Matrigel was also inhibited relative to controls. We then also examined the relative expression levels of several proteins involved in cell cycle control and motility, in addition to PKCα activity, as before (see Fig. 3C and D). LN-229, T98G, and U87 cells in which rictor expression was specifically inhibited had marked inhibition of cyclin D1, CDK2, integrin β1, and integrin β3 expression, whereas Rb levels were increased relative to control cells (Fig. 4C). LN-229/shRNA, T98G/shRNA, and U87/shRNA cells also had reduced levels of phospho-Ser473 Akt, PKCα levels relative to controls (Fig. 4D). These results suggested that reductions in rictor levels result in the inhibition of cell growth and migration via the inhibition of TORC2 activity and subsequent PKCα/integrin function.

**Alterations in rictor expression affect tumor xenograft growth.** Because H4 cells do not form tumors in nude mice, we initially determined whether H4 cells overexpressing rictor were tumorigenic in vivo. Interestingly, H4/riotor cells did form tumors in nude mice with a take rate of ~65% (10 of 15), unlike the parental or H4.neo cells (Fig. 5A). Paraffin-embedded sections of harvested tumors displayed high levels of rictor and phospho-Ser473 Akt, suggesting that the transgene was still present and active in situ (not shown). We then carried out similar experiments using the U87/riotor LN-229/shRNA and appropriate paired control cell lines in severe combined immunodeficient (SCID) mice. As shown in Fig. 5B, U87/riotor tumor xenografts grew markedly faster and with a significant shorter latency period (~28 days following injection; \( P < 0.05 \)) as compared with tumors formed by U87 or U87.neo cells. Tumors harvested at autopsy also showed elevated rictor expression and increased phospho-Ser473 Akt levels relative to extracts from control tumors. Conversely, xenografts of LN-229/shRNA cells formed significantly smaller tumors with a much longer latency period (~30 days following injection; \( P < 0.05 \)) as compared with tumors formed by LN-229 or LN-229/shRNA cells (Fig. 5C). Extracts of LN-229/shRNA tumors (day 63) had markedly reduced levels of rictor and phospho-Ser473 Akt as compared with control tumors. These data showed that overexpression of rictor in H4 cells was sufficient to induce tumorigenesis in vivo, and in addition, the overexpression or targeted knockdown of rictor in xenografted cells promotes or inhibits tumor growth, respectively.

**Discussion**

Here, we have identified the mTORC2 component rictor as being overexpressed in glioma cell lines and primary samples. In addition, our data suggest that overexpression of rictor results in increased mTORC2 assembly and activity and promotes glioma cell proliferation, migration, and invasiveness. We began our study by examining the expression of TORC components in vitro in several cell lines that showed overexpression of rictor relative to normal brain. We also determined that mTORC2 activity was significantly higher in these lines as well. We subsequently determined that rictor was overexpressed in a majority of primary gliomas and correlated with enhanced mTORC2 activity. We further determined that modulation of rictor levels had marked effects on several oncogenic properties both in vitro and in vivo. Taken together, these data support the hypothesis that overexpression of rictor in gliomas results in increased mTORC2 complex formation and elevated activity, which contributes to glioma cell cycle progression, mobility, and invasive character.

Whereas we observed increased mTORC2 activity in gliomas, which correlated with overexpression of rictor and enhanced complex formation, the proximal signaling events driving TORC2 activity are not well defined. It is known that TORC2 activity is responsive to growth factor stimulation mediated via tyrosine kinase receptors (34) and the abnormal activation of these...
receptors is a hallmark of malignant gliomas (35). Thus, it is possible that, for example, overexpressed EGFR or ligand-independent EGFRvIII may mediate upstream signaling to mTORC2, which is further amplified by the overexpression of rictor in gliomas. The overexpression of rictor would result in a larger pool of assembled mTORC2 that is competent to transduce upstream signaling.

The forced overexpression of rictor in cell lines resulted in prominent effects on growth, migration, and invasion. H4 clones overexpressing rictor also showed marked effects on cell morphology (Supplementary Fig. S1D). This is most likely due to the effects on both Akt and PKCα signaling, which were shown to be enhanced in these clones and have been shown to regulate these parameters (36, 37). In clones that stably expressed shRNA targeting rictor, mTORC2 activity was significantly reduced and resulted in the inhibition of glioma cell growth, migration, and invasion. Interestingly, we did not observe effects on the actin cytoskeleton as had previously been reported (ref. 14; data not shown); however, this was consistent with the lack of cytoskeletal effects in rictor-deficient mouse embryonic fibroblasts (38). Furthermore, we did not observe significant alterations in Sin1 protein levels in rictor-knockdown clones as have been observed in rictor-null cells, and this may be attributable to differences in cell types used in these studies (refs. 39, 40; data not shown).

Our data also showed that PTEN status did not correlate with rictor overexpression (Fig. 1A and B) as LN-229 cells, which harbor wild-type PTEN and have normal expression levels of the protein, also overexpressed rictor (41). This suggests that rictor overexpression can lead to elevated Akt activity even in the presence of PTEN. However, it would be expected that cells overexpressing rictor and also containing a loss-of-function PTEN mutation would have markedly elevated Akt activity. Indeed, T98G, U-138MG, and U87 glioblastoma cell lines, all of which express a mutated PTEN (41) and overexpressed rictor, showed the highest levels of activated Akt in our analysis. Loss of PTEN has been reported in H4 glioma cells (42), but these cells displayed significantly reduced amounts of Ser473 phosphorylated Akt, comparable to levels present in LN-229 cells. However, H4 cells did not overexpress rictor to the same degree as the other cell lines tested (Fig. 1B).

We also noted that the relative association of raptor and mTOR was unaffected by modulation of rictor expression levels (Fig. 3A; Supplementary Fig. S3). Currently, the mechanisms that regulate the formation of individual TORCs are unknown. Recent data suggest that under steady-state conditions, rictor and raptor associate with mTOR at near stoichiometric levels and that the ratios of rictor to raptor bound to mTOR seem to be inversely related (14, 20). In previous studies, however, cells expressing shRNAs targeting rictor did not seem to significantly influence the amount of raptor subsequently associated with mTOR (20). Our
data are consistent with this finding and may be due to affects on mTOR, which preclude binding of raptor to mTOR under these conditions.

Integrin $\beta_1$ and $\beta_3$ expression was also markedly effected by modulation of rictor levels, and these proteins play an important role in tumor cell migration and invasion (43). Indeed, it has been shown that integrin $\alpha_V\beta_3$ is overexpressed in glioblastoma multiforme, particularly at the invasive edges of the tumor (44). Additionally, antibodies and peptide ligands of $\alpha_V\beta_3$, which function as antagonists, led to tumor regression in animal models of glioblastoma multiforme (45). However, other studies have shown that overexpression of integrin $\beta_3$ suppresses glioma cell
growth *in vivo* (46) and have further shown that host β3 expression and the tumor microenvironment play critical roles in modulating glioma growth (47). Both our *in vitro* and *in vivo* data support a role for increased β1 and β3 expression in glioma cell growth and migration. The effects on β1 and β3 expression are likely due to signaling via PKCα as the β1 integrin subunit directly associates with PKCα and its overexpression induces surface up-regulation of β1 attributable to the PKCα regulatory subunit (48).

A recent study by Qiao et al. (49) showed that mTORC2 activity regulates the metastatic potential of a series of human breast cancer lines established from successive biopsies from a single patient. This study supported a role for rictor in the control of mTORC2 activity and showed that rictor was required for elevated Ser\(^{473}\) phosphorylated Akt levels, which correlated with increased anchorage-independent growth and migration, consistent with our experiments in gliomas. However, the authors concluded that rictor expression was not significantly different in these cell lines and could not account for the differences in Akt activity observed, and proposed that dysregulation of PH domain leucine-rich repeat protein phosphatase (PHLPP) expression may be one mechanism by which Akt activity is regulated in these cell lines. PHLPP blunts Akt signaling by directly dephosphorylating Ser\(^{473}\) on Akt (50). Indeed, PHLPP levels are reduced in several glioblastoma lines resulting in elevated levels of Ser\(^{473}\) phosphorylated Akt, including in LN-229 cells (50), which we used in our studies. Our data clearly support a role for altered levels of rictor expression in the regulation of mTORC2 activity in gliomas and are consistent with previous reports that show varied levels of rictor protein abundance in different cell lines (14). Thus, it is likely that both of these mechanisms can influence the overall balance of Akt activity in the cell.

In summary, our data support a role for elevated mTORC2 activity in glioma cell proliferation, mobility, and invasive character. Rictor expression was found to be elevated in glioma lines and primary

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**Figure 5.** Growth of rictor-overexpressing or rictor-knockdown clones *in vivo*.  
*A*, growth of H4/rictor cells in xenografts in nude mice (*n* = 10) was monitored for tumor formation for up to 70 d.  
*B*, growth of U87 (○), U87/neo (▲), or U87/neo (□) xenografts in SCID mice (*n* = 4–5 per group).  
*C*, growth of LN-229 (○), LN-229/scr shRNA (■), or LN-229/shRNA (▲) cells *in vivo* in SCID mice (*n* = 4–5 per group). 

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Hyperactivated mTORC2 and Rictor Overexpression in Glioma
tumors, resulting in increased mTORC2 formation and activity. Modulation of rictor had pronounced effects on growth, migration, and invasiveness. Future experiments will focus on the mechanisms mediating rictor overexpression in this tumor type.

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