Acyl-CoA Synthetase VL3 Knockdown Inhibits Human Glioma Cell Proliferation and Tumorigenicity

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Abstract

The contribution of lipid metabolic pathways to malignancy is poorly understood. Expression of the fatty acyl-CoA synthetase ACSVL3 was found to be markedly elevated in clinical malignant glioma specimens but nearly undetectable in normal glia. ACSVL3 levels correlated with the malignant behavior of human glioma cell lines and glioma cells propagated as xenografts. ACSVL3 expression was induced by the activation of oncogenic receptor tyrosine kinases (RTK) c-Met and epidermal growth factor receptor. Inhibiting c-Met activation with neutralizing anti-hepatocyte growth factor monoclonal antibodies reduced ACSVL3 expression concurrent with tumor growth inhibition in vivo. ACSVL3 expression knockdown using RNA interference, which decreased long-chain fatty acid activation, inhibited anchorage-dependent and anchorage-independent glioma cell growth by ~70% and ~90%, respectively. ACSVL3-depleted cells were less tumorigenic than control cells, and subcutaneous xenografts grew ~60% slower than control tumors. Orthotopic xenografts produced by ACSVL3-depleted cells were 82% to 86% smaller than control xenografts. ACSVL3 knockdown disrupted Akt function as evidenced by RTK-induced transient decreases in total and phosphorylated Akt, as well as glycogen synthase kinase 3β, via a caspase-dependent mechanism. Expressing constitutively active myr-Akt rescued cells from the anchorage-dependent and anchorage-independent growth inhibitory effects of ACSVL3 depletion. These studies show that ACSVL3 maintains oncogenic properties of malignant glioma cells via a mechanism that involves, in part, the regulation of Akt function.

Introduction

Gliomas account for more than 50% of all primary brain tumors, and nearly two thirds of gliomas are highly aggressive with "malignant" pathologic features (WHO grade 3 or 4; ref. 1). Despite advances in the neurosurgical, radiotherapeutic, and chemotherapeutic treatment of gliomas, the prognosis generally remains poor (1).

A high rate of lipid synthesis is needed to support membrane biogenesis required for tumor growth. Lipids also play key roles in second messenger pathways that are dysregulated in malignant cells, and elevations in specific lipid messengers are associated with malignancy (2). The primary source of the fatty acid (FA) constituents of tumor lipids is de novo synthesis and not uptake from extracellular sources; thus, cancer cells typically have increased rates of FA synthesis (3). The observation that a breast cancer biomarker, OA-519, was identical to a key enzyme in the FA synthesis pathway, fatty acid synthase (FASN; ref. 4), inspired renewed interest in tumor cell lipid metabolism. Subsequently, high FASN levels have been found in many human cancers, including primary brain cancer (5, 6), and this finding is associated with a poor prognosis (4).

FAs must be "activated" by thioesterification to CoA for further metabolism. Acyl-CoA synthetases (ACS) catalyzing this reaction thus play a central role in providing activated FAs for complex lipid synthesis, energy-yielding catabolic pathways, protein acylation, and other processes (7). Humans have 26 genes encoding ACSs that differ in their ability to activate short-, medium-, long-, and very-long-chain FAs (8). The six-member very-long-chain ACS (ACSVL) family includes ACSVL3 [SLC27A3; also known as FA transport protein (FATP)-3], an ACS that activates long-, and very-long-chain FAs (8). The six-member very-long-chain ACS (ACSVL) family includes ACSVL3 [SLC27A3; also known as FA transport protein (FATP)-3], an ACS that activates saturated FAs containing 16 to 24 carbons (9). The biological basis for the numerous ACS enzymes is only partially understood. In the adult mouse brain, ACSVL3 protein is detectable at low levels in neurons but not glia (9). In this report, we show that human malignant gliomas abundantly overexpress ACSVL3, and that depletion of this enzyme decreases the malignant phenotype of glioma cells in vitro and of glioma xenografts in mice. These effects are mediated, in part, by changes in receptor tyrosine kinase (RTK)–dependent signaling via Akt.

Materials and Methods

Cell culture. Human U87 MG (ATCC #HTB-14) and U373 MG (ATCC #HTB-17) glioblastoma cell lines have been maintained in our laboratory for >10 y and were cultured as previously described (10). These cell lines are tested at least twice yearly for expression of molecular and biochemical markers characteristic of these cells. U87 cells stably expressing epidermal growth factor receptor variant III (EGFvrIII; ref. 11) and corresponding control line were from Dr. Gregory Riggins (Johns Hopkins University, Baltimore, MD). Mayo 22 xenografts were obtained from Mayo Foundation for Medical Research and Education (Rochester, MN) and were maintained by serial passage as subcutaneous xenografts (12). Cell proliferation, anchorage-independent growth, and tritiated thymidine incorporation were measured as previously described (13, 14).

ACSVL3 knockdown. Transient ACSVL3 knockdown (KD) was achieved using previously described methods (9). Clones with stable KD of ACSVL3, FATP4, and ACSF2 were produced using the pSilencer 4.1-CMV hygro vector (Ambion). Complete methodologic details can be found in Supplementary Methods.

Quantitative reverse transcription-PCR. RNA from normal human brain and glioblastomas was a gift from Dr. Charles Eberhart (Johns Hopkins University, Baltimore, MD). Forward primer 5′-CCCA-GAGTTTCCTGGTGGCTTCT-3′ and reverse primer 5′-GGACACTTCCAGGCCGCAAT-3′ amplify a 256-bp intron-spanning ACSVL3 fragment. One-step
reverse transcription-PCR (RT-PCR) was done using the iScript SYBR Green RT-PCR kit and the iQ5 Real-time PCR Detection System (Bio-Rad Laboratories). Amplification of 18S RNA was done as a control. Analysis was done with iQ5 and Prism (GraphPad Software) software using the ∆∆Ct method.

**Immunohistochemistry, immunofluorescence, and Western blotting.** Immunohistochemical staining and immunofluorescence analysis were done using affinity-purified antibodies as described (9, 15). ACSVL3 was detected on Western blots with SuperSignal West Pico reagents (Pierce Biotechnology). Antibody to total Akt was from BD Biosciences; antibodies were done using affinity-purified antibodies as described (9, 15). ACSVL3 staining of normal adult mouse brain revealed low expression but is not increased in gliomas. Vertical pairs of micrographs represent consecutive slices from the same biopsy. B, quantitative RT-PCR. ACSVL3 mRNA from normal brain (N1 and N2) and glioblastomas (G1–G5) was quantitated by RT-PCR. Columns, mean relative ratio of ACSVL3 to 18S RNA from triplicate determinations; bars, SE. For N1 and N2, the ratio was <0.005 and is thus not visible on the scale of the plot.

**Results**

**ACSVL3 protein expression is low in normal human brain but increased in malignant gliomas.** Immunohistochemical staining of ACSVL3 in adult mouse brain revealed low expression that was limited to neurons; little or no protein was detected in glia (9). A similar staining pattern was found in normal adult human brain (Fig. 1A, top row). In contrast, all 79 tumors on a human glioma tissue array that included astrocytoma, oligodendroglioma, anaplastic astrocytoma, and glioblastoma multiforme (Fig. 1A, top row), as well as tumors of mixed etiology (not shown), showed increased ACSVL3 expression, with ~80% exhibiting robust expression. Expression of ACSBG1, another ACS expressed in adult neurons (Fig. 1A, bottom row), was not detected in gliomas (Fig. 1A, bottom row), confirming the specificity of ACSVL3 overexpression. ACSVL3 mRNA levels were also significantly greater in glioblastomas than in normal brain as determined by quantitative RT-PCR (Fig. 1B).

**ACSVL3 expression correlates with malignant phenotype in human glioma cells in vitro and in xenografts.** ACSVLS3 was readily detectable in established human glioma cell lines and in human glioma cells maintained as xenografts (Fig. 2A). The highly tumorigenic U87 human glioblastoma cell line and cells derived from tumorigenic primary glioblastoma xenografts (Mayo 22) expressed ACSVL3 at high levels (Fig. 2A). Less tumorigenic U373 glioma cells (13) expressed ACSVL3 at lower levels than either U87 or Mayo 22 cells (Fig. 2A). Hepatocyte growth factor (HGF), which enhances glioma cell tumorigenicity, induced ACSVL3 expression in U373 and Mayo 22 cells (Fig. 2A). Treatment with the EGFR ligand, epidermal growth factor (EGF), also increased ACSVL3 expression (Fig. 2A). Whereas ACSVL3 expression in U87 cells was not increased above its high basal expression levels by HGF or EGF (Fig. 2A), U87 cells engineered to express the constitutively active EGFR deletion mutant EGFRVIII had considerably more ACSVL3 expression when compared with control-transfected cells (Fig. 2B).

Increased ACSVL3 protein was observed in U373 cells by 2 hours after addition of HGF (Supplementary Fig. S1). Maximal levels were
observed at 4 to 8 hours. Addition of either an inhibitor of transcription (actinomycin D) or an inhibitor of protein synthesis (cycloheximide) prevented the HGF-induced increase in ACSVL3 level, suggesting that RTK activation stimulated ACSVL3 mRNA synthesis (Supplementary Fig. S2).

To assess the role of HGF in ACSVL3 expression in vivo, mice bearing preestablished U87 xenografts were treated with neutralizing anti-HGF monoclonal antibody (mAb; L2G7) under conditions that inhibit HGF/c-Met pathway activation and tumor growth (17). Control and anti-HGF treated tumors were resected and tumor extracts assessed for ACSVL3 expression. HGF/c-Met pathway inhibition led to reduced ACSVL3 expression (Fig. 2C) concurrent with tumor growth inhibition. Thus, ACSVL3 is expressed by human glioma cell lines; activation of multiple oncogenic RTK pathways induces ACSVL3 expression; and inhibiting oncogenic RTK signaling inhibits ACSVL3 expression in human glioma xenografts.

**Knockdown of ACSVL3 inhibits glioma cell growth and tumorigenicity.** Clonal lines of U87 cells with stable KD of ACSVL3 expression were developed using two short hairpin-producing plasmids, ACSVL3-3 and ACSVL3-4, which targeted different regions of ACSVL3 mRNA (Supplementary Methods). Six clonal lines had nearly undetectable levels of ACSVL3, as judged by indirect immunofluorescence and Western blot (Fig. 3). ACSVL3 KD was verified by measuring ACS enzyme activity using [1-14C]C16:0 as substrate. Control U87 cell ACS activity was 2.45 nmol/min/mg protein, whereas activities of 1.90, 2.07, and 1.68 nmol/min/mg protein were measured in ACSVL3-3, ACSVL3-4, and ACSVL3-3 + ACSVL3-4 KD cells, respectively. To determine the effect of ACSVL3 KD on cellular phenotype, adherent cell growth rates and anchorage-independent clonogenic growth were assessed. ACSVL3 KD inhibited anchorage-dependent growth by ~67% (Fig. 4A; P < 0.001; day 9) and inhibited clonogenicity in soft agar by 87% (Fig. 4B; P < 0.001).

ACSVL3 expression was also inhibited in tumor cells derived from Mayo 22 glioblastoma xenografts using ACSVL3-specific small interfering RNA (siRNA). Anchorage-dependent growth of Mayo 22 cells was decreased by 31% (Fig. 4C; P < 0.001), and colony formation in soft agar was reduced by 40% (P < 0.01) compared with cells treated with control RNA (Fig. 4D). DNA synthesis, as measured by tritiated thymidine incorporation 3 days after transfection, was reduced by 70% to 85% (P < 0.001) in ACSVL3 KD Mayo 22 cells (Supplementary Fig. S3).

We examined the effects of inhibiting two other ACS family members on glioma cell growth. FATP4 (SLC27A4), an ACSVL3 family member (20), and ACSF2, which activates medium-chain FAs (8); both are expressed in U87 cells, and KD of either FATP4 or ACSF2 (Supplementary Fig. S4) did not appreciably affect U87 cell anchorage-dependent growth (Fig. 4A; P > 0.05) or clonogenic growth in soft agar (Fig. 4B; P > 0.05). These findings support our hypothesis that ACSVL3 expression is specifically required to support glioma cell growth in vitro.

To investigate the effects of ACSVL3 KD on glioma cell tumorigenicity, control-transfected and stable ACSVL3 KD U87 cells were implanted s.c. and tumor growth was evaluated. Control-transfected U87 cells generated tumors at all implantation sites (12 of 12) and ACSVL3 KD cells generated palpable tumors only 58% (7 of 12) of the time (Fig. 5A). No evidence of tumors was found at the other five sites when mice were sacrificed 26 days after injection. The average growth rate of the seven ACSVL3 KD xenografts was reduced by ~60% (P < 0.001, day 23) relative to control tumors as assessed by serial caliper measurements (Fig. 5B). ACSVL3 KD tumors resected on post-implantation day 26 weighed 64% less than control xenografts (2.1 ± 0.5 versus 0.8 ± 0.4 g; g; P < 0.001). Western blot analysis verified that ACSVL3 levels remained low in ACSVL3 KD xenografts compared with control tumors (Fig. 5C). KD tumor homogenates had reduced ACS enzyme activity when assayed with radiolabeled C16:0 (1.16 ± 0.09 nmol/min/mg protein for KD xenografts versus 1.87 ± 0.13 nmol/min/mg for control tumors). ACSVL3 KD similarly inhibited the growth of orthotopic glioma xenografts generated by implanting control-transfected or ACSVL3 KD U87 cells in the right caudate/putamen. Mice implanted with control U87 cells developed large (23.5 ± 6.2 mm3) tumors (Fig. 5D), whereas xenografts produced from ACSVL3-3 KD
or ACSVL3-3 + ACSVL3-4 KD U87 cells were >80% smaller than control tumors (Fig. 5D; \( P < 0.001 \)).

**Aberrant Akt signaling results from ACSVL3 KD.** We hypothesized that the tumor-suppressing effects of ACSVL3 KD might result from defects in lipid-dependent oncogenic signaling pathways. RTK signaling pathways support glioma cell growth *in vitro* and tumorigenicity *in vivo* (21, 22). Phosphatidylinositol 3-kinase (PI3K) activation by RTKs plays a prominent role in maintaining the malignant glioma phenotype, and a key downstream target of PI3K activation by RTKs plays a prominent role in maintaining tumorigenicity *in vivo* (23). When treated with HGF in the presence of a cell-permeable pan-caspase inhibitor, Z-VAL-FMK, the aberrant Akt response of ACSVL3 KD cells was normalized (Fig. 6C). In contrast, caspase inhibition had no effect on the level or phosphorylation state of Akt in HGF-treated control U87 cells (Supplementary Fig. S5).

We hypothesized that if Akt instability contributes to the diminished malignant phenotype of ACSVL3 KD glioma cells, then constitutive Akt activation should reverse the effects of ACSVL3 KD on glioma cell growth. Therefore, the effects of constitutively active myr-Akt on the growth of control and ACSVL3 KD cells were examined. Vector-transfected ACSVL3 KD U87 cells displayed significantly diminished anchorage-dependent (Fig. 6D, left; \( P < 0.01 \)) and anchorage-independent (Fig. 6D, right; \( P < 0.001 \)) growth as compared with control U87 cells, consistent with the results shown in Fig. 4. In contrast, both anchorage-dependent and anchorage-independent growth rates were restored to near normal by myr-Akt (\( P > 0.05 \) for control myr-Akt versus KD ACSVL3-3 myr-Akt). Myr-Akt had no appreciable effect on the anchorage-dependent or anchorage-independent growth (\( P > 0.05 \)) of control U87 cells.

**Discussion**

Brain malignancies are responsible for significant morbidity and mortality in both adults and children. Lipogenic and FA synthetic pathways are hyperactivated in rapidly growing tumors and cancer cells, including human gliomas and glioma cell lines, and several studies indicate that targeting FASN and other enzymes in FA and lipid metabolic pathways may be of therapeutic value in human malignancies (3, 5, 6, 24). Because of their central position in FA metabolism, ACSs are also rational choices for investigation as therapeutic targets. Whereas there is but a single FASN isoform, there are 26 known ACSs, reflecting significant metabolic complexity at the level of FA activation (8). This complexity is due to known differences in ACS substrate specificities and other less understood functions such as enzyme-specific targeting to support specialized subcellular lipogenic requirements. In this report, we show that ACSVL3, an enzyme not normally found in glia, is expressed at extraordinarily high levels in human malignant gliomas. We also found that ACSVL3 levels were elevated in tumorigenic glioma cells and that expression levels correlated with more aggressive tumorigenic phenotypes. We previously found that ACSVL3 mRNA is high in embryonic brain and decreases to very low levels in adult brain (9). Together, these findings suggested that ACSVL3 might function during periods of mitogenic pathway activation and rapid cell growth, and that depleting cells of this enzyme could have antitumor effects. Our findings that ACSVL3 depletion inhibits glioma cell tumorigenicity and the growth rates of glioma cell lines and glioma xenografts support this hypothesis.

We used RNA interference to deplete glioma cells of ACSVL3. Targeting two distinct regions (nucleotides 397–415 and 1,863–1,881) of ACSVL3 mRNA yielded nearly complete KD of ACSVL3 expression in U87 cells, as judged by indirect immunofluorescence, Western blot, and enzyme activity. Although off-target effects cannot be completely ruled out, their likelihood is reduced by...
our findings that two distinct ACSVL3-specific constructs, but not scrambled constructs or constructs targeting two other ACS family members, were tumor suppressive.

We questioned whether overexpression of ACSVL3 alone was sufficient to transform less malignant U373 cells into cells exhibiting a more malignant phenotype. The growth rate of U373 cells, comparable to that of ACSVL3-deficient U87 cells (see Fig. 4), did not increase when transiently transfected with ACSVL3 cDNA; however, overexpressed ACSVL3 is mistargeted to the endoplasmic reticulum (9), obscuring the significance of this negative result.4 For similar reasons, expressing short hairpin RNA (shRNA)-resistant ACSVL3 cDNA to reverse KD effects was not feasible because mistargeted ACSs exhibit altered enzymatic properties (9, 15).

The specific role(s) of ACSVL3 in cellular lipid metabolism has not yet been elucidated. The products of this enzyme are long- to very-long-chain fatty acyl-CoAs (9), which have numerous potential fates, including degradation for energy production, elongation, incorporation into complex lipids, and protein acylation. These possibilities are under investigation in our laboratory. ACSVL3 and the other five ACSVL family members have also been investigated as FATPs (reviewed in ref. 25) because, when overexpressed, several members of this family facilitate cellular uptake of fluorescent or radiolabeled long-chain FAs (26, 27). Although we (9) and others (27) reported that ACSVL3 does not have a FA transporter function, studies to determine whether ACSVL3 stimulates FA uptake in glioma cells are currently under way.

Distinct from our findings with ACSVL3, the glioma malignant phenotype was not associated with or dependent on at least three other ACS family members. ACSBG1 expression was not increased in clinical glioma specimens, and KD of either FATP4 or ACSF2 did not ameliorate the malignant phenotype of glioma cells in vitro. It remains possible that other ACSs may contribute to the malignancy of glioma or other cancers. Few studies of ACSs in human cancers have been reported; most investigate ACSL5, a long-chain ACS. ACSL5 expression was variably associated with malignancy, being increased in many colorectal tumors but decreased in some intestinal tumors including adenocarcinomas (28, 29). ACSL5 expression was also increased in well-differentiated, but not in poorly differentiated, endometrial adenocarcinomas (30). ACSL5 mRNA levels were high in primary human gliomas and A172 glioma cells but, in contrast to our findings with ACSVL3, were not detectable in either U87 or U373 glioma cells (31). To our knowledge, gain-of-function or loss-of-function studies directed at determining the role of ACSL5 in the cancer phenotype have not been reported.

Few inhibitors of the ACS reaction are known. The most studied is triacsin C, a nonspecific compound that inhibits ACSL1, ACSL3, ACSL4, and ACSVL1 (FATP2), but not ACSL5, ACSL6, ACSVL5 (FATP4), or ACSBG2 (32–37). Mashima and colleagues reported that triacsin C induced cell death and caspase activity in human lung (NCI-H23), colon (HCT-15), and brain (SF268) cancer cell lines (38). Cardiolipin, an inner mitochondrial membrane lipid that is important for retaining cytochrome c, was decreased by triacsin C treatment, suggesting this as a possible mechanism by which ACS inhibition promotes apoptosis. These investigators also reported that triacsin C treatment of mice suppressed the growth of NCI-H23 lung carcinoma xenografts by about 50%, suggesting that ACSs might serve as therapeutic targets. The effect of triacsin C on ACSVL3 activity is not known, and the lack of triacsin specificity prevents its use to investigate the functions of specific ACS family members. Our data showing a caspase-mediated Akt-dependent mechanism by which ACSVL3

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**Figure 4.** Growth properties of control and ACSVL3 KD glioma cells. A and B, U87 cells. A, growth in culture of two control (scrambled shRNA), one ACSVL3-3 KD, one ACSVL3-4 KD, one ACSF2 KD, and one FATP4 KD U87 cell line were measured. Cells (5,000 per well) were seeded into six-well plates; at the indicated time points, cells from triplicate wells were harvested and each was counted in duplicate using a hemacytometer. B, anchorage-independent growth of control, ACSVL3-3 KD, and FATP4 KD U87 cells. Cells (5,000 per well) were mixed with soft agar and seeded into six-well plates. On day 20, cells from triplicate wells were examined microscopically for colony formation. C and D, Mayo 22 cells. Cells were transiently transfected with either ACSVL3-3 siRNA or control (scrambled) siRNA as described in Supplementary Methods. Anchorage-dependent growth was measured on day 7 after transfection (C) and anchorage-independent growth was measured on day 18 after transfection (D) as described above for U87 cells. Points and columns, mean; bars, SE. Statistical significance was determined by one-way ANOVA with Bonferroni’s multiple comparison test (A and B) or Student’s t test (C and D). ***, P < 0.001; **, P < 0.01; n.s., P > 0.05 (not significant).

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KD alters glioma cell growth and clonogenicity may reflect, in part, the observations of Mashima and colleagues. However, our results also differ substantially from those of Mashima and colleagues because we observed little, if any, cell death in response to ACSVL3 KD.

Overactivation of tumor RTKs (e.g., EGFR, platelet-derived growth factor, and c-Met) and their downstream signaling pathways is closely associated with malignant progression and poor patient survival (22, 39–42). Our findings show a relationship between ACSVL3 expression, functional RTK signaling, and the malignant phenotype in the human glioma models examined. The most tumorigenic models examined (U87 and Mayo 22) expressed much higher basal levels of ACSVL3 compared with the least tumorigenic U373 glioma cells. Activating c-Met and expressing EGFRvIII, which enhance glioma cell malignancy by multiple criteria (11, 13), were found to induce ACSVL3 expression. Conversely, inhibiting c-Met signaling in animals bearing glioma xenografts was associated with ACSVL3 expression inhibition concurrent with tumor regression. RTKs such as c-Met exert their oncogenic effects via multiple downstream signaling pathways, among which Akt activation (i.e., phosphorylation) by PI3K plays a prominent role (43). Depleting glioma cells of ACSVL3 was found to significantly alter Akt function as evidenced by a rapid but transient decrease in both the phosphorylated and the nonphosphorylated forms of Akt in response to either c-Met or EGFR agonists. The caspase dependency of this activation-induced aberrant Akt response is consistent with a proteolytic mechanism (44, 45). Whereas it is well known that Akt phosphorylation requires protein translocation to membrane, details about the phospholipids involved are currently undefined. Although somewhat speculative, our findings suggest that ACSVL3 enzymatic activity is necessary to maintain phospholipid membrane specializations required for optimal Akt stability. Possibilities include production of lipidic signaling molecules, such as specific phosphoinositide species and diacylglycerol, or the generation of acylated proteins known to participate in RTK signaling pathways. That expression of constitutively active Akt reversed the glioma growth-inhibiting effects of ACSVL3 KD strongly supports a functional interaction between ACSVL3-dependent metabolic and Akt-dependent oncogenic signaling. Identifying the precise biochemistry by which ACSVL3 supports the malignant phenotype and Akt function should provide new insights into the interrelationships between FA metabolism and molecular oncogenesis.

The results obtained in this study suggest that ACSVL3 is a potential therapeutic target in glioma. An important consideration is whether ACSVL3 is needed for the growth and survival of normal cells. Current evidence suggests that this is not the case. Essentially complete KD of ACSVL3 has been achieved in human U87 glioma cells (this study), mouse MA-10 Leydig cells (9), and mouse Neuro2a neuroblastoma cells.1 Whereas lack of ACSVL3 decreases the growth rate of all of these tumor cell lines, cell survival is unaffected, and the cells continue to propagate in culture. Several mouse tissues were found to lack detectable ACSVL3 mRNA by Northern blot analysis, including skeletal muscle, heart, lung, liver, kidney, and intestine (9), further suggesting that this enzyme is not required for normal cell function. However, further investigation of pharmacologic intervention is currently limited by the lack of specific ACSVL3 inhibitors.

Disclosure of Potential Conflicts of Interest

B. Lal and J. Laterra: licensing agreement with Galaxy Biotech. The other authors disclosed no potential conflicts of interest.
Figure 6. Akt signaling in control and ACSVL3 KD U87 cells. A and B, effect of HGF treatment on Akt function and stability. Control and ACSVL3-3 KD U87 cells were incubated with HGF (20 ng/mL) for the indicated time period before analysis for total Akt, phospho-Akt, and phospho-GSK3β levels by Western blot. All samples were run on a single gel. A, LiCOR Odyssey images; control and KD images were obtained simultaneously. B, quantitation of the LiCOR data. C, effect of caspase inhibition on Akt stability. Control and ACSVL3-3 KD U87 cells were preincubated with or without Z-VAD-FMK (50 μmol/L; Sigma) for 2 h; HGF (20 ng/mL) was then added at the indicated time point, followed by analysis for total Akt and phospho-Akt levels by Western blot. Left, LiCOR Odyssey images; right, quantitation of the LiCOR data for KD cells. Corresponding data for control U87 cells are presented in Supplementary Fig. S5. D, constitutively active Akt reverses the growth inhibitory effects of ACSVL3 KD. Control or KD U87 cells were transfected with myr-Akt (a gift of Dr. Michael J. Quon, NIH, NHLBI, Bethesda, MD; ref. 46) or empty vector using Nucleofector Kit T with the Nucleofector apparatus (Amaxa). Left, adherent growth was measured 11.5 d after transfection as described in the legend to Fig. 4A. Right, anchorage-independent growth was measured by colony formation in soft agar on day 17 after transfection as described in the legend to Fig. 4B. Columns, mean; bars, SE. Statistical significance was determined by one-way ANOVA with Bonferroni’s multiple comparison test. **, P < 0.01, versus control vector; ***, P < 0.001, versus control vector; n.s., P > 0.05 (not significant), versus control myr-Akt.
Acknowledgments

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