Neoplasia • Vol. 7, No. 9, September 2005, pp. 862–872 862

www.neoplasia.com

Regulation of Glioma Cell Migration by Serine-Phosphorylated P311[1]

Wendy S. McDonough[2], Nhan L. Tran[2] and Michael E. Berens

The Translational Genomics Research Institute, Phoenix, AZ 85004, USA

Abstract

P311, an 8-kDa polypeptide, was previously shown to be highly expressed in invasive glioma cells. Here, we report the functional characteristics of P311 with regard to influencing glioma cell migration. P311 is constitutively serine-phosphorylated; decreased phosphorylation is observed in migration-activated glioma cells. The primary amino acid sequence of P311 indicates a putative serine phosphorylation site (S59) near the PEST domain. Site-directed mutagenesis of *S59A* retarded P311 degradation and induced glioma cell motility. In contrast, S59D mutation resulted in the rapid degradation of P311 and reduced glioma cell migration. Coimmunoprecipitation coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis identified Filamin A as a binding partner of P311, and immunofluorescence studies showed that both proteins colocalized at the cell periphery. Moreover, P311-induced cell migration was abrogated by inhibition of β1 integrin function using *TACβ1*$_A$, a dominant-negative inhibitor of β1 integrin signaling, suggesting that P311 acts downstream of β1 signaling. Finally, overexpression of P311 or P311 *S59A* mutant protein activates Rac1 GTPase; small interfering RNA–mediated depletion of Rac1 suppresses P311-induced motility. Collectively, these results suggest a role for levels of P311 in regulating glioma motility and invasion through the reorganization of actin cytoskeleton at the cell periphery.

Neoplasia (2005) **7,** 862–872

Keywords: glioma, migration, P311, integrin, phosphorylation.

Introduction

Glioblastoma multiforme (GBM) is the most common primary malignant tumor of the adult central nervous system and has a median survival time of < 1 year. The highly lethal nature of this tumor partly derives from the acquisition of an invasive phenotype, which allows the tumor cells to infiltrate surrounding brain tissues [1,2]. Accordingly, localized treatments of GBM are palliative, but there is lack of success in eradicating this invasive disease. Consequently, identification and characterization of proteins that drive the invasive behavior of GBM may serve as reliable diagnostic and prognostic markers, as well as potential targets for effective therapy.

Gene expression profiling using mRNA differential display and cDNA-based microarrays has been used to identify the spectrum of genes differentially expressed coincident to glioma migration [3,4]. Among these invasion-regulated genes was *P311* (PTZ17) [5]. P311 codes for an 8-kDa intracytoplasmic protein initially identified in neurons and muscles [6]. P311's amino acid sequence contains a conserved PEST domain (Pro, Glu, Ser, and Thr) [7], which plays a role in targeting proteins for degradation by the ubiquitin/proteasome system or plays a function in protein–protein interactions [8]. The rapid decay of P311 protein has been described in both smooth muscle and neural cells [9]. The half-life of P311 is 5 minutes or less, with the rapid degradation being directed by the ubiquitin/proteasome system and an unidentified metalloprotease [9].

The identification of *P311* as a glioma invasion candidate gene is supported by expression levels that correlate with glioma motility [5]. Antisense oligonucleotides downregulated P311 mRNA and protein levels, and resulted in the reduction of glioma cell migration. Immunohistochemical staining of human glioblastoma specimens indicated elevated staining of P311 in glioma cells at the invasive edge of the tumor, compared to the absent expression in normal brain cells [5].

In this study, the role of P311 protein and its potential interactions with the cytoskeleton as a mediator and/or prognostic marker of glioma invasion are described. Overexpression of P311 enhances glioma cell motility; P311 activity and stability are regulated by the phosphorylation of serine 59 located near the PEST domain. Site-directed mutagenesis of S59A stabilizes the P311 protein and induces glioma cell migration. In addition, immunoprecipitation coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis identified Filamin A (a β1 integrin actin binding protein that regulates cell motility) as a P311 binding partner. Both P311 and Filamin A colocalize at

Abbreviations: ECM, extracellular matrix; FBS, fetal bovine serum; GBM, glioblastoma multiforme; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; BIM-1, bisindolylmaleimide-1; Pak1, p21-activated kinase 1; GST, glutathione *S*-transferase; PKC, protein kinase C

Address all correspondence to: Michael E. Berens, Division of Neurogenomics, The Translational Genomics Research Institute, 445 North Fifth Street, Phoenix, AZ 85004.
E-mail: mberens@tgen.org

[1]This work was supported by National Institutes of Health grant NS42262.

[2]Wendy S. McDonough and Nhan L. Tran contributed equally to this work.

Received 17 February 2005; Revised 31 May 2005; Accepted 1 June 2005.

DOI 10.1593/neo.05190

the leading edge of migrating glioma cells, and overexpression of P311 induces Rac1 activation. Depletion of Rac1 expression by small interfering RNA (siRNA) oligonucleotides abrogates P311-induced migration. Thus, these results indicate that P311 may function in the reorganization of the actin cytoskeleton at the cell periphery necessary for cell migration and that P311-induced cell migration and protein stability are dependent on S59 phosphorylation.

Materials and Methods

Cell Culture Conditions and Extracellular Matrix (ECM) Preparation

Human glioma cell lines G112 [10], SF767 [11], T98G, and U118MG (American Type Culture Collection, Manassas, VA) were maintained in minimum essential medium (MEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT) in a 37°C, 5% CO_2 atmosphere at constant humidity. U118–P311–FLAG and U118 vector-only stably transfected glioma cells (Dr. Gregory Taylor; Duke University, Durham, NC) were cultured under G418 selection (200 mg/ml) in MEM supplemented with 10% heat-inactivated FBS in a 37°C, 5% CO_2 atmosphere at constant humidity. Glioma-derived ECM was prepared as previously described [11].

Radial Cell Migration Assay

Quantification of cellular migration was performed using a microliter scale migration assay [12]. Approximately 2000 cells were plated onto 10-well slides precoated with glioma-derived ECM using a cell sedimentation manifold (CSM, Inc., Phoenix, AZ) to establish a confluent 1-mm-diameter monolayer. Cells were allowed to disperse for 24 to 48 hours. Measurements were taken of the area occupied by the cells at regular intervals over 48 hours. The average migration rate of 10 replicates was calculated as the increasing radius of the entire cell population over time.

Site-Directed Mutagenesis and Adenovirus Production and Infection

P311 cDNA (pRcCMV, human P311) was excised and ligated into pcDNA3.1 (Invitrogen Corp.) using *Hin*dIII and *Apa*I. Mutagenic primers were designed specifically for the region of the gene-flanking serine codon 59 (TCC) as follows: GCC (coding for alanine), GAC (coding for aspartate), and GAG (coding for glutamate) (Table 1). The Quick Change Kit (Stratagene, La Jolla, CA) was used according to the manufacturer's instructions on copying this region of the gene. These primers were designed: to flank the site of mutation by 10 to 15 bases, to have a 62.5% GC content, to terminate in one or more G or C bases, and to have a calculated melting temperature of 82.1°C as per manufacturer's instructions. After the primers were used to generate double-stranded copies of the plasmid containing mutated P311 insert, the original vector/insert DNA was digested using *Dpn*I to destroy the methylated parental template, leaving a new circular, nicked dsDNA copy of the plasmid containing the mutated gene of interest. This plasmid was transformed into XL-1 Blue supercompetent cells to repair the nicks in the mutated plasmid. The cells were scaled up, and new plasmids were isolated for subsequent transformation or shuttling into mammalian expression vectors containing a CMV promoter for the transformation of glioblastoma cells with the mutated *P311* gene.

Primers were designed to add the FLAG sequence and to incorporate an *Xho*I restriction site to the existing plasmids (forward 5′-CAG AAT TCC GGA CCA TGG TTT ATT ACC CAG AAC TCT TT-3′ and reverse 5′-CAT CTA GAC TGA TGT TCC TGC TGC TAC TGT TCA TCA GAT CTA C-3′). All sequences were verified by DNA sequencing. Restriction digestion using *Xho*I and *Bgl*II was performed to subclone the plasmids into the pShuttle-CMV to prepare recombinant E1-deleted adenoviruses using the Ad-Easy system, as described [13]. Viruses were propagated in HEK 293 cells (ATCC CRL 1573), clonally isolated, and titered as previously described [14]. Dominant-negative TAC$\beta 1_A$ integrin adenoviral construct [15] was obtained from Dr. Joseph Loftus (Mayo Clinic, Scottsdale, AZ). Cells were infected at matched multiplicity of infection (MOI), as noted in the Results section.

Antibodies and Immunoblotting

A mouse monoclonal antibody, anti–FLAG M2, was obtained from Eastman Kodak Co. (Rochester, NY). Polyclonal anti-FLAG antibodies were purchased from Sigma (St. Louis, MO). Monoclonal antibodies to the human β1 integrin subunit were purchased from Chemicon International (Temecula, CA). Polyclonal antibodies to phosphoserine were obtained from Zymed (South San Francisco, CA). Monoclonal antibodies to protein kinase C (PKC) ζ and polyclonal antibodies specific to PKCα, PKCβ1, PKCδ, PKCε, and

Table 1. Site-Directed Mutagenesis of P311.

Mutation	Strand	Primer or Gene Sequence (all 5′ to 3′)
None	Complimentary P311 strand	cc gtcgtcactt gaggcg*a*ggg gttcttagtc
	Sense P311 strand (locate at base 359)	gg cagcagtgaa ctccgc*t***cc**c caagaatcag
Serine→alanine (S59A)	Forward mutagenic primer	gg cagcagtgaa ctccgc*G***cc**c caagaatcag
	Reverse mutagenic primer	ctgattcttg g**gg***C*gcggag ttcactgctg cc
Serine→aspartate (S59D)	Forward mutagenic primer	gg cagcagtgaa ctccgc**GAC**c caagaatcag
	Reverse mutagenic primer	ctgattcttg g**GT***C*gcggag ttcactgctg cc
Serine→glutamate (S59E)	Forward mutagenic primer	gg cagcagtgaa ctccgc*GAG*c caagaatcag
	Reverse mutagenic primer	ctgattcttg g**CT***C*gcggag ttcactgctg cc

PKCγ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to alpha tubulin were purchased from Upstate Biotechnology (Lake Placid, NY). Anti–mouse IgG and anti–rabbit IgG antibodies coupled to horseradish peroxidase (HRP) were purchased from Promega (Madison, WI). Immunoblotting and protein determination experiments were preformed as previously described [16]. Briefly, cells were washed in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), as described above. The cells were then lysed in 2× sodium dodecyl sulfate (SDS) sample buffer (0.25 M Tris–HCl, pH 6.8, 2% SDS, and 25% glycerol) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 mM NaF, and 1 mM PMSF. Protein concentrations were determined using the BCA assay procedure (Pierce, Rockford, IL), with bovine serum albumin as a standard. Thirty micrograms of total cellular protein was loaded per lane, separated by 12% SDS polyacrylamide gel electrophoresis (PAGE) or 16% Tricine gels for P311 analysis, and then transferred to nitrocellulose (Schleichter&Schuell, Keene, NH) by electroblotting. The nitrocellulose membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (pH 8.0) with 0.1% Tween-20 prior to addition of the primary antibody and then HRP-conjugated anti–mouse/rabbit IgG (Promega). Bound secondary antibodies were detected using a chemiluminescence system (NEN Life Science Products, Boston, MA). Densitometric analysis of the bands was conducted using Scion Image.

PKC Inhibitors and Phosphatase Inhibitors

Cells were plated at 50% confluency. PKC-specific inhibitor bisindolylmaleimide-1 (BIM-1), Go6976, or R0-32-0432 was added to the cells 30 minutes prior to cell lysis at 10 μM—an IC_{50} that has been previously shown to inhibit selective PKC isoforms [17]. BIM-1 inhibits classic Ca^{2+}-dependent PKC isoforms α and βI, as well as novel PKC isoforms δ, ε, and ζ with this same ranked order of potency [18]. Go6976 inhibits PKC with particular specificity for the classic PKC isoforms α, βI, and βII but also for atypical PKCμ [19], whereas Ro-32-0432 selectively inhibits isoforms α and βI [20]. In certain experiments, Calyculin A (20 nM) and okadaic acid (100 nM) were added to cells 30 minutes prior to cell lysis to inhibit protein phosphatases PP1 and PP2A, respectively. Each experiment was performed in triplicate.

Immunofluorescence Microscopy

Cells were grown on 10-well glass slides previously coated with glioma-derived ECM until they reached about 50% confluency, fixed for 5 minutes in 4% paraformalydehyde in PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS. After washing with PBS, cells were blocked with 1% bovine serum albumin and 3% goat serum, and incubated with primary antibody for 1 hour at 25°C. Following washing with 0.1% BSA in PBS and incubation with Cy3-conjugated anti–mouse IgG or fluorescein isothiocyanate–conjugated antirabbit Ig, cell nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) from (Sigma) in PBS for 15 minutes at 37°C, and mounted with SlowFade from Molecular Probes (Eugene OR). Immunofluorescent samples were examined under a laser scanning confocal microscope (LSM 410) equipped with helium, neon, and argon lasers (Zeiss, Thorton, NY) using appropriate filters.

Immunoprecipitation and MALDI-TOF MS

Cells were plated onto 60-mm plates, grown to 70% confluency, and, in certain experiments, labeled in Cys/Met–free Dulbecco's modified Eagle's medium (Invitrogen Corp.) supplemented with 10% FBS (vol/vol) and 0.5 mCi [^{35}S]EXPRE^{35}S^{35}S protein labeling mix (NEN Life Science Products). Prior to lysis, cells were incubated for 30 minutes with lactacystin (10 μm) and *o*-phenanthroline (1.26 mM) to block P311 proteosome degradation [9], or left untreated. Cell were washed in PBS containing 1 mM PMSF and then lysed in NP40 buffer (10 mM Tris–HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 2 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Protein concentrations were determined using the BCA assay procedure (Pierce), with bovine serum albumin as a standard. Equivalent amounts of proteins were precleared and immunoprecipitated from the lysates, and washed with lysis buffer followed by S1 buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1.5% Triton X-100, 0.5% deoxycholate, and 0.2% SDS) (Tran NL 2002). Samples were resuspended in 2× SDS sample buffer (0.25 M Tris–HCl, pH 6.8, 2% SDS, and 25% glycerol) and boiled in the presence of 2-mercaptoethanol (Sigma). Samples were separated on 16% tricine gels and silver-stained or blotted onto nitrocellulose for immunoblot analysis.

For MALDI-TOF MS, protein bands were stained with the silver stain kit from (Bio-Rad, Hercules, CA) [21]. Briefly, proteins in gels were fixed with 50% methanol and 10% acetic acid, then oxidized in a solution of potassium dichromate in diluted nitric acid, washed with water, and treated with silver nitrate solution. Color development was stopped with 5% acetic acid once the desired staining intensity had been achieved. Unique bands were excised from the gel and washed twice in 100 nM NH_4HCO_3 buffer, followed by soaked in 100% acetonitrile for 5 minutes, aspiration of the acetonitrile, and drying for 30 minutes. After rehydration of the pellet, the proteins were digested using 20 μg/ml trypsin (Promega) suspended in 25 mM NH_4HCO_3 buffer, incubated at 37°C for 20 hours, and dried under vacuum. Trifluoroacetic acid (TFA) (5%, 120 μl) was added to the tube and incubated at 40°C for 1 hour in a water bath. After collecting the supernatant, another 120 μl of TFA solution (2.5% TFA and 50% acetonitrile) was added to the tube and incubated at 30°C for 1 hour in a water bath. The supernatants were combined and dried to a powder under vacuum. The samples of generated peptides were dissolved in 5 μl of 0.5% TFA and measured by MALDI-TOF MS analysis at the University of Arizona Proteomic Core Facility (Tucson, AZ). Data searches were performed using the NCBI protein data bank with a minimum matching peptide setting of four, a mass tolerance setting of 50 to 200 ppm, and a single trypsin miss cut setting.

siRNA Preparation and Transfection

siRNA oligonucleotides specific for Rac1 and GL2 luciferase were previously described [22]. siRNA oligonucleotides specific for PKCα, PKCε, and PKCζ were designed according to Elbashir et al. [23] and purchased from Qiagen (Valencia, CA). siRNA sequences to PKC isoforms used were: PKCα (regions 494–514, 5′-CTG CGG AAT GGA TCA CAC TGA), PKCε (regions 2631–2651, 5′-AAC TCA TTG GGT CAG CAA TTA), and PKCζ (regions 482–502, 5′-AAC GGG AGA AGA CAA ATC TAT). Transient transfection of siRNA was carried out using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's protocol. Cells were plated in a six-well plate at 2.0×10^5 cells/well in 1.5 ml of MEM, supplemented with 10% serum without antibiotics. Transfections were carried out according to the manufacturer's protocol after cells were fully spread (6 hours postplating). Cells were infected with Rac1 siRNA oligonucleotides at a concentration of 20 nM, and PKCα, PKCε, and PKCζ siRNA oligonucleotides at a concentration of 40 nM. No cell toxicity was observed at these concentrations of siRNA. Using quantitative PCR, we verified that the siRNA oligonucleotides to PKCα, PKCε, and PKCζ specifically inhibited the expression of PKCα, PKCε, and PKCζ, respectively. In addition, Rac1 siRNA was previously shown to specifically inhibit the expression of Rac1 [22]. Maximum inhibition was achieved by days 2 to 3 after transfection, and cells were assayed on day 3 or day 4 posttransfection.

Rac1 and Cdc42 Activity Assays

The activity assays for Rac1 and Cdc42 were performed according to the manufacturer's protocol (Pierce). Briefly, equal numbers of cells were seeded and infected with the P311 wild-type (wt) or mutant adenoviral constructs. Cells were serum-starved for 16 hours, lysed, and centrifuged for 10 minutes. The supernatant (500 μg of protein) was added to 20 μg of glutathione *S*-transferase (GST) human p21-activated kinase 1 (Pak1) binding, rotated at 4°C for 1 hour, and followed by three washes of protein complexes with lysis buffer. Pak1-bound proteins were dissociated and denatured by heating in sample buffer at 98°C for 5 minutes and subjected to gel electrophoresis. Bound proteins were visualized using either anti–Rac1 or anti–Cdc42 antibody (Pierce) and chemiluminescence techniques.

Results

P311 Enhances Glioma Cell Migration and Localizes at the Leading Edge of Cell Migration

Previous data from antisense oligonucleotide studies suggested that P311 plays a role in migrating glioma cells [5]. To examine the effect of P311 expression on cell migration, stably expressing P311–FLAG–tagged U118 glioma cells and their matched vector controls were deposited using a cell sedimentation manifold onto 10-well glass slides previously coated with glioma-derived ECM [24]. Glioma-derived ECM has been previously shown to enhance the motility behavior of these cells [4,24]. The U118–P311–FLAG–tagged glioma cells migrated one and a half times faster than the vector-only control cells (Figure 1*A*). P311-transfected glioma cells show a reduction of stress fiber actin cytoskeleton compared to controls and an increase in cortical actin deposits (Figure 1*B*, *a* and *d*). In addition, immunocytochemistry revealed P311 localization at the leading edge of cell migration (Figure 1*B*). Thus, the data corroborate the previous study suggesting that overexpression of P311 induces glioma cell motility.

Actively Migrating Glioma Cells Display Decreased Serine Phosphorylation of P311

Analysis of P311 amino sequence using the *Ex*pert *P*rotein *A*nalysis *Sy*stem (ExPASy) Prosite proteomics database (http://au.expasy.org/prosite/) predicted that codon 59 serine is a putative PKC phosphorylation site. U118 glioma cells respond to different substrates with different migration behaviors. On glioma-derived ECM, the cells

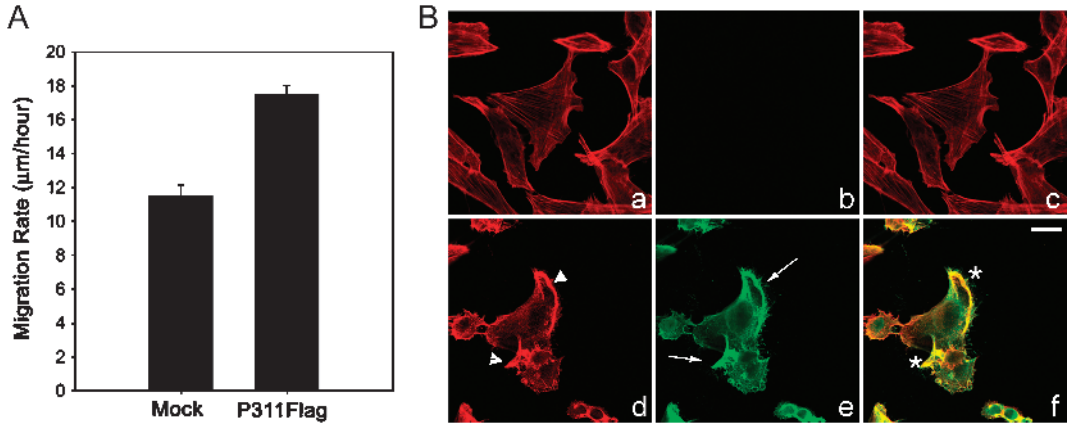

Figure 1. *P311 expression increases glioma cell motility. (A) U118–P311–FLAG–transfected cells and U118 mock-transfected cells were plated onto 10-well glass slides precoated with glioma-derived ECM. Cell migration was assessed over a period of 48 hours. (B) Mock-transfected U118 cells (panels a, b, and c) and U118–P311–FLAG–tagged cells (panels d, e, and f) were grown on 10-well glass slides, fixed, and costained for F-actin (phallodin–Cy3 conjugate; panels a and d) and FLAG-tagged P311 using anti–FLAG M2 antibodies (fluorescein isothiocyanate–stained). Arrowhead represents cortical actin. Arrow represents P311 at the cell periphery. Coimmunolocalizations of cortical actin and P311 are indicated by asterisks. Bar = 20 μm.*

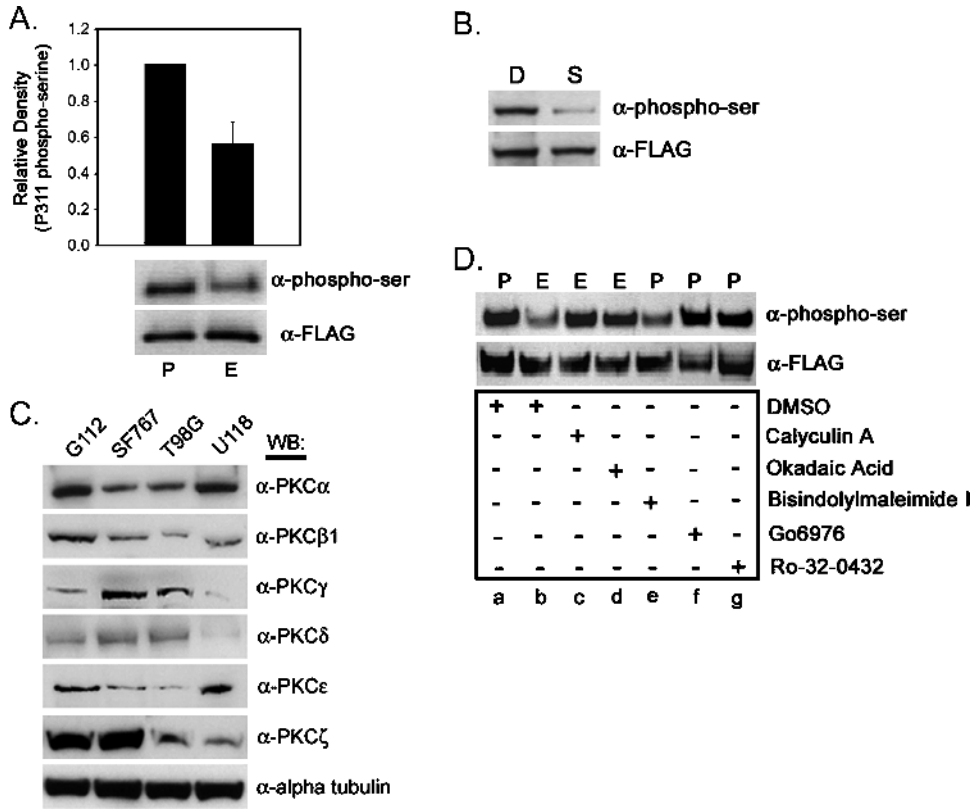

Figure 2. *Actively migrating cells display reduced serine phosphorylation of P311. (A) U118–P311–FLAG–transfected cells were cultured on tissue culture plastic (P) or glioma-derived ECM (E). Cells were then preincubated with proteosome inhibitors lactacystin and o-phenanthroline for 30 minutes prior to cell lysis. P311 was immunoprecipitated with an anti–FLAG M2 antibody and immunoblotted with phosphorylated serine or FLAG M2–specific antibodies. Relative protein densities of P311 phosphoserine to total P311 ratio were determined. Each relative protein value was further normalized to the P311 phosphorylation levels in cells cultured on tissue culture plastic. Data represent the average of three independent experiments. (B) U118–P311–FLAG–transfected cells were cultured on glioma-derived ECM at high cell density (1×10^5 cells/cm^2; D, dense) or low cell density (1×10^4 cells/cm^2; S, sparse), and phosphoserine levels of P311 were evaluated as described above. (C) Immunoblot analysis of various PKC isoforms on whole cell lysates from four glioma cell lines. (D) U118–P311–FLAG–transfected cells were cultured on tissue culture plastic (P) or glioma-derived ECM (E). Cells were then incubated with the indicated PKC inhibitors at 10 μM or solvent DMSO control and proteosome inhibitors for 30 minutes prior to cell lysis, and phosphoserine levels of P311 were evaluated as described above.*

migrate, whereas on standard tissue culture, plastic cell motility is minimal. Cells were grown either on unmodified culture dishes (stationary nonmotile) or in flasks precoated with glioma-derived ECM (migration-promoting environment). Total cellular lysates were prepared, and P311 was immunoprecipitated using an anti–FLAG M2 antibody, followed by an immunoblot analysis for phosphoserine. Serine phosphorylation of P311 was two-fold higher for stationary cells compared to cells plated on a motility-activating substrate (Figure 2*A*), suggesting that phosphorylation modification of P311 accompanies glioma cell motility. To control for effects related to the composition of the ECM, we examined the serine phosphorylation levels of P311 in glioma cells plated on glioma-derived ECM at high cell density, which prevents cell movement, and at low density, which promotes cell motility. A three-fold decrease of serine phosphorylation was detected in actively migrating glioma cells compared to migration-restricted cells on the identical substrate (Figure 2*B*). This suggests that altered serine phosphorylation of P311 is, in part, a migration-associated effect.

Because a putative PKC phosphorylation site is identified at codon 59 of P311, we investigated whether the serine phosphorylation of P311 is a consequence of PKC activation. Western blot analysis from four glioma cell lines revealed differential expression of various PKC isoforms in the different glioma cell lines (Figure 2*C*). Specifically in U118 cells, PKCα and PKCε are expressed at high levels, and PKCβ1 and PKCζ are detected at moderate levels, whereas minimal expression of PKCγ and PKCδ was detected (Figure 2*C*). The activity of different PKC family members was targeted using selective PKC inhibitors Go6976, Ro-32-0432, and BIM-1 [18–20]. U118 cells were grown sparsely on standard tissue culture flasks then treated with the various PKC inhibitors at 10 μM concentration. P311 was immunoprecipitated from cell lysates using anti–FLAG M2 antibodies. Western blot analysis using specific antiphosphoserine antibodies showed that serine phosphorylation of P311 was reduced three-fold in the presence of BIM-1 (Figure 2*D*; *cf. lanes A and E*). Additionally, no phosphorylation changes were affected by Go6976 and Ro-32-0432 (Figure 2*D*, *lanes F and G, respectively*). Moreover, because decreased serine phosphorylation is observed in migrating glioma cells, we examined the effect of specific phosphatase inhibitors of PP1 and PP2A [17]. Treatment of cells cultured on glioma-derived ECM with Calyculin A (Figure 2*D*, *lane c*) and okadaic acid (Figure 2*D*, *lane d*) prevented the loss of

P311 serine phosphorylation levels compared to solvent treatment (Figure 2*D*, *lane b*). These outcomes suggest a role for phosphatases such as PP1 or PP2A in P311 activity, and further support the regulated serine phosphorylation of P311 impacting the migration of glioma cells.

siRNA-Mediated Depletion of PKCε and PKCζ Results in Loss of P311 Serine Phoshorylation

The effect of BIM-1 on P311 suggests that the novel class of PKC isoforms, particularly δ, ε, and ζ, may play a role in P311 serine phosphorylation. Because PKCε and PKCζ are detected in U118 cells, we examined whether these two isoforms are critical for the serine phosphorylation of P311. siRNA oligonucleotide duplexes were specifically designed against either PKCα, PKCε, or PKCζ. The degree of protein knockdown in U118–P311–FLAG–tagged glioma cells was ~85% to 90%, as verified by Western blot analysis using antibodies specific to either PKCα (Figure 3*A*), PKCε (Figure 3*B*), or PKCζ (Figure 3*C*). As expected, high serine phosphorylation of P311 was detected in migration-restricted cells (Figure 3*D*, *lane a*), whereas decreased levels of P311 serine phosphorylation were detected in migration-activated cells (Figure 3*D*, *lane b*). Interestingly, siRNA-mediated depletion of either PKCε (Figure 3*D*, *lane d*) or PKCζ (Figure 3*D*, *lane e*) resulted in a decrease in P311 serine phosphorylation in migration-restricted cells, whereas no loss of P311 serine phosphorylation was detected in PKCα-depleted cells. Taken together, our results show that the serine phosphorylation of P311 is dependent on the function of both PKCε and PKCζ.

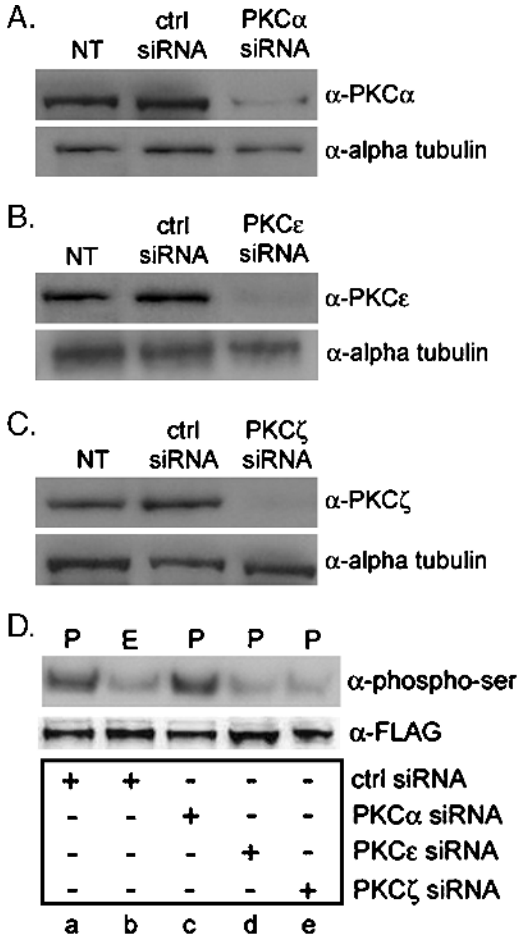

Figure 3. *Depletion of either PKCε or PKCξ inhibits P311 serine phosphorylation in migration-restricted cells. Western blot analysis determining the protein expression of PKCα (A), PKCε (B), and PKCζ (C) after siRNA transfection, as described in Materials and Methods. (D) U118–P311–FLAG–tagged cells were plated on either tissue culture plastic (P) or glioma-derived ECM (E). Cells were then transfected with siRNA directed against either PKCα, PKCε, PKCζ, or control luciferase (ctrl siRNA), and cultured for an additional 24 hours. Proteosome inhibitors were then added 30 minutes prior to cell lysis, and P311 was immunoprecipitated with an anti–FLAG M2 antibody and immunoblotted for phosphorylated serine or FLAG M2–specific antibodies.*

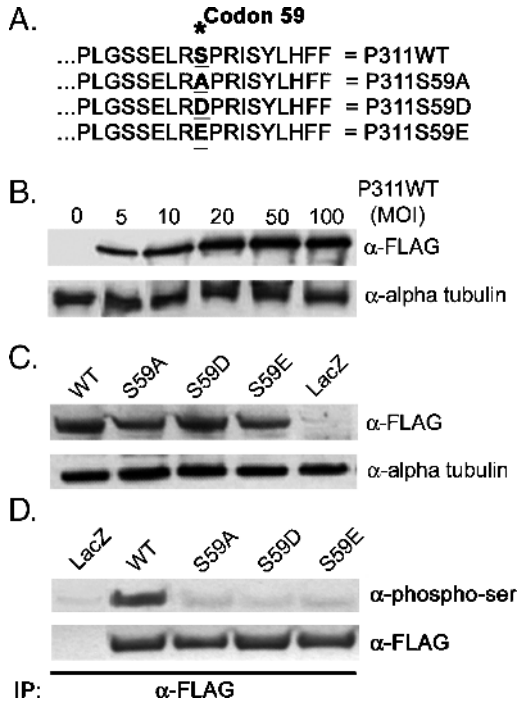

Figure 4. *Site-directed mutagenesis of P311 at a putative PKC serine phosphorylation domain at codon 59. (A) Site-directed mutagenesis of serine codon 59 (serine to alanine, S59A; serine to aspartate, S59D; and serine to glutamate, S59E, respectively). (B) Titer-dependent expression of P311wt adenovirus at various MOI concentrations. U118 cells were infected with various concentrations of adenovirus expressing FLAG-tagged P311wt for 48 hours. Cells were incubated with the proteosome inhibitors lactacystin and o-phenanthroline, then total cellular lysates were collected and immunoblotted using anti–FLAG M2 antibody. (C) Confirmation of expression of P311–FLAG adenoviral constructs in U118 cells infected for 48 hours with an MOI of 20 by immunoblotting with anti–FLAG M2 antibody. Lysates were collected in the presence of the proteosome inhibitor. (D) Serine codon 59 is the sole active phosphorylation site of P311. U118 cells were infected with the different adenoviral constructs for 24 hours. Cells were incubated with the proteosome inhibitors lactacystin and o-phenanthroline, then cellular lysates were immunoprecipitated with anti–FLAG M2 antibody, immunoblotted, and probed for phosphorylated serine or FLAG, as indicated.*

Serine Phosphorylation at S59 Is Important for P311-Induced Cell Migration and P311 Protein Stability

To determine whether serine phosphorylation at S59 of P311 influences migration, site-directed mutants of P311 were generated and propagated into replication-deficient adenoviruses (Figure 4*A*). Each construct also contained a FLAG epitope at the carboxyl terminus. Alanine (S59A)

substitution was constructed to eliminate phosphorylation at S59, whereas aspartate (S59D) and glutamate (S59E) were designed to mimic a constitutively phosphorylated serine at S59 (Figure 4*A*). U118 glioma cells infected with varying MOIs of P311wt showed a titer-dependent induction of the P311 protein (Figure 4*B*). In addition, expression of all mutated constructs was verified by immunoblot analysis for the FLAG epitope (MOI = 20; Figure 4*C*). The substitution of P311 S59 for *S59A*, *S59D*, and *S59E* eliminated the detection of any serine phosphorylation (Figure 4*D*), demonstrating that S59 is the only active phosphoregulated serine in P311.

To determine the effect of P311 S59 mutations on glioma cell migration, we examined the migratory capacity of cells expressing either wild-type or phosphorylation site mutants of P311. Cells expressing wild-type P311 or the *S59A* mutant displayed enhanced migration rates in four different glioma cell lines, ranging from 1.4- to 2-fold induction (Figure 5*A*). Glioma cells expressing P311 *S59D* or *S59E* mutants showed a decreased migration rate relative to uninfected or LacZ-infected controls (Figure 5*A*). These results indicate that glioma cell migration, in part, requires serine phosphorylation of P311 and that serine phosphorylation of P311 at S59 is a potential mediator of P311-regulated glioma cell migration.

A previous study by Taylor et al. [9] showed that P311 was targeted for degradation by multiple proteolytic pathways, including the ubiquitin/proteasome pathway, resulting in an extremely short protein half-life. Because S59 is flanked by a PEST sequence, we investigated the effect of the S59 mutational substitution on P311 protein stability. U118 glioma cells were infected with the adenoviral *P311* mutant constructs and cultured in the presence or absence of the proteosome inhibitors lactacystin and *o*-phenanthroline. Whole cell lysates were collected and immunoblotted for anti–FLAG M2. In the absence of proteosome inhibitors, P311wt was rapidly degraded (Figure 5*B*). Blocking the ubiquitin/proteosome degradation pathway resulted in a nearly two-fold rise in P311wt protein levels. In contrast, the levels of P311 *S59A* and *S59E* mutants were unaffected by the absence of the proteosome inhibitors; the P311 *S59D* mutant showed degradation of the protein, similar to P311wt (Figure 5*B*). The stability of the P311wt and each P311 mutant protein was observed in the presence of the ubiquitin/proteosome inhibitors. Confirmation of the effect of S59 mutations on P311 protein stability was performed by ^{35}S methionine labeling of proteins in U118 cells. Densitometric analysis of the autoradiogram revealed an approximately two-fold ($P < .001$) increase in the protein stability of P311 *S59A* and *S59E* compared to P311wt and *S59A* mutant in the absence of the ubiquitin/proteosome inhibitor (data not shown).

Identification of Filamin A as a Binding Partner to P311

Because P311 is not a member of any known family of proteins, clues suggesting function based on analogy are not available. To determine possible mechanisms by which P311 regulates cell migration, we sought potential binding partners of P311 in an immunoprecipitation pulldown experiment coupled with MALDI-TOF MS analysis. U118–P311 cells were cultured under migration-restricted or migration-activated states. Total cellular lysates were prepared and P311 protein was immunoprecipitated using an anti–FLAG M2 epitope antibody. Coimmunoprecipitated proteins accompanying P311 showing differential abundance under

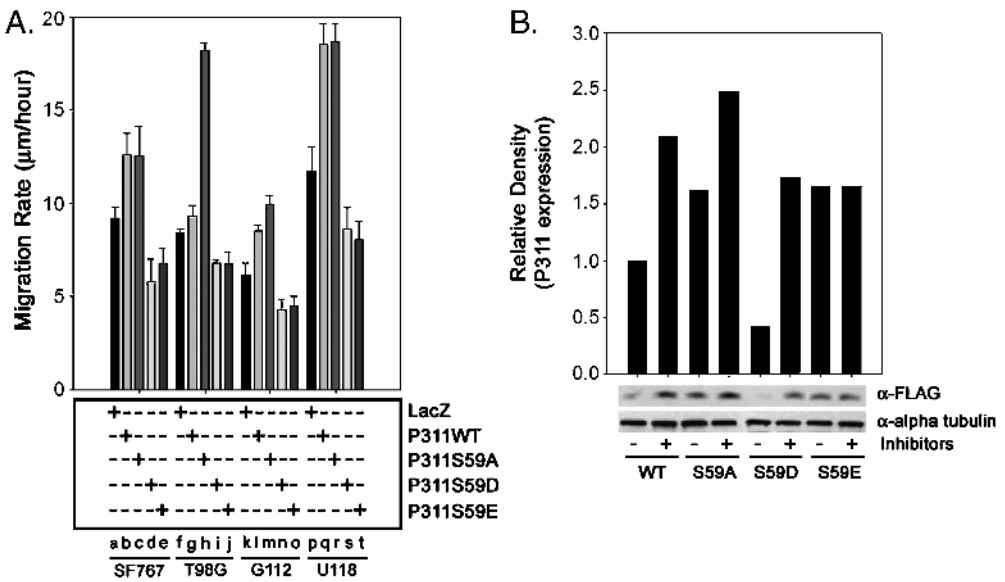

Figure 5. *Site-directed mutation of P311 S59A induces glioma cell migration and P311 protein stability. (A) Glioma cells were infected with the indicated P311 mutant constructs at an MOI of 20 for 24 hours prior to migration analysis. Cell motility was assessed over an additional 48 hours. (B) Evaluation of protein stability of P311 mutant constructs. U118 cells were infected with the indicated P311 mutant constructs (MOI of 20) in the presence or absence of the ubiquitin inhibitors (lactocystin and o-phenanthroline). Total cell lysates were collected and analyzed for FLAG and alpha tubulin expression. Relative protein blot densities of P311 were normalized to alpha tubulin and further corrected to P311wt without inhibitor treatment. Data are representations of three independent experiments. WB, Western blot; IP, immunoprecipitation.*

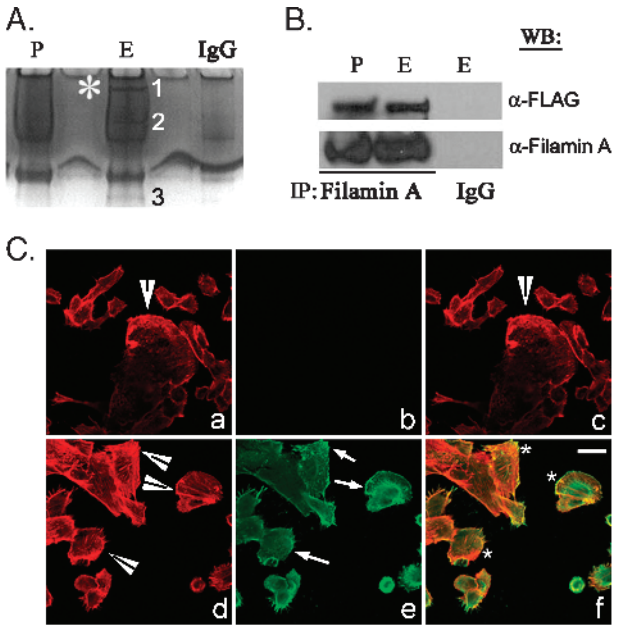

Figure 6. *Identification of Filamin A as a binding partner to P311 in migration-activated cells using peptide mass fingerprinting and MALDI-TOF MS analysis. (A) U118 cells stably expressing P311–FLAG were seeded on tissue culture plastic (P) or glioma-derived ECM (E). Cells were incubated with the proteosome inhibitors lactacystin and o-phenanthroline prior to total cellular lysate collection. P311 was immunoprecipitated using anti–FLAG M2 antibody, separated on 10% SDS-PAGE gel, and silver-stained. A 230-kDa band (*), present only in actively migrating cells, was isolated and identified by MALDI-TOF MS as Filamin A. (B) U118 cells were cultured on tissue culture plastic or glioma-derived ECM, and infected with P311wt adenoviruses. Cells were preincubated with the proteosome inhibitors, and Filamin A was immunoprecipitated using anti–Filamin A antibody and immunoblotted for P311 with anti–FLAG M2 antibody. (C) Coimmunolocalization of P311 (fluorescein isothiocyanate–stained) and Filamin A (Cy3-stained) in control mock-transfected U118 cells (panels a, b, and c) and U118–P311–FLAG–tagged cells (panels d, e, and f). Arrowheads represent positive Filamin A staining. Arrows represent P311 positive staining. Asterisks represent colocalization of P311 and Filamin A (panel f). Bar = 20 μm.*

migration-restricted or migration-activated states were detected on a standard one-dimensional SDS-PAGE system (Figure 6*A*). Three putative P311-binding proteins were uniquely detected in migration-activated glioma cells. Band 2 was a contaminant and band 3 was identified by MALDI-TOF MS analysis as a GAPDH homolog protein. Interestingly, band 1, a 230-kDa protein, was identified as Filamin A, a β1 integrin cytoskeletal-binding protein (Figure 6*A*). Coimmunoprecipitation of P311 with Filamin A was verified by immunoprecipitation with a Filamin A antibody, suggesting that Filamin A interacts with P311. Interestingly, Filamin A immunoprecipitation studies showed that P311 binds equally to Filamin A in migration-activated and migration-restricted cells (Figure 6*B*). To further confirm the association of P311 with Filamin A, P311 and Filamin A were immunolocalized in glioma cells. P311 immunofluorescence showed strong localization at the leading edge of cell migration in lamellipodial structures as anticipated (Figure 6*C*; *panel e*, *arrow*). When cells were coimmunolabeled for Filamin A and P311, Filamin A was also detected at the leading edge of cell migration (Figure 6*C*; *panel d*, *arrowhead*). Merging of the two confocal images revealed identical patterns of colocalization (Figure 6*C*; *panel f*, *asterisk*). Thus, these data support an association between the two proteins, P311 and Filamin A.

Inhibition of β1 Integrin Function Suppresses P311-Induced Cell Motility

β1 Integrin has been previously shown to play a role in glioma cell motility and invasion [25–28]; blocking β1 integrin function resulted in decreased glioma adhesion and migration [26,27]. Because Filamin A is a cytoskeletal protein that interacts with the β1 integrin, we investigated whether the inhibition of β1 integrin function by a dominant-negative β1 protein, TACβ1$_A$, would suppress P311-induced cell motility. The TACβ1$_A$ chimeric receptor contains the β1 integrin intracellular domain coupled to a reporter consisting of the transmembrane and extracellular domains of the small, nonsignaling subunit of the interleukin-2 receptor [29]. TACβ1$_A$ was previously shown to disrupt β1 integrin adhesion and signaling [15,29]. Expression of the TACβ1$_A$ protein in U118 cells resulted in a two-fold decrease in cell migration on glioma-derived ECM compared to LacZ-infected control (Figure 7*A*). As expected, overexpression of P311wt enhances glioma migration (Figure 7*A*). However, coexpression of P311wt and TACβ1$_A$ suppresses P311-induced cell migration, suggesting that regulation of P311-induced cell motility is dependent on functional β1 integrins.

We next determined whether interfering with the β1 integrin function by TACβ1$_A$ expression alters P311 serine phosphorylation status. U118–P311 glioma cells plated

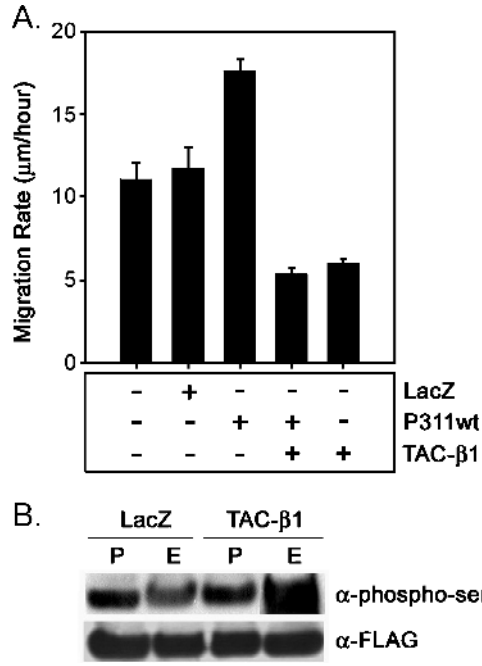

Figure 7. *Inhibition of β1 integrin function with TACβ1$_A$ suppresses P311-induced cell migration and prevents the reduction of P311 serine phosphorylation. (A) U118 cells were infected with adenoviruses expressing control LacZ, P311wt, or TACβ1$_A$. Cell migration was assessed on glioma-derived ECM over 48 hours. (B) U118–P311–FLAG–transfected cells were infected with adenoviruses expressing TACβ1$_A$ or control LacZ, and cultured for 24 hours. Cells were then cultured on tissue culture plastic or glioma-derived ECM, and phosphoserine levels of P311 were evaluated as described above.*

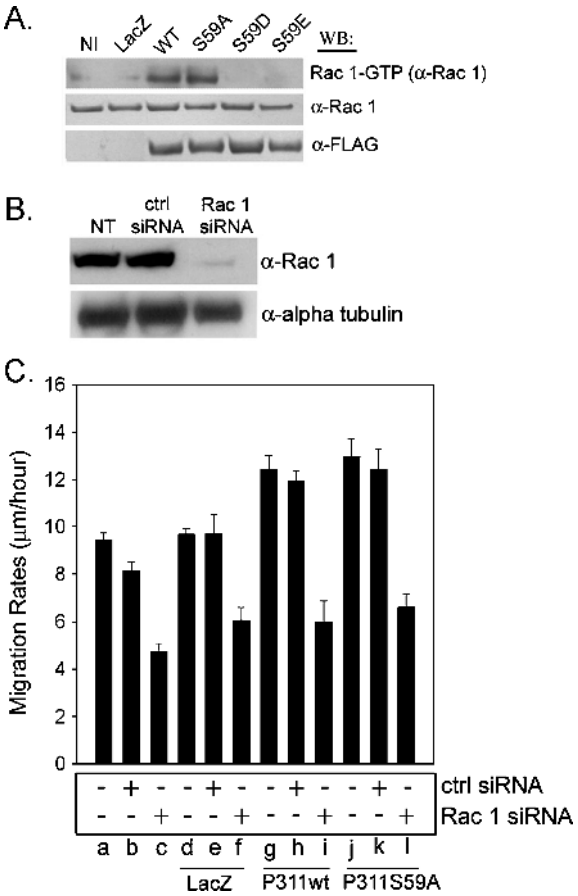

Figure 8. *P311 overexpression induces Rac1 activation, and depletion of Rac1 inhibits P311-induced cell migration (A). U118 cells were infected with P311wt or P311 mutant adenoviruses (NI, no infection). Cells extracts were assayed for Rac1 activation using the PBD assay, as described in Materials and Methods. (B) Western blot analysis determining the protein expression of Rac1 after siRNA transfection, as described in Materials and Methods. NT, no transfection. (C) U118 cells were transfected with siRNA directed against control luciferase (trl siRNA) or Rac1. Cells were then infected with adenoviruses expressing control LacZ, P311wt, or P311 S59A. Cell migration was assessed on glioma-derived ECM over 48 hours.*

sparsely or densely on glioma-derived ECM were infected with adenoviruses expressing dominant-negative TAC$\beta 1_A$ integrin or control TAC$\alpha 5$ integrin. Cells infected with control LacZ showed a decrease in P311 serine phosphorylation levels in migration-stimulated cells compared to migration-restricted cells (Figure 7*B*). However, disruption of $\beta 1$ integrin function by TAC$\beta 1_A$ not only inhibited the loss of P311 serine phosphorylation in migration-activated glioma cells, but appeared to lead to hyperphosphorylation of P311 (Figure 7*B*). These data support the notion that P311-induced migration is dependent on $\beta 1$ integrin function.

P311-Induced Glioma Migration Is Dependent on Rac1 Activation

The small GTPase, Rac1, is important for the migration and invasion of tumor cells [30,31]. Because P311 is localized at the cell periphery, we sought to determine whether P311-induced glioma migration is dependent on Rac1 activation. We examined the effect of P311wt and mutant proteins on Rac1 activation. For these experiments, we utilized the Rac1-binding domain of Pak1 fused to GST to affinity-precipitate active Rac1 [32,33]. Cells infected with either P311wt or P311 *S59A* showed high Rac1 activation compared to uninfected cells or control cells (LacZ infection) (Figure 8*A*). No Rac1 activation was detected in cells infected with P311 *S59D* and P311 *S59E*. Immunoprecipitation results indicated that Rac1 does not directly associate with P311 (data not shown), arguing for other intermediate regulatory proteins serving as activating links between P311 and Rac1. In addition, overexpression of P311wt or P311 *S59A* had no effect on Cdc42 activation (data not shown). Specific siRNA oligonucleotides knocked down endogenous Rac1 protein by ~90% (Figure 8*B*) and suppressed glioma migration (Figure 8*C*, *lane c*). Interestingly, Rac1 depletion abrogated P311wt- and P311 *S59A*–induced cell motility (*cf. lanes g versus i and j versus l*); luciferase control siRNA had no effect on P311-induced cell migration (*lanes h and k*). These results suggest that P311-induced cell migration is dependent on Rac1.

Discussion

To date, the function of P311 in glioma biology is unknown. P311 has been previously characterized by the presence of a conserved PEST domain that is targeted by the ubiquitin/proteosome system, resulting in a protein half-life of about 5 minutes [7]. In this study, we demonstrated the importance of the phosphorylation on S59 in the regulation and stability of P311 in glioma cells. The phosphorylation status of S59 is largely dependent on upstream PKC activity and active cell motility. Mutational analysis of S59 indicates that this serine residue is the solitary active phosphoserine within P311. Consequently, mutation of P311 *S59A* resulted in loss of P311 serine phosphorylation, heightened stability of the P311 protein, and induction of glioma cell migration.

Regulation of cell migration is a dynamic process involving, among other reactions, modulation of focal adhesion dynamics and actomyosin polymerization and contraction [34,35]. Currently, it is not known how P311 participates in the regulation of cell migration. Comparative analysis of P311 serine phosphorylation status indicated that glioma cells actively migrating on glioma-derived ECM displayed a reduction in P311 serine phosphorylation. This decreased level of serine phosphorylation appears to be independent of the glioma-derived ECM because migration-restricted confluent glioma cells on glioma-derived ECM also demonstrated a decreased level in P311 serine phosphorylation (data not shown). Accordingly, mutation of P311 *S59A* results in the loss of serine phosphorylation and an enhanced cellular migration similar to overexpression of the P311wt protein. In contrast, both *S59E* and *S59D* mutations did not change cell motility. The difference between alanine substitution and aspartate and glutamate substitution is that both aspartate and glutamate substitutions were designed to mimic a constitutive phosphorylated serine by weight and charge, suggesting that the phosphorylated serine residue regulates P311-induced cell migration. The findings in this

report support this interpretation. In addition, maintenance of P311 in its dephosphorylated form is dependent on β1 integrin signaling, as indicated by maintenance of a constant (elevated) level of serine phosphorylation when TACβ1$_A$ is expressed, which inhibits β1 integrin function. Inhibition of β1 integrin signaling also abrogated P311-induced cell migration, suggesting that P311 acts in concert with signaling that is downstream of β1 integrin.

Site-directed mutagenesis of S59A prevents the P311 protein from being degraded by ubiquitin-mediated proteolysis. Interestingly, although the P311 *S59E* mutant did not enhance glioma motility, protein stability was similar to the P311 *S59A* mutant, whereas P311 *S59D* resulted in rapid ubiquitin-mediated proteolysis. It is likely that tertiary protein conformations from the site-directed mutants may explain the differential binding of ubiquitin proteins to the PEST sequence. Interestingly, pharmacologic inhibitor data suggest that a novel class of PKC isoforms, particularly δ, ε, and ζ, regulates P311 serine phosphorylation status. In the present study, BIM-1 reduced P311 serine phosphorylation, whereas Go6976 and Ro-32-0432 had no effect. BIM-1 inhibits the δ, ε, and ζ isoforms of PKC, distinct from the other inhibitors [18]. PKC activity has been correlated with growth rates [36,37] and migration behavior [38–40] in gliomas *in vitro*. Specifically, PKCε has been shown to positively regulate β1 integrin-dependent adhesion, spreading, and motility of human glioma cells [40]. In fact, the current findings support the notion that both PKCε and PKCζ are upstream mediators of P311 serine phosphorylation. Depletion of either PKCε or PKCζ expression resulted in the loss of P311 serine phosphorylation in migration-restricted glioma cells. However, P311 serine phosphorylation is not completely abrogated by BIM-1 treatment or siRNA depletion of PKCε or PKCζ, suggesting that other signaling pathways besides PKC are involved in P311 serine phosphorylation.

Immunoprecipitation of P311, coupled with MALI-TOF MS analysis, revealed that Filamin A is directly or indirectly a binding partner of P311. Filamin A is a large actin-binding protein that stabilizes three-dimensional actin webs. Filamin A links the actin cytoskeleton to cellular membranes, thereby regulating cellular, architectural, and signaling functions essential for cell motility [41]. Filamin A binds to at least 20 other marcromolecules, including β1 integrin [42], which has been shown to play a crucial role in glioma cell migration and invasion [25,27,28] and has multiple functions, including its role in cortical stability necessary for lamellipodial extension and cell crawling [43]. We demonstrated that P311 and Filamin A colocalize at the leading edge of migrating glioma cells and that both P311 and Filamin A are localized at lamellipodial structures, suggesting a possible influence on the activity of the small GTPase, Rac1. We report that overexpression of P311 in glioma cells induces Rac1 activation. Notably, activation of Rac1 occurs through the exchange of bound GDP for GTP, catalyzed by one of multiple Rac guanine exchange factors (GEFs) [44]. Likewise, inhibition of Rac1 expression by siRNA oligonucleotides suppresses P311-induced migration. It is possible that P311 may modulate cytoskeleton dynamics by influencing one of the Rac GEF proteins involved in Rac1 activation and subsequently regulating cell migration. In fact, Rac1 has been shown to play an important role in various malignant carcinoma cells and in gliomas [22,30,45]. Interestingly, immunoprecipitation studies showed no evidence of a direct physical interaction between P311 and Rac1, further supporting the notion that P311 activation of Rac1 is mediated by intermediate soluble protein(s). In summary, these findings highlight the importance of P311 in the regulation of glioma cell migration. Importantly, we believe that the nonphosphorylated form of P311 on S59 results in increased protein stability, which subsequently promotes cytoskeleton dynamics essential for migration. We further speculate that P311 turnover in cells may impact the regulated action of actin-binding proteins interacting with signaling molecules such as Rac1. Thus, understanding the biologic role of P311 and its potential effectors is likely to open new possibilities for therapeutic interventions aimed at invading glioma cells.

Acknowledgements

We thank Gregory Taylor for providing the U118–P311 cells. We also thank Joseph Loftus for providing TACβ1$_A$ adenoviruses, Jean Kloss for assistance with adenoviral constructs, and Marc Symons for providing the control luciferase and Rac1 siRNA oligonucleotides. We also thank Don Weaver and Spyro Mousses for assistance with the design of the PKC isoform siRNA oligonucleotides, and Mitsutoshi Nakada and Dominique Hoelzinger for the critical review of the manuscript.

References

[1] Giese A, Bjerkvig R, Berens ME, and Westphal M (2003). Cost of migration: invasion of malignant gliomas and implications for treatment. *J Clin Oncol* **21,** 1624–1636.

[2] Keles GE and Berger MS (2004). Advances in neurosurgical technique in the current management of brain tumors. *Semin Oncol* **31,** 659–665.

[3] McDonough W, Tran N, Giese A, Norman SA, and Berens ME (1998). Altered gene expression in human astrocytoma cells selected for migration: I. Thromboxane synthase. *J Neuropathol Exp Neurol* **57,** 449–455.

[4] Mariani L, Beaudry C, McDonough WS, Hoelzinger DB, Demuth T, Ross KR, Berens T, Coons SW, Watts G, Trent JM, et al. (2001). Glioma cell motility is associated with reduced transcription of proapoptotic and proliferation genes: a cDNA microarray analysis. *J Neuro-Oncol* **53,** 161–176.

[5] Mariani L, McDonough WS, Hoelzinger DB, Beaudry C, Kaczmarek E, Coons SW, Giese A, Moghaddam M, Seiler RW, and Berens ME (2001). Identification and validation of P311 as a glioblastoma invasion gene using laser capture microdissection. *Cancer Res* **61,** 4190–4196.

[6] Studler JM, Glowinski J, and Levi-Strauss M (1993). An abundant mRNA of the embryonic brain persists at a high level in cerebellum, hippocampus and olfactory bulb during adulthood. *Eur J Neurosci* **5,** 614–623.

[7] Varshavsky A (1997). The ubiquitin system. *Trends Biochem Sci* **22,** 383–387.

[8] Dice JF (1987). Molecular determinants of protein half-lives in eukaryotic cells. *FASEB J* **1,** 349–357.

[9] Taylor GA, Hudson E, Resau JH, and Vande Woude GF (2000). Regulation of P311 expression by Met-hepatocyte growth factor/scatter factor and the ubiquitin/proteasome system. *J Biol Chem* **275,** 4215–4219.

[10] Westphal M, Meissner H, Matschke J, and Herrmann HD (1998). Tissue culture of human neurocytomas induces the expression of glial fibrillary acidic protein. *J Neurocytol* **27,** 805–816.

[11] Berens ME, Rief MD, Loo MA, and Giese A (1994). The role of extra-

cellular matrix in human astrocytoma migration and proliferation studied in a microliter scale assay. *Clin Exp Metastasis* **12,** 405–415.
[12] Berens ME and Beaudry C (2004). Radial monolayer cell migration assay. *Methods Mol Med* **88,** 219–224.
[13] He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, and Vogelstein B (1998). A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* **95,** 2509–2514.
[14] Lipinski CA, Tran NL, Bay C, Kloss J, McDonough WS, Beaudry C, Berens ME, and Loftus JC (2003). Differential role of proline-rich tyrosine kinase 2 and focal adhesion kinase in determining glioblastoma migration and proliferation. *Mol Cancer Res* **1,** 323–332.
[15] Keller RS, Shai SY, Babbitt CJ, Pham CG, Solaro RJ, Valencik ML, Loftus JC, and Ross RS (2001). Disruption of integrin function in the murine myocardium leads to perinatal lethality, fibrosis, and abnormal cardiac performance. *Am J Pathol* **158,** 1079–1090.
[16] Tran NL, Adams DG, Vaillancourt RR, and Heimark RL (2002). Signal transduction from N-cadherin increases Bcl-2. Regulation of the phosphatidylinositol 3-kinase/Akt pathway by homophilic adhesion and actin cytoskeletal organization. *J Biol Chem* **277,** 32905–32914.
[17] Sakoff JA and McCluskey A (2004). Protein phosphatase inhibition: structure based design. Towards new therapeutic agents. *Curr Pharm Des* **10,** 1139–1159.
[18] Hers I, Tavare JM, and Denton RM (1999). The protein kinase C inhibitors bisindolylmaleimide I (GF 109203x) and IX (Ro 31-8220) are potent inhibitors of glycogen synthase kinase-3 activity. *FEBS Lett* **460,** 433–436.
[19] Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, and Johannes FJ (1996). Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase C isoenzymes. *FEBS Lett* **392,** 77–80.
[20] Birchall AM, Bishop J, Bradshaw D, Cline A, Coffey J, Elliott LH, Gibson VM, Greenham A, Hallam TJ, Harris W, et al. (1994). Ro-32-0432, a selective and orally active inhibitor of protein kinase C, prevents T-cell activation. *J Pharmacol Exp Ther* **268,** 922–929.
[21] Merril CR, Dunau ML, and Goldman D (1981). A rapid sensitive silver stain for polypeptides in polyacrylamide gels. *Anal Biochem* **110,** 201–207.
[22] Chuang YY, Tran NL, Rusk N, Nakada M, Berens ME, and Symons M (2004). Role of synaptojanin 2 in glioma cell migration and invasion. *Cancer Res* **64,** 8271–8275.
[23] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411,** 494–498.
[24] Giese A, Rief MD, Loo MA, and Berens ME (1994). Determinants of human astrocytoma migration. *Cancer Res* **54,** 3897–3904.
[25] Treasurywala S and Berens ME (1998). Migration arrest in glioma cells is dependent on the alpha v integrin subunit. *Glia* **24,** 236–243.
[26] Joy AM, Beaudry CE, Tran NL, Ponce FA, Holz DR, Demuth T, and Berens ME (2003). Migrating glioma cells activate the PI3-K pathway and display decreased susceptibility to apoptosis. *J Cell Sci* **116,** 4409–4417.
[27] Giese A, Laube B, Zapf S, Mangold U, and Westphal M (1998). Glioma cell adhesion and migration on human brain sections. *Anticancer Res* **18,** 2435–2447.
[28] Uhm JH, Gladson CL, and Rao JS (1999). The role of integrins in the malignant phenotype of gliomas. *Front Biosci* **4,** D188–D199.
[29] Akiyama SK, Yamada SS, Yamada KM, and LaFlamme SE (1994). Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. *J Biol Chem* **269,** 15961–15964.
[30] Shaw LM, Rabinovitz I, Wang HH, Toker A, and Mercurio AM (1997). Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell* **91,** 949–960.
[31] Sahai E and Marshall CJ (2002). RHO-GTPases and cancer. *Nat Rev Cancer* **2,** 133–142.
[32] Benard V, Bohl BP, and Bokoch GM (1999). Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J Biol Chem* **274,** 13198–13204.
[33] Sander EE, van Delft S, ten Klooster JP, Reid T, van der Kammen RA, Michiels F, and Collard JG (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell–cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J Cell Biol* **143,** 1385–1398.
[34] Feng Y and Walsh CA (2004). The many faces of filamin: a versatile molecular scaffold for cell motility and signalling. *Nat Cell Biol* **6,** 1034–1038.
[35] Upadhyaya A and van Oudenaarden A (2004). Actin polymerization: forcing flat faces forward. *Curr Biol* **14,** R467–R469.
[36] Couldwell WT, Uhm JH, Antel JP, and Yong VW (1991). Enhanced protein kinase C activity correlates with the growth rate of malignant gliomas *in vitro*. *Neurosurgery* **29,** 880–886 (discussion 886–887).
[37] Donson AM, Banerjee A, Gamboni-Robertson F, Fleitz JM, and Foreman NK (2000). Protein kinase C zeta isoform is critical for proliferation in human glioblastoma cell lines. *J Neuro-Oncol* **47,** 109–115.
[38] Zhang W, Law RE, Hinton DR, and Couldwell WT (1997). Inhibition of human malignant glioma cell motility and invasion *in vitro* by hypericin, a potent protein kinase C inhibitor. *Cancer Lett* **120,** 31–38.
[39] Cho KK, Mikkelsen T, Lee YJ, Jiang F, Chopp M, and Rosenblum ML (1999). The role of protein kinase C alpha in U-87 glioma invasion. *Int J Dev Neurosci* **17,** 447–461.
[40] Besson A, Wilson TL, and Yong VW (2002). The anchoring protein RACK1 links protein kinase C epsilon to integrin beta chains. Requirements for adhesion and motility. *J Biol Chem* **277,** 22073–22084.
[41] Stossel TP and Hartwig JH (2003). Filling gaps in signaling to actin cytoskeletal remodeling. *Dev Cell* **4,** 444–445.
[42] Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, and Shapiro SS (2001). Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol* **2,** 138–145.
[43] Cunningham CC, Gorlin JB, Kwiatkowski DJ, Hartwig JH, Janmey PA, Byers HR, and Stossel TP (1992). Actin-binding protein requirement for cortical stability and efficient locomotion. *Science* **255,** 325–327.
[44] Nobes CD and Hall A (1995). Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem Soc Trans* **23,** 456–459.
[45] O'Connor KL and Mercurio AM (2001). Protein kinase A regulates Rac and is required for the growth factor–stimulated migration of carcinoma cells. *J Biol Chem* **276,** 47895–47900.