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The Ras-related Protein, Rap1A, Mediates Thrombin-stimulated, Integrin-dependent Glioblastoma Cell Proliferation and Tumor Growth*

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Background: Thrombin and RhoA are linked to cancer cell proliferation.
Results: Thrombin induces sustained RhoA-mediated Rap1 activation leading to integrin signaling and glioblastoma cell proliferation.
Conclusion: GPCRs that signal through RhoA can engage a Rap1/integrin pathway promoting proliferation.
Significance: Rap1 is regulated by endogenous GPCRs and is required for tumor cell growth.

Rap1 is a Ras family GTPase with a well documented role in ERK/MAP kinase signaling and integrin activation. Stimulation of the G-protein-coupled receptor PAR-1 with thrombin in human 1321N1 glioblastoma cells led to a robust increase in Rap1 activation. This response was sustained for up to 6 h and mediated through RhoA and phospholipase D (PLD). Thrombin treatment also induced a 5-fold increase in cell adhesion to fibronectin, which was blocked by down-regulating PLD or Rap1A or by treatment with a β_1 integrin neutralizing antibody. In addition, thrombin treatment led to increases in phospho-focal adhesion kinase (tyrosine 397), ERK1/2 phosphorylation and cell proliferation, which were significantly inhibited in cells treated with β_1 integrin antibody or Rap1A siRNA. To assess the role of Rap1A in tumor formation *in vivo*, we compared growth of 1321N1 cells stably expressing control, Rap1A or Rap1B shRNA in a mouse xenograft model. Deletion of Rap1A, but not of Rap1B, reduced tumor mass by >70% relative to control. Similar observations were made with U373MG glioblastoma cells in which Rap1A was down-regulated. Collectively, these findings implicate a Rap1A/β_1 integrin pathway, activated downstream of G-protein-coupled receptor stimulation and RhoA, in glioblastoma cell proliferation. Moreover, our data demonstrate a critical role for Rap1A in glioblastoma tumor growth *in vivo*.

Glioblastoma multiforme is the most prevalent and aggressive type of central nervous system tumor. The invasive nature of glioblastoma underscores the need to better understand the molecular mechanisms involved in glial tumor growth. G-protein-coupled receptors (GPCRs)[6] constitute the largest class of cell surface receptors, but the involvement of GPCR signaling in regulating glial tumor growth has not been explored extensively. Thrombin, a peptide hormone and GPCR ligand, is detected in cancer cells, including glioblastoma (1–5), and enhanced signaling through the PAR-1 thrombin receptor has been reported to cause cell transformation and to increase tumor cell invasiveness (6–8). One proposed transduction mechanism by which PAR-1 receptor stimulation leads to enhanced proliferation is through activation of the low molecular weight G-protein RhoA (9–11). During the past decade, the mechanism by which GPCRs induce RhoA activation has been elucidated. A subset of GPCRs, including the PAR-1 receptor and receptors for the lysophospholipids S1P and lysophosphatidic acid (LPA), couple to and signal through the $G_{12/13}$ family of heterotrimeric G-proteins (12–17). The $G\alpha_{12}$ protein was originally isolated as an oncogene (18) and more recently determined to bind to and regulate guanine nucleotide exchange factors (GEFs) for RhoA (19–22). Our laboratory has demonstrated that PAR-1 stimulation with thrombin induces mitogenesis in 1321N1 glioblastoma cells through activation of the heterotrimeric $G_{12/13}$ protein (12) and the low molecular weight small GTPase RhoA (13, 23).

PAR-1 activation in platelets has also been shown to activate another Ras family GTPase, Rap1 (24, 25). This response is mediated through phospholipase D and a Rap1 guanine nucleotide exchange factor CALDAG-GEF (26–28). Rap1 can interact with B-Raf, leading to activation of an ERK/MAPK signaling cascade (29, 30). In addition, Rap1 regulates integrins through actions initiated from inside the cell and can thus affect integrin-mediated cell adhesion and integrin signaling (31–33). Rap1 has been implicated in cancer cell proliferation and tumor cell growth based on studies using overexpression or genetic

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[6] The abbreviations used are: GPCR, G-protein-coupled receptor; FAK, focal adhesion kinase; LPA, lysophosphatidic acid; GEF, guanine nucleotide exchange factor; RBD, Rap1-binding domain; PAR-1, protease-activated receptor-1.

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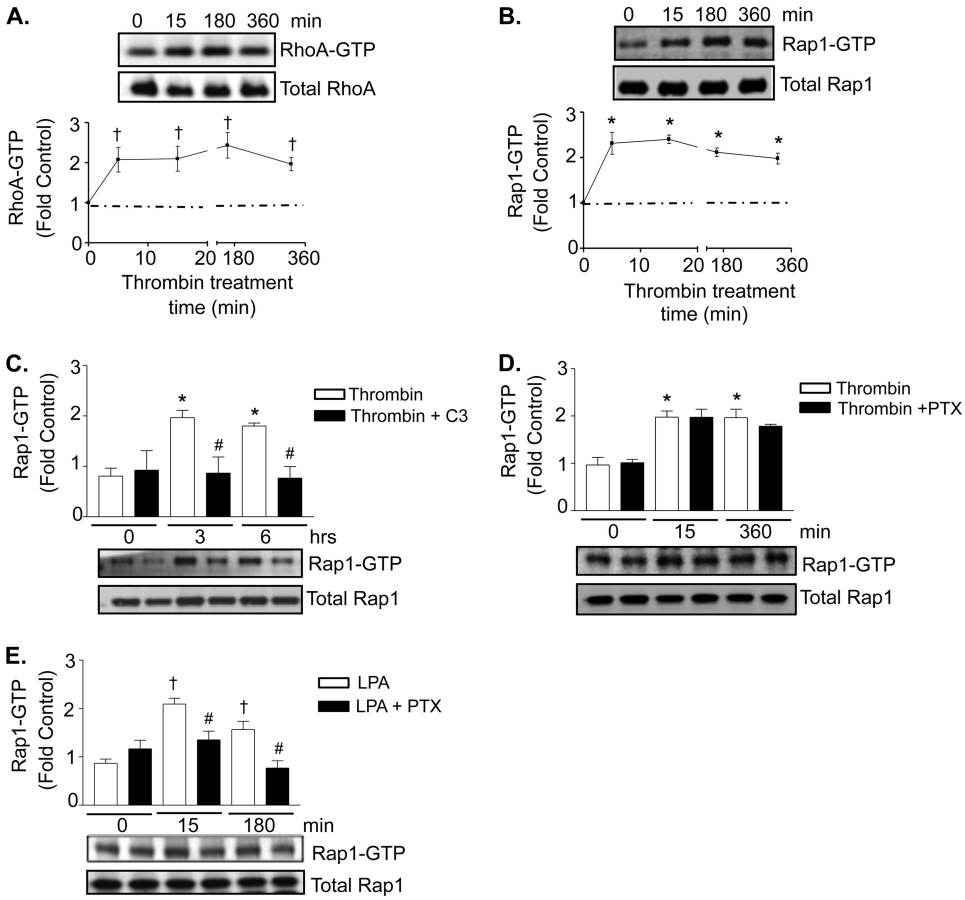

FIGURE 1. **Thrombin induces sustained Rap1 activation that is RhoA- but not G_i-dependent.** Cells (1321N1) were serum-starved for 24 h and subsequently treated with 0.5 units/ml thrombin for the indicated times. *A*, active RhoA was pulled down using the RhoA-binding domain of the RhoA effector rhotekin, and total RhoA was visualized using an anti-RhoA antibody. †, $p < 0.05$ *versus* time zero. *B*, active Rap1 was precipitated with a GST-fused Rap1 binding domain of RalGDS (RalGDS RBD)-agarose beads and probed with anti-Rap1 antibody. Total Rap1 was determined using an anti-Rap1 antibody. *, $p < 0.01$ *versus* time zero. Cells were pretreated with 1 μg/ml C3 for 4 h (*C*) or 100 ng/ml pertussis toxin (*PTX*) (*D*) overnight then treated with vehicle or thrombin for the indicated times, and GTP-bound Rap1 was assessed by a Rap1 pulldown assay. Whole-cell lysates were immunoblotted for total Rap1. Values are means ± S.E. *, $p < 0.01$ *versus* time zero control (*C* and *D*). #, $p < 0.05$ *versus* thrombin (*C*). *E*, cells were pretreated with 100 ng/ml pertussis toxin and subsequently stimulated with 10 μM LPA. †, $p < 0.05$ *versus* time zero control. #, $p < 0.01$ *versus* LPA. All data are means ± S.E. from four independent experiments.

deletion of molecules that regulate Rap1 activation (Rap1GEFs and GTPase-activating proteins) (26, 34–36). However, the question of how extracellular signals activate Rap1 in tumor cells and the role of GPCRs and integrins in regulating this process have not been explored.

We examined the possibility that Rap1 is regulated through PAR-1 activation in glioblastoma cells and that Rap1 serves as a downstream mediator of RhoA signaling. Our findings demonstrate that thrombin elicits a sustained increase in Rap1 activation, which is required for integrin signaling and proliferation of glioblastoma cells. Furthermore, we use shRNA to down-regulate and demonstrate a requirement for Rap1A in glioblastoma tumor growth *in vivo*. We suggest that GPCRs that signal through RhoA induce a signaling cascade leading to Rap1 and integrin activation and to enhanced glioblastoma cell growth.

EXPERIMENTAL PROCEDURES

Materials—C3 exoenzyme was purchased from Upstate Biotechnology (Charlottesville, VA). Pertussis toxin was purchased from Calbiochem (San Diego, CA). Human α thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). LPA was purchased from Avanti Polar Lipids (Avanti Polar Lipids (Alabaster, AL). 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) was a kind gift from Dr. Michael Frohman (Stony Brook University School of Medicine, Stony Brook, New York). The pGEX-2T vector encoding a GST fusion protein of the rhotekin, which is the RhoA-binding domain, was originally provided by M. Schwartz (Yale University, New Haven, CT). The pGEX-2T/GST-Ral-GDS-RBD, which is the Rap1-binding domain, was described previously (37). Control scrambled siRNA and ON-TARGETplus SMARTpool siRNA targeting Rap1A and phospholipase D (PLD1) were purchased from Dharmacon (Lafayette, CO). Rabbit antiserum against human Rap1B protein was raised against peptide sequence TPVPGKARKKSS conjugated to keyhole limpet hemocyanin (Quality Control Biochemicals (QCB), Hopkinton, MA). Anti-Rap1 and actin antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-p44/42 MAPK, p44/42 MAPK, and PLD1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phospho-Tyr^{397}-focal adhesion kinase (FAK) antibody was purchased from Invitrogen. Total FAK and Rho antibodies were purchased from BD Biosciences. For blocking studies, antibody to

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integrin β_1 was from Chemicon (Temecula, CA), and normal mouse IgG was from Santa Cruz Biotechnology.

Cell Culture—1321N1 cells, a subclone of human U118MG glioblastoma cells, were originally isolated from primary cultures of a human cerebral glioblastoma multiforme (38). The U373MG cell line provided by Frank Furnari (University of California at San Diego) was derived from a patient classified as having malignant anaplastic astrocytoma grade 111 glioblastoma (39). Both cell lines were maintained in DMEM containing 5% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine in a 37 °C, 10% CO_2 humidified environment. Cells were starved for at least 24 h before the start of experiments.

SDS-PAGE and Western Blotting—Cells were washed with two volumes of ice-cold PBS and lysed in buffer containing 50 mM Tris HCl (pH 7.6), 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 1 mM disodium 4-nitrophenylphosphate, 100 μM sodium orthovanadate, and 10 μg/ml leupeptin. Laemmli buffer (4×) was added to cleared protein lysates, and samples were boiled and separated by SDS-PAGE, and membranes were probed with primary antibodies. A 1:1000 dilution of primary antibody and a 1:4000 dilution of secondary IgG-horseradish peroxidase in 3% BSA in Tris-buffered saline containing 0.1% Tween 20 were used. Proteins were visualized using enhanced chemiluminescence and quantitated using gel documentation software (Alpha Innotech, Corp., San Leandro, CA).

RhoA and Rap1 Activation Assay—RhoA and Rap1 activation were assessed using affinity pulldown assays as described (40, 41). Cells were serum-starved for 24 h, treated with appropriate stimuli and then lysed. Cleared lysates were incubated with the Sepharose-bound GST-rhotekin-RhoA-binding domain or the GST-fused Rap1-binding domain of RalGDS (RalGDS-RBD) for 50 min at 4 °C and then centrifuged to pellet the agarose beads. Beads were washed, pellets were resuspended in 2× Laemmli sample buffer, and samples were boiled and resolved by SDS-PAGE.

siRNA Transfection of Glioblastoma Cells—Cells grown to 80% confluency were transfected with control scrambled siRNA or siRNA targeting Rap1A or PLD1 using DharmaFect 4 transfection reagent (Dharmacon, Lafayette, CO) per the manufacturer's instructions. A 20 μM concentration of SMARTpool (Dharmacon) siRNA targeting Rap1A or PLD1 or a non-targeting control siRNA was used, per the manufacturer's instructions. A single verified Rap1A siRNA (Qiagen) was also used to control for off-target silencing. The efficacy of Rap1 or PLD1 knockdown was assessed 48 h following transfection.

Cell Proliferation Assay—Glioblastoma cell proliferation was determined using the CyQUANT NF assay (Invitrogen), which is based on measurement of cellular DNA content via fluorescent dye binding. Briefly, cells were plated on 24-well plates, serum-starved for 24 h, and treated with appropriate agonist. Growth medium was removed from cells, 200 μl of 1× dye binding solution was added to wells, and plates were incubated at 37 °C for 40 min. The fluorescence intensity of each sample was measured using a fluorescence microplate reader

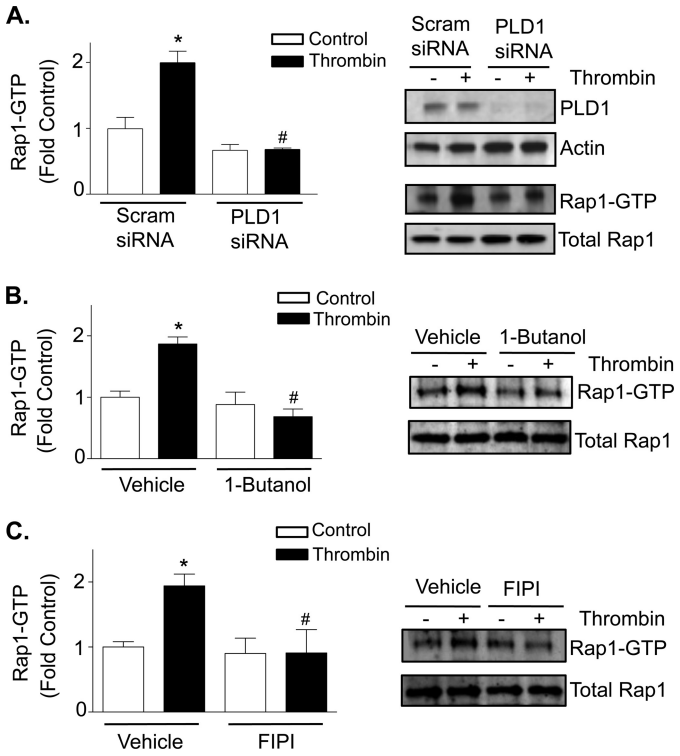

FIGURE 2. **Thrombin induces Rap1 activation through phospholipase D.** *A*, 1321N1 cells were transfected with either 4 μg control scrambled (*Scram*) or PLD1 siRNA. Cells were serum-starved for 24 h following transfection and then treated with 0.5 units/ml thrombin for 3 h. PLD1 expression and Rap1 activity were determined by Western blot analysis and a Rap1 pulldown assay, respectively. Representative blots are shown. Rap-GTP was normalized to total Rap; quantitated values are means ± S.E. averaged from four independent experiments. *, $p < 0.01$ *versus* untreated scrambled siRNA. #, $p < 0.01$ *versus* scrambled siRNA + thrombin. *B* and *C*, cells were pretreated with vehicle, 0.4% 1-butanol for 15 min or 1 μM FIPI for 4 h followed by treatment with 0.5 units/ml thrombin for 3 h. Active Rap1 was assessed via a Rap1 pulldown assay. Data are means ± S.E. *, $p < 0.01$ *versus* non-stimulated control. #, $p < 0.01$ *versus* thrombin + vehicle. Representative blots are shown.

(TECAN) with excitation at 485 nm and emission detection at 530 nm.

Cell Adhesion Assay—Forty-eight well plates were coated with 1.2 μg/ml fibronectin overnight at 4 °C. Plates were washed twice with PBS (pH 7.6) and blocked with a solution of 1% BSA/PBS (pH 7.6) for 1 h at 37 °C. 1321N1 cells (2.5×10^5 cells/ml, 200 μl/well) were added to plate and allowed to attach for 1 h at 37 °C. Unbound cells were aspirated, and cells remaining adherent were stained and fixed with 0.1% crystal violet (Sigma) in PBS (pH 7.6) and 20% methanol solution for 20 min. Unbound dye was removed by washing with water, and the plate was dried overnight. Cell-bound dye was reconstituted with 300 μl of 100% methanol and quantified by measuring absorbance at 600 nm.

shRNAs, Lentivirus Production, and Infection of 1321N1 and U373MG Glioblastoma Cells—The specificity of the Rap1A and non-targeting shRNA in pLKO vector (Sigma) was confirmed using Rap1 and Rap1b-specific antisera (42). The Rap1A shRNA sequence used for this study was 5′-GCAAAGTCAAAGATCAATGTT-3′ and the Rap1B shRNA sequence was 5′-GCACAACAGTGTATGCTTGAA-3′. Recombinant lentiviruses were generated by co-transfecting HEK293T cells with pCMVΔ8.2vpr (43), pCMV-VSV-G, and the shRNA/pLKO

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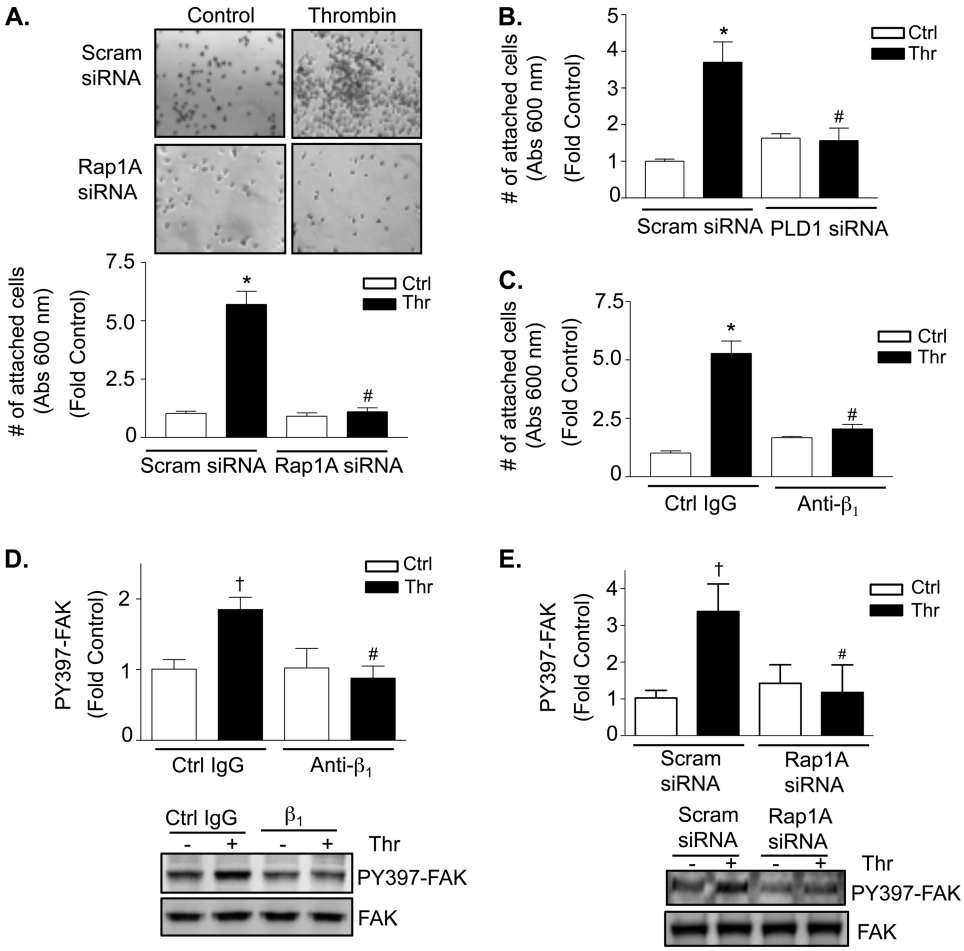

FIGURE 3. **Thrombin induces cell adhesion and FAK phosphorylation through Rap1A and β_1 integrin.** Adhesion of 1321N1 cells to fibronectin substrate was evaluated in cells transfected with either scrambled control (*Scram*), Rap1A (*A*), or PLD1 siRNA (*B*) or in cells pretreated for 30 min with 5 μg/ml of anti-integrin β_1 antibody or IgG control (*C*) and subsequently treated with 0.5 units/ml thrombin for 3 h. Data are means ± S.E. averaged from three separate experiments performed in triplicate. *, $p < 0.01$ *versus* non-stimulated scrambled siRNA or non-stimulated control IgG. #, $p < 0.01$ *versus* scrambled siRNA + thrombin or control IgG + thrombin (*Thr*). Cells pretreated with 5 μg/ml of IgG or anti-integrin β_1 antibody (*D*) or transfected with scrambled or Rap1A siRNA (*E*) were stimulated with thrombin for 3 h and FAK tyrosine 397 phosphorylation was determined by immunoblotting with phospho-Tyr^{397}-FAK antibody. Values are means ± S.E. from four separate experiments. †, $p < 0.05$ *versus* non-stimulated control (*Ctrl*) IgG (*D*) or scrambled siRNA (*E*); #, $p < 0.05$ *versus* thrombin.

vector employing methods established previously (44). Virus was harvested 72 h post-transfection and 1321N1 or U373MG cells were infected in the presence of 6 μg/ml polybrene (Sigma). Following infection, cells were selected with 5 μg/ml puromycin and cultured as above. Rap1A or Rap1B expression was measured in cells to identify the stable clone with the highest knockdown efficiency for the study.

In Vivo Growth of 1321N1 and U373MG Cells—Two million 1321N1 or U373MG cells treated with lentiviral control scrambled, Rap1A, or Rap1B lentiviral shRNA were suspended in 200 μl of 1:2 Matrigel in DMEM then injected subcutaneously in Nu/Nu mice. Tumor growth was monitored and tumor mass assessed 22 days post-implantation.

RT-PCR of Rap1—Total RNA was extracted from 1321N1 or U373MG cells infected with scrambled control shRNA or Rap1A shRNA using an RNeasy kit (Invitrogen), and cDNA was generated using the SuperScript II first-strand synthesis kit for RT-PCR (Invitrogen) according to the manufacturer's instructions. The sequences of the PCR primers for Rap1A were as follows: forward, 5′-GCGTTGGGAAGTCTGCTCTGGTAA-3′; reverse, 5′-TGTCTCCCACATCCACTCTGCC-3′.

Statistical Analysis—Statistical significance was determined by either analysis of variance followed by the Tukey's post hoc test or a Student's *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Thrombin Treatment Leads to Sustained RhoA-dependent Rap1 Activation—Our laboratory has shown previously that thrombin induces $G_{12/13}$ and RhoA-dependent proliferation of human glioblastoma multiforme (1321N1) cells. We examined the temporal aspects of RhoA activation and observed that 5 nM (0.5 units/ml) thrombin induced an ~2-fold increase in activated RhoA, observed at as early as 5 min, which was sustained for at least 6 h (Fig. 1*A*). Thrombin also induced a concomitant 2.5-fold increase in Rap1 activation, also significant as early as 5 min and sustained for at least 6 h (Fig. 1*B*). The temporal relationship between these responses suggested their possible association. To ascertain whether RhoA signaling is required for Rap1 activation, 1321N1 cells were pretreated for 4 h with C3 exoenzyme to ribosylate and inactivate RhoA. Thrombin-stimulated Rap1 activation was fully abolished by C3 treatment (Fig.

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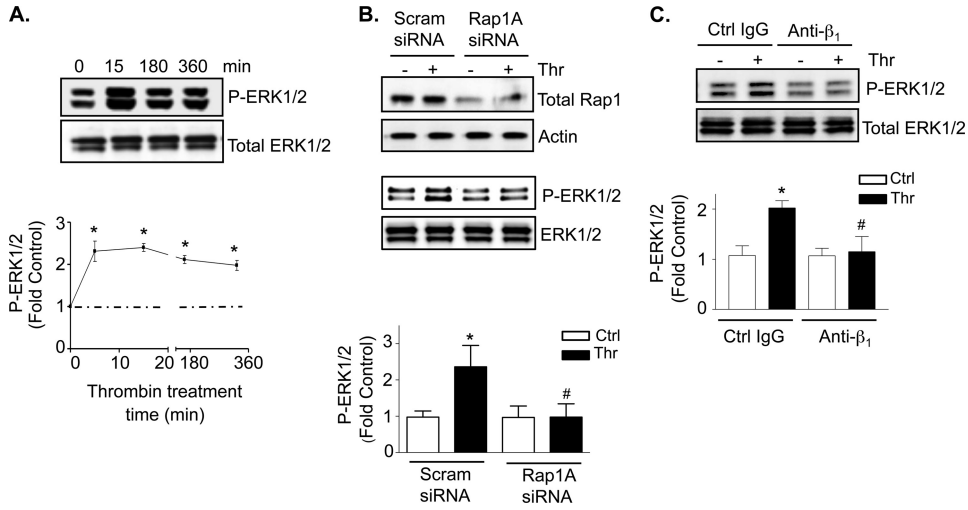

FIGURE 4. **Thrombin-stimulated ERK1/2 activation is mediated by Rap1A and β_1 integrin activation.** *A*, serum-starved 1321N1 cells were treated with vehicle or thrombin (*Thr*) for the indicated times. Phosphorylated ERK1/2 and total ERK1/2 were determined by immunoblotting with p-p44/42 MAPK or p44/42 antibody, respectively. P-ERK1/2 and total ERK1/2 levels were quantitated and normalized to total ERK1/2. Data are means ± S.E. of four separate experiments. *, $p < 0.01$ *versus* time zero. *B*, 1321N1 cells transfected with either control scrambled (*Scram*) or Rap1A siRNA were stimulated with 0.5 units/ml of thrombin for 3 h. Rap1 knockdown was determined by immunoblotting with Rap1 antibody, and actin served as a loading control (*Ctrl*). Lysates were immunoblotted for p-ERK1/2 or total ERK1/2 and p-ERK1/2 was normalized to total ERK1/2. Quantitated values were averaged from four independent experiments and presented as means ± S.E. *, $p < 0.01$ *versus* non-stimulated scrambled siRNA. #, $p < 0.01$ *versus* scrambled siRNA + thrombin. *C*, cells were pretreated for 30 min with 0.5 μg/ml of anti-integrin β_1 antibody and stimulated with thrombin for 3 h, and cell lysates were immunoblotted for p-ERK1/2 or total ERK1/2. Data are means ± S.E. from three independent experiments. *, $p < 0.01$ *versus* non-stimulated control IgG. #, $p < 0.01$ *versus* control IgG + thrombin. Representative blots are shown (*A–C*).

1*C*). Pertussis toxin, which inhibits G_i signaling, had no significant effect on thrombin-stimulated Rap1 activation (Fig. 1*D*) but was effective at blocking the more transient activation of Rap1 by LPA (Fig. 1*E*).

Thrombin Activates Rap1 through Phospholipase D—We then tested the hypothesis that PLD, which is regulated by RhoA (45–48), is a mediator of thrombin stimulated Rap1 activation. This was addressed using three distinct approaches. First, we demonstrated that when PLD1 was down-regulated with siRNA, thrombin-stimulated Rap1 activation was abolished (Fig. 2*A*). Next, we treated 1321N1 cells with 1-butanol to disrupt PLD signaling and demonstrated that this fully inhibited thrombin-induced Rap1 activation (Fig. 2*B*). Finally, blocking PLD activity using the pharmacological inhibitor FIPI abolished thrombin-induced Rap1 activation (Fig. 2*C*).

Thrombin-induced Rap1 Activation Enhances Cell Adhesion and Integrin Signaling—Because Rap1 mediates "inside-out" activation of integrins to promote cell adhesion (31, 49, 50), we asked whether thrombin increased 1321N1 cell adhesion through Rap1. Cells were plated on fibronectin for 1 h and then treated with vehicle or thrombin for 3 h. Thrombin treatment led to a 5-fold increase in cell adhesion to fibronectin (Fig. 3*A*). When Rap1A was down-regulated using SMARTpool siRNA targeting Rap1A (Fig. 3*A*), the thrombin-induced cell adhesion was abolished. This finding was confirmed using a verified Rap1A siRNA from a different source (data not shown). Knockdown of PLD1 with siRNA also abolished thrombin induced cell adhesion (Fig. 3*B*). To demonstrate that thrombin-induced cell adhesion to fibronectin was mediated by β_1 integrins, cells were treated with a β_1-neutralizing antibody for 20 min prior to thrombin stimulation. Thrombin failed to increase cell adhesion in cells treated with the β_1 integrin antibody compared with cells treated with control IgG (Fig. 3*C*).

Integrin ligation promotes phosphorylation of FAK, which can contribute to glioblastoma cell growth (51–54). Because our studies examining cell adhesion suggested that thrombin promotes integrin activation, we further assessed the ability of thrombin and Rap1 to elicit integrin signaling to FAK. As demonstrated in Fig. 3, *D* and *E*, thrombin treatment significantly increased FAK Tyr^{397}, and this response was blocked by the β_1 integrin-neutralizing antibody (Fig. 3*D*) and by down-regulating Rap1A with siRNA (Fig. 3*E*).

Sustained ERK1/2 activation is required for proliferation of many cell types. Thrombin stimulation led to a nearly 2.5-fold increase in ERK1/2 phosphorylation by 5 min, which remained at 2-fold control for at least 6 h (Fig. 4*A*). We assessed involvement of Rap1 and β_1 integrin in ERK1/2 phosphorylation at 3 h. of thrombin treatment. Down-regulation of Rap1A (Fig. 4*B*) or treatment with β_1-neutralizing antibody (Fig. 4*C*) prevented sustained ERK1/2 activation, implicating Rap1-mediated integrin signaling in this response.

Thrombin Induces Proliferation through Rap1A and β_1 Integrin—We postulated that activation of Rap1 and the integrin-signaling pathway delineated above play a role in thrombin-stimulated 1321N1 cell proliferation. Indeed, when Rap1A was down-regulated by a SMARTpool siRNA targeting Rap1A, thrombin-induced cell proliferation was fully inhibited (Fig. 5*A*). In a separate set of experiments using a different source of siRNA targeting Rap1A, we confirmed that thrombin stimulated cell proliferation was blocked (data not shown). Notably, proliferation of 1321N1 cells in response to LPA, which induced more transient G_i-mediated Rap1 activation (Fig. 1*E*) was Rap1A-independent (Fig. 5*B*). Treatment with β_1-neutralizing antibody also significantly inhibited thrombin-induced 1321N1 cell proliferation (Fig. 5*C*). Blocking ERK1/2 activation with the MEK inhibitor U0126 inhibited thrombin-stimulated

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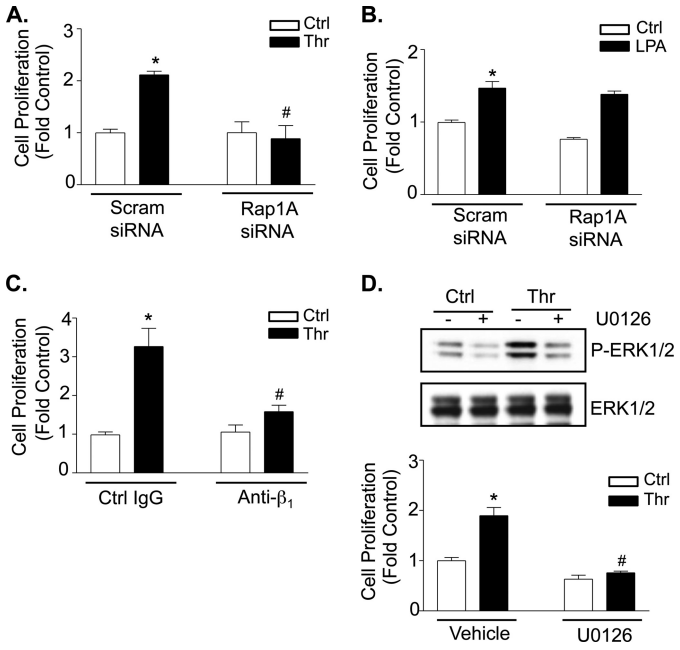

FIGURE 5. **Thrombin-stimulated 1321N1 cell proliferation is Rap1A-, β_1 integrin-, and ERK1/2-mediated.** 1321N1 cells were transfected with Rap1A siRNA (*A* and *B*) or pretreated for 30 min with 5 μg/ml anti-integrin β_1 antibody (*C*) or 10 μM U0126 (*D*) and subsequently stimulated with thrombin (*Thr*; *A*, *C*, and *D*) or 10 μM LPA (*B*) for 24 h. Cell proliferation was assessed by Cyquant NF (Invitrogen) as described under "Experimental Procedures." Inhibition of ERK1/2 phosphorylation by U0126 was determined by immunoblotting with anti-phospho-ERK1/2 antibody. Data are means ± S.E. from four independent experiments performed in triplicate. *, $p < 0.01$ *versus* non-stimulated scrambled (*Scram*) siRNA (*A* and *B*), control (*Ctrl*) IgG (*C*), or vehicle control (*D*). #, $p < 0.01$ *versus* thrombin treatment.

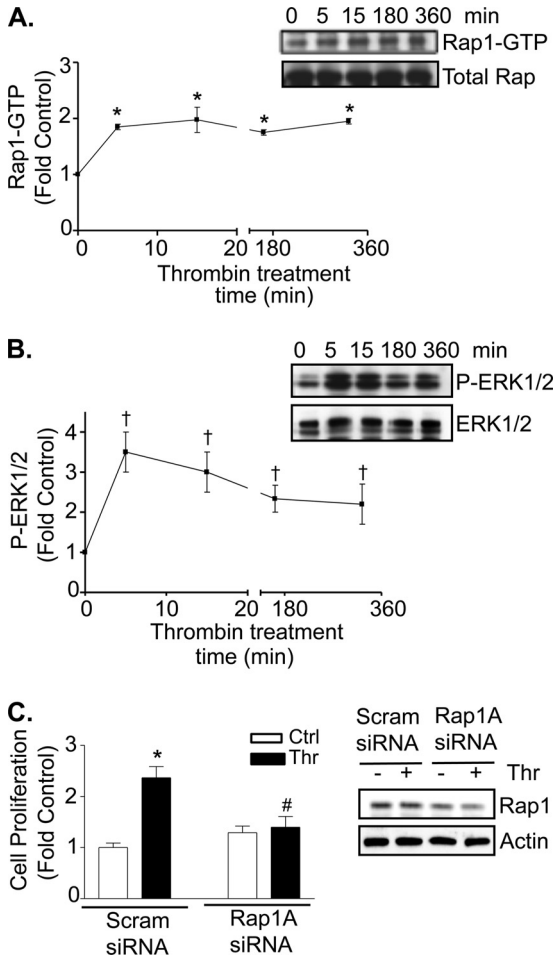

FIGURE 6. **Thrombin also stimulates sustained Rap1 and ERK1/2 activation and Rap1-dependent proliferation in U373MG glioblastoma cells.** U373MG cells were treated with vehicle or 0.5 units/ml thrombin for the indicated times, and Rap1 pulldown assays were performed (*A*), or whole cell lysates were immunoblotted for ERK1/2 phosphorylation (*B*). Representative immunoblots are means ± S.E. from four independent experiments. *, $p < 0.01$ *versus* time zero (*A*). †, $p < 0.05$ *versus* time zero (*B*). U373MG cells transfected with either control (*Ctrl*) scrambled (*Scram*) or Rap1A siRNA were treated with 0.5 units/ml thrombin (*Thr*) for 24 h, and cell proliferation was determined by Cyquant assay (*C*). Data are means ± S.E. from three experiments performed in triplicate. Values are means ± S.E. *, $p < 0.01$ *versus* non-stimulated scrambled siRNA. #, $p < 0.05$ *versus* scrambled siRNA + thrombin. A representative immunoblot of Rap1 knockdown is shown.

ERK1/2 phosphorylation and prevented thrombin induced 1321N1 cell proliferation (Fig. 5*D*). Taken together, these findings suggest that thrombin induces glioblastoma cell proliferation through its ability to activate RhoA and subsequent Rap1-mediated integrin-dependent FAK and ERK1/2 activation.

Thrombin-induced Rap1 Activation and Signaling in U373 MG Cells—We then extended our studies to an additional glioblastoma cell line. Thrombin treatment of U373MG cells led to a 2-fold increase in Rap1 activation, which was also sustained for up to 6 h (Fig. 6*A*). This was accompanied by a sustained increase in ERK1/2 activation (Fig. 6*B*). Rap1A was required for thrombin-stimulated U373MG cell proliferation because this response was lost in cells in which Rap1 was down-regulated relative to cells expressing scrambled siRNA (Fig. 6*C*). Thus, Rap1 is critical for thrombin-induced cell proliferation in several glioblastoma cell lines.

Rap1A Is Required for Glioblastoma Tumor Growth—To test the role of Rap1 in glioblastoma tumor growth, shRNA to the Rap1A or Rap1B isoforms was introduced into glioblastoma cells by lentiviral transduction. Puromycin-resistant colonies were expanded and selected to obtain clones with Rap1A and Rap1B mRNA knockdown of >80%. We used a mouse xenograft model in which Nu/Nu mice were injected with cells expressing either control scrambled shRNA or Rap1A or Rap1B shRNA. Tumor mass was assessed 22 days following injection, and four mice per group were examined in each of three separate experiments. The average tumor mass in mice injected with 1321N1 cells expressing Rap1A shRNA was reduced by >70% compared with that in mice injected with cells expressing control scrambled shRNA (Fig. 7, *A* and *B*). The decrease in Rap1A mRNA was maintained in the xenograft as confirmed via PCR analysis of the resected tumors (Fig. 7*C*). To further establish the importance of Rap1A in glioblastoma tumor growth *in vivo*, we examined the growth of U373MG glioblastoma cells in which Rap1A expression was down-regulated through shRNA expression. Rap1A knockdown also had a profound effect on the growth of these cells, with tumor mass reduced by 90% relative to control (Fig. 7, *D* and *E*). The decrease in Rap1A mRNA was maintained in tumors (Fig. 7*F*). Remarkably, knockdown of Rap1B did not significantly alter 1321N1 tumor growth (Fig. 7, *G* and *H*), although Rap1B protein expression was significantly down-regulated in the resected tumors (Fig. 7*I*). We also confirmed that Rap1B expression was unaffected in cells expressing Rap1A shRNA (Fig. 7*I*). These

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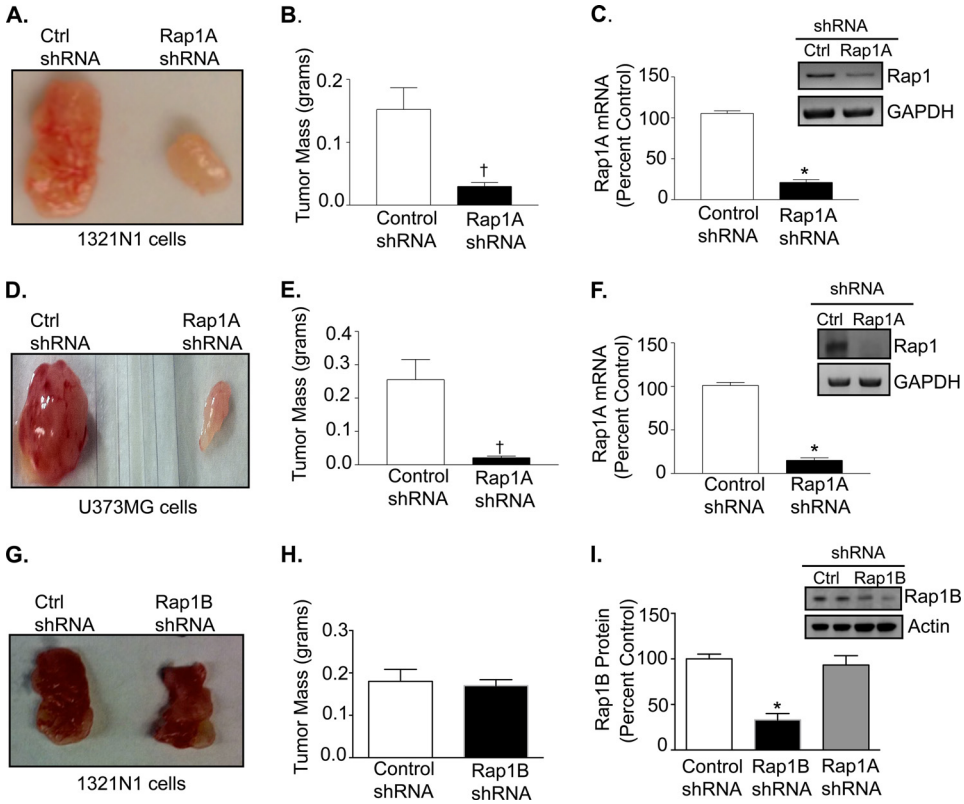

FIGURE 7. **Rap1A is necessary for glioblastoma cell growth *in vivo*.** 1321N1 (*A* and *G*) or U373MG (*D*) cells expressing control, Rap1A, or Rap1B shRNA were suspended in Matrigel and implanted into the flanks of Nu/Nu mice. Mice were sacrificed 22 days following implantation, and tumors were resected. Representative solid tumors are shown. Mean tumor mass from mice injected with 1321N1 (*B* and *H*) or U373MG (*E*) cells expressing Rap1A, Rap1B shRNA, or control (*Ctrl*) scrambled shRNA is expressed as mean ± S.E. averaged from three (*B* and *H*) or two (*E*) separate experiments with four mice per group. †, $p < 0.05$ *versus* control shRNA (Student's *t* test). Rap1A mRNA levels in resected tumors (*C* and *F*) were determined by PCR analysis. Rap1B protein expression levels in resected tumors (*I*) were determined by immunoblotting with a Rap1B antibody. Data in *C* and *F* and *I* are means ± S.E. ($n = 4$). *, $p < 0.01$ *versus* control shRNA (Student's *t* test).

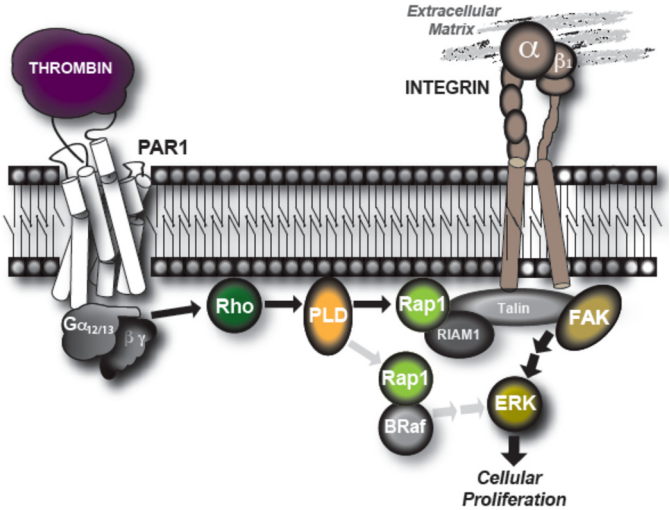

FIGURE 8. **A proposed mechanism for thrombin-stimulated glioblastoma cell proliferation.** Stimulation of $G_{12/13}$-coupled PAR1 receptor with thrombin results in sustained Rap1 activation through RhoA and PLD. Active Rap1-induces formation of a complex containing RIAM and talin, resulting in talin recruitment to integrins and consequent inside-out β_1 integrin activation, which stimulates integrin signaling to activate FAK and ERK1/2. Rap1 also interacts with B-Raf to activate ERK signaling, through an already established pathway (*light arrows*). The downstream result of this pathway is enhanced glioblastoma cell proliferation.

findings demonstrate that Rap1A plays a critical role in glioblastoma tumor growth *in vivo* and that Rap1B does not subserve a redundant function.

The model presented in Fig. 8 summarizes our findings and hypothesis. Thrombin induces sustained Rap1 activation through RhoA and PLD1. Rap1 enhances β_1 integrin activation and signaling through FAK and ERK. The repertoire and duration of GPCR stimulation is extended through involvement of RhoA, Rap1, and integrin signaling pathways, which can contribute to glioblastoma cell proliferation and tumorigenesis.

DISCUSSION

We and others have shown that thrombin signaling through the PAR-1 receptor and the small G-protein RhoA contributes to cancer cell proliferation (9, 13, 55, 56). Among the best described effects of RhoA on cell growth is the ability to facilitate cell cycle progression by inhibiting expression of the cyclin-dependent kinase regulators p21(Cip1) and p27(Kip1) (57–59). RhoA can also enhance cell proliferation through transcriptional regulation of genes, including the matricellular protein CCN1 (56, 60). Here, we demonstrate that activation of RhoA also initiates growth signals through Rap1 and integrin signaling and that this signaling cascade can be regulated by stimulation of the PAR-1 receptor with thrombin.

Similar to the PAR1 receptor, the G-protein-coupled receptor for LPA has been extensively associated with tumor cell growth (61), and concentrations of both thrombin and LPA are elevated in the tumor cell environment (62, 63). Notably, however, LPA elicits only transient Rap1 activation, which is mediated through G_i and Rap1 and is not required for LPA-induced

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mitogenesis. Thus, the pathway we describe is one that would appear to be utilized by the subset of mitogenic GPCRs that predominantly signal through $G_{12/13}$ and RhoA, rather than the canonical G_i/Ras pathway.

We showed previously that thrombin induces the expression and secretion of the matricellular protein CCN1, which signals and mediates cell proliferation through its effects on β_1 integrin (56). This led us to consider the possibility that there could be a coordinate inside-out activation of integrins through thrombin signaling to Rap1. Although the role of Rap1 in mediating inside-out activation of integrins via a RIAM1 and talin-dependent mechanism has been well documented, there is limited evidence that GPCR agonists acting at endogenous cell surface receptors initiate Rap1-mediated integrin activation (31, 49, 50). Here, we demonstrate that thrombin treatment leads to integrin activation, as assessed by increased 1321N1 cell adhesion to fibronectin and that this is blocked by siRNA-mediated knockdown of Rap1A and its upstream activator PLD. Concomitantly, knockdown of Rap1A prevents thrombin induced FAK phosphorylation and sustained ERK activation, both downstream effects of β_1 integrin signaling. Finally, Rap1A knockdown and β_1 integrin neutralization significantly reduce thrombin-induced 1321N1 cell proliferation. Thus, our studies provide compelling evidence that the ability of thrombin to activate Rap1 provides an inside-out signal that promotes integrin activation and subsequent signaling from the extracellular matrix to regulate the growth of glioblastoma cells.

Dysregulation of RhoA and Rap signaling in cancer has been largely associated with alterations or mutations in their regulatory proteins, the GEFs, or GTPase-activating proteins (21, 26, 34, 64, 65). There is considerable evidence that altered expression of RapGEFs or GTPase-activating proteins affects cell proliferation and *in vivo* tumor growth (26, 34, 35, 66, 67). An earlier paper also demonstrated that overexpression of Rap1B in Swiss 3T3 cells was sufficient to enhance proliferation and tumorigenicity (68). Although these studies clearly demonstrate that elevated Rap1 activation can lead to enhanced cell growth, whether increased proliferation also results from receptor-mediated extracellular signals that stimulate Rap1 activation is less clear. Indeed, a recent study examining EGF-induced Rap1 activation demonstrated a requirement for Rap1 in pancreatic and prostate cancer cell metastasis and angiogenesis but not in the growth properties of these tumor cells (69). Our study is, to our knowledge, the first to use knockdown of Rap1A to demonstrate that this small G-protein is required for tumor cell growth *in vivo*.

All of our *in vitro* studies examined the effects of siRNA-mediated knockdown of the Rap1A isoform to inhibit downstream responses. Our studies using the *in vivo* mouse xenograft model went on to compare the role of the Rap1A and Rap1B isoforms. Rap1A and Rap1B isoforms were both effectively down-regulated by lentiviral-mediated shRNA expression, and the knockdown was retained over several weeks of tumor cell growth. Notably, whereas Rap1A knockdown abolished *in vivo* tumor cell growth, blunting Rap1B expression had no significant effect. In addition, Rap1B was not decreased in the cells in which Rap1A was down-regulated; thus, its expression clearly did not compensate for the loss of Rap1A in supporting tumor cell growth. Rap1A and Rap1B are 95% homologous, but they have been reported to have distinct biological functions as evidenced by phenotypic differences in Rap1A and Rap1B knock-out cells and mice (70, 71). Differences in subcellular localization of Rap1A and Rap1B (70) or in their mechanisms of activation may underlie their distinct functions and roles in glioblastoma cell proliferation. We suggest that thrombin and other endogenous GPCR agonists act *in vivo* to activate GEFs specific for, and/or localized to the same subcellular compartment as Rap1A and that GPCR signaling to Rap1A, by virtue of its mechanism, localization, or kinetics, is uniquely linked to regulating tumor cell growth.

In conclusion, our studies implicate integrin activation and signaling downstream of Rap1A as critical players in glioblastoma cell proliferation. A further implication of our work is that enhanced GPCR signaling effected through either genetic changes or via elevated levels of GPCR ligands such as thrombin could lead to aberrant cell growth through sustained Rap1 activation.

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