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Role of Synaptojanin 2 in Glioma Cell Migration and Invasion

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ABSTRACT

The small GTPase Rac1 is thought to play an important role in cell migration and invasion. We have previously identified synaptojanin 2, a phosphoinositide phosphatase, as an effector of Rac1. Here, we show that small interfering RNA-mediated depletion of either Rac1 or synaptojanin 2 inhibits invasion of SNB19 and U87MG glioblastoma cells through Matrigel and rat brain slices. Depletion of Rac1 or synaptojanin 2 also inhibits migration of SNB19 and U87MG cells on glioma-derived extracellular matrix. In addition, we found that depletion of Rac1 or synaptojanin 2 inhibits the formation of lamellipodia and invadopodia, specialized membrane structures that are thought to be involved in extracellular matrix degradation. These results suggest that synaptojanin 2 contributes to the role of Rac1 in cell invasion and migration by regulating the formation of invadopodia and lamellipodia. This study also identifies synaptojanin 2 as a novel potential target for therapeutic intervention in malignant tumors.

INTRODUCTION

Synaptojanins are a family of phosphatidylinositol (PI) phosphatases that contain two distinct phosphatase domains that are arranged in tandem, a *Sac*I homology domain that acts on PI-3-P, PI-4-P, and PI-3,5-P_2 and an inositol 5′-phosphatase domain, acting both on PI-4,5-P_2 and PI-3,4,5-P_3 (1). There are two synaptojanins in the human genome. The major splice isoform of synaptojanin 1 is brain specific (2), whereas synaptojanin 2 is expressed in a wide range of tissues (3).

The synaptojanins play key roles in clathrin-mediated endocytosis. Synaptojanin 1 appears to specifically function in the decoating of synaptic vesicles (4, 5), which is necessary for efficient synaptic vesicle recycling. In contrast, we have found a critical role for synaptojanin 2 in the formation of clathrin-coated pits (6). It is possible, however, that synaptojanin 2 also functions in subsequent steps of clathrin-mediated endocytosis, such as vesicle decoating.

Synaptojanin 2 specifically binds to the small GTPase Rac1 (7), whereas synaptojanin 1 does not (8). Rac1 is a member of the Rho family of small GTPases. Rho GTPases essentially function as switches; they are "on" in the GTP-bound and "off" in the GDP-bound state. In the active state, these GTPases relay signals from growth factors and cytokines to numerous effector proteins. Rho GTPases are activated by guanine nucleotide exchange factors and inactivated by GTPase-activating proteins (9). Rho GTPases regulate a large number of cellular functions ranging from actin cytoskeletal organization and cell adhesion to the control of mitogenesis and cell survival (10, 11).

Importantly, work from several laboratories has indicated important roles for the Rho family members Rac, Cdc42, and Rho in cell transformation, tumor cell invasion, and metastasis (12–15). Rho GTPases have also been implicated in the formation of invadopodia (16), which appear to be the main cellular structures that are responsible for the degradation of extracellular matrix by tumor cells (17, 18).

Synaptojanin 2 binds to Rac1 in a GTP-dependent fashion, suggesting that it mediates Rac1-regulated functions (7). Here, we report on the role of synaptojanin 2 in tumor cell invasion. We have focused on the invasive properties of glioblastoma cells (19). We have shown recently a critical role for Rac1 in glioblastoma cell invasion *in vitro*.[5] In addition, Rac1 protein expression has been positively associated with histologic grading of gliomas (20). To analyze the functions of synaptojanin 2 in glioma cell migration and invasion, we used RNA interference (21) to specifically inhibit synaptojanin 2 expression. We show that RNA interference-mediated depletion of synaptojanin 2 strongly inhibits glioma cell invasion and migration. We also show that depletion of either Rac1 or synaptojanin 2 inhibits the formation of invadopodia and lamellipodia. These results suggest that synaptojanin 2 mediates Rac1-regulated cell invasion and migration by regulating invadopodia and lamellipodia formation.

MATERIALS AND METHODS

Small Interfering RNA Preparation. Small interfering RNA oligonucleotides specific for Rac1, synaptojanin 2, and GL2 luciferase were designed according to Elbashir *et al.* (22) and purchased from Dharmacon (Lafayette, CO). The small interfering RNA sequences used are: Rac1 (coding region 439–459; 5′AAGGAGATTGGTGCTGTAAAA), SJ2–1 (coding region 1612–1633; 5′AACGTGAACGGAGGAAAGCAG), SJ2–2 (region 5419–5440 in the 3′ untranslated region; 5′CTCTTGCTGATACGCGATATT), and GL2 luciferase (coding region 153–173; 5′ AACGTACGCGGAATACTTCGATT). Small interfering RNA duplex formation was performed according to the manufacturer's instructions.

Cell Culture and Small Interfering RNA Transfections. Cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and penicillin/streptomycin. Transient transfection of small interfering RNA was carried out using either Oligofectamine (Invitrogen, Carlsbad, CA) or Lipofectamine 2000 (Invitrogen). Cells were plated in a 24-well plate at 1.2×10^5 cells/well for Oligofectamine and 1.7×10^5 cells/well for Lipofectamine 2000 transfections in 0.5 mL of DMEM, supplemented with 10% serum but without antibiotics. Transfections were carried out as soon as the cells were fully spread, using the protocol provided by the manufacturer. Synaptojanin 2-directed small interfering RNA was transfected at 100 nmol/L; Rac1-directed small interfering RNA was transfected at 20 nmol/L. Synaptojanin 2 expression was determined by Western blot analysis using polyclonal antibodies raised against the COOH-terminal domain of human synaptojanin 2 (7). Rac and tubulin expression levels were determined using monoclonal antibodies against Rac and β3-tubulin (Upstate Biotechnology, Lake Placid, NY). Using quantitative PCR, we verified that Rac1 small interfering RNA inhibits the expression of Rac1, but not Rac3, the Rho family member that is most similar to Rac1 (23). Maximum inhibition was achieved by day 2 to 3 after transfection, and cells were assayed at day 3 or 4 post-transfection.

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Note: Y-y. Chuang, N. L. Tran, and N. Rusk contributed equally to this study. Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

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[5] A. Chan *et al.*, Role of Rac proteins in human tumor cell invasion, manuscript in preparation.

Immunofluorescence. Cells were plated on either gelatin- or laminin-coated coverslips (human placental laminin, Sigma-Aldrich, St. Louis, MO). Subsequently, cells were washed with PBS, fixed in 4% formaldehyde/PBS, permeabilized with 0.1% Triton X-100 dissolved in PBS, followed by FITC-conjugated phalloidin (Sigma-Aldrich) staining or indirect immunofluorescence using a mouse monoclonal anticortactin antibody (clone 4F11, Upstate Biotechnology) or rabbit polyclonal antibodies that we generated against human synaptojanin 2 (7). Images were collected using an IX70 Olympus (Melville, NY) inverted microscope equipped with a ×60 (1.4 numerical aperture) objective, an Orca II cooled charge coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ), and ESee (Inovision, Raleigh, NC) image analysis software.

Cell Migration Assay. Cells were plated on glioma-derived matrix and assayed for migration as described previously (24, 25). In brief, glioma-derived matrix was obtained by growing SF-767 cells on 10-well slides (CSM, Phoenix, AZ). The migration assay uses a cell sedimentation manifold (CSM) to establish a confluent monolayer 1 mm in diameter. After removal of the manifold, cells were allowed to migrate for 24 hours. The average migration rate was calculated as the increasing radius of the entire cell population over time.

Matrigel Invasion Assay. Invasion was assayed using Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA). Two days after transfection with small interfering RNA, cells were placed in the top chamber in whole medium (DMEM and 10% fetal calf serum); 4×10^4 cells for SNB19, U87MG, and BT549; and 2×10^4 cells for NCI-H23. Whole medium (500 μL) was added to the bottom chamber. After 24 hours of incubation at 37°C, cells on the top surface of the filter were wiped off with a Q-tip, and the filter was fixed in 4% formaldehyde/PBS. After staining with Crystal Violet, all of the cells on the bottom of the chamber were counted using an IX70 Olympus inverted microscope.

Brain Slice Invasion Assay. The *ex vivo* invasion assay on rat brain slices was carried out as described previously (26). Briefly, 400-μm-thick sections were prepared from Wister rat brain cerebrum. Approximately 1×10^6 glioma cells stably expressing green fluorescence protein (GFP; provided by Dr. Joseph Loftus, Mayo Clinic Scottsdale, Scottsdale, AZ) were gently placed in a pinhole (0.5-μl transfer volume), created with a micropipette on the putamen of the brain slice. Imaging of specimens was performed at ×10 magnification using a Macro-Fluorescent Imaging System (SZX12-RFL3, Olympus) equipped with a GFP barrier filter (DP50, Olympus) at 0, 24, and 48 hours after seeding the cells. Images were processed using Adobe Photoshop.

Glioma cell invasion into the rat brain slices was quantitated using a LSM 5 Pascal Laser scanning confocal microscope (Zeiss, Thornwood, NY) to observe GFP-labeled cells on the tissue insert with the micropore filter membrane. Serial sections were obtained every 5 μm downward from the surface plane to the bottom of the slice. The invasion rate was calculated as described previously (27). In brief, for each focal plane, the area of fluorescent cells was calculated and plotted as a function of the distance from the top surface. The extent of glioma cell invasion was calculated as the depth corresponding to half of the maximum area.

Quantification of Lamellipodia Formation. Digital images of phalloidin-stained cells were collected in a random fashion (see under Immunofluorescence). Lamellipodia were traced using ESee image analysis software (Inovision). For each cell, lamellipodia formation was quantified by calculating the total length of the lamellipodia, divided by the cell circumference.

Invadopodia Formation Assay. FITC-gelatin–coated coverslips were prepared as described previously (28) and modified for SNB19 cells. Briefly, FITC (Sigma-Aldrich) was added (final concentration of 0.2 mg/mL) to 300 bloom gelatin [Sigma-Aldrich; 20 mg/mL dissolved in 50 mmol/L $Na_2B_4O_7$/40 mmol/L NaCl (pH 9.3)] and incubated for 2 hours. The FITC-gelatin was dialyzed against PBS for 2 to 3 days at 37°C. A thin layer of FITC-conjugated gelatin was placed on coverslips and cross-linked with 0.8% glutaraldehyde for 10 minutes on ice. Cross-linking was continued at room temperature for another 30 minutes. Coverslips were rinsed with PBS, incubated with 5 mg/mL sodium borohydride for 3 minutes at room temperature, rinsed again with PBS and incubated with 70% EtOH for 10 minutes, and left dry for ≥15 minutes in the tissue culture hood. One hour before plating cells, coverslips were quenched with DMEM containing 10% fetal bovine serum at 37°C. Cells were cultured on FITC-gelatin-coated coverslips in DMEM without serum for 6 hours, for the synaptojanin 2 localization studies, or 18 hours, for quantifying invadopodia formation. Invadopodia formation was determined by counting the percentage of cells that had an associated area of matrix degradation.

RESULTS

To examine the role of synaptojanin 2 in glioma cell invasion, we inhibited the expression of synaptojanin 2 using transient transfection of small interfering RNA oligonucleotide duplexes in two different glioblastoma grade IV lines, SNB19 and U87MG. We first determined the effect of synaptojanin 2 depletion on the invasion of SNB19 and U87MG cells using a transwell Matrigel invasion assay (29). Small interfering RNA -mediated depletion of synaptojanin 2 by ~75% causes a very strong inhibition (>70%) in SNB19 cell invasion (Fig. 1, *A* and *C*). The level of inhibition is similar to that caused by depletion of Rac1 by ~80%. Similar results were obtained using two independent synaptojanin 2-directed small interfering RNA oligos, indicating that the inhibitory effect of the oligos is specific. Notably, the invasion assay was performed with serum present both in the top and bottom wells of the transwell chamber, implying that synaptojanin 2 depletion causes the inhibition of invasive behavior, rather than of chemotaxis. Similar results were obtained using U87MG cells (Fig. 1, *B* and *D*), indicating that synaptojanin 2 plays a critical role in glioblastoma cell invasion. We also found that depletion of synaptojanin 2 inhibited Matrigel invasion of two different carcinoma cell lines, NCI-H23 lung adenocarcinoma and BT549 ductal breast carcinoma (Supplementary Data, Fig. 1), indicating that

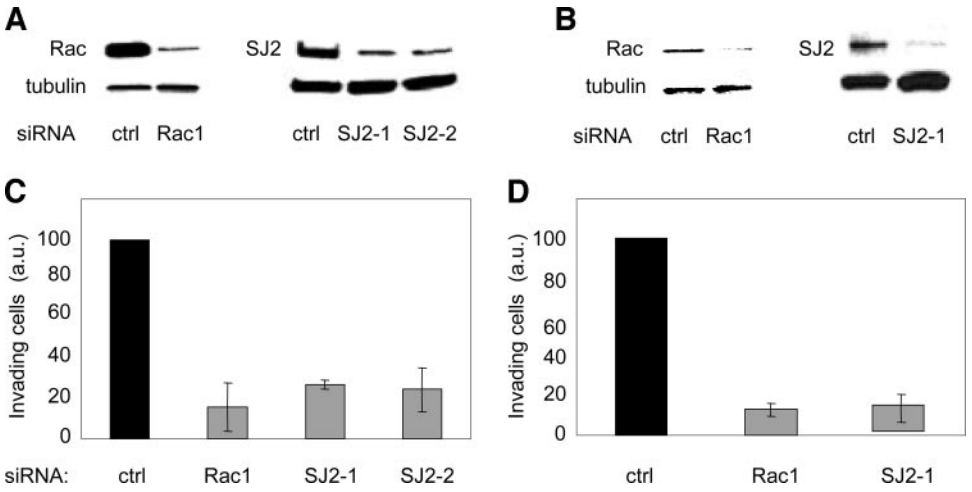

Fig. 1. Depletion of either synaptojanin 2 or Rac1 inhibits invasion of glioblastoma cells through Matrigel. *A* and *B*, Western blot analysis determining the protein expression of Rac and synaptojanin 2 after small interfering RNA transfection. SNB19 cells (*A*) and U87 cells (*B*) were transfected with 100 nmol/L of the indicated small interfering RNA oligos using Oligofectamine. Three days post-transfection, cell lysates were separated by SDS-PAGE and transferred onto PVDF membrane, and proteins were visualized using Rac antibody or synaptojanin 2 antiserum. Membranes were stained for tubulin to demonstrate equal loading. *C* and *D*, invasion of SNB19 (*C*) and U87 (*D*) cells through Matrigel. Two days after transfection with the indicated small interfering RNA oligos, cells were plated in transwell invasion chambers coated with Matrigel, and 24 hours later, cells that had migrated through the filter were stained and counted. Shown are the mean of at least two independent experiments; *bars,* ±SE. (PVDF, polyvinylidene difluoride)

synaptojanin 2 may play a role in the invasive behavior of a wide range of tumor cells.

To examine whether the roles of Rac1 and synaptojanin 2 in glioma cell invasion extend to conditions that more closely resemble the *in vivo* situation, we determined whether RNA interference-mediated depletion of Rac1 or synaptojanin 2 inhibits the invasion of GFP-expressing human glioma cells into rat brain slices, a well-established organotypic model for glioma invasion (30, 31) that we have modified recently (26). These brain slice cultures mimic the *in vivo* setting, and the extracellular matrix and architecture of the neural tissue can be preserved for several weeks. Invasion was quantified by confocal microscopy, as detailed in Materials and Methods. Depletion of either synaptojanin 2 or Rac1 causes an ~50% decrease in cell invasion, both in the SNB19 (Fig. 2, *A* and *B*) and U87MG cells (Fig. 2*B*).

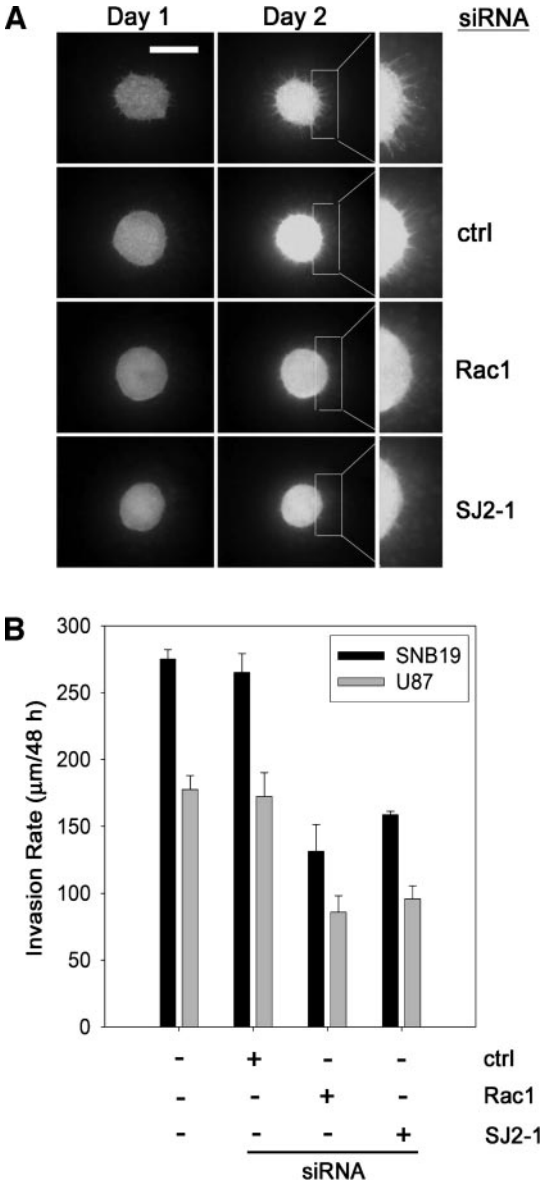

Fig. 2. Depletion of either synaptojanin 2 or Rac1 inhibits invasion of glioblastoma cells in rat brain slices. *A*, SNB19 glioma cells stably expressing GFP were transfected with small interfering RNAs directed against luciferase (control), Rac 1, or synaptojanin 2, as described in Fig. 1, 2 days before implantation into the bilateral putamen on rat organotypic brain slice and observed at the indicated time (*bar*, 500 μm). In *B*, invasion rates of SNB19 and U87MG cells treated with the indicated small interfering RNA constructs were calculated from *Z*-axis images collected by confocal laser scanning microscopy; bars, ±SE. The mean value of invasion rates was obtained from six independent experiments.

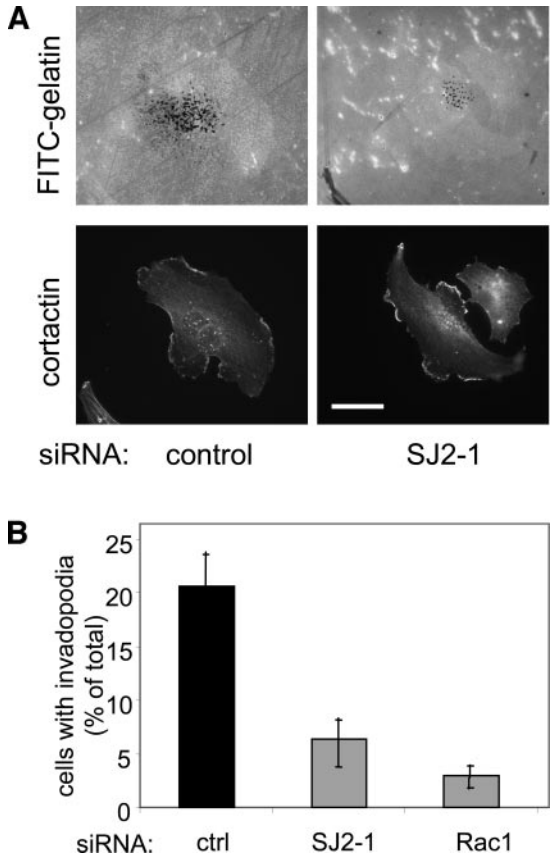

Fig. 3. Depletion of either synaptojanin 2 or Rac1 inhibits invadopodia formation. *A*, fluorescence micrographs of FITC-gelatin and cortactin immunofluorescence of SNB19 cells. Areas of extracellular matrix degradation show up as dark spots in the FITC-gelatin micrographs. Representative images are shown. *Bar*, 20 μm. *B*, quantification of invadopodia formation. SNB19 cells were transfected with the indicated small interfering RNAs 72 hours before plating on FITC-gelatin-coated coverslips for 6 (*A*) or 18 (*B*) hours. Invadopodia were quantified as described in Materials and Methods. For each condition, shown are the means of 24 fields comprising a total of ~100 cells; *bars,* ±SE. Data shown are representative of four independent experiments.

These results confirm that Rac1 and synaptojanin 2 play important roles in glioblastoma cell invasion.

Because invadopodia contribute to extracellular matrix degradation by tumor cells, we also examined whether synaptojanin 2 plays a role in the formation of these structures. We visualized invadopodia by culturing SNB19 cells on a thin layer of cross-linked fluorophore-conjugated gelatin matrix (28, 32). Areas of matrix degradation appear as dark holes in this fluorescent matrix (Fig. 3*A*). A subset of the degraded areas colocalizes with cortactin, an actin-binding protein that is known to localize to invadopodia (18, 33). Although ≤20% of control small interfering RNA-transfected cells make invadopodia, only ~5% of synaptojanin 2-depleted cells show invadopodia (Fig. 3*B*). Rac1 depletion also strongly inhibits invadopodia formation (Fig. 3*B*).

Rac proteins have been implicated in the regulation of cell migration (34, 35). Migration of glioma cells strongly depends on the type of extracellular matrix and is optimal on laminin or matrix derived from the SF767 glioma cell line (24). Using a microliter scale migration assay (25), we showed that depletion of synaptojanin 2 inhibits the migration of SNB19 and U87MG cells on glioma-derived matrix by >70%, which is similar to the level of inhibition caused by depletion of Rac1 (Fig. 4).

Rac1-stimulated cell migration is thought to be mediated, at least in part, by regulating the formation of lamellipodia (11, 36). Depletion of synaptojanin 2 significantly inhibits serum-induced lamellipodia for-

mation in SNB19 cells, although this inhibitory effect is not as strong as that caused by depletion of Rac1 (Fig. 5).

We have shown (Figs. 3 and 5) that synaptojanin 2 regulates the formation of two distinct cellular structures that are thought to play a role in cell migration and invasion, lamellipodia and invadopodia. To determine whether endogenous synaptojanin 2 localizes to these structures, we performed immunofluorescence using a polyclonal antibody that we described previously (7). SNB19 cells plated on laminin-coated coverslips display wide and flat lamellipodia, as visualized by fluorescent phalloidin. These lamellipodia are enriched in synaptojanin 2 (Fig. 6). Synaptojanin 2 is also significantly enriched in invadopodia, visualized by areas of gelatin degradation (Fig. 6).

DISCUSSION

In this study, we demonstrate a novel role for synaptojanin 2 in glioma cell invasion. We show that RNA interference-mediated depletion of synaptojanin 2 strongly inhibits invasion of glioblastoma cells in both Matrigel and rat brain slices. We also show that synaptojanin 2 depletion inhibits the formation of lamellipodia and invadopodia, two cellular structures that are thought to play an important role in tumor cell invasion.

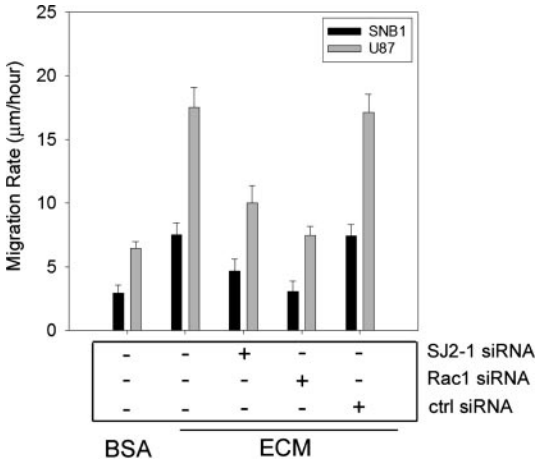

Fig. 4. Depletion of either synaptojanin 2 or Rac1 inhibits glioblastoma cell migration on glioblastoma-derived matrix. SNB-19 and U87MG cells were transfected with small interfering RNA oligos as described in Fig. 1. Two days after transfection, cells were plated on glioma-derived matrix and assayed for migration using a cell sedimentation manifold as described in Materials and Methods. Cells were allowed to migrate for 24 hours. The average migration rate of five replicates was calculated as the increasing radius of the entire cell population over 24 hours. BSA-coated slides were used as control substrate; bars, ±SE. (BSA, bovine serum albumin)

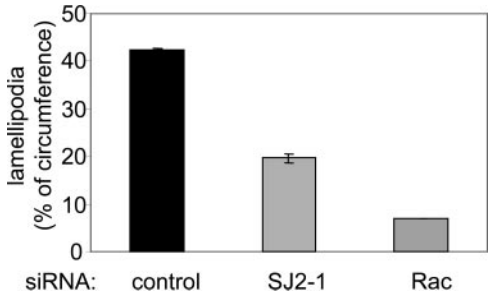

Fig. 5. Depletion of either synaptojanin 2 or Rac1 inhibits lamellipodia formation. SNB19 cells were transfected with the indicated small interfering RNA oligos and 24 hours later plated on glass coverslips coated with laminin. After 30 hours, the cells were serum starved for 18 hours and subsequently stimulated with 2% FBS. Filamentous actin was visualized with fluorescent phalloidin. Lamellipodia were quantified as the percentage of the total cell periphery as described in Materials and Methods. Data are the mean of two independent experiments; *bars,* ±SE. For each experiment, 10 cells were quantified. (FBS, fetal bovine serum)

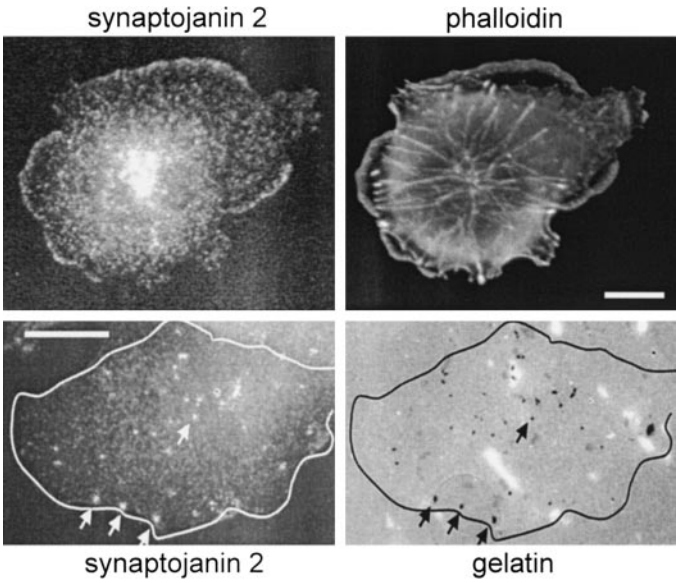

Fig. 6. Synaptojanin 2 localizes to lamellipodia and invadopodia. *Top*, fluorescence micrographs showing localization of endogenous synaptojanin 2 to lamellipodia, visualized by phalloidin staining. *Bottom*, fluorescence micrographs showing localization of endogenous synaptojanin 2 to invadopodia, visualized by dark areas in FITC-conjugated gelatin. SNB19 cells were plated on laminin-coated coverslips for 16 hours (*top*) or on FITC-gelatin-coated coverslips for 6 hours (*bottom*). Cells were fixed and processed for immunofluorescence as described in Materials and Methods. *Bars*, 10 μm.

The molecular mechanisms that underlie the roles of synaptojanin 2 in the formation of lamellipodia and invadopodia remain to be elucidated. The role of vesicular trafficking in cell migration has been hotly contested for a long time (37, 38). One possible mechanism that we envisaged for the role of synaptojanin 2 in cell migration is that synaptojanin 2 may regulate the internalization and recycling of cell adhesion molecules to support membrane protrusion. However, we found that RNA interference-mediated depletion of α-adaptin, an essential component of the AP-2 clathrin adaptor complex (39), did not have any effect on the migratory or invasive properties of SNB-19 cells, although it inhibited clathrin-mediated internalization of both transferrin and epidermal growth factor receptors in these cells.[6]

We propose that a more likely mechanism that mediates the functions of synaptojanin 2 in cell migration and invasion is its control of actin dynamics. PI metabolism is thought to modulate the activities of numerous actin-regulatory proteins, including cofilin and cortactin, proteins that have been implicated in actin filament nucleation and actin filament branching, respectively (40, 41). Both proteins bind to and are inhibited by PI-4,5-P_2 (42, 43), and synaptojanin 2-mediated PI-4,5-P_2 hydrolysis may therefore stimulate their activity.

We showed previously that a recombinant version of synaptojanin 2 directly binds to recombinant Rac1 in a GTP-dependent fashion (7). Thus, synaptojanin 2 appears to be a bona fide effector of Rac1. Moreover, here, we have identified several functions that are similarly affected by depletion of either Rac1 or synaptojanin 2, cell migration and invasion, and the formation of lamellipodia and invadopodia. Therefore, these findings strongly suggest that synaptojanin 2 functions downstream of Rac1 to mediate these functions. We found that synaptojanin 2 also binds to Rac3[7] and thus may also act downstream of this GTPase. The specific functions of Rac3 in cell migration and invasion remain to be clarified, however.

[6] N. Rusk and M. Symons, unpublished observations.
[7] A. Chan *et al.,* unpublished observations.

In summary, we have identified a critical function for synaptojanin 2 in glioma cell migration and invasion. Our findings also indicate that these novel functions of synaptojanin 2 may be mediated by the roles of synaptojanin 2 in the formation of lamellipodia and invadopodia. Furthermore, our results suggest that synaptojanin 2 may be a novel target for therapeutic intervention in malignant glioma.

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