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Regulation of synaptojanin 2 5'-phosphatase activity by Src

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Abbreviations: LC-MS/MS, tandem mass spectrometry coupled to liquid chromatography; PI, phosphatidylinositol; PRD, proline-rich domain; SYNJ2, synaptojanin 2

Synaptojanin 2 (SYNJ2) is a phosphatidylinositol (PI) phosphatase that controls two distinct functions, clathrin-mediated endocytosis and tumor cell invadopodia formation and invasion. Here, we identify a number of novel SYNJ2 binding partners, several of which have previously been shown to be necessary for invadopodia formation or clathrin-mediated endocytosis. We focus on Src family kinases. We found that Src phosphorylates SYNJ2 on Tyr^{490}, thereby stimulating SYNJ2 5′-phosphatase activity in vitro. We also provide evidence that Src-mediated phosphorylation of SYNJ2 contributes to invadopodia formation.

Introduction

Synaptojanin (SYNJ) phosphatidylinositol (PI) phosphatases contain two distinct phosphatase domains, a Sac1 homology domain that acts on PI(3)P, PI(4)P and PI(3,5)P_2 and an inositol 5'-phosphatase domain that acts 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 SYNJ1 is brain-specific,[2] whereas SYNJ2 is expressed in a wide range of tissues.[3] The C-terminal domains of SYNJ 1 and 2 diverge in sequence. However, both contain proline-rich domains (PRDs) that mediate binding to SH3 domain-containing proteins. The C-terminal domain of SYNJ2, but not SYNJ1, also contains a Rac1-binding domain.[4]

SYNJ1 and 2 play diverse roles in clathrin-mediated endocytosis. The primary role of SYNJ1 appears to be the uncoating of clathrin-coated vesicles,[5] although it also contributes to vesicle internalization.[6,7] In contrast, synaptojanin 2 plays a critical role at an early step of clathrin-mediated endocytosis,[8] although it is possible that SYNJ2 also functions in vesicle decoating.

We also have shown that SYNJ2 is necessary for the invasive behavior of a number of different tumor cell types.[9] The role of SYNJ2 in tumor cell invasion is independent from its role in clathrin-mediated endocytosis and is likely to be mediated by its function in the formation of invadopodia.[9] SYNJ2 also is strongly enriched in invadopodia.[9] Tumor cell invadopodia are specialized membrane structures at which extracellular matrix degradation is concentrated.[10-12] We note that invadopodia are similar in composition and structure to podosomes.[10] The term podosome is usually reserved for such structures found in normal cells and in Src-transformed fibroblasts, whereas the term invadopodium is restricted to cancer cells. How SYNJ2 regulates invadopodia formation remains to be elucidated. A number of proteins that are regulated by PI metabolism play key roles in invadopodia. These include Tks5 and cortactin.[13,14] Thus, likely functions of SYNJ2 in invadopodia include regulating the localization and/or activity of these proteins.

SYNJ2 binds to a number of SH3 domain-containing proteins, including endophilin, amphiphysin and CIN85, all of which play key roles in the regulation of clathrin-mediated endocytosis.[15-18] In this paper, we identify a number of novel SYNJ2 binding partners, several of which have been shown to be necessary for invadopodia formation. We focus on Src family kinases, as Src is a central regulator of invadopodia formation.[10,12]

Results

In order to identify novel SYNJ2 binding partners, we screened a library of purified SH3 domains immobilized on a membrane (TranSignal SH3 Domain Arrays, Panomics) using a His-tagged C-terminal domain of SYNJ2 that contains all five PRDs (**Fig. 1**). The list of positives (**Table 1**) included two SH3 domain-containing protein that are known to interact with both SYNJ2 and SYNJ1, endophilin and Grb2, and several that previously have been shown to bind to SYNJ1, intersectin, Nck1 and

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SNX9.[15,19-22] Remarkably, five members of the Src family of kinases (SFK) were among the positives, Fyn, Hck, Lck, Lyn and Src. Src is known to be a key regulator of invadopodia.[10,12] Cortactin, another protein that is necessary for invadopodia formation[14] also was among the positives.

To confirm the interaction of SYNJ2 with Src family kinases in a cellular setting, we performed co-expression of full length SYNJ2 together with full length Lyn or Fyn in HEK293 cells. Immunoprecipitation of Lyn or Fyn from lysates of co-expressing cells, but not from cells in which SYNJ2 was overexpressed on its own, showed specific interaction between SYNJ2 and these kinases (**Fig. 2A and B**). We obtained similar results for the interaction of SYNJ2 with intersectin (**Fig. 2C**). We also confirmed the interaction between SYNJ2 and cortactin, using two different approaches. First, endogenous cortactin could be precipitated from SNB19 glioblastoma cell lysates with an immobilized SYNJ2 C-terminal domain GST-fusion protein, but not GST itself (**Fig. 2D**). Second, SYNJ2 co-immunoprecipitated with cortactin from HEK293 cells co-expressing full length versions of both SYNJ2 and cortactin (**Fig. 2E**). We also identified intersectin and cortactin using mass spectrometry, as endogenous proteins associated with SYNJ2 that was overexpressed in HEK293 cells (data not shown).

The interaction of SYNJ2 with Src family kinases suggested to us that Src may be phosphorylating SYNJ2 and thereby modulating its activity. To examine whether Src phosphorylates SYNJ2, we co-expressed full length SYNJ2 together with constitutively active Src in 293T cells, immunoprecipitated SYNJ2 with an anti-Myc antibody and determined tyrosine phosphorylation of SYNJ2 using an anti-phospho-tyrosine antibody. We observed robust Src-stimulated phosphorylation of SYNJ2 in these conditions (**Fig. 3A**). We confirmed these findings by examining tyrosine phosphorylation of endogenous SYNJ2 by immunoprecipitating SYNJ2 from Src-transformed or control SNB19 glioblastoma cells (**Fig. 3B**).

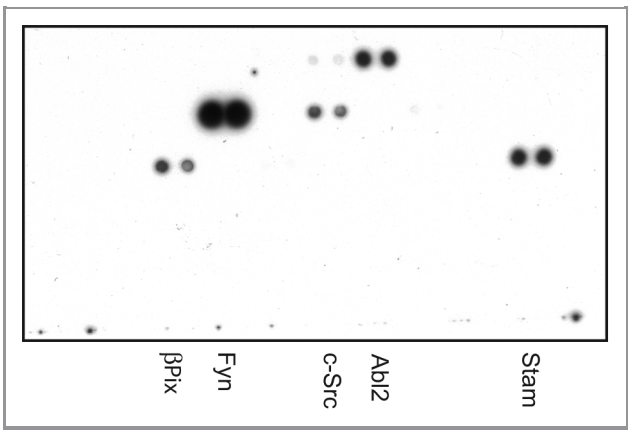

Figure 1. Chemoluminescence detection of SYNJ2-interacting SH3 domains. TransSignal SH3 Domain Array membranes were incubated with a His-tagged C-terminal domain of SYNJ2 (GST-SYNJ2-C-His) and developed as described in Materials and Methods. Shown is SH3 Domain Array-I out of four arrays examined.

Table 1. Candidate SYNJ2 binding partners identified using protein arrays

Symbol	Description
ABL2	Abelson-related protein, Arg
ARHGEF7	Rho guanine nucleotide exchange factor 7 (βPIX)
CTTN	Cortactin
FYN	Proto-oncogene tyrosine kinase Fyn
GRAP2	GRB2-related adaptor protein 2, SH3 domain #2
GRB2	Growth factor receptor-bound protein 2, SH3 domains #1,2
HCK	Hemopoietic cell kinase
ITSN1	Intersectin, SH3 domains #3,4
LCK	Lymphocyte-specific protein tyrosine kinase
LYN	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog
NCK1	Nck adaptor protein 1, SH3 domain #2
NPHP1	Juvenile nephronophthisis 1 protein
PACSIN3	Protein kinase C and casein kinase substrate in neurons 3
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 1 (α)
PLCG2	Phospholipase C-γ-2
PPP1R13B	Protein phosphatase 1, regulatory subunit 13B
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1
SH3GL2	SH3 domain-containing GRB2-like protein 2
SORBS2	Sorbin and SH3 domain containing 2, SH3 domain #2 (ARGBP2)
SORBS3	Sorbin and SH3 domain containing 3, SH3 domain #3 (vinexin)
SRC	Cellular Rous Sarcoma viral oncogene homolog, c-Src
SNX9	Sorting nexin 9
STAM	Signal transducing adaptor molecule
TEC	Tec tyrosine kinase

List of candidate SYNJ2 binding partners identified using TransSignal SH3 Domain Array membranes. See Materials and Methods for description of the assay.

To identify SYNJ2 tyrosine residues that are phosphorylated by Src, we performed an in vitro phosphorylation assay using Myc-tagged SYNJ2 purified from 293T cells and recombinant His-tagged Src purified from Sf21 insect cells. Phosphorylated SYNJ2 peptides resulting from a trypsin digest were analyzed by tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS). The only tyrosine-phosphorylated residue detected was Tyr^{490} (**Fig. 4A**) located between the Sac1 and 5'-phosphatase domains. To confirm that Tyr^{490} is indeed a substrate for Src phosphorylation, we mutated this residue to Ala using site-directed mutagenesis. We observed that the Src-stimulated in vitro tyrosine phosphorylation of the Y490A mutant was strongly inhibited in comparison to that of wild-type SYNJ2 (**Fig. 4B and C**).

To examine the functional consequences of Src-mediated phosphorylation of SYNJ2, we determined whether Src stimulates

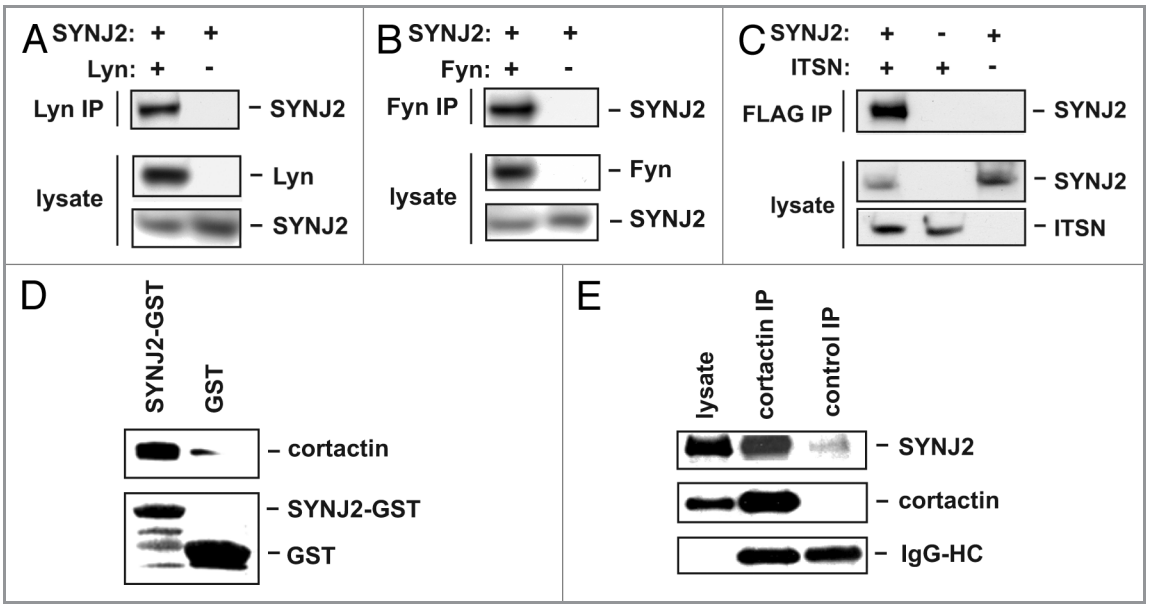

Figure 2. Confirmation of SYNJ2 interaction with select binding partners. (**A**) SYNJ2 interacts with Lyn. HEK293 cells were transiently co-transfected with expression plasmids for Lyn or SYNJ2 or transfected with a SYNJ2 plasmid alone. Cells were lysed 24 h post-transfection. Cell lysates were incubated with an anti-Lyn antibody, followed by protein A-agarose. Expression of Lyn and SYNJ2 in lysates and immunoprecipitated SYNJ2 were visualized by western blotting. (**B**) SYNJ2 interacts with Fyn. The same methodology was used as in (**A**), except that Lyn was replaced by Fyn. (**C**) SYNJ2 interacts with intersectin. HEK293 cells were transiently transfected with the indicated combinations of expression plasmids for FLAG-tagged intersectin-1 (ITSN) or SYNJ2. Cells were lysed 24 h post-transfection and lysates were incubated with an anti-FLAG antibody, followed by protein G-agarose. Expression of intersectin and SYNJ2 in lysates and immunoprecipitated SYNJ2 were visualized by western blotting. (**D and E**) SYNJ2 interacts with cortactin. (**D**) Co-immunoprecipitation experiment. HEK293 cells were transiently co-transfected with cortactin and SYNJ2 expression plasmids. Cells were lysed 24 h post-transfection and lysates were incubated either with an anti-cortactin antibody followed by protein G-sepharose or with control IgG followed by protein G-sepharose. Expression of cortactin and SYNJ2 in lysates and immunoprecipitated SYNJ2 were visualized by western blotting. Immunoglobulin heavy chain (IgG-HC) was visualized by anti-mouse HRP. (**E**) GST-pulldown experiment. SNB19 cell lysates were incubated with immobilized GST or GST-SJ2 (GST-SYNJ2-C). Precipitated cortactin was visualized by western blotting using an anti-cortactin antibody (upper panel) and bound GST proteins were visualized by Ponceau staining (lower panel). Data shown are representative of at least 2 experiments.

SYNJ2 5'-phosphatase activity using a fluorescence polarization-based, competitive assay that quantifies the production of $PI(3,4)P_2$ from $PtdInd(3,4,5)P_3$. We found that incubation with Src significantly enhanced SYNJ2 5'-phosphatase activity (**Fig. 5**). In addition, basal 5'-phosphatase activity of SYNJ2-Y490A was lower than that of wild-type SYNJ2 and Src only marginally stimulated this activity. These results demonstrate that Src-stimulated phosphorylation of Tyr^{490} enhances SYNJ2 5'-phosphatase activity.

To further examine whether Src-mediated phosphorylation of SYNJ2 contributes to the formation of invadopodia, we simultaneously depleted endogenous SYNJ2 from SNB19 cells and reconstituted with wild-type or mutant versions of SYNJ2. SiRNA-mediated depletion of SYNJ2 was accomplished using a 3' UTR-targeting oligo that effectively depletes endogenous SYNJ2 (by approximately 90%), but does not target the respective reconstituted proteins. We observed that wild-type SYNJ2 largely reversed the inhibitory effect of depleting endogenous SYNJ2, whereas the Y490A mutant was much less effective (**Fig. 6**). In contrast, the 5'-phosphatase-deficient mutant was completely ineffective in reconstituting invadopodia formation. Reconstitution by the Sac1-deficient mutant was not significantly different from that of wild-type SYNJ2. These results strongly suggest that Src-mediated phosphorylation of Tyr^{490} contributes to invadopodia formation by stimulating SYNJ2 5'-phosphatase activity.

Discussion

In this paper, we identify a number of novel binding partners of the SYNJ2 PI phosphatase and focus on Src family kinases. We show that Src phosphorylates SYNJ2, thereby enhancing its 5'-phosphatase activity. We also provide evidence that Src-mediated stimulation of SYNJ2 activity contributes to invadopodia formation.

Src is a central regulator of invadopodia and a number of Src substrates have been shown to be essential for invadopodia formation, including cortactin and Tks5/Fish[10-12] Cortactin is an F-actin-binding protein and activates the Arp2/3 actin-nucleation complex.[23] Cortactin also is thought to stimulate protease secretion by regulating vesicular trafficking.[24] We note that cortactin also plays a role in clathrin-mediated endocytosis.[25] Tks5 is a scaffold protein that contains five SH3 domains and is targeted to invadopodia via its PX domain that binds to PtdIns(3)P and $PtdIns(3,4)P_2$.[13] Tks5 binds to members of the ADAM family of transmembrane proteases and to proteins involved in the regulation of actin dynamics, including cortactin and N-WASP.[13,26] The data presented in

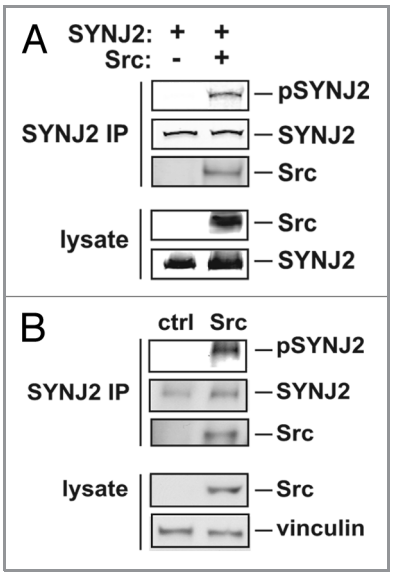

Figure 3. Src phosphorylates SYNJ2. (**A**) Tyrosine phosphorylation of SYNJ2 in Src-SYNJ2 co-expression conditions. Myc-tagged SYNJ2 was co-expressed with or without constitutively active Src (SrcY527F) in 293T cells and immunoprecipitated with an anti-Myc antibody. Tyrosine phosphorylated SYNJ2 in the immunoprecipitate was visualized by western blotting using an anti-phospho-tyrosine antibody (pSJ2). Total SYNJ2 and Src were visualized with their respective antibodies. (**B**) Tyrosine phosphorylation of SYNJ2 in Src-transformed cells. Endogenous SYNJ2 was immunoprecipitated from Src-transformed SNB19 glioblastoma cells or parental cells using an anti-SJ2 polyclonal antibody. Samples from cell lysates and immunoprecipitate were immunoblotted with the indicated antibodies. Data shown are representative of at least two experiments.

this paper add SYNJ2 to the array of Src substrates that control invadopodia formation.

Interestingly, kinetic analysis of Src-induced podosome formation in NIH-3T3 cells has indicated that clustering of PtdIns(3,4)P_2 is an early and necessary event in podosome formation.[26] In addition, both PI3K and SYNJ2 have been shown to be necessary for the formation of podosomes and invadopodia.[26,27] Importantly, we also showed that it is the 5'-phosphatase, but not Sac1 enzymatic activity of SYNJ2 that regulates invadopodia formation. Thus, these findings, together with our observation that Src stimulates SYNJ2 5'-phosphatase activity, strongly suggest that SYNJ2 stimulates invadopodia/podosome formation by producing PtdIns(3,4)P_2 through hydrolysis of PtdIns(3,4,5)P_3 and subsequent recruitment of Tks5. It is also possible however that SYNJ2 contributes to invadopodia formation by hydrolyzing PtdIns(4,5)P_2. Indeed, type-I phosphatidylinositol 4-phosphate 5-kinase α (PIP5KI α) has been shown to be essential for invadopodia formation[28] and a number of proteins that have been implicated in invadopodia formation are regulated by PtdIns(4,5)P_2, including the actin filament severing protein cofilin and N-WASP, a protein that regulates actin nucleation via Arp2/3.[29,30]

We also note that both Src and SYNJ2 play critical roles in clathrin-mediated endocytosis.[8,31,32] Thus, it is likely that Src-stimulated SYNJ2 5'-phosphatase activity also contributes to this function.

In addition to Src family members and cortactin, we have identified two other potential binding partners of SYNJ2 that have been implicated in the formation of invadopodia and/or podosomes, including Abl2/Arg and PSTPIP1. The tyrosine kinase Arg has been shown to function downstream of Src in the functional maturation of invadopodia, at least in part by phosphorylating cortactin and stimulating actin nucleation.[33] PSTPIP1 is an adaptor protein that is primarily expressed in hematopoietic cells and also controls the actin cytoskeleton, in part via its interaction with the protein tyrosine phosphatase PTP-PEST and the actin nucleating protein WASP.[34] Mutations in PSTPIP1 that disrupt binding to PTP-PEST are responsible for pyogenic sterile arthritis, pyoderma gangrenosum and acne (PAPA) syndrome, an auto-inflammatory disorder.[34] Recently, PSTPIP1 has been shown to function in podosome formation and macrophage migration.[35]

Our screen also identified intersectin and SNX9, two proteins that have been implicated in the regulation of clathrin-mediated endocytosis, as novel binding partners of SYNJ2. Intersectin is a multi-domain adaptor protein that is thought to stabilize an endocytic multi-protein complex.[36,37] SNX9 is a member of the sorting nexin family and plays multiple roles in clathrin-mediated endocytosis, including regulation of dynamin acitivity and membrane curvature.[38] Whether intersectin and SNX9 also function in the formation of podosomes and invadopodia remains to be established.

In conclusion, our findings shed new light on the molecular mechanisms that regulate SYNJ2. In addition, the identification of a large number of SYNJ2 binding partners strongly suggests that, in addition to regulating PI metabolism, SYNJ2 may function as a scaffold to coordinate signaling events that regulate clathrin mediated endocytosis and the formation of podosomes and invadopodia.

Materials and Methods

Reagents. *Plasmids.* A Myc-tagged wild-type SYNJ2 plasmid (pcDNA-Myc-SYNJ2) was constructed by inserting a PCR product containing the full length SYNJ2 gene into the ClaI/EcoRV sites of the pCAN-Myc1 vector. The following mutant versions of SYNJ2 were introduced into pcDNA-Myc-SYNJ2 using the QuickChange Site-Directed Mutagenesis Kit (Stratagene): Src-phosphorylation mutant (SYNJ2-Y490A), Sac1 mutant (SYNJ2-C386A) and 5'-phosphatase mutant (SYNJ2-R796A/R803F).

GST-SYNJ2-C, containing most of the SYNJ2 C-terminal domain (aa 1,023–1,497), was subcloned from pEXV-2H28[4] into the EcoRI site of pFASTBac (Invitrogen) and subsequentially into the BamHI/NotI sites of pET-42 (Novagen). GST-SYNJ2-C-His, a shorter version of the SYNJ2 C-terminal domain that still contains all five PRDs (aa 1,051–1,381), was generated by PCR and subcloned into pET-42b. pRK5-intersectin-1 was a gift from Nathalie Lamarche-Vane.[39] The cortactin-expression plasmid pEMS1 was a gift from Ed Schuuring. pRK5-Fyn was purchased from Adgene. pDONR223-Lyn was purchased from Adgene and subcloned into pRN and subsequently into pCDNA.

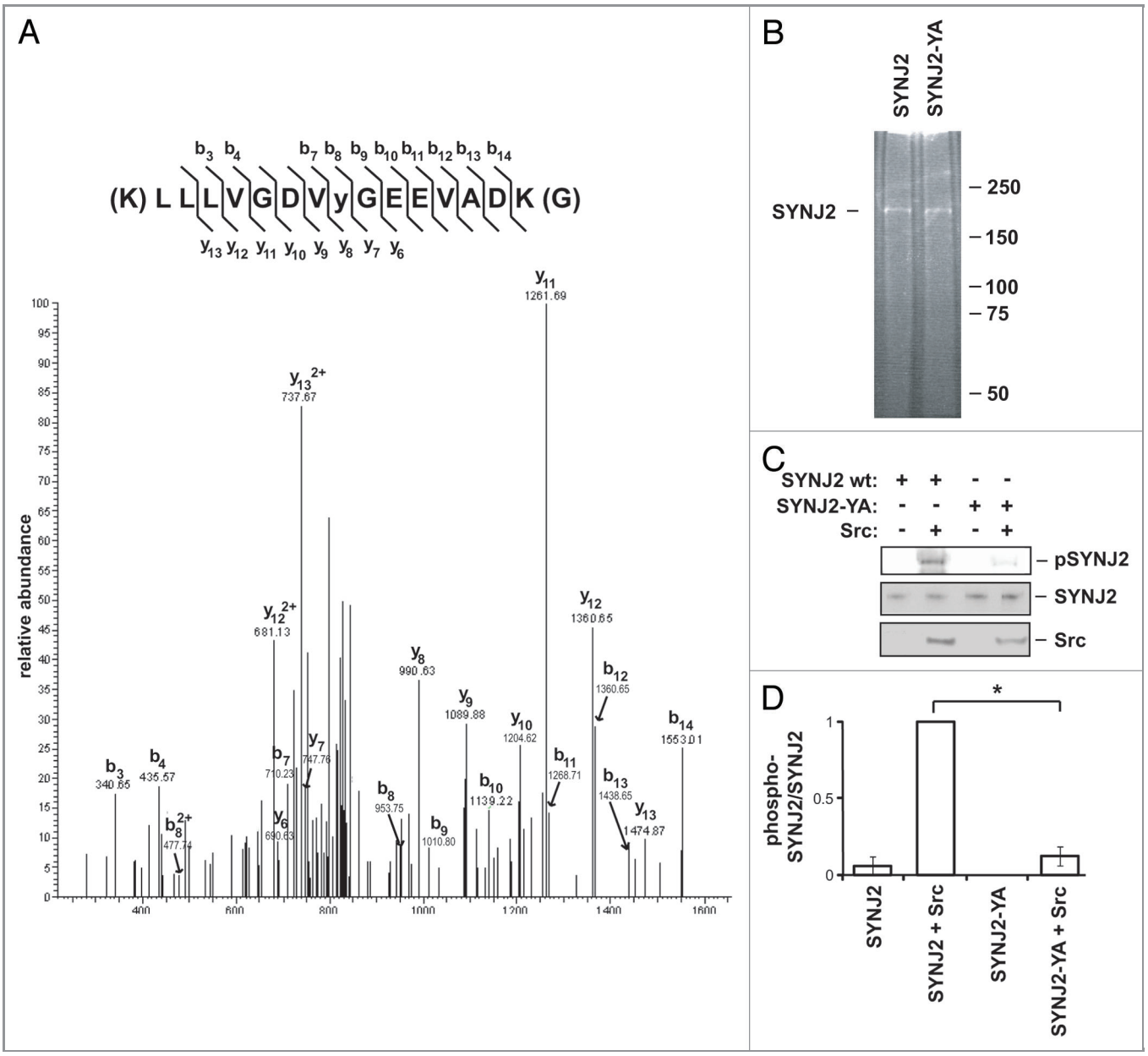

Figure 4. Src phosphorylates SYNJ2 on Tyr490. (**A**) Tandem mass spectrum of tryptic peptide containing Tyr490 where the phosphorylated tyrosine is denoted as "y." SYNJ2 phosphorylated by Src in vitro was analyzed by LC-MS/MS as described in Materials and Methods. (**B**) Sypro Ruby-stained SDS-PAGE gel illustrating the purity of recombinant SYNJ2 and SYNJ2-Y490A purified from 293T cells. (**C**) Phosphorylation of wild-type SYNJ2 and SYNJ2-Y490A by Src in vitro. (**D**) Quantification of western blot data. Bars represent the average (± SEM) of three independent experiments (* = $p < 0.005$, two-tailed t-test).

Small interfering RNA. An siRNA targeting the SYNJ2 3' UTR (target sequence: 5'-AAUCACCAGGCCAGUGUUUCU) was designed using the Dharmacon web tool and obtained from Dharmacon. An siRNA directed against GL2 luciferase with target sequence 5'- AACGTACGCGGAATACTTCGATT was purchased from Ambion.

Proteins. Recombinant His-tagged Src protein was obtained from Millipore (14-326). Myc-SYNJ2 and Myc-SYNJ2-Y490A were expressed in 293T cells and purified using the c-Myc tagged Protein Mild Purification Kit (MBL) following the protocol provided by the manufacturer.

Antibodies. Polyclonal antibodies against SYNJ2 are as previously described.[4] Anti-cortactin (4F11, 05-180) and anti-Src (GD11, 05-184) antibodies were obtained from Millipore. Anti-Fyn (Sc-16) and anti-Lyn (Sc-15) antibodies were obtained from Santa Cruz Biotechnology. Anti-HA (1 666 606) and anti-His6-Peroxidase (1 965 085) antibodies were from Roche. The anti-FLAG M2 antibody (200472) was from Stratagene. The

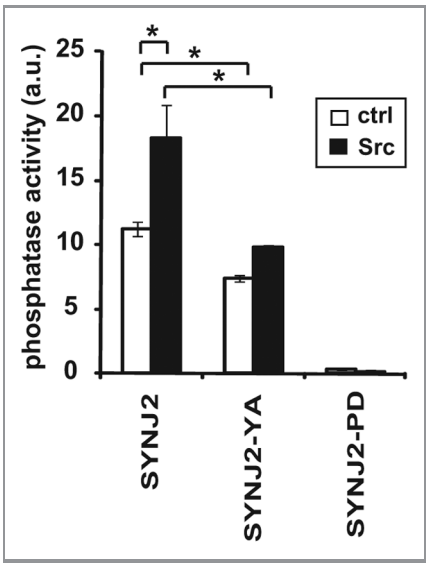

Figure 5. Src stimulates SYNJ2 5′-phosphatase activity. Wild-type SYNJ2 and SYNJ2-Y490A were phosphorylated in vitro by Src. Subsequently, the 5′-phosphatase activities of wild-type and mutant SYNJ2 proteins were determined using a fluorescence polarization assay as described in Materials and Methods. A 5′-phosphatase-deficient mutant of SYNJ2 (SJ2-PD) was used as negative control. Data shown are the average (± SEM) of triplicate wells and are representative of at least three independent experiments (*, $p < 0.05$, two-tailed t-test).

anti-Myc antibody (1 667 203) used for the immunofluorescence experiments was from Roche. To visualize phospho-tyrosine we used a mixture of antibodies, 4G10 (05-321, Millipore) and PY20 (610000, BD Biosciences).

Cell culture. SNB19 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO_2. SNB19 cells that stably express constitutively active chicken Src were established by co-transfection of the pSGT-SrcY527F plasmid (gift from Sara Courtneidge) and pRL-puro (Clontech) for puromycin selection. Clonal populations were selected in 0.75 μg/ml of puromycin. Isolated clones were characterized by Src immunoblotting.

SH3 domain array screening. TransSignal SH3 Domain Array membranes (Panomics) were incubated with bacterial lysates containing GST-SYNJ2-C-His protein. The membranes were washed and developed according to the protocol provided by the manufacturer.

In vitro SYNJ2 phosphorylation assay. Two micrograms of Myc-SYNJ2 or Myc-SYNJ2-Y490A protein were incubated with 20 ng of Src in kinase buffer (60 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 3 μM Na_3VO_4) with 1.25 mM DTT and 20 μM of ATP for 30 min at room temperature. The reaction was stopped by addition of Laemmli sample buffer (Bio-Rad Laboratory). Subsequently, proteins were visualized using 1D SDS-PAGE, followed by western blotting or Sypro Ruby staining (for mass spectrometric analysis).

Mass spectrometry. The Sypro Ruby-stained band corresponding to SYNJ2 was digested in trypsin (10 μg/mL) at 37°C

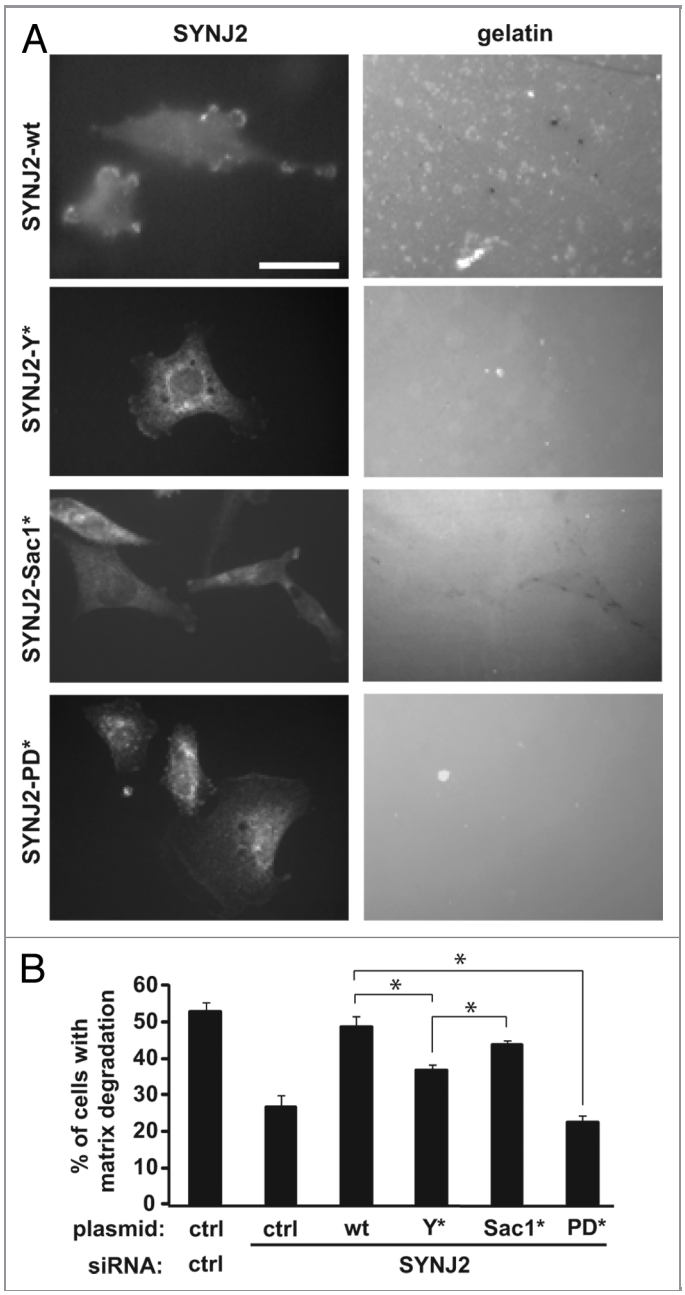

Figure 6. Src-dependent stimulation of SYNJ2 5′-phosphatase activity regulates invadopodia formation. (**A**) Representative micrographs showing reconstitution of depleted endogenous SYNJ2 from SNB19 cells with either wild-type or mutant versions of SYNJ2 (left panels) and cell-associated matrix degradation (right panels). SNB19 cells were first transfected with an siRNA that targets the SYNJ2 3′ UTR and the next day transfected with the indicated plasmids. Control cells were similarly transfected with control siRNA (targeting luciferase) and a pcDNA-Myc plasmid. Two days after transfection, cells were plated on FITC-gelatin-coated coverslips and fixed 21 h later to assay invadopodia formation, as described in Materials and Methods. (**B**) Quantification of invadopodia formation. For each experiment and condition, the means were determined for at least 10 fields comprising a total of at least 25 cells. Data shown are the average values (± SEM) obtained from at least three independent experiments (*, $p < 0.05$, two-tailed t-test).

overnight and tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) analysis was performed as described previously.[40] Following positive protein identification of the SYNJ2 2B2 isoform by protein database searching as described previously,[40] the tandem mass spectra were also searched in a biased way using the primary sequence of SYNJ2 2B2, considering additional variable modifications (addition of +79.999 on serine, threonine and tyrosine residues). The tandem mass spectra of modified peptides from this search were visually inspected for good quality. In addition, their appropriate data files were further re-searched in an unbiased way using the ipihuman v 3.65 protein database, considering the same additional variable modifications, in order to ensure that these spectra did not correlate better to other human proteins.

5'-phosphatase assay. SYNJ2 5'-phosphatase activity was measured using the 5'-PI(3,4,5)P_3 Phosphatase Fluorescence Polarization Activity Assay kit (Echelon) according to the manufacturer's protocol. The assay is a competitive assay for the product of the phosphatase reaction PI(3,4)P_2. Briefly, 50 ng of purified wild-type SYNJ2 or mutant SYNJ2-Y490A, was first in vitro phosphorylated (or not) by Src (7 ng Src protein in kinase buffer + 1 mM DTT, 100 μM ATP) for 30 min at room temperature. Control reaction was performed in kinase buffer without Src protein. Next, samples were diluted into the kit-provided reaction buffer and subsequently mixed with PIP3 substrate for 30–40 min at room temperature. The 5'-phosphatase reaction was stopped by heating the sample at 95°C for 3 min. Subsequently, the PI(3,4)P_2 detector and the fluorescent PI(3,4)P_2 probe were added and incubated for 30 min. Fluorescence polarization was examined using a VictorX plate reader provided with 550 nm excitation and 580 nm polarizing emission filters.

Invadopodia formation assay. FITC-gelatin-coated coverslips were prepared as described previously[41] and modified for SNB19 cells.[9] Cells were cultured on FITC-gelatin-coated coverslips in DMEM with 0.25% FBS for 24 h. Subsequently, cells were fixed in 4% formaldehyde/PBS and subsequently, permeabilized with 0.1% Triton X-100/PBS. Control cells were stained with Rhodamine-conjugated phalloidin and cells that express recombinant proteins were stained with an anti-Myc antibody, followed by a Texas Red anti-mouse antibody. Coverslips were mounted in ProlongGold mounting medium (Invitrogen) and images were collected using an IX70 Olympus inverted microscope equipped with a 60× objective, an Orca II CCD camera (Hamamatsu) and ESee (Inovision) image analysis software. Invadopodia formation was determined by counting the percentage of cells that had associated matrix degradation. For the reconstitution studies, only cells that express low to medium levels of the respective SYNJ2 constructs were analyzed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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