Construction of expression vectors for spinophilin

Full-length SPL was subcloned into the pCR®-Blunt II-TOPO® vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and sequenced on both strands. SPL was additionally subcloned into pCMV-3B expression vector, using restriction sites for *HindIII* and *SalI* incorporated by synthetic oligonucleotides. Spinophilin fragments were generated by PCR as shown below. The amplified fragments were subcloned into the pCR®-Blunt II-TOPO® vector and sequenced. The resulting constructs were digested with *HindIII* (or *PstI*) and *SalI* and ligated to pCMV-3B.

Fragment	Forward primer	Reverse primer
Full length	AAAAAGCTTATGATGAAGACGGAG	AAAGTCGACTTAAGTAGAATTGGA
SPL177-817	AAACTGCAGATGATGAAGACGGAG	AAAGTCGACTTAAGTAGAATTGGA
SPL1-151	AAAAAGCTTATGATGAAGACGGAG	AAAGTCGACTTAGAACAGCTTCCGCGT
SPL151-444	AAAAAGCTTATGTTCGAACGGAGCGTC	AAAGTCGACTTAGGCTGGGTCCTCTTC
SPL444-586	AAAAAGCTTATGCCGAGCCGGAAGATC	AAAGTCGACTTATCCAGGCCGCTCCCG
SPL586-817	AAAAAGCTTATGGAACAGAGTGAAGTG	AAAGTCGACTTAAGTAGAATTGGA
SPL1-444	AAAAAGCTTATGATGAAGACGGAG	AAAGTCGACTTAGGCTGGGTCCTCTTC
SPL1-586	AAAAAGCTTATGATGAAGACGGAG	AAAGTCGACTTATCCAGGCCGCTCCCG
SPL444-817	AAAAAGCTTATGCCGAGCCGGAAGATC	AAAGTCGACTTAAGTAGAATTGGA
SPL664-817	TATAAGCTTCCTGTGGAGATGGAG	ACGCGTCGACTTAAGTAGAATTGGA
SPL586-664	CCCAAGCTTGGAGAACAGAGTGAAG	AAACGTCGACCTCCATCTCCACAG

Binding of RGS18 to $G_{q\alpha}$ and spinophilin

GST-RGS18 (full length) and GST in pGEX-4T were expressed in BL-21 cells and purified on glutathione-Sepharose (GE Healthcare, Piscataway, NJ). In the binding studies, bead-bound GST-RGS18 and GST were incubated for 2 hrs at 4°C with either 1) mouse platelet lysates (prepared with 50mM HEPES, pH8.0/300mM NaCl/1mM DTT/6mM $MgCl_2$/1% Triton X-100 with protease inhibitor) in the presence of GDP (30 µM), $AlCl_3$ (30 µM) and NaF (20 mM) as indicated or 2) lysates from CHO cells transfected with Myc-SPL (lysed 48 hrs after transfection in ice-cold 50 mM Tris-HCl/100 mM NaCl/5 mM EDTA/1% Triton X-100, pH 7.4 with protease inhibitor). After 3 washes, the beads were boiled in sample buffer, subjected to SDS PAGE and immunoblotted with either anti-$G_{q\alpha}$ or anti-Myc.

Cell culture and transfection

Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were transfected at 60% confluence using FuGENE®6 reagent (Roche Applied Science). Cells were harvested and assayed 48 h after transfection. When indicated, they were serum-starved overnight starting approximately 30 hours post transfection.

Immunoblotting

Platelets or cells were lysed in NP-40 or Triton X-100 lysis buffer in the presence of protease inhibitors. The lysates were boiled in sample buffer before SDS PAGE analysis. Phosphotyrosine immunoblots were performed with antibody 4G10 alone or in combination with antibody PY20.

Co-precipitation experiments

CHO cells were lysed 48 h after transfection in ice-cold lysis buffer (50 mM Tris-HCl/100 mM NaCl/5 mM EDTA/1% Triton X-100, pH 7.4) containing Complete™ protease inhibitor cocktail and 2 mM Na_3VO_4. Washed or gel-filtered platelets were incubated in an aggregometer cuvette at 37°C, and lysed with ice-cold 5× Nonidet P-40 buffer (1% NP-40 in 50 mM Tris/150 mM NaCl with protease inhibitor and 2 mM Na_3VO_4). When indicated, platelets were preincubated with aspirin (ASA, 1 mM for 30 min) and/or apyrase (10 units/ml for 10 minutes). After centrifugation at 16,000 × *g* for 20 min at 4°C, supernatants were precleared with protein A agarose (for rabbit antibodies) or protein G agarose (for goat and mouse antibodies) for an hour and incubated overnight at 4°C with an immunoprecipitating antibody (2 μg) or normal rabbit, goat or mouse IgG. Protein/antibody complexes were isolated with protein A- or G-agarose for 2 h at 4°C. After 6 washes with lysis buffer, the beads were boiled in sample buffer (2% SDS/1% 2-mercaptoethanol/0.008% bromophenol blue/80 mM Tris/1 mM EDTA, pH 6.8).

Tyrosine phosphatase assay

SHP-1 phosphatase activity was measured with the fluorescent-based Rediplate™ 96 Enzchek® kit R-22067 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Myc-SPL and SHP-1 transfected CHO cells lysates were incubated overnight at 4°C with 2 μg mouse anti-Myc antibody or normal mouse IgG. Protein/antibody complexes were isolated with protein G Magnetic beads (Millipore, Temecula, CA) for 2 h at 4°C. No-PTPase controls were also included. After 6 washes with lysis buffer, the beads were added into microplate well containing 80 μl reaction buffer. The reactions were incubated at room temperature for 20 min. Fluorescence was then measured at 355 nm excitation and emission at 460 nm.

Figure S1. Mapping the RGS18 binding site on spinophilin
The top part of the panel shows a representation of full length spinophilin. Tyrosine residues are indicated as lollipops (Y398 and Y483 are marked in red) as the reported binding domains (BD) for actin, G protein coupled receptors (GPCR), protein phosphatase 1 (PP1), and RGS2. The spinophilin PDZ domain and coiled-coil domain are also marked. The lower part of the panel indicates the Myc-tagged SPL constructs that were co-expressed with RGS18 in CHO cells. Lysates were probed with anti-Myc to detect expression or precipitated with anti-RGS18 and probed with anti-Myc to detect complex formation. Each fragment was studied at least twice. The gray box, which spans residues 586–664, indicates the region within SPL that is necessary and sufficient for its interaction with RGS18.

Figure S2. Binding sites for SHP-1 in spinophilin
(A) CHO cells were transfected with Myc-tagged full length spinophilin and either wild type SHP-1 or SHP-1 Y398/483F. Proteins were precipitated with anti-Myc, and then probed for SHP-1 and spinophilin. The bar graph summarizes results obtained in 3 studies (mean ± SEM). (B) CHO cells were transfected with SHP-1 and either full length Myc-tagged spinophilin or Myc-tagged SPL 586–817. Proteins were precipitated with anti-Myc, and then probed for SHP-1. The bar graph summarizes results obtained in 3 studies (mean ± SEM). See also Figure 4F.

Figure S3. SHP-1, SHP-2, and phosphatase activity in CHO cells
(A) Expression of SHP-1 is required for thrombin-induced dephosphorylation of spinophilin in CHO cells. CHO cells were transfected with Myc-tagged spinophilin and SHP-1 (top) or with Myc-tagged spinophilin alone (bottom). Thrombin was added after an overnight incubation in serum-free medium. Lysates were precipitated with the phosphotyrosine-specific antibody, 4G10, and then probed with Myc-SPL. The example shown is representative of 2 experiments. (B) Endogenous expression of SHP-2 in CHO cells. Brain, platelet, and CHO cell lysates were probed for SHP-2. The example shown is representative of 2 experiments. (C) CHO cells were transfected as in (A) and then stimulated with thrombin for the times indicated. Lysates were precipitated with anti-Myc and then assayed for tyrosine phosphatase activity. The results shown are the mean ± 1 S.D. from two experiments.

Figure S4. Phosphorylation of SHP-1(Ser591)
CHO cells were transfected with Myc-tagged spinophilin and human SHP-1. Lane 1, total cell lysate. Except for the sample in lane 2 (labeled “NS” or non-starved), the cells were incubated overnight in serum-free medium beginning approximately 30 hours after transfection. Thrombin was added for the times indicated. Except for the sample in lane 1, proteins were then precipitated with anti-Myc and probed for (*top*) SHP-1 phospho-Ser591, (*middle*) SHP-1 and (*bottom*) Myc-SPL. Results are representative of two experiments. The results show that although some of the SHP-1 in CHO cells is phosphorylated on Ser591, the fraction associated with spinophilin is phosphorylated neither before nor after the addition of thrombin.

Figure S5. RGS10 and RGS18 expression in SPL (−/−) platelets
(A) Western blots of RGS18 expression. The blots were re-probed for actin showing equal loading. *Top.* Representative experiment. *Bottom.* Summary of RGS18 expression in 8 SPL(−/−) mice and 8 matched WT controls (mean ± SEM). (B) Western blots of RGS10 expression. The blots were re-probed for actin showing equal loading. *Top.* Representative experiment. *Bottom.*

Summary of RGS10 expression in 3 SPL(−/−) mice and 4 matched WT controls (mean ± SEM). The results show that knocking out spinophilin has no effect on RGS10 and RGS18 expression.

Figure S6. Ca^{2+} mobilization in SPL (−/−) platelets
Left. Fura-2–loaded platelets from matched wild-type and SPL(−/−) mice stimulated with 10 μg/ml collagen in the presence of aspirin and apyrase. *Right.* Summary of data from 3 studies (mean ± SEM).

Figure S7. A model for the binding of SHP-1 to spinophilin
In quiescent cells, spinophilin is phosphorylated on Y398 and Y483. The data indicate that SHP-1 binds to spinophilin *via* pY398 and a secondary binding site located within SPL(586–817) (denoted as "2° binding domain" in the figure), a combination that is sufficient for binding, but not activating SHP-1. Activation occurs when Y536 in SHP-1 is phosphorylated. As indicated in the figure, we propose that 1) Y398 binds to the C-terminal SH2 domain in SHP-1, leaving the N-terminal SH2 domain to engage (and inhibit) the SHP-1 catalytic domain and 2) phosphorylation of Y536 disengages the N-terminal SH2 domain from the catalytic domain, allowing activation of the phosphatase, dephosphorylation of Y398 and Y483, and dissociation of the SPL/RGS/SHP1 complex.

Figure S1

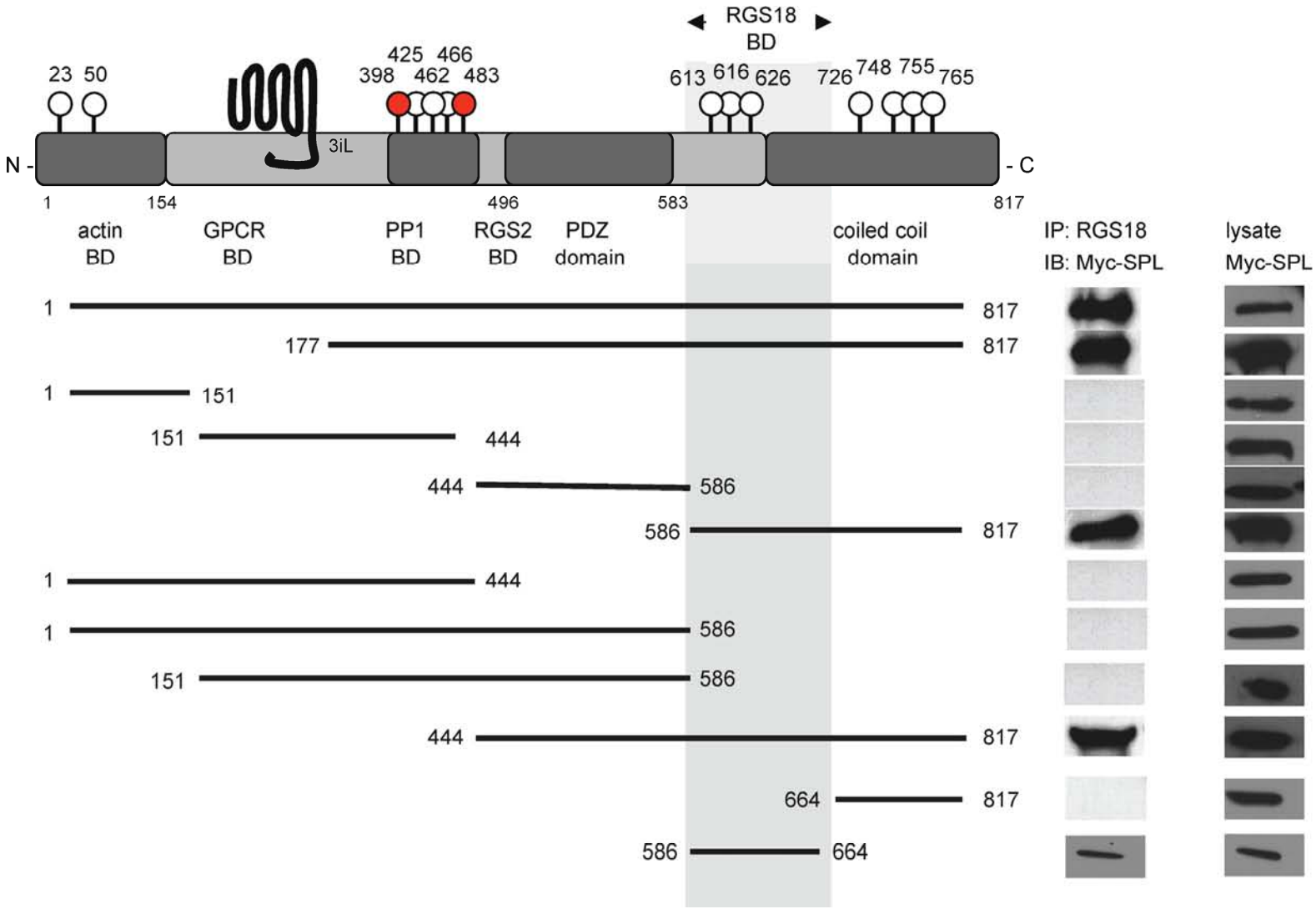

RGS18
BD
23 50
425 466
398 462 483
613 616 626
726 748 755 765
3iL
N -
- C
1
154
496
583
817
actin
BD
GPCR
BD
PP1
BD
RGS2
BD
PDZ
domain
coiled coil
domain
IP: RGS18
IB: Myc-SPL
lysate
Myc-SPL
1 817
177 817
1 151
151 444
444 586
586 817
1 444
1 586
151 586
444 817
664 817
586 664

Figure S2

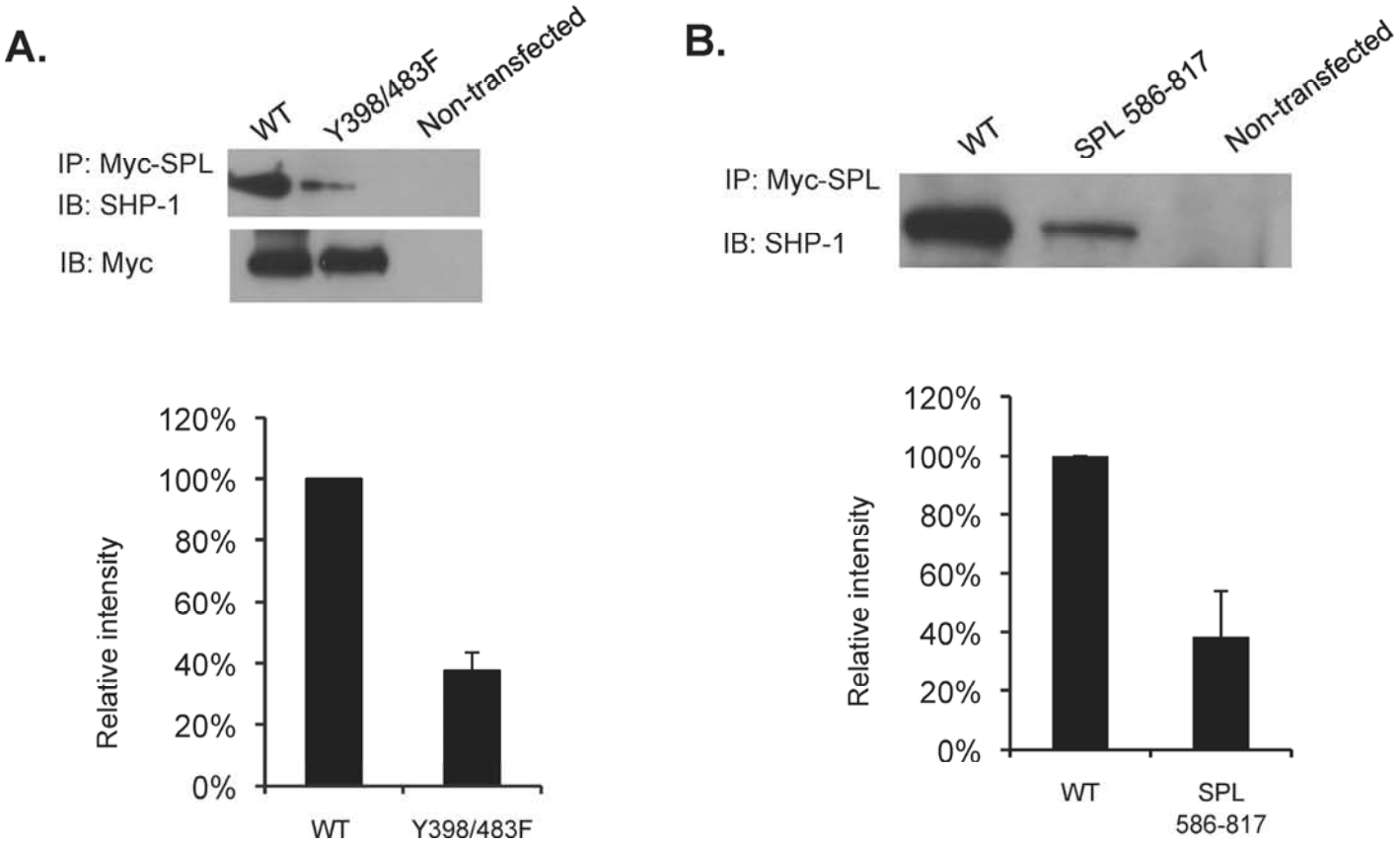

A.
WT
Y398/483F
Non-transfected
IP: Myc-SPL
IB: SHP-1
IB: Myc
120%
100%
80%
60%
40%
20%
0%
Relative intensity
WT
Y398/483F
B.
WT
SPL 586-817
Non-transfected
IP: Myc-SPL
IB: SHP-1
120%
100%
80%
60%
40%
20%
0%
Relative intensity
WT
SPL
586-817

Figure S3

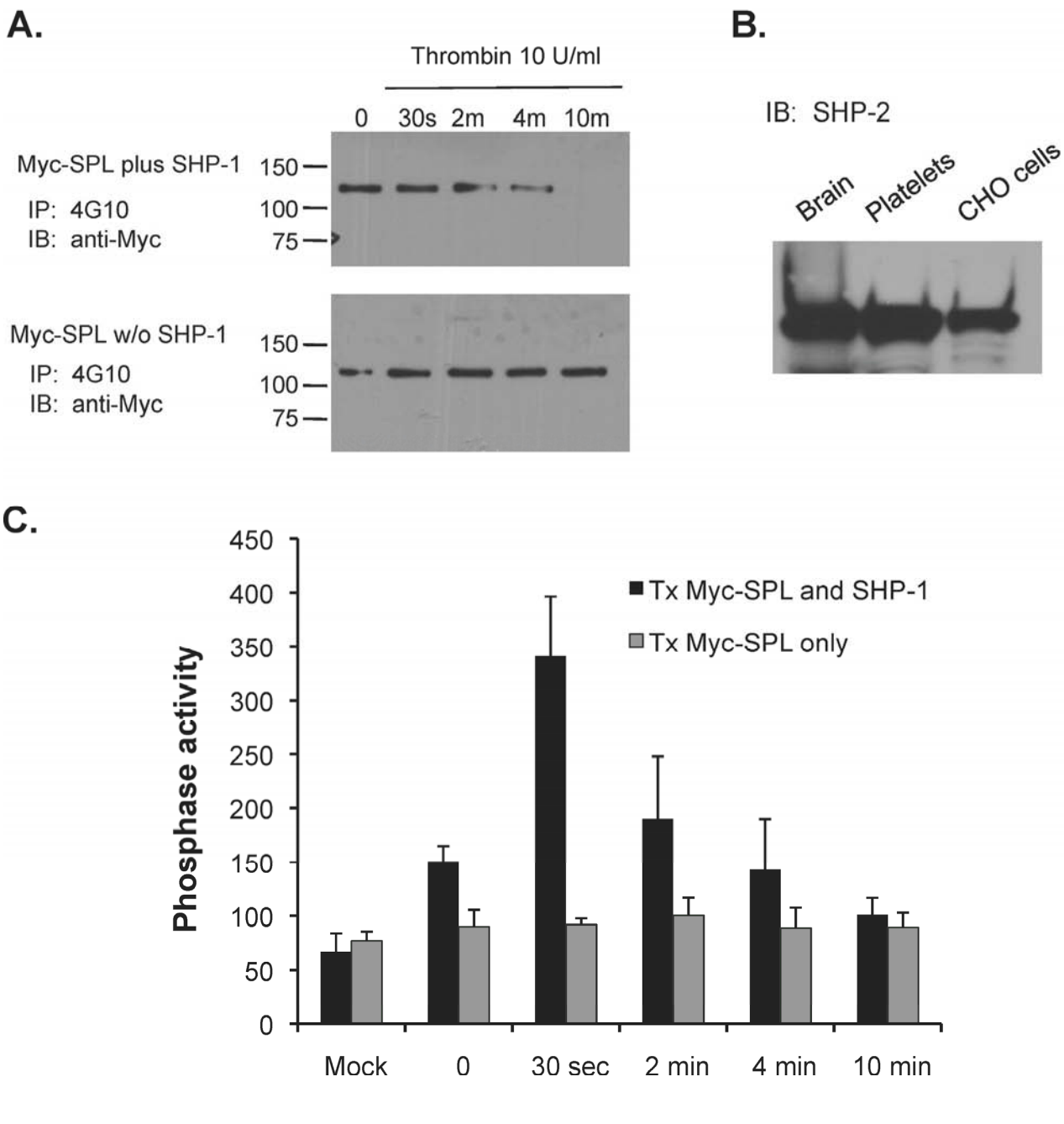

A.
Thrombin 10 U/ml
0
30s
2m
4m
10m
Myc-SPL plus SHP-1
IP: 4G10
IB: anti-Myc
150
100
75
Myc-SPL w/o SHP-1
IP: 4G10
IB: anti-Myc
150
100
75
B.
IB: SHP-2
Brain
Platelets
CHO cells
C.
Phosphase activity
450
400
350
300
250
200
150
100
50
0
Tx Myc-SPL and SHP-1
Tx Myc-SPL only
Mock
0
30 sec
2 min
4 min
10 min

Figure S4

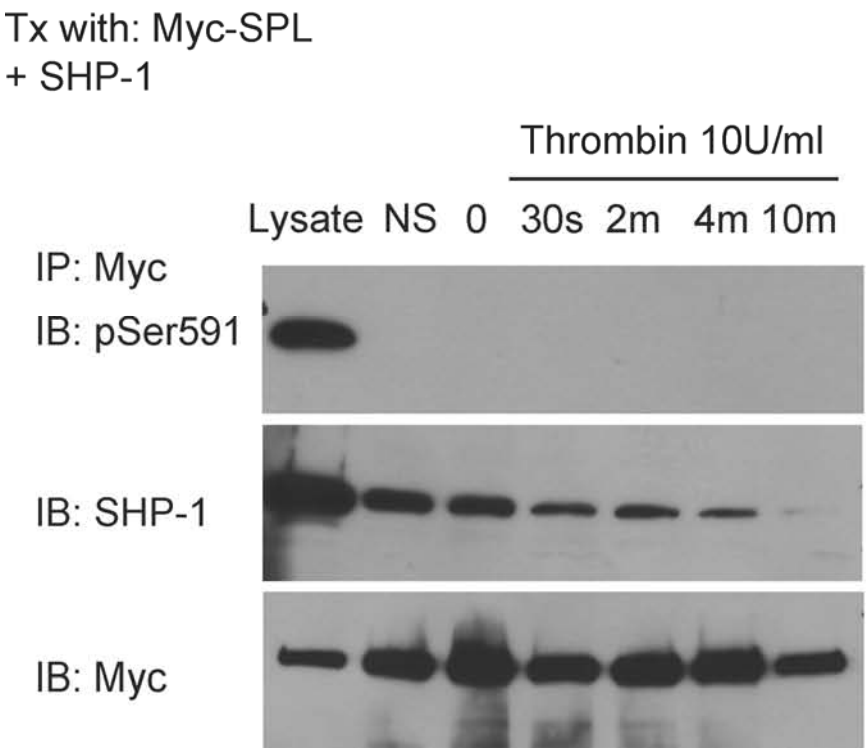

Tx with: Myc-SPL
+ SHP-1
Thrombin 10U/ml
Lysate NS 0 30s 2m 4m 10m
IP: Myc
IB: pSer591
IB: SHP-1
IB: Myc

Figure S5

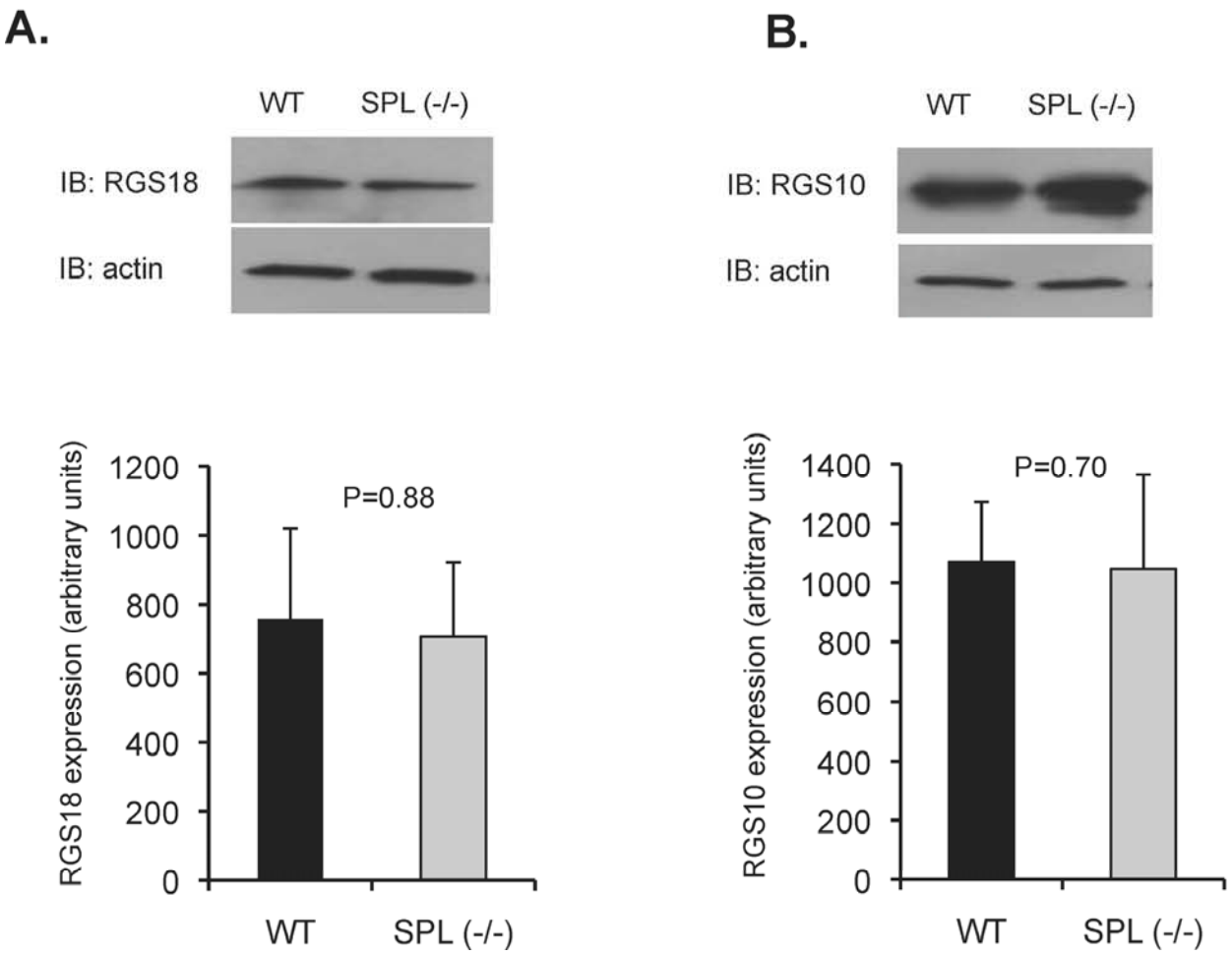
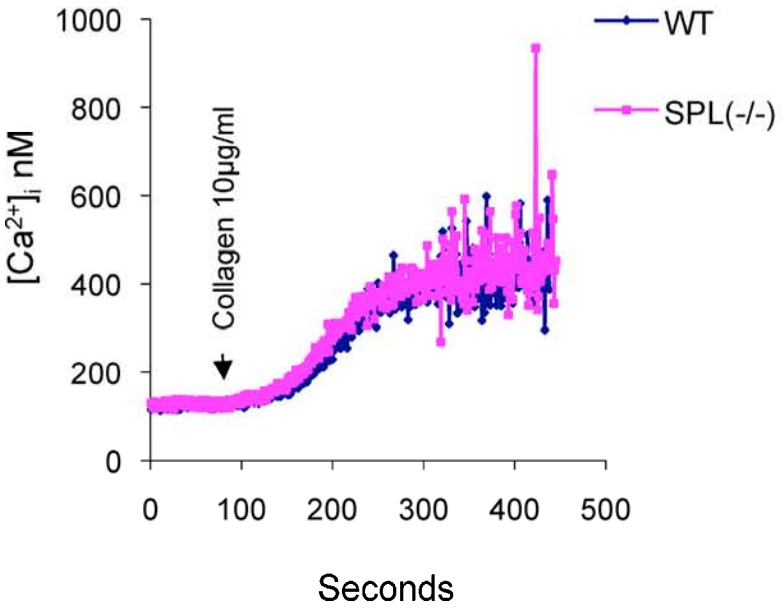

A.
WT
SPL (-/-)
IB: RGS18
IB: actin
RGS18 expression (arbitrary units)
1200
1000
800
600
400
200
0
P=0.88
WT
SPL (-/-)
B.
WT
SPL (-/-)
IB: RGS10
IB: actin
RGS10 expression (arbitrary units)
1400
1200
1000
800
600
400
200
0
P=0.70
WT
SPL (-/-)

Figure S6


1000
800
600
400
200
0
[Ca2+]i nM
Collagen 10µg/ml
WT
SPL(-/-)
0
100
200
300
400
500
Seconds

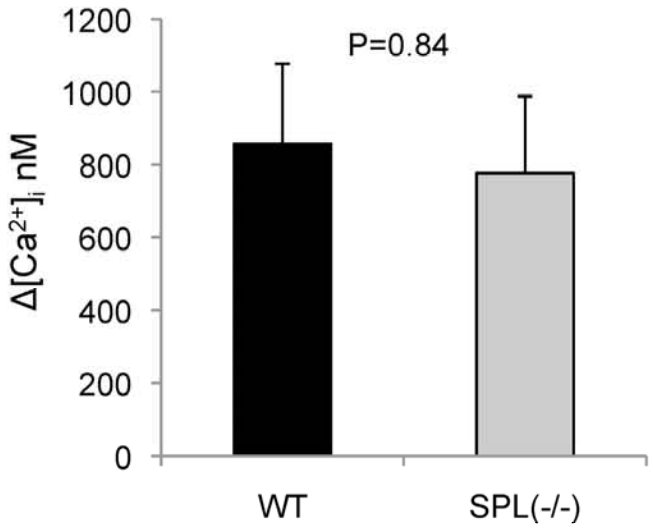
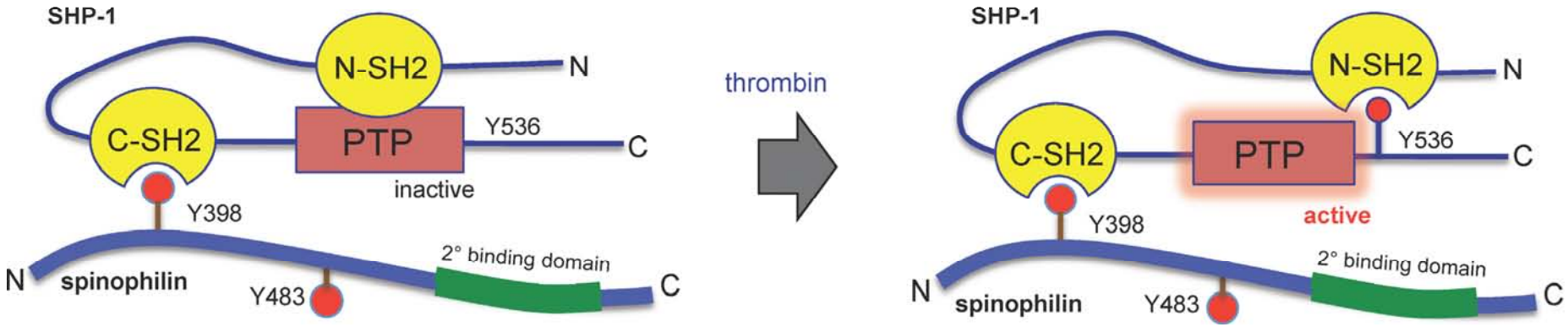

1200
1000
800
600
400
200
0
Δ[Ca2+]i nM
P=0.84
WT
SPL(-/-)

Figure S7


SHP-1
N-SH2
N
C-SH2
PTP
Y536
C
inactive
Y398
N
spinophilin
Y483
2° binding domain
C
thrombin
SHP-1
N-SH2
N
C-SH2
PTP
Y536
C
active
Y398
2° binding domain
N
spinophilin
Y483
C