Synaptotagmin-like protein 4 and Rab8 interact and increase dense granule release in platelets

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Running head: Role of Slp4 and Rab8 in dense granule release

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Summary

Background: Platelets are highly specialized cells which regulate haemostasis and thrombosis in the vasculature. Upon activation platelets release various granules which impact on platelets, the coagulation system, other blood cells and on the vessel wall, however the mechanisms controlling granule release are only partially known. We have shown previously that synaptotagmin-like protein 1 (Slp1) decreases dense granule release in platelets.

Objectives: To determine the role of other synaptotagmin-like proteins and their binding partners on platelet dense granule release.

Methods: RT-PCR and immunoblot were used to identify Slps in human platelets. Interaction between Slp4 and Rab8 was investigated by pull-down assay, co-immunoprecipitation and by confocal microscopy. Secretion assays on permeabilized platelets were performed to investigate the effects of Slp4 and Rab8 on dense granule release.

Results: Slp4 mRNA and protein are expressed in human platelets. Slp4 interacts with Rab8 in transfected cells and at endogenous protein levels in platelets. We mapped the Rab interaction site to the SHD domain of Slp4 and we show preferential binding of Slp4 to the GTP-bound form of Rab8. Live microscopy showed co-localization of GFP-Slp4 and mCherry-Rab8 at the plasma membrane of transfected cells. Endogenous platelet Slp4 and Rab8 co-localized in the center of activated platelets where granule secretion takes place. Secretion assays revealed that Slp4 and Rab8 enhance dense granule release and we show that the Slp4 effect is dependent on Rab8 binding.

Conclusions: Slp4 and Rab8 are expressed and interact in human platelets and might be involved in dense granule release.

Keywords: granule, granuphilin, platelet, Rab8, Slp4, synaptotagmin

Introduction

Platelets play a central role in haemostasis and are a critical component in the development of atherothrombosis [1]. Platelets contain dense and alpha granules and upon activation release their granule contents to promote thrombus formation. Dense granules contents include ADP, 5-hydroxytryptamine (5-HT, serotonin), Ca^{2+}-ions and pyrophosphate, whereas alpha granules enclose chemokines, coagulation factors, growth factors and other proteins [2]. Inherited defects in platelet granule formation lead to bleeding disorders as for example the Hermansky-Pudlak or Chediak-Higashi syndromes affecting dense granules or the gray platelet syndrome for alpha granules [3]. Platelet granule release is thought to depend on mechanisms that are similar to synaptic vesicle secretion [4, 5]. Granules are transported to the plasma membrane where they are tethered followed by docking and fusion of granule and plasma membranes resulting in the release of granule contents into the extracellular space. The fusion of granules with the plasma membrane requires soluble NSF-sensitive attachment protein receptors (SNARE). SNARE family proteins suggested to play a role in platelet granule release include VAMP-2, -3, -8, syntaxin 2, 4, 7, 11 and SNAP-23, -29 [6]. In addition to the SNARE proteins, membrane fusion requires members of the Sec1/Munc18 (SM) family which direct SNARE function [7] and platelets have been shown to express various isoforms of Munc18 [8]. Another group of proteins that are emerging as important regulators of the membrane fusion process are the synaptotagmins and related multiple C2 domain proteins. Synaptotagmins are characterized by two tandem C-terminal C2 domains which interact with membrane phospholipids and are involved in membrane curvature induction [9, 10]. Synaptotagmins have also been described to interact with SNAREs and to act as Ca^{2+}-sensors. Synaptotagmin-like proteins (Slp) constitute a related group of proteins containing tandem C2 domains and a N-terminal Slp-homology domain (SHD) that binds small GTP-binding proteins of the Rab family [11-13]. Slp1 has been shown to inhibit dense granule release [14] and Munc13-4, a protein with separate N- and C-terminal C2 domains, positively regulates alpha and dense granule release in platelets [15]. Slp1 and Munc13-4 interact with Rab27, a small G-protein involved in dense granule biogenesis and release [16]. Rab proteins control vesicle trafficking by binding to internal membranes as well as to numerous effector proteins [17].

In this paper we describe the identification and characterization of Slp4 (also called granuphilin) in platelets. We show that Slp4 interacts with Rab8 and that both proteins might play a role in the regulation of dense granule release.

Materials and methods

Antibodies, Constructs, Materials

A polyclonal rabbit antibody against human Slp4 was developed by OpenBiosystems (Lafayette, CO) using the peptide AEGTLQLRSSMAKQKLGL at position 654 of Slp4. Other antibodies used in this study include: anti-Slp4 (HPA001475, rabbit, Atlas), anti-Slp1 [14], anti-Rab8A Human (H00004218-M02, mouse, Tebu-Bio), anti-FLAG tag (M2, Sigma- Aldrich), Alexa Fluor 488 donkey anti-rabbit IgG (A-21206, Invitrogen), Alexa Fluor 568 donkey anti-mouse IgG (A10037, Invitrogen), horseradish peroxidase-coupled donkey anti-rabbit and donkey anti-mouse (Jackson ImmunoResearch Europe).

Constructs and protein purification

Full-length human Slp4-a was obtained from Origene (SC120377, SYTL4). Slp4 was FLAG- or GFP-tagged at the N-terminus and expressed using the mammalian expression vector pcDNA4/TO (Invitrogen). HA-tagged Rab8A (N-terminus) was purchased from the University of Missouri-Rolla and mCherry-Rab8 was expressed in pmCherryC1 (Invitrogen). Site-directed mutagenesis was performed by polymerase chain reaction amplification of Rab8 constructs using mutagenic primer pairs, Pfu DNA polymerase (Fermentas), digestion with DpnI (Fermentas) and transformation into TOP10 bacteria (Invitrogen). All constructs were verified by DNA sequencing (Eurofins MWG Operon). Slp4, Slp1 and Rab8 Glutathione-S-transferase fusion proteins were generated using the pGEX-4T3 vector (GE Healthcare) expressed in *Escherichia coli* BL21 and purified as described [14, 18].

Cell Preparation, Transfection, Lysis, Immunoprecipitation and Pull-down experiments

HEK293T and HeLa cells were cultured using DMEM supplemented with 10% FCS and 1% penicillin/streptomycin, at 37 ºC and 5% CO_2 in air. Cells were transfected using Metafectene (Biontex, Martinsried, Germany), or Fugene (Promega) according to manufacturer's instructions. Venous blood was drawn from healthy volunteers taking no medications who gave their informed consent according to the declaration of Helsinki. 40 ml freshly drawn venous blood from healthy volunteers was collected into 10 ml pre-warmed CCD-EGTA buffer (100 mM tri-sodium-citrate, 7 mM citric acid, 140 mM glucose, 15 mM EGTA) and centrifuged at 150 x *g* and RT for 15 minutes. Platelet rich plasma (PRP) was recovered and platelets were pelleted at 600 x *g* for 10 min and resuspended in resuspension buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, pH 7.4) to a final concentration of $2x10^8$ platelets/ml. Cell lysis, immunoprecipitation and pull-down assays were performed as described [19] using 5 µl of Anti-FLAG M2 Affinity Gel (Sigma-Aldrich), 10

µl of anti-Slp4 polyclonal antibody or 5 µl of Gluthatione SepharoseTM 4B suspension (GE Healthcare) saturated with GST fusion proteins.

RT-PCR analysis

Total cellular RNA was isolated from 1 ml of freshly washed platelets (~1x10E9 platelets) using 40ml Trizol reagent, as described by the manufacturer (Invitrogen). Total RNA from human brain was obtained from BD Biosciences. Total RNA was reverse transcribed using RevertAid™ H Minus Reverse Transcriptase (Fermentas) and amplified using Random Hexamer Primers (Fermentas). The cDNA was then used for RT-PCR amplification using specific sense and antisense primers for Slp2 (Forward: CAGCACCAGCAAGCCCGAGT, reverse: CCCTCTCGGAGCCCCTCTCG), Slp3 (Forward: GGCAGCTGCAGGTCTCGGTG, reverse: CAAGGTGCCATCTGGCCGCA), Slp4 (Forward: GCCTGGGCCGTTTGAGTCCC, reverse: CACTGGGCTCCTTCTGCCGC) and Slp5 (Forward: AGGCCCCAATGGCAGCTGGA, reverse: CGTGTGCTTGGGGCTGGTGA). PCR amplification was performed for 25 cycles at an annealing temperature of 55-65°C. Positive bands were excised and verified by sequencing.

Confocal microscopy

HeLa cells were grown on 8 well micro-slides (Ibidi) and transfected with GFP-Slp4 and mCherry-Rab8. After 18-24hrs, living cells were visualized using an Andor (Nikon) spinning disc microscope using 488 nm and 594 nm lasers and Plan Fluor 100x/1.30 oil objective. The obtained images were analyzed using Andor IQ2 software. Platelets were let spread on glass coverslips and fixed and stained as described [20] using rabbit Slp4 and mouse Rab8 primary antibodies followed by anti-rabbit Alexa 488 and anti-mouse Alexa 564 secondary antibodies and mounted onto slides using Fluoromount (Sigma). Stained platelets were visualized as described above. Cross-reactivities of secondary antibodies were ruled out by swapping of secondary antibodies in single-labelling experiments. For unactivated platelets, an equal volume of CCD-EGTA blood was mixed with 3.7% of room temperature paraformaldehyde prepared in PBS and fixed for two hours at room temperature. Subsequent steps were carried out at 4 °C. Platelet-rich plasma supernatant was obtained by centrifugation at 300 x *g* for 15 minutes. The platelet-rich plasma was centrifuged at 1300 × *g* for 12 minutes to pellet the platelets. The pellet was resuspended in resuspension buffer and an equal volume of 4% paraformaldehyde in PBS was added to the final platelet suspension for 30 minutes at room temperature. Platelets were then diluted in 990 µl PBS, dropped on coverslips, permeabilized and stained as described above.

Dense Granule Secretion Assay

Washed platelets were permeabilized with 0.6 µg/mL streptolysin-O, kindly provided by S. Bhakdi, Mainz [21], incubated with purified GST-fusion proteins on ice and secretion assays were performed as described [14] using 18 mM Ca^{2+}, which corresponds to a final concentration of approximately 1.3 µM free Ca^{2+} [22], at 30 ºC for 2 min to stimulate granule release. Recombinant proteins had no effect on 5-HT secretion in the absence of Ca^{2+} (supporting information, Fig. S1). The average efficacy of 5-HT release ranged between 30 and 50% of total 5-HT. The secreted levels of 5-HT were normalized against samples incubated with GST only and stimulated with Ca^{2+}. Data are the mean of at least 3 independent experiments performed in triplicate. The statistical significance of the means was analyzed by analysis of variance and Bonferroni post-test (95% confidence interval) (Fig. 1, 4A, 4B) and by 2-tailed T-test (Fig. 4C) using GraphPad Prism software, Version 5.0. *P* values below 0.05 were considered statistically significant.

Results

Slp4 is present in human platelets and increases dense granule release

We had shown previously that Slp1 controls dense granule release in platelets [14]. To investigate a potential role of other Slp family members in platelets we isolated RNA from washed human platelets and performed RT-PCR analysis. In addition to Slp1, only Slp4 but not Slp 2, 3 or 5 was found to be expressed in platelets (Fig. 1A and data not shown). Following the detection of Slp4 mRNA, a Slp4 antibody was used to confirm the presence of endogenous Slp4 in platelets at the protein level by immunoblotting. The antibody recognized Slp4 both in cells transfected with FLAG-tagged Slp4 and in human platelet lysate with no Slp4 being detected in non-transfected HEK293T cells (Fig. 1B). Our previous work had suggested that Slp1 inhibits dense granule release [14]. To analyze a possible role of Slp4 in granule release we performed a secretion assay using streptolysin-O permeabilized platelets and purified GST-tagged Slp4. Slp4 significantly enhanced the Ca^{2+}-stimulated release of 5-HT, a marker for dense granules (Fig. 1C), whereas GST alone had no significant effect (supporting Fig. S2). The enhancing action of Slp4 was dose-dependent (supporting Fig. S3). In the absence of Ca^{2+} Slp4 did not stimulate 5-HT release (supporting Fig. S1). Deletion of the SHD domain of Slp4 abolished the enhancing effect of Slp4 (Fig. 1C, 2nd bar). Since SHD domains of Slps have been shown to interact with Rab proteins these data suggest that Slp4 might require its SHD domain to interact with a Rab protein to increase dense granule release.

Slp4 interacts with Rab8 in platelets

To identify Rab proteins that might bind to Slp4 in platelets we performed GST pull-down assays using purified GST-Rab fusion proteins. Previous extensive screening experiments had shown that Slp4 can bind to Rab8A, Rab27A and Rab27B, and weakly to Rab3A [23, 24] and human platelets are known to express Rab8 and Rab27 [2]. Therefore GST-Rab8A, GST-Rab27A and GST-Rab27B were generated and Slp4 binding was analyzed. Endogenous Slp4 in platelet lysates bound strongly to GST-Rab8, whereas almost no binding to Rab27 isoforms could be detected (Fig. 2A, upper panels). Since Slp1 had been shown to bind to Rab8 previously [25] we investigated the interaction of Slp1 with Rab8 and Rab27. Slp1 was able bind Rab8 and Rab27A and B equally well (Fig. 2A, lower panels). Next we compared binding of Slp1 and Slp4 to endogenous platelet Rab8. Pull-down assays using GST-Slp4 and GST-Slp1 indicated a stronger interaction of Slp4 and Rab8 compared to Slp1 (Fig. 2B). To verify the binding of Slp4 to Rab8 at completely endogenous levels we performed an immunoprecipitation using a Slp4 antibody. Analysis of the precipitates by immunoblotting revealed that Rab8 was only present in Slp4 antibody samples but not in IgG controls indicating that endogenous Slp4 and Rab8 interact in human platelets (Fig. 2C). This binding was further confirmed by co-immunoprecipitation using HEK293T cells transfected with FLAG-tagged Slp4 and HA-tagged Rab8. Rab8 could only be detected in precipitates from cells co-expressing both proteins (Fig. 2D). To verify the role of the SHD domain of Slp4 for interaction with Rab8 GST-Slp4ΔSHD and GST-Slp4 were used in pull-down experiments from human platelets and transfected HEK293T cells. Subsequent immunoblotting revealed that the Slp4 mutant lacking the SHD domain did not bind to Rab8 (Fig. 2E, F). From these data we conclude that Slp4 binds to Rab8 in platelets and that the SHD domain of Slp4 is required for Rab8 binding.

Subcellular localization of Slp4 and Rab8

To investigate the subcellular localizations of Slp4 and Rab8, HeLa cells were transfected with either GFP-tagged-Slp4 or mCherry-tagged-Rab8. In living cells GFP-Slp4 showed strong localization to the plasma membrane with no nuclear or cytoplasmic staining present (Fig. 3A1). Most mCherry Rab8 was observed on cytosolic vesicle-like structures with no membrane or nuclear staining (Fig. 3A2). We next determined the effect of co-expression of GFP-Slp4 and mCherry-Rab8 on their localization. As in singly transfected cells, co-transfected Slp4 was present predominantly in the plasma membrane (Fig 3B1). Interestingly in co-transfected cells there was an enrichment of mCherry-Rab8 to the plasma membrane (Fig. 3B2). Overlaid images confirmed co-localization of GFP-Slp4 and mCherry-Rab8 at the plasma membrane (Fig. 3B3). Next, we investigated locations of endogenous Slp4 and Rab8 in human platelets using specific antibodies. Immunofluorescence staining revealed a partial co-localization of Slp4 and Rab8 in unactivated platelets (Fig. 3C,1-3). In activated platelets,

Slp4 was present predominantly in the center and to some extent in the outer plasma membrane. Rab8 was present in the center of activated platelets where it co-localized with Slp4 (Fig. 3C,4-6). Quantitation of co-localisation of Slp4 and Rab8 in platelets and transfected cells indicated approximately 30-40 % co-localisation of both proteins (supplementary Fig. S4). These data support our findings that Slp4 and Rab8 interact in transfected cells and in platelets.

Rab8 increases dense granule release

We had already shown that Slp4 is able to augment platelet dense granule release (Fig. 1C). To investigate the potential role of Rab8 and of a combination of Slp4 and Rab8 on dense granule release we performed secretion assays using permeabilized platelets. Rab8 alone as well as combined addition of Slp4 and Rab8 were able to increase $Ca2^{+}$-induced dense granule release significantly (Fig. 4A). No effects of Rab8 were observed in the absence of Ca^{2+}-stimulation (supplementary Fig. S1).

Rab GTPases function as molecular switches which can alternate between GTP-bound active and GDP-bound inactive forms. To investigate if Rab8 effects on granule release were dependent on the GTP- or GDP-bound form two mutants were created, a constitutively active GTP-binding mutant, Rab8Q67L and a constitutively inactive GDP-binding mutant, Rab8T22N, as described [26]. On their own both Rab8 mutants were able to enhance dense granule release similar to the wildtype form of Rab8 (Fig. 4B). We next tested the ability of Rab8 mutants to enhance Slp4 induced granule release. The GTP-Rab8 mutant (Q67L) had a significantly stronger effect on granule release than the GDP-Rab8 mutant (T22N) (Fig. 4C). To investigate the possible reason for this difference in more detail we studied the binding of Rab8 mutants to Slp4. The GTP-bound Rab8 mutant was capable of pulling down endogenous Slp4 whereas the inactive mutant was not (Fig. 4D). These differences in binding were confirmed by co-immunoprecipitation from HEK293T cells transfected with FLAG-tagged Slp4 and either HA-tagged Rab8Q67L or HA-tagged Rab8T22N (Fig. 4E). We conclude that Slp4 interacts preferentially with the GTP-bound form of Rab8 resulting in enhanced dense granule release. Rab8 might also be able to increase granule release independent of Slp4 binding (Fig. 4B).

Discussion

We have identified Slp4 as new platelet protein involved in dense granule release. Slp4 interacts with the GTP-bound form of the small G-protein Rab8. Studies of living cells suggest interaction of Slp4 and Rab8 at the plasma membrane and both proteins localize to the granule-containing center of activated spread platelets indicating a possible role for Slp4 and Rab8 in the granule release process. In secretion assays Slp4 and Rab8 were able to augment dense granule release. Preliminary

experiments using platelet factor 4 as marker of alpha granules did not show any significant role for Slp4 in alpha granule release (supporting Fig. S5).

The exact mechanism of Slp4 action remains to be determined. Previously, Slp4 has been described as regulator of the release of insulin granules from pancreatic beta cells [27], amylase granules from parotid gland acinar cells [28] and dense core granules in PC12 neuronal cells [23]. In pancreatic beta cells Slp4 is thought to act as a bridging molecule which enables the docking of secretory granules containing membrane-bound Rab27 to SNARE proteins like syntaxin in the plasma membrane [29]. Slp4 was shown to interact with syntaxin 2 in parotid acinar cells [28]. Very recently both munc18-2 and syntaxin-11 have been described to have important functions in platelet secretion [30, 31] and possible links with Slp4 need to be investigated. Slp4 might be involved in granule transport by recruitment of the motor protein myosin Va [32]. Slp4 could also facilitate the membrane fusion event as described for synaptotagmin and other multiple C2 domain proteins [10]. A recently generated mRNA expression database suggests that Slp4 is the most highly expressed protein of the C-type tandem C2 protein family in human platelets followed by Slp1 as the second most highly expressed protein, whereas classical synaptotagmins are expressed at much lower levels [33]. Slp4 function differs from the closely related protein Slp1 in a number of ways. Slp1 attenuates dense granule release and interacts with Rap1GAP2, a GTPase-activating protein of Rap1 [14], whereas Slp4 enhances granule release and does not bind to Rap1GAP2 (data not shown). Furthermore, Slp1 interacts with Rab27, whereas Slp4 binds to Rab8 preferentially (Fig. 2).

Our experiments suggest that Slp4 requires Rab8 to increase granule release in platelets (Fig. 1C). Granule-attached Rab8 and plasma membrane-bound Slp4 could help dock granules to the plasma membrane as has been proposed for Rab27 previously [34]. This concept is supported by our findings that Rab8 alone localizes to vesicular structures whereas co-expression of Slp4 leads to membrane targeting of Rab8 (Fig. 3B). Slp4 appears to be a classic effector of Rab8 since Slp4 interacts preferentially with GTP-Rab8. Interestingly, Rab8 is probably in the GTP-bound state already in resting platelets, as is indicated by the interaction of Slp4 and Rab8 in resting platelets (Fig. 2C). Thrombin treatment does not affect the Slp4/Rab8 interaction (data not shown), although thrombin has been shown to induce phosphorylation of Rab8. [35]. Also Rab27 is present in its GTP-bound form in resting platelets [36]. Our data further indicate that GTP-Rab8 is more effective in stimulating granule release compared to GDP-Rab8, however only in combination with Slp4 (Fig. 4C). In addition, Rab8 might enhance granule release independent of its nucleotide binding state (Fig. 4B) which indicates Slp4-independent functions of Rab8. Studies in other cells have shown that Rab8 regulates membrane-recycling and the docking and fusion of exocytotic vesicles [37]. Rab8 facilitates the export of cholesterol from macrophages [38] and regulates translocation of GLUT4 glucose

transporter containing vesicles in muscle cells [39]. Rab8 also plays an essential role in ciliogenesis by binding to the exocyst complex of secretory vesicles [40] The exocyst complex has been shown to regulate dense granule release in platelets [41] suggesting possible links between Rab8, the exocyst complex and Slp4. Further studies using isolated reconstituted liposomes, cellular models, and genetically modified platelets are required to define the exact mechanisms through which Slp4 and Rab8 control platelet granule secretion.

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Figure legends

Figure 1. Slp4 is expressed in human platelets and enhances dense granule release. (A) Expression of Slp4 mRNA in human platelets. RNA was isolated from washed platelets and RT-PCR was carried out using specific primers for Slp4. Brain RNA was used as a positive control, with the negative control containing no template. The indicated band corresponds to the expected size of the PCR product of 336bps and DNA sequencing confirmed the identity of this band as Slp4. The lower band in the brain sample might represent a splice isoform of Slp4. (B) Expression of Slp4 protein in human platelets. Non-transfected, FLAG-Slp4 transfected HEK293T cells and human platelets were lysed and expression of Slp4 was analyzed by SDS-PAGE and immunoblotting using a specific anti-Slp4 antibody (Rabbit, peptide purified, OpenBiosystems). A band of expected size was detected in transfected cells and in platelets but not in non-transfected cells. (C) Effects of Slp4 on dense granule release. Platelets were permeabilized using streptolysin-O and incubated with 1 µM (final concentration) purified recombinant GST alone, GST-Slp4 fusion protein lacking the SHD domain, and GST-wild-type-Slp4. Granule release was stimulated by addition of Ca^{2+} ions and released serotonin (5-HT) was measured as indicator of dense granule release as described in Materials and Methods. Data was normalized to stimulated platelets incubated with GST. The results shown are expressed as mean of three independent experiments performed in triplicate. *P<0.05 and ***P<0.001 (statistically significant).

Figure 2. Slp4 interacts with Rab8 in platelets and transfected cells. (A) Pull-down assay of endogenous Slp4 and Slp1. Equal amounts of purified recombinant GST, GST-Rab8, GST-Rab27A and B coupled to GSH-Sepharose beads were used to pull down endogenous Slp4 and Slp1 from lysates of washed human platelets. Presence of Slp4 and Slp1 in the precipitates was analyzed by SDS-PAGE and immunoblotting. Total platelet lysates were analyzed in parallel to verify equal levels of Slp4 and Slp1 in the lysate samples. (B) Pull-down of endogenous Rab8 from human platelet lysate using GST-Slp1 and GST-Slp4. Equal amounts of GST as control, GST-Slp1 and GST-Slp4 coupled to GSH-Sepharose beads were used for precipitation. Bound Rab8 was visualized by immunoblotting using an anti-Rab8 antibody. Total platelet lysates were analyzed to verify equal loading. (C) Co-immunoprecipitation of endogenous Slp4 and Rab8. Washed human platelets were lysed and a specific antibody against Slp4 (Atlas) was used to immunoprecipitate endogenous Slp4. Non-specific IgG was used as negative control. The precipitates were analyzed for the presence of bound Rab8 by immunoblot using anti-Rab8 antibody. (D) Co-immunoprecipitation of transfected FLAG-Slp4 and HA-Rab8. HEK293T cells were transfected with FLAG-tagged Slp4, HA-tagged Rab8 or FLAG-tagged Slp4 together with HA-tagged Rab8. Empty vector was transfected as a negative control. After lysis,

Slp4 was precipitated using an anti-FLAG antibody. The precipitates were analyzed for the presence of bound Rab8 by immunoblot using anti-HA antibody and the lysates were analyzed for the expression of equal levels of Slp4 and Rab8 (totals). (E) Pull-down of endogenous Rab8 with GST-Slp4 and GST-Slp4ΔSHD from human platelets. Equal amounts of GST, GST-Slp4 and GST-Slp4ΔSHD coupled to GSH-Sepharose beads were incubated with human platelet lysate. Bound endogenous Rab8 protein was visualized with an anti-Rab8 antibody. (F) Pull-down of transfected HA-tagged Rab8. Lysates of HeLa cells overexpressing HA-Rab8 were subjected to pull-down experiments using equal amounts of GST-Slp4 and GST-Slp4ΔSHD beads. The precipitates were analyzed for the presence of bound Rab8 by immunoblot with anti Rab8 antibody. Non-transfected and pcDNA4T0 transfected cells were used as controls. Shown are representative data of independent experiments performed at least three times.

Figure 3. Slp4 and Rab8 co-localize in transfected cells and in platelets. (A) Localization of GFP-Slp4 and mCherry-Rab8 in singly transfected cells. HeLa cells were transfected with either GFP-Slp4 (A1) or mCherry-Rab8 (A2) fusion constructs. One day after transfection living cells were imaged using a confocal microscope. Green shows Slp4 staining; red shows Rab8 staining. (B) Co-localization of co-transfected Slp4 and Rab8. HeLa cells were transfected with both, GFP-Slp4 and mCherry-Rab8 and the localization of the proteins was analyzed by live-cell microscopy. Co-localization of Slp4 and Rab8 is shown in yellow (B3). (C) Localization of endogenous Slp4 and Rab8 in non-activated and activated platelets. Resting human platelets were fixed in solution (C1-3) or platelets were allowed to spread on glass (C4-6) followed by fixation. Platelets were then permeabilized and dual-labelled with primary antibodies directed against Slp4 (green, rabbit, OpenBiosystems) or Rab8 (red, mouse, Tebu-Bio) followed by Alexa-Fluor conjugated secondary antibodies. Overlay of green/red images indicate co-localization of fluorochromes (yellow, C3, C6). Scale bar equals 10µm. Shown are representative images of independent experiments performed three times.

Figure 4. GTP-Rab8 interacts with Slp4 and enhances dense granule release. (A) Effects of Rab8 and Slp4 on dense granule release. Permeabilized platelets were incubated with 1 µM GST, 1 µM GST-Rab8 or with 0.5 µM GST-Slp4 together with 0.5 µM GST-Rab8. Ca^{2+}-induced dense granule secretion was measured using 5-HT. Data was normalized to GST control. (B) Secretion assays as described in (Fig. 4A) were performed using 1 µM of a constitutively active GTP-bound mutant of Rab8 (Q67L) or an inactive GDP-bound mutant (T22N). (C) Effects of combinations of GST-Slp4 with active or inactive mutants of Rab8 were analyzed in secretion assays as described in (A). Data was normalized to the combination of Rab8-Q67L with Slp4. The results shown in (A) to (C) are expressed as mean of seven

(A), four (B) and seven (C) independent experiments performed in triplicate. *P<0.05 and **P<0.01 (statistically significant). (D) Pull-down of endogenous Slp4 from human platelet lysate using GST-Rab8-Q67L and GST-Rab-T22N coupled to GSH-Sepharose beads. Bound Slp4 was analyzed by SDS-PAGE and immunoblotting using an anti-Rab8 antibody. Total platelet lysates were analyzed to verify equal loading. (E) Co-immunoprecipitation of transfected Slp4 and Rab8 mutants. HEK293T cells were transfected with FLAG-tagged Slp4 together with wild-type and mutants of HA-tagged Rab8. Cells were lysed, Rab8 was precipitated using anti-HA antibodies and precipitates and lysates were analyzed by immunoblotting. Data shown in panels D and E are representative of three independent experiments.

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Figure 1

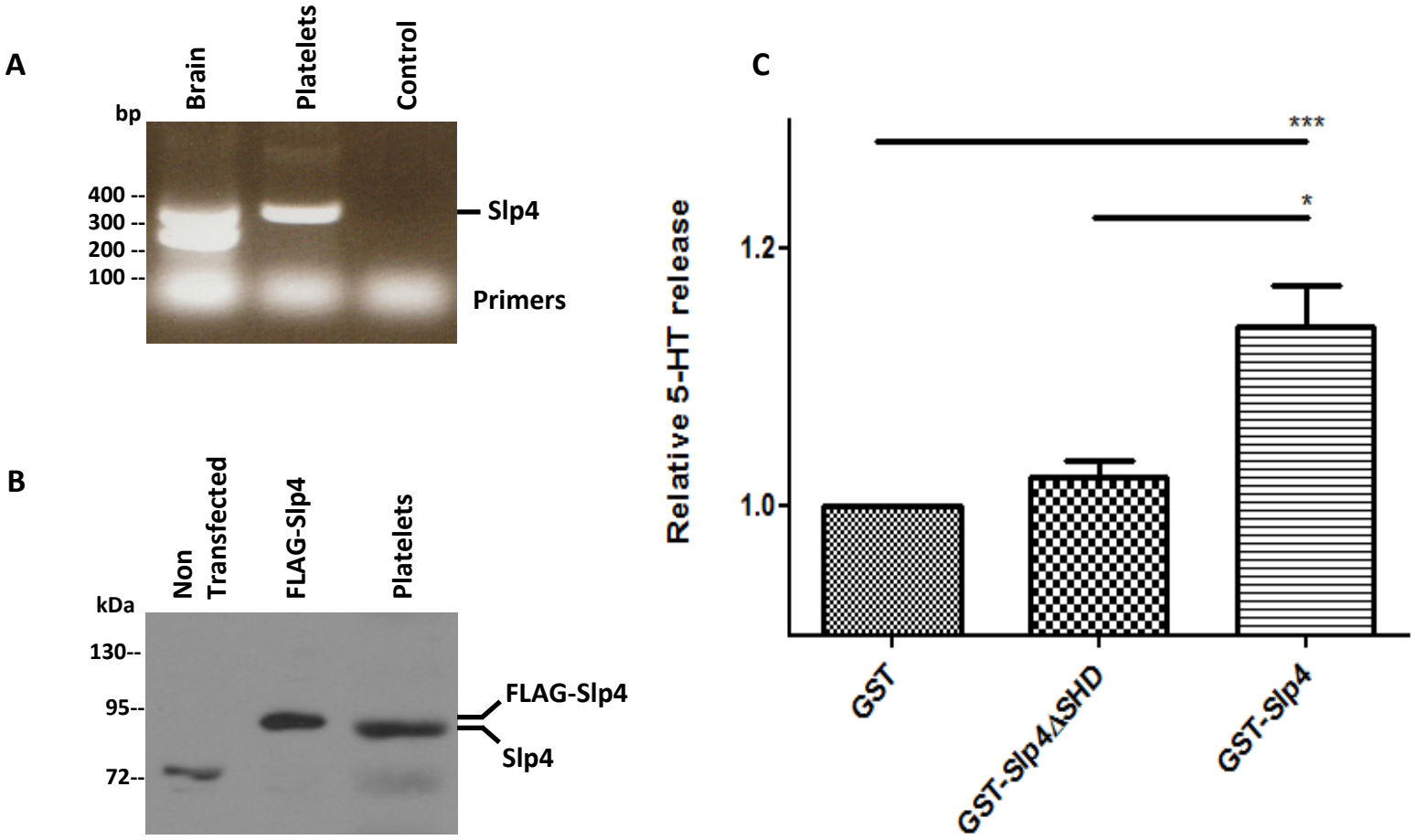

A
Brain
Platelets
Control
bp
400 --
300 --
200 --
100 --
Slp4
Primers
B
Non Transfected
FLAG-Slp4
Platelets
kDa
130--
95--
72--
FLAG-Slp4
Slp4
C
Relative 5-HT release
1.2
1.0
***
*
GST
GST-Slp4ΔSHD
GST-Slp4

Figure 2

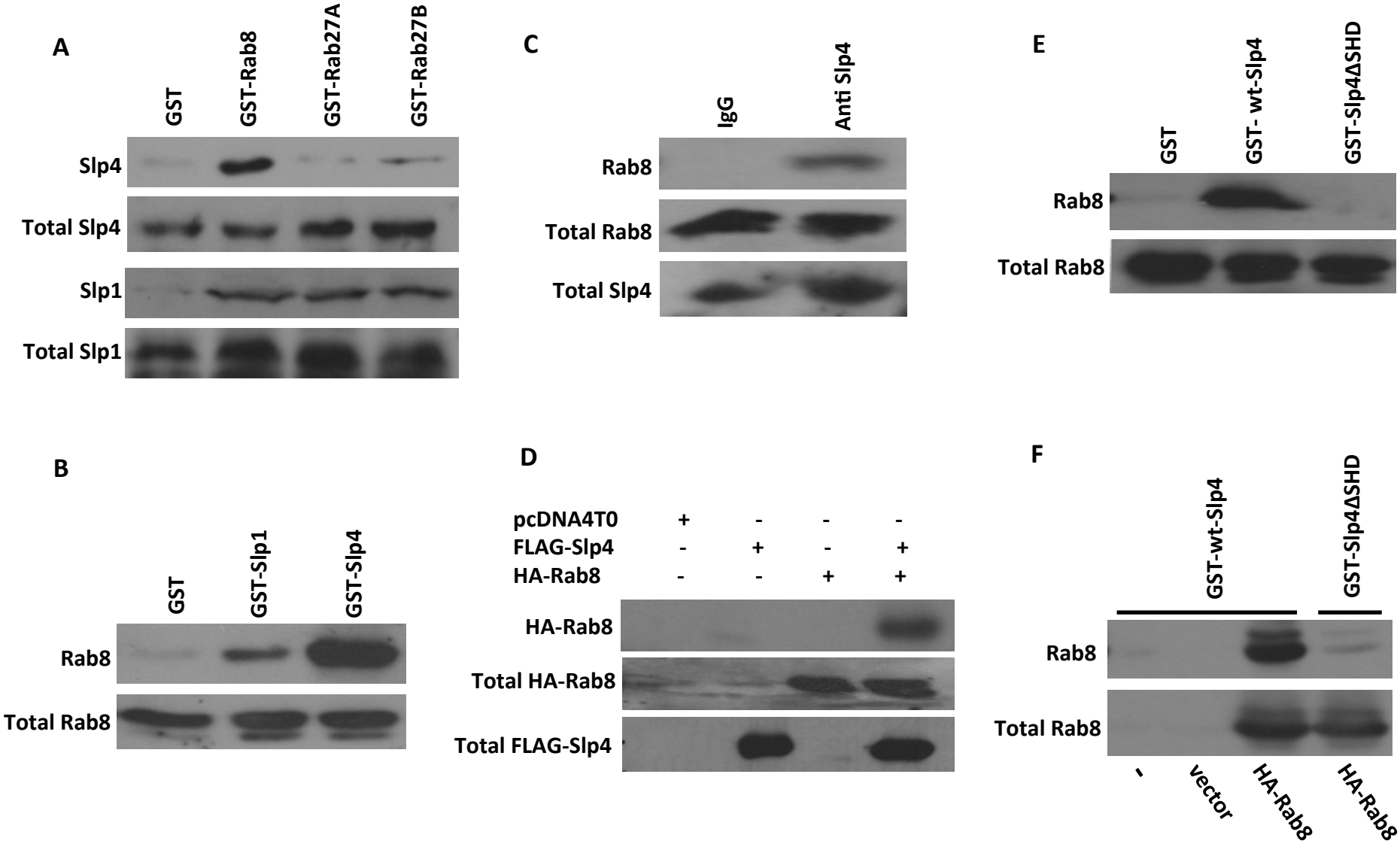

A
GST
GST-Rab8
GST-Rab27A
GST-Rab27B
Slp4
Total Slp4
Slp1
Total Slp1
B
GST
GST-Slp1
GST-Slp4
Rab8
Total Rab8
C
IgG
Anti Slp4
Rab8
Total Rab8
Total Slp4
D
pcDNA4T0 + - - -
FLAG-Slp4 - + - +
HA-Rab8 - - + +
HA-Rab8
Total HA-Rab8
Total FLAG-Slp4
E
GST
GST- wt-Slp4
GST-Slp4ΔSHD
Rab8
Total Rab8
F
GST-wt-Slp4
GST-Slp4ΔSHD
Rab8
Total Rab8
-
vector
HA-Rab8
HA-Rab8

Figure 3

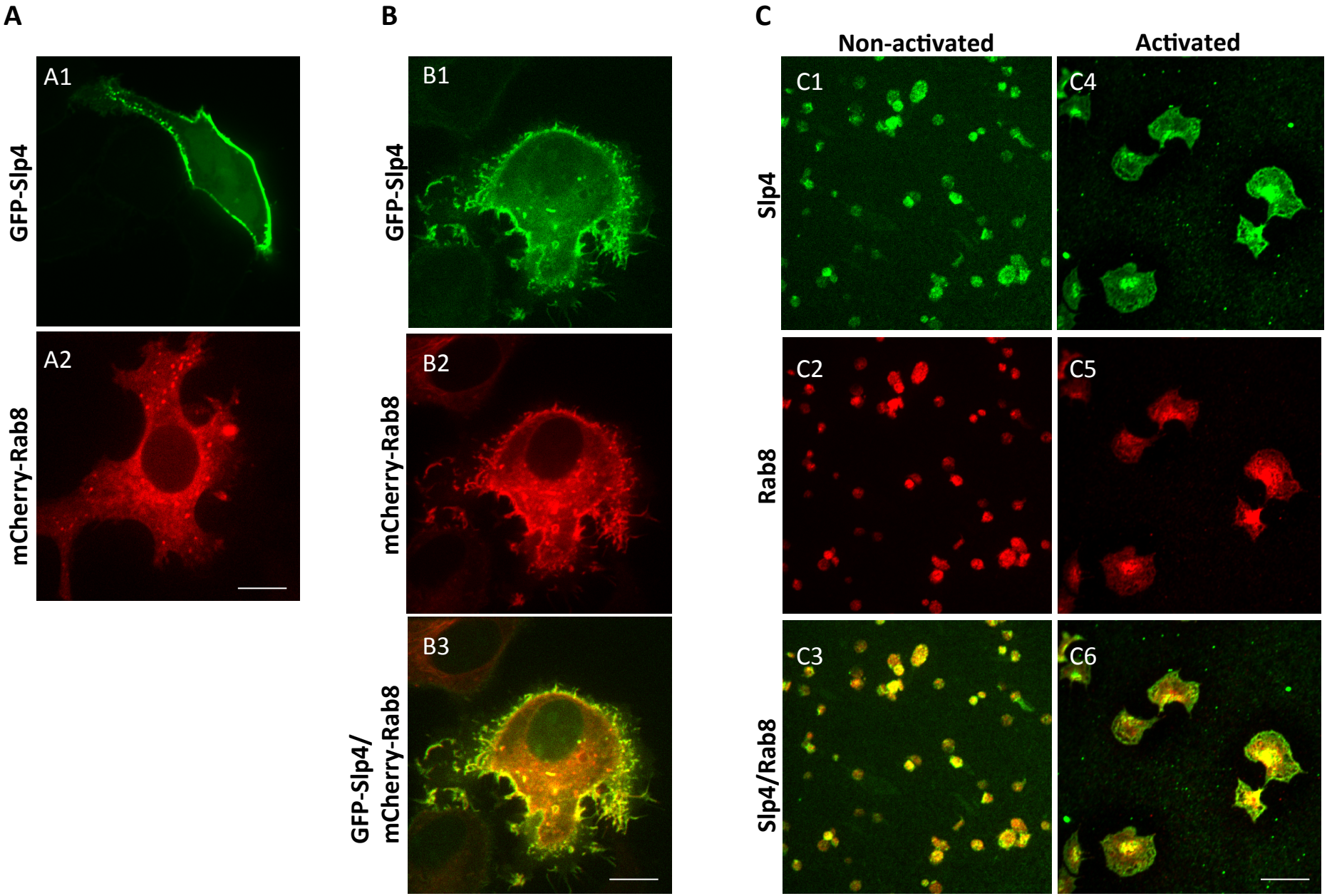
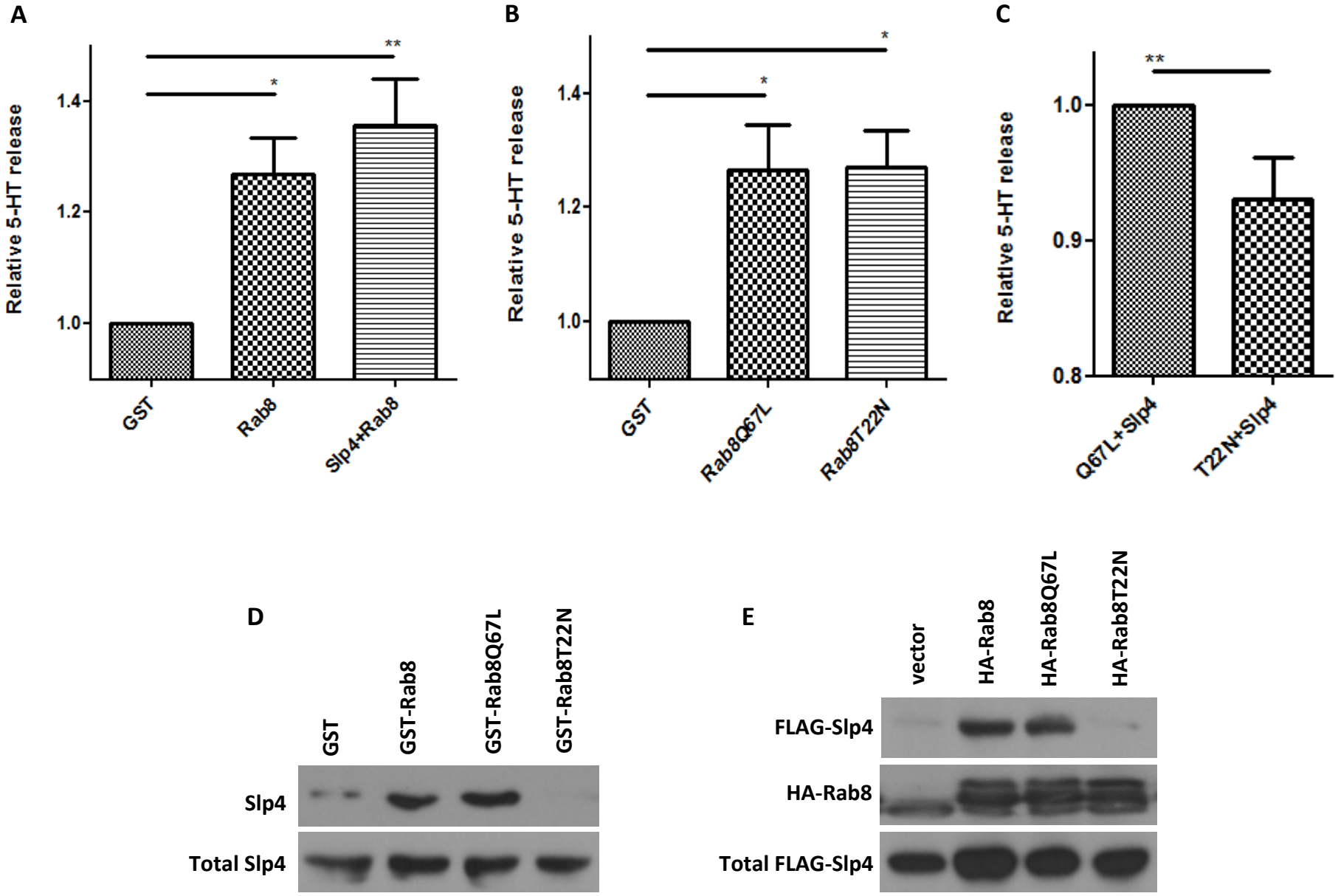

A
B
C
Non-activated
Activated
A1
A2
B1
B2
B3
C1
C2
C3
C4
C5
C6
GFP-Slp4
mCherry-Rab8
GFP-Slp4/
mCherry-Rab8
Slp4
Rab8
Slp4/Rab8

Figure 4


A
Relative 5-HT release
1.4
1.2
1.0
*
**
GST
Rab8
Slp4+Rab8
B
Relative 5-HT release
1.4
1.2
1.0
*
*
GST
Rab8Q67L
Rab8T22N
C
Relative 5-HT release
1.0
0.9
0.8
**
Q67L+Slp4
T22N+Slp4
D
GST
GST-Rab8
GST-Rab8Q67L
GST-Rab8T22N
Slp4
Total Slp4
E
vector
HA-Rab8
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HA-Rab8T22N
FLAG-Slp4
HA-Rab8
Total FLAG-Slp4

Supporting Information for Hampson et al.

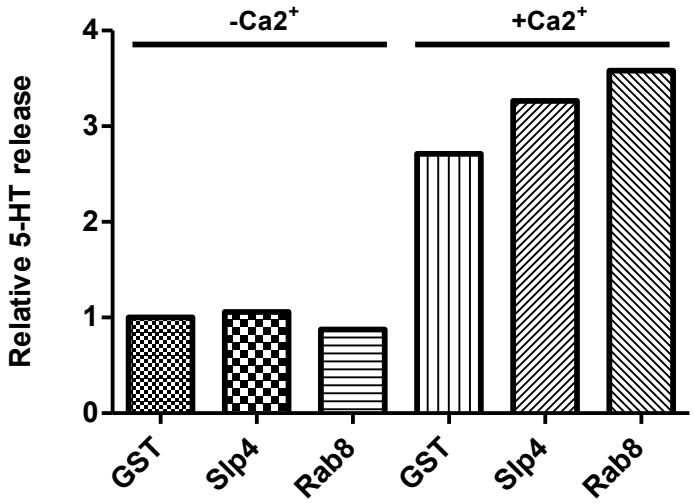

Figure S1. Effects of Slp4 and Rab8 on granule release in the absence of Ca^{2+}.
Permeabilized platelets were incubated with 1 μM final concentration of recombinant GST, GST-Slp4, or GST-Rab8 as indicated. Platelets were then incubated in the absence or presence of approximately 1.3 μM free Ca^{2+} at 30 degrees for 2.0 minutes as indicated. Released 5-HT was determined and normalized to the GST control (without Ca^{2+}) (n=1, performed in triplicate).

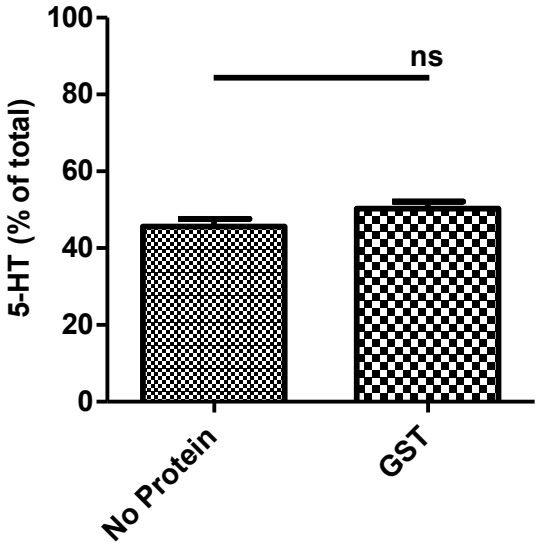

Figure S2. Effects of GST alone on Ca^{2+}-induced granule release. Permeabilized platelets were incubated without (no protein) or with 1 μM final concentration of recombinant GST (GST). Platelets were then incubated for 2.0 minutes at 30 degrees in the presence of approximately 1.3 μM free Ca^{2+}. 5-HT release was determined and expressed as a percentage of total 5-HT. The results shown represent means of three independent experiments performed in triplicate. Addition of GST alone had no significant effect on Ca^{2+}-induced 5-HT release.

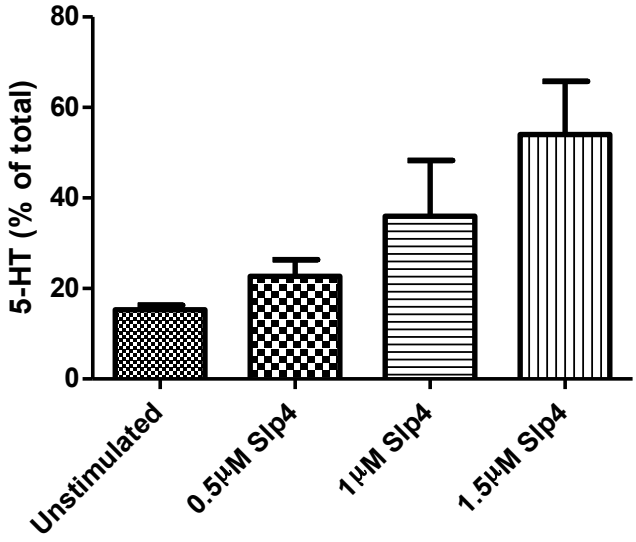

Figure S3. Effects of increasing concentrations of Slp4 on 5-HT release.
Permeabilized platelets were incubated without or with increasing concentrations of GST-Slp4. Granule release was induced by addition of approximately 1.3 μM free Ca^{2+} to Slp4-treated samples. Data from four independent experiments is shown.

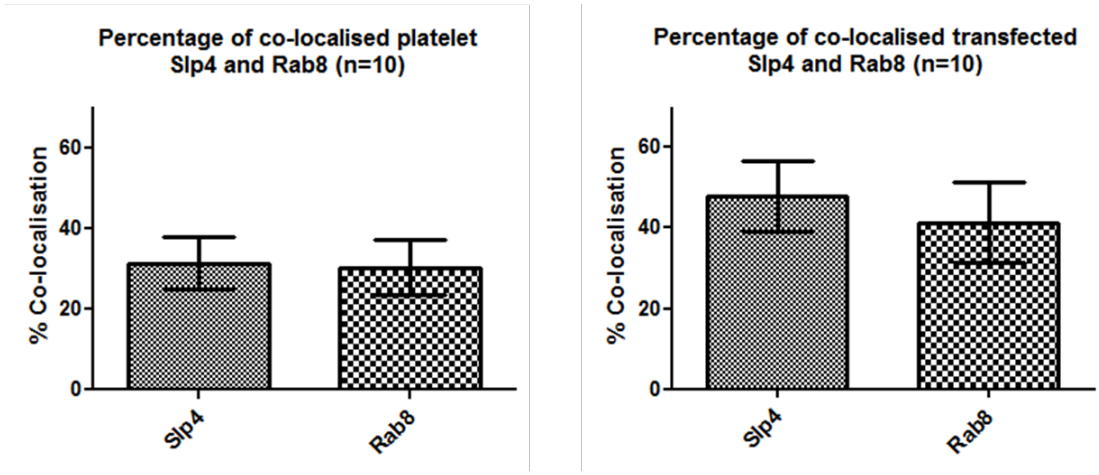

Figure S4. Quantitation of co-localisation of Slp4 and Rab8 in platelets and transfected cells.
Platelets spread on glass were fixed and permeabilized, and Slp4 and Rab8 were stained using specific antibodies. HeLa cells were transfected with GFP-Slp4 and mCherry-Rab8. Images were obtained and the raw intensities of Slp4 and Rab8 signals in single cells were measured in the green and red channels by manually drawing regions of interests around the stained cellular areas using the ImageJ program. The raw intensity of the yellow co-localised signal was then measured in each channel and the percentage co-localisation was calculated.

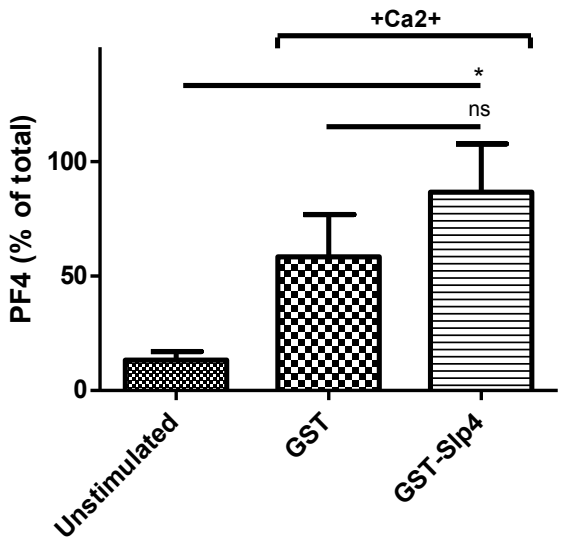

Figure S5. Effects of Slp4 on PF4 release.

Permeabilized platelets were incubated with GST or GST-Slp4. Granule release was induced by addition of Ca^{2+} as indicated. Levels of released Platelet factor 4 (PF4) were determined in the supernatant using an ELISA kit (Human CXCL4/PF4 kit, R&D Systems). Shown data represents five independent experiments.