CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES INHIBIT BINDING OF 14-3-3 TO THE GTPASE-ACTIVATING PROTEIN RAP1GAP2 IN PLATELETS

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GTPase-activating proteins are required to terminate signaling by Rap1, a small guanine-nucleotide binding protein that controls integrin activity and cell adhesion. Recently, we identified Rap1GAP2, a GTPase-activating protein of Rap1 in platelets. Here we show that 14-3-3 proteins interact with phosphorylated serine 9 at the N-terminus of Rap1GAP2. Platelet activation by ADP and thrombin enhances serine 9 phosphorylation and increases 14-3-3 binding to endogenous Rap1GAP2. Conversely, inhibition of platelets by endothelium-derived factors nitric oxide and prostacyclin disrupts 14-3-3 binding. These effects are mediated by cGMP- and cAMP-dependent protein kinases that phosphorylate Rap1GAP2 at serine 7, adjacent to the 14-3-3 binding site. 14-3-3 binding does not change the GTPase-activating function of Rap1GAP2 *in vitro*. However, 14-3-3 binding attenuates Rap1GAP2 mediated inhibition of cell adhesion. Our findings define a novel crossover point of activatory and inhibitory signaling pathways in platelets.

Platelets are involved in many physiological and pathological events in the vascular system including hemostasis and thrombosis as well as inflammation, angiogenesis and metastasis. Endothelium-derived messenger molecules nitric oxide (NO) and prostacyclin (PGI_2) initiate two major inhibitory signaling pathways in platelets. NO and PGI_2 activate platelet guanylyl- and adenylyl cyclases to produce cGMP and cAMP. In consequence cGMP and cAMP activate cGMP- and cAMP-dependent protein kinases (cGK/PKG and cAK/PKA) that phosphorylate substrate proteins leading to inhibition of platelet activation, adhesion and aggregation (1-3). To date only few substrates of cGK/cAKs in platelets have been characterized and the mechanisms mediating platelet inhibition downstream of the substrates are largely unknown. We and others have recently detected that cGK and cAK efficiently block agonist-induced formation of Rap1-GTP in platelets (4,5). Rap1 is a guanine-nucleotide binding protein that regulates integrin functions and plays a pivotal role in adhesion of many cell types (6-9). In platelets Rap1 is required for adhesion, aggregation and thrombus formation (10). Since Rap1 controls integrin function and platelet aggregation, Rap1 inhibition might play a central role in platelet inhibition by NO and PGI_2. Rap1 cycles between an active GTP-bound and an inactive GDP-bound conformation. The transition between these two states is controlled by unique guanine-nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP).

In a previous study we identified Rap1GAP2, the only known GAP of Rap1 in platelets (11). Furthermore, *in vitro* experiments provided prelimary evidence for cGK- and cAK-mediated phosphorylation of Rap1GAP2 (11). Rap1GAP2 contains a central conserved GAP domain as well as large N- and C-terminal regions of unknown function. We hypothesized that these regions might be involved in protein/protein interactions and performed a genetic screening in yeast to identify Rap1GAP2-interacting proteins. In the present work we establish the phosphoserine/phosphothreonine-binding protein 14-3-3 as major interaction partner of Rap1GAP2 and map its binding site to the N-terminus of Rap1GAP2. We show that the formation of the Rap1GAP2/14-3-3 protein complex is regulated by two phosphorylation events in platelets. ADP and thrombin stimulate phosphorylation of the 14-3-3 binding serine residue, whereas the previously described phosphorylation of Rap1GAP2 by cGK

and cAK inhibits 14-3-3 binding. Our data suggest that Rap1GAP2 represents an intersection point of activatory and inhibitory pathways in platelets.

EXPERIMENTAL PROCEDURES

Materials

Human thrombin, ADP, wortmannin, YC-1, DEA-NOate, DETA-NOate, sodium-nitroprusside (SNP) and forskolin were obtained from Sigma (Taufkirchen, Germany). Human fibronectin was purchased from Calbiochem (Merck, Darmstadt, Germany). 8-pCPT-cGMP, 8-Br-cAMP, Rp-8-pCPT-cGMPS and Rp-8-Br-cAMPS were from Biolog (Bremen, Germany), prostacyclin (PGI_2) was from Cayman Chemical (Ann Arbor, USA), LY294002 and triciribine were from Biomol (Hamburg, Germany), bisindolylmaleimide I, rottlerin, PKCα and PKCδ were from Axxora (Lörrach, Germany), PKB was from Jena Bioscience (Jena, Germany) and protein phosphatase 1 was obtained from New England Biolabs (Frankfurt, Germany).

To detect Rap1GAP2 a previously described polyclonal antibody (11) was used. Commercially available antibodies were used for detection of 14-3-3 (H-8, K-19, Santa Cruz), HA-tag (HA.11, Covance, Princeton, New Jersey), myc-tag (4A6 from Upstate, Dundee, UK or 9E10 from Santa Cruz Biotechnology, Santa Cruz, California) and FLAG-tag (M2, Sigma). HRP-coupled goat-anti-rabbit and goat-anti-mouse were from Dianova (Hamburg, Germany).

Cloning of Rap1GAP2 was described before (11). In the present work only the Rap1GAP2a isoform was used. Rap1GAP2 was FLAG-tagged at the C-terminus and expressed using the mammalian expression vector pcDNA4/TO (Invitrogen, Karlsruhe, Germany). Site-directed mutagenesis was performed by polymerase chain reaction amplification of Rap1GAP2-FLAG-pcDNA4/TO using mutagenic primer pairs, Pfu DNA polymerase (Stratagene, La Jolla, USA), digestion with DpnI (Fermentas, St. Leon-Rot, Germany) and transformation in XL-10 Gold bacteria. Full size 14-3-3β cDNA, obtained from the yeast-two-hybrid screen, was cloned together with a C-terminal myc-epitope into the mammalian expression vector pcDNA4/TO (Invitrogen). To produce purified GST-tagged 14-3-3β and 14-3-3ζ, full-size cDNAs, obtained from the yeast-two-hybrid screen, were cloned into pGEX-4T3 vector (GE Healthcare, Freiburg, Germany). For GST pull-down assay, full-length GST-14-3-3β and GST-14-3-3ζ were expressed in *E. coli* BL21. The resultant GST fusion proteins were purified on GSH-Sepharose.

Rap1 and Rap1GAP1 constructs were kindly provided by J. Bos (Department of Physiological Chemistry, Utrecht, The Netherlands).

Yeast-two-hybrid screening

The MATCHMAKER Two-Hybrid System 3 (Clontech-Takara Bio, Saint-Germain-en-Laye, France) was used for yeast-two-hybrid screening. Full-length Rap1GAP2 was subcloned into pGBKT7 bait vector, creating a fusion protein with the Gal4 DNA-binding domain. The bait vector and 3.5 x 10^6 cfu of a human adult brain cDNA library cloned into pACT2 prey vector were introduced into the AH109 yeast strain. A high-stringency screen was performed according to the manufacturer's instruction.

Cell culture and transfection

COS-1 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C and ambient air supplemented with 5% CO_2. Transient transfections of COS-1 cells were performed with DEAE-dextran. In brief, 6x10^5 cells were seeded per 10 cm dish. On the following day cells were washed with PBS, and expression plasmids were applied in 5.7 ml serum-free medium mixed with 300 µl of DEAE-dextran (1 mg/ml stock) and 12 µl chloroquine (100 mM stock). After incubation for 2 hours, the transfection mixture was removed and the cells were cultured in DMEM with 10% FCS for 24-48 hours prior to use. HeLa cells were transiently transfected using Metafectene (Biontex, Martinsried, Germany) according to the manufacturer's instructions.

Platelet preparation

Venous blood was drawn from healthy volunteers taking no medications, who gave their informed consent according to the declaration of Helsinki. Washed platelets were obtained by sequential centrifugation as previously described (4).

Antibody production

The anti-phospho-Rap1GAP2 pS7 and pS9 antibodies were produced using the phosphorylated MFGRKRpSVSFGGFC or MFGRKRSVpSFGGFC peptides conjugated to KLH via cysteine obtained from Schafer-N (Copenhagen, Denmark). Immunization of rabbits

and subsequent purification of the antipeptide antibody with immunizing peptides and the non-phosphorylated MFGRKRSVSFGGFC peptide was performed by ImmunoGlobe Antikörpertechnik GmbH (Himmelstadt, Germany).

Preparation of cell lysates

Adherent COS-1 or HeLa cells were washed 3 times with PBS and detached by scraping with a rubber policeman. The lysates were prepared by resuspending washed platelets or washed cells in ice-cold lysis-buffer (50 mM Tris-HCl pH 7.5, 1% Triton, 150 mM NaCl, 5 mM $MgCl_2$ with phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF, 5 mM $Na_4P_2O_7$) and protease inhibitors (2 µg/µl aprotinin, 1 µg/µl leupeptin, 0.1 mM PMSF). After 20 minutes, the lysates were centrifuged at 14000*g* for 20 minutes to remove insoluble material.

Immunoprecipitation, in vitro phosphorylation and pull-down experiments

Proteins were immunoprecipitated from 500 µl lysate by addition of 5 µl of ANTI-FLAG M2 Affinity Gel (Sigma) or 2 µg of anti-myc antibody (Upstate) at 4 °C for 2 h. In case of immunoprecipitation with anti-myc after 1h 7 µl of Protein A/G Agarose (Santa Cruz Biotechnology) were added. In vitro phosphorylation of precipitated proteins was performed as described (11) using purified recombinant protein kinases. In pull-down experiments 5 µl of glutathione sepharose bead suspension (GE Healthcare) saturated with GST-14-3-3β, GST-14-3-3ζ or GST-Rip2 were added to 500 µl of platelet or cell lysate and incubated at 4 °C for 2 hours or overnight. The beads were washed 5 times with lysis-buffer before adding 20 µl 3x SDS-buffer and boiling for 5 minutes. Proteins were separated by SDS-PAGE and identified by immunoblotting as described before (11). Shown data are repesentative of at least 3 independent experiments.

Phospho-peptide binding assays

Peptides with the sequences MFGRKRpSVSFGGFC (pS7), MFGRKRSVpSFGGFC (pS9) and MFGRKRpSVpSFGGFC (pS7-pS9) corresponding to the N-terminus of Rap1GAP2 coupled to sepharose beads via cysteine were obtained from Immunoglobe Antikörpertechnik GmbH. Peptide beads were incubated with 100 ng of purified recombinant GST-14-3-3β in 500 µl PBS buffer with 0.1 % Tween 20, 0.3 % Triton-X-100 and 1 % BSA for 15 min at 4 °C. Then beads were washed with the same buffer lacking BSA and bound GST-14-3-3 was eluted by boiling in SDS-buffer and analyzed by SDS-PAGE and immunoblot. Shown data are repesentative of 3 independent experiments.

In vitro GAP assay

The coding sequence of amino acids 1-167 of human Rap1B was fused at the N-terminus to a hexahistidine tag in the bacterial expression vector pET28 (Merck). Recombinant His-Rap1B was purified from *E.coli* using Ni-NTA. For GTP loading 25 µM His-Rap1B was incubated with 125 nM ^{32}P-GTP (800 Ci/mmol, 10 µCi/µl, obtained from Hartmann Analytic, Braunschweig, Germany) in the presence of 25 µM cold GTP, 10 mM EDTA, 1 mM DTE and 30 mM Tris/HCl pH 7.5 for 1 h at RT. The exchange reaction was stopped by adding 20 mM $MgCl_2$ and non-incorporated nucleotides were removed by gel filtration using NAP-5 columns (GE Healthcare). FLAG-tagged Rap1GAP2 proteins were purified from transfected HeLa cells using ANTI-FLAG M2 Affinity Gel. Rap1GAP2 proteins bound to beads were incubated with purified ^{32}P-GTP-loaded His-Rap1B in a buffer containing 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$ and 1 mM DTE in 50 µl aliquots at 25°C. After different times 5 µl aliquots were removed and mixed with 395 µl of a cold 5 % (w/v) charcoal suspension in 20 mM phosphoric acid. After centrifugation 200 µl supernatant was removed, mixed with scintillation fluid and counted.

Adhesion assays

96-well plates were coated overnight at 4 °C with 20 µg/ml fibronectin in PBS, washed with PBS and blocked for 1 h at room temperature with 0.5% BSA in PBS. After washing 50 µl DMEM was added per well. HeLa cells were transiently transfected with pRluc (BioSignal Packard, Meriden, USA) and wild-type or mutant Rap1GAP2. Cells were washed, detached with 0.05% ETDA in PBS, spun down and resuspended in DMEM containing 10% FCS at a concentration of 2 x 10^5 cells/ml. 50 µl of cell suspension was added per well of prepared plate. After spinning down the cells for 1 minute at 10*g*, they were allowed to adhere for 50 minutes at 37 °C. Non-adherent cells were removed by washing with prewarmed 0.5% BSA in PBS.

Adherent cells were lysed and subjected to luciferase assay using Renilla Luciferase Assay System (Promega, Mannheim, Germany) according to the manufacturer's instructions. Expression levels of transfected constructs were confirmed by immunoblotting. Numbers of bound cells were calculated and corrected for transfection efficiency by measuring luciferase activity of total input cells.

RESULTS

Rap1GAP2 interacts with 14-3-3

To identify Rap1GAP2-associated proteins we performed yeast two-hybrid screening. We used full-length wild-type Rap1GAP2a, the predominant splice isoform of Rap1GAP2 in platelets, as a bait in a screen of a two-hybrid human adult brain cDNA library. Out of 112 positive clones 18 were identified as 14-3-3β and 2 clones were 14-3-3ζ. 14-3-3 proteins are phosphoserine/phosphothreonine-binding molecules that mediate phosphorylation dependent effects of signaling (12-16). To verify that Rap1GAP2 and 14-3-3 interact in intact mammalian cells we performed co-immunoprecipitation experiments. COS-1 cells were transfected with epitope tagged versions of Rap1GAP2 and 14-3-3β. Immunoprecipitates of 14-3-3 were examined for the presence of Rap1GAP2 by immunoblot. Rap1GAP2 was present only in samples containing 14-3-3 (Fig. 1A, first lane). We confirmed binding to 14-3-3 in a reverse experiment, using FLAG-tagged Rap1GAP2 (Fig. 1A, third lane).

Rap1GAP2 is the so far only known GAP of Rap1 in platelets (11). To verify the Rap1GAP2/14-3-3 interaction in platelets we constructed GST-fusion proteins of 14-3-3β and ζ isoforms (both isoforms were identified in the initial yeast two-hybrid screening) and performed pull-down experiments. Both, 14-3-3β and ζ, bound to endogenous Rap1GAP2 with equal potency whereas a control GST-GFP fusion protein did not bind Rap1GAP2 (Fig. 1B). To determine if 14-3-3 proteins also bind to other RapGAPs we tested Rap1GAP1, a closely related protein that exhibits about 50% overall identity with Rap1GAP2 (11,17). No interaction between Rap1GAP1 and 14-3-3β could be detected in co-immunoprecipitation experiments in transfected HeLa cells (Fig. 1C).

To determine the 14-3-3 binding site(s) within Rap1GAP2 we generated mutants containing either the N-terminal 121 residues (N-term) or residues 121 to 715 (C-term) of Rap1GAP2. Only the N-terminal Rap1GAP2 construct, but not the C-terminal construct, co-precipitated with 14-3-3 from transfected HeLa cell lysates (Fig. 1D, second and third lane). To define the 14-3-3 interacting region of Rap1GAP2 more precisely we looked for previously described consensus 14-3-3 binding sites (13,18). The N-terminus of Rap1GAP2 exhibits a region resembling a mode I consensus motif for 14-3-3 binding (RSXpSXP, where „X" denotes „any amino acid residue" and pS represents phosphorylated serine). The Rap1GAP2 sequence RSVSFG (residues 6-11) differs only in the final glycine residue from the mode I consensus. We tested the role of this motif for 14-3-3 binding in co-immunoprecipitation experiments. Deletion of the 13 N-terminal residues of Rap1GAP2 resulted in loss of 14-3-3 binding (ΔN13 in Fig. 1D). We conclude that Rap1GAP2 contains one 14-3-3 binding site that localizes to the very N-terminus.

Characterization of the 14-3-3 binding motif of Rap1GAP2

Alignment of the Rap1GAP2 N-terminal sequence with mode I and mode II consensus sequences for 14-3-3 binding is shown in Figure 2A. The mode I motif matches best to the Rap1GAP2 N-terminus and both motifs predict serine 9 as the critical phosphorylated serine residue required for 14-3-3 binding. To test this prediction we mutated serine 9 of Rap1GAP2 and performed co-immunoprecipitation experiments with 14-3-3β. Mutation of serine 9 to alanine (S9A) completely abolished 14-3-3 binding (Fig. 2B, third lane). Neither mutation of Rap1GAP2 serine 9 to glutamic acid (S9E, Fig. 2B, fourth lane) nor to aspartic acid (not shown) restored 14-3-3 binding. This observation agrees with the notion that only phosphorylated serine or threonine residues interact with the basic pocket in the 14-3-3 binding groove (19).

The 14-3-3 binding motif of Rap1GAP2 includes serine 7 that we have previously identified as phosphorylation site for cyclic nucleotide dependent protein kinases cGK and cAK *in vitro* (11). We generated point mutants to determine if Rap1GAP2 serine 7 phosphorylation affects 14-3-3 binding. A serine 7 to alanine mutant (S7A) coprecipitated 14-3-3β similar to wild-type Rap1GAP2 (Fig. 2C). In contrast, a glutamic acid mutant (S7E) did not bind 14-3-3 at all (Fig. 2C). These results indicate that serine 7 is

not essential for 14-3-3 binding, however, phosphorylation of serine 7 might inhibit binding. To verify the roles of phosphorylated serines 7 and 9 of Rap1GAP2 for 14-3-3 binding we generated short peptides corresponding to the N-terminus of Rap1GAP2 containing either phosphorylated serine 7 or 9, or both residues phosphorylated. These peptides were coupled to beads and used to pull-down purified recombinant 14-3-3β. Only the serine 9 phosphorylated peptide bound to 14-3-3, whereas peptides containing phosphorylated serine 7 either alone or in combination with phosphorylated serine 9, did not bind to 14-3-3 (Fig. 2D). To test if binding of endogenous 14-3-3 to Rap1GAP2 would also depend on serines 7 and 9 we transfected HeLa cells with wild-type or mutant Rap1GAP2. S7A mutant Rap1GAP2 precipitated endogenous 14-3-3 similar to wild-type Rap1GAP2, whereas phosphomimetic mutants of serine 7 either to glutamic acid (Fig. 2E) or to aspartic acid (data not shown) did not bind to 14-3-3. Mutation of serine 9 abolished 14-3-3 binding, as observed before (Fig. 2E). To confirm the role of serines 7 and 9 of Rap1GAP2 for 14-3-3 binding in a vice versa aproach we performed pull-down experiments using 14-3-3β and ζ to precipitate the different Rap1GAP2 mutants. Both 14-3-3 isoforms precipitated wild-type and S7A mutant Rap1GAP2 from cell lysates equally well (Fig. 2F). All other mutations of serines 7 or 9 abolished binding of 14-3-3β and ζ. These data confirm that serine 9 is required for binding of 14-3-3 proteins to Rap1GAP2. Furthermore, serine 7 appears to represent a regulatory site that inhibits 14-3-3 binding to Rap1GAP2 in case of phosphorylation of this site.

Platelet activation induces Rap1GAP2 serine 9 phosphorylation and stimulates 14-3-3 binding

To confirm the physiological significance of our findings on the regulation of the Rap1GAP2 14-3-3 interaction by phosphorylation we developed specific antibodies against the phosphorylated serine 7 and serine 9 sites. To test the specificity of the phospho-serine 9 antibody we expressed wild-type Rap1GAP2 and phosphorylation site mutants in HeLa cells. Immunoblotting with the pS9 antibody resulted in a single strong band in HeLa cells transfected with wild-type Rap1GAP2 which indicates that Rap1GAP2 is phosphorylated on serine 9 already in the basal state (Fig. 3A). In case of mutation of serine 9 to alanine or to glutamic acid the pS9 antibody did not detect Rap1GAP2 (Fig. 3A). Mutations of serine 7 reduced the signal of the pS9 antibody, especially if phospho-mimetic residues were introduced. The reduction of the signal can be explained by the fact that serine 7 is included in the epitope recognized by the pS9 antibody (Fig. 3A). Treatment of purified Rap1GAP2 from transfected HeLa cells with protein phosphatase 1 resulted in a loss of the pS9 signal (Fig. 3B). Taken together the data confirm that the pS9 antibody is specific for serine 9 phosphorylated Rap1GAP2, even if the absence of a serine residue at position 7 might reduce binding of the pS9 antibody.

Next we tested the pS9 antibody in freshly isolated human platelets. Immunoblotting of resting platelets revealed a consistent basal serine 9 phosphorylation of Rap1GAP2 (Fig. 3C, top panel, first lane). Treatment of platelets with ADP or thrombin increased the phosphorylation of serine 9 (Fig. 3C, top panel, second and third lane). In parallel, we performed pull-down experiments using GST-14-3-3 to precipitate endogenous Rap1GAP2 from lysates of ADP or thrombin treated platelets. Increased phosphorylation of Rap1GAP2 on serine 9 correlated with an increased binding of Rap1GAP2 to 14-3-3 (Fig. 3C, third panel). GST alone did not pull down any Rap1GAP2 which confirms the specificity of the observed effect (Fig. 3C, bottom).

To obtain conclusive evidence that endogenous 14-3-3 and Rap1GAP2 proteins interact we developed a novel precipitation approach. Since neither our Rap1GAP2 antibody nor any of the tested commercially available 14-3-3 antibodies were able to immunoprecipitate their antigens efficiently from platelets, we used a second Rap1GAP2 interacting protein (Rip2) characterized in our group (unpublished data) to precipitate sufficient amounts of endogenous Rap1GAP2. In a control experiment in COS-1 cells we showed that a GST-Rip2 fusion protein precipitates 14-3-3 only in the presence of Rap1GAP2 (Fig. 3D, left panel). This result confirms that Rip2 cannot bind to 14-3-3 by itself. Accordingly, we used GST-Rip2 to pull-down endogenous Rap1GAP2 from platelet lysates and were able to co-precipitate endogenous 14-3-3 (Fig. 3D, right panel). A control experiment confirmed that GST alone did not bind Rap1GAP2 or 14-3-3. Thrombin treatment of platelets resulted in an increased interaction of 14-3-3 and Rap1GAP2 at the endogenous level (Fig. 3D, right panel). From these experiments we

conclude that an increased level of Rap1GAP2 serine 9 phosphorylation during platelet activation leads to enhanced binding of 14-3-3 to Rap1GAP2.

Kinases involved in Rap1GAP2 serine 9 phosphorylation

The serine/threonine kinases known to be activated in platelets in response to ADP and thrombin are PKCs, including PKCδ (20,21), PKB/Akt, a downstream kinase of phosphatidylinositol-3-kinase (PI3K) (22-24), PKD, a PI3K and PKC-dependent kinase (25), and GSK3, which is also PI3K-dependent (26). Treatment of platelets with the PI3K inhibitors wortmannin or LY294002 did not affect levels of Rap1GAP2 serine 9 phosphorylation (Fig. 4A). Also, PKB inhibitor triciribine and PKC inhibitors bisindolylmaleimide I and rottlerin, a compound more specific for PKCδ, did not induce any significant differences in serine 9 phosphorylation (Fig. 4A and B). Phosphorylation experiments using purified proteins showed that a N-terminal construct of Rap1GAP2 containing the 14-3-3 binding site was only phosphorylated by cAMP-dependent protein kinase, on serine 7 as shown before (11), but not by PKB, PKCα or PKCδ (Fig. 4C). The scansite algorithm (27) predicted calmodulin-dependent protein kinase II (CaMK II) as another possible serine 9 kinase. However, the CaMK inhibitors KN62 and KN93 did not inhibit serine 9 phosphorylation (data not shown). We conclude that Rap1GAP2 is phosphorylated on serine 9 by a so far uncharacterized kinase activated by ADP and thrombin signaling pathways in platelets.

Phosphorylation of Rap1GAP2 serine 7 in platelets

To elucidate the role of serine 7 phosphorylation in respect of the Rap1GAP2/14-3-3 protein complex a phosphospecific antibody against the serine 7 site of Rap1GAP2 was generated. The specificity of this new antibody was tested using lysates of HeLa cells transfected with different phosphorylation site mutants of Rap1GAP2. Before lysis cells were treated without or with forskolin to activate endogenous cAMP production by adenylyl cyclase and thus to induce Rap1GAP2 phosphorylation by endogenous cAK. In immunoblots the pS7-antibody detected some wild-type Rap1GAP2 already in untreated cells and addition of forskolin strongly enhanced the signal (Fig. 5A). Mutation of serine 7 either to alanine or to glutamic acid abolished binding of the pS7-antibody, whereas mutations of serine 9 did not (Fig. 5A). Basal serine 7 phosphorylation in the absence of forskolin was detected in wild-type and S9E but not in S9A mutants suggesting that phosphorylated serine 9, present in wild-type (Fig. 3A) and mimicked by the S9E mutant, might enhance secondary phosphorylation of serine 7 by cAK. Preabsorption of the pS7-antibody with the antigenic peptide resulted in loss of the signal obtained with wild-type Rap1GAP2 (data not shown). These experiments prove the specificity of the pS7-antibody towards serine 7 phosphorylated Rap1GAP2.

Next, we used the pS7-antibody to monitor the phosphorylation state of endogenous Rap1GAP2 in freshly isolated human platelets. In untreated platelets no pS7 signal could be detected by immunoblot (Fig. 5B, control lanes). To investigate the contribution of various components of the NO/cGMP pathway to Rap1GAP2 serine 7 phosphorylation we tested effects of NO-donors and an activator of soluble guanylyl cyclase. Both the fast-releasing nitric oxide-donor DEA-NO as well as DETA-NO, a long acting NONOate, induced a dose-dependent increase in Rap1GAP2 serine 7 phosphorylation in platelets (Fig. 5B, first two panels). NO has been shown to exert its inhibitory functions in platelets via cGMP-dependent as well as cGMP-independent pathways. Treatment of platelets with 3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), a specific activator of soluble guanylyl cyclase induced Rap1GAP2 serine 7 phosphorylation (Fig. 5B, third panel) suggesting that NO effects are cGMP-dependent. 8-pCPT-cGMP, a membrane permeable PDE-resistant cGMP analog and activator of cGK, dose dependently induced the phosphorylation of Rap1GAP2 on serine 7 (Fig. 5C, first panel). Direct activation of cAK using 8-Br-cAMP had the same effect (data not shown). Furthermore, Rp-8-pCPT-cGMPS and Rp-8-Br-cAMPS, two inhibitors of cGK and cAK, attenuated serine 7 phosphorylation induced by the NO-releasing substance sodium-nitroprusside (SNP) and by forskolin, respectively. We conclude that NO/cGMP and cAMP signaling pathways induce Rap1GAP2 serine 7 phosphorylation via cGK and cAK in platelets.

We studied the time course of Rap1GAP2 serine 7 phosphorylation in platelets induced by endothelium-derived cyclic nucleotide activators NO and prostacyclin (PGI_2). After treatment of platelets with the NO-releasing substance SNP a pS7 signal appeared after 1 minute reaching a

maximum at 10 minutes followed by a gradual decline (Fig. 5D). PGI_2 induced a similar increase in Rap1GAP2 serine 7 phosphorylation (Fig. 5E). The pS7 signal was already visible after 30 seconds corresponding to a fast receptor-mediated action of PGI_2. Maximum signal intensity was reached after 5 minutes and then Rap1GAP2 phosphorylation diminished until is was completely lost after 90 minutes (Fig. 5E). These data indicate that endogenous Rap1GAP2 is reversibly phosphorylated in response to NO and PGI_2.

Inhibition of 14-3-3 binding to Rap1GAP2 by cyclic nucleotide dependent protein kinases in platelets

To investigate if serine 7 phosphorylation would affect 14-3-3 binding in platelets we performed pull-down assays using recombinant GST-tagged 14-3-3β. SNP treatment drastically reduced binding of 14-3-3 to Rap1GAP2 which correlated with increased serine 7 phosphorylation (Fig. 6A, left panel). A similar result was obtained using the direct cGK activator 8-pCPT-cGMP (Fig. 6A, right panel). Stimulation of cAMP production using PGI_2 or forskolin inhibited the interaction of Rap1GAP2 and 14-3-3 (Fig. 6B). Reduced binding to 14-3-3 was accompanied by increased Rap1GAP2 serine 7 phosphorylation, as before. Since platelet activators enhance binding of 14-3-3 to Rap1GAP2 we asked if cyclic nucleotides could inhibit 14-3-3 binding in the presence of ADP or thrombin. We preincubated platelets with a cGMP analog or with forskolin before adding ADP and thrombin. Both cGMP and cAMP pathways inhibited 14-3-3 binding to Rap1GAP2 in the presence of ADP or thrombin (Fig. 6C). This inhibition again correlated with increased serine 7 phosphorylation of Rap1GAP2. Of note, we observed that thrombin alone induced a slight serine 7 phosphorylation possibly via cross activation of cAMP pathways (Fig. 6C). We conclude that cyclic nucleotides block interaction of 14-3-3 and Rap1GAP2 by phosphorylation of serine 7 via cyclic nucleotide dependent protein kinases.

Binding of 14-3-3 does not affect the catalytic GTPase-activating function of Rap1GAP2 in vitro

Next, we investigated the functional consequences of 14-3-3 binding to Rap1GAP2. Since it is known that 14-3-3 proteins modulate the catalytic activity of some of their targets (13,28), we performed *in vitro* GAP assays. We analyzed if wild-type and S7A mutant Rap1GAP2 bound to 14-3-3 would exhibit differences in their GAP function compared to the S7E, S9A and S9E mutants that cannot bind 14-3-3. During purification of Rap1GAP2 proteins from transfected HeLa cells, wild-type and S7A Rap1GAP2 but not the other mutants remained bound to endogenous HeLa cell derived 14-3-3 (Fig. 7A). In all Rap1GAP2 containing samples Rap1 GTPase activity was detectable, however, no differences between the various mutants were observed (Fig. 7B). Addition of an excess of purified recombinant 14-3-3 did not change the outcome of the GAP assay (data not shown). Also, measurements of Rap1-GTP levels in total lysates of transfected HeLa cells by pull-down assay (4,11) did not reveal any differences between Rap1GAP2 mutants (data not shown). We conclude that 14-3-3 binding does not affect the catalytic activity of Rap1GAP2 *in vitro*.

Binding of 14-3-3 modulates Rap1GAP2 effects on cell adhesion

Rap1GAP proteins are known to inhibit Rap1 and integrin mediated adhesion of intact cells (29). Therefore, we studied effects of 14-3-3 binding to Rap1GAP2 on cell adhesion. HeLa cells expressing endogenous Rap1 and 14-3-3 were seeded onto fibronectin-coated dishes to stimulate integrin-mediated adhesion. As expected, Rap1GAP2 reduced cell adhesion to 50% of mock-transfected cells (Fig. 8, wt). To determine the role of 14-3-3 binding for Rap1GAP2 activity we used the previously described Rap1GAP2 point mutants. Equal expression of Rap1GAP2s was confirmed by immunoblotting. As a further control we checked the expression of integrin β1 which is required for adhesion to fibronectin. Integrin expression was not altered by overexpression of the different Rap1GAP2 mutants (Fig. 8, inset). We observed striking differences in Rap1GAP2 effects on cell adhesion dependent on the capacity of Rap1GAP2 to bind 14-3-3. The S7A mutant of Rap1GAP2 that binds 14-3-3 constitutively, was less active and attenuated cell adhesion only to 80% of control cells (Fig. 8, S7A). In contrast, S7E, S9A and S9E mutants of Rap1GAP2 that do not interact with 14-3-3 inhibited cell adhesion to 30% of control cells. The differences in adhesion between S7E, S9A, S9E and S7A mutant Rap1GAP2 were statistically significant ($P < 0.05$, Bonferroni post-test). Wild-type Rap1GAP2 exhibited an intermediate phenotype compared to S7A and S7E/S9A/S9E mutants. This is probably the result of basal phosphorylation of wild-type Rap1GAP2

on serine 7 in HeLa cells (Fig. 5A) followed by a reduction of 14-3-3 binding. From these experiments we conclude that release of 14-3-3 stimulates Rap1GAP2 mediated inhibition of cell adhesion.

DISCUSSION

We identified 14-3-3 proteins as interaction partners of Rap1GAP2. We show that 14-3-3 binding to Rap1GAP2 is a dynamic process. Both, activatory and inhibitory pathways in platelets converge on the regulation of 14-3-3 binding to Rap1GAP2. Platelet activation by thrombin or ADP promotes 14-3-3 binding by phosphorylation of serine 9 of Rap1GAP2. Endothelium-derived platelet inhibitors NO and PGI_2 induce phosphorylation of Rap1GAP2 on serine 7 resulting in the release of 14-3-3 from Rap1GAP2.

Interaction of Rap1GAP2 and 14-3-3

In the present study we characterize 14-3-3 proteins as interaction partners of Rap1GAP2. All members for the 14-3-3 protein family are highly conserved and share high amino acid identity (30,31). With pull-down experiments we show that Rap1GAP2 binds to 14-3-3β and ζ. In immunoprecipitations from HeLa cells also endogenous 14-3-3ε emerges to interact with Rap1GAP2. Platelets are known to express 14-3-3 isoforms β, γ and ζ and to lower extent also isoforms ε and η (32,33). We did not test if Rap1GAP2 also interacts with γ and η, but it is well possible that these isoforms also interact with Rap1GAP2 since the used 14-3-3 antibodies (K19 and H8) recognize the 14-3-3 family.

Regulation of 14-3-3 binding

14-3-3 binding to proteins is generally thought to be induced by protein kinases phosphorylating specific serine or threonine residues (15). For example, cAK-mediated phosphorylation of the GPIbβ subunit of the von Willebrand receptor has been described to enhance 14-3-3 binding (34). Our experiments prove that serine 9 of Rap1GAP2 forms the phosphorylated residue required for binding of 14-3-3 and that platelet activation by ADP and thrombin further enhances the phosphorylation of serine 9. We excluded possible roles of PI3K, PKB, PKC, PKD, GSK3 and CaMK in Rap1GAP2 serine 9 phosphorylation. Thus, other uncharacterized kinases appear to be involved in ADP and thrombin signaling and platelet activation. The human genome is predicted to express around 400 serine/threonine kinases (35) and only few of these have been studied in detail so far. Serine 7 of Rap1GAP2 represents an additional regulatory site that modulates 14-3-3 binding. We observed an inhibitory effect of cAK- and cGK-mediated phosphorylation of Rap1GAP2 serine 7 on 14-3-3 binding. Serine 7 is located at the -2 position of the 14-3-3 binding serine 9 of Rap1GAP2. The type I consensus motif for 14-3-3 binding contains a serine at the -2 position and a regulatory function of this residue has been described before. For example, 14-3-3 binding to the tumor suppressor protein p53 was abolished by serine phosphorylation at the -2 position (36). Bulavin et al. observed that phosphorylation of the -2 serine residue prevented phosphorylation of the 14-3-3 binding serine in the protein phosphatase Cdc25C resulting in decreased binding of 14-3-3 (37). Our findings on the regulation of the Rap1GAP2/14-3-3 complex via serine 7 phosphorylation substantiate that phosphorylation of the -2 serine residue might represent a more general regulatory mechanism for 14-3-3 binding, as suggested before (14). The 14-3-3 binding site of Rap1GAP2 resembles a type I consensus motif for 14-3-3 binding. Only the proline residue at the +2 position is changed to glycine in Rap1GAP2. The presence of glycine instead of proline at this position was shown to reduce the affinity by around 4-fold (18). This reduced affinity might facilitate regulation of 14-3-3 binding by serine 7 phosphorylation.

Cyclic nucleotide signaling in platelets

cGK and cAK mediate many effects of NO and PGI_2 in platelets. However, only few substrates of cGK/cAK have been verified at the level of endogenous proteins in intact cells. Using a phospho-specific antibody we clearly prove that Rap1GAP2 is a substrate of both kinases in platelets. The kinetics of Rap1GAP2 phosphorylation induced by SNP or PGI_2 closely resemble the time-course of phosphorylation of the actin-associated vasodilator-stimulated phosphoprotein (VASP), a previously established cGK/cAK substrate in platelets and endothelial cells (38,39). This suggests that Rap1GAP2 and VASP are equally accessible for cGK and cAK, in spite of their different subcellular localization. Rap1GAP2 exhibits a granular distribution (11) whereas VASP localizes to actin filaments and focal adhesions in spread platelets (38). Similar to

VASP, Rap1GAP2 phosphorylation is rapidly reversible suggesting a tight control by specific phosphatases. Previously, we established phospho-specific antibodies against VASP as tools for monitoring cyclic nucleotide signaling in platelets and other cells (2,38). In a similar way our phospho-specific antibody for the Rap1GAP2 serine 7 phosphorylation site may be used to detect the endogenous kinase activity of cGK and cAK in platelets. Furthermore, platelet activation can be monitored on the same protein via serine 9. In a recent screen for 14-3-3 binding partners VASP and LASP (40), another cytoskeleton-associated cGK substrate in platelets, were detected (41). These data together with our Rap1GAP2 data hint towards a more general role of 14-3-3s in cyclic nucleotide mediated signaling in platelets.

Function of 14-3-3 binding to Rap1GAP2

14-3-3 binding attenuates Rap1GAP2 effects on cell adhesion. Rap1GAP2 mutants deficient in 14-3-3 binding block cell adhesion more efficiently than 14-3-3 bound Rap1GAP2. However, the mechanism of action of 14-3-3 remains to be determined. We show that 14-3-3 does not impact directly on the catalytic actvity of Rap1GAP2 in vitro. Furthermore, the effects of 14-3-3 binding on cell adhesion are not reflected by changes in total cellular Rap1-GTP levels. We speculate, that 14-3-3 binding affects the subcellular distribution of Rap1GAP2 resulting in local changes of Rap1-GTP levels. Cyclic nucleotide regulated binding of 14-3-3 represents a unique property of Rap1GAP2 since the N-terminal 14-3-3 binding motif is absent in other RapGAPs. This might provide a means for tissue-specific regulation of cell adhesion by cyclic nucleotides. In platelets release of 14-3-3 binding might contribute to the inhibition of thrombus formation by endothelium-derived protective factors.

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FOOTNOTES

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The abbreviations used are: GAP, GTPase-activating protein; NO, nitric oxide; PGI_2, prostacyclin; cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; YC-1, 3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole.

FIGURE LEGENDS

Figure 1. Rap1GAP2 and 14-3-3 associate in intact cells.
A) Co-immunoprecipitation of Rap1GAP2 and 14-3-3β in transfected COS-1 cells. COS-1 cells were transfected with FLAG-tagged Rap1GAP2 and myc-tagged 14-3-3β. After lysis Rap1GAP2 and 14-3-3 were precipitated using tag-specific antibodies (mouse-α-FLAG, mouse-α-myc). Precipitates were analyzed by immunoblot using mouse anti-myc or rabbit anti-Rap1GAP2. The upper panel shows

precipitation results and the two lower panels show controls for transfection efficiency. The broad band of Rap1GAP2 is probably due to extensive posttranslational modifications. Immunoglobulin heavy and light chains (marked with *) are detected in precipitates only where antibodies from the same species (mouse) were used for precipitation as well as for blotting.
B) 14-3-3β and ζ bind to endogenous Rap1GAP2. Equal amounts of GST-fusion proteins of 14-3-3β, 14-3-3ζ and GFP as control were coupled to GSH-Sepharose beads and incubated with platelet lysates. Bound proteins were eluted from the beads and probed with anti-Rap1GAP2. The lower panel shows a Rap1GAP2 expression control.
C) 14-3-3 does not bind to Rap1GAP1. HeLa cells transfected with an expression vector for HA-tagged Rap1GAP1 and myc-tagged 14-3-3β were lysed, and Rap1GAP1 was precipitated using an antibody against the HA tag. Precipitates were analyzed for the presence of 14-3-3 by immunoblot with anti-myc (upper panel). Efficient precipitation of Rap1GAP1 was verified by immunoblot (second panel from top). The lower two panels show expression levels of Rap1GAP1 and 14-3-3 in total lysates.
D) 14-3-3 interacts with the N-terminus of Rap1GAP2. HeLa cells were cotransfected with myc-tagged 14-3-3β and without or with different FLAG-tagged constructs of Rap1GAP2. N-term comprises amino-acids 1-121, C-term contains amino acids 121-715, and ΔN13 lacks the first 13 residues of Rap1GAP2. Cells were lysed, FLAG-containing proteins were precipitated and precipitates were analyzed for the presence of 14-3-3 by immunoblot (upper panel). The lower panel shows expression controls for the transfected constructs.

Figure 2. 14-3-3 binds to serine 9 of Rap1GAP2 and binding is blocked by phosphorylation of serine 7.
A) Alignment of Rap1GAP2 N-terminal sequence with type I and type II consensus motifs for 14-3-3 binding. pS indicates phosphorylated serine. Matching residues are boxed and identical residues are additionally shaded in grey.
B) Serine 9 of Rap1GAP2 is required for binding to 14-3-3. HeLa cells were cotransfected with myc-tagged 14-3-3 and FLAG-tagged wild-type Rap1GAP2 or mutants of Rap1GAP2 having the serine 9 residue mutated either to alanine (S9A) or to glutamic acid (S9E). Cells were lysed, Rap1GAP2 was precipitated and 14-3-3 binding was analyzed as described in Fig. 1D.
C) Mutation of Rap1GAP2 serine 7 to glutamic acid blocks 14-3-3 binding. HeLa cells were cotransfected with myc-tagged 14-3-3 and FLAG-tagged wild-type or mutant Rap1GAP2. 14-3-3 binding was analyzed as described (Fig. 1D).
D) 14-3-3 binds to phosphorylated serine 9 containing peptides and additional phosphorylation of serine 7 abolishes binding. Synthetic peptides containing phosphorylated serines 7, 9 or both were coupled to sepharose beads and used to pull-down GST-14-3-3β. Sepharose beads alone were used as control. Binding was determined by immunoblot using anti-14-3-3.
E) Serines 7 and 9 of Rap1GAP2 regulate binding to endogenous 14-3-3. HeLa cells transfected with wild-type or mutant FLAG-tagged Rap1GAP2 were lysed and FLAG-containing proteins were precipitated using specific antibodies. Co-precipitated endogenous 14-3-3 was visualized by immunoblot (upper panel). The lower panel shows expression controls for transfected Rap1GAP2 and for endogenous 14-3-3.
F) Serines 7 and 9 of Rap1GAP2 regulate binding to 14-3-3β and ζ isoforms. COS-1 cells were transfected with wild-type FLAG-tagged Rap1GAP2 or with various point mutants having serines 7 and 9 changed to alanine or glutamic acid alone and in combination as indicated. Equal amounts of GST-fusion proteins of 14-3-3β (upper panel) and 14-3-3ζ (middle panel) coupled to GSH-Sepharose beads were used for precipitation. Bound proteins were visualized by immunoblot analysis with a FLAG-specific antibody. The lower panel shows the expression levels of Rap1GAP2 proteins.

Figure 3. Platelet activation stimulates phosphorylation of Rap1GAP2 at serine 9 and increases 14-3-3 binding.
A) Characterization of a phospho-specific antibody against serine 9 phosphorylated Rap1GAP2. HeLa cells were transfected with FLAG-tagged wild-type and various point mutants of Rap1GAP2. Cells were lysed, total cell lysates were separated by SDS-PAGE and immunoblotted using a purified

antibody specific for Rap1GAP2 phosphorylated on serine 9 (pS9-Rap1GAP2) and for total Rap1GAP2 using an antibody against the FLAG-tag as control.
B) 14-3-3 binding to Rap1GAP2 is abolished upon phosphatase treatment. HeLa cells were transfected with FLAG-tagged Rap1GAP2. One day after transfection cells were lysed and Rap1GAP2 was immunoprecipitated using an antibody against the FLAG-tag. The precipitates were incubated without or with 15 units of protein phosphatase 1 (PP1) for 1.5 h at 30 °C and analyzed with the pS9-Rap1GAP2 antibody and for total Rap1GAP2 (anti-FLAG) as control.
C) ADP and thrombin enhance Rap1GAP2 serine 9 phosphorylation and 14-3-3 binding to Rap1GAP2 in platelets. Platelets were treated without or with 10 μM ADP for 1 min or with 0.1 U/ml thrombin for 30 sec. Then cells were lysed and total cell lysates were analyzed by immunoblot using anti-pS9-Rap1GAP2 and anti-Rap1GAP2 (first and second panels from top). The same lysates were incubated with purified recombinant GST-14-3-3β or GST alone as control to pull down endogenous Rap1GAP2. Precipitates were washed and bound proteins were analyzed by immunoblot using anti-Rap1GAP2 (third and fourth panels from top).
D) Thrombin enhances binding of endogenous 14-3-3 and Rap1GAP2 in platelets. In a control experiment COS-1 cells (left panels) were transfected with 14-3-3 and without or with Rap1GAP2. Cells were lysed and a GST-fusion protein of the Rap1GAP2 interacting protein 2 (Rip2) was used to pull down Rap1GAP2 and indirectly also 14-3-3 bound to Rap1GAP2. Precipitated 14-3-3 was detected by SDS-PAGE and immunoblotting of 14-3-3 using anti-14-3-3 (blot: 14-3-3). Amounts of precipitated Rap1GAP2 were controlled by immunoblotting of the precipitates using anti-Rap1GAP2 (blot: Rap1GAP2). In parallel total cell lysates were analyzed for the expression of 14-3-3 (total 14-3-3) and Rap1GAP2 (total Rap1GAP2). Platelets (right panels) were treated with thrombin as indicated followed by lysis and pull-down assay using either GST alone or GST-Rip2. The presence of endogenous 14-3-3 and Rap1GAP2 in the precipitates as well as total protein levels were analyzed by SDS-PAGE and immunoblotting using specific antibodies.

Figure 4. PI3K, PKB and PKC are not involved in Rap1GAP2 serine 9 phosphorylation.
A) and B) Freshly isolated human platelets were treated with 0.1 μM wortmannin for 20 min (wo) followed by ADP stimulation, with 50 μM LY294002 (LY) or 10 μM triciribine (tri) for 60 min, or with 1 μM bisindolylmaleimide I (bis) or 1 μM rottlerin (rot) for 20 min. Cells were lysed and serine 9 phosphorylation was analyzed by immunoblot using the pSer9-specific antibody and total amounts of Rap1GAP2 were determined as control.
C) An epitope-tagged construct of Rap1GAP2 containing the N-terminus and the GAP domain was expressed in HeLa cells. Cells were lysed and the Rap1GAP2 protein was purified using tag-specific antibodies. Rap1GAP2 was incubated with purified recombinant protein kinases cAK (A), PKB (B), PKCα (Cα) and PKCδ (Cδ) in the presence of ^{32}P-labelled ATP. Proteins were analyzed by SDS-PAGE, blotted to nitrocellulose and exposed to film (^{32}P) followed by immunodetection of total Rap1GAP2 levels (total Rap1GAP2).

Figure 5. Endogenous cyclic nucleotide signaling pathways induce Rap1GAP2 serine 7 phosphorylation in platelets.
A) Characterization of a phospho-specific antibody against serine 7 phosphorylated Rap1GAP2. HeLa cells were transfected with FLAG-tagged Rap1GAP2 constructs as indicated. One day after transfection cells were treated without or with 10 μM forskolin for 30 minutes and lysed. Total cell lysates were subjected to SDS-PAGE analysis followed by immunoblotting using an antibody specific for Rap1GAP2 phosphorylated on serine 7 (pS7-Rap1GAP2) and for total Rap1GAP2 using anti-FLAG.
B) NO-induced Rap1GAP2 serine 7 phosphorylation is mediated by cGMP. Platelets were treated with indicated concentrations of NO-donors DEA-NONOate or DETA-NONOate for 1 minute or guanylyl cyclase activator YC-1 for 5 minutes. Cells were lysed and immunoblot analysis was performed using antibodies specific for serine 7 phosphorylated Rap1GAP2 (pS7) as well as total Rap1GAP2.
C) Cyclic nucleotide-induced Rap1GAP2 serine 7 phosphorylation is mediated by cGK and cAK. Platelets were treated with the cGK activator 8-pCPT-cGMP for 30 minutes as indicated (left panels). In inhibitor studies (right panels) platelets were preincubated with 0.5 mM Rp-8-pCPT-cGMPS or 0.5

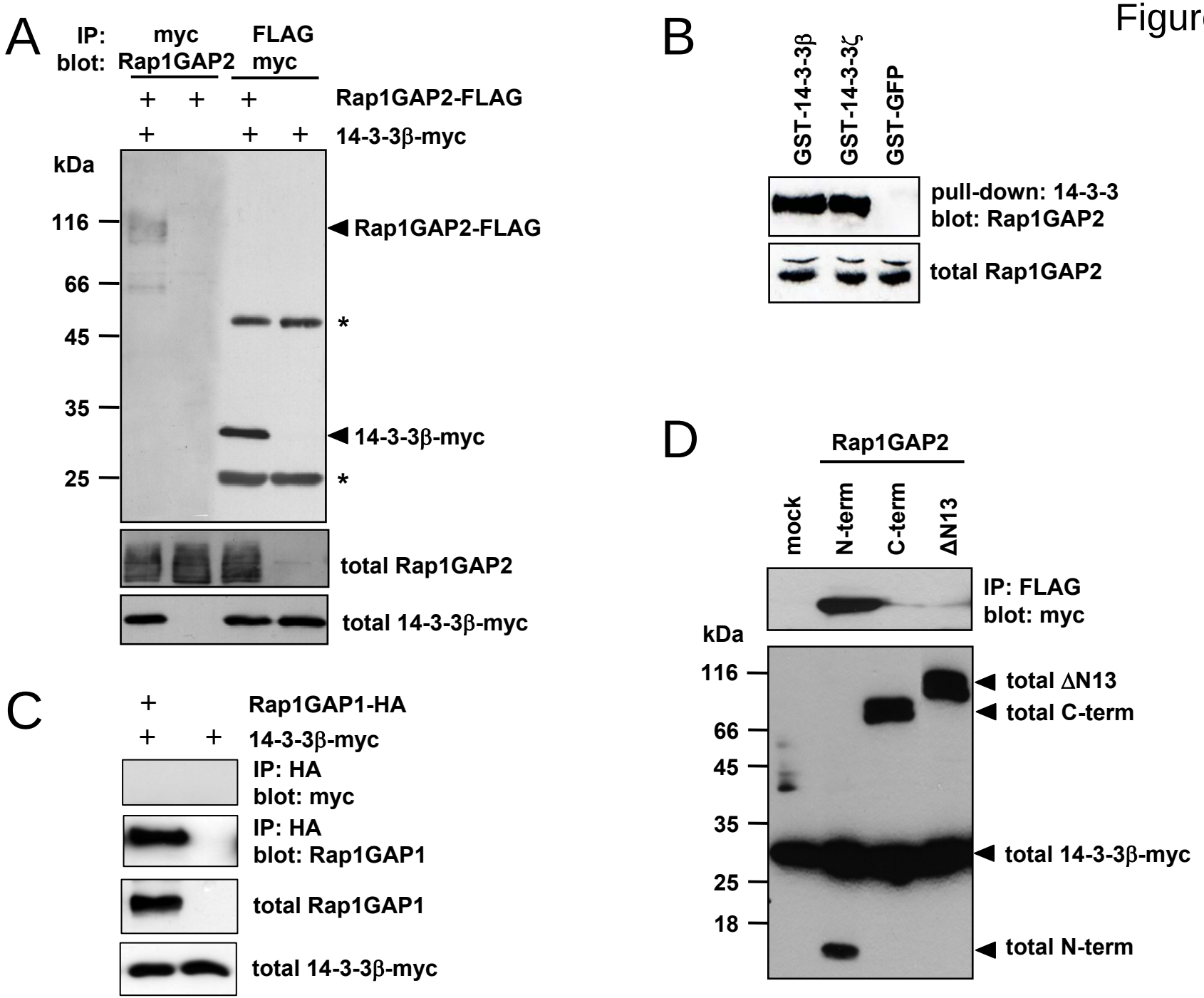
mM Rp-8-Br-cAMPS for 20 min at 37°C . Then 1 μM SNP or 1 μM forskolin were added for 1.5 min, followed by lysis, SDS-PAGE and immunoblot analysis of serine 7 phosphorylated and total Rap1GAP2.
D) The NO-donor SNP induces reversible Rap1GAP2 phosphorylation in platelets. Aliquots of platelets treated without or with 10 μM SNP were removed after indicated time points, mixed with SDS-buffer and boiled. Immunoblots were performed using antibodies specific for serine 7 phosphorylated Rap1GAP2 (pS7) as well as total Rap1GAP2. Obtained bands were scanned and quantitated by densitometry using the ImageJ program (NIH). Results from 3 independent experiments were plotted. Shown data represent the means (±S.D.).
E) Prostacyclin (PGI_2) induces reversible Rap1GAP2 phosphorylation in platelets.
Experiments were performed as described in the legend to Fig. 5D, using 1 μM PGI_2.

Figure 6. Cyclic nucleotide pathways inhibit 14-3-3 binding to Rap1GAP2 in platelets.
A) cGMP signaling blocks 14-3-3 binding to Rap1GAP2. Platelets treated with SNP or 8-pCPT-cGMP as indicated were lysed and pull-down assays using GST-14-3-3β were performed as described in the legend to Figure 1B. Total cell lysates were analyzed by immunoblot to determine total amounts of Rap1GAP2 and serine 7 phosphorylated Rap1GAP2.
B) cAMP signaling blocks 14-3-3 binding to Rap1GAP2. Platelets treated with either PGI_2 or forskolin were lysed and analyzed as described in A.
C) Cyclic nucleotide pathways inhibit 14-3-3 binding to Rap1GAP2 in the presence of ADP and thrombin. Platelets were incubated with 0.5 mM 8-pCPT-cGMP for 20 min of with 10 μM forskolin for 10 min followed by treatment with 10 μM ADP for 1 min or with 0.1 U/ml thrombin for 30 sec. Then cells were lysed and pull-down assays using GST-14-3-3β were performed as described.

Figure 7. 14-3-3 binding does not affect the GAP activity of Rap1GAP2 *in vitro*.
(A) Wild-type FLAG-tagged Rap1GAP2 and the S7 and S9 point mutants of Rap1GAP2 were expressed in HeLa cells and purified using anti-FLAG agarose beads. As control a mock purification from non-transfected HeLa cells was performed and used in the GAP assay. Wild-type and S7A mutant Rap1GAP2 were purified in a complex together with endogenous 14-3-3 from HeLa cells. Presence of 14-3-3 in wt and S7A samples only, was confirmed by SDS-PAGE and immunoblot analysis. The used 14-3-3 antibody (H8) recognizes more than one 14-3-3 isoform. The upper 14-3-3 band represents 14-3-3ε (the only 14-3-3 isoform that migrates at an apparent Mr of 33kDa) and the lower 14-3-3 band (apparent Mr of 30 kDa) may consist of different 14-3-3 isoforms endogenously expressed in HeLa cells.
(B) His-tagged Rap1B was purified from *E.coli* and loaded with ^{32}P-GTP as described in experimental procedures. Equal amounts of Rap1GAP2 proteins, as shown by immunoblot in A, were added to the GTP-loaded Rap1 and reactions were incubated at 25°C. Aliquots were removed at indicated time points, amounts of released ^{32}P-phosphate were determined by liquid scintillation counting and plotted as percentage of input Rap1-bound ^{32}P-GTP counts. Shown data represent the means (±S.E.) of four independent experiments.

Figure 8. Disruption of the Rap1GAP2/14-3-3 complex leads to reduced cell adhesion.
HeLa cells were transfected without or with FLAG-tagged wild-type or serine 7/serine 9 mutants of Rap1GAP2 together with a vector expressing luciferase. One day after transfection cells were seeded onto fibronectin-coated dishes. Adherent cells were quantified as described in experimental procedures. The percentage of adherent cells was plotted relative to the total amount of seeded cells. Summarizing data of five independent experiments performed in quintuplicate are shown with error bars representing standard error of the mean. The differences in adhesion between S7A and S7E as well as S7A and S9A mutants and also between S7A and S9E were statistically significant ($P < 0.05$, Bonferroni post-test). In all experiments equal levels of Rap1GAP2 expression were confirmed by immunoblots using FLAG-specific antibodies (inset, top). Furthermore, expression levels of the β1 integrin were determined by immunoblot (inset, bottom).

Figure 1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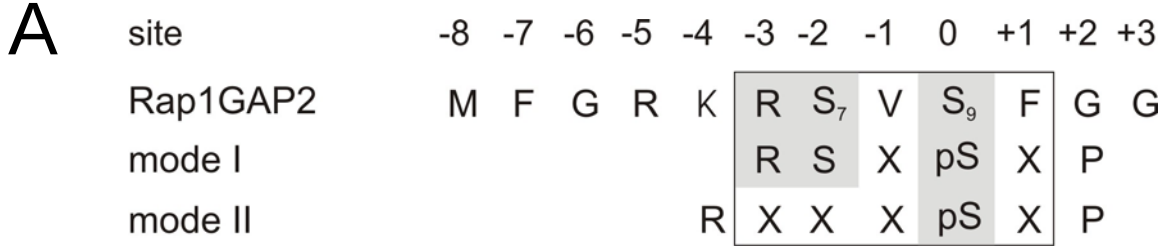
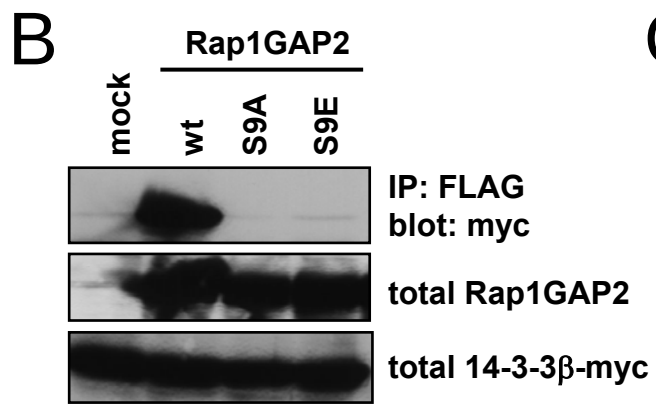
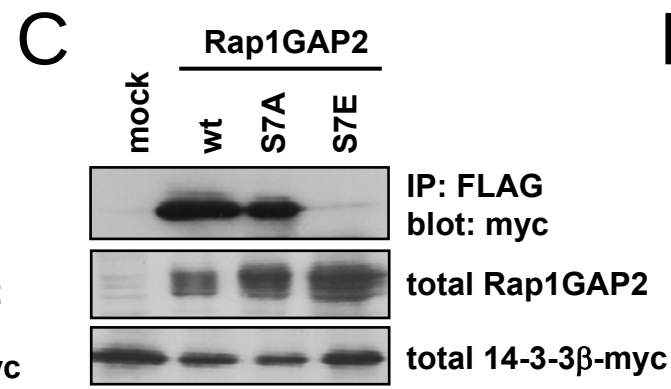
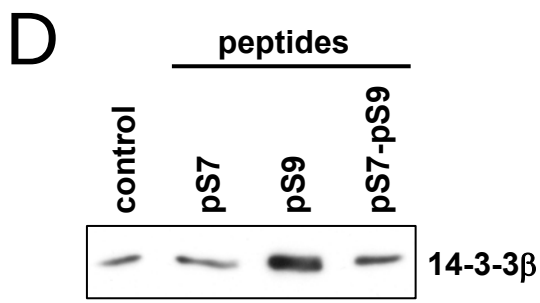
35
25
Rap1GAP2-FLAG
*
14-3-3β-myc
*
total Rap1GAP2
total 14-3-3β-myc
B
GST-14-3-3β
GST-14-3-3ζ
GST-GFP
pull-down: 14-3-3
blot: Rap1GAP2
total Rap1GAP2
C
Rap1GAP1-HA
14-3-3β-myc
IP: HA
blot: myc
IP: HA
blot: Rap1GAP1
total Rap1GAP1
total 14-3-3β-myc
D
Rap1GAP2
mock
N-term
C-term
ΔN13
IP: FLAG
blot: myc
kDa
116
66
45
35
25
18
total ΔN13
total C-term
total 14-3-3β-myc
total N-term

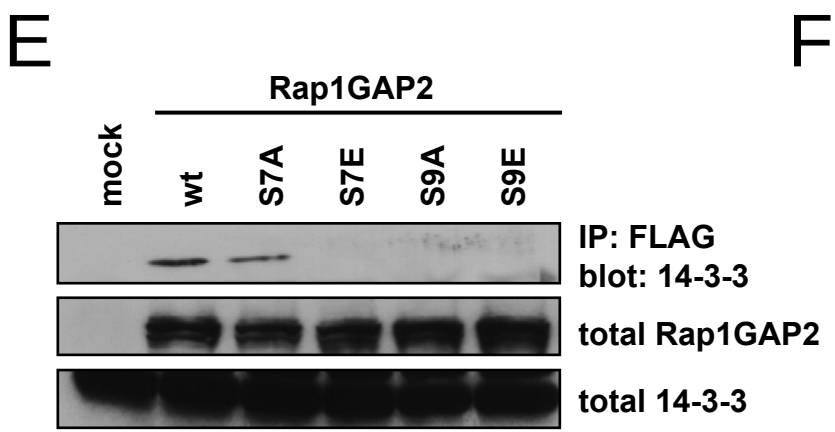
Figure 2


A
site
-8 -7 -6 -5 -4 -3 -2 -1 0 +1 +2 +3
Rap1GAP2
M F G R K R S7 V S9 F G G
mode I
R S X pS X P
mode II
R X X X pS X P


B
Rap1GAP2
mock
wt
S9A
S9E
IP: FLAG
blot: myc
total Rap1GAP2
total 14-3-3β-myc


C
Rap1GAP2
mock
wt
S7A
S7E
IP: FLAG
blot: myc
total Rap1GAP2
total 14-3-3β-myc


D
peptides
control
pS7
pS9
pS7-pS9
14-3-3β


E
Rap1GAP2
mock
wt
S7A
S7E
S9A
S9E
IP: FLAG
blot: 14-3-3
total Rap1GAP2
total 14-3-3

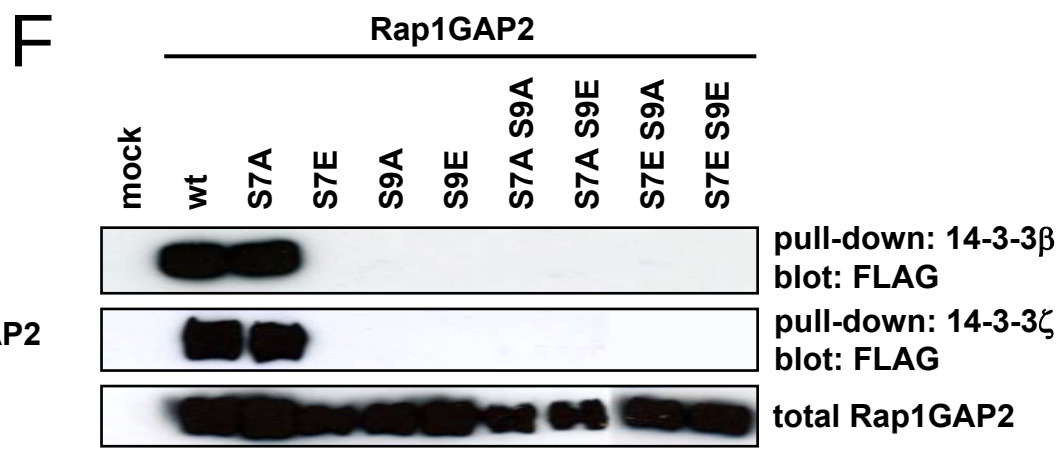
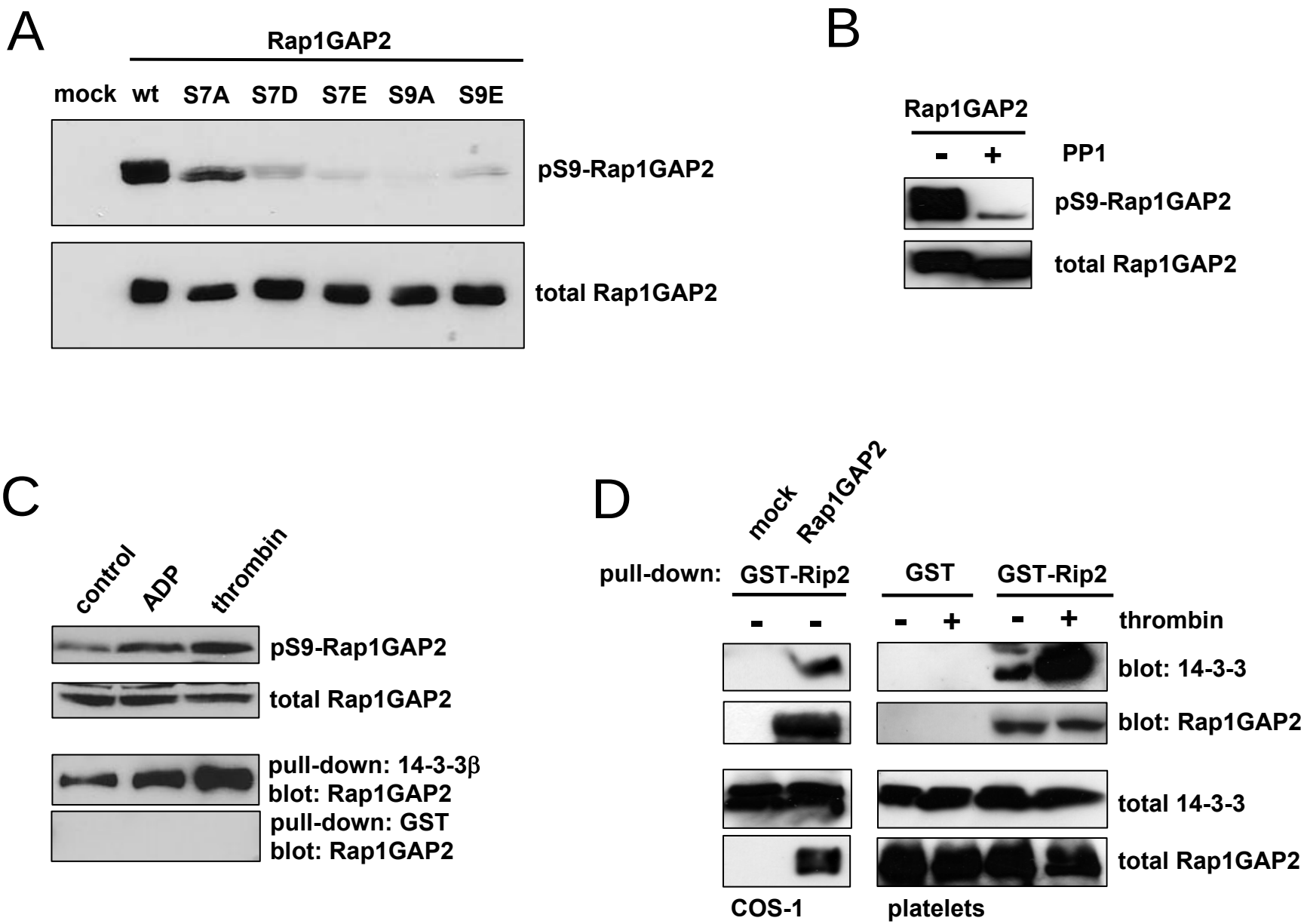

F
Rap1GAP2
mock
wt
S7A
S7E
S9A
S9E
S7A S9A
S7A S9E
S7E S9A
S7E S9E
pull-down: 14-3-3β
blot: FLAG
pull-down: 14-3-3ζ
blot: FLAG
total Rap1GAP2

Figure 3


A
Rap1GAP2
mock
wt
S7A
S7D
S7E
S9A
S9E
pS9-Rap1GAP2
total Rap1GAP2
B
Rap1GAP2
-
+
PP1
pS9-Rap1GAP2
total Rap1GAP2
C
control
ADP
thrombin
pS9-Rap1GAP2
total Rap1GAP2
pull-down: 14-3-3β
blot: Rap1GAP2
pull-down: GST
blot: Rap1GAP2
D
mock
Rap1GAP2
pull-down:
GST-Rip2
GST
GST-Rip2
thrombin
blot: 14-3-3
blot: Rap1GAP2
total 14-3-3
total Rap1GAP2
COS-1
platelets

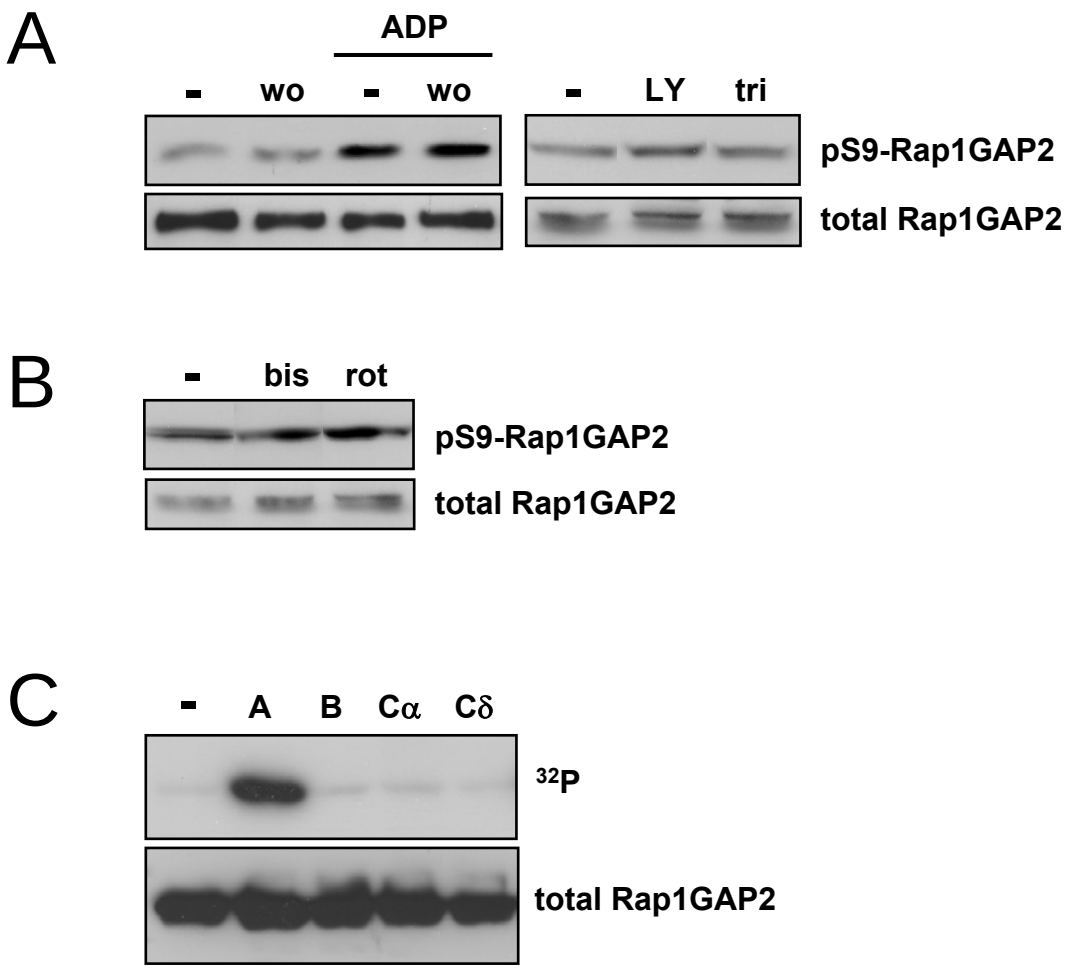

A
ADP
-
wo
-
wo
-
LY
tri
pS9-Rap1GAP2
total Rap1GAP2
B
-
bis
rot
pS9-Rap1GAP2
total Rap1GAP2
C
-
A
B
Cα
Cδ
32P
total Rap1GAP2

Figure 5

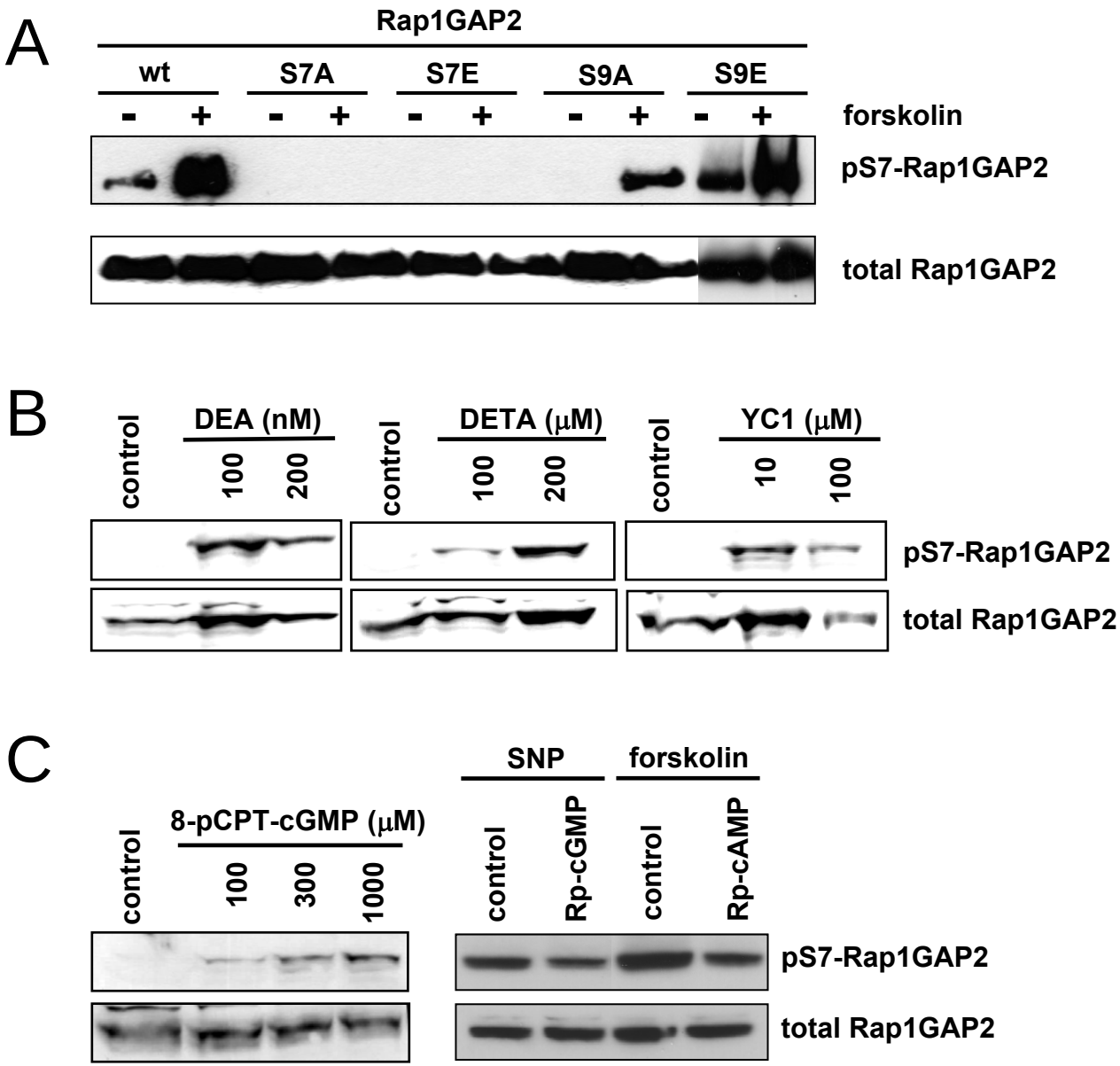

A
Rap1GAP2
wt
S7A
S7E
S9A
S9E
- + - + - + - + - +
forskolin
pS7-Rap1GAP2
total Rap1GAP2
B
control
DEA (nM)
100
200
control
DETA (μM)
100
200
control
YC1 (μM)
10
100
pS7-Rap1GAP2
total Rap1GAP2
C
8-pCPT-cGMP (μM)
control
100
300
1000
SNP
forskolin
control
Rp-cGMP
control
Rp-cAMP
pS7-Rap1GAP2
total Rap1GAP2

Figure 5

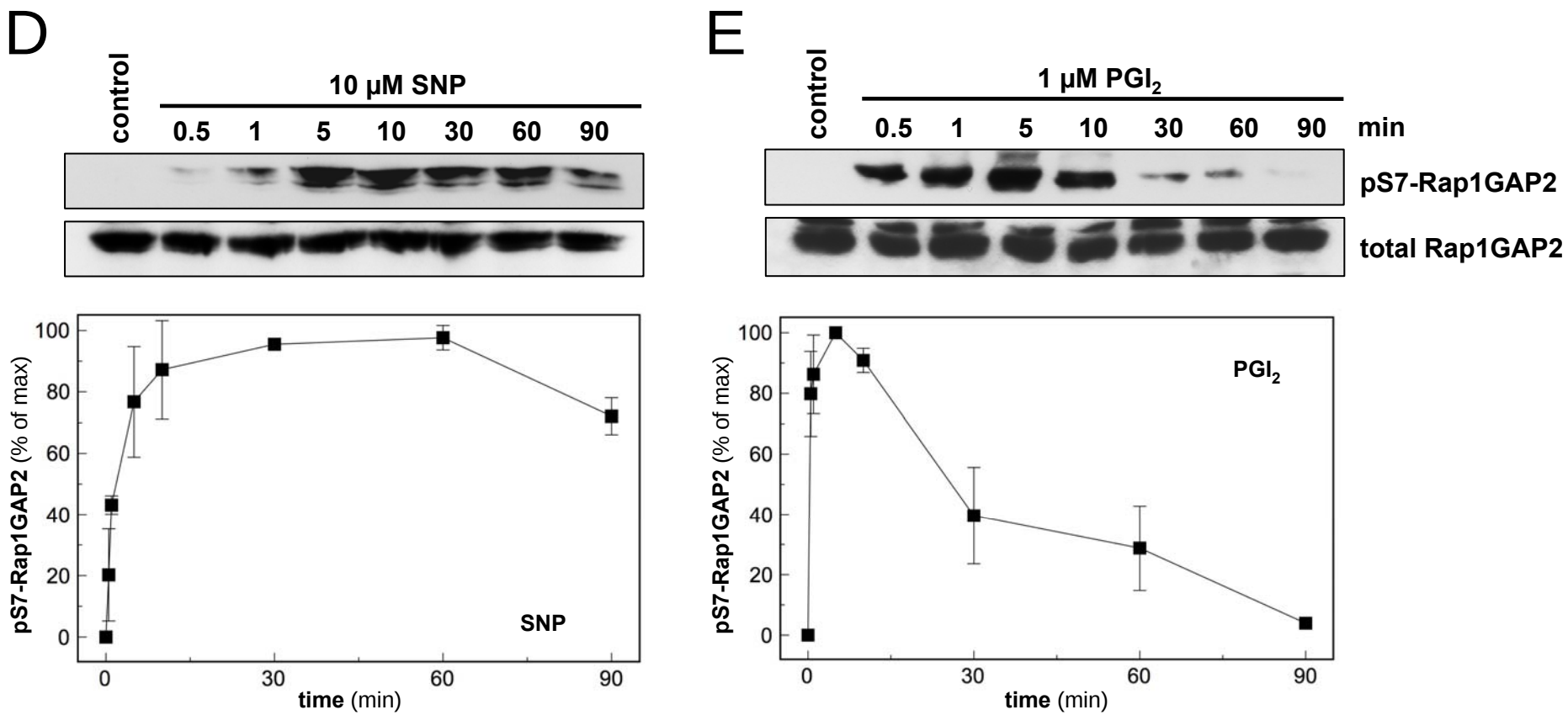
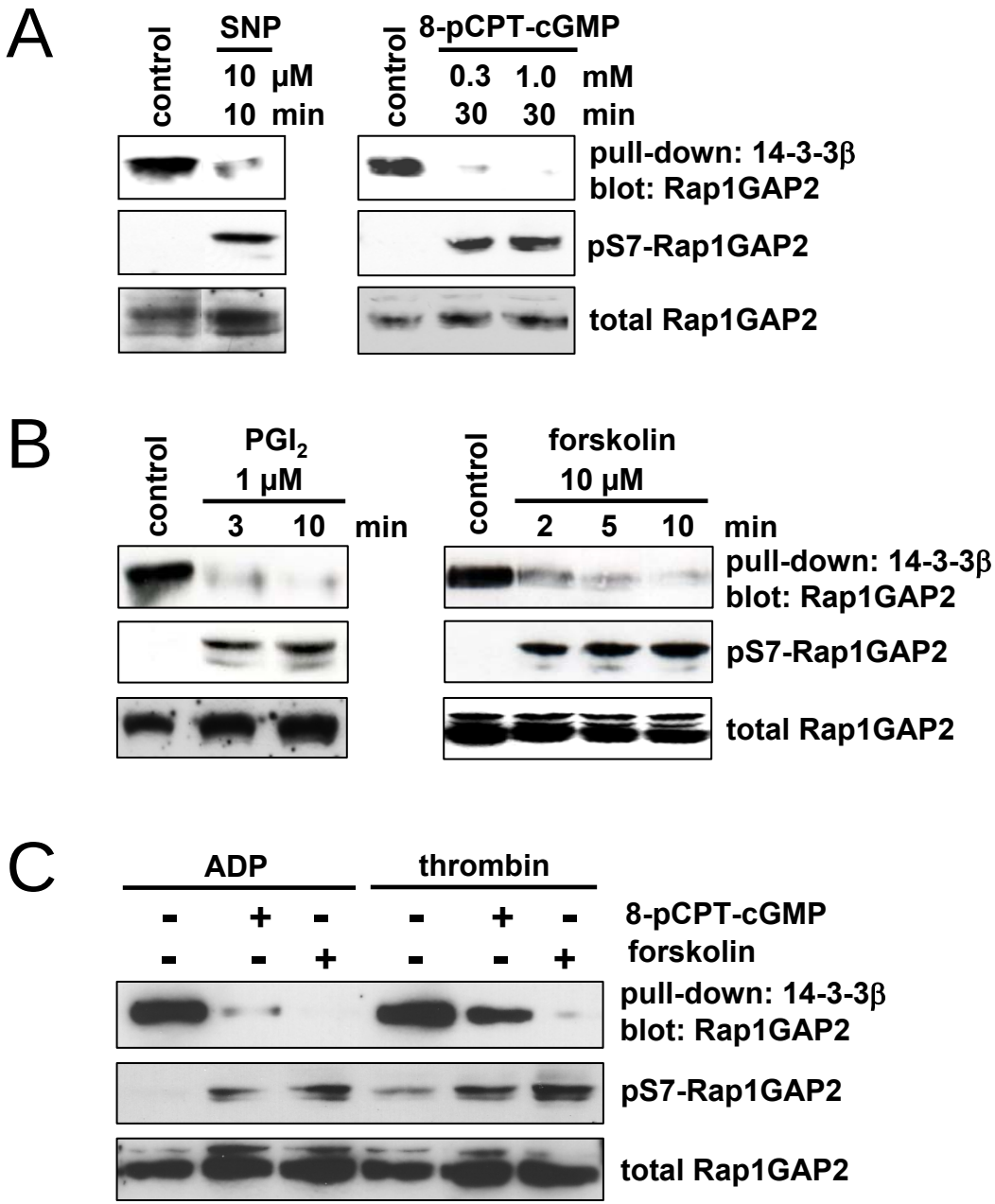
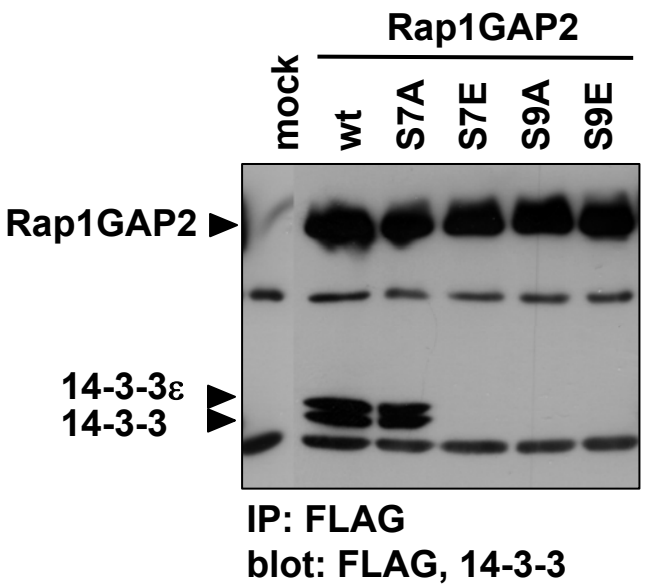

D
control
10 µM SNP
0.5 1 5 10 30 60 90
pS7-Rap1GAP2 (% of max)
SNP
time (min)
E
control
1 µM PGI2
0.5 1 5 10 30 60 90 min
pS7-Rap1GAP2
total Rap1GAP2
PGI2
time (min)

Figure 6


A
control
SNP
10 μM
10 min
control
8-pCPT-cGMP
0.3 1.0 mM
30 30 min
pull-down: 14-3-3β
blot: Rap1GAP2
pS7-Rap1GAP2
total Rap1GAP2
B
control
PGI2
1 μM
3 10 min
control
forskolin
10 μM
2 5 10 min
pull-down: 14-3-3β
blot: Rap1GAP2
pS7-Rap1GAP2
total Rap1GAP2
C
ADP
thrombin
- + - - + -
8-pCPT-cGMP
- - + - - +
forskolin
pull-down: 14-3-3β
blot: Rap1GAP2
pS7-Rap1GAP2
total Rap1GAP2

A


Rap1GAP2
mock
wt
S7A
S7E
S9A
S9E
Rap1GAP2
14-3-3ε
14-3-3
IP: FLAG
blot: FLAG, 14-3-3

B

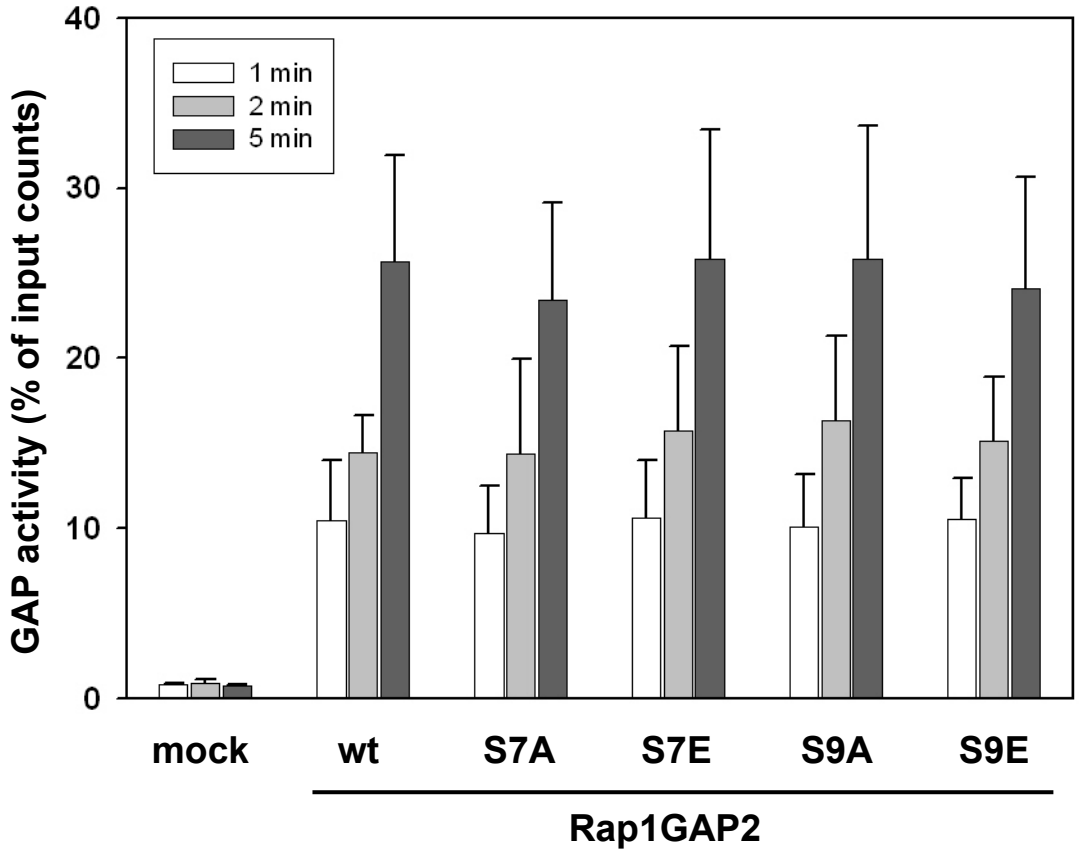
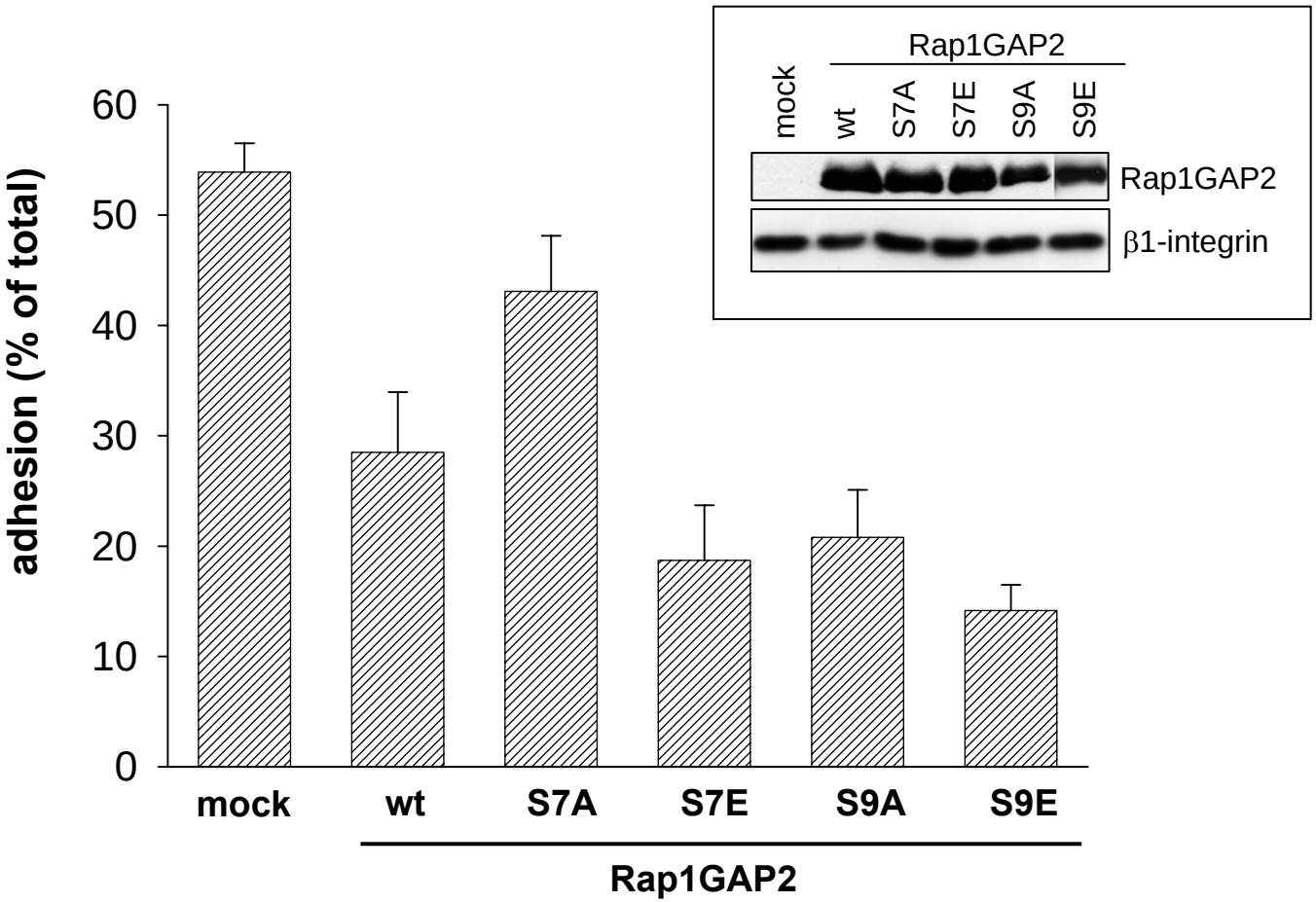

GAP activity (% of input counts)
40
30
20
10
0
1 min
2 min
5 min
mock
wt
S7A
S7E
S9A
S9E
Rap1GAP2

Figure 8


adhesion (% of total)
60
50
40
30
20
10
0
mock
wt
S7A
S7E
S9A
S9E
Rap1GAP2
Rap1GAP2
mock
wt
S7A
S7E
S9A
S9E
Rap1GAP2
β1-integrin