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# **Rap1GAP2 is a new GTPase activating protein of Rap1 expressed in human platelets**

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Running title: Identification of Rap1GAP2

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## **Abstract**

The Ras-like guanine-nucleotide-binding protein Rap1 controls integrin  $\alpha_{IIb}\beta_3$  activity and platelet aggregation. Recently, we have found that Rap1 activation can be blocked by the NO/cGMP signaling pathway via type I cGMP-dependent protein kinase (cGKI). In search of possible targets of NO/cGMP/cGKI we studied the expression of Rap1-specific GTPase-activating proteins (GAPs) and guanine nucleotide-exchange factors (GEFs) in platelets. We could detect mRNAs for a new protein most closely related to Rap1GAP as well as for PDZ-GEF1, CalDAG-GEFs I and III. Using 5'-RACE we isolated the complete cDNA of the new GAP encoding a 715 aminoacid protein, which we have termed Rap1GAP2. Rap1GAP2 is expressed in at least three splice variants, two of which are detectable in platelets. Endogenous Rap1GAP2 protein partially colocalizes with Rap1 in human platelets. In transfected cells we show that Rap1GAP2 exhibits strong GTPase-activating activity towards Rap1. Rap1GAP2 is highly phosphorylated and we have identified cGKI as a Rap1GAP2 kinase. cGKI phosphorylates Rap1GAP2 exclusively on serine 7, a residue present only in the platelet splice variants of Rap1GAP2. Phosphorylation of Rap1GAP2 by cGKI might mediate inhibitory effects of NO/cGMP on Rap1. Rap1GAP2 is the first GTPase activating protein of Rap1 found in platelets and is likely to have an important regulatory role in platelet aggregation.

## Introduction

Platelets are of great physiological importance as regulators of clot formation and inflammation in the vasculature and have been established as major therapeutic targets in cardiovascular disease<sup>1</sup>. Platelets contain high levels of Rap1, a Ras-like guanine-nucleotide-binding protein. Recently Rap1 has been identified as potent regulator of integrin function<sup>2-5</sup>. For example, regulation of lymphocyte and macrophage adhesion via integrins  $\alpha_L\beta_2$ <sup>6</sup> and  $\alpha_M\beta_2$ , respectively, is controlled by Rap1<sup>7</sup>. Rap1 also facilitates the activation of platelet integrin  $\alpha_{IIb}\beta_3$ , which is required for fibrinogen binding and aggregation<sup>8,9</sup>. Very recently, Rap1 was shown to control aggregation of mouse platelets<sup>10</sup>.

Many different platelet agonists including thrombin, ADP, collagen and thromboxane induce Rap1 activation<sup>11,12</sup>. However, the exact pathways involved in Rap1 activation in platelets are unknown. Data from other cell types suggest that receptor-mediated formation of second messengers can lead to the activation of Rap1-specific guanine nucleotide-exchange factors (GEFs). Calcium and diacylglycerol activate CalDAG-GEFI (also termed RasGRP2), a protein detected in mouse megakaryocytes and platelets<sup>10,13</sup> and CalDAG-GEFIII (also termed RasGRP3), involved in neuronal differentiation and B-cell development<sup>14,15</sup>. cAMP activates cAMP-dependent Epacs<sup>16,17</sup> and C3G is regulated by tyrosine phosphorylation<sup>18</sup> and by binding to adaptor proteins<sup>19,20</sup>. PDZ-GEF1 is a ubiquitously expressed GEF of Rap1 and contains a negative regulatory domain although the physiological activator is currently unknown<sup>21,22</sup>. Finally, DOCK4 has been described to activate Rap1<sup>23</sup>. So far, platelet expression has only been reported for mouse CalDAG-GEFI<sup>10</sup>.

Inactivation of Rap1 requires specific GTPase-activating proteins (GAPs) and two major groups of Rap1-specific GAPs have been identified. The first one is comprised of Rap1GAP<sup>24</sup> and its splice variant Rap1GAPII<sup>25</sup>. Rap1GAP is expressed in brain<sup>24,26</sup>, thyroid cells<sup>27</sup> and some other tissues<sup>28</sup>. The activity of Rap1GAP can be regulated by direct interaction

with G $\alpha$  proteins<sup>25,29-31</sup>. The second group of Rap1-specific GAPs encompasses the structurally related proteins SPA-1, found in lymphoid tissues<sup>28</sup> and E6TP1 $\alpha$  (SPAR/SPAL), expressed in hippocampal neurons and other tissues<sup>32-34</sup>. SPA-1 activity is controlled by the cytoskeleton-anchoring protein AF-6<sup>35</sup>.

Platelet activation and aggregation are negatively regulated by the nitric oxide (NO)/cGMP pathway<sup>36-39</sup>. Many antiplatelet effects of NO/cGMP are mediated by type I cGMP-dependent protein kinase (cGKI)<sup>40,41</sup> and we have recently observed that cGKI potently inhibits agonist-induced activation of Rap1 in human platelets (Thrombosis and Haemostasis, in press). Therefore, we were interested to identify possible substrates of cGKI mediating this effect on Rap1 activity.

In this study we provide evidence that human platelets express a distinct subgroup of Rap1-GEF proteins composed of PDZ-GEF1 and CalDAG-GEFs I and III. Furthermore, only one GAP of Rap1 appears to be present in platelets. We have characterized this new protein, termed Rap1GAP2, as a potent Rap1-specific GAP. We show colocalization of endogenous Rap1GAP2 and Rap1 in platelets. Additionally, we show that Rap1GAP2 is phosphorylated by cGKI and we have mapped the single phosphorylation site to serine 7 of the Rap1GAP2a and 2b splice variants.

## **Materials and Methods**

### **Materials**

Eukaryotic expression vectors for HA-tagged Rap1B, Rap1GAP1, Ras and PDZ-GEF1 as well as GST-RalGDS-RBD and GST-Raf1-RBD for bacterial expression were kindly provided by J. Bos, Utrecht. KIAA1039 partial cDNA clone and CalDAG-GEFIII (KIAA0846) full length cDNA clone were obtained from Kazusa DNA Research Institute,

Japan. The eukaryotic expression vector pSG8, containing N-terminal His6- and VSV-epitope tags, was a kind gift from S. Gross, Frankfurt.

### Preparation of platelet RNA

Leukocyte reduced platelet concentrate (a gift from the local blood bank) was subjected to three low-speed spins (150 g) to remove aggregated platelets and residual contaminating red and white blood cells. The resulting platelets were pelleted at 1500 g and washed three times in Tyrode's Buffer/EDTA, selecting only the top 66% of the pellet with each cycle. Total RNA extraction was done using Tri-Reagent as described by the manufacturer (MRC). Total RNA from human brain and spleen was obtained from BD Biosciences.

### RT-PCR analysis

First strand synthesis was performed with 0.5 to 1.0 µg of total RNA and Superscript II (Invitrogen) at 42°C for 1 h, using specific primers for the target mRNAs and oligo(d)T primers in parallel. PCR with specific primers was done for 30 cycles at different annealing temperatures (50°C, 55°C and 60°C). PCR-downstream primers were selected with a minimum distance of 200 bp to the respective first strand primers. In addition, control-PCRs were performed with upstream and first strand primers in oligo(d)T primed first strand reactions.

The following list summarizes used primer sequences (all in 5'-3' orientation):

name	Acc. number	1 <sup>st</sup> strand primer	PCR-primer downstream	PCR-primer upstream
Rap1GAP1a/ Rap1GAP	M64788	AGCCTGCACCACGACGTA	GCACAGGATAGAACCGATC	CATTCCATACCCGAGCGT
Rap1GAP1b/ Rap1GAP1I	AB003930	AGCCTGCACCACGACGTA	GCACAGGATAGAACCGATC	CTGGCAGAACACAGATCTAT
SPA-1	AB005666	GCAATTGAACACTACGCG	GTAGTGGTCACCTCGTTG	CTAGACACCAAAACGGATT
E6TP1α	AF090989	TCTCTGTCTCCAAAGTCCGAT	GAACCAGTATCACTGGATGAA	CCTCAAAATCTTCTATGAACG
Tuberin	X75621	GAAGTTGGAGAAGACGTATCG	CATGTAGTCGCAGACGCAGTA	TGGTGTCTTATGAGATCGTCC
KIAA1039	AB028962	GCGGAATTTTCAGGTTCTGA	CATGGAGTGGCTGCGTAC	AGCTGCCATTTACCGACG

KIAA1389	AB037810	TACTCCTGCCTCGTTTGA	TTGTCTAGCTGAGCTCGATA	TGTCCCTCAATACCGTGC
KIAA0545	AB011117	TGGGTGCTTATTGCGTAC	TGGGTGCTTATTGCGTAC	GCTGCTCTTTTCAATCGT
p761J1523 (DKFZ)	AL136573	CATCAAATTGGTGTGATAA	CATATCCAGGGTACGCCG	TGTCCCTCAATACCGTGC
CD-GEFI	AF081194	TGGAAGTTGTGTACGAAGC	TCCACTCCTCCAATACCG	CAGGACTATCACAGTTTCGT
CD-GEFIII	AB020653	CCTCACAGAGATCTTGCG	CAAAGGCCTTGC GGTAATTG	CCTTGGAAAGATCGATTGCT
DOCK4	AY233380	TTGAAGAGCTCGCGGTAT	GTCTATCTGTCAATTGTGTAAT	ATGGTACGTGGACCGGAT
Epac	AF103905	GAGGGCCAGTCGGTATAC	CTCCAGTCGTGGTCCGTC	GAGGCAGCAGATCTTGCG
Epac2	U78516	TGAAATGTCTATGGTCGACG	AAACTTTCAAATCCGCATAG	ATGTTTCAGTATTTACGACGC
Repac	D87467	GGTCTGCGACAGTCGACT	CCAGGTCTTTCCGGTAGA	TCAGATGTTCCCGGTAGG
PDZ-GEF1	AB002311	AGAAAGGAAC TACGACGTAC	TGGAATGGAGTAGCGACT	AGGCTGTTGAATATCCGG
PDZ-GEF2	AF478567	TCCAATACTCTCCGTTTAAAC	CAGTGACCTCTGTGCAAAC	AAATTGCACTTCATTGTGAGAGA
C3G	D21239	CATTGAAGAAGTTTATAATGTCG	ATGGCTGTGAAAGTCGTGC	ATTCAAGAAGCGCGTCAGC
smg GDS	X63465	GCGAAGTTTGGATACGAC	TTTGTGATTTACTGTGTCGTATAAG	TGTTACGATAGCCATTCGCT

## Cloning of Rap1GAP2

The complete 5'-end of the mRNA coding for Rap1GAP2a was obtained with the GeneRacer Kit from Invitrogen. The following gene specific primers (gsp) were selected from the sequence of KIAA1039: gsp1 5'-GCGGAATTCAGGTTTCA-3', gsp2 5'-CTCCATGGAGTGGCTGCGTACGCGGAT-3' and gsp3 5'-CACGTCCAGGCCTCCTCGGAAAC -3'. 5'-RACE produced a 981 bp fragment, which was cloned into pCR2.1 vector (Invitrogen). A full length sequence construct of Rap1GAP2a was obtained by modified asymmetric PCR from the 5'-RACE product and the KIAA1039 clone as described <sup>42</sup> using 5'-TTAAGCGGCCCGCCATGTTTGGCCGGAAGCGCAGTGT-3' and gsp3 for the 5'-RACE product and 5'-GTTTCCGAGGAGGCCTGGACGTG-3' and 5'-CCCGTCTGACTTAGTGACCCGCACCAGAGCTGG-3' for the KIAA1039 clone. Full length Rap1GAP2a and CalDAG-GEFIII were cloned into pSG8 expression vector. The products of 5'-RACE, full length asymmetric PCR and pSG8-constructs were verified by sequencing of both strands.

For analysis of splice variants of Rap1GAP2 in platelets, following primers

were used: 5'-CAGACAGACATCGGCACGTA-3' (Exon 1),  
5'-ATCATGTTTGGCCGGAAGCGCAGTGTCTCC-3' (Exon 2), 5'-  
CCGGCTTCTGCTCTTCAA-3', 5'-GGACCCCAGAAGAACAAGGA-3' (Exon 6), 5'-  
GAAAATGCAGGACGACTATAT-3', 5'-ATATAGTCGTCCTGCATTTTC-3' (Exon 6  
spliced out).

### **Sequence comparisons**

Multiple alignments of the deduced amino acid sequences were constructed by CLUSTALX version 1.81<sup>43</sup>. The program package PHYLIP version 3.6a (obtained from J. Felsenstein, Seattle) was used for phylogenetic analyses. Distances between pairs of protein sequences were calculated according to the Jones Taylor Thornton substitution model<sup>44</sup>. Tree construction was performed by the neighbor joining method<sup>45</sup>. The reliability of the trees was tested by bootstrap analysis<sup>46</sup> with 100 replications (SEQBOOT program from the PHYLIP package). Trees were drawn using the TREEVIEW software program<sup>47</sup>.

### **Northern blotting**

Northern blots of multiple human tissues (Clontech) were carried out by overnight hybridization using modified Church buffer (0.5 M Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 7% SDS, pH 7.2). Probes, coding for Rap1GAP1 and Rap1GAP2 were generated from the expression vectors by EcoRI/XhoI-digestion, gel purification and [ $\alpha$ -<sup>32</sup>P]CTP-labeling.

### **Antibody preparation**

Female New Zealand White Rabbits were immunized with a peptide corresponding to amino acids 1-32 of human Rap1GAP2a/b (MFGRKRSVSFGGFGWIDKTMLASLKVKKQELANH<sub>2</sub> obtained from Schafer-N, Copenhagen). Four injections in 30 d intervalls were performed using 100  $\mu$ g of peptide in 0.5 ml PBS mixed with 0.5 ml of Freund's Adjuvant

(Sigma, complete and incomplete adjuvant at a ratio of 1:2) per injection. After 149 days the animals were killed and serum was prepared. Antiserum 644.4 (diluted 1:500 in blocking solution containing 4% non-fat dry milk) was used to detect Rap1GAP2a/b in various cells by Western blotting. For preabsorption, the antigenic peptide was incubated with serum (2 mg peptide/ml serum) for 4 h at RT. Antiserum against human CalDAG-GEFI was prepared similarly using the peptide MAGTLDLKDGCTVEELLRGCIEAFDDSG from the N-terminus of the protein. To detect Rap1GAP1 and SPA-1 commercially available antibodies were used (Santa-Cruz and BD Transduction Laboratories).

### **Immunofluorescence**

Washed platelets were diluted 1:100 in resuspension buffer and allowed to attach to glass coverslips for 30 min at RT. Alternatively COS-1 cells were grown on glass coverslips and transfected with plasmids containing Rap1GAP2 cDNA. Cells were fixed with 3.7% paraformaldehyde in PBS for 15 min on ice followed by permeabilization with 0.2% Triton-X-100 in PBS. To detect Rap1GAP2 the antiserum 644.4 was affinity-purified using the antigenic peptide and the purified serum was used at a concentration of 2.5 µg/ml followed by Cy3-anti-rabbit (COS-1 cells) or Cy5-anti-rabbit (platelets) secondary antibodies. In platelets Rap1 was labeled in parallel using a monoclonal antibody (Transduction Laboratories) diluted 1:50 followed by Cy3-anti-mouse secondary antibody. COS-1 cell staining was observed with a Zeiss-Axiovert 200 microscope and images were obtained with a digital camera (Hamamatsu C4742-95) using OPENLAB software (Improvision). Platelets were analyzed using a Zeiss LSM 510 confocal laser scanning microscope and LSM 510 META software.

### **Rap1-GTP assay**

COS-1 cells were seeded onto 10 cm dishes ( $1 \times 10^6$  cells per plate) and transfected on the following day with cDNAs for Rap1B and various combinations of GEFs and GAPs using up

to 6  $\mu\text{g}$  of DNA, 300  $\mu\text{g}$  DEAE-dextran and 200  $\mu\text{M}$  chloroquine in 6 ml serum-free medium for three hours. Two days later cells were washed twice with ice cold PBS, lysed by addition of 1 ml of lysis buffer (200 mM NaCl, 2.5 mM  $\text{MgCl}_2$ , 50 mM Tris-HCl pH 7.4, 1% NP-40, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1  $\mu\text{M}$  leupeptin, 2  $\mu\text{M}$  aprotinin) to a 10 cm plate and scraped off with a rubber policeman. After incubation for 15 min at 4°C lysates were clarified by centrifugation at maximum speed for 15 min. Then a sample of 50  $\mu\text{l}$  was removed for analysis of the total amounts of transfected proteins. 75  $\mu\text{l}$  of a 10% (v/v) glutathione sepharose beads suspension (Amersham Bioscience) saturated with GST-RalGDS-RBD were added to the supernatant and incubated at 4°C for 45 min rotating. Beads were washed four times in 1x lysis buffer and finally boiled in 3x SDS sample buffer. Samples were analyzed by Western blot using nitrocellulose membranes (Schleicher & Schuell) and monoclonal antibodies directed against the HA-tag (HA.11, Covance) were used to detect transfected Rap1B, PDZ-GEF and Rap1GAP1. An antibody against the VSV-tag (P5D4, Sigma) was used to detect Rap1GAP2 and CalDAG-GEFIII followed by HRP-coupled secondary antibodies and enhanced chemiluminescence detection (Amersham). All experiments shown were performed at least three times with similar results.

### **Phosphorylation of Rap1GAP2**

COS-1 cells were transfected with cDNAs for wild-type Rap1GAP2, N-terminally tagged with VSV, as well as various mutants of Rap1GAP2 generated with the transformer site-directed mutagenesis kit (Stratagene). After two days cells were washed twice with ice-cold PBS and lysed in MIPP-buffer (1% Na-deoxycholate, 20 mM Tris/HCl pH 7.4, 75 mM NaCl, 0.1% SDS, 1% Triton-X-100, 10 mM EDTA, 10 mM Na-pyrophosphate, 50 mM NaF, 5 mM p-nitro-phenyl-phosphate, 2  $\mu\text{g/ml}$  aprotinin, 1  $\mu\text{g/ml}$  leupeptin). Rap1GAP2 was precipitated using anti-VSV antibody P5D4 (Sigma) and ProteinA/G-Sepharose (Santa-Cruz) and phosphorylated *in vitro* with purified cGK I and catalytic subunit of cAMP-dependent protein

kinase as described <sup>48</sup>. Samples were subjected to SDS-PAGE followed by blotting on nitrocellulose. Blots were exposed first to film to detect <sup>32</sup>P-incorporation. Then Rap1GAP2 expression was analyzed using anti-VSV antibodies and ECL.

## Results

### GEFs and GAPs of Rap1 expressed in human platelets

In our analysis of Rap1 inhibition by NO/cGMP/cGKI we set out to identify the relevant proteins controlling Rap1 activity in human platelets. We performed systematic RT-PCR analysis of known GEFs and GAPs of Rap1 expressed in platelets. For RNA isolation, platelets were purified extensively to avoid contamination by lymphocyte RNA. To verify that the primer sets were able to detect the mRNA for the protein of interest we performed control experiments using RNA from tissues in which the particular proteins are known to be expressed (Fig. 1A and B, lower panels). Platelets contain considerable amounts of RNA including PolyA<sup>+</sup> mRNA and active protein synthesis is carried out in these cells <sup>49,50</sup>. A recent comparison of transcriptome as well as proteome information from human platelets revealed a high degree of correlation between both types of data <sup>51</sup>.

Surprisingly, none of the established Rap1-GAPs including Rap1GAP, SPA-1 and E6TP1 $\alpha$  were detectable in platelets (Fig. 1A). Therefore we searched human EST and cDNA databases for sequences homologous to the GAP domain of Rap1GAP. Four cDNAs were identified, one of which, KIAA1039 (GenBank Acc.# AB028962 <sup>52</sup>), was found to be strongly expressed in human platelets (Fig. 1A). We detected mRNAs for PDZ-GEF1 and CalDAG-GEFIII and weakly also CalDAG-GEFI (Fig. 1B) but not DOCK4, any of the different Epacs, C3G or smgGDS. Recently, CalDAG-GEFI was shown to be expressed in mouse megakaryocytes and platelets <sup>10,13</sup> and we confirmed CalDAG-GEFI protein expression in human platelets by Western blot (not shown). As observed before on the mRNA

level, the Western blot signal for CalDAG-GEFI was very weak, indicating low expression levels of CalDAG-GEFI in human platelets.

### **Cloning of a new Rap1GAP**

The KIAA1039 sequence represented only a partial cDNA sequence lacking the 5'-end. To obtain the complete cDNA we performed a 5'-RACE experiment using purified platelet mRNA as template. A complete cDNA was isolated encoding a protein of 715 residues with a predicted molecular weight of 78.4 kDa. Comparison with the Pfam protein motif database showed the presence of a conserved Rap\_GAP domain (residues 262-449). The closest relative of the new GAP is Rap1GAP exhibiting 52% identity (Fig. 2B). Two splice variants of Rap1GAP have been described and named Rap1GAP and Rap1GAPII, the latter containing 31 additional amino acids at the N-terminus<sup>25</sup>. To comply with standard nomenclature we have named the new protein Rap1GAP2. Accordingly, we would like to suggest that the splice isoforms Rap1GAP and Rap1GAPII be renamed Rap1GAP1a and Rap1GAP1b. During the course of this study, a new full-length cDNA derived from cerebellum (AK124640) was published containing the KIAA1039 sequence and confirming part of our RACE product. However, the N-terminus of the encoded protein differed from our sequence. Database searches revealed that the gene for Rap1GAP2 is localized on chromosome 17 (17p13.3) spanning 261 kilobases and the coding sequence is distributed over 26 exons (Fig. 2A). Comparison of genomic and cDNA sequences showed that both, our platelet sequence as well as the cerebellar sequence, were products of alternative splicing (splice variants “a” and “c” in Fig. 2A, respectively). Using exon specific primers we confirmed variant “a” as the predominant form in human platelets. In addition we discovered a third splice variant present in human platelets (designated “b” in Fig. 2A). In summary, three splice variants of the new Rap1GAP2 encoding alternative N-termini have been identified: variant “a” lacking exons 1 and 6 prevalent in platelets, variant “b” including exon 6 and variant “c” corresponding to the

cerebellar clone lacking exon 2 (Fig. 2A). Interestingly, a putative GoLoco domain found in Rap1GAP1b/Rap1GAPII<sup>25</sup> appears to be conserved in Rap1GAP2b and c (Fig. 2B). Orthologs of Rap1GAP2 are present in mouse (AK122424) as well as in rat (XP\_220692). Sequences of Rap1GAP2a and b have been submitted to the GenBank/EMBL/DDBJ database under the accession numbers AJ628447 and AJ628446.

Rap1GAP1 and Rap1GAP2 proteins constitute a distinct subgroup in the family of Rap1-specific GAPs (Fig. 2C). Two other putative Rap1GAPs found by database searching belong to the SPA-1 family (KIAA0545 and KIAA1389) and the DKFZp761J1523 clone might represent a novel subgroup (Fig. 2C).

### **RNA expression of Rap1GAP2 and Rap1GAP1 in human tissues**

Single transcripts of 6.7 kb for Rap1GAP2 and 3.4 kb for Rap1GAP1 were detectable by Northern blot analysis of polyA<sup>+</sup> RNA from different human tissues. Expression levels varied between tissues and both genes tended to be expressed in different locations. For example, Rap1GAP2 is specific for heart, testis and blood leukocytes whereas Rap1GAP1 is strongly expressed in brain, kidney and prostate (Fig. 3). Overlapping expression is observed in pancreas and gastrointestinal tract with prominent signals in stomach. The expression pattern observed for Rap1GAP1 is consistent with previous data<sup>26,28</sup>.

### **Rap1GAP2 protein is expressed in human platelets**

A polyclonal antiserum was raised against a peptide derived from the N-terminus of Rap1GAP2a/b. Western blotting with the obtained antiserum showed expression of a 95 kDa protein in COS-1 cells transiently transfected with an expression vector for epitope-tagged Rap1GAP2a but not in the cells transfected with Rap1GAP1 (Fig. 4). In platelets and less also in lymphocytes a protein of approximately 90 kDa was detected (Fig. 4), corresponding to endogenous Rap1GAP2a/b. The epitope tag included in the transfected protein results in

additional 4.4 kDa of calculated molecular weight, which matches almost exactly the difference observed between endogenous and transfected proteins. Preabsorption of the antiserum with the antigenic peptide abrogated labeling of the 90/95 kDa Rap1GAP2 band (data not shown). To further verify mRNA data we analyzed Rap1GAP1 and SPA-1 protein expression in platelets and lymphocytes. As expected, Rap1GAP1 was not detectable (Fig. 4) whereas SPA-1 protein was expressed in lymphocytes but not in platelets (data not shown).

### **Rap1GAP2 and Rap1 partially colocalize in human platelets**

To analyze the subcellular localization of Rap1GAP2 we transfected the cDNA of Rap1GAP2 into COS-1 cells. Staining with the antiserum against Rap1GAP2 revealed a cytosolic and perinuclear localization of overexpressed Rap1GAP2 (Fig. 5A). To confirm specificity of the signal we stained non-transfected cells with the Rap1GAP2 antibody. The signal was absent in non-transfected cells indicating that the unspecific background protein detected in Western blot (Fig. 4) was not labeled in immunofluorescence (Fig. 5B). We next investigated the localization of endogenous Rap1GAP2 in human platelets spread on glass coverslips. Rap1GAP2 staining was observed in central, granular structures (Fig. 5C, F) and this staining overlapped with the localization of endogenous Rap1 (Fig. 5D, G and merged images in E, H). Rap1 tended to exhibit a more spread localization whereas Rap1GAP2 was confined to the central structures.

### **Activation of Rap1 GTPase activity by Rap1GAP2**

Since no physiological activator of endogenous Rap1GAP2 is known and since platelets are not amenable to transfection studies we decided to analyze GTPase activity of Rap1GAP2 in cell lines. We transiently expressed the Rap1GAP2a variant in COS-1 cells and measured Rap1 GTPase activity with the pull-down assay developed by J. Bos <sup>11</sup>. Coexpression of Rap1B together with the platelet GEFs CalDAG-GEFIII (Fig. 6A) or PDZ-GEF1 (not shown)

strongly increased amounts of Rap1B-GTP. Additional expression of either Rap1GAP2 or Rap1GAP1, almost completely blocked the formation of Rap1B-GTP (Fig. 6A). Using increasing concentrations of both GAPs we show that Rap1GAP2 and 1 have similar Rap1-GTPase activating potential (Fig. 6A and quantitation in Fig. 6B). Recently the crystal structure of the catalytic domain of Rap1GAP1 was solved indicating an important role of an asparagine residue for catalytic activity<sup>53</sup>. This asparagine residue is conserved in Rap1GAP2 (N342 of Rap1GAP2a) and mutation of this site to alanine completely abolished GAP activity, as expected (data not shown). To verify that the Rap1GAP domain conveys Rap1-specific GAP activity we studied effects of Rap1GAP2 on Ras-GTP levels using the Ras-binding domain of Raf-kinase as an activation-specific probe. No influence of Rap1GAP2 overexpression on CalDAG-GEFIII-stimulated Ras activity could be observed (data not shown). These data confirm that Rap1GAP2 is indeed a functional and Rap1-specific GTPase-activating protein as predicted from the sequence homology with known Rap1GAPs.

### **Rap1GAP2 is phosphorylated by cGKI on serine 7**

Rap1GAP2 contains a great number of serine residues, which could be substrates for protein phosphorylation. *In vivo*-labeling of transfected COS1-cells with <sup>32</sup>P-ortho-phosphate followed by precipitation of expressed Rap1GAP2 revealed a very strong basal phosphorylation of Rap1GAP2 (data not shown). Rap1GAP2a and 2b contain two serine residues that correspond to the consensus sequence  $-(R/K)_2-X-S/T-$  for phosphorylation by cyclic nucleotide regulated protein kinases: serine 7 (-RKRS-) and serine 549 (564 in 2b) (-KRRS-). To evaluate if Rap1GAP2a/b was a substrate of cyclic nucleotide regulated protein kinases we performed *in vitro* kinase assays with purified cGKI and the catalytic subunit of cAMP-dependent protein kinase (cAK). Both kinases strongly phosphorylated Rap1GAP2a (Fig. 7A). To identify the exact phosphorylation sites candidate serine residues were mutated

to alanine. Mutation of serine 7 completely abolished Rap1GAP2a phosphorylation by cGKI, whereas phosphorylation by cAK was only marginally reduced (Fig. 7A). Mutation of serine 549 did not change cGKI- nor cAK-mediated Rap1GAP2a phosphorylation. From these results we conclude that cGKI phosphorylates Rap1GAP2a/b on a single serine residue. To identify the functional consequences of phosphorylation we compared GTPase activities of wild-type and serine 7 to alanine, as well as phospho-mimetic serine 7 to aspartate or glutamate mutants of Rap1GAP2a. However, no significant differences in GTPase activity could be detected in transfected COS-1 cells (Fig. 7B). These data might suggest that serine 7 phosphorylation does not directly regulate Rap1GAP2a activity. However, considering the very strong activity of Rap1GAP2a in our COS-cell assay (Fig. 6), subtle changes in catalytic activity cannot be excluded.

## **Discussion**

### **Identification of a new Rap1GAP in human platelets**

In the present work we have identified and characterized Rap1GAP2, a new GTPase activating protein of Rap1. Rap1GAP2 is the first GTPase-activating protein of Rap1 to be described in platelets and our data suggest that it might be the only one. We show that Rap1GAP2 colocalizes with Rap1 in platelets and can potently inhibit Rap1-GTP formation in COS-cells. Thus Rap1GAP2 most likely also inhibits endogenous Rap1 in platelets.

### **Expression patterns of Rap1GAPs and GEFs**

The Rap1GAP family of proteins can be grouped into at least two subfamilies, the Rap1GAP- and the SPA-1-family. Expression patterns of the various Rap1-GAPs are tissue-specific, whereas Rap1 itself is ubiquitously expressed<sup>54</sup>. Rap1GAP1 is prominent in kidney, prostate and brain, Rap1GAP2 is found in platelets and lymphocytes and SPA-1 is strongly expressed

in lymphoid tissue<sup>28</sup>, but not in platelets. Rap1GAP2 and SPA-1 are also present together in heart and testis. Rap1GAP1 and 2 are both found in pancreas and in the gastrointestinal tract these three GAPs appear to be coexpressed. Of course, the tissue distribution needs to be further analyzed on the cellular level. Variations in Rap1GAP expression patterns suggest that Rap1 signaling is differentially regulated depending on the cell type. These patterns might also reflect cell type-specific functions of Rap1.

We have identified three Rap1-activating proteins in platelets, namely PDZ-GEF1, CalDAG-GEFI/RasGRP2 and CalDAG-GEFIII/RasGRP3. PDZ-GEF1 was known before to be ubiquitously expressed, although platelet expression had not been analyzed<sup>21</sup>. CalDAG-GEFI has been studied in mouse platelets<sup>10</sup>, however, CalDAG-GEFIII expression was observed only in glial cells of brain, in kidney mesangial cells and in B-cells<sup>14,15</sup>. We conclude that Rap1 is activated in a cell type-specific way by different sets of GEFs. Little is known about the regulation of PDZ-GEF1 and CalDAG-GEFs. PDZ-GEF1 function is restricted towards activation of Rap1 and Rap2<sup>22</sup>, whereas CalDAG-GEFIII can activate a broad range of Ras family GTPases including Rap1, Ha-Ras and R-Ras<sup>14</sup>. CalDAG-GEFIII is regulated mainly by DAG<sup>15</sup> and CalDAG-GEFI is considered to be Ca<sup>2+</sup>-dependent<sup>2,10</sup>. CalDAG-GEFIII can also be phosphorylated by PKC and phosphorylation is thought to enhance its GEF activity in lymphocytes<sup>15,55</sup>.

### **Phosphorylation of Rap1GAP2**

In the present study we show that Rap1GAP2 is phosphorylated by both cyclic nucleotide regulated kinases, cAK and cGKI. Cyclic nucleotide-dependent protein kinases often exhibit similar substrate specificities, at least *in vitro*, and Polakis et al.<sup>56</sup> provided indirect evidence that Rap1GAP1 could be phosphorylated by cAK on serine residues 490 and 499<sup>57</sup>. Serine 490, but not serine 499, is conserved in Rap1GAP2, however, mutation of the corresponding serine 549 of Rap1GAP2a does not affect phosphorylation by cAK or cGKI. Instead we have

identified serine 7 as phosphorylation site of Rap1GAP2a, which is preferred by cGKI over cAK. Interestingly, serine 7 is only present in the two platelet splice variants Rap1GAP2a and 2b, but not in the Rap1GAP2c isoform found in cerebellum. This might suggest important regulatory functions of the additional N-terminal sequences derived from exon 2 (Fig. 2A). cGKI can inhibit platelet aggregation<sup>40,41</sup> and under certain conditions also activating effects of cGKI on platelet function have been described<sup>58,59</sup>. However, none of the hitherto identified cGKI substrates in platelets have been conclusively shown to mediate platelet inhibition/activation by NO/cGMP/cGKI. For example deletion of VASP, an established substrate of cGKI involved in platelet adhesion<sup>60,61</sup>, only marginally affects NO/cGMP functions in platelets<sup>62,63</sup>. The new protein RIAM might be a link between VASP and Rap1 although platelet functions of RIAM have not been studied<sup>64</sup>. Early studies also showed direct phosphorylation of Rap1 by cGKI, but neither GTP-binding nor GTPase activity were influenced by phosphorylation<sup>65,66</sup> and no correlation between Rap1 phosphorylation and inhibition of platelet activation could be detected<sup>67</sup>. Rap1GAP2a/b is a new effector of the NO/cGMP pathway and we speculate that Rap1GAP2 phosphorylation by cGKI could enhance its GAP activity thereby leading to Rap1 inactivation and ultimately to the inactivation of integrin  $\alpha_{IIb}\beta_3$ . However, serine 7-phosphorylation does not appear to change the GAP activity of Rap1GAP2a/b directly. Instead, serine 7 phosphorylation could regulate the association of Rap1GAP2a/b with binding proteins. The identity of such proteins will need to be determined in future studies. Rap1GAP2 contains a high number of serine and threonine residues that might represent additional phosphorylation sites for different kinases. *In vivo* labeling of transfected COS-1 cells indeed revealed a very strong basal phosphorylation of Rap1GAP2. Since COS-1 cells do not express cGKI, phosphorylation could be mediated in part by cAK. GSK3 was recently shown to phosphorylate Rap1GAP1 on serine 525 resulting in enhanced proteasomal degradation<sup>27</sup>. However, this residue is not conserved in Rap1GAP2.

The present work suggests that Rap1 activity in human platelets is controlled by PDZ-GEFI, CalDAG-GEFs I and III and Rap1GAP2. Additionally, we have identified Rap1GAP2 as new target of the NO/cGMP/cGKI signaling pathway. The mechanisms of Rap1GAP2 regulation deserve further study considering the important role of Rap1 in platelet activation and aggregation.

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## Figure Legends

### **Figure 1. Expression of GAPs and GEFs of Rap1 in human platelets.**

RT-PCR analysis of GAP (A) and GEF (B) expression in washed human platelets was done using transcript-specific primers for reverse transcription as well as PCR. As positive control human brain RNA was used in most reactions except for SPA-1, KIAA1039, KIAA0545 and CalDAG-GEFI (CD-GEF I), which were detected in spleen (lower panels in A and B). Shown results were confirmed using oligo dT priming for first strand synthesis. None of the known GAPs could be detected in platelets. Database searches disclosed a number of cDNAs encoding new potential Rap1GAPs and of these, KIAA1039 was found to be expressed in platelets. The only GEFs of Rap1 detectable in platelets were CalDAG-GEFI (CD-GEF I)/RasGRP2, CalDAG-GEFIII (CD-GEF III)/RasGRP3 and PDZ-GEF1. Shown are representative results from at least three (platelets) or two (control) similar experiments.

### **Figure 2. Gene structure and protein sequence of Rap1GAP2.**

A complete cDNA corresponding to KIAA1039 was cloned from platelet RNA encoding a protein most closely related to Rap1GAP. Consequently the new protein was named Rap1GAP2. A: Rap1GAP2 is expressed in three different splice variants. The originally cloned variant “a” is the predominant form in platelets. Rap1GAP2a is lacking exons 1 and 6, Rap1GAP2b is lacking only exon 1 and Rap1GAP2c (AK124640, cloned from cerebellum) is generated by splicing of exon 2 which results in the appearance of a new start codon within exon 3 (\* indicates start codons including Kozak initiation sequences). Black shading indicates non-coding regions. Exon 26 comprises 4351 bp. To the right, the results of RT-PCR analysis of variant expression in platelets are shown. Only the splice variants Rap1GAP2a and Rap1GAP2b are detectable in human platelets. This experiment was performed twice with similar results. B: Sequence comparison between Rap1GAP1 and 2 reveals a conserved Rap1GAP domain (solid line), as well as a putative GoLoco domain

(dotted line). Differences between variants are marked in grey: the box in Rap1GAP1 marks additional sequences found only in the Rap1GAP1b/Rap1GAPII splice variant, the first N-terminal box in the Rap1GAP2 sequence is missing in variant “c”, the second box in Rap1GAP2 indicates additional sequences derived from exon 6 present only in Rap1GAP2b and c. Asterisks, double dots and single dots indicate different degrees of amino acid conservation. C: A phylogenetic tree of all presently known Rap1GAPs including related uncharacterized cDNAs shows Rap1GAP1 and Rap1GAP2 as distinct subgroup. Accession numbers of used amino acid sequences are presented in the Materials and Methods section. The numbers at the branches represent the confidence limits computed by the bootstrap procedure. The bar indicates 0.1 substitutions per site.

**Figure 3. Tissue distribution of Rap1GAP2 and Rap1GAP1.**

Northern blots containing PolyA<sup>+</sup> RNA from multiple tissues were probed with <sup>32</sup>P-labeled probes specific for Rap1GAP2 or Rap1GAP1. The size of the detected mRNAs is indicated. Shown are representative results from two experiments.

**Figure 4. Expression of Rap1GAP2 protein.**

COS-1 cells transiently transfected without or with epitope-tagged Rap1GAP2 or Rap1GAP1, isolated peripheral blood lymphocytes and isolated washed human platelets were lysed in SDS-containing stop solution, total protein was separated by SDS-PAGE and immunoblotted with rabbit antiserum against an N-terminal peptide derived from Rap1GAP2 (left panel) or with an antibody specific for Rap1GAP1 (right panel). Arrows indicate tagged overexpressed (95 kDa) as well as endogenous Rap1GAP2 (90 kDa). The antiserum cross-reacts with an unknown protein of 110 kDa. Platelets, and to a lesser extent also lymphocytes, contain endogenous Rap1GAP2 but not Rap1GAP1 protein. Shown are examples of experiments performed at least four times.

**Figure 5. Localization of Rap1GAP2 in transfected COS-1 cells and in platelets.**

A, B: COS-1 cells transiently transfected with Rap1GAP2 (A) and non-transfected control cells (B) were stained with an antibody specific for Rap1GAP2. Rap1GAP2 localized predominantly to cytosolic and perinuclear structures in transfected cells. Non-transfected control cells were not labeled indicating specificity of the antibody. C - H: Human platelets spread on glass were double-labeled with antibodies specific for Rap1GAP2 (C, F) and Rap1 (D, G and merged images in E, H). In the center of panels F-H a single large spread platelet is shown. Both proteins partially colocalized in central vesicular structures. Shown are examples of experiments performed at least five times. *Bar* = 10  $\mu$ M.

**Figure 6. GTPase activity of Rap1GAP2.**

COS-1 cells were transiently transfected with HA-tagged Rap1B together with VSV-tagged CalDAG-GEFIII (CD-GEF III). In addition increasing amounts of VSV-tagged Rap1GAP2a or HA-tagged Rap1GAP1 were expressed as indicated. Two days after transfection cells were lysed and pull-down assays with an activation specific probe were performed to determine the amounts of Rap1B-GTP (panel A). Levels of total Rap1B, Rap1GAP2, Rap1GAP1 and CalDAG-GEFIII were determined with tag-specific antibodies. Blots from four independent pull-down experiments were scanned and quantified (panel B). To compensate for differences in total Rap1 expression levels ratios of Rap1-GTP to total Rap1 signals were calculated. Shown are means  $\pm$  SEM. Both, Rap1GAP2 and Rap1GAP1, blocked CalDAG-GEFIII-induced activation of Rap1B with similar potency.

**Figure 7. Phosphorylation of Rap1GAP2 by cyclic nucleotide regulated kinases.**

A: COS-1 cells were transiently transfected with VSV-tagged wild-type Rap1GAP2a as well as mutants of Rap1GAP2a containing serine to alanine mutations of serine 7 and serine 549,

either singly (S7A and S549A) or in combination (S7A/S549A). Expressed proteins were precipitated with anti-VSV antibody and *in vitro* kinase assays were performed using [ $\gamma$ - $^{32}$ P]ATP and purified cGKI or catalytic subunit of cAK. To detect  $^{32}$ P incorporation samples were separated by SDS-PAGE, blotted onto nitrocellulose and exposed to film ( $^{32}$ P). Then protein amounts were determined using anti-VSV antibodies and ECL detection (WB). Both kinases strongly phosphorylated Rap1GAP2a and mutation of serine 7 to alanine abolished Rap1GAP2a phosphorylation by cGKI. B: Rap1-GTP levels were analyzed in COS-1 cells transiently transfected with Rap1, CalDAG-GEFIII (CD-GEF III), wild-type Rap1GAP2a as well as mutants of Rap1GAP2a containing mutations of serine 7 to alanine (S7A), aspartate (S7D) or glutamate (S7E) as described in the legend to Figure 6. Mutation of serine 7 did not change Rap1GAP2 activity in COS-1 cells. Shown are representative results from at least three independent experiments.

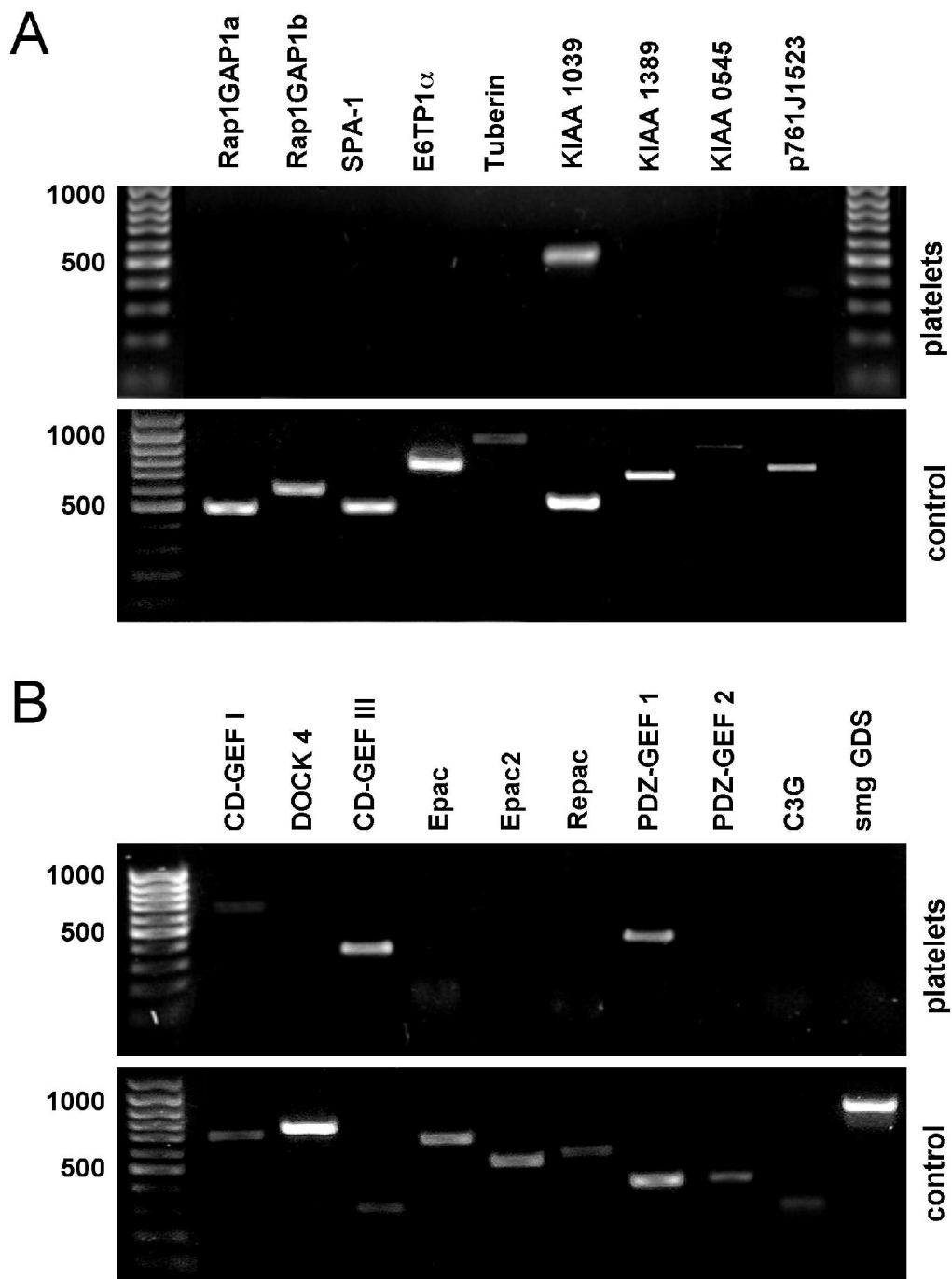


Figure 1

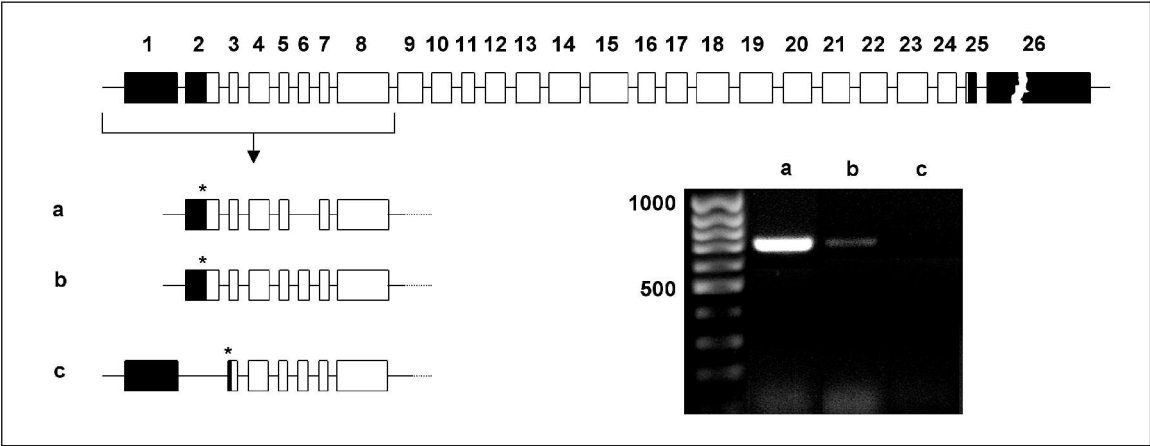


Figure 2A



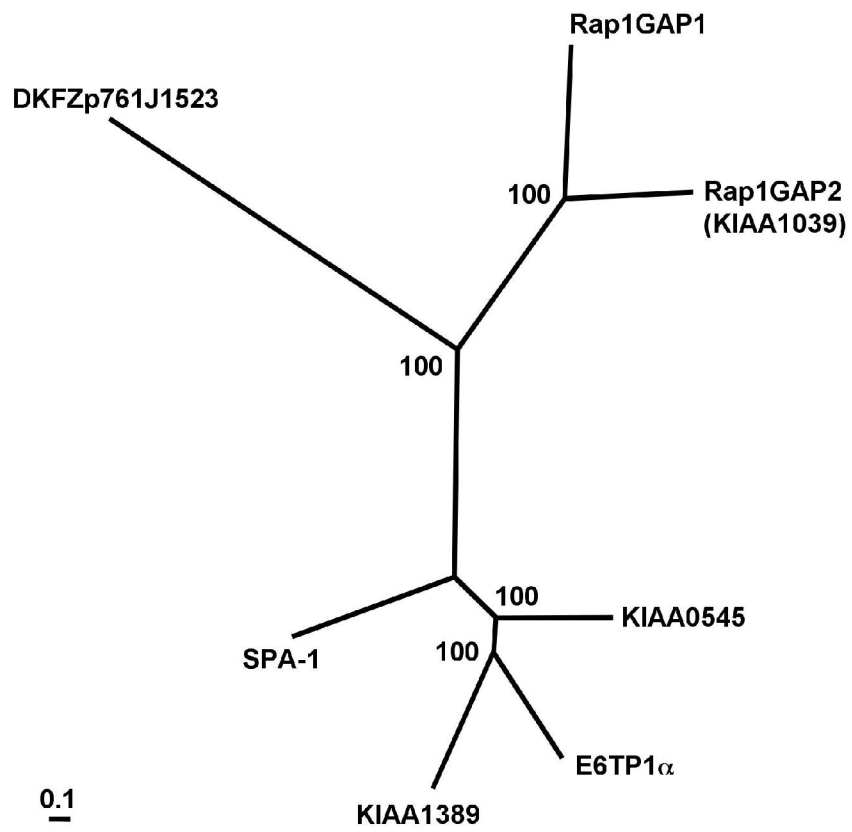


Figure 2C

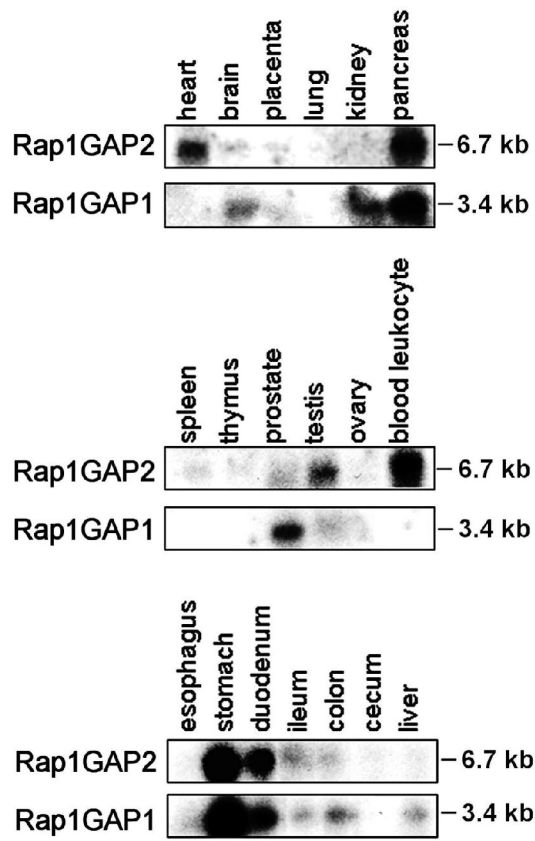


Figure 3

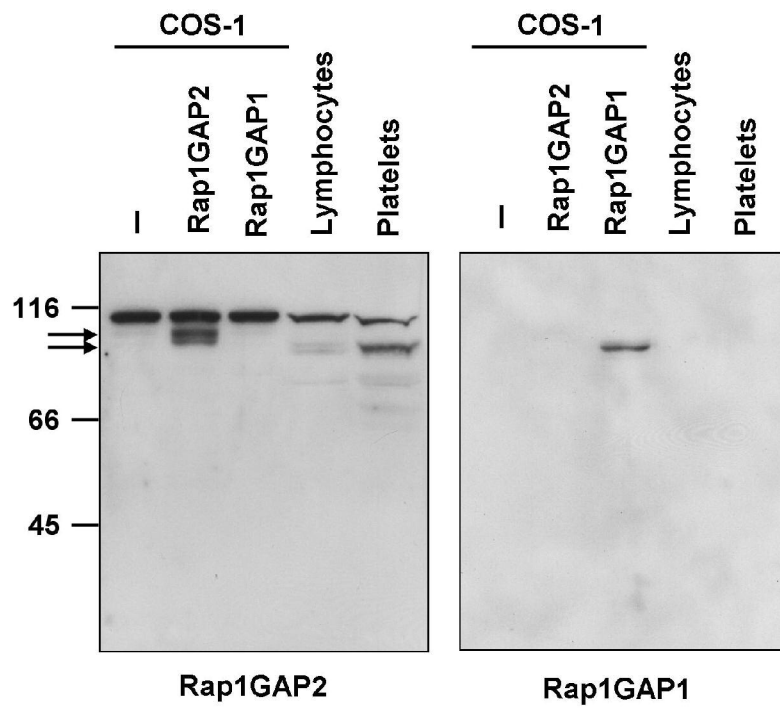


Figure 4

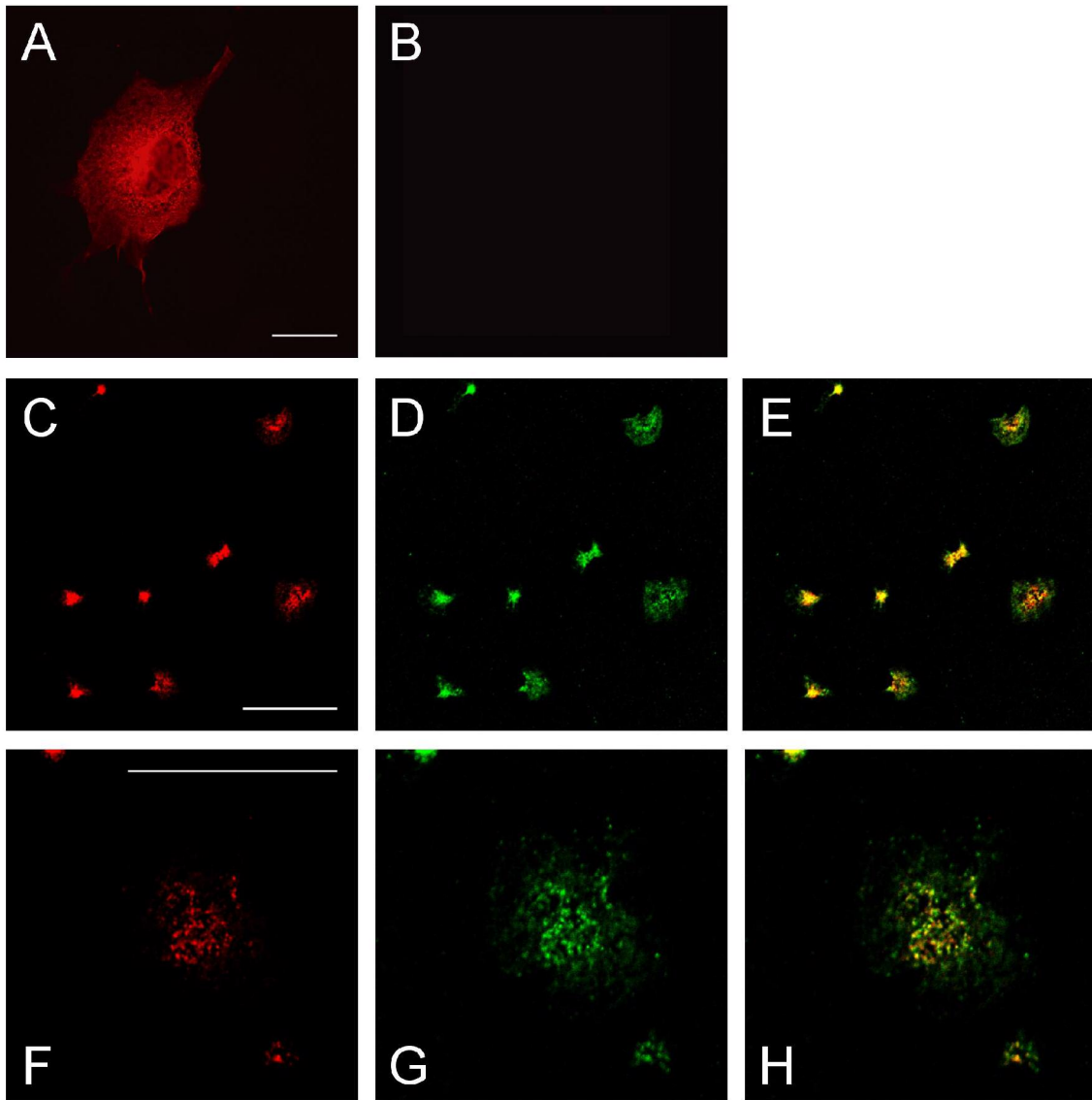


Figure 5

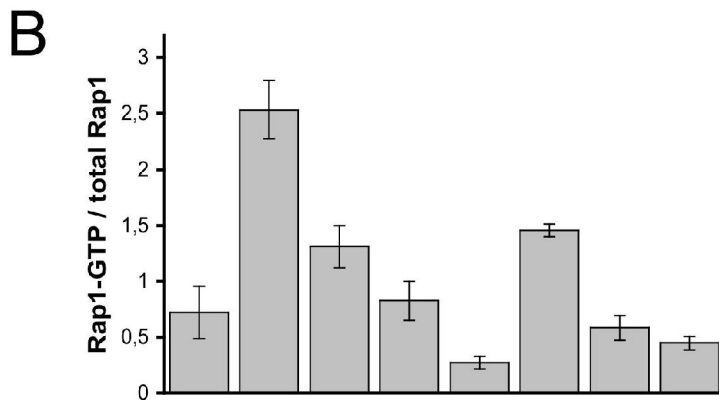
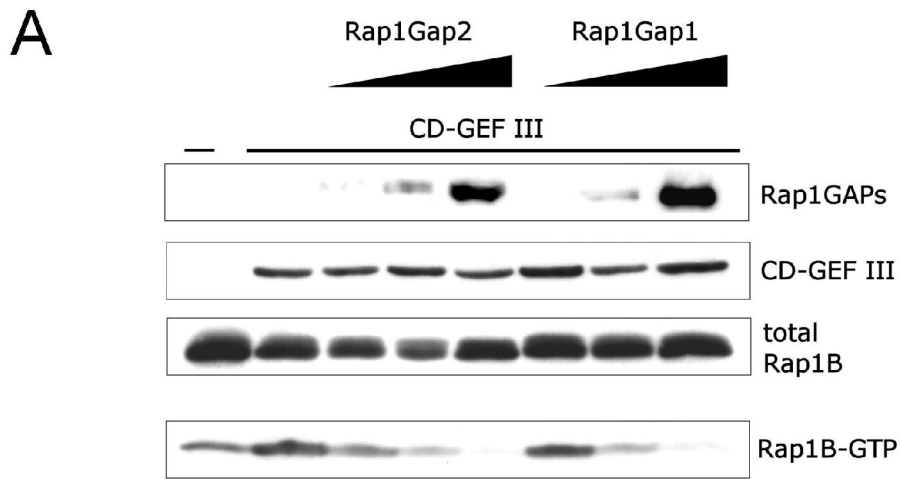


Figure 6

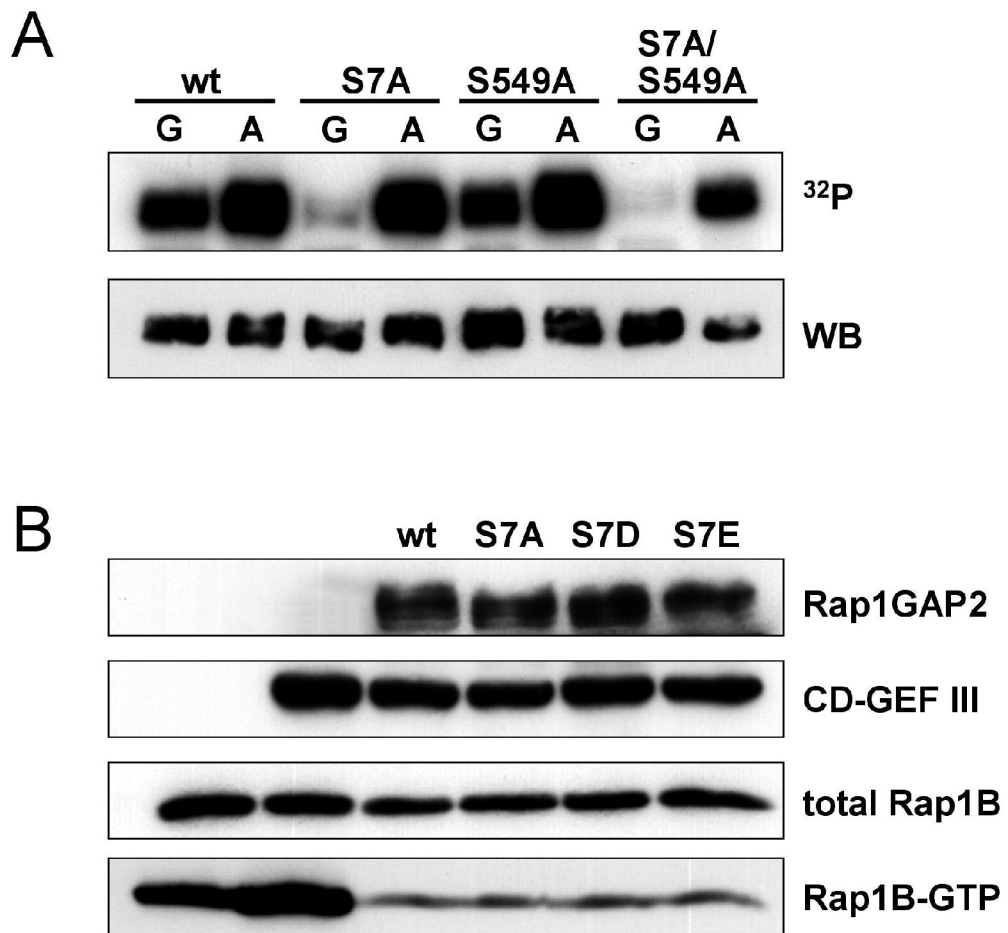


Figure 7