**Protein** – A PCR amplified rat synGAP fragment spanning the C2 and GAP domains (residues 229-725) was ligated in a pET derived expression vector in frame with an N-terminal His6-tag followed by the tobacco etch virus (TEV) protease cleavage site and expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Novagen). Bacteria were grown in TB medium to an OD\(_{600}\) of 0.9 at 37°C and induced with 0.2 mM isopropyl-β-thiogalactoside (IPTG) at 25°C for 18h. Bacterial pellets were resuspended in 20 mM Tris-HCl, 500 mM NaCl, 5 mM MgCl\(_2\), 15 mM imidazole, 1 mM β-mercaptoethanol, 0.2 % Tergitol, type NP-40, pH 7.5 and lysed with an EmulsiFlex homogenizer (Avestin). The lysate was loaded onto a Ni-NTA resin (Qiagen) (2 ml) and eluted with an imidazole gradient (15-250 mM). The fractions containing the SynGAP protein were pooled and TEV protease added. The solution (10-15 ml) was dialyzed against 20 mM Tris-HCl, 200 mM NaCl, 5 mM MgCl\(_2\), 1 mM β-mercaptoethanol, pH 7.5 and the digested sample further purified by Ni\(^{2+}\) affinity chromatography. The protein was finally dialyzed against 20 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl\(_2\), 2 mM di-thiothreitol (DTT), pH 7.5 concentrated to 20 mg/ml and flash frozen in liquid N\(_2\) and stored to –80 °C. The same protocol was used for the proteins comprising PH-C2-GAP (residues 103 - 725), for GAP domain (residues 393 - 725) and C2-GAP mutants, which were generated by site directed mutagenesis using the overlap extension method (Ho et al., 1989), and confirmed by DNA sequencing.
**Biochemical analysis** – Rap1B and H-Ras were prepared according to (Brinkmann et al., 2002; Tucker et al., 1986) and the fluorescent derivative of GTP, 2′(3′)-O-(N-ethylcarbamoyl-(5′″-carboxytetramethylrhodamine amide)-GTP (tamra-GTP), was synthesized as described (Eberth et al., 2005). Stopped-flow experiments using tamraGTP-bound Rap were performed to measure individual rate constants for the interaction of the various SynGAP mutants with Rap in an Applied Photophysics SX18MV stopped-flow apparatus (Ahmadian et al., 1997; Eberth et al., 2005). All reactions were performed at 25 °C in 30 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2 mM DTT, using an excitation wavelength of 546 nm for tamra. SynGAP mutants in the indicated concentrations were mixed with 0.1 μM Rap•tamraGTP in the stopped-flow apparatus and the fluorescence was monitored. For measurements no longer than 100 sec the fluorescence traces presented are the average of at least three individual measurements. Exponential fits to the data were done with Grafit (Erithacus software). HPLC experiments were performed to analyze the nucleotide concentrations and the GTP hydrolysis as described (Ahmadian et al., 1999).

**Crystallization and data collection** – Crystals of C2-GAP were grown in hanging drops at room temperature (~20°C). 4 μl of protein solution (10 mg/ml) were supplemented with 1 μl EDTA (0.1 mM), mixed with 5 μl of crystallization buffer (80 mM ammonium sulfate, 11% PEG 3000, 0.1 M sodium phosphate/citrate pH 4.9) and suspended over a reservoir containing 500 μl crystallization buffer. The crystals were cryoprotected by stepwise transfer into stabilizing solutions containing crystallization buffer with increasing amounts of PEG 400 (to a final 20 %) and shock frozen in liquid nitrogen. The best crystals diffracted to a resolution of 2.7 Å in a synchrotron beam. A
native data set was collected at the European Synchrotron Radiation Facility (ESRF, France), beam line ID 14-2. Data were processed with XDS (Kabsch, 1993) (Table 2).

**Structure determination and refinement** – The structure was solved by molecular replacement using the program EPMR (Kissing et al., 2001) with the central portion of the catalytic domain of p120GAP (residues 766-974) as a search model, in which all the non-identical non-glycine residues were truncated to alanine using the program MOLEMAN (Kleywegt et al., 2001).

The correct space group was identified P6₁ and the best solution contained two molecules in the asymmetric unit with a reasonable crystal packing. The unaveraged map at a resolution of 3 Å was interpretable and the two GAP components in the asymmetric unit were built independently and refined. The model was subjected to alternate rounds of model building using O (Jones and Kjeldgaard, 1997) and refinement in CNS (Brunger et al., 1998) and REFMAC (Murshudov et al., 1997), gradually including data to 2.7 Å resolution. Composite simulated annealing omit maps were routinely calculated to facilitate model correction. Of the C2 domain only β-strands in proximity of the GAP portions could be traced (supplementary Figures S5). However, this allowed determining the approximate orientation of C2 relative to the GAP domain (see below). The final model consists of two chains, comprising 348 (chain A) and 358 (chain B) amino acids. The Ramachandran plot depicts 98.3 % of main chain torsion angles in the most favored and allowed regions, with no residues in disallowed regions. We generally refer to chain A in the structure description in this study. Structural figures were prepared with the programs MOLSCRIPT (Kraulis, 1991) and POVSCRIPT (Fenn et al., 2003) or PYMOL (http://pymol.sourceforge.net).
**Structure Modeling** – GenTHREADER (Jones, 1999) was used for protein fold recognition and the structures ranked with a high confidence (protein data bank codes 1gmi and 1wfj) were used as templates. Homology models for the C2 domain (residues 242 - 393) were built with MODELLER (Sali and Blundell, 1993), based on the alignment provided by GenTHREADER, and the stereochemistry validated with PROCHECK (Laskowski et al., 1993).

**Supplementary figures:**

**Figure S1**

![Graph](image.png)

**Legend to Figure S1:**
Stimulation of the Rap GTPase reaction by C2-GAP wild type (○) and N472T (△). The observed rate constants obtained using 0.1 μM Rap•tamraGTP are plotted against...
increasing concentrations of C2-GAP on a logarithmic scale to derive the $k_{cat}$ and the $K_d$ values (Table 1).

**Figure S2**

Legend to Figure S2:
Single turnover stopped-flow measurements of 0.1 µM Ras•tamraGTP and 5 µM SynGAP proteins as indicated. The observed rate constants determined from single exponential fitting are 0.00132 s$^{-1}$ for GAP, 0.013 s$^{-1}$ for C2-GAP and 0.0031 s$^{-1}$ for C2-GAP R470K. The intrinsic GTPase reaction (0.0001 s$^{-1}$), shown in black, is up to 2-fold slower than for C2-GAP mutant R470P. Data presented are the average of at least three individual measurements.
Legend to Figure S3:

Multiple sequence alignment of C2-GAP with representative homologues, constructed with Clustal X (Thompson et al., 1997). Assignment of secondary structure elements is according to the program DSSP (Kabsch and Sander, 1983) and is included for SynGAP (colored) above and for p120GAP (gray) below the alignment blocks. α-helical regions are depicted in red (GAPc) or magenta boxes (GAPex), the secondary structure of the
C2 assigned region in dark yellow. The numbering of the β-strands is according to the C2 domains used for modeling. The residues mutated in this study are marked with ‘+’.
The interdomain region comprising the hydrophobic interface is boxed. Abbreviations of species are as follows: m, Rattus norvegicus; hs, Homo sapiens; sc, Saccharomyces cerevisiae.

Figure S4

Legend to Figure S4
Model of a hypothetical C2-GAP-Rap complex obtained by aligning the GAPc portion of SynGAP with that of p120GAP from the Ras-RasGAP complex (Scheffzek et al., 1997) and aligning Rap (from PDB code 1gua) with Ras. The two molecules from the asymmetric unit are superimposed, showing different orientations of the C2 portions as far as they are visible in the electron density (see text and Fig. 2).
Figure S5

Legend to Figure S5

Omit map (contoured at $1\sigma$) of a representative region showing a $\beta$-sheet of the C2 domain. The map has been calculated after refinement of the model that did not include the coordinates for the respective segment.
References


