The C2 domain of SynGAP is essential for stimulation of the Rap GTPase reaction

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The brain-specific synaptic guanosine triphosphatase (GTPase)-activating protein (SynGAP) is important in synaptic plasticity. It shows dual specificity for the small guanine nucleotide-binding proteins Rap and Ras. Here, we show that RapGAP activity of SynGAP requires its C2 domain. In contrast to the isolated GAP domain, which does not show any detectable RapGAP activity, a fragment comprising the C2 and GAP domains (C2–GAP) stimulates the intrinsic GTPase reaction of Rap by approximately 1×10^4 . The C2–GAP crystal structure, complemented by modelling and biochemical analyses, favours a concerted movement of the C2 domain towards the switch II region of Rap to assist in GTPase stimulation. Our data support a catalytic mechanism similar to that of canonical RasGAPs and distinct from the canonical RapGAPs. SynGAP presents the first example, to our knowledge, of a GAP that uses a second domain for catalytic activity, thus pointing to a new function of C2 domains.

Keywords: synaptic plasticity; Ras; X-ray crystallography; long-term potentiation; GTP hydrolysis; C2 domain

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INTRODUCTION

The strength of synaptic connections between neurons is an important factor in the formation of memory. Synaptic efficacy is regulated by a complex biochemical machinery, termed post-synaptic density (PSD), at the cytosolic part of the postsynaptic membrane (Kennedy, 2000). The PSD contains several proteins involved in synaptic plasticity, including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), PSD-95, ionotropic glutamate type receptors (*N*-methyl-D-aspartate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and synaptic guanosine triphosphatase (GTPase)-activating protein (SynGAP; Chen *et al*, 1998; Kim *et al*, 1998), a 143 kDa protein that interacts with PSD-95 by a carboxy-terminal PDZ-binding motif and is phosphorylated by CaMKII (Oh *et al*, 2004). SynGAP is involved in neuronal development (Kim *et al*, 2003; Vazquez *et al*, 2004) and apoptosis (Knuesel *et al*, 2005), glutamate receptor transport (Zhu *et al*, 2002; Rumbaugh *et al*, 2006) and in the induction of hippocampal long-term potentiation (Komiya *et al*, 2002).

On the basis of sequence similarity, SynGAP was identified as a Ras-specific GTPase-activating protein (RasGAP; Chen *et al*, 1998; Kim *et al*, 1998). GAPs are known to regulate the biological activity of guanine nucleotide-binding proteins, usually with high substrate specificity (Vetter & Wittinghofer, 2001; Scheffzek & Ahmadian, 2005). In the SynGAP sequence, the RasGAP domain is preceded by a C2 and a pleckstrin homology (PH) domain, a domain scheme present in several RasGAPs (Bernards, 2003). Many PH domains bind to phospholipids and might act as membrane recruitment modules (Lemmon, 2004). C2 domains have been described as Ca^{2+} -dependent phospholipid-binding modules (Nalefski & Falke, 1996) in various proteins involved in signal transduction with considerable variations in the binding ligands, which range from small molecules to proteins (Rizo & Sudhof, 1998; Cho, 2001; Benes *et al*, 2005). Little is known about the roles of C2 domains in RasGAPs, in which they typically occur between a PH domain at the amino-terminal end and a RasGAP module at the C-terminal end (Bernards, 2003). In CAPRI (Ca^{2+} -dependent RasGAP), two C2 domains are required for Ca^{2+} -dependent membrane association *in vivo* (Lockyer *et al*, 2001).

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Fig 1 | Structural overview and comparative kinetic analysis of synaptic guanosine triphosphatase-activating proteins. (A) Schematic representation of the SynGAP fragments used for biochemical and structural analysis. (B) Single-turnover measurements for the reaction of 0.1 μM Rap●tamraGTP and different SynGAP proteins at 5 μM concentration. (C) Ribbon diagram showing the overall structure of C2-GAP. The GAP region corresponding to the p120GAP domain responsible for catalysis (GAPc) with the helices presenting the Ras-binding groove highlighted in light yellow, the GAP region dispensable for catalysis (GAPex) and the C2 domain portions are shown in red, magenta and dark yellow, respectively. The C2 domain body in grey was modelled using Modeller and superposition onto the well-defined β-sheet near the domain interface. (D) Stereo view of the C2-GAP interface. The backbone (Cα trace) of the C2 domain is shown in yellow and that of the GAP domain in red. The composite omit map is contoured at 1.1σ. PH, pleckstrin homology; SynGAP, synaptic guanosine triphosphatase-activating protein; tamraGTP, 2'/(3')-O-(N-ethylcarbamoyl-(5''-carboxytetramethylrhodamine) amide)-GTP.

SynGAP activation by Ca^{2+} -dependent CaMKII phosphorylation (Oh *et al*, 2004) might couple the increase in intracellular calcium during excitatory synaptic transmission to the regulation of the Ras/ERK pathway. Recently, it was found that SynGAP, despite its close homology to RasGAPs, stimulates the GTPase of the Ras-related protein Rap much more potently than that of Ras itself (Krapivinsky *et al*, 2004). Although Rap and Ras belong to the same family, their roles in cellular processes are different (Reuther & Der, 2000).

Here, we have investigated the structural requirements for the RapGAP activity of SynGAP. By using biochemical analysis of proteins containing the GAP module in different domain environments, we have identified the C2 domain as essential for the RapGAP activity of SynGAP, which otherwise shares common features with canonical RasGAPs. By structural analysis of C2-GAP crystals, we suggest a model to explain how the C2 domain might approach the nucleotide-binding region of Rap to contribute to catalysis.

Table 1 | Rate constants for the reaction of tamraGTP-bound Rap with SynGAP proteins

SynGAP proteins	k_{cat} (s^{-1})	K_d (μM)
C2-GAP (wild type)	0.8	10
C2-GAP N472T	0.045	5.43
C2-GAP R470P	ND	ND
GAP (wild type)	ND	ND

Kinetic parameters were determined by fluorescence spectroscopy according to the model described by Eberth *et al* (2005).
ND, not determinable under the condition of C2-GAP wild type; SynGAP, synaptic guanosine triphosphatase-activating protein; tamraGTP, 2'-(3')-O-(N-ethylcarbamoyl-(5''-carboxytetramethylrhodamine) amide)-GTP.

RESULTS AND DISCUSSION

RapGAP activity of SynGAP requires its C2 domain

We measured the kinetics of the GAP-catalysed GTP hydrolysis reactions of Ras and Rap proteins in a fluorescence-based GAP assay by using 2'-(3')-O-(N-ethylcarbamoyl-(5''-carboxytetramethylrhodamine) amide)-GTP (hereafter designated as tamra-GTP) and rat SynGAP proteins comprising the GAP domain only, C2-GAP and PH-C2-GAP (Fig 1A). In contrast to other fluorescent nucleotides including 2'-(or 3')-O-(N-methylanthraniloyl) GTP, which does not show any signal in the case of Rap, tamraGTP allows real-time monitoring of the kinetics of GAP-catalysed GTP hydrolysis reactions (Eberth *et al*, 2005). The isolated GAP domain did not show detectable RapGAP activity, whereas the C2-GAP protein showed a marked increase in RapGAP activity, reflected in a rapid decrease of fluorescence on mixing Rap●tamraGTP with the GAP proteins. The curves were fitted to a single exponential decay (Fig 1B). The rate constants increased in a hyperbolic manner as a function of C2-GAP concentration (supplementary Fig S1 online). Fitting a hyperbolic curve to the data points (Eberth *et al*, 2005) led to an apparent dissociation constant (K_d) of 10 μM and a maximal rate (k_{cat}) of 0.8 s^{-1} (Table 1). The rate acceleration is 1.6×10^4 with respect to the basal GTPase activity. The presence of the PH domain did not influence RapGAP activity significantly, as indicated by kinetic analysis of the PH-C2-GAP protein (Fig 1B). This indicates that SynGAP uses the C2 domain as an integral element of the GAP machinery, pointing to a new function of C2 domains. GAP activity was not sensitive to Ca^{2+} , and the C2 domain did not bind to Ca^{2+} in isothermal calorimetry experiments (data not shown).

In contrast to the data obtained with Rap●tamraGTP, fluorescence measurements with Ras●tamraGTP (supplementary Fig S2 online) indicated a RasGAP activity that was approximately 3,600-fold lower than that with the GAP domain of neurofibromin under similar conditions (data not shown). Nevertheless, the C2-GAP activity towards Ras●tamraGTP was tenfold higher than that of the isolated GAP domain (supplementary Fig S2 online). Our results indicate that the C2 domain is crucial for its RapGAP activity and that SynGAP is an ineffective RasGAP under our experimental conditions, which is consistent with previously published data (Kim *et al*, 1998; Krapivinsky *et al*, 2004).

Interdomain communication in the C2-GAP structure

The crystal structure of the RasGAP-related C2-GAP fragment (residues 229–725; see Methods; Table 2; Fig 1C) confirms the

Table 2 | Summary of the crystallographic analysis

<i>Data collection</i>	
Space group	$P6_1$
<i>Cell dimensions</i>	
a, b, c ($\text{\AA}$)	113.4, 113.4, 166.28
α, β, γ ($^\circ$)	90, 90, 120
Wavelength ($\text{\AA}$)	0.933
Resolution ($\text{\AA}$)	20–2.7 (2.86–2.70)
R_{meas}^*	10.7 (70.4)
$I/\sigma(I)^*$	14.7 (1.86)
Completeness (%)	99.6 (100.0)
Number of observed reflections	167,932 (16,576)
Redundancy	5.10 (3.29)
<i>Refinement</i>	
Resolution ($\text{\AA}$)	20–2.7
Number of reflections	31,232
$R_{work}/R_{free}^{\dagger}$	0.243/0.288
<i>Number of atoms</i>	
Protein	5,493
<i>B-factors</i>	
Protein	39.426
<i>R.m.s. deviations</i>	
Bond lengths ($\text{\AA}$)	0.015
Bond angles ($^\circ$)	1.55

*As specified in XDS (Kabsch, 1993).
[†]As specified in REFMAC (Murshudov *et al*, 1997).
Values for the highest resolution shell are given in parentheses.

RasGAP fold (Scheffzek *et al*, 1996) for the GAP portion (root mean square deviation of $\sim 1.2 \text{\AA}$, comparing 153 out of 207 C α positions) and shows a tight local interaction between the two domains (Fig 1D). Although a major portion of the C2 domain could not be traced in the electron density, its C-terminal and adjacent β -strands (residues 343–348, $\beta 7$, and 387–398, $\beta 8$; amino acids 238–249, $\beta 1$, and 295–300, $\beta 4$; see the supplementary information online) could be assigned, thus clearly allowing determination of the relative domain orientation with homology modelling indicating the approximate extension of the domain (see the supplementary information online; Fig 1C). Extensive attempts to improve crystals or to obtain alternative crystal forms with the C2 domain ordered were unsuccessful. Disordered domains have been observed previously in crystal structures; we do not have an explanation for the partial disorder in our crystals apart from local stabilization by the GAP extra (GAPex) domain. We believe it is likely that the remaining portion of the C2 domain will become structurally ordered upon Rap binding. Our results suggest that a glycine-rich region (residues 353–381) form an extended peptide segment within the C2 domain rather than a flexible linker connecting C2 with the GAP domain, which sequence comparison might suggest (supplementary Fig S3

Fig 2 | Structural regions crucial for the C2-GAP-stimulated GTPase of Rap. (A) Ribbon representation of the catalytic interface between SynGAP and Rap, as modelled by aligning Rap1 and C2-GAP based on the Ras-RasGAP complex (see panel C; Scheffzek *et al*, 1997). GAPc is shown in red, GAPex in magenta, the finger loop in yellow and Rap in grey. Residues that are thought to be important in catalysis are shown as sticks. (B) Single-turnover measurements of 0.1 μM Rap•tamraGTP and 5 μM SynGAP proteins as indicated. The observed rate constants determined from single exponential fitting are 0.28 s^{-1} for C2-GAP wild type, 0.023 s^{-1} for C2-GAP (N472T), 0.0031 s^{-1} for C2-GAP (R470K) and 0.0002 s^{-1} for C2-GAP (R470P). The intrinsic GTPase reaction rate is 0.0001 s^{-1} . Data are the average of at least three individual measurements. (C) Hypothetical complex of C2-GAP with Rap based on a structural alignment of GAPc and Rap with the corresponding portions of the Ras-RasGAP complex (Scheffzek *et al*, 1997). The two monomers from the asymmetric unit were superimposed to show the shift of the GAPex (coil) and C2 domain (β -strands) towards the presumed Rap-GAPc interface. The body of the C2 domain is included as an ellipsoid to visualize the potential conformational shift. GAPc is depicted as a dark pink surface. GTPase, guanosine triphosphatase; sw II, switch II region; SynGAP, synaptic guanosine triphosphatase-activating protein; tamraGTP, 2'/(3')-O-(N-ethylcarbamoyl)-(5''-carboxytetramethylrhodamine) amide)-GTP.

online). This results in an unexpected tight local interaction between the two domains at the domain boundaries. As the GAPex domain is known not to be required for catalysis in RasGAPs, one might speculate that it stabilizes the central catalytic (GAPc) domain and might act as a docking platform for the C2 domain in GAPs with the C2-GAP domain organization.

Implications for the interaction with Rap/Ras targets

Structural alignment of the RasGAP domain of SynGAP with that of p120GAP in complex with Ras (Scheffzek *et al*, 1997) indicates an approximate model for the Rap-SynGAP complex (Fig 2A; supplementary Fig S4 online). In this model, the superimposed Rap (from Protein Data Bank code 1gua) interacts with the GAP

portion in a manner similar to Ras, with the C2 domain being in the proximity of the switch II region (see below). The model is supported by the observation that the RasGAP compromising mutations of the catalytic arginine to lysine or proline (Arg 470 $\rightarrow$ Lys/Pro in SynGAP; Ahmadian *et al*, 1997; Klose *et al*, 1998) reduce RapGAP activity 100-fold in the former and almost entirely in the latter (Fig 2B). In keeping with this model, Val12Rap, which is sensitive towards canonical RapGAPs (Brinkmann *et al*, 2002), is—similar to Val12Ras—not sensitive to SynGAP (data not shown).

Although Rap is a close homologue of Ras, it has a threonine residue instead of the highly conserved catalytic glutamine at position 61, which is believed to be responsible for the lower rate

of GTP hydrolysis observed in Rap proteins and also seems to be a determinant of substrate specificity with respect to p120GAP (Hart & Marshall, 1990). Rap1GAP uses a catalytic mechanism that uses an asparagine residue provided by the GAP component, presumably to supply the missing functional group of the canonical Gln 61 (Daumke *et al*, 2004). However, replacing Thr61 by glutamine does not significantly affect the kinetics of SynGAP-mediated GTPase activation of Rap (data not shown). We considered whether an asparagine close to the catalytic site, Asn472, might have a role similar to that of the catalytic asparagine in Rap1GAP (Fig 2A). Interestingly, the mutation of Asn472 to threonine, as found in the p120GAP homologue, decreased RapGAP activity by approximately 20-fold but did not affect the binding affinity of C2–GAP overall (Fig 2B; Table 1). This indicates that, in addition to Arg 470, Asn472 might also have a catalytic role in the SynGAP-stimulated GTPase reaction of Rap.

Next, we addressed how the C2 domain of SynGAP might affect GAP activity. In our model of the Ras/Rap–SynGAP complex (supplementary Fig S4 online), the C2 domain is in the vicinity of the nucleotide-binding region of the G-protein target. Alignment of the two molecules of the asymmetric unit, with respect to the GAP domain, indicated that the C2 domain might move by approximately 10 Å in the direction of the catalytic site in a structural rearrangement that would also involve portions outside the GAPc domain (Scheffzek *et al*, 1997; Fig 2C). Although the differences between the conformations of the two monomers in the asymmetric unit might be due to crystal packing, they indicate flexibility, which might become functionally important in the context of C2 domain-assisted GTP hydrolysis.

The C2–GAP tandem is a feature of several canonical RasGAP proteins, some of which do not show RapGAP activity (Frech *et al*, 1990; Noto *et al*, 1998). Sequence comparisons of such RasGAPs, with respect to the C2 domain, do not show candidate determinants that would allow the prediction of dual or switched substrate specificity (supplementary Fig S3 online). Dual substrate specificity has been reported for GAP1 family members, which activate the GTPase of Ras and Rap (Cullen *et al*, 1995; Kupzig *et al*, 2006). Their domain scheme indicates an N-terminal tandem of two C2 domains, followed by a RasGAP domain and a PH domain. Although it is tempting to speculate that in GAP1^{IP4BP} the C2 domains might have a role similar to that of SynGAP observed in this study, sequence comparison does not explain how specificity might be mediated. It has recently been shown that residues outside the GAP domain are required for the RapGAP activity of GAP1^{IP4BP} (Kupzig *et al*, 2006).

Conclusions

It is evident from the data presented in this study that SynGAP requires its C2 domain for GAP activity, which inactivates Rap much more efficiently than Ras. This provides an unprecedented mode of action, adding another level of complexity on how inactivation of Ras-related proteins can be achieved. Further studies of complexes with target guanine nucleotide-binding proteins will be required to define the precise mechanism of this novel feature of GAP action.

METHODS

A detailed description of the experimental procedures is provided in the supplementary information online.

Protein expression, purification, crystallization and structural analysis. SynGAP fragments comprising GAP (residues 393–725), C2–GAP (residues 229–725) and PH–C2–GAP (residues 103–725) were PCR amplified, cloned in pET-derived vectors, expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Novagen, Merck, Nottingham, UK) and purified by Ni²⁺ affinity chromatography. Rap1B and H-Ras were prepared as described previously (Tucker *et al*, 1986; Brinkmann *et al*, 2002). Crystals of C2–GAP were grown by using the vapour diffusion technique, and X-ray analysis and modelling were carried out as described in the supplementary information online.

Biochemical analysis. The fluorescent derivative of GTP (tamraGTP) was synthesized as described previously (Eberth *et al*, 2005). Stopped-flow experiments using tamraGTP-bound Rap were performed to measure individual rate constants (see the supplementary information online).

Atomic coordinates accession codes. Atomic coordinates and structural factors have been deposited with the Protein Data Bank (www.rcsb.org), accession code 3BXJ.

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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