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► **To cite this version:**

Meera Soundararajan, Xiaowen Yang, Jonathan M Elkins, Frank Sobott, Declan A Doyle. The centaurin gamma-1 GTPase-like domain functions as an NTPase. *Biochemical Journal*, 2006, 401 (3), pp.679-688. 10.1042/BJ20060555 . hal-00478577

HAL Id: hal-00478577

<https://hal.science/hal-00478577>

Submitted on 30 Apr 2010

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The Centaurin Gamma-1 GTPase-like domain functions as an NTPase

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Short title: Structure of the CENTG1 GLD domain

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Summary

Centaurins are a family of proteins that contain GTPase activating protein domains with the gamma family members containing in addition a GTPase-like domain. Centaurins reside mainly in the nucleus and are known to activate PI-3 kinase, a key regulator of cell proliferation, motility and vesicular trafficking. In this report, through X-ray structural analysis, enzymatic assays and nucleotide binding studies, we show that for centaurin gamma-1 (CENTG1) the GTPase-like domain has broader tri-nucleotide specificity. Alterations within the G4 motif of CENTG1 from the highly conserved NKxD found in typical GTPases to TQDR result in the loss of specificity, a lower affinity for the nucleotides and higher turnover rates. These results indicate that the centaurins could be more accurately classified as NTPases and point to alternative mechanisms of cell signalling control.

Introduction

Cell signalling involves the detection, amplification and regulation of a specific signal that frequently starts when a ligand binds to its cell surface receptor. In the case of the family of small GTPases, activation of the signal pathway involves a switch of the GTPase enzyme from an inactive GDP-bound form to the active GTP-bound state [1-3]. The ability of the GTPases to function as molecular switches is regulated through differential action of Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs). GEFs convert the GTPases to the active state by replacing bound GDP for GTP while GAPs reverse this process and down regulate the signalling pathway by enhancing the GTPase activity thus converting the enzyme into the inactive GDP-bound state. Differences in structure between the GDP- and GTP-bound states of the GTPases are the basis for this signal transmission. The main changes centre on two loop regions designated Switch I and Switch II [4-8]. There are five subfamilies based on sequence alignment (Ras, Rho, Rab, Arf/Sar1 and Ran) that comprise the small GTPase family [9].

All of the centaurin proteins, like many proteins involved in signalling, are composed of multiple domains. The domains that the centaurin family members have in common include an Arf-GAP domain, Ankyrin repeats and Pleckstrin homology (PH). Both the cytohesin and centaurin PH domains bind the lipid phosphatidylinositol 3,4,5-triphosphate [10, 11] thus

localising them to the membrane surface where their target protein is located. The centaurin gamma subfamily stands out from the rest of the centaurins for having an unusual domain organisation with a GTPase-like domain (GLD) on the N-terminus (Figure 1A). Based on sequence identity, this GTPase domain is actually more closely related to the Rab and Ras subfamilies than to the Arf family of small GTPases. The human centaurin gamma (CENTG) subfamily is represented by three isoforms: centaurin gamma-1 (CENTG1), centaurin gamma-2 (CENTG2) and centaurin gamma-3 (CENTG3) which are highly similar in primary structure (Figure 1B). Due to the sequence similarity of the GLD domain to the classical GTPase family and the presence of an Arf GAP domain it has been assumed that the CENTG GLD domains are functionally GTPases.

The rat Phosphoinositide 3-kinase enhancer (rPIKE) proteins are recognised to be the closest homologues to the centaurin gamma proteins by gene structure and sequence (Figure 1). rPIKE is a brain specific nuclear GTPase protein, which binds PI 3-kinase and stimulates its lipid binding activity [12]. Three different alternatively spliced forms of PIKE have been reported: PIKE-L the long isoform (gi: 25989575), PIKE-S, the short isoform and the recently identified isoform called PIKE-A (gi:1531538). The PIKE-A isoform is the product of a different initiation transcript of PIKE-L and is identical to the human centaurin gamma-1. CENTG1 has been previously labelled GGAP2, while CENTG2 has been previously labelled GGAP1. We refer to these proteins as CENTG1 or CENTG2 within this paper.

CENTG1 was shown to interact directly with activated protein kinase B/Akt in a GTP-dependent manner and stimulate Akt kinase activity [13]. CENTG1 is co-amplified with cyclin-dependent kinase 4 in a variety of human cancers [14, 15]. Recent studies have shown that CENTG1 is a physiological regulator of apoptosis [16] and an oncogenic effector of cell invasion through its activation of Akt kinase function [13].

The signalling pathways mediated by the Ras superfamily in the cell cytoplasm have been extensively characterised. However, the mode of action of signalling GTPases in the nucleus remains largely obscure. In this study, we present the crystal structure of the N-terminal GLD of CENTG1 in the absence of bound nucleotide. Significant structural differences exist between the CENTG1 GLD domain and the archetypal small GTPases, such as H-ras, that called into question the nucleotide specificity for this GLD domain. To verify its activity, we performed enzymatic assays that indicated that the domain is able to catalyse the hydrolysis of a selection of

triphosphate nucleotides thereby classifying the CENTG1 GLD domain as an NTPase. No other human GTPase-like domain showing NTPase activity has been characterised before. This study provides a beginning for the understanding of the structure and function of this family of nuclear NTPases that actively participates in the promotion of cancer cell invasion and prevention of apoptosis.

EXPERIMENTAL PROCEDURES

Cloning

A sequence containing the GLD of centaurin gamma-1 (GenBank: gi:7661962) was amplified by PCR from DNA in the Mammalian Gene Collection (I.M.A.G.E. Consortium Clone ID 5218187, GenBank: BC028020.1) and subcloned into an in-house vector carrying ampicillin resistance, pLIC-SGC, using ligation-independent cloning. The resulting plasmid expresses residues 66-241 of the GLD with an N-terminal hexahistidine tag and TEV protease tag cleavage site (extension MHHHHHSSGVDLGTENLYFQ*SM-). After digestion with TEV protease, the protein retains an additional serine and methionine on the N-terminus. The point mutants of CENTG1 were created in a two-step overlapping PCR process and subcloned into pLIC-SGC in an identical manner. The sequences of all constructs were confirmed by DNA sequencing.

Expression and purification

Plasmids encoding the CENTG1 GLD domain or the mutants were transformed into BL21(DE3) competent cells and the transformants used to inoculate 10 ml of Luria-Bertani media with 100 µg/ml ampicillin which was incubated overnight at 37 °C. This culture was used to inoculate 1 L of Terrific Broth media with 100 µg/ml ampicillin and grown at 37 °C until an OD₆₀₀ of 0.6 was reached. The protein was induced with 1 mM IPTG for 12 hours at 18 °C. The cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5 % glycerol, 10 mM imidazole) and frozen at -80 °C until further use.

The frozen cell pellet was thawed in the presence of one EDTA-free protease inhibitor tablet (Roche). The cells were lysed using an Emulsiflex C5 high pressure homogenizer (Avestin), polyethylenimine added to a final concentration of 0.15 % and the insoluble debris removed by centrifugation for 45 min at 15,000 rpm. The CENTG1 GLD was extracted from the

clarified supernatant by affinity tag purification using Ni-NTA resin (Qiagen): the supernatant was bound to Ni-NTA and washed with 30 column volumes of lysis buffer and 5 column volumes of wash buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5 % glycerol, 20 mM imidazole). The protein was eluted from the resin with 5 column volumes of elution buffer (20 mM Tris pH 8.0, 200 mM NaCl, 5 % glycerol, 150 mM imidazole). The eluted protein was further purified by gel filtration chromatography (S75 16/60) in either 50 mM Tris pH 8.0, 150 mM NaCl (for the *C2* space group) or 50 mM Tris pH 8.0, 150 mM NaCl, 100 μ M GDP, 10 mM NaF, 20 μ M AlF₃, 5 mM DDT (for the *P2₁2₁2₁* space group). Fractions containing the CENTG1 GLD were identified using SDS-PAGE, pooled and concentrated to 10 mg/ml using a 10 kDa cutoff concentrator. The protein was incubated overnight at 4 °C with TEV protease and purified from the tag by passing over Ni-NTA. The purified CENTG1 GLD was concentrated to 30 mg/ml. The identities of the CENTG1 GLD proteins were confirmed by mass spectrometry under denaturing conditions using an Agilent LC-MS system with a reverse phase column (WT expected 19581.1 Da, observed 19581.4 Da; T101A expected 19551.0 Da, observed 19550.8 Da; T101P expected 19577.1 Da, observed 19577.0 Da).

Crystallisation, data collection and structure solution

Crystals of CENTG1 GLD were obtained from 150 nl sitting drops containing a 1:1 ratio of protein to reservoir solution at 20 °C. The reservoir solution consisted of either 50 mM Tris pH 8.0, 150 mM NaCl, 0.1 M SPG pH 7.0, 30 % PEG₁₀₀₀, 0.5 % DMSO (*C2* space group) or 0.1 M propionate:cacodylate:Bis Tris propane pH 6.0, 30 % PEG₁₀₀₀ (*P2₁2₁2₁* space group). Crystals were mounted in nylon loops and cryo-cooled by transfer to a 100 K nitrogen stream. Data was collected on a Rigaku/MSR FR-E rotating anode generator equipped with an R-Axis HTC image plate.

The data was processed with MOSFLM [17] and the CCP4 suite [18]. 5 % of the reflections were excluded for calculation of R_{free} . A molecular replacement solution was found for the *C2* space group using PHASER [19] and an ensemble of search models with primary sequence identity to CENTG1 between 25 % and 27 %. There was one molecule in the asymmetric unit. The model was rebuilt using O [20] and refinement was performed using CNS [21] and finally Refmac5 [22]. The *P2₁2₁2₁* space group was solved by molecular replacement

using the refined structure from the C2 space group as a search model. There was one molecule in the asymmetric unit. Data processing and refinement statistics can be found in Table 1.

Relative activity of gel filtration fractions

The purified protein was gel filtrated and the eluate fractions were 99 % pure when analysed on SDS denaturing gels. The protein from each fraction was analysed for GTP and NTP hydrolysis using Malachite Green Phosphate Assay Kit (BioAssay Systems), according to the manufacturer's instruction. The release of phosphate was quantified using a standard curve and the relative GTPase and NTPase activity of each fraction was determined and plotted as shown in Figure 2. As the altered nucleotide specificity was unusual for GTPases this procedure was carried out in order to ensure that there was no contaminating enzyme contributing to the NTPase activity.

GTPase and NTPase activity Assay

Purified CENTG1 GLD (100 nM) was incubated with 12 μ M to 5 mM of nucleotide triphosphates in reaction buffer (20 mM HEPES pH 7.0, 150 mM NaCl and 5 mM MgCl₂) at 30 °C in an 80 μ l reaction mix. The assays performed at 1 and 2 mM MgCl₂ did not show any difference in activity. The reactions were repeated at pH 6.0 and pH 8.0. The experiment was performed according to the manufacturer's instructions. The product formation was determined by recording the absorption at 650 nm using a SPECTRAMax spectrophotometer (Molecular Devices) 1 minute after the initiation of the reaction. The experiment was repeated with different substrate concentrations. Blanks containing corresponding nucleotide concentrations in the reaction buffer with malachite green solution were subtracted from each recording. To determine values for V_{\max} and K_m , the data were fitted to the Michaelis-Menten equation using non linear regression (Kaleidograph software). All nucleotides and analogues were purchased from Sigma.

Non-covalent mass spectrometry

Non-covalent mass spectrometry was performed on a Q-ToF Micro (Waters) instrument equipped with a Nanomate spray chip (Advion) nano-electrospray ionisation source. Samples were buffer-exchanged into aqueous ammonium acetate buffer (10 - 25 mM, pH 7) using microbiospin chromatography columns (Biorad). Pressure settings on the instrument were around 0.6

mbar (source, speedivalve) with sample and extractor cone voltages chosen such that non-covalent complexes are preserved intact [23, 24].

Isothermal Calorimetry

ITC measurements were carried out at 20°C using a VP-ITC MicroCalorimeter (MicroCal). CENTG1 and the nucleotides were in a solution of 20 mM HEPES pH 7.4, 150 mM NaCl, 5mM MgCl₂ and 5 mM DTT which was degassed in a ThermoVac apparatus (MicroCal). ITC experiments were performed by stepwise titration of the non-hydrolysable NTP analogue GTPγS or ATPγS (1 mM solution) into an adiabatic cell containing CENTG1 (100 μM), and the heat energy change accompanying the reaction was detected upon each injection by comparison with a reference cell. Protein solution was placed in the 1.4 ml calorimeter cell and stirred to ensure rapid mixing, and 10 μl aliquots of the nucleotide analogue (titrant) were injected over 10 sec with a 5 min interval between each injection until saturation. The titrant injected into buffer alone was used as a negative control. The heat changes were integrated after subtracting values obtained when CENTG1 was titrated into buffer, plotted against the molar ratio CENTG1: NTP and analysed as a non-linear least squares fit. Data were analysed with the ORIGIN software package supplied by MicroCal.

Results and Discussion

Structure of the Centaurin Gamma-1 NTPase domain

The crystal structure of the GLD domain (residues 66-241) of CENTG1 was determined in two different space groups, in *C2* to a resolution of 2.1 Å and in *P2₁2₁2₁* to a resolution of 1.6 Å. The structures were very similar to each other (r.m.s.d. 0.69 Å over 166 Cα atoms), with significant differences only in the region of the loop connecting helix H1 with β-strand B2 (the G2 nucleotide binding motif also known as Switch I). For both structures all of the important loops involved in nucleotide binding and hydrolysis are fully resolved. Figure 3A shows the overall fold and important residues for the *C2* crystal form. In this structure the Thr101 side chain points away in solution, however, in the in *P2₁2₁2₁* crystal form this Thr side chain flips down close to but beneath the expected Mg²⁺ ion binding site. Notably, in neither space group

was any nucleotide or Mg^{2+} ion present in the nucleotide binding site, with the electron density revealing only water molecules where the nucleotide would be expected to bind. In the analysis and discussion below the *C2* space group structure was used unless specified otherwise. A summary of the structure refinement and model statistics is given in Table 1.

The overall structure of the domain resembles that of other structurally characterised small GTPases, for example c-H-ras (Figure 3B). The protein adopts a classical nucleotide binding fold consisting of a six-stranded β -sheet surrounded by five α -helices. The five α -helices (H1-H5) and six β -strands (B1-B6) connect with a B1-H1-B2-B3-H2-B4-H3-B5-H4-B6-H5 topology (Figure 1B). Superimposition of CENTG1 with the structure of c-H-ras bound to GDP (PDB ID 4Q21) results in a r.m.s.d. of 1.26 Å over 135 C α atoms (Figure 3B, 3C). The GTPase domain of CENTG1 shares moderate sequence identity with the Ras (28 % RAP2A gi:10518344; 24 % M-ras gi:32189357; 23 % v-Ha-ras gi:4885425) and Rab (25 % RAB5A gi:19923262; 25 % RAB11B gi:4758986; 25 % Rab9A gi:4759012) subfamily members of small GTPases thus rendering the classification of the CENTG1 N-terminal GLD domain unclear.

There are three regions that show significant differences in their conformation in comparison with benchmark GTPases such as H-ras. They are the Switch I loop, the loop connecting β -strand B3 with H2 (the G3 nucleotide binding motif or Switch II) and residues Thr179 to Arg182 (equivalent to the G4 nucleotide binding motif, Figure 1B) (Figure 3). These differences can only be partially explained by the lack of a nucleotide in the active site since there are significant sequence differences in these regions between CENTG1 and typical GTPases.

Active site structure

In the absence of nucleotide, parts of the protein obscure the volume in which the nucleotide would normally reside. These regions of the protein chain are presumably in different conformations in the presence of bound nucleotide. Figure 3C shows the structural differences between CENTG1 GLD and the inactive form of H-ras at the nucleotide binding site. Included in the superimposition is the location of the GDP molecule present only in the H-ras structure.

In both space groups the N-terminal part of the Switch I loop has a similar conformation in which Leu98 and Glu99 are positioned such that the backbone carbonyl group of Leu98 occludes the binding pocket for the α -phosphate of a bound nucleotide. The C-terminal part of

Switch I is one residue shorter than its equivalent in H-ras or Rab9 (Figure 1B). This part of Switch I occupies a different conformation in each of the structures. It is unlikely that either conformation represents the conformation when bound to GTP or GDP but it is interesting to note that in the *C2* space group the side chain of the conserved Thr101 is in a similar position to the equivalent Thr (Thr35 in H-ras) in the classical GDP-bound inactive state (Figure 3B, 3C) [5].

The role of this Switch I threonine in binding, hydrolysis, and biological function including effector mediated interaction is well established. It plays an important nucleotide binding role when the GTPase is in the active GTP-bound state, where it coordinates the Mg^{2+} ion with its side chain and the γ -phosphate with its main chain NH. As such, it is important for interactions with effector proteins, such as in RhoA where the equivalent threonine to asparagine mutation prevented activation of phospholipase D [25] and in Ras where a Thr35Ala mutant reduced the binding affinity for the Ras binding domain of the Raf-1 protein kinase by a factor of 188 [26]. The threonine is most likely invariant in Ras-related proteins, and even Thr35Ser mutants show loss of function. For instance, a Thr35Ser mutant of Ras showed different dynamic behaviour in its Switch I loop and a more prevalent non-effector-binding conformation [27].

The lack of any bound nucleotide came as a surprise, not least since small GTPases typically have a low nanomolar affinity for GDP [28], and a number of unusual structural features called into question the ability of this GLD to bind a specific nucleotide. Generally for the family of small GTPases, the guanine nucleotide binding site fits tightly around the ring system with a number of hydrophobic as well as charged interactions combining to stabilise the nucleotide. The residues that form the classical small GTPase guanine binding site, as exemplified by H-ras, are the conserved NKxD sequence of the G4 motif [29-31], the phenylalanine side chain immediately C-terminal to H1 on the loop between H1 and B2, and the conserved TSAK sequence of the G5 motif (Figure 1B and 3). All of these residues are highly conserved throughout the family of small GTPases. The NKxD motif interacts with the ring system via the lysine side chain forming a hydrophobic interaction by running parallel with the ring system plane and, more importantly, the Asp side chain coordinating the two nitrogen groups of the purine (Figure 3C). The aspartate-guanine interaction is primarily responsible for achieving guanine nucleotide specificity. The phenylalanine packs at right angles to the ring

system plane on the opposite side to the NKxD lysine side chain (Figure 3B) while the conserved residues of the G5 motif interact both with the G4 motif and with the base of the nucleotide.

For CENTG1 GLD the equivalent of the NKxD motif is TQDR (Figure 1B). In both space groups the side chain of the aspartate residue from this loop (Asp181) occupies part of the place where the guanine ring would reside (Figure 3C). It is plausible that a rearrangement of this loop could allow a similar interaction between the Asp181 residue and a guanine nucleotide as in typical GTPases; however the layered interaction equivalent to the NKxD Lys is likely to be lost. Mutational analyses in Ras to determine the role of the Asp181 equivalent in many small GTPases have resulted in a protein that has a very high turnover of GTP and an extremely low affinity for nucleotides [32]. The mutation to alanine resulted in loss of function [33]. The mutation to asparagine resulted in a change of specificity of nucleotide recognition from guanine to xanthine with out any loss of hydrolytic function or effector interaction [34]. For CENTG1, a tyrosine residue (Tyr95) replaces the phenylalanine after H1 and the extra polar hydroxyl group could also destabilise any bound nucleotide by steric hindrance or electronic interaction (Figure 3C). Only the G5 motif is relatively conserved in CENTG1.

The Switch I & II regions of small GTPases undergo a conformational change depending on whether GDP (inactive state) or GTP (active state) is bound. While the Switch I loop of CENTG1 adopts different conformations in the two crystal forms that we have determined, the Switch II loop adopts the same conformation in both (Figure 3C). Examination of the sequence alignment (Figure 1B) indicates a five residue deletion with respect to the sequences of H-Ras or Rab9, which results in the shorter Switch II loop in the structure (Figure 3B, 3C). This is likely to have implications for binding to effector proteins, but in addition CENTG1 GLD is missing a Gln residue in this switch II region (equivalent to Gln61 in Ras), a highly conserved residue in other small GTPases and known to play an important role in GTP hydrolysis [4, 28, 35]. Gln61 has been shown to play a vital role in GAP mediated GTP hydrolysis by positioning a water molecule in the transition state aided by the arginine finger contributed by the GAP molecule [35-38]. Interestingly, the tRNA modification GTPase MnmE also lacks this glutamine residue and has relatively high GTPase activity (K_{cat} 10.2 min⁻¹, K_m > 0.5 mM) [39].

Taken together, these sequence and structural differences prompted us to examine the enzymatic activity and specificity of the CENTG1 GLD.

Nucleotide hydrolysis by CENTG1

CENTG1 GLD actively catalyses the hydrolysis of GTP, ATP, UTP and CTP (Figure 4A). Multiple turnover conditions at differing substrate concentrations were used to analyse the catalytic properties of the enzyme (Table 2). The kinetic values show that the enzyme is active and the intrinsic nucleotide turnover rate is relatively high compared to typical rates of GTPases. K_{cat} of CENTG1 with the NTPs is between 16 - 26 min^{-1} depending on the nucleotide (Table 2). Though the rate of hydrolysis is not comparable to that in the presence of GAPs, it is higher than the “quick” turnover small GTPases such as Rab5 and Ras for which K_{cat} values of $2 \times 10^{-1} \text{ min}^{-1}$ and $2.8 \times 10^{-2} \text{ min}^{-1}$ have been reported [40, 41]. The efficient hydrolysis of UTP, ATP and CTP shows that CENTG1 is an NTPase and not simply a GTPase as previously reported [42]. To confirm that the hydrolysis was effected only by CENTG1 and not by a contaminating protein the GTP and UTP hydrolysis was measured for each of the individual fractions eluted from a gel filtration. This clearly showed that the activity was proportional to the enzyme concentration present in each fraction (Figure 2).

The CENTG1 GLD showed hydrolytic activity of all the ribonucleotide triphosphates in the analysed range of pH (6.0 to 8.0) and substrate concentrations (Table 2). These observations, combined with the sequence differences discussed above, in particular the absence of the classical NKxD G4 motif; the substitution of Tyr95 for Phe on Switch I and the shorter Switch I loop suggest the protein should be classified as a member of the P-Loop NTPase superfamily rather than part of the small GTPase family.

Two papers describe the characterization of the full length and the GLD domain of centaurins with opposing results. Nie et al [43] were neither able to detect GDP, GTP, ADP or ATP binding nor GTPase activity for both the GLD domain and the full length mouse CENTG2 which is identical in sequence over the GLD domain to human CENTG2. On the other hand, Xia et al. [42] were able to measure GTPase activity for the GLD domain and the full length protein of human CENTG2 with the GLD enzymatic activity increasing ~10 fold in the presence of its C-terminal GAP domain. They also recorded a high GDP dissociation rate for the GLD domain alone. Neither study examined nucleotide selectivity for either the full length or GLD domain. From the published papers, it is unclear why for essentially the same domain GTPase activity was recorded by Xia et al [42] but not by Nie et al [43].

Our results agree more with Xia et al. in that our centaurin GLD domain is active. However, the turnover rates are dramatically different: 0.01 min^{-1} [42] compared to 21.4 min^{-1} for GTP (Table 2). We attribute this large difference partly to the different pH values at which the experiments were recorded: pH 8.0 by Xia et al. [42] as compared to our study at pH 7.0 which is closer to the optimal pH for catalytic activity (Table 2), but mostly due to the differences between the two centaurin isoforms: CENTG2 in the previously published work and CENTG1 for this study. Interestingly, the reported 10 fold acceleration in the rate of hydrolysis by CENTG2 due to its GAP domain is unusually low. Typically, GAP domains activate the hydrolysis of small GTPases by a factor of 10^4 to 10^5 [44, 45]. The switch I loop is known to play a central role in the catalytic process of GTPases as an almost invariant Thr side chain within the loop complexes the Mg^{2+} ion and interacts with the γ -phosphate group in the active GTP bound state only. CENTG1 has a Thr residue in the equivalent position (Figure 1B, 3C) whereas CENTG2 has a Pro residue, thus making it likely that catalysis for CENTG2 would be different from CENTG1.

In order to test this hypothesis, we created Thr101Ala and Thr101Pro point mutations of CENTG1 to assess the importance of this residue for GTP hydrolysis. Overall, we observed a 30 to 40-fold reduction in K_{cat} for Thr101Ala and Thr101Pro compared to wild-type CENTG1 (Figure 4B, Table 2), illustrating the importance of Thr101 in the hydrolysis mechanism, where by analogy with Ras it would be expected to contribute to the octahedral coordination of the Mg^{2+} in the transition state. However, our measured rate of hydrolysis at pH 8.0 for CENTG1 is still an order of magnitude greater than that determined for CENTG2 [42].

Nucleotide affinity of the CENTG1 GLD domain

The values of K_m of between 400 and 600 μM obtained in the activity measurements (Table 2) are very different from the typical picomolar range of dissociation constants found in Ras family GTPases [41]. Therefore, we attempted to confirm nucleotide binding to the CENTG1 NTPase domain by non-covalent mass spectrometry [46]. ATP, GTP and GDP were screened for binding in the presence and absence of magnesium. Figure 5A shows a nano-electrospray ionisation mass spectrum of the CENTG1 NTPase (9 μM) in the presence of a 10-fold excess of GTP. The experiments were performed using sample and instrument parameters which preserve non-covalent complexes intact in the mass spectrometer The main peak series

corresponding to charge states from 7+ to 10+ and a mass of 19575 +/-8 Da (calculated from amino acid sequence: 19581.1 Da) indicates that the CENTG1 NTPase does not have any nucleotide bound under these conditions. No evidence for ATP, GTP or GDP bound to the protein was obtained.

We then used isothermal calorimetry (ITC) to measure the real affinity of CENTG1 for GTP γ S and ATP γ S nucleotide analogues in the presence and absence of Mg²⁺ ions. Two parameters are measured by this technique, the change in the heat content of the system (enthalpy, ΔH), the tendency being negative, and the change in disorder (entropy, ΔS), the tendency being positive [47]. The best fit was obtained by applying a one site binding model involving exothermic reaction phases (negative enthalpy changes) with similar favourable free energy changes. The binding assay determined a very weak binding for both the NTP analogues. Analysis of the data indicates that complete saturation of the binding site is not achieved (Figure 5B, 5C). This is likely due to the high dissociation rate from the active site. The stoichiometry of CENTG1 binding to these NTP analogues is identical (1:1 ratio) and the association constant K_a is similar showing no preferential binding to either of the nucleotides (Table 3). The K_m/K_{cat} which indicates substrate specificity determined from the hydrolytic experiments also imply no preferential substrate selectivity among the analysed NTPs (Table 2). No binding of CENTG1 with ATP γ S and GTP γ S was measurable in the absence of Mg²⁺ (data not shown) thus showing that the affinity for NTPs is completely lost in the absence of Mg²⁺. Assays to measure association constants for CENTG1 with all of the nucleotide diphosphates in the presence or absence of Mg²⁺ ions did not show any binding implying extremely low association rates and/or a very high dissociation rates. The low affinity for the nucleotides explains the absence of bound nucleotide in the crystal structures but also highlights the unusual nucleotide binding properties of CENTG1 in comparison with typical small GTPases.

Kinetic properties of CENTG1 in comparison to other non-classical GTPases

The high affinity of Ras GTPases for GDP results from a low dissociation rate in the range of 10⁻⁵ s⁻¹ [48]. The lower affinity of CENTG1 for nucleotides presumably accounts for its higher turnover rate. The high intrinsic NTPase activity and low affinity for nucleotide di- or tri-phosphates resemble the properties of the large GTPase dynamin, involved in membrane vesicular trafficking, and related enzymes (reviewed in [49]). The GTPase properties of dynamin

are distinct from classical Ras GTPases [50], with the dissociation rate of GTP from dynamin 10^4 times faster than from Ras GTPases [51, 52]. The K_{cat} of CENTG1 with the different NTPs ($16\text{--}28\text{ min}^{-1}$) is comparable to that of wild type dynamin turnover on lipid tubule ($105 \pm 47\text{ min}^{-1}$) [50]. As mentioned above, the atypical GTPase MnmE which controls the modification of uridine at the wobble position of certain tRNAs [53, 54] also exhibits nucleotide activity similar to CENTG1 GLD [39].

Conclusions

In human glioblastoma cells, CENTG1 over-expression prevented apoptosis which was not the case for a dominant-negative mutant of CENTG1 [13]. It was found that the dominant-negative mutant has two mutations (K83A and S84N; Figure 1B) that reside in the P-loop of the NTPase domain. This is consistent with our structural and functional data, as the double mutation will reduce the ability of the NTPase domain to bind the γ -phosphate of GTP and have little or no effect on the binding site for the ring systems. Hence the structure provides an explanation for the inability of the double mutant to bind GTP.

Due to the relatively high affinity that typical small GTPases generally have for the guanine nucleotide, another protein, functionally known as a guanine exchange factor (GEF), is usually required to aid nucleotide displacement. For CENTG1, the SH3 domain of phospholipase $\text{C}\gamma 1$ has been identified as a GEF [55]. However, as we have shown CENTG1's GLD has a naturally low affinity for the nucleotide triphosphates, the need for a GEF is debatable which has implications for the cellular action of CENTG1 as it is clearly different from the classical small GTPases in terms of specificity and activity. The smaller Switch II loop (Figure 3B) of CENTG1 as compared to the classical small GTPase family would also be expected to offer a smaller enzyme-GEF interaction surface [56]. Hence, more research is required to uncover the structural and functional roles that known modulatory proteins, such as phospholipase $\text{C}\gamma 1$ and even CENTG1's own GAP domain, play.

The five signature sequences (G1 – G5, Figure 1B) of the small GTPases define the binding site for both the guanine ring system and the phosphate groups. Lack of structural information on other GTPases or GTPase-like proteins in their apo form and bound to alternative nucleotides prevents a comprehensive explanation of the mechanisms for NTP binding and hydrolysis by CENTG1, but this study provides important information towards that explanation.

The CENTG1 NTPase structure has revealed the important residues involved in nucleotide specificity and along with enzymatic assays has demonstrated that this domain should be classified not as a GTPase but as an NTPase.

It therefore appears that CENTG1 is not a classical small GTPase, and in its catalytic properties is related to some atypical large GTPases, exhibiting a rate of hydrolysis as if in presence of a GEF, with a correspondingly low nucleotide binding affinity. The unusual nature of this enzyme is confirmed by its broad nucleotide hydrolysis efficiency which is likely the result of its unusual G4 motif. These results point to an alternative control mechanism for this enzyme opening up new avenues of research for this family of proteins.

Acknowledgements

We thank Neil Oldham, Chemistry Department, University of Oxford for access to the mass spectrometer. The Structural Genomics Consortium is a registered charity (number 1097737) funded by the Wellcome Trust, GlaxoSmithKline, Genome Canada, the Canadian Institutes of Health Research, the Ontario Innovation Trust, the Ontario Research and Development Challenge Fund, the Canadian Foundation for Innovation, VINNOVA, The Knut and Alice Wallenberg Foundation, The Swedish Foundation for Strategic Research and Karolinska Institutet.

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Table 1: Data Collection and Refinement**Data Collection**

Spacegroup	<i>C</i> 2	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit Cell (<i>a</i> , <i>b</i> , <i>c</i> (Å), β (°))	62.2, 31.1, 81.9, 102.2	36.3, 62.1, 67.5, 90.0
Resolution range ^a (Å)	30.63-2.10 (2.21-2.10)	20.72-1.50 (1.58-1.50)
No. of unique observations ^a	8653 (1180)	24972 (3424)
No. of total observations ^a	43169 (5102)	217987 (14791)
Completeness ^a (%)	95.1 (89.9)	99.3 (95.6)
Multiplicity ^a	5.0 (4.3)	8.7 (4.3)
<i>R</i> _{merge} ^a (%)	6.8 (22.1)	6.0 (33.7)
<I/σ(I)> ^a	16.9 (5.6)	24.8 (2.7)

Refinement

<i>R</i> factor (%)	16.3	15.0
<i>R</i> _{free} (%)	22.5	21.4
Rmsd bond length (Å) (angle (°))	0.014 (1.53)	0.011 (1.47)
PDB ID	2BMJ	2IWR

^a: Numbers in parentheses refer to the highest resolution shell

Table 2: Kinetic and binding parameters of CENTG1 GLD and mutants

Substrate	pH	$V_{\max}(\mu\text{M min}^{-1})$	$K_{\text{cat}}(\text{min}^{-1})$	$K_{\text{m}}(\mu\text{M})$	$K_{\text{cat}}/K_{\text{m}}(\mu\text{M}^{-1} \text{min}^{-1})$
GTP	7.0	2.16	21.40	541	0.039
	8.0	0.43	4.62	442	0.010
	6.0	0.47	4.67	427	0.010
ATP	7.0	1.91	19.60	412	0.047
	8.0	0.45	4.89	378	0.013
	6.0	0.48	4.53	412	0.011
UTP	7.0	2.81	28.62	517	0.055
	8.0	0.65	6.73	467	0.014
	6.0	0.36	3.57	449	0.007
CTP	7.0	1.71	16.14	601	0.026
	8.0	0.33	3.06	516	0.006
	6.0	0.34	3.41	505	0.006
T101P					
GTP	7.0	0.076	0.74	990	
	8.0	0.012	0.12	889	
	6.0	0.017	0.14	901	
T101A					
GTP	7.0	0.089	1.08	2681	
	8.0	0.037	0.41	4026	
	6.0	0.030	0.34	3062	

Table 3: Thermodynamic parameters of CENTG1 interaction

NTP	N	K_a (M^{-1})	ΔH (Kcal M^{-1})	ΔS (cal $M^{-1}K^{-1}$)	ΔG (Kcal M^{-1})
GTP γ S	0.98	$4.13 \pm 1.0 \times 10^4$	-4.56 ± 0.3	5.56	-4.56
ATP γ S	0.98	$9.33 \pm 2.5 \times 10^4$	-9.15 ± 0.7	-8.462	-9.32

Figure Legends

Figure 1. Domain and sequence comparison of CENTG1. (A) Domain architecture of the centaurins and small GTPases. PI3 kinase enhancer isoform-A or CENTG1 is shown in comparison with long isoform PIKE-L and small GTPases. The proline rich domain present only in PIKE-L is shown in black rectangles at the N terminus. The GTPase like domains (GLD) of CENTG1 and PIKE-L, and GTPase domain of a small GTPase are shown in light grey. The plextrin homology domain is highlighted in dark grey. The Arf-GAP domain is depicted as a dotted grey box. Two ankyrin repeats in the C-terminus are shown as black dotted filled boxes. **(B) Sequence alignment of human centaurin gamma with small GTPases.** ClustalW based sequence alignment of CENTG1 (gi: 6176569), CENTG2 (gi: 51338837), CENTG3 (gi: 16799069) with the small GTPases c-H-Ras (gi: 231061) and Rab9A (gi: 55670684), with editing to reflect the structural superimposition. Identical amino acids are indicated by an asterisk. Highly conserved small GTPase functional loops, G1 - G5, are highlighted in grey. The secondary structure depiction at the bottom of the sequences is according to CENTG1 GLD domain structure determined here (PDB ID 2BMJ). Alpha helices are represented as red rectangles and beta sheets as green arrows.

Figure 2. GTPase and NTPase activity of the gel filtration fractions of the CENTG1 GLD. Chromatogram of the superdex75 gel filtration run with the Coomassie Blue stained SDS-PAGE gel corresponding to the fractions displayed beneath. The extreme left of the gel shows the molecular weight marker. The measured activity was plotted for every fraction. The black and the grey bars represent the percentage of GTPase and NTPase activity of each fraction, respectively.

Figure 3. (A) Overview of the CENTG1 GLD structure. Ribbon representation with the secondary structural elements labelled. The Switch I region is in green and the Switch II region in yellow. **(B) Superimposition of CENTG1 (blue) GLD and the inactive form of c-H-Ras (PDB ID 4Q21).** The GDP (in yellow) and magnesium ion (red) of c-H-ras are also included. The Switch I and Switch II loops of c-H-ras are coloured as for CENTG1 GLD with the addition of black stripes. **(C) Close up view of the nucleotide binding site.** Ras is shown in thicker lines,

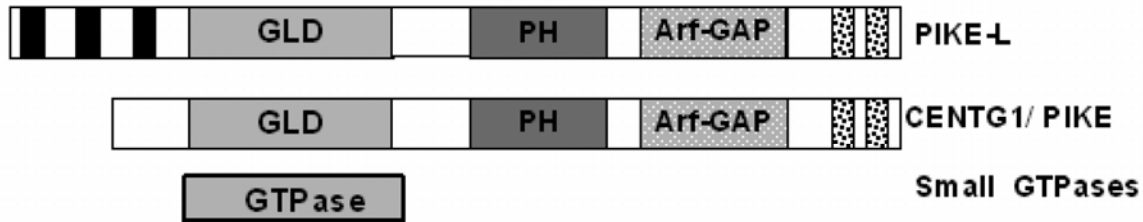
with its Switch I loop in pink, the GDP bound to Ras in yellow and the Mg^{2+} a pink sphere. The two crystal forms of CENTG1 are shown in dark blue (C2, 2BMJ) and light blue (P2₁2₁2₁, 2IWR) thinner lines. Relevant CENTG1 residues are numbered in blue and the NKxD G4 motif of Ras is marked with red letters.

Figure 4. Hydrolysis activity of the wild type and mutants CENTG1 NTPase domain. (A) Plot of the initial rate of reaction V_o against concentration of nucleotide triphosphates (mM), fitted to the Michaelis-Menten equation using Kaleidograph software. Hydrolytic rates determined in the absence of enzyme or nucleotides are shown in pink spheres and blue diamonds respectively. (B) GTP hydrolysis of CENTG1 mutants. Plot of V_{max} against substrate concentration (mM) fitted as for wild type.

Figure 5. Nucleotide binding analysis. Nano-electrospray ionisation mass spectrometry analysis of the CENTG1 NTPase domain in the presence of a 10 fold excess of ribonucleotide triphosphates does not show any nucleotide bound to the protein. The highlighted peaks correspond to charge states of the protein (calculated mass 19581.1 Da, measured 19575 +/-8 Da). The inset shows Na^+ attachment to the 9+ peak. (B).ITC of interactions between CENTG1 and GTP γ S. The upper panel displays raw energy changes during titration (time), and in the lower panel the derived integrated total energy changes as a function of the molar ratio of the interactants. The yielded thermodynamic parameters are listed in Table 3. (C). ITC interactions between CENTG1 and ATP γ S. The representation is same as for figure 5b.

Figure 1

A



B

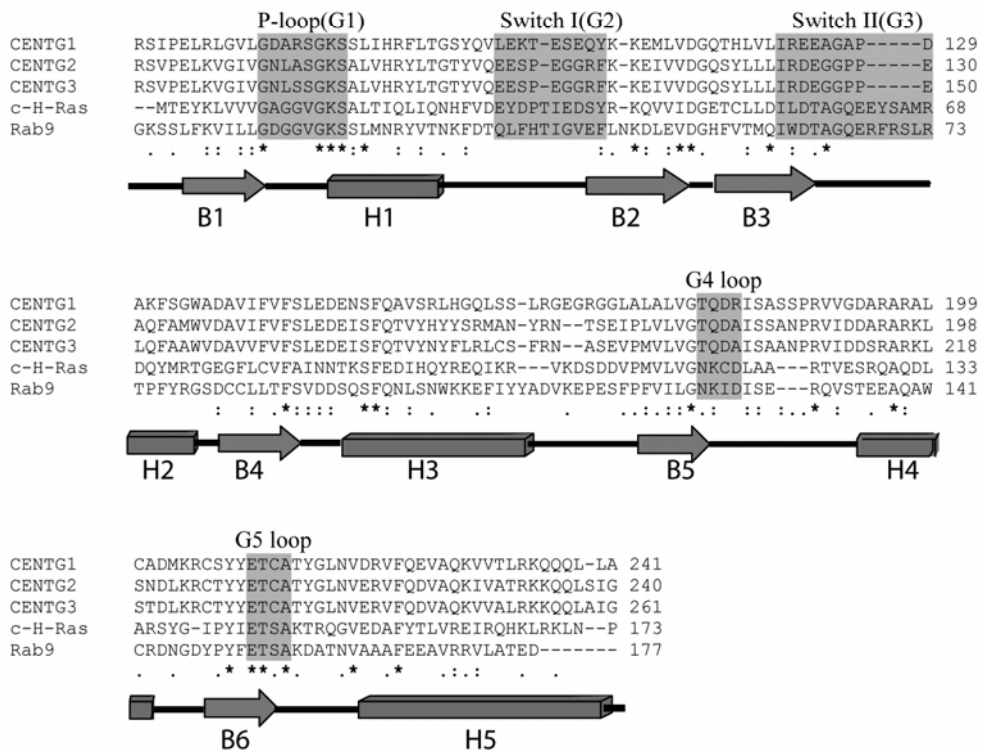


Figure 2

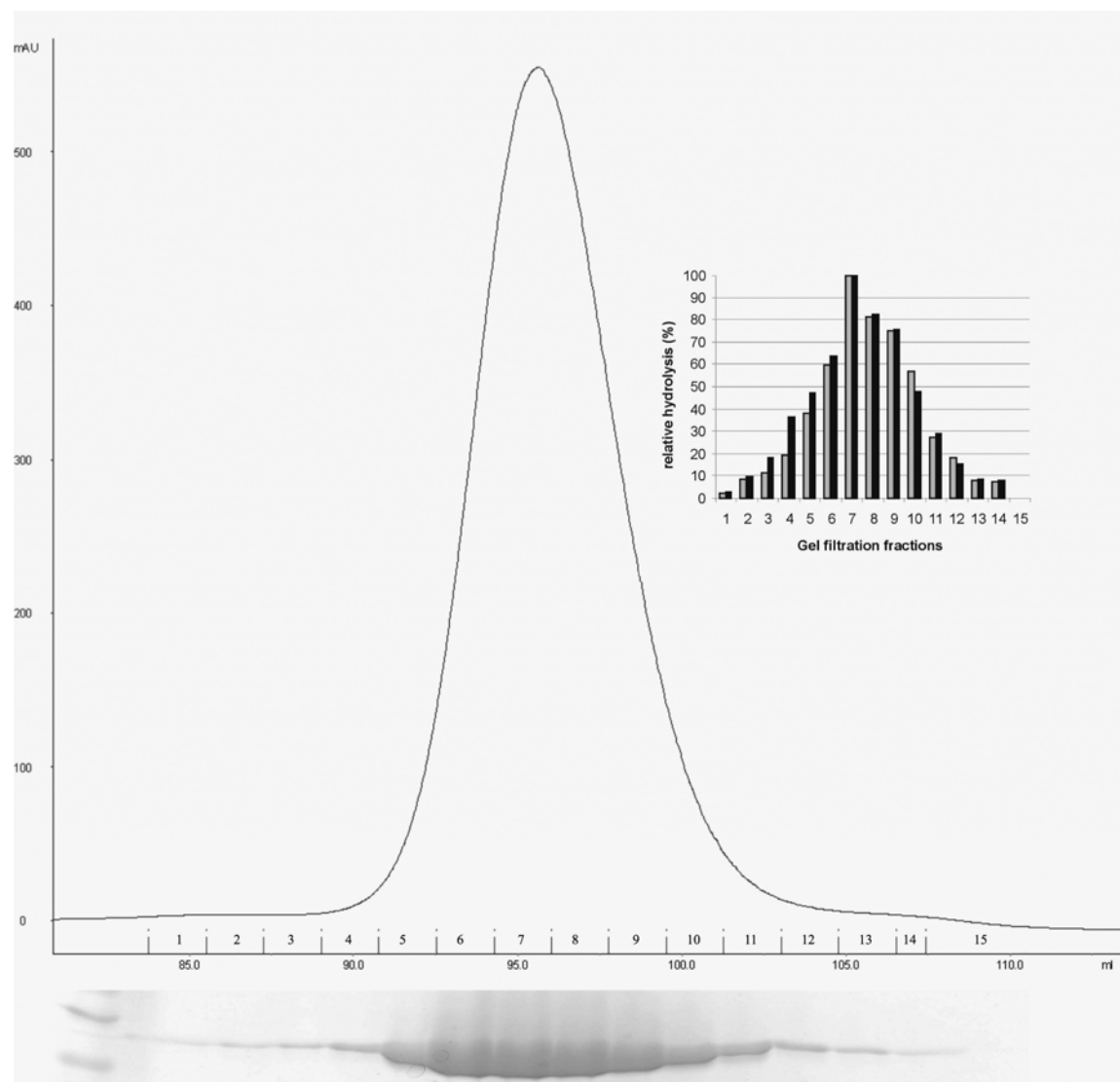


Figure 3

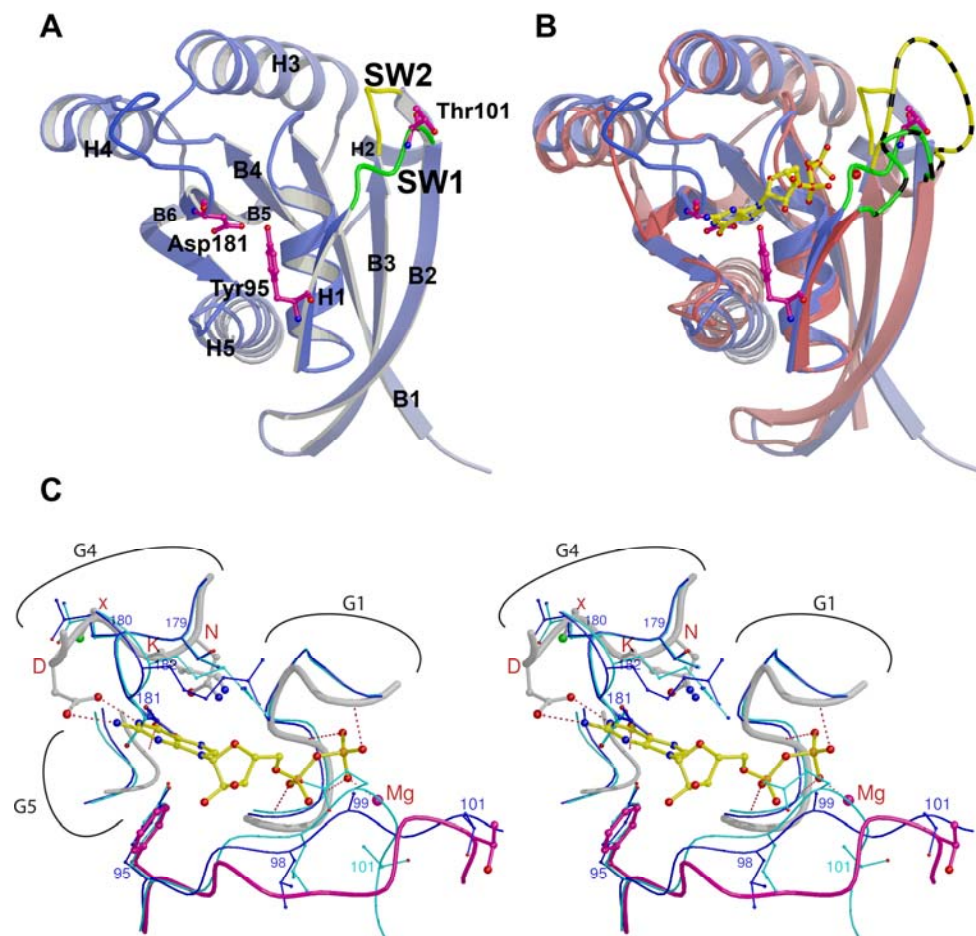


Figure 4

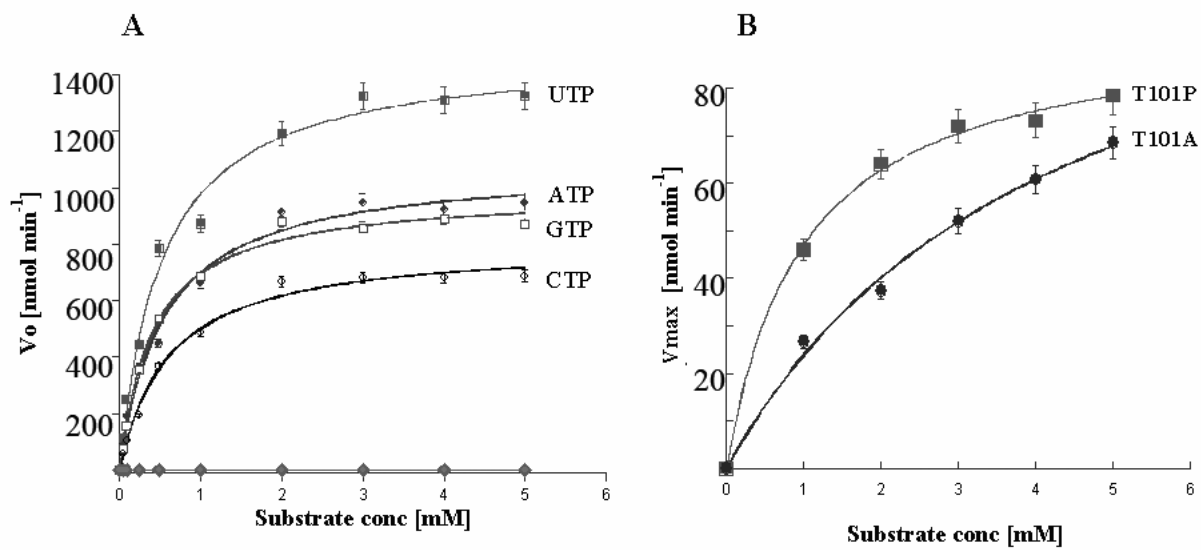


Figure 5

