

# Nucleotide binding by the synapse associated protein SAP90

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**Abstract** The rat synapse associated protein SAP90 is a member of a superfamily of potential guanylate kinases localized at cell–cell contact sites. This superfamily includes the synapse associated protein SAP97, a close relative of SAP90, the *Drosophila* tumor suppressor gene product *dlg*-Ap, the mammalian zonula occludens proteins ZO-1 and ZO-2 and the erythrocyte protein p55. Here we show that SAP90 specifically binds GMP in the micromolar range while binding to ATP, GDP and ADP is at a much lower affinity (10–25 mM), whether or not binding is detected for other guanine and adenine nucleotides. No guanylate kinase activity of SAP90 was detected under our experimental conditions. The importance of the GMP binding capacity per se and an evolutionary role for conserving of the guanylate kinase domain in this superfamily are discussed.

**Key words:** Dlg-A; Guanylate kinase; Nucleotide binding; Synaptic protein

## 1. Introduction

A group of proteins which are localized at cell–cell contact sites were shown recently to constitute a novel superfamily [7]. These proteins are expected to play a role in the assembly, stabilization and maintenance of such sites. Members of this superfamily are the *Drosophila lethal* (1) *discs-large-1* (*dlg*) tumor suppressor gene product [1], the mammalian zonula occludens proteins ZO-1 and ZO-2 [2,3] and the erythrocyte p55 [4]. The first neuronal member of this family to be discovered is the rat brain protein PSD95/SAP90 [5,6]. SAP90 is a member of a small family of proteins (here referred to as SAPs) which are localized at the neuronal cell–cell contact structure, the synapse. Using immunocytochemistry on rat brain sections, we showed that SAP90 was localized in terminals of a certain subset of synapses in the cerebellum, specifically the inhibitory synapses of basket cells on Purkinje cells [6]. SAP90 is also found in other regions of the rat brain (Kistner et al., in preparation). We recently identified a close relative of SAP90, SAP97 [7], that was found to exhibit a broader expression pattern in different rat tissues including epithelial tissues. Remarkably, SAP97 in addition to its localization in nerve terminals of excitatory synapses is also found in the unmyelinated

axons in the rat CNS [7]. An additional related protein, SAP102, was recently cloned (unpublished results) and its distribution in the rat brain is currently being studied.

All members of this novel superfamily contain: (i) repeats of a 90 aa segment situated in the amino terminal domain. The function of these repeats, referred as GLGF [6] or DHR (for Discs-large Homology Region) [8], is still unknown. (ii) A src homology 3 (SH3) domain, found in a variety of proteins involved in signal transduction processes which are mediated by direct protein–protein interactions (see review [9]). (iii) A carboxy-terminal region similar to the guanylate kinases (GK) from yeast [10] and from mammals [11,12]. GK (ATP:GMP phosphotransferase EC 2.7.4.8) catalyzes the phosphorylation of GMP at the expense of ATP according to the reaction  $\text{GMP} + \text{ATP} + \text{Mg}^{2+} = \text{GDP} + \text{ADP} + \text{Mg}^{2+}$ .

The important role of the GK domain in the protein's function is evident from mutations in the *Drosophila dlg* gene [13,14]. The *dlg* gene product (*dlg*-Ap) is localized to the septate junctions between epithelial cells of the imaginal discs in the *Drosophila* larvae as well as at synaptic sites. Mutations in *dlg-A* result in a failure of adhesion between the developing epithelial cells and in an improper formation of septate junctions. The loss of growth control results in a neoplastic growth of epithelial cells in the imaginal discs and brain [13]. In addition, striking changes in neuromuscular synapses are observed in *dlg* mutants [14]. In both studies, mutations in the GK domain account for the majority of the mutant phenotypes. This indicates an important role of this domain in cellular adhesion, in growth control and in determining synaptic structure. However, as of yet, an enzymatic activity for *dlg*-Ap or any of its related proteins has not been demonstrated.

In this study, we analyzed SAP90 for its ability to bind various purine nucleotides as well as its GK activity. We show that bacterially expressed SAP90 binds GMP already at the micromolar range, while binding to ATP is observed only at the mM range. No GK activity of SAP90 was detected under our experimental conditions. The importance of nucleotide binding to SAP90 and its relatives and the evolutionary conservation of the GK domain throughout the superfamily are discussed.

## 2. Materials and methods

### 2.1. Bacterial expression of SAP90

SAP90 was expressed and purified from bacteria either as a fusion protein with glutathione-S-transferase (GST) via the pGEX-2T expression system (Pharmacia), or in the pRK174 expression vector in the absence of a carrier protein. The entire open reading frame of SAP90 (nucleotides 203–2376, [6]) was cloned into the pGEX-2T or pRK174 expression vector. The SAP90-GST fusion protein or GST alone were purified with glutathione agarose (Sigma) as described [15]. The yield and purity were analyzed by 8% SDS-PAGE and Comassie blue staining. The yield of SAP90-GST fusion protein was 10–15 mg/l culture and its purity was greater than 95% as determined by silver staining (Bio-Rad). The expression of SAP90 in pRK174 under the

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**Abbreviations:** aa, amino acid; ABTS, 2,2'-Azinobis (3-ethylbenzthiazoline) sulfonic Acid; ELISA, enzyme-linked immunosorbent assay; GK, guanylate kinase; GST, glutathione-S-transferase; PAGE, polyacrylamide gel electrophoresis, SDS, sodium dodecyl sulfate.

control of the T7 promoter was performed in *E. coli* strain BL21(DE3) as described [16].

## 2.2. Acetone fractionation of bacterial cell extract

For a large scale protein preparation 400 ml of BL21(DE3) cells in the logarithmic phase were induced as described [16]. The yield of SAP90 expressed protein varied between 2–10% of the total proteins. Following induction, the cells were pelleted and resuspended in 5 ml of buffer A (1 M potassium-phosphate buffer, pH 7.2, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ M leupeptin, 10  $\mu$ M pepstatin, 10  $\mu$ M aprotinin) and lysed by sonication (4 times, 20 s with 40 s cooling intervals). Membranous and unlysed material were discarded by centrifugation (20 min, 20,000  $\times$  g, 4°C). To enrich the native form of SAP90 while providing optimal conditions for the GK activity acetone fractionation was performed according to [17] with 3 discrete steps of precipitation (fractions I–III). Most of SAP90 was recovered in fraction II (80%) and fraction III (20%) as determined by quantitative immunoblot analysis.

## 2.3. Nucleotide binding assay

The nucleotide binding specificity of SAP90 was assessed by testing the ability of various free nucleotides to release SAP90-GST fusion protein bound to affinity column. SAP90-GST or the carrier protein GST alone were overexpressed and purified from *E. coli* as described above. 100–200  $\mu$ g of the SAP90-GST (>95% purity) or GST alone (>98% purity) were loaded onto an agarose-beads column (300–400  $\mu$ l volume) covalently linked to Cibacron Blue F3GA (Affi-Gel Blue, Bio-Rad, capacity >11 mg/ml). The column was equilibrated with 50 mM imidazol, pH 6.8, 350 mM NaCl, 1 mM DTT and 2 mM MgCl<sub>2</sub>. For maximal loading, freshly prepared SAP90-GST (or the GST alone) was loaded repeatedly and the loaded material was extensively washed with equilibration buffer (2–3 column volumes, 10 times). Bound proteins were subsequently eluted (rate of elution was 250–300  $\mu$ l/min) by stepwise addition of various free nucleotides (buffered to pH 7.0) in increasing concentration. The fractions were analyzed by ELISA. Each elution step was measured in triplicate using an affinity purified antibody against GST (gift of Ruediger Veh, Hamburg). The variations between the measurements were under 8% in all cases. A peroxidase based detection system with ABTS as substrate was used according to the manufacturer's instruction (Zymed). The elution profiles of all experiments were independent of the initial amount of protein loaded on the column (in the range of 20  $\mu$ g to 1 mg protein/ml of column).

## 2.4. Assay of GK activity

Enzymatic activity of the SAP90-GST fusion protein and acetone fractions of SAP90 expressed in BL21(DE3) cell were measured spectrophotometrically as described [18]. The activity was calculated from the decrease in absorbance ( $A_{340}$ ) of NADH reflecting the formation of both ADP and GDP from ATP and GMP by a coupled reaction with pyruvate kinase and lactate dehydrogenase. The assay was started by mixing all reagents (including 1 mM ATP) and equilibration for 30 seconds all reagents excluding GMP, followed by adding the protein fraction. The reaction was initiated by the addition of GMP. At the end of each experiment (500–600 s) we included purified GK from porcine brain (Sigma) as a positive control. A residual activity of about 2% of the control is still expected to be detectable. In the situation where acetone extracts were used the amount of SAP90 in each reaction mixture was estimated to be higher than 5  $\mu$ g.

## 2.5. Miscellaneous techniques

Staining of gels following SDS-PAGE by silver staining was done according to the manufacturer's instruction (Bio-Rad). Quantitative protein determination was done by the BCA system (Pierce). Immunoblotting was according to standard protocols using the peroxidase based luminescence method (ECL, Amersham) as the detection system.

# 3. Results

## 3.1. GMP binding activity

SAP90 contains in its carboxy-terminus (aa 534–724) a region that is very similar to the yeast and the mammalian GKs [10–12]. This region of GK in SAP90 share 37 and 39% identical

aa to the GK from yeast and from bovine retina, respectively. The similarity is increased to 56% by including conserved amino acids. To analyze the nucleotide binding properties of SAP90, purified SAP90-GST fusion protein was loaded on an affinity chromatography column (Affi-Gel Blue beads), which is characterized by its high affinity to many nucleotide binding proteins. 80–90% of the bacterial expressed protein was retained on the column following extensive washing. Specificity of binding was determined by eluting the bound protein with varying concentration of the following nucleotides: GMP, GDP and GTP.

As shown in Fig. 1A, most (>60%) of the SAP90-GST fusion protein is readily eluted with 1  $\mu$ M GMP which reflects a high affinity for GMP. Although the remaining SAP90 could not be eluted in any range between 10  $\mu$ M and 1 mM of GMP, an additional fraction is eluted with 10 mM to 25 mM (5% and 25%, respectively) of GMP. Selectivity of binding to the mono- versus di- and tri-phosphate forms of the guanine nucleotide was analyzed by repeating the elution experiment using GDP (Fig. 1B) and GTP (Fig. 1C). A small but reproducible fraction

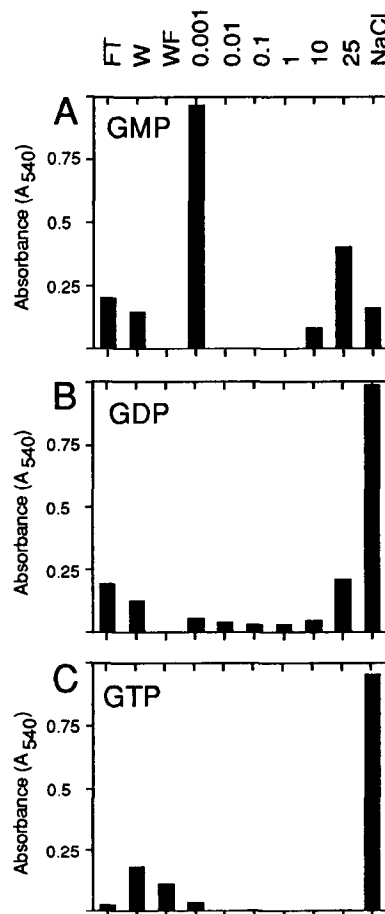


Fig. 1. Elution profiles for SAP90 by guanine nucleotides. 100  $\mu$ g of SAP90 fusion protein were loaded on a affinity column (Affi-Gel Blue) and eluted with either GMP (A), GDP (B) or GTP (C). SAP90 was detected in the eluates by ELISA (absorbance  $A_{340}$ ). The bars represent the absorbance of the different fractions along the column: FT, flow through; W, first wash; WF, final wash; NaCl, final elution by 1 M NaCl. Increasing amount (in mM) of the appropriate nucleotide used in the experiment are as indicated.

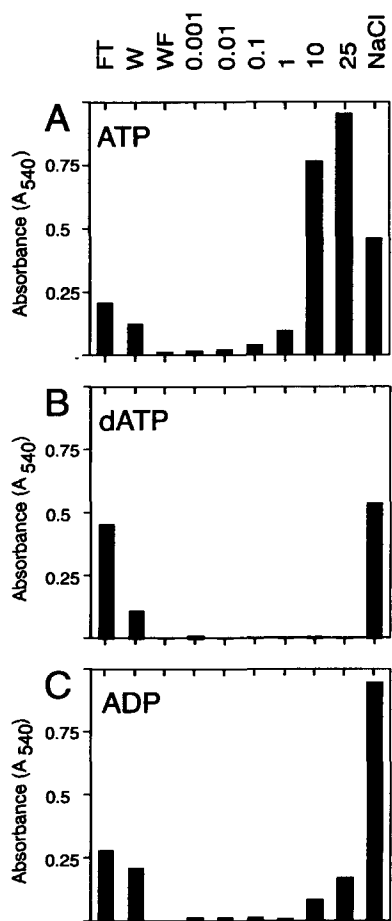


Fig. 2. Elution profiles for SAP90 by adenine nucleotides. 100  $\mu$ g of SAP90 fusion protein were loaded on an affinity column (Affi-Gel Blue), eluted with ATP (A), dATP (B) or ADP (C) and detected as in Fig. 1.

of the fusion protein is eluted with 10 mM GDP (15%) suggesting a lower affinity for this form (Fig. 1B). However, no specific binding was observed for GTP since this guanine nucleotide was not able to elute the bound SAP90-GST (Fig. 1C). We conclude that although the binding of GDP is very weak it may reflect the use of GDP in the reverse reaction of the GK [19]. The results from quantitative immunoblot analysis following electrophoresis of the eluted fractions were similar to the ELISA analysis (not shown).

### 3.2. ATP binding activity

The GMP binding site is highly conserved between all SAPs and yeast GK. Moreover, binding of GMP to the SAP90-GST fusion protein is in the mM range (Fig. 1). In contrast, the classical ATP binding motif GxxGxGK (x represents any aa) present in GKs is altered and 3 central aa of the 7 aa motif are missing (see also Fig. 3). Although this motif is not the only one known to bind ATP, it is common to many ATP binding proteins including kinases and ATPases [19]. To analyze the ability of SAP90 to bind ATP we performed a similar set of experiments as described above using the following adenine nucleotides: ATP, dATP and ADP.

As shown in Fig. 2, the SAP90-GST fusion protein can be specifically eluted with ATP. Elution begins already at about 1 mM ATP, however, the majority of SAP90-GST (>75%) is eluted at 10 and 25 mM ATP. The final elution step with high salt removes the remaining protein (Fig. 2A). No elution with dATP is observed and the protein can be eluted only by the high salt step (Fig. 2B). While no elution was observed with dATP, a partial elution is achieved with 25 mM ADP (20%, Fig. 2C). This low affinity binding for ADP may also reflect the use of this substrate in the reverse reaction of the GK as mentioned above. In order to check the potential effect of the carrier protein on the binding of SAP90 to the Affi-gel blue column, a similar set of experiments were performed by loading purified GST protein on the column. Most of the loaded GST (90%) was

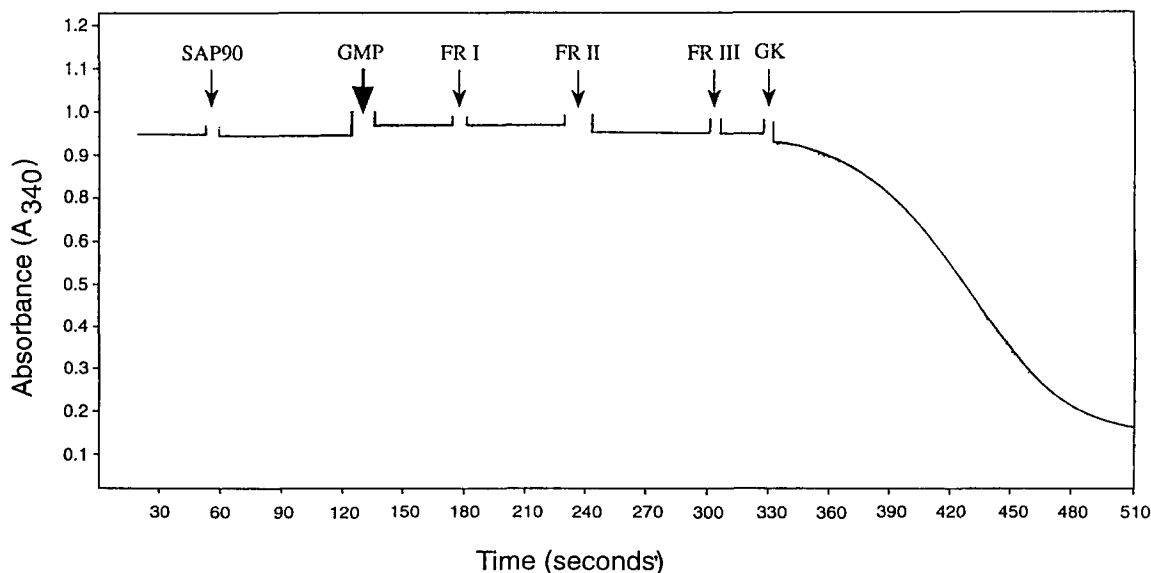


Fig. 3. GK assay. The rate of the change of  $A_{340}$  with time is plotted. Protein was added only after obtaining a stable baseline. Arrows mark the addition of different protein samples to the reaction mixture. According to the activity of the control GK the decrease in absorbance  $\Delta A_{340} = 0.1/\text{min}$  resembles a catalytic activity of 0.08 u (as defined [19]). 20  $\mu$ g of purified fusion protein and 150  $\mu$ g of fractions (FR I, II and III) were added. GK, 2.5  $\mu$ g of GK from porcine brain.

not bound to the column and the minor bound fraction showed no preference to any of the nucleotides (not shown).

### 3.3. Enzymatic activity

To examine the GK activity of SAP90 the purified fusion protein was used in spectrophotometric coupled enzymatic assay reflecting the formation of GDP and ADP using SAP90–GST fusion protein. To avoid a potential steric influence of the GST fused to SAP90 on its enzymatic activity, SAP90 was also expressed under control of the T7 promoter in *E. coli* BL21(DE3). Protein fractions were prepared by acetone extraction, according to the purification protocol for porcine GK [17], 50 to 250  $\mu$ g protein of the different fractions (>5  $\mu$ g expressed SAP90) were tested in the assay. To avoid any loss of activity due to misfolding, degradation or partial denaturation, in all experiments freshly prepared proteins were used.

A combined experiment showing the results for the purified fusion protein and three different acetone protein fractions is shown in Fig. 3. After adding the fusion protein to the reaction mixture the reaction was initiated by the addition of GMP. The acetone fractions were added subsequently. The experimental reaction was challenged by addition of the commercial GK from porcine brain as positive control. For none of the protein samples, neither the acetone precipitated fractions nor the purified fusion protein or using immunoprecipitated SAP90 from rat brain extracts (using antibodies against SAP90 amino-terminal domain), a catalytic activity could be detected. For each of the protein samples an individual experiment was performed for the whole time of 6–8 min, which also had no detectable residual GK activity.

### 3.4. Structural similarities between SAP90 and the GK from yeast

Our biochemical study on the GK domain in SAP90 (Figs. 1–3) is evaluated with respect to the structural level by analyzing

the similarities of this domain to the yeast GK. The X-ray structure of the yeast protein was already solved at 2.0 Å resolution [21]. Ten of eleven aa involved in the binding of the GMP nucleotide in the yeast GK (aa 34, 38, 41, 44, 50, 69, 78, 80, 100–102) are conserved (Fig. 4). Surprisingly a high degree of similarity is extended towards the carboxy-terminal of the proteins (up to aa 150 and aa 680 of the yeast GK and SAP90, respectively). Marked differences in the sequence are found in the domain including the glycine-rich loop building the anion hole in the yeast protein (between  $\beta$ 1 and  $\alpha$ 1). This implies that while the GMP binding site in SAP90 is a conserved core structure, around this core evolutionary changes were accumulated.

The alignment of the aa sequences of the GK domains of all family members is shown in Fig. 4. It is evident that in SAP90, as well as in SAP97, *dlg*-Ap and in the erythrocyte protein p55 [4] all nine GMP binding aa known to interact directly with the nucleotide are conserved. It is worth mentioning that the conservation of the ZO proteins is lower. Only two of these essential aa are conserved and in addition a significant part of the GK domain is deleted (Fig. 4). The high conservation in the GMP binding site found for the SAPs, *dlg*-Ap and p55 is in contrast to a lack of conservation in the ATP binding site. The classical glycine-rich ATP binding motif (aa 9–15 in the yeast) is altered in the SAPs, *dlg*-Ap and the ZO proteins. In contrast p55 contains the entire GxxGxGK motif found in the GKs. Additional residues (aa 131, 135, 146 in yeast GK) are expected to coordinate the phosphate groups of the ATP during phosphoryl transfer, based on structural studies on adenylate kinases [20,21]. Among these aa in GK which are believed to be essential for the catalysis, only one is conserved in the SAPs and in *dlg*-Ap (Fig. 4). Based on the different levels of conservation of the nucleotide binding sites this superfamily may be divided to subfamilies: (i) the SAPs and *dlg*-Ap, which contain all GMP binding aa and are missing the glycine-rich motif,

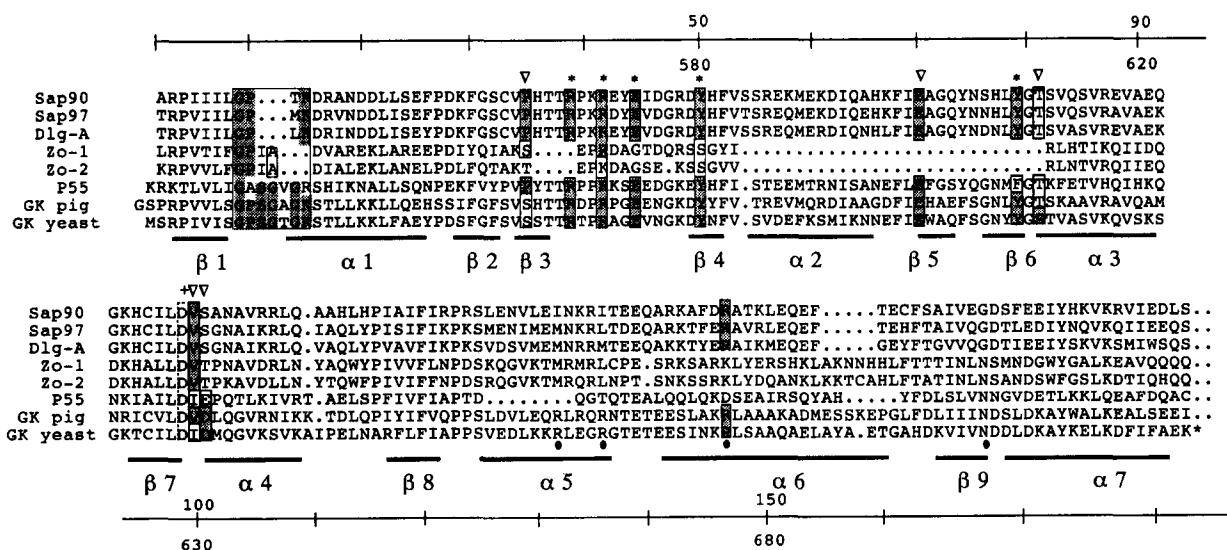


Fig. 4. Sequence alignment of the GK domains of the superfamily members. The alignment was done using PILEUP (GCG software package). The glycine-rich ATP binding motif and conserved aa involved in binding the guanine ring (\*) and the phosphates (V) of GMP in yeast GK are boxed, the residue binding  $Mg^{2+}$  (+) and aa involved in catalysis identical between adenylate kinases and GK (●) are marked. Identical aa are in gray. Based on the three dimensional coordinates for the yeast GK a secondary structure is deduced using DSSP [26]. Only  $\alpha$  helices and  $\beta$  sheets are marked. Position of aa for the yeast GK and for SAP90 are indicated above and below the line, respectively.

- (ii) the ZO proteins, which are missing both binding sites and
- (iii) p55, in which most aa of both nucleotide binding sites are conserved.

#### 4. Discussion

GK is a key enzyme in the metabolism of guanine nucleotides. During purine nucleotide biosynthesis the levels of these nucleotides affect each other. An important example are the GTP binding proteins (G-proteins), whose activity is regulated by GTP/GDP levels. G-proteins regulate ion channel activation, vesicle targeting, receptor mediated responses, cell growth, and differentiation (see review [22]). The disruption of the GK domain in *Drosophila dlg* gene account for the majority of the phenotypes of *dlg* mutants; both the loss of growth control and cellular adhesion [1] and the striking alteration in synapse structure [14]. It has been suggested that *dlg* mutants reflect a disruption in a signal transduction pathway via GTP/GDP levels that regulate local G-proteins [13].

In this report we show that the synapse associated protein SAP90 binds GMP already at the  $\mu\text{M}$  range. Since the protein is localized in nerve terminals, we suggest that SAP90 may affect synaptic activity by buffering guanine nucleotide levels. This might affect G-proteins such as the small GTP binding proteins of the rab family. Rab proteins were shown to play a central role in neurotransmitter release, in synaptic vesicle biogenesis and in regulating endocytosis [22].

While SAP90 binds GMP already at the  $\mu\text{M}$  range, the binding of ATP is observed only at the mM range. Kinetic analysis of mammalian GKs [20] revealed that the calculated  $K_m$  values (in mM) for GMP are between 6 and 18 and only 10–20-fold lower for ATP (100–200  $\mu\text{M}$ ). Our results on GMP binding in SAP90 (Fig. 1A) are consistent with the calculated  $K_m$  values for mammalian GKs. On the other hand for ATP binding the apparent affinity of SAP90 is lower – in the range of 10–25 mM (Fig. 2A), probably a non-physiological range. The binding characteristics of SAP90 differ therefore from those of mammalian GKs. These marked differences suggest that while ATP binding and GMP binding in mammalian GK occur in tandem, they may be decoupled in the context of SAP90. The GK domain may provide a novel function that may require the GMP binding, but not the enzymatic activity.

Further clues on the role of the GK domain may be obtained by observing its varying degrees of conservation throughout the superfamily (see section 3.4). This raised the possibility that the GK domain was conserved as a structural module rather than a functional one. A non-conventional evolutionary route was proposed to explain the surprising similarity among different taxon-specific crystallins and their enzyme counterparts [23]. Most crystallins (e.g. from birds, reptiles and mammals) show a great deal of similarity to unexpected proteins, most of which are related to key metabolic enzymes. However, the expected enzymatic activity of the related protein is often lost or reduced [23,24]. The puzzling case of the crystallins evoked many attempts to explain this evolutionary phenomena [23]. The most plausible explanations suggest structural conservation, referred to as gene sharing [25]. The domains that are adopted by the crystallins may be preserved because of physical properties of the enzymes, e.g. their ability to reach high concentrations

without precipitation or their thermodynamic properties which predict their stability and a very low turn over [25]. A surprising sequence similarity with no apparent functional conservation was also shown between a synaptic vesicle protein and a family of crystallin/enzyme [27]. Along the same line of reasoning we would like to speculate that also in the SAPs (and probably in other member of the superfamily), conservation of the GK domain provides a novel structural feature. Indeed the localization of members of this superfamily to discrete sites of cell–cell contacts may require common structural and physical properties of such proteins.

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