Ras-binding domains: predicting function versus folding

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Abstract Ras interacts with a number of effector molecules to achieve its prolific signalling. Based on iterative sequence profile and motif searches of databases a novel family of Ras-binding domains was recently identified (Ponting and Benjamin, Trends Biochem. Sci. 21: 422–425, 1996). Among them the rat unconventional myosin and Rho-GTPase-activating protein myr 5 was predicted to contain a Ras-binding domain at its N-terminus. Here we report that direct binding experiments between the proposed Ras-binding domain of myr 5 and Ras failed to demonstrate any interaction. Molecular modelling suggests that this domain in myr 5 adopts a similar folding topology as the Ras-binding domain of Raf kinase. However, unlike the Ras-binding domain of Raf kinase, the myr 5 domain lacks the positive surface charges necessary for binding the negatively charged Ras contact site. This result exemplifies the functional diversity of similar structures and suggests that the identified Ras-binding motif does not reliably predict Ras-binding domains.

Key words: Ras-binding domain; Ras-effector; Unconventional myosin; Myr 5; Rho-GAP; Diacylglycerol kinase

1. Introduction

A large number of bona fide and putative effector proteins that bind to members of the Ras family of GTPases has now been identified [1]. At first sight these proteins displayed no obvious sequence homology. However, in a recent publication Ponting and Benjamin [2] derived a consensus sequence for a 'Ras-association' (RA) motif to predict Ras-binding proteins. The identification of this RA-motif was based on iterative sequence profile and motif searches of databases. In addition to several known Ras-binding domains this search identified a number of sequences not previously recognized as Ras-binding domains. Among these, the unconventional myosin myr 5 (a class IX myosin) was predicted to contain a Ras-binding domain at its N-terminus. Because myr 5 also exhibits a Rho-GAP domain [3], this prediction offered the fascinating possibility that myr 5 might couple Ras and Rho signal transduction in relation to the organization of the actin cytoskeleton. Therefore, we determined Ras-binding by the predicted myr 5 Ras-binding domain directly. No detectable interaction between activated Ras and the predicted myr 5 Ras-binding domain was observed.

2. Materials and methods

2.1. Two hybrid analysis

The two hybrid system employed in this study was developed by Chevray and Nathans [4] and modified here by using the yeast strain Y190. Ras-G12V (amino acids 1–166) was cloned into the pPC97 GAL4 DNA-binding domain fusion vector using the *Sma*I and *Sal*I sites. The cDNAs encoding Raf-RBD (amino acids 51–131) and Myr 5 (amino acids 2–117) were cloned into the *Bgl*II and *Sal*I sites of the pPC86 GAL4 activation domain vector which contained a modified multiple cloning site. Yeast strains Y190 transformed with the respective plasmids were plated on synthetic medium lacking leucine and tryptophane to show efficient transformation. Yeast strains were further plated to synthetic medium lacking histidine, leucine and tryptophane, but containing 25 mM 3-amino-1,2,4-triazole. Colonies were grown for 5 days at 30°C before being transferred to a nitrocellulose filter. The nitrocellulose filter was soaked in liquid nitrogen for 20 s, was allowed to warm to room temperature again and then placed on a filter that had been prewetted in Z buffer [5] containing 0.75 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Filters were incubated for 3 h at 37°C and analysed for the development of blue colour that is indicative of β-galactosidase activity.

2.2. In vitro Ras-binding

H-Ras (amino acids 1–166) and GST-Raf-RBD (amino acids 51–131 of Raf) were expressed and purified as described previously [6]. Ras was preloaded with GppNp [7]. cDNA encoding amino acids 2–117 of myr 5 was amplified by PCR and cloned into the *Bam*HI and *Sal*I sites of pGEX-4T-1. GST and GST-myr 5 (2–117) were expressed and purified as recommended by the manufacturer (Pharmacia). Binding of Ras to immobilized GST-myr 5 (2–117), GST-Raf-RBD and GST alone, respectively, was assayed essentially as described by Zhang et al. [8]. Briefly, 3 nmoles of GST fusion protein was mixed with glutathione agarose beads preequilibrated in buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 1% Triton X-100) in a volume of 300 μl and incubated for 30 min on ice. The beads were washed four times with cold buffer A. 3 nmoles of GppNp loaded Ras were added to the washed beads and the volume adjusted to 600 μl with buffer B (buffer A plus 5 mM $MgCl_2$, 0.2% BSA). The bead suspension was allowed to rotate slowly at 4°C for 5 h. After five washes with cold buffer B, proteins bound to the beads were eluted by boiling in gel electrophoresis sample buffer. Eluates were analysed for the presence of Ras by immunoblotting with the monoclonal Ras antibody Y13-259.

2.3. Molecular modelling

Structure prediction for the myr 5 RA homology sequence consisting of residues 16–114 was performed using the program Threader [9]. The three-dimensional model was constructed using the Composer program that is part of the modelling package of Sybyl (Tripos, Inc.). Further refinement was done employing energy minimization and a molecular dynamics run at 300 K for 100 ps followed by another energy minimization. This model was used for calculation of the surface potential using GRASP [10].

3. Results and discussion

A yeast two-hybrid system was used to test for the inter-

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Abbreviations: DAG, diacylglycerol; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GppNp, guanylyl-5′-yl imidodiphosphate; GST, glutathione S-transferase; RBD, Ras binding domain

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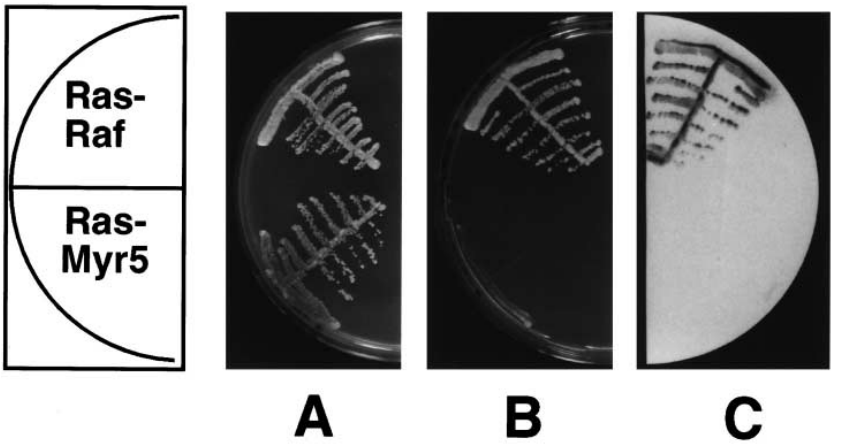

Fig. 1. Yeast two hybrid analysis of the Ras-Myr 5 interaction. Yeast cells transformed with either Ras/Raf as a positive control or Ras/Myr 5 N-terminus were streaked to synthetic medium lacking leucine and tryptophane (A). To test for interaction cells were streaked to selective medium lacking histidine, leucine, and tryptophane (B). Cells from this plate were transferred to a nitrocellulose filter and assayed for β-galactosidase activity (C). Both markers show an interaction between Ras and Raf, but no detectable interaction between Ras and the N-terminus of myr 5.

action of the predicted myr 5 Ras-binding domain with Ras. A strong signal was observed for the interaction of Ras with Raf-RBD (amino acids 51–131) used as a positive control, but no signal was obtained with the predicted myr 5 RA-motif (Fig. 1). Furthermore, in contrast to GST-Raf-RBD, a purified GST-myr 5 RA-motif fusion protein did not detectably interact with activated Ras even at high micromolar concentrations using either a bead immobilization assay (Fig. 2) or the BIAcore method (data not shown). Since dissociation constants for binding between Ras family proteins and Ras-binding proteins are generally in the nanomolar range, these results exclude a functional interaction between the myr 5 RA-motif and Ras family proteins.

Computer modelling of the predicted myr 5 Ras-binding domain performed in parallel also supports our notion that myr 5 is not a Ras-binding protein. The predicted myr 5 Ras-binding domain will adopt with high probability a folding similar to the Ras-binding domain of Raf (Raf-RBD) according to the program Threader [9] (Fig. 3). However, homology modelling of the myr 5 domain to Raf-RBD and calculation

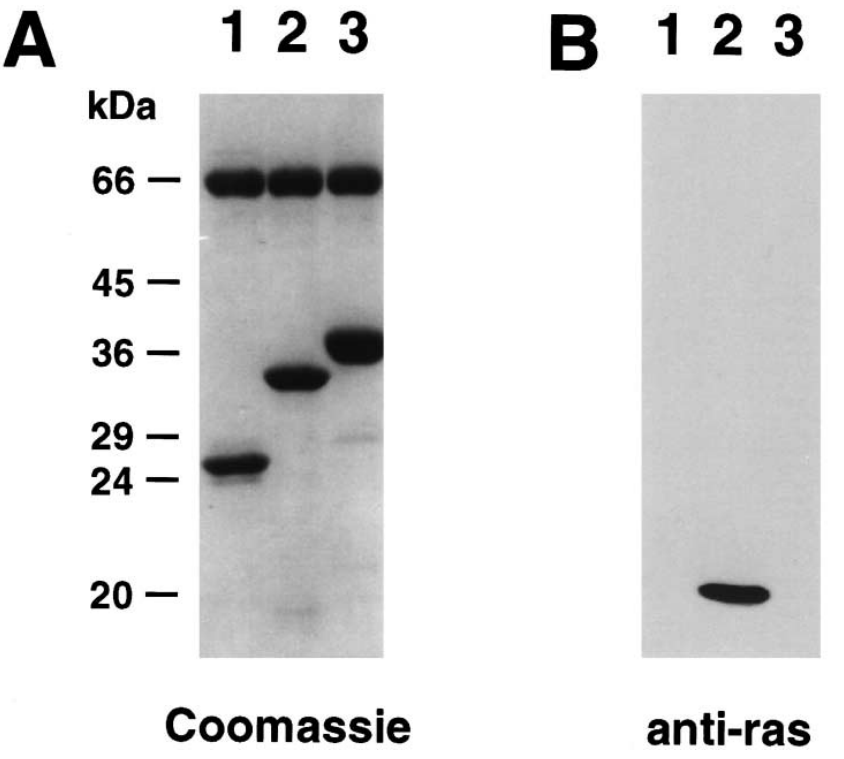

Fig. 2. Ras does not bind to the N-terminal domain of myr 5. Equal amounts of GST (lane 1), GST-Raf-RBD (lane 2), or GST-myr 5 N-terminus (lane 3) were incubated with glutathione beads and GppNp loaded Ras. GST fusion proteins eluted from the beads were detected by Coomassie staining (A) and bound Ras was detected by immunoblotting with anti-Ras antibody (B), respectively. BSA included in the wash buffer appears as a band at 66 kDa in (A).

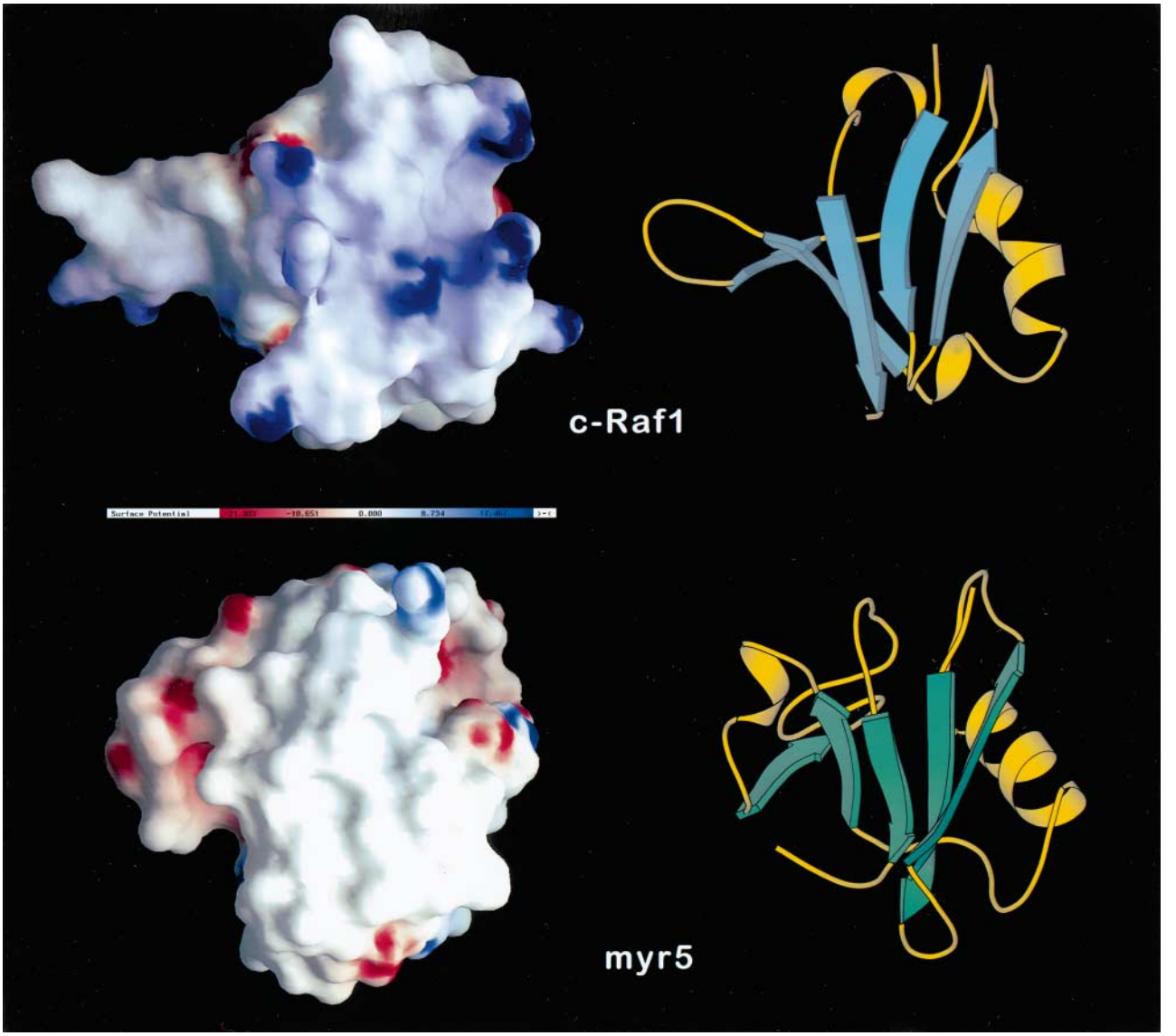

Fig. 3. Comparison of the proposed myr 5 'Ras-association' domain with the Raf-RBD. The view is directed onto the binding surface of the Raf-RBD. For comparison the equivalent surface of the presumably structurally homologous domain of myr 5 is shown. The folding topology and orientation of these domains is shown with Molscript [14]; negative potential is coloured red and positive potential blue.

of the surface potential revealed striking differences between the two domains. The Raf-RBD contact surface is clearly basic providing a favourable interaction surface with the negatively charged Ras contact site [11]. Indeed, two basic residues in Raf-RBD have been demonstrated to confer the major contribution to the binding affinity [12]. These characteristics of the Ras contact site also prove to be conserved in other Ras-binding domains such as the RBD of RalGEF [13]. In marked contrast, the myr 5 domain exhibits no charges and is likely to adopt a surface shape different from Raf-RBD (Fig. 3). Since all Ras family members (e.g. R-Ras, Rap) contain a conserved negatively charged effector-binding region, we can exclude that myr 5 will bind to any other Ras family member.

To test whether the described failure in predicting a Ras-binding domain is restricted to myr 5, we also modelled the consensus motif found in human DAG kinase. As found for the myr 5 domain, also this domain is likely to adopt a similar folding topology as Raf-RBD, but its surface structurally equivalent to the contact site of Raf-RBD does not fulfil the requirements for Ras-binding. In conclusion, the proposed consensus motif is not suitable to predict reliably novel Ras-binding domains. It rather predicts domains having the Raf-RBD folding topology of which some are Ras-binding domains. This exemplifies the functional diversity of similar structures.

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