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# Characterization of the Ras Binding Domain of the RalGDS-Related Protein, RLF

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**The Ras binding domain (RBD) of Rlf, a member of the RalGDS family of proteins, was characterized. Using an ELISA-based technique, the relative binding affinity of Rlf for a variety of mutant Ras proteins was determined. Rlf had significantly different binding characteristics than the Raf-1 RBD. The minimal effective Ras binding domain was defined as residues 657-778 using N- and C-terminal deletions of Rlf. Using the PHD algorithm, the secondary structure of this domain was predicted to be similar to the ubiquitin superfold previously identified in the Raf-1 RBD. When the predicted secondary structure of the Rlf-RBD was aligned with the known secondary structure of the Raf-RBD, amino acids in Raf-1 essential for Ras binding were found to also be conserved in Rlf. Consistent with this observation, alanine substitution of one of these residues (K687) in Rlf significantly reduced affinity for Ras-GTP.** 

The Ras proteins are 21 kDa, membrane-associated proteins which function as mitogenic switches coupled to growth factors and tyrosine kinases [1]. The best studied pathway regulated by Ras is the mitogen-activated protein kinase (MAPK) pathway [2]. Activated receptor-tyrosine kinases recruit guanine-nucleotide exchange factors to the plasma membrane, which induce the Ras proteins to release GDP and bind GTP. When bound to GTP, Ras assumes an activated conformation capable of binding to the regulatory domain of the Raf protein kinases. When associated with Ras at the plasma membrane, Raf itself becomes activated through a multistep process, and in turn phosphorylates and activates the MAPK kinase, Mek. Activated Mek then phosphorylates the Erk1 and 2 MAP kinases on tyrosine and threonine, resulting in their activation. While Erk1 and 2 are sufficient alone in some systems to provide a mitogenic signal, growing evidence suggests that Ras regulates other target proteins, termed "effectors", which also contribute to the Ras mitogenic signal [3]. Other potential effectors include PI-3 kinase and the RalGDS family of proteins.

The RalGDS proteins are guanine-nucleotide exchange factors specific for the Ral proteins [4]. The Ral proteins are Ras-related, guanine-nucleotide binding proteins whose function has been linked to regulation of Src and other GTPase signaling pathways [5, 6]. RalGDS and its homologue RGL, have been reported to associate with the GTP-bound conformation of Ras, suggesting a possible regulator-effector relationship. Numerous experiments suggest that RalGDS and RGL may play a role in Ras-mediated cellular transformation, although in vitro binding studies indicate that RalGDS is more likely to physically associate with the Ras-related Rap proteins than with Ras [7, 8]. No direct homology has been identified between the Ras binding domain (RBD) of Raf and RalGDS.

Using Rap1B as bait in a yeast two-hybrid screen, we have identified and isolated a partial clone of a third member of the RalGDS family. Upon DNA sequence analysis, the cloned gene was found to be identical to the RalGDS-like factor or Rlf [9]. Rlf shares 30% identity with RalGDS. The C-terminal 91 amino acids of Rlf was observed to bind equally well to both Ras-GTP and Rap1A-GTP, suggesting that the protein might interact similarly with both GTPases in vivo. Our fragment of the Rlf gene consists of an open reading frame coding for the 122 carboxy-terminal amino acids of the protein (residues 657-778). Subcloning the Rlf fragment into a GST vector permitted us to purify and characterize the GST-Rlf[657-778] fusion protein using

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an ELISA-based method. Here we report that the Rlf-RBD and the Raf-1 RBD interact similarly with a variety of Ras effector mutant proteins and probably share a similar secondary structure.

## METHODS

*Two-hybrid library cloning and screening for Ras binding proteins.* A murine T-cell library, yeast strain Y190, and the bait vector pAS-CYH-II were provided by Dr. Stephen J. Elledge, Baylor College of Medicine, Houston, TX. The vector pAS-CYH-II harboring cDNA encoding mouse Rap1B lacking the last four carboxyterminal amino acids was transformed into Y190 and stable transformants were selected on SD $Trp^-$ plates as described [10].

*Construction of GST-Rlf fusion vectors and protein purification.* The two-hybrid clone PTP.31 contained an open reading frame for the 122 C-terminal amino acids of Rlf. This fragment was subcloned as an XhoI fragment into pGEX-4T-2 producing GST-Rlf[657-778]. Truncation mutants were generated using polymerase chain reaction and the parental PTP.31 clone. All constructs were DNA sequenced. Purification of GST fusion proteins were as described [10].

*Measurement of the binding affinity of GST-Rlf proteins to Raf.* An ELISA method was used to assess the binding affinity of Rlf to Ras. The method used was identical to that already published for Ras and p120RasGAP except that GST-Raf[51-149] and GST-Rlf were substituted for GST-GAP [11]. Fusion proteins expressed from two independent clones of each construct were used.

*Mutagenesis of GST-Rlf 1-122.* Rlf was excised from the original PTP.31 plasmid as a *Bgl*II fragment and cloned into the *BamH*I site of pAlter-1 (Promega, Madison, WI). Lysine codon 687 was mutated to encode an alanine using the Altered Sites mutagenesis kit according to the manufacturer's protocol (Promega, Madison, WI). Mutations were verified by DNA sequencing, and Rlf[K687A] was further subcloned as an *Xho*I fragment into pGEX-4T-2.

*Profile network prediction of the secondary structure (PHDsec).* The secondary structure of the Rlf RBD was predicted using a homology/neural network-based algorithm known as PHDsec [12, 13]. The prediction was obtained for Rlf residues 644 to 737 using an internet submission protocol found at URL:/www.embl-heidelberg.de/predictprotein/predictprotein.html.

## RESULTS

*Isolation of a cDNA encoding the C-terminal 122 AA ORF of Rlf.* A yeast two-hybrid screen was performed to identify proteins which bound to Rap 1B. Forty-five large colonies grew on the SD $His^-$ plates and showed strong $\beta$-galactosidase expression. DNA sequencing revealed that 14 out of 15 positives were identical to the RalGDS homologue previously designated Rlf [9]. The sequence of clone PTP.31 was identical to the sequence of Rlf encoding the 122 C-terminal amino acids (data not shown).

*Determination of relative Ras/Raf and Ras/Rlf binding affinities.* Amino acid substitutions within the Ras effector region have been previously shown to impair the ability of Ras to transform cells and to associate with the Raf-1 RBD [14]. We wished to determine if the mechanism of Rlf-Ras association was similar to Raf-1. Using an ELISA assay we compared the abilities

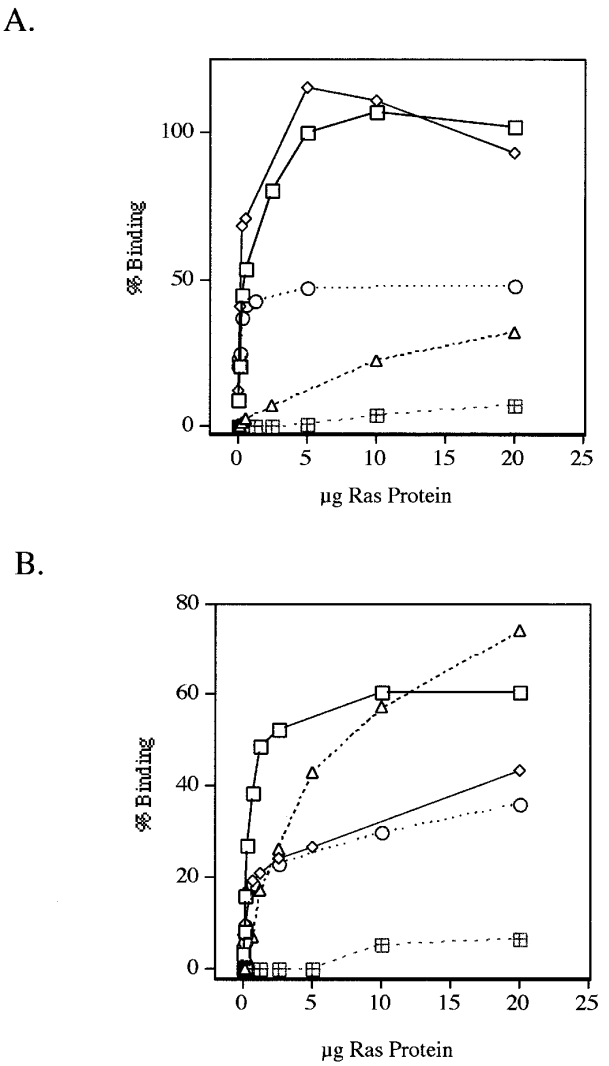


**FIG. 1.** Association of the Raf-1 and Rlf RBDs with Ras mutant proteins. The relative binding affinities of the RBDs of Raf-1 amino acids 51-149 and Rlf residues 657-778 were determined for the GTP-bound form of the following mutants: Ras[V12] (♦), Ras[L61] (□), Ras[V12G26I27] (○), Ras[E30K31] (△) and Ras[V12E45] (ii/home2/milesgrsym/8point/gs91,l(3,0)). (A) GST-Raf-1[51-149]. (B) GST-Rlf[657-778]. Purified GST and H-Ras[L61]-GDP were used in all ELISA experiments as negative controls. Ras[L61]-GTP at 5 $\mu$g was used as the 100% binding control. Results are representative of four separate assays.

of GST-Rlf[657-778] and GST-Raf[51-149] to bind the GTP-forms of the Ras[V12G26I27], Ras[E30K31] and Ras[V12E45] effector mutants. Binding of the Rlf and Raf-1 RBDs to oncogenic Ras[V12] and Ras[61] proteins bound to GTP was also measured. As seen in Figure 1, both Rlf and Raf-1 RBDs bound to the oncogenic forms of Ras. Interestingly, GST-Rlf bound more strongly to the Ras[L61] protein than to the Ras[V12] protein while GST-Raf bound both equally well. Furthermore, the GST-Rlf protein associated equally well

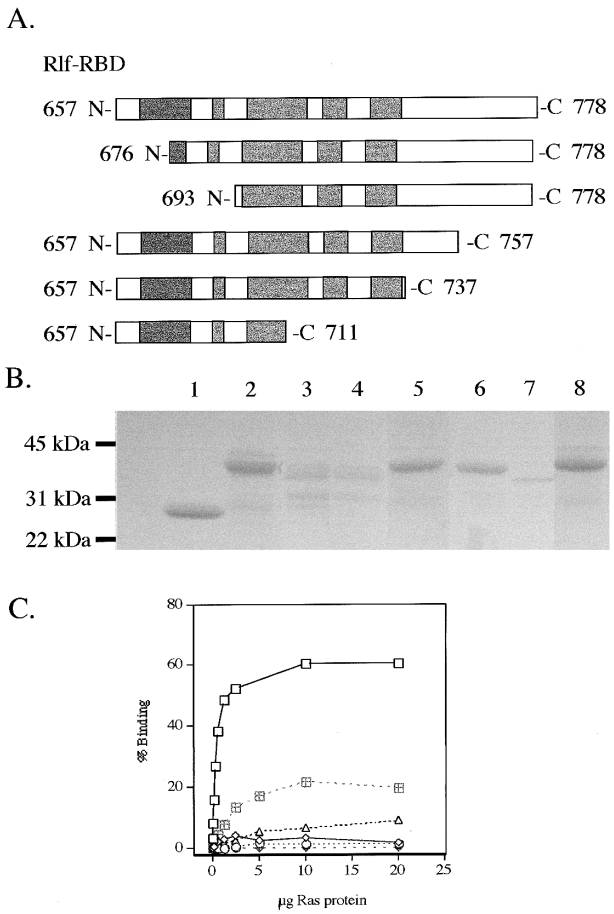


**FIG. 2.** Deletion analysis of the Rlf RBD. (A) Schematic representation of each Rlf deletion used in the analysis. (B) SDS-PAGE analysis of equivalently purified mutant Rlf proteins. (1) GST, (2) GST-Rlf[657-778], (3) GST-Rlf[676-778], (4) GST-Rlf[693-778], (5) GST-Rlf[657-757], (6) GST-Rlf[657-737], (7) GST-Rlf[657-711], (8) GST-Rlf[K687A]. (C) Association of Rlf deletion mutants with Ras[L61]-GTP. The N-terminal deletion mutants GST-Rlf[676-778] (◇) and GST-Rlf[693-778] (□) and C-terminal deletion mutants GST-Rlf[657-757] (△), GST-Rlf[657-737] (⊞) and GST-Rlf[657-711] (⊕) were tested for binding to H-RasL61-GTP. Controls were the same as in Figure 1. Results are representative of three separate assays.

with both the Ras[E30K31] mutant and oncogenic Ras[V12]. GST-Raf bound poorly to the Ras[E30K31] mutant.

Localization of the minimal Rlf-RBD by N- and C-terminal deletion truncation. The 122 amino acids contained within the GST-Rlf[657-778] fusion protein contained regions not conserved with the either RalGDS or RGL. We wished to identify the smallest protein fragment still capable of binding to Ras-GTP. Using polymerase chain reaction, the Rlf RBD coding region was sequentially truncated from both the N- and C-terminus as shown in Figure 2A. The N-terminal region of the Rlf RBD is highly conserved with the other RalGDS proteins. Deletion of these N-terminal homologies (GST-Rlf 676-778 and GST-Rlf 693-778) resulted in unstable proteins unable to bind to Ras (Fig. 2B and C). The C-terminal 50 amino acids of the Rlf RBD is not well conserved with other RalGDS family members. However, successive truncations of this region significantly reduced the ability of the Rlf-RBD to associate with Ras (Figure 2C). Curiously, the GST-Rlf 657-737 mutant bound to Ras better than the larger GST-Rlf 657-757 mutant. Further truncation of the C-terminus resulted in a less stable protein (GST-Rlf 657-711) with no detectable Ras binding activity (Fig. 2B and C).

*Secondary structure analysis of the Rlf-RBD predicts a ubiquitin fold.* Although sequence alignment of the Raf and RafGDS RBDs has failed to identify extensive sequence conservation, the Ras binding region of each protein may be similar at the level of secondary and tertiary structure. The PHDsec algorithm was used to predict the secondary structure for the Rlf-RBD and is shown in Figure 3. The predicted structure for the Rlf-RBD is almost identical to the known ubiquitin-like secondary structure of the Raf-1-RBD in that it is predicted to contain three beta strands and two alpha helices in the same order with nearly identical spacing.

The amino acid sequence for the Raf-1-RBD was aligned to Rlf using the predicted and known secondary structures to guide the alignment. Examined in the light of the secondary structure alignment, significant conservation was observed between Rlf and Raf-1, especially in regions of conserved structure (Fig. 3). The strongest regions of sequence conservation were between the regions defining the first beta strand and the first alpha helix of the RBDs. Although beta strand 1 does not play a direct role in Ras-Raf interactions (and is actually missing from our two-hybrid-derived clone), helix 1 does. The basic residues in Raf-1 helix 1 have been shown to be directly involved in Ras binding. Similarly, the predicted Rlf helix 1 also contains conserved basic residues which we predicted would also be involved in Ras binding. In order to test this hypothesis, we changed the most conserved of these residues, lysine 687, to an alanine. The K687A mutant was tested as a GST fusion protein for the ability to bind to Ras-GTP (Fig. 4). In support of our model, the Rlf[K687A] mutant had significantly impaired association with Ras.

## DISCUSSION

We have compared the abilities of the RalGDS-related protein, Rlf, with Raf-1 to associate with a variety of oncogenic and effector mutants of H-Ras [15]. Although the Raf-RBD bound with similar affinity to both Ras[V12] and Ras[L61], the Rlf-RBD showed significantly increased association with Ras[L61] relative to

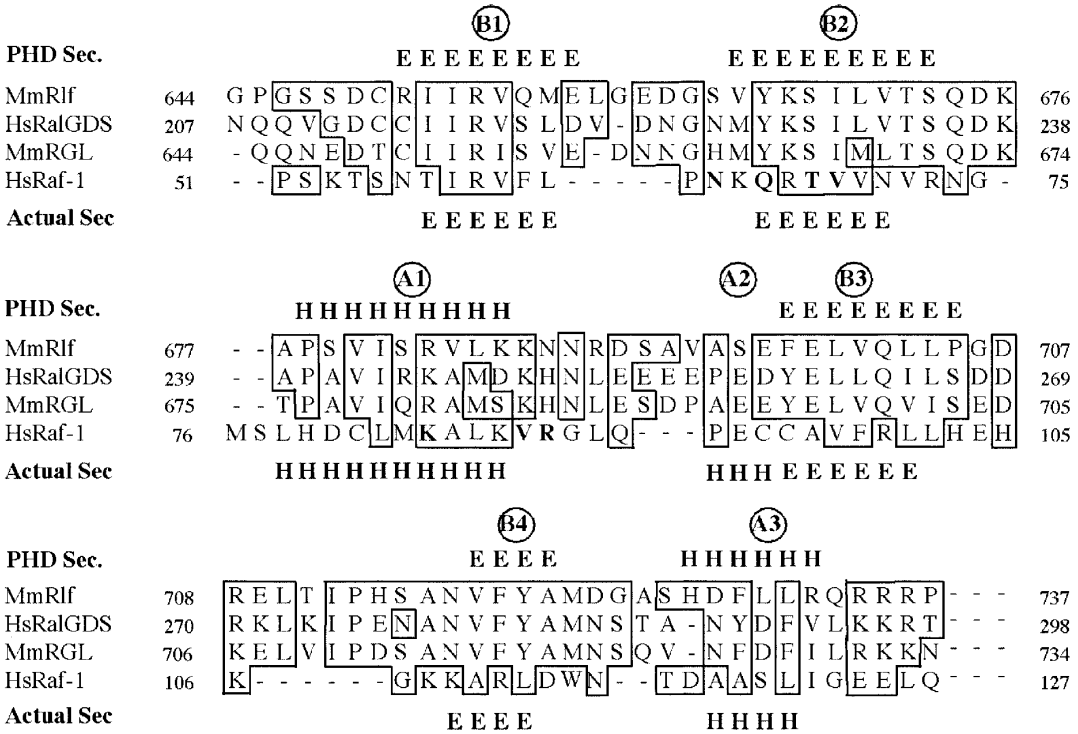


**FIG. 3.** Sequence and structural alignment of the Rlf and Raf-1 RBDs. The secondary structure of Rlf residues 644-737 was predicted by PHDsec [13]. The Rlf sequence was aligned by clustal with the sequences of RalGDS and RGL. Raf-1 sequence alignment was performed manually using common secondary structural elements as guides. E=beta strand. H=alpha helix.

Ras[V12]. Both p120 RasGAP and neurofibromin have also been observed to bind to Ras[L61] with an affinity 10-100-fold greater than for Ras[V12] [16, 17]. This result suggests a role for the Ras switch 2 region in Rlf binding [18]. Clear differences were also found between Raf-1 and Rlf in their relative binding affinities for different Ras effector mutants. The Ras effector mutants used in this study have reduced biological potency as measured by a decreased ability to transform fibroblast cells and to induce neuronal cell differentiation [15]. The G26I27, E30K31 and E45 mutants are respectively reduced in transformation efficiency of 25-, 7- and >1000-fold. This is reflected in the relative binding affinities of these mutant proteins for the Raf-1 RBD (Fig. 1). The correlation between Ras effector mutant association and transformation efficiency did not hold true for the Rlf-RBD. Although the E45 mutant was defective for binding, the G26I27 mutant bound comparably to wild type Ras. The E30K31 mutant was unique in that it appeared to bind better to the Rlf-RBD than wild type Ras. The presence of glutamic acid and lysine at positions 30 and 31 have been reported as being important for promoting a preferred association between Rap1A protein and the RalGDS RBD [8]. These results are consistent with Rlf not being required for Ras-dependent fibroblast transformation and in functioning as preferred effectors for the Rap family of proteins.

By constructing deletion mutants of the C-terminal portion of Rlf we were able to tentatively residues which might be important for the binding of Rlf to Ras. Although the last 50 amino acids of the Rlf[657-778] RBD are non-homologous with other RalGDS family proteins, deletion of this region had a detrimental effect upon Ras binding. We hypothesize that the extreme C-terminal region plays a role in stabilizing the active binding conformation of the small Rlf-RBD. The structural importance of the N-terminus of the Rlf-RBD was demonstrated by the instability of E. coli-expressed N-terminal deletion mutants.

Possibly the most usefully result of this study, was the successful application of the PHDsec algorithm to identify a putative ubiquitin superfold in the Rlf-RBD. Although previous investigation has failed to identify significant homology between the Raf and RalGDS RBDs, the apparent conservation of secondary structure has permitted us to identify residues in the Rlf-RBD which are conserved with characterized Ras-binding residues in Raf-1. Crystallographic and mutagenic studies of the Raf-1 RBD have identified residues in beta strand 2 and alpha helix 1 as directly interacting with Ras [14, 19]. Particularly important for the recog-

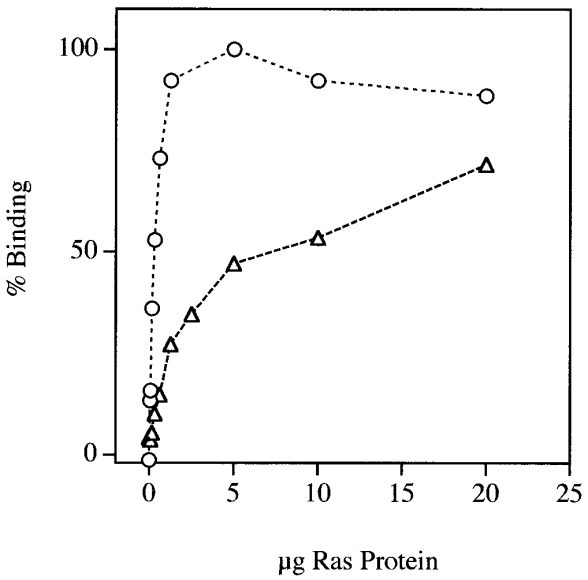


**FIG. 4.** Mutation of Rlf lysine 687 impairs association with Ras. The Ras binding ability of the wild type GST-Rlf[657-778] protein (○) was compared with the GST-Rlf[657-778] K687A mutant (△) by ELISA. Results are representative of four separate assays.

nition of the acidic residues in the Ras effector loop are the basic residues in Raf-1 RBD alpha helix 1. The predicted alpha helix 1 of the Rlf-RBD similarly contains conserved basic residues with lysine 687 completely conserved among all the RalGDS family of proteins. This lysine would be considered analogous to arginine 89 in the Raf-1 RBD, which is essential for Ras association [14]. In support of this hypothesis, substitution of lysine 687 with alanine severely reduced the ability of Rlf to bind to Ras.

In summary, the Ras binding domain of the RalGDS protein, Rlf, appears to consist of a ubiquitin superfold and interacts with Ras through residues conserved with Raf-1. The careful use of deletions, mutations and secondary structure prediction can be useful tools to identify the conserved ubiquitin-like Ras binding domain in potential Ras effector proteins without the use of more difficult techniques such as NMR or X-ray crystallography.

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