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A SMALL PEPTIDE DERIVED FROM THE AMINOTERMINUS OF c-RAF-1 INHIBITS c-RAF-1/RAS BINDING

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Various domains of the aminoterminal part of c-Raf-1 expressed as glutathione-S-transferase fusion proteins were analyzed for Ras binding. The binding site was localized at the aminoterminus outside of the cysteine-rich region. A single aminoacid exchange at aminoacid residue 89 (Arg89 to Leu) of c-Raf-1 inhibits binding. A small synthetic peptide corresponding to c-Raf-1 aminoacids 77 to 101 comprising Arg89 in a central position competes for Ras binding and thereby characterizes the relevant binding domain of Ras on c-Raf-1.

The cytoplasmatic serine/threonine kinase c-Raf-1 and the small GTP-binding protein Ras are acting as central intermediates in the signal transduction pathway transmitting signals from ligand activated growth factor receptors to the nucleus and thereby play a central role in control of cell growth and differentiation (1,2). Upon stimulation with various agents c-Raf-1 becomes rapidly phosphorylated and activated (3). The aminoterminal regulatory domain comprising aminoacids 1 to 331 modulates the function of the carboxyterminal catalytic domain (1). Ras cycles between an inactive GDP-bound and an active GTP-bound conformation and a wide variety of extracellular signals can stimulate the formation of the active GTP-bound state (4). c-Raf-1 acts downstream of Ras (5) and recently interaction between active GTP-bound Ras and the aminoterminal domain of c-Raf-1 has been described *in vitro* and in yeast two hybrid systems (6-10). Coimmunoprecipitation of both proteins in stimulated mammalian cells has also been reported (11,12). However, this interaction does

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Abbreviations: GSH, glutathion; GST, glutathion-S-transferase; kD, kiloDalton; SDS-PAGE, sodiumdodecylsulphate polyacrylamide gel electrophoresis; wt, wild-type.

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not correlate with elevated kinase activity of c-Raf-1. Ras seems to function in localizing c-Raf-1 to the plasma membrane where c-Raf-1 remains attached independently of Ras (13-15). There a separate, Ras-independent, activation of c-Raf-1 by not identified kinases or other unknown factors occurs. Recently we described specific association of a 28 kD cellular protein and of a c-Raf-1 phosphorylating kinase with the aminoterminus of c-Raf-1 with possible functions in further activation (16,17).

One candidate domain for Ras interaction in the aminoterminal part of c-Raf-1 is the cysteine-rich region. It is postulated to bind a putative c-Raf-1 activator (18) and has been shown to interact with Ras *in vitro* (10). However, screening of a cDNA library with Ras using the two hybrid system identified a more aminoterminal region for interaction comprising aminoacids 51 to 131 (8). In this study we used a peptide corresponding to c-Raf-1 aminoacids 77 to 101 to compete c-Raf-1/Ras binding and thereby restrict the relevant binding domain.

MATERIALS AND METHODS

Cloning and expression of recombinant GST-Raf fusion proteins. pGEX derived expression plasmids producing GST-Raf fusion proteins GST-Raf 1-331, GST-Raf 1-180 and GST-Raf 181-331 have been published (17,19). pGEX derived expression plasmids producing GST-Raf fusion proteins GST-Raf 1-148, GST-Raf 148-331 and GST-Raf 52-331 were constructed using single restriction sites (HindIII and XhoII, respectively). Mutation of Arg89 to Leu (R89L) was introduced by recombinant PCR. GST-fusion proteins were expressed in *E.coli* JM101 and affinity purified on glutathione (GSH)-agarose (Sigma) as described (20). Purified GST-Raf proteins remained on the beads for *in vitro* binding assay. Protein content and purity were controlled by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining.

***In vitro* binding assay.** Each assay contained 1 μg purified recombinant bacterially expressed H-Ras G12V protein, a generous gift of A. Wittinghofer (21). Nucleotide loading of Ras was done in 50 mM Tris-HCl, pH 7.5, 7.5 mM EDTA, 0.5 mg/ml bovine serum albumin, 2 mM DTT, 1 mM GDPβS or 1 mM GTPγS (a 25 x molar excess to Ras) for 15 min at 37°C. The reaction was stopped on ice by the addition of $MgCl_2$ to a final concentration of 12.5 mM. The binding assay was performed in 300 μl NETN buffer (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM DTT, 1 mM PMSF, 200 KIE/ml Trasylol) with 3 μg GST-Raf fusion protein affinity purified on GSH-agarose beads for 2 h at 4°C with end over end rotation. After association beads were washed five times with 1 ml ice cold NETN buffer, subjected to 15% SDS-PAGE, immunoblotted for Ras using pan-Ras monoclonal antibody (Oncogene Science) and detected using enhanced chemiluminescence (Amersham). For peptide competition GTPγS loaded Ras was preincubated with a 1000 x molar excess of peptide relating to GST-Raf fusion protein in NETN buffer for 2 h at 4°C with end over end rotation. Peptides were synthezised chemically on an automated peptide synthesizer AB430A (Applied Biosystems).

RESULTS AND DISCUSSION

In order to analyze binding of Ras to c-Raf-1 we used a glutathione-S-transferase (GST) fusion protein containing aminoacids 1 to 331 of the human c-Raf-1 (GST-Raf 1-331) which represents the aminoterminal regulatory domain. Various domains of the aminoterminal part

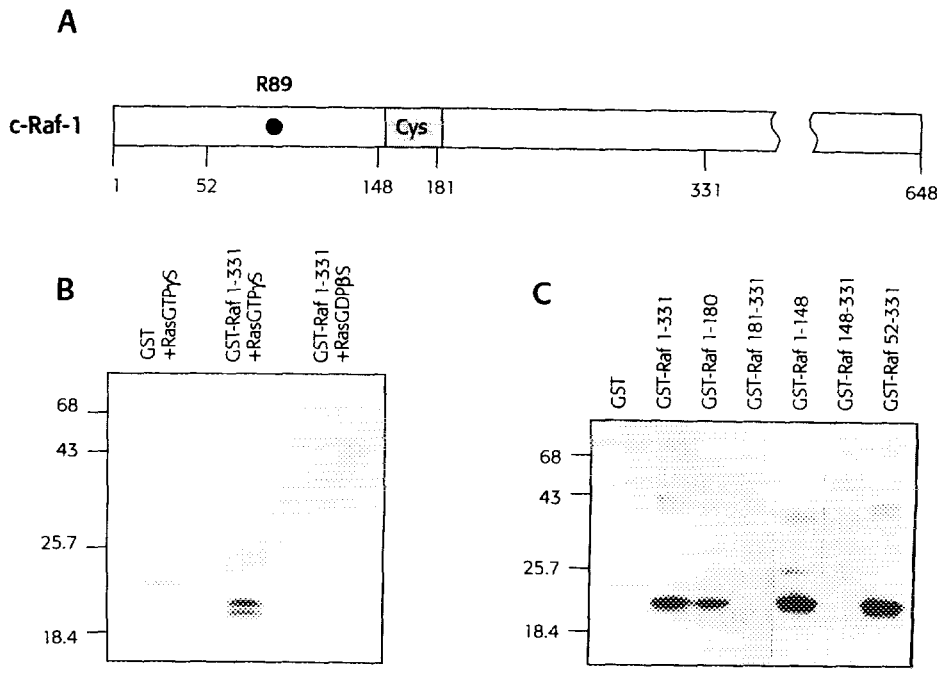

Figure 1. Binding of GST-Raf 1-331 to Ras. (**A**) Schematic drawing of c-Raf-1. Numbers refer to aminoacid residues. The cysteine-rich region (Cys) and the conserved Arg 89 (R89) are indicated. (**B**) Recombinant GST and GST-Raf 1-331 proteins immobilized on GSH-agarose beads were incubated with GTPγS-loaded or GDPβS-loaded recombinant Ras. After washing GSH-agarose beads were analyzed by SDS-PAGE followed by immunoblotting with anti-Ras antibody. Molecular weight markers are shown in kD. (**C**) Various length GST-Raf proteins immobilized on GSH-agarose beads were incubated with GTPγS-loaded Ras and analyzed as in B.

of c-Raf-1 schematically outlined in Figure 1A were expressed as GST fusion proteins and used to identify the binding site for Ras. The GST-Raf fusion proteins were efficiently purified from bacterial lysates by affinity chromatography and used in binding assays with purified recombinant GDPβS or GTPγS loaded H-Ras G12V. After washing the beads were subjected to SDS-PAGE and immunoblotted for Ras. Binding occurred to GST-Raf 1 - 331 only in the presence of GTPγS loaded activated Ras (Figure 1B). No binding is observed between GST-Raf 1-331 and GDPβS loaded Ras. GST protein in the presence of activated GTPγS loaded Ras is shown for control. GST-Raf 1-180, GST-Raf 1-148 and GST-Raf 52-331 also associate with GTPγS loaded Ras (Figure 1C). No binding is observed with GST-Raf 148-331, comprising the cysteine-rich region, and with GST-Raf 181-331. GST-Raf 1-331 point-mutated in Cys 168 to Ser in comparison to GST-Raf 1-331 wild-type (wt) showed reduced binding affinity to Ras (data not shown). This might be due to the structural importance of Cys 168 for the aminoterminal domain of c-Raf-1. These data characterize a binding domain between aminoacids 52 and 147 beyond the cysteine-rich region of c-Raf-1 consistent with the data resulting from the two hybrid system described by Vojtek et al. (8).

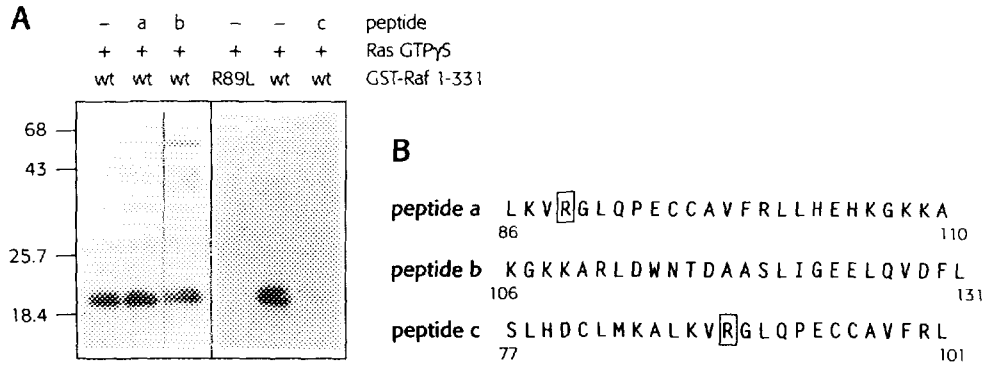

Figure 2. Binding of GST-Raf 1-331 to Ras in the presence of various peptides derived from c-Raf-1. **(A)** GTPγS-loaded Ras was first incubated with the respective peptide and then with GST-Raf 1-331 (wt) protein immobilized on GSH-agarose beads. Incubation without peptide was performed with GST-Raf 1-331 (wt) and GST-Raf 1-331 (R89L) protein. The GSH-agarose beads were analyzed as in Figure 1B. Molecular weight markers are shown in kD. **(B)** Aminoacid sequence of peptide a, b and c derived from human c-Raf-1. Peptide a corresponds to c-Raf-1 aminoacids 86 to 110, peptide b to aminoacids 106 to 131 and peptide c to aminoacids 77 to 101. Arg 89 is marked by a square.

The alteration of Arg 217 to Leu in *Drosophila* Raf led to a partial loss of Raf function (22). This aminoacid residue corresponds to Arg 89 in human c-Raf-1. To examine the importance of this conserved position we constructed a point mutant in GST-Raf 1-331 (wt), Arg 89 to Leu (R89L), and compared both proteins in the binding assay. No binding of GTPγS loaded Ras to GST-Raf 1-331 (R89L) was observed (Figure 2A). While this work was in progress Fabian et al. (23) have identified Arg 89 as a critical residue involved in c-Raf-1/Ras interaction *in vitro*, in the two hybrid system and in *Xenopus laevis* oocyte maturation. To obtain a more precise restriction of the relevant binding epitope we chose three different peptides corresponding to c-Raf-1 aminoacids 86 to 110 (peptide a), aminoacids 106 to 131 (peptide b) and aminoacids 77 to 101 (peptide c) shown in Figure 2B and analyzed them for competing the binding between GTPγS loaded Ras and GST-Raf 1-331 (wt). Preincubation of activated Ras with peptide a or with peptide b did not disturb the binding to GST-Raf 1-331 (wt) (Figure 2A). However, preincubation of GTPγS loaded Ras with peptide c prevents binding to GST-Raf 1-331 (wt). Arg 89 is located at position 4 in peptide a, at the central position in peptide c and is absent from peptide b. Only peptide c is able to prevent the interaction between GST-Raf 1-331 (wt) and Ras. This defines the relevant binding epitope at both sides of Arg 89 to aminoacids 77 to 101. This peptide could be a good tool to analyze further the activation of c-Raf-1 in different systems.

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