Characterization of the interaction of Raf-1 with *ras* p21 or 14-3-3 protein in intact cells

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Abstract Several deletion mutants of Raf-1 were expressed with v-*ras* p21 or 14-3-3 protein in COS-7 cells and Sf9 cells and the interaction of Raf-1 with *ras* p21 or with 14-3-3 protein in intact cells was examined. Raf(1–135) (residues 1–135) and Raf(1–322) interacted with v-*ras* p21, but other deletion mutants such as Raf(136–322) or Raf(321–648) did not. Raf(1–322) interacted with 14-3-3 protein much more efficiently than Raf(321–648) did. While Raf(1–135) did not interact with 14-3-3 protein, Raf(136–322) did. These results clearly indicate that Raf-1 simultaneously interacts with both *ras* p21 and 14-3-3 protein through the distinct binding domains in intact cells.

Key words: 14-3-3 Protein; *ras* p21; Raf-1; COS cell; *Spodoptera frugiperda* cell (Sf9 cell)

1. Introduction

Protein kinase Raf-1 is believed to be a key component of the signal transduction pathway leading to MAP kinase activation by extracellular signals [1,2]. Among the intracellular signals which activate Raf-1, the signal through *ras* p21 has been well studied [1,2]. Extracellular signals induce the conversion of the GDP-bound inactive form of *ras* p21 to the GTP-bound active form [3]. The GTP-bound form of *ras* p21 interacts with Raf-1 [4–10]. The 81-amino acid fragment (residues 51–131) of Raf-1 is a minimal *ras* p21-binding domain [8,11–14]. However, it has been reported that the interaction of Raf-1 with *ras* p21 is not sufficient for Raf-1 activation, suggesting that other factors are required for *ras* p21-dependent Raf-1 activation [1,10].

Several groups including us have found that the 14-3-3 family of proteins (14-3-3 protein) interacts with Raf-1 and stimulates this protein kinase activity [15–17]. 14-3-3 protein has been originally identified as a series of very abundant acidic proteins with a M_r of 30,000 in brain tissue [18]. At least seven mammalian isoforms have been identified, and 14-3-3 protein exhibits multiple functions such as an activator of tyrosine and tryptophan hydroxylase or a potent inhibitor of protein kinase C [18–22]. Our results have shown that 14-3-3 protein associates with and activates Raf-1 in *Xenopus* oocytes and that 14-3-3 protein induces oocyte maturation through Raf-1 [17]. The N-terminal domain of Raf-1 binds to 14-3-3 protein more efficiently than the C-terminal domain in yeast two-hybrid system [15–17]. However, 14-3-3 protein-binding domain of Raf-1 has not yet been characterized in intact cells.

To understand how *ras* p21 and 14-3-3 protein activate Raf-1, we have analyzed the binding domains of Raf-1 for *ras* p21 and 14-3-3 protein using intact cells. Here we demonstrate that the binding domain of Raf-1 for *ras* p21 is distinct from the 14-3-3 protein-binding domain in intact cells.

2. Materials and methods

2.1. Materials

Mammalian expression vectors, pCGN and pBJ-1, and the mouse anti-influenza virus HA1 monoclonal antibody 12CA5 [23] were provided by Dr. Q. Hu (University of California, San Francisco, CA, USA). pCGN was designed to express an influenza virus HA epitope fused protein which was recognized by the antibody 12CA5. The baculovirus carrying the Myc-14-3-3ζ cDNA, pBJ/Myc-14-3-3ζ, and the mouse anti-Myc monoclonal antibody 9E10 were provided by Dr. A. Klippel (Chiron Corp., CA, USA). 14-3-3ζ protein was tagged at the N-terminus with a Myc epitope which was recognized by the antibody 9E10. COS-7 cells were obtained from the American Type Culture Collection. 14-3-3ζ cDNA was isolated as a Raf-interacting molecule by yeast two-hybrid method [17]. v-*ras* p21, Raf-1, GST fused to the N-terminal domain of Raf-1 (amino acids 1–322) (GST-N-Raf), and GST fused to the C-terminal domain of Raf-1 (amino acids 321–648) (GST-C-Raf) were expressed in Sf9 cells as described [10,24]. Other materials were obtained as described [10,25].

2.2 Plasmid constructions

The fragments of the human Raf-1 cDNA sequence corresponding to amino acid residues 1–60, 1–135, 1–196, 61–322, 81–322, 106–322, 136–322, 1–322, and 321–648 were synthesized by a PCR. These PCR fragments were designed to have *Xba*I and *Pst*I sites at the 5′-end and the 3′-end, respectively. These PCR fragments were digested with *Xba*I and *Pst*I and the *Pst*I site was blunted with Klenow. Then the PCR fragments were inserted in the *Xba*I and *Sma*I cut pCGN. To construct pBJ-1 encoding v-*ras* p21, the 0.6 kb fragments containing v-*ras* p21 with the *Xba*I and *Bam*HI sites was synthesized by PCR. This fragment was digested with *Xba*I and *Bam*HI and inserted into the *Xba*I and *Bam*HI cut pBJ-1.

2.3. Interaction assay of Raf-1 with ras p21 and 14-3-3 protein in COS-7 cells and Sf9 cells

COS-7 cells were cultured as described [26]. COS-7 cells were transfected with pCGN- and pBJ-derived constructs described above by the DEAE-dextran method [27]. Sixty hours after transfection, the cells were lysed [26]. The proteins of the lysates (160 μg of protein) were immunoprecipitated with the anti-*ras* p21 (Y13-238) or anti-Myc antibody [25,26]. The precipitates were probed with the anti-HA antibody. Monolayers of Sf9 cells were infected with high-titer recombinant baculoviruses [24]. Seventy-two hours after infection, the cells were lysed [10]. The proteins of the lysates (240 μg of protein) were immunoprecipitated with the anti-Raf-1 antibody or precipitated with glutathione Sepharose 4B [10]. The precipitates were probed with the anti-Raf-1, anti-*ras* p21, anti-HA, or anti-Myc antibody.

2.4. Other determinations

Protein concentrations were determined with bovine serum albumin as a standard [28].

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Abbreviations: MAP kinase, mitogen-activated protein kinase; HA, hemagglutinin; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction; Sf9 cells, *Spodoptera frugiperda* cells; IP, immunoprecipitation.

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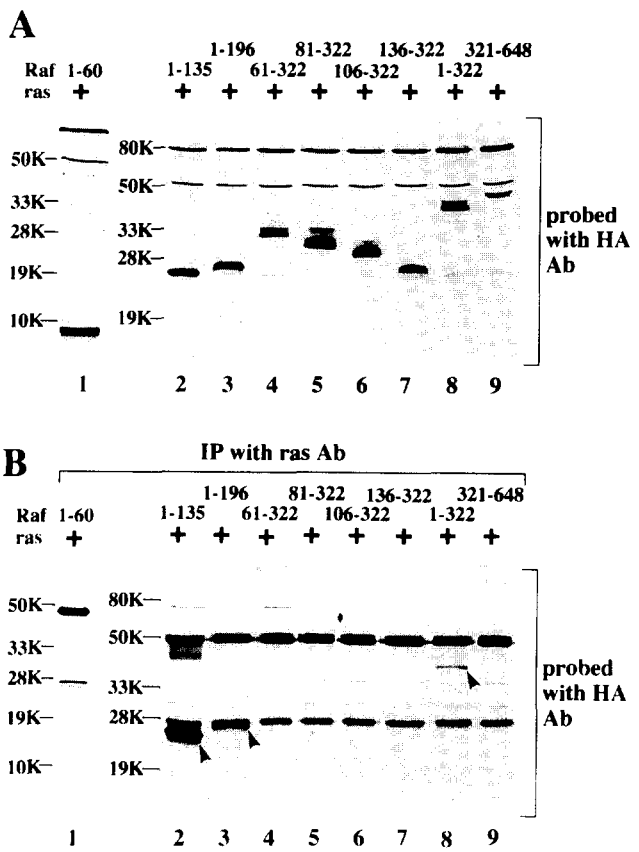

Fig. 1. Interaction of Raf-1 deletion mutants with *ras* p21 in COS cells. (A) Expression of Raf-1 deletion mutants. Aliquots (10 μl each) of the lysates expressing v-*ras* p21 and Raf(1–60) (lane 1), Raf(1–135) (lane 2), Raf(1–196) (lane 3), Raf(61–322) (lane 4), Raf(81–322) (lane 5), Raf(106–322) (lane 6), Raf(136–322) (lane 7), Raf(1–322) (lane 8), or Raf(321–648) (lane 9) were probed with the anti-HA antibody. (B) Interaction of Raf-1 deletion mutants with *ras* p21. The lysates expressing v-*ras* p21 and Raf-1 mutants described above were immunoprecipitated with the anti-*ras* p21 antibody (Y13-238). Arrow heads indicate the precipitated Raf-1 mutants. The results shown are representative of three independent experiments.

3. Results

To characterize the *ras* p21-binding domain of Raf-1 in intact cells, we made a series of the N- and C-terminal deletion mutants of Raf-1 and expressed them with v-*ras* p21 in COS cells. These Raf-1 deletion mutants were expressed as HA-tagged proteins. The expression levels of transfected Raf-1 deletion mutants were similar as assessed by immunoblotting using the anti-HA antibody (Fig. 1A). The expression levels of transfected v-*ras* p21 were also similar in these cells (data not shown). When the lysates expressing the Raf-1 deletion mutants and v-*ras* p21 were immunoprecipitated with the anti-*ras* p21 antibody, Raf(1–135), Raf(1–196), and Raf(1–322) were detected in the *ras* p21 immune complex (Fig. 1B, lanes 2, 3 and 8). However, none of Raf(1–60), Raf(61–322), Raf(81–322), Raf(106–322), Raf(136–322), or Raf(321–648) was immunoprecipitated with *ras* p21 (Fig. 1B, lanes 1, 4–7, and 9).

Next we characterized the interaction of Raf-1 and 14-3-3 protein in Sf9 cells. 14-3-3 protein was expressed as a Myc-tagged protein. The expression level of transfected 14-3-3 protein alone was similar to that in the cells expressing Raf-1 and 14-3-3 protein as assessed by immunoblotting using the anti-Myc antibody (Fig. 2A). When the lysates expressing Raf-1 and 14-3-3 protein were immunoprecipitated with the anti-Raf-1 antibody, both Raf-1 and 14-3-3 protein were detected in the Raf-1 immune complex (Fig. 2B, lane 3). When the lysates expressing Raf-1 alone or 14-3-3 protein alone were immunoprecipitated with the anti-Raf-1 antibody, 14-3-3 protein was not detected (Fig. 2B, lanes 1 and 2). Neither Raf-1 nor 14-3-3 protein was immunoprecipitated with non-immune serum from the lysates expressing both proteins (Fig. 2B, lane 4). To characterize the interaction of Raf-1 and 14-3-3 protein further, we expressed 14-3-3 protein with GST-N-Raf or GST-C-Raf in Sf9 cells. GST-N-Raf and GST-C-Raf were expressed as HA-tagged proteins. The expression levels of transfected GST-N-Raf and GST-C-Raf were similar as assessed by immunoblotting using the anti-HA antibody (Fig. 2C, lanes 1 and 2). The expression levels of transfected 14-3-3 protein were also similar in these cells (Fig. 2C, lanes 1 and 2). When the lysates expressing GST-N-Raf and 14-3-3 protein were precipitated with glutathione Sepharose 4B, GST-N-Raf was precipitated with 14-3-3 protein (Fig. 2C, lane 3). However, GST-C-Raf bound to 14-3-3 protein very weakly (Fig. 2C, lane 4). These results are consistent with the recent observations of the interaction of Raf-1 and 14-3-3 in vitro [29]. Furthermore, we examined the interaction of Raf-1, v-*ras* p21, and 14-3-3 proteins in Sf9 cells. Both v-*ras* p21 and 14-3-3 protein were immunoprecipitated with Raf-1 (Fig. 2D, lane 3). The binding of *ras* p21 to Raf-1 was not found to affect that of 14-3-3 protein to Raf-1 (Fig. 2D, lanes 1–3).

These observations prompted us to examine whether the *ras* p21-binding and 14-3-3 protein-binding domains of Raf-1 were separate. We expressed 14-3-3 protein and Raf-1 deletion mutants in COS cells. 14-3-3 protein was expressed as a Myc-tagged protein. The expression levels of transfected 14-3-3 protein were similar in these cells (data not shown). Raf(1–322) and Raf(136–322) were immunoprecipitated with 14-3-3 protein,

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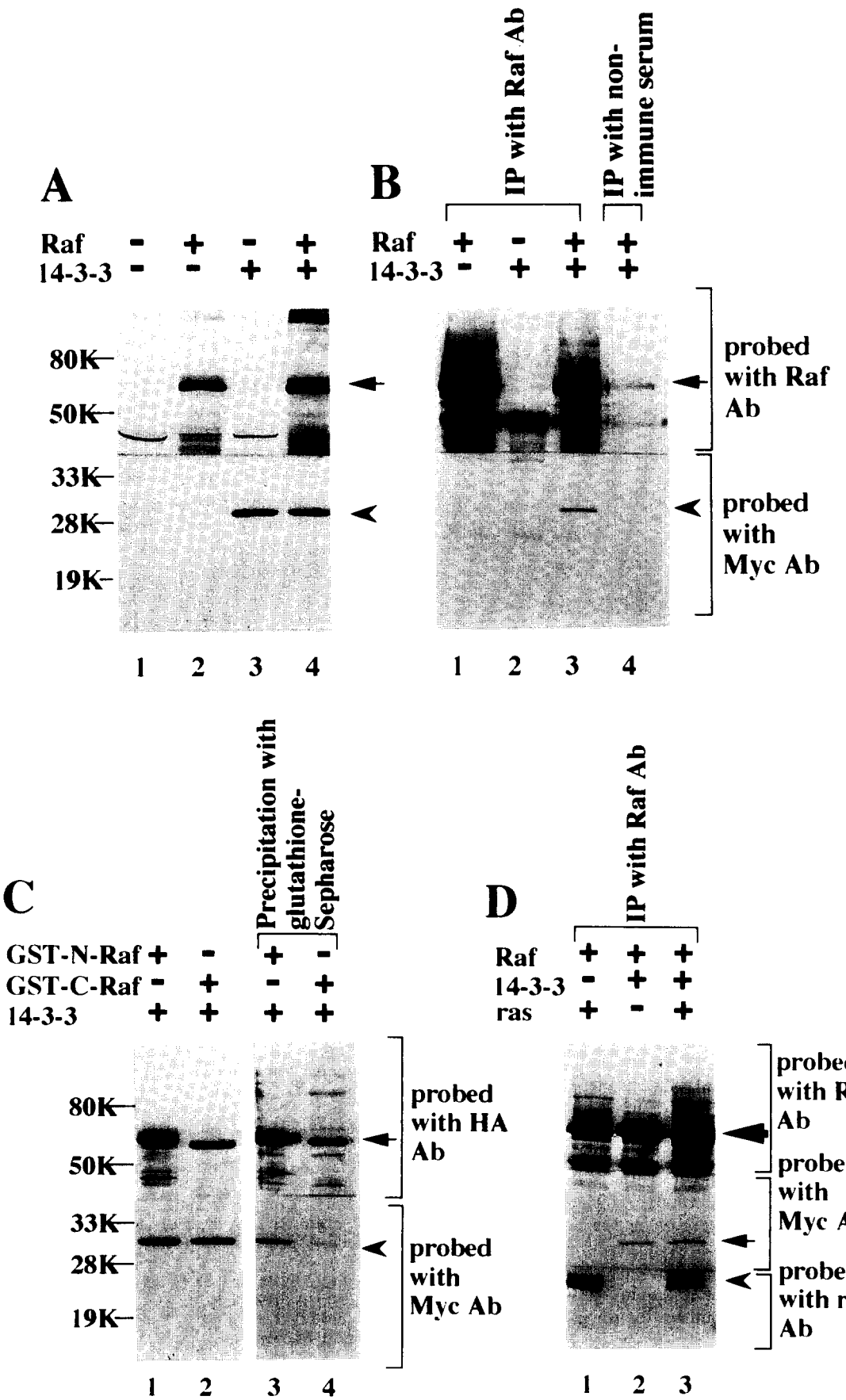
Fig. 2. Interaction of Raf-1 with 14-3-3 protein in Sf9 cells. (A) Expression of Raf-1 and 14-3-3 protein. Aliquots (10 μl) of Sf9 cells expressing no protein (lane 1), Raf-1 alone (lane 2), 14-3-3 protein alone (lane 3), or both proteins (lane 4) were probed with the anti-Raf-1 and anti-Myc antibodies. An arrow and an arrowhead indicate the positions of Raf-1 and 14-3-3 protein, respectively. (B) Interaction of Raf-1 with 14-3-3 protein. The lysates expressing Raf-1 alone (lane 1), 14-3-3 protein alone (lane 2), or both proteins (lanes 3 and 4) were immunoprecipitated with the anti-Raf-1 antibody (lanes 1–3) or non-immune serum (lane 4). (C) Interaction of 14-3-3 protein with N-terminal domain of Raf-1. The lysates expressing GST-N-Raf and 14-3-3 protein (lanes 1 and 3) or GST-C-Raf and 14-3-3 protein (lanes 2 and 4) were lysed and aliquots (10 μl each) of the lysates were probed with the anti-HA and anti-Myc antibodies (lanes 1 and 2). The proteins of the lysates were precipitated with glutathione Sepharose 4B (lanes 3 and 4). An arrow indicates the positions of GST-N-Raf and GST-C-Raf. An arrowhead indicates the position of 14-3-3 protein. (D) Complex formation of Raf-1, 14-3-3 protein, and *ras* p21. The lysates expressing Raf-1 and v-*ras* p21 (lane 1), Raf-1 and 14-3-3 protein (lane 2), or Raf-1, 14-3-3 protein, and v-*ras* p21 (lane 3) were immunoprecipitated with the anti-Raf-1 antibody. A big arrow, a small arrow, and an arrowhead indicate the positions of Raf-1, 14-3-3 protein, and *ras* p21, respectively. The results shown are representative of three independent experiments.


A
Raf - + - +
14-3-3 - - + +
80K
50K
33K
28K
19K
1 2 3 4
B
IP with Raf Ab
IP with non-immune serum
Raf + - + +
14-3-3 - + + +
probed with Raf Ab
probed with Myc Ab
1 2 3 4
C
Precipitation with glutathione-Sepharose
GST-N-Raf + - + -
GST-C-Raf - + - +
14-3-3 + + + +
80K
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1 2 3 4
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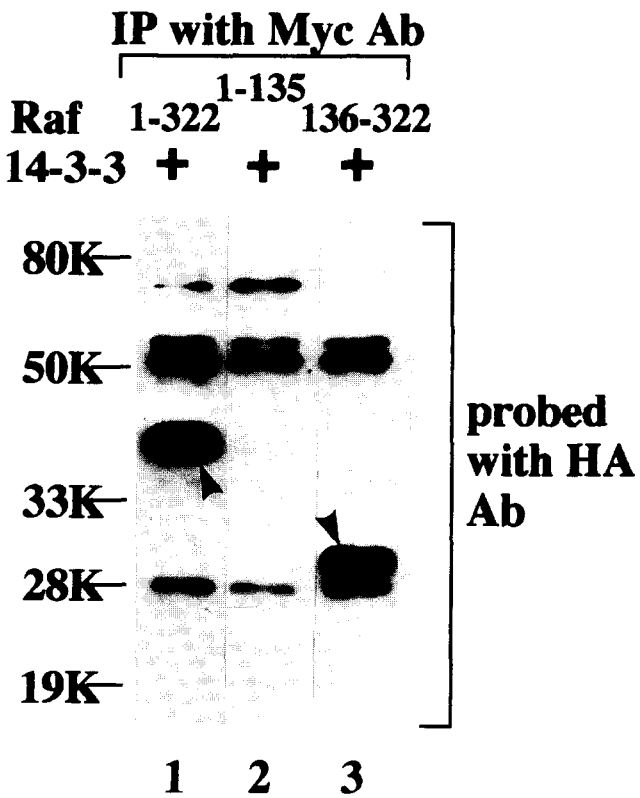

Fig. 3. Interaction of Raf(136–322) with 14-3-3 protein in COS cells. The lysates expressing 14-3-3 protein and Raf(1–322) (lane 1), 14-3-3 protein and Raf(1–135) (lane 2), or 14-3-3 protein and Raf(136–322) (lane 3) were immunoprecipitated with the anti-Myc antibody. Arrows indicate the precipitated Raf-1 mutants. The results shown are representative of three independent experiments.

but Raf(1–135) was not (Fig. 3). These results indicate that residues 1–135 and 136–322 constitute the binding domains for *ras* p21 and 14-3-3 protein, respectively.

4. Discussion

The experiments to determine the binding domain of Raf-1 for *ras* p21 and 14-3-3 protein have been carried out using yeast two-hybrid and in vitro systems [4–9,11–17]. The experiments to show the physical interaction of these proteins in intact cells have been little done. Here we have characterized the binding domains of Raf-1 for *ras* p21 and 14-3-3 protein using intact cells.

We have demonstrated that Raf(1–135) interacts with *ras* p21 and that neither Raf(1–60) nor Raf(61–322) interacts with *ras* p21. These results suggest that residues 1–135 of Raf-1 contain *ras* p21-binding domain and that residues 1–60 lack a *ras* p21-binding domain but may be necessary for the interaction of residues 61–135 with *ras* p21. These results are consistent with the observations in vitro that residues 52–64 are essential for the interaction of Raf-1 with *ras* p21 although this region does not represent a direct binding domain for *ras* p21 [13]. It is conceivable that residues 1–60 are necessary to keep the *ras* p21-binding domain of Raf-1 in a binding conformation. Our results using intact cells taken together with the previous observations [8,11–14] provide the basis of developing agents that may effectively inhibit the interaction of Raf-1 with *ras* p21 in vivo.

We have shown for the first time that 14-3-3 protein mainly interacts with the N-terminal domain of Raf-1 in intact cells. Since the N-terminal domain of Raf-1 is believed to be its regulatory domain, 14-3-3 protein may regulate Raf-1 activity. In fact, microinjection of 14-3-3 protein in *Xenopus* oocyte activates Raf-1 and overexpression of 14-3-3 protein in yeast stimulates the function of Raf-1 [15–17]. Therefore, 14-3-3 protein may be at least a cofactor involved in the activation of Raf-1 by binding to the N-terminal domain of Raf-1. Furthermore, we have demonstrated that Raf-1 simultaneously binds to both *ras* p21 and 14-3-3 protein and that the binding-domain of Raf-1 for *ras* p21 is distinct from the 14-3-3 protein-binding domain. It is known that *ras* p21 or 14-3-3 protein alone is not sufficient for Raf-1 activation [10,30]. Our results suggest that *ras* p21 and 14-3-3 protein may cooperate to activate Raf-1 by their binding to the specific domains of Raf-1. Although it is not clear how *ras* p21 and 14-3-3 protein activate Raf-1, one possibility is that 14-3-3 protein could act to contribute the translocation of Raf-1 from the cytosol to the plasma membrane where the active form of *ras* p21 binds to Raf-1. Our observations favor this model. However, it has been recently reported that 14-3-3 protein weakly binds to and directly activates the catalytic domain of Raf-1 whose the N-terminal domain is deleted [29]. Indeed, our results have shown that the C-terminal domain of Raf-1 weakly binds to 14-3-3 protein. It is intriguing to speculate that 14-3-3 protein may activate Raf-1 by two different ways. Further studies are necessary to understand the mode of activation of Raf-1 by *ras* p21 and 14-3-3 protein.

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