The 14-3-3 protein binds its target proteins with a common site located towards the C-terminus

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Received 26 June 1997; revised version received 10 July 1997

Abstract The 14-3-3 protein family binds a variety of proteins in cell-signaling pathways, but the structural elements necessary for the ligand binding are poorly understood. Here we demonstrate that the 'box-1' region, which spans residues 171–213 in the η-isoform and was previously identified as the binding site of 14-3-3 to the phosphorylated tryptophan hydroxylase, plays a critical role in the interaction with many target proteins. Using a series of truncated 14-3-3 mutants, we show that the mutant 167–213 carrying box-1 binds bacurovirus-expressed Raf-1 and Bcr protein kinases to the similar extent as the full-length 14-3-3 in a phosphorylation-dependent manner, while the mutants lacking this region abolish the binding activity. Furthermore, the box-1 region also appears essential for binding of 14-3-3 to more than 40 phosphoproteins found in the brainstem extract. These results suggest that the box-1 region, consisting of helices 7 and 8 in the tertiary structure, is a common structural element whereby the 14-3-3 protein binds many, if not all, target proteins.

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Key words: Signal transduction; Phosphorylation; 14-3-3 protein; Oncogene; Protein structure

1. Introduction

The 14-3-3 protein is a family of acidic, dimeric proteins with a relative molecular mass of ~60 kDa distributed widely among eukaryotic cells. This protein family binds a variety of proteins in intracellular signaling pathways and participates in the regulation of cell proliferation, differentiation, and functions (reviewed in [1] and [2]). Although the precise role of this family is not fully elucidated at a molecular level, its binding specificity is beginning to emerge from recent studies. It has been shown that the interaction of 14-3-3 with tyrosine (TH) and tryptophan hydroxylases (TPH) depends on the phosphorylation of these hydroxylases with calmodulin (CaM)-dependent protein kinase [3–5], and likewise, the complex of 14-3-3 with Raf-1 and with Bcr protein kinases dissociates by phosphatase treatment [6]. Furthermore, analysis of the primary site of phosphorylation in Raf has led to the identification of a 14-3-3 binding sequence, RSxS(*p*)xP, where S(*p*) is phosphoserine [7]. These results suggest that phosphorylation of the target protein at a single serine residue triggers 14-3-3 binding.

On the other hand, little is known about the region(s) of 14-3-3 responsible for target binding. We have previously shown, using a series of truncation mutants of the 14-3-3 η-isoform expressed in *Escherichia coli*, that the COOH-terminal region, especially restricted in residues 171–213 (termed box 1), is essential and sufficient for the association of 14-3-3 with phosphorylated TPH [8]. From this result, together with the general phosphorylation-dependent mechanism of the 14-3-3/ligand interaction, we assumed that box-1 might also be a site of interaction with other target proteins [8]. In this study we have examined this assumption and demonstrate that box-1 indeed acts as a common binding site of 14-3-3 for many target proteins including Raf-1, Bcr, and brainstem phosphoproteins.

2. Materials and methods

2.1. Plasmid constructions

The cDNAs for the full-length glutathione-*S*-transferase (GST)–14-3-3 η and its truncation forms were generated by a PCR, and ligated into the cloning site of the bacterial expression vector pGEX-3X (Pharmacia Biotech Inc.) as described [8]. The full-length cDNA for human Raf-1 [9] was synthesized by a PCR using oligonucleotides (5′-TGGATCCCAATGGAGCACATACAGG-3′ and 5′-AGAATTCCTAGAAGACAGGCAGC-3′). The PCR fragment was digested with *Bam*HI and *Eco*RI and the *Eco*RI site was blunted with Klenow. Then the PCR fragment was inserted into the *Bam*HI and *Hind*III sites (where the *Hind*III site was blunted) of bacurovirus expression vector pBlueBacHisC (Invitrogen). Bcr cDNA was made by cleaving the plasmid pGD′210 [10] with *Eco*52I and *Bgl*II followed by blunting both sides with Klenow. The cDNA fragment was cloned into the blunted *Bam*HI and *Hind*III sites of pBlueBacHisC. The resulting cDNA encodes the region of first exon Bcr kinase (amino acids 1–412) which contains the intrinsic 14-3-3 binding site [11].

2.2. Expression and purification of proteins

GST fusion proteins were expressed in *Escherichia coli* strain JM109 and purified on glutathione–Sepharose 4B beads (Pharmacia Biotech Inc.) as described [8]. Raf-1 and Bcr protein kinases were expressed in Sf21 insect cells and purified on Ni-NTA agarose beads (Qiagen) according to the procedure provided by the supplier.

2.3. Binding assay

Raf-1 or Bcr protein (~0.1 μg each) was incubated for 20 min at 30°C with appropriate amounts of GST–fusion proteins (see figure legends) in a buffer containing 25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 0.5 mM ATP (for Raf-1) or containing 20 mM HEPES, pH 7.4, 20 mM $MnCl_2$, and 0.5 mM ATP (for Bcr) in a final volume of 200 μl. Glutathione–Sepharose beads (~50 μl) that had previously been incubated in 5% (w/v) skim milk were then added to the mixture and incubated for 60 min at 4°C. The protein

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Abbreviations: TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase; CaM, calmodulin; GST, glutathione-*S*-transferase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; 2D, two-dimensional

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PII S0014-5793(97)00910-1

complexes bound to the beads were washed 3 times with 500 μl of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM dithiothreitol and solubilized in a SDS-sample buffer. The bound Raf-1 and Bcr were analyzed by SDS-PAGE followed by Western blotting using an antibody to each protein kinase (Santa Cruz Biotechnology). The binding assay was also performed with the rat brainstem extract. In this experiment, the extract previously fractionated with 25–55% saturated $(NH_4)_2SO_4$ (500 μg protein) was incubated at 30°C for 20 min in a buffer (200 μl) containing 50 mM HEPES, pH 7.6, 5 mM $Mg(CH_3COO)_2$, 0.1 mM $CaCl_2$, 0.05 mM ATP, 10 μCi [γ-^{32}P]ATP (Amersham Corp.), 4 μg of CaM [9] and 5 μg of GST–fusion proteins. After GST–fusion protein was recovered as described above, the γ-^{32}P-labeled proteins bound to the fusion protein were analyzed by autoradiography following 2D-PAGE [12].

2.4. Others

Phosphatase treatment was performed essentially according to the procedure described in [6]. Briefly, Raf-1 or Bcr protein (~0.1 μg each) was incubated for 20 min at 30°C with or without 3 U of type VII potato acid phosphatase (Sigma) in a buffer containing 40 mM PIPES, pH 6.0, 10 mM $MgCl_2$, and 10 mM $MnCl_2$ (for Raf-1) or in a buffer containing 40 mM PIPES, pH 6.0, and 20 mM $MnCl_2$ (for Bcr) in a final volume of 200 μl. The amounts of recombinant proteins were evaluated from the densitometric quantitation of the protein bands after SDS-PAGE and Coomassie Blue staining.

3. Results

A series of COOH-terminal truncation mutants of the 14-3-3 η-isoform expressed as the GST–fusion proteins (illustrated in Fig. 1A) were examined for their binding activities to Raf-1 and Bcr protein kinases. Raf-1 or Bcr was incubated with the truncated GST–fusion proteins and glutathione–Sepharose beads were added to the mixture to collect the fusion mutants. Raf-1 or Bcr bound to the mutants was then assayed by Western blot with a specific antibody to each protein kinase. This analysis revealed that the deletion mutants carrying the box-1 region (mutants 1–237 and 1–213) bound these protein kinases, while the mutants lacking box-1 (mutants 1–78 and 1–170) did not (Fig. 1B). This suggests that box-1 is involved in the Raf/Bcr binding.

To confirm the above observation, similar experiments were performed using two additional mutants (167–213 carrying box-1 and Δ171–213 lacking box-1; see Fig. 1A). This analysis proved that box-1 is the primary site for interaction of the η-isoform with Raf-1 and Bcr, because the mutant 167–213 bound these proteins to the similar extent to the full-length η-protein (compare Fig. 2A, lanes 1 and 3) and the deletion mutant lacking 171–213 (Δ box-1) bound neither Raf-1 nor Bcr (lane 2).

Since the complex formation between 14-3-3 and Raf-1 and between 14-3-3 and Bcr had been shown to be prevented by the treatment of these kinases with protein phosphatase [6], we examined whether the mutant 167–213 binds the phosphatase-treated Raf-1 and Bcr. As shown in Fig. 2B, the binding of Raf-1 or Bcr to the η-protein (lanes 1 and 2) as well as to the mutant 167–213 (lanes 3 and 4) was apparently inhibited by the phosphatase treatment. After phosphatase treatment, both Raf-1 and Bcr exhibited slight mobility shifts on SDS-PAGE (see Fig. 2B, lower panel), probably due to the removal of phosphate from the bacurovirus-expressed proteins. Thus, like the full-length 14-3-3 proteins, interaction between box-1 and the target proteins is phosphorylation-dependent.

Although the η-protein was identified to be an isoform that bound preferentially Raf in 293T cells [13], other isoforms such as β and ζ also bind Raf in vivo [14] and in vitro [7]. We have prepared the mutants with and without box-1 on the basis of the β sequence and demonstrated that this isoform also requires the box-1 (166–208 in the β sequence) for Raf binding (Ichimura et al., unpublished results). Likewise, the mutants carrying box-1 (residues 166–208) of the τ-isoform, a rat counterpart of mouse Bcr-binding protein Bap-1 [11], bound Bcr, while the mutants lacking box-1 did not.

To further evaluate the box-1 function in the 14-3-3/ligand interaction, we performed the following experiment. A crude extract was prepared from the rat brainstem, and to the extract was added the full-length η-protein or the mutant Δbox-1 fused to GST. After the mixture was incubated in the presence of Ca^{2+}, CaM and [γ-^{32}P]ATP, the GST–protein was precipitated with glutathione–Sepharose and the co-precipitated γ-^{32}P-labeled proteins were analyzed by autoradiography following 2D-PAGE (Fig. 3). From the extract supplemented with the full-length η-protein more than 40 phosphoproteins were co-precipitated, while a few were detected with the mutant Δbox-1 (Fig. 3, compare A and B). In addition, most of the phosphoproteins co-precipitated with the mutant Δbox-1

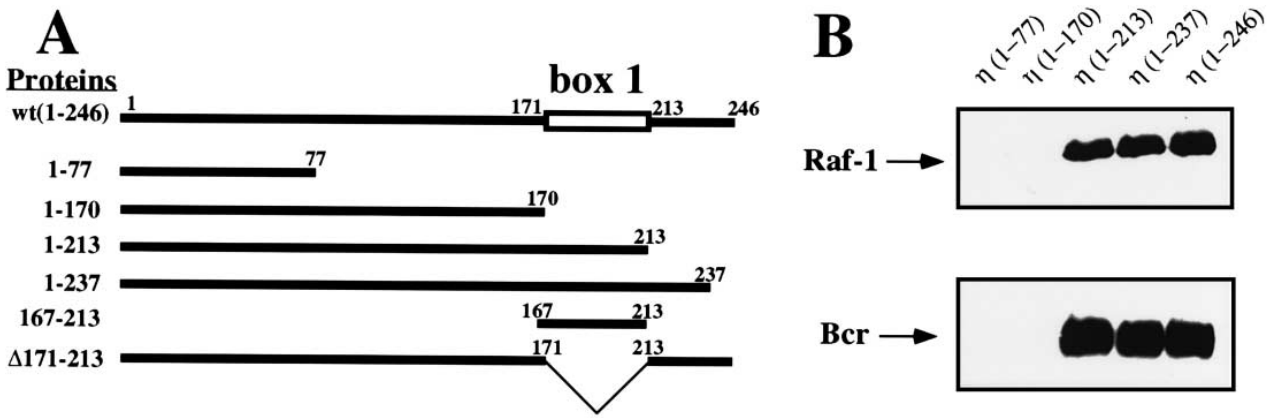

Fig. 1. Deletion analysis of the functional region of 14-3-3 η. A: Schematic illustration of wild-type and mutant forms of 14-3-3 η. For simplicity, only 14-3-3 regions of the fusion proteins are shown. For wild-type 14-3-3, the box-1 region is indicated by a hatched box. For mutants, dotted lines are used to depict the regions in the η sequence, where the numbers indicate the residue numbers of NH_2 and COOH termini of each mutant. B: Binding of the deletion mutants to Raf-1 and Bcr. Raf-1 or Bcr (~0.1 μg each) was incubated with 25 pmol of each protein and processed as described in Section 2. Raf-1 or Bcr bound to the fusion protein was detected by the immunoperoxidase-ECL (Amersham Corp.).

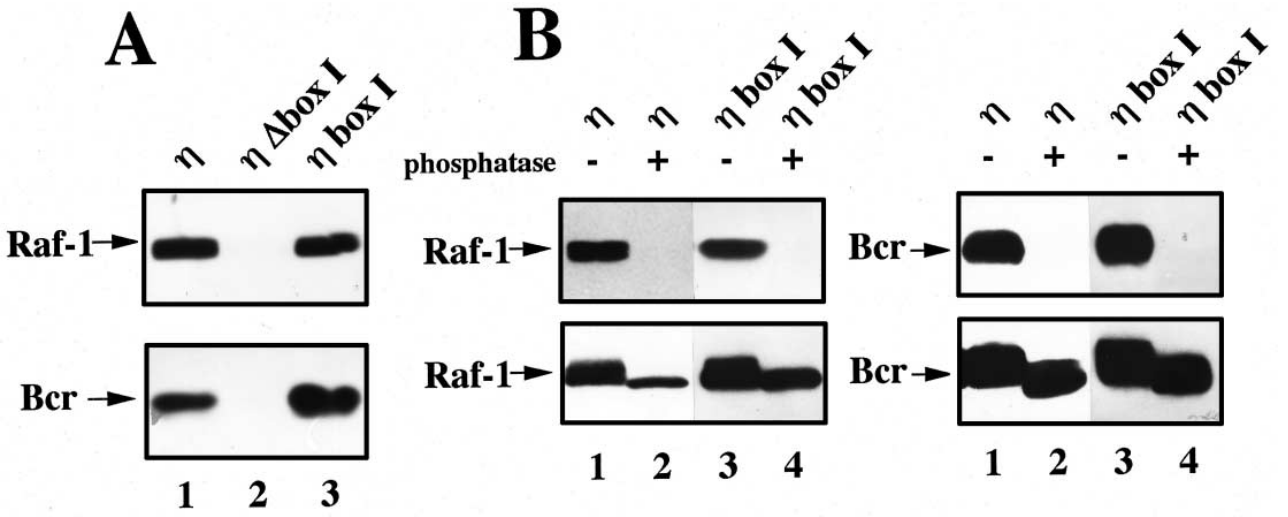

Fig. 2. A: Binding of mutants 167–213 (vboxI) and Δ171–213 (vΔboxI) to Raf-1 and Bcr. Raf-1 or Bcr (~0.1 μg each) was incubated with 40 pmol of each protein and analyzed as in Fig. 1B. B: Effect of phosphatase treatment. Raf-1 or Bcr (~0.1 μg each) was incubated with (+) or without (−) potato acid phosphatase for 20 min and examined for binding to the full-length η or to the mutant 167–213 (vboxI) (upper panel). After phosphatase treatment, an aliquot of the reaction mixture was examined by Western blotting (lower panel) to exclude the possibility of proteolytic cleavage during the reaction.

appeared to result from non-specific binding to the GST moiety of the fusion protein (data not shown). Although most of the phosphoproteins associated with the GST–η-protein were not identified in the present study, we detected TPH as one of those proteins by Western blotting (indicated by an arrow in Fig. 3). Therefore, the box-1 region appears to be a site of interaction in 14-3-3 for a variety of proteins including TPH, Raf-1, Bcr, TH (Ichimura et al., unpublished results), and many potential target proteins in the brainstem extract.

4. Discussion

In this study we have shown that box-1 represents a common structural element of the 14-3-3 protein for its ligand binding. This region consists of helices 7 and 8 located near the edge of the groove-like tertiary structure, and has an amino acid sequence which is highly conservative among the members of the 14-3-3 family found in yeast to man [8]. This may explain why several mammalian 14-3-3 isoforms bind in vitro to human Raf-1 and Bcr protein kinases ([8,11], and data not shown) and the yeast 14-3-3 proteins activate the human Raf-1 kinase [9]. Recent findings, however, show that some isoforms of this family have different affinities to several target proteins, suggesting an isoform-specific ligand binding. For example, A20, an inhibitor of tumor necrosis factor-induced apoptosis, was bound preferentially to

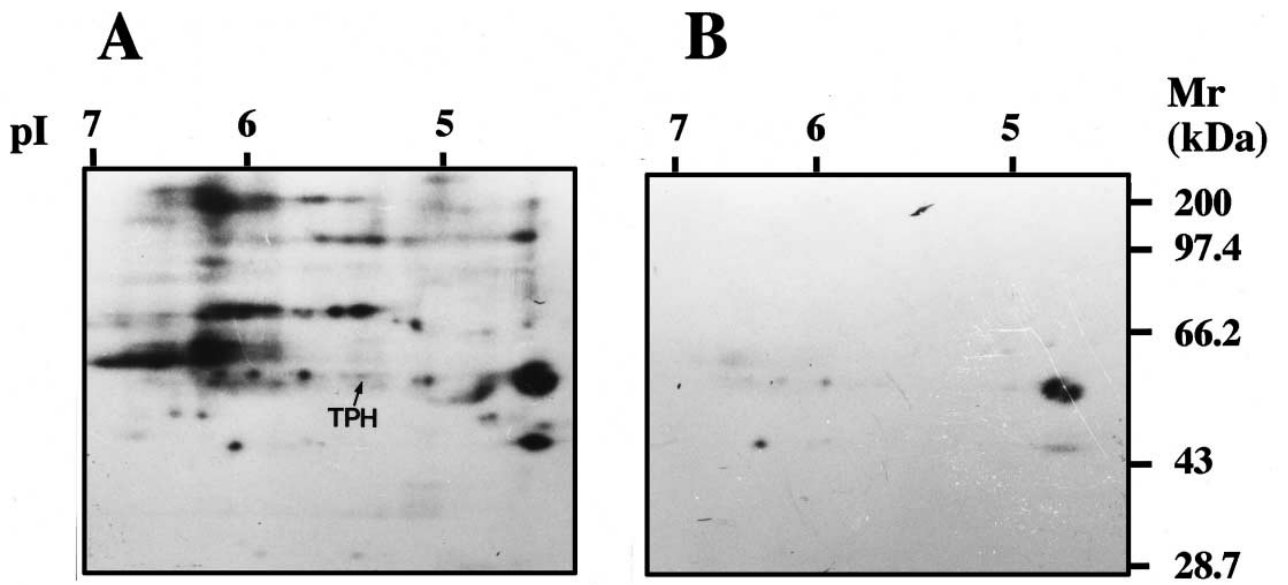

Fig. 3. Two-dimensional analysis of brainstem phosphoproteins bound to the full-length 14-3-3 (A) and the mutant Δ171–213 (B) fused to GST. The rat brainstem extract (500 μg protein) supplemented with 5 μg of each protein was phosphorylated as described in Section 2, and the phosphorylated γ-^{32}P-labeled proteins bound to each fusion protein were analyzed by autoradiography after 2D-PAGE. TPH (indicated by an arrow) was identified by Western blotting with an antibody to TPH [8].

the endogenous η-isoform when A20 was transfected into 293T cells [13]. The β- and ζ-isoforms bound A20 much weakly, and the ε-isoform had an intermediate affinity. It has also been shown that the η-isoform binds preferentially Raf-1 in 293T cells [13]. It is possible, therefore, that the variation in a few amino acids within the structure of box-1 or an additional region to box-1 may determine the isoform specificity in the 14-3-3/ligand interaction. However, we were not able to detect the isoform-specific interaction in the present assay performed.

Recently, a consensus sequence motif, RSxS(*p*)xP, has been proposed for the sequence that binds 14-3-3 [7]. In fact, the synthetic peptide containing this motif binds 14-3-3 in a phosphoserine-dependent manner, disrupts the 14-3-3/Raf complex, and inhibits the maturation of *Xenopus laevis* oocyte. It has also been shown that this motif is found in common with many 14-3-3 target proteins including Bcr, two apoptosis-related proteins A20 [13] and Bad [15], and plant nitrate reductase [16]. The present study demonstrates that box-1 is a site of interaction with Raf/Bcr kinases and that the interaction is dependent on phosphorylation (Fig. 2). However, whether the box-1 structure directly recognizes the binding motif remains to be established. Further studies are also needed to specify residues, within box-1, which are directly involved in the ligand binding. Such a study will facilitate the analysis of the 14-3-3/ligand interaction at a tertiary structure level and aid studies of the 14-3-3 function, for instance by assisting a design of drugs that modulate 14-3-3-mediated intracellular signaling.

Acknowledgements: We thank Dr. K. Matsumoto (Nagoya University, Nagoya) for Raf-1 cDNA and Dr. M.L. Scott (Whitehead Institute, Cambridge) for Bcr cDNA. This work was supported in part by Grant (08458254 to T. Isobe) from the Ministry of Education, Science, Sports and Culture of Japan.

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