Identification of the Site of Interaction of the 14-3-3 Protein with Phosphorylated Tryptophan Hydroxylase*

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The 14-3-3 protein family plays a role in a wide variety of cell signaling processes including monoamine synthesis, exocytosis, and cell cycle regulation, but the structural requirements for the activity of this protein family are not known. We have previously shown that the 14-3-3 protein binds with and activates phosphorylated tryptophan hydroxylase (TPH, the rate-limiting enzyme in neurotransmitter synthesis, Ca2+-dependent stimulation of noradrenaline secretion, and the regulation of the activity of Ca2+-phospholipid-dependent protein kinase C (2)). Recent findings, particularly in fission yeast, have shown that the 14-3-3 family is associated with the products of proto-oncogenes and oncogenes such as Raf-1, Bcr, Bcr-Abl, and Polyomavirus middle tumor antigen, suggesting that this family of proteins is also involved in cell transformation and mitogenic signaling pathways (for a review, see Ref. 3). We have previously shown that the 14-3-3 protein activates TPH in concert with phosphorylation of the hydroxylase by CaM Kinase II (4, 5) and demonstrated that this activation results from the binding of 14-3-3 protein to the phosphorylated hydroxylase (6). From these results, together with the structural features of the 14-3-3 protein (4), we proposed that the acidic COOH-terminal region of the 14-3-3 protein might be involved in the interaction with phosphorylated TPH. However, little evidence has been provided in this and other systems on the structural requirements of the 14-3-3 protein for its biological activities.

In this study, we used the TPH system as a model to assess the functional region of the 14-3-3 protein and showed that the acidic COOH-terminal region, especially restricted in residues 171–213, is a primary site for the interaction of 14-3-3 with phosphorylated TPH.

EXPERIMENTAL PROCEDURES

Materials—TPH was purified from rat brainstem by pteridine affinity chromatography as described (7). CaM and CaM kinase II were purified from bovine brain (6). Anti-TPH antibody was kindly provided by Dr. H. Hasegawa (The Nishi-Tokyo University). The plasmid pGEX-3X and glutathione-agarose beads were purchased from Pharmacia Biotech Inc. Oligonucleotides were obtained from Biotech International. The oligonucleotides were as follows: n1, 5′-GGGATCCGCAT-GGGAGGACCCGACAGCTGCTG-3′; n167, 5′-AGGATCCACAC-ACCCCATCAGG-3′; n214, 5′-ACCCGGGATCTTAAAGGACTC-3′; c77, 5′-GGAATTCTCCGTTAGTGTCCAG-3′; c190, 5′-TGATATCTCTTCTTCTATCCATC-3′; c213, 5′-GGGATCCGCCGTCATTTCTTCTATCCATC-3′; c246, 5′-GGAATTCTCTTGACCCGGTGCAGGACGACAAGACG-3′; c237, 5′-GGATCTCCTGCTGCTGAGAGC-3′; c246, 5′-GGAATTCTTATCATGTCCTGCTGAGAGC-3′.

Construction of Expression Plasmids—The cDNAs for the full-length GST-14-3-3 protein were completely amplified by polymerase chain reaction using the synthetic oligonucleotides (n1–c246 for pG14a; n1–c190 for pG14b; n1–c170 for pG14c; n1–c237 for pG14d; n1–c213 for pG14e; n1–c167 for pG14f; n167–c246 for pG14g; n167–c213 for pG14h; see Fig. 2A) and inserted into the cloning site of the expression vector pGEX-3X. The resulting products were digested with BamHI and EcoRI and cloned into the cloning site of the expression vector pGEX-3X. The plasmid pG14a (see Fig. 2A) was made by polymerase chain reaction using the oligonucleotides n214–c246. The resulting product was digested with Smal and EcoRI and cloned into the SmaI/EcoRI site of pG14b.

Expression and Purification of GST Fusion Proteins—Cultures of Escherichia coli strain JM109, transformed by 14-3-3 plasmids, were grown and induced with isopropyl-1-thio-β-D-galactopyranoside for expression as described (8). The bacteria were collected by centrifugation and resuspended in buffer A (20 mM Tris-HCl, 150 mM NaCl, 0.1 mM dithiothreitol, pH 7.5). Vigorous sonication was performed before centrifugation at 12,000 × g for 20 min. The resulting supernatant was loaded onto a column (1.4 × 3 cm) packed with 3 ml of cross-linked cyanogen bromide-agarose.
glutathione resin. The column was washed extensively with buffer A, and bound GST-fused proteins were eluted with buffer A containing 50 mM glutathione. To remove glutathione, the eluate was further applied to a DEAE-SPW column (0.75 × 10 cm, Tonen) that had been pre-equilibrated with 20 mM Tris/HCl, 50 mM NaCl, pH 7.5. The eluted proteins were stored at −80 °C until use.

Phosphorylation and Binding Assay—Phosphorylation of TPH (−1 μg) was carried out at 30 °C for 20 min in a reaction mixture (50 mM Hepes, pH 7.6, 5 mM Mg(CH₃COO)₂, 0.1 mM CaCl₂, 0.5 mM ATP, 1 μM CaM kinase II, and various amounts of GST-fused protein (see figure legends)) in a final volume of 200 μl. The control experiment was performed under the same conditions described above except that CaM kinase II was removed from the reaction mixture. For phosphorylation of rat brainstem extract (25–55% saturated (NH₄)₂SO₄ fraction), the extract (500 μg) was incubated in a buffer (200 μl) containing 50 mM Hepes, pH 7.6, 5 mM Mg(CH₃COO)₂, 0.1 mM CaCl₂, 0.5 mM ATP, 4 μg of GST, and 5 μg of GST-fused protein at 30 °C for 20 min. Control experiments were performed without ATP. For binding assay, glutathione-agarose beads (−50 μl) were added to the reaction mixture and incubated for 30 min at 4 °C, and the protein complexes bound to the beads were washed three times with buffer A and solubilized in SDS sample buffer. The bound TPH was analyzed by SDS-PAGE followed by Western blotting using a TPH antibody. In some experiments, bound TPH was measured with its enzymatic activity using the beads or by Western blot with TPH antibody (inset). The cross-reacting band was visualized by horseradish peroxidase-conjugated goat antibodies against rabbit IgG and ECL reagent (Amersham Corp.). The arrowhead indicates the position of TPH. C, formation of a complex between the recombinant 14-3-3 protein and the phosphorylated TPH in the brainstem extract. The rat brainstem extract (500 μg) was incubated under nonphosphorylating (lanes 1 and 3) or phosphorylating (lanes 2 and 4) conditions in the presence of GST (5 μg, lanes 1 and 2) or GST-fused η protein (5 μg, lanes 3 and 4) and was then analyzed as in B. ND, not detected.

RESULTS AND DISCUSSION

We used the bacterial expression system that produces proteins in E. coli as fusions with an affinity tag, GST, to prepare recombinant proteins, because this system permits affinity purification of active proteins. Bovine brain 14-3-3 η isoform was expressed in this system, and the GST-fused η protein was purified by affinity chromatography on cross-linked glutathione resin. The purified protein showed a single protein band on SDS gel with an expected molecular mass of ∼55 kDa (see Fig. 2B, lane 1), and this protein cross-reacted with polyclonal antibodies to bovine brain 14-3-3 protein (data not shown).

Before truncation of the recombinant protein, we examined, using TPH system, whether the GST-fused η protein produced in E. coli indeed shares similar properties with bovine brain η isoform (Fig. 1). The fused η protein added to the TPH assay mixture stimulated the activity of TPH about 2-fold more than the level of TPH measured in the absence of the fused protein (Fig. 1A). This effect was dose-dependent, and the concentration of the η protein necessary for half-maximal activation (V₅₀/2) of TPH was about 20 nM. These values were almost equal to the published values with bovine brain 14-3-3 η (4, 5).

In addition, no stimulation of TPH activity was observed, as analyzed in the absence of CaM kinase II or in the presence of GST alone (Fig. 1A, dotted lines), confirming the previous data (4, 5) that this activation of TPH needs both phosphorylation of TPH and the 14-3-3 protein.

We next examined whether the fused η protein can interact with TPH in a phosphorylation-dependent manner. TPH was incubated with the fused protein under its phosphorylating or nonphosphorylating conditions (see “Experimental Procedures”), and glutathione-agarose beads that bind with the fused protein were added to the mixtures. Bound TPH with the fused η protein immobilized on the beads was then assayed by its enzymatic activity and by Western blot with a TPH antibody. As illustrated in Fig. 1B (lanes 3 and 4), TPH bound with the fused η protein only under its phosphorylating condition. Incorporation of phosphate to TPH under this condition was confirmed using [γ⁻³²P]ATP (data not shown, see Ref. 6), suggesting that, like the bovine brain protein (6), the recombinant protein binds with TPH in a phosphorylation-dependent manner.

We also performed similar experiments using crude brainstem extract supplemented with the recombinant η protein (Fig. 1C). As shown in Fig. 1C, lane 4, TPH present in the brainstem extract also bound to the added η protein, indicating that this binding occurs in the crude extract. Again, no interaction of TPH to the η protein was detected under nonphosphorylating conditions (lane 3). In both experiments, GST alone did not bind to TPH (Fig. 1, B and C, lanes 1 and 2). All these properties of the expressed fusion protein were same as the reported characteristics of brain 14-3-3 protein. Furthermore, like the bovine brain protein (9, 10), the expressed protein...
activated protein kinase C. Thus, we concluded that the expression system used in this study can produce the protein that is suitable for analysis of the functional region of the 14-3-3 protein.

A series of truncation mutants were made (Fig. 2A), and the expressed proteins were purified with the same procedure described above. As judged by SDS-PAGE (Fig. 2B), the mutants were almost pure with a major band of the expected sizes. The truncated GST-fused proteins were then examined in a similar way to that described in the legend of Fig. 1B for their ability to bind with phosphorlated TPH (see also the legend to Fig. 3). This analysis confirmed the importance of the COOH-terminal acidic region of the 14-3-3 protein (residues 170–246) for the interaction with TPH, because the deletion mutants lacking this region (mutants 1–170 and 170–213, Fig. 2A) were found no longer bound with TPH (Fig. 3). These mutants were analyzed in terms of TPH binding. This analysis revealed that the two mutants, 167–246 and 167–213, bound to TPH to a similar extent (Fig. 3B, lanes 1 and 2), but the mutant 171–213 did not (lane 3). These results proved the above assumption and provided direct evidence that the structural region consisting of residues 171–213, which we termed 14-3-3 box I, is a primary site for the interaction of the 14-3-3 protein to the phosphorlated TPH. We also observed that all of the mutants which lacked the box I and failed to bind phosphorylated TPH, such as the mutants 1–170 and 171–213, had no activities toward TPH even with the excess amount over TPH. This suggests that the box I structure is also essential for the activity of the 14-3-3 protein.

The amino acid sequence of the 14-3-3 box I is one of the highly conservative sequences extended among the members of the 14-3-3 family. Fig. 4 displays the alignment of corresponding sequences of the 14-3-3 box I from bovine brain 14-3-3 isoforms with a known sequence as well as the plant and yeast counterparts, GF14 and BMH1, respectively. The sequence similarity suggests that the 14-3-3 box I represents a common structural element, which may be involved in the association of these 14-3-3 isoforms to TPH. Consistent with this assumption, the previous data have shown that the rat brain TPH could be activated by all these bovine isoforms as well as by the plant GF14 (5, 11), although at present whether the yeast BMH1 protein may be necessary for the interaction with the phosphorlated TPH.

To test this assumption, deletions were made to produce three additional mutants, the mutant 167–246 (carring the complete COOH-terminal region), the mutant 167–213 (carring the 171–213 region), and the mutant lacking the 171–213 region (Δ171–213, Fig. 2A), and these mutants were analyzed in terms of TPH binding. This analysis revealed that the two mutants, 167–246 and 167–213, bound to TPH to a similar extent (Fig. 3B, lanes 1 and 2), but the mutant Δ171–213 did not (lane 3). These results proved the above assumption and provided direct evidence that the structural region consisting of residues 171–213, which we termed 14-3-3 box I, is a primary site for the interaction of the 14-3-3 protein to the phosphorlated TPH. We also observed that all of the mutants which lacked the box I and failed to bind phosphorylated TPH, such as the mutants 1–170 and Δ171–213, had no activities toward TPH even with the excess amount over TPH. This suggests that the box I structure is also essential for the activity of the 14-3-3 protein. Our results, however, cannot exclude the possibility that an additional region(s) to the box I participates in the interaction with TPH because the truncation of the box I might induce conformational changes in another part of the molecule and thereby prevent the mutants lacking the box I from the interaction with the hydroxylase.

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protein activates TPH is not known. We also note that the 14-3-3 box I includes Ser184, which has been reported to be the site of phosphorylation in the β and γ 14-3-3 isoforms by proline-directed protein kinase (12).

Recently, the crystal structures of the homodimeric proteins of the 14-3-3 γ (13) and τ (14) isoforms have been reported. These proteins have a similar tertiary fold consisting of a bundle of nine anti-parallel α-helices of each monomer, and the dimers form a large negatively charged channel or groove. Both reports have suggested that this groove may represent the ligand binding surface of the 14-3-3 molecules. In the tertiary structure, the 14-3-3 box I represents helices 7 and 8 and a part of the linker between helices 8 and 9, which are located near the edge of the groove. In this viewpoint, our results are consistent with the proposal from the crystal structure studies and further emphasize the important role of the COOH-terminal structure including helices 7 and 8 in the ligand/14-3-3 protein interaction.

Increasing evidence suggests that the phosphorylation may be a common mechanism that regulates the binding of 14-3-3 to its target molecules. For example, the interaction of 14-3-3 with Raf-1 and Bcr protein kinases was ultimately prevented by the treatment of these kinases with protein phosphatase (15). It has also been shown that the 14-3-3 γ reduces the dephosphorylation of Raf-1 with protein phosphatases through the interaction with Raf-1 and blocks the dephosphorylation-induced enzymatic inactivation of Raf-1 (16). The facts that the mode of interaction between these protein kinases and 14-3-3 is similar to the TPH/14-3-3 interaction described here and that the 14-3-3 box I is a highly conserved structure from yeast to mammals (Fig. 4) imply that the box I structure may serve as a common binding site for many target proteins including these protein kinases, but this awaits further investigation.

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