Two Regions Responsible for the Actin Binding of p57, a Mammalian Coronin Family Actin-Binding Protein

Teruaki Oku, a Saotomo Itoh,b Masamitsu Okano, a Akiko Suzuki,a Kensuke Suzuki,b Shizuo Nakajin,a Tsutomu Tsujia,b,d William Michael Nauseef,c and Satoshi Toyoshimad

a Hoshi University School of Pharmacy and Pharmaceutical Sciences; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan; b Pharmaceutical Frontier Research Laboratories, Japan Tobacco, Inc.; 1–13–2 Fukura, Kanazawa-ku, Yokohama 236–0004, Japan; c The Inflammation Program and Department of Medicine, University of Iowa and Veterans Affairs Medical Center; Iowa City, Iowa 52242, U.S.A.; and d Pharmaceuticals and Medical Devices Evaluation Center, National Institute of Health Science; 3–8–21 Toranomon, Minato-ku, Tokyo 105–8409, Japan.

Received August 22, 2002; accepted December 17, 2002

The actin-binding protein p57, a member of the coronin protein family, is expressed in a variety of immune cells. It has five WD repeats and a coiled-coil motif containing a leucine zipper, both of which are known to mediate protein–protein interactions. In order to identify the precise actin-binding regions in p57, and to assess the contribution of these structural motifs, we prepared various truncated p57 as fusion proteins with glutathione S-transferase (GST) and examined their actin-binding activity. A co-sedimentation assay demonstrated that p57(1–371) (C-terminal truncated p57) had the ability to bind F-actin, but p57(372–461) (a fragment containing the coiled-coil motif) did not. A segment consisting of the N-terminal 34 amino acids of p57 (p57(1–34)) was found to bind to F-actin in the co-sedimentation assay. Furthermore, fluorescence microscopic observation showed that p57(1–34) was co-localized with F-actin in COS-1 cells after the transfection with the p57(1–34) construct. Deletion of 10KFRHVF15, a sequence conserved among coronin-related proteins, from p57(1–34) abolished its actin-binding activity, suggesting that this sequence with basic and hydrophobic amino acids is crucial for p57 to bind to F-actin. However, the N-terminal deletion mutant p57(35–461) retained the binding ability to F-actin. This result suggests the presence of a second actin-binding region. Further deletion analysis revealed that p57(111–204), which includes the second and third WD repeats, also exhibited weak actin-binding activity in the co-sedimentation assay. Taken together, these data strongly suggest that at least two regions within Met-1 to Asp-34 and Ile-111 to Glu-204 of p57 are responsible for its binding to the actin cytoskeleton.

Key words actin-binding protein; coronin; cytoskeleton; WD repeats; leucine zipper

As the primary effector cells in the innate immune system, phagocytes such as neutrophils and macrophages contribute to host defense by engulfing and destroying invading microbes. Various actin-binding proteins have been implicated in the regulation of actin–cytoskeleton interactions, essential for properties integral to phagocytic cell function, including chemotaxis, phagocytosis, and degranulation. As described previously, we have identified an immune cell-specific actin-binding protein p57 that has significant homology with coronin, an actin-binding protein of Dictyostelium discoideum. Coronin plays crucial roles in various cell functions, including cell locomotion, phagocytosis, and cytokinesis of Dictyostelium. Homologous proteins to coronin have been identified in many eukaryotes from yeast to human, and are assigned an important role in cell motility. Recent reports have suggested that p57 participates as well in the formation of phagosome and its maturation in neutrophils and macrophages.

Most coronin protein family members, including p57, consist of 450–500 amino acid residues and share common structural features, five WD (tryptophan-aspartic acid) repeats located at the center of the molecule and a coiled-coil motif containing a leucine zipper at the C-terminus. These motifs are known to mediate protein–protein interactions, but their specific function in the coronin family of actin-binding proteins has not been elucidated. Since coronin has no significant homology with other actin-binding proteins, the region(s) responsible for the binding to actin remains undetermined. Recently, two groups have reported putative actin binding regions in coronin family members of two distinct species. In Crn1p, a coronin in budding yeast, the N-terminal part containing WD repeats, has been shown to have actin-binding activity. In Xcoronin, a coronin homologue in Xenopus oocytes, the deletion of N-terminal 63 amino acids or of C-terminal 65 amino acids reduced its actin-binding activity, although the WD repeats themselves were not directly involved in the binding to actin. Thus, there is an argument concerning the region(s) responsible for actin binding of the coronin family of proteins.

To identify the region(s) responsible for actin binding of p57, we prepared various mutants of p57 in E. coli and assessed their ability to bind to actin by two approaches. We evaluated their abilities to co-sediment with F-actin after actin polymerization, and to co-localize with actin filaments in cells after transfection with the plasmid constructs for the mutant. The results obtained suggest that there are at least two regions responsible for actin binding of p57.

MATERIALS AND METHODS

Expression of Glutathione S-Transferase (GST) Fusion Proteins of Full-Length, and Deletion Mutants of p57

Full-length and partial coding sequences of p57 were expressed in E. coli as GST fusion proteins using the vector pGEX-5X-1 (Pharmacia, Uppsala, Sweden). Expression plasmids pGEX-p57 (full-length), pGEX-p57(1–371) (Met-1 to Asp-371), and pGEX-p57(372–461) (Pro-372 to Lys-461) (Fig. 1) were prepared by restriction endonuclease digestion and
The expression plasmids of pGEX-p571—71 (Met-1 to Asp-71) and pGEX-p577—34 (Met-1 to Thr-34) (Fig. 1), were generated from pGEX-p571—34 (Met-1 to Thr-34) and pGEX-p571—371 by introducing stop codons. The primers used were: E300Z, sense primer 5'-GAT CCG TGG CTG TCC T-3' and antisense primer 5'-GGT ACT TTG AGA TCA CTT CCT AGG AAG TGA-3'. Deletion of amino acid residues 10 to 15 from p571—15 and anti-sense primer 5'-C CTT GGC CGG CTG GCC GAG CAC C-3'.

The expression plasmids of pGEX-p571—371 (Met-1 to Asp-71) and pGEX-p577—34 (Met-1 to Thr-34) (Fig. 1), were generated from pGEX-p571—34 (Met-1 to Thr-34) and pGEX-p571—371 by introducing stop codons. The primers used were: E300Z, sense primer 5'-GAT CCG TGG CTG TCC T-3' and antisense primer 5'-GGT ACT TTG AGA TCA CTT CCT AGG AAG TGA-3'. Deletion of amino acid residues 10 to 15 from p571—15 and anti-sense primer 5'-C CTT GGC CGG CTG GCC GAG CAC C-3'.

The upper panel indicates the schematic representation of p57. Hatched boxes indicate WD repeats and a shaded box indicates a coiled-coil motif.

Fig. 1. Structures of p57 and its Deletions
were induced by overnight culture of the bacteria with 0.5 mM isopropylthio-β-d-galactoside. After the culture, the bacteria were pelleted, suspended in a sonication buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA), and incubated with 1.0 mg/ml of lysozyme for 20 min on ice. The suspensions were sonicated and centrifuged at 10000×g for 30 min. Then, the supernatants were incubated with 0.25 ml of glutathione-Sepharose beads (Pharmacia) overnight at 4 °C. The beads were washed three times with a sonication buffer. The bound GST fusion proteins were eluted with 50 mM reduced glutathione in 50 mM Tris–HCl buffer. The beads were washed three times with a sonication buffer. The bound GST fusion proteins were eluted with 50 mM reduced glutathione in 50 mM Tris–HCl buffer, pH 8.0.

**Actin Co-sedimentation Assay** An actin co-sedimentation assay was performed to investigate the ability of fusion proteins to bind actin. Each fusion protein, with or without G-actin (30 µg, Sigma, St. Louis, MO, U.S.A.) was incubated in an F-actin buffer (20 mM Tris–HCl, pH 8.0, 160 mM KCl, and 0.2 mM ATP) for 1.5 h at 25 °C. After the incubation, the reaction mixtures were ultracentrifuged at 198000×g for 1.5 h at 4 °C. The reaction mixture before ultracentrifugation is referred to as the total reaction mixture in the text. The supernatants, pellets, and total reaction mixtures were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (10% gel), and gels were stained with Coomassie brilliant blue (CBB). To confirm the presence of fusion proteins, immunoblot analysis using anti-p57 polyclonal antibody was performed. Immunoblot analysis using anti-p57 polyclonal antibody was also performed.

**Expression Plasmids of p57 and Its Deletions** To construct plasmids for the expression of p57 in mammalian cells, the DNA fragment encoding full-length p57 was amplified by PCR. The primers used were (restriction enzyme sites are underlined): 5′-GGG GAA TTC AGT CCA GGC CAA GGC GAG CCC CAA GCT TGA ATT CTG C-3′ and 5′-GCA GAA TTC AAG CTT GGG GCT CTA CTT GGC CTG G-3′. The amplified DNA fragments were digested with EcoRI and cloned into pEF1/Myc-His A (Invitrogen, Carlsbad, CA, U.S.A.). Then, TAG stop codons were replaced by GCG to generate a fusion protein with Myc-His tag using a QuikChange Site-directed mutagenesis kit (Stratagene). The primers used were: 5′-GGG GAA TTC AGT CCA GGC CAA GGC GAG CCC CAA GCT TGA ATT CTG C-3′ and 5′-GCA GAA TTC AAG CTT GGG GCT CTA CTT GGC CTG G-3′. The amplified DNA fragments were digested with EcoRI and cloned into pEF1/Myc-His A (Invitrogen, Carlsbad, CA, U.S.A.). Then, TAG stop codons were replaced by GCG to generate a fusion protein with Myc-His tag using a QuikChange Site-directed mutagenesis kit (Stratagene). The primers used were: 5′-GGG GAA TTC AGT CCA GGC CAA GGC GAG CCC CAA GCT TGA ATT CTG C-3′ and 5′-GCA GAA TTC AAG CTT GGG GCT CTA CTT GGC CTG G-3′. The amplified DNA fragments were digested with EcoRI and cloned into pEF1/Myc-His A (Invitrogen, Carlsbad, CA, U.S.A.).

For expression of p57111—204, the DNA fragments were subcloned into pCDNA3.1/V5-His A (Invitrogen). For expression of p571—371 and p57372—461, the corresponding DNA fragments were subcloned into pCDNA3.1/V5-His A (Invitrogen). For expression of p57111—204, the DNA fragments were subcloned into pCDNA3.1/V5-His A (Invitrogen).

**Identification of Region(s) in p57 Responsible for Actin Binding** To identify the region(s) of p57 responsible for actin binding, we examined the abilities of GST-fusion proteins with full-length and several truncated forms of p57 to co-sediment with F-actin in vitro. GST-p57 (a fusion protein of full-length p57 with GST) and GST-p57111—34 (a fusion protein containing five WD repeats but lacking a C-terminal coiled-coil motif) (Fig. 1) co-precipitated with F-actin (Fig. 2A, B). Although some impurities were present in the GST-p57 preparation, the identity of GST-p57 was confirmed by immunoblotting using an anti-p57 antibody, and the impurities remained in the supernatant after ultracentrifugation. In contrast, GST-p57372—461 (a fusion protein containing a C-terminal fragment with a coiled-coil motif) (Fig. 1) did not co-sediment with F-actin (Fig. 2C). These results suggested that the region responsible for p57 binding to actin was located in the N-terminal part of p57 that includes WD repeats, but does not include the coiled-coil motif at the C-terminus. We then prepared two additional truncated proteins containing the N-terminus of p57 but lacking the WD repeats, GST-p571—71 and GST-p57111—204 (Fig. 1). These deletion mutants were grown in Iscove’s Modified Dulbecco’s Medium (IMDM, GIBCO) supplemented with 10% heat inactivated FCS (Sanko Junyaku Co., Ltd., Tokyo), penicillin (50 U/ml), and streptomycin (50 µg/ml) under the standard cell culture condition (37 °C, humidified 5% CO2 in air).
co-precipitated with F-actin (Figs. 3B, C), suggesting that the N-terminal 34 amino acids of p57 possessed the capacity to bind F-actin. Moreover, the deletion of six amino acid residues, \(10^{10}KFRHV15\), from p57\(^{1-34}\) (GST-p57\(^{1-34}\)) abolished the actin-binding ability (Fig. 3D), implicating a sequence rich in basic and hydrophobic amino acids as essential for actin binding of p57\(^{1-34}\).

**Further Analysis of Region(s) in p57 Responsible for Actin Binding**

To evaluate the role of the central region of p57 molecules containing WD repeats in actin binding, we analyzed the F-actin binding capacity of six truncated fusion proteins of p57 that lack the putative N-terminal actin-binding sequence, namely GST-p57\(^{63-461}\), GST-p57\(^{63-299}\), GST-p57\(^{297-429}\), GST-p57\(^{63-127}\), GST-p57\(^{111-204}\) and GST-p57\(^{205-296}\) (Fig. 1). GST-p57\(^{63-461}\), which lacks the N-terminal 62 amino acids, co-precipitated with F-actin (Fig. 4A), suggest-
ing the presence of a second region responsible for actin-binding of p57 in addition to the N-terminal sequence (p57\(^{1-34}\)). Furthermore, GST-p57\(^{63-299}\) and GST-p57\(^{111-204}\) also co-precipitated with F-actin (Figs. 4B, C), whereas GST-p57\(^{63-127}\), GST-p57\(^{205-296}\), and GST-p57\(^{297-429}\) did not (Figs. 4D—F). Based on these results on the differential representation of the WD repeats among these mutant forms of p57 (Fig. 1), it is strongly suggested that p57 contains at least two regions that mediate actin binding; i.e., the N-terminal region (p57\(^{1-34}\)) and the central region containing the second and third WD repeats (p57\(^{111-204}\)).

**Binding of p57 Deletions to Actin in COS-1 Cells**  To extend our analysis of the putative actin-binding regions of p57 to intact cells, we expressed in COS-1 cells full-length and deletion mutants of p57 (p57\(^{1-371}\), p57\(^{372-461}\), p57\(^{1-71}\), p57\(^{1-34}\) and p57\(^{111-204}\)). Expressed forms of p57 constructs were visualized in transiently transfected cells using immunofluorescence microscopy (Fig. 5). Full-length p57 and its deletions that exhibited actin-binding activity *in vitro*, including p57\(^{1-371}\), p57\(^{1-71}\), p57\(^{1-34}\) and p57\(^{111-204}\), were localized in cortical F-actin rich regions. In contrast, p57\(^{372-461}\), which did not exhibit actin-binding activity *in vitro*, was diffusely distributed in cytosol and did not show cortical localization. These results are in good agreement with those of the co-sedimentation assay *in vitro*.
DISCUSSION

Our previous study demonstrated that the transient periphagosomal association of p57 plays an essential role in the maturation of phagolysosome in phagocytes.\(^{24}\) Considering that reorganization of the actin cytoskeleton is required for phagocytosis, the interaction between p57 and F-actin is one of the key steps during phagocytic processes. In this study, we have demonstrated that a p57 molecule possesses two regions responsible for its binding to F-actin. One is located in a short stretch of N-terminal 34 amino acids, and the other is located in the central region of the molecule containing WD repeats. Our results are partly consistent with a recent report.

Fig. 5. Intracellular Localization of p57 and Its Deletions Expressed in COS-1 Cells

Expression plasmids pcDNA3.1/p57V5His (p57), pcDNA3.1/p571–371 (p571–371), pcDNA3.1/p57372–461 (p57372–461), pcDNA3.1/p571–71 (p571–71), pcDNA3.1/p571–34 (p571–34), and pcDNA3.1/p57111–204 (p57111–204) were transiently expressed in COS-1 cells. Deletion mutants of p57 and F-actin were stained by anti-p57 antibodies or anti-Xpress antibody, followed by secondary antibody conjugated with FITC and by rhodamine-labelled phalloidin, respectively, and they were observed by fluorescence microscopy. Experiments were performed more than three times, and representative data are shown. Scale bars, 20 μm.
by Goode et al. They demonstrated that the actin-binding region of a yeast homologue of coronin, Crn1p, was located in the N-terminal part containing WD repeats, but not in its C-terminal region with a coiled-coil motif. By contrast, Mishima and Nishida reported that the deletion of N-terminal 63 amino acids or of C-terminal 65 amino acids from Xcoronin, a Xenopus homologue of coronin, reduces, but does not eliminate, its actin-binding activity. The deletion of actin-binding activity in Xcoronin lacking its C-terminal coiled-coil motif is not consistent with the results from p57 and Crn1p. This apparent conflict may be reflected by the differences among coronin species, and coronin homologues do not necessarily share common characteristics in the actin-binding activity.

A co-sedimentation assay and fluorescent microscopic observation strongly suggest that the N-terminal fragment of p57 with 34 amino acids (p571-34) was bound to F-actin (Figs. 3, 5). Deletion of the sequence KFRHVF abolished the actin-binding activity of p571-34, suggesting that the stretch of these six amino acids is essential for actin binding by the actin-binding activity of p571-34, indicating that the presence of two actin-binding regions in p57 molecule would increase its affinity for F-actin, as multiple actin-binding regions in several actin-binding proteins are believed to augment their interaction with actin. This hypothesis is supported by the result that the interaction of intact p57 with F-actin seems to be stronger than those of deletions containing only one of two actin-binding regions (Figs. 2, 4).

Acknowledgements This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labour and Welfare of Japan, and the U.S. Public Health Service (RO1AI34879).

REFERENCES


Fig. 6. Alignment of the Putative Actin-binding Region at the N-terminus of p57 and the Corresponding Regions of Other Coronin Family Actin-Binding Proteins

Asterisks indicate basic amino acid residues conserved in the coronin family of actin-binding proteins.