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

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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 ^{10}KFRHVF^{15}, 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$^{63—461}$ 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.[1] 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.[2,3] As described previously,[4] 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.[5—7] Homologous proteins to coronin have been identified in many eukaryotes from yeast to human,[8—17] and are assigned an important role in cell motility.[18] Recent reports have suggested that p57 participates as well in the formation of phagosome and its maturation in neutrophils and macrophages.[19—21]

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[4] at the C-terminus.[8] These motifs are known to mediate protein–protein interactions,[22,23] 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 pu-

tative actin binding regions in coronin family members of two distinct species.[8,13] In Crn1p, a coronin in budding yeast, the N-terminal part containing WD repeats, has been shown to have actin-binding activity.[8] 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.[13] 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

adapter ligation.[4] Briefly, the construct for the expression of p57 was prepared by digestion of the full-length cDNA of p57 with *Pma*CI and *Sac*I, and was ligated with both adapter I and adapter II (adapter I, upper strand 5′-GAT CCG AAT GAG CCG GCA GGT GGT CCG CTC CAG CAA GTT CCG CCA C-3′ and lower strand 5′-GTG GCG AAA CTT GCT GGA GCG GAC CAC CTG CCG GCT CAT TCG-3′; adapter II, upper strand 5′-CCA GAA GCG CTT GGA CAG GCT GGA GGA GAC AGT CCA GGC CAA GTA GG-3′ and lower strand 5′-TCG ACC TAC TTG GCC TGG ACT GTC TCC TCC AGC CTG TCC AAG CGC TTC TGG AGC T-3′). The construct for the expression of p57[1—371] was prepared by digestion of the full-length cDNA with *Pma*CI and *Bgl*I, and ligated with adapter I and adapter III (adapter III, upper strand 5′-CGG CTG AGG AGT GGC TGG CTG GGG GGT CGG GAT GCT GGG TAG G-3′ and lower strand 5′-AGT GCC GAC TCC TCA CCG ACC CCC AGC CCC TAC GAC CCA TCC AGC T-3′). The construct for the expression of p57[373—461] was prepared by digestion of the full-length cDNA with *Bgl*I and *Sac*I, and ligated with adapter IV and adapter II (adapter IV, upper strand 5′-GAT CCC TGC CCT CA-3′ and lower strand 5′-GGA CGG G-3′). The ligated fragments and adapters were subcloned into pGEX-5X-1, that had been digested with *Bam*HI and *Sal*I.

The expression plasmids of pGEX-p57[1—71] (Met-1 to Asp-71) and pGEX-p57[1—34] (Met-1 to Thr-34) (Fig. 1), were generated from pGEX-p57[1—371] by introducing stop codons using a QuikChange™ Site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The sequences of mutagenic primers are as follows (the substituted bases are underlined): K72Z, sense primer 5′-GGC AAG ACT GGA CGT GTG GAC TAG AAT GCG CCC ACG TCT GTG GG-3′ and anti-sense primer 5′-CCA CAG ACC GTG GGC GCA TTC TAG TCC ACA CGT CCA GTC TTG CC-3′; and W35Z, sense primer 5′-CGT CTC ACA GAC CAC CTA GGA CAG TGG CTT CTG TGC-3′ and anti-sense primer 5′-GCA CAG AAG CCA CTG TCC TAG GTG GTC TGT GAG ACG-3′. Deletion of amino acid residues 10 to 15 from p57[1—34] was also performed by a QuikChange Site-directed mutagenesis kit. The primers used were: Δ(10—15), sense primer 5′-G GTG GTC CGC TCC AGC GGA CAG CCG GCC AAG G-3′ and anti-sense primer 5′-C CTT GGC CGG CTG TCC GCT GGA GCG GAC CAC C-3′.

The other deletions of p57, pGEX-p57[63—461] (Pro-63 to Lys-461), pGEX-p57[63—127] (Pro-63 to Leu-127), pGEX-p57[111—204] (Ile-111 to Glu-204), pGEX-p57[205—296] (Pro-205 to Glu-296), and pGEX-p57[297—429] (Ile-297 to Ala-429) (Fig. 1), were obtained by polymerase chain reaction (PCR), followed by digestion and subcloning in expression vector pGEX-5X-1. The amplified DNA fragments were digested with *Bam*HI and *Eco*RI, then cloned into pGEX-5X-1. The primers used are as follows (restriction enzyme sites are underlined): *Bam*HI-p57[63—], 5′-GG GGG ATC CCC CTG GGC AAG ACT GGA CGT GTG G-3′ and *Eco*RI-*Hind*III-p57[461—] reverse, 5′-GGG GAA TTC AAG CTT GGG GCT CTA CTT GGC CTG G-3′ for p57[63—461]; *Bam*HI-hp57[63—] and *Eco*RI-p57[127—] reverse, 5′-GGG GAA TTC CAG GGT GAC GAC GGG CTC CCG CAG G-3′ for p57[63—127]; *Bam*HI-p57[111—], 5′-GGG GGG ATC CCC ATC CCG GAT GGG GGC CTG AT-3′ and *Eco*RI-p57[204—] reverse, 5′-GGG GAA TTC CTC GAT GAT GCG CAC GCG CT-3′ for p57[111—204]; *Bam*HI-p57[205—], 5′-GGG GGG ATC CCC CCC CGC AAA GGC ACT GTC GT-3′ and *Eco*RI-p57[296—] reverse, GGG GAA TTC CTC AAA GTA CCG GAT TGA GC for p57[205—296]; and *Bam*HI-p57[297—], GGG GGG ATC CCC ATC ACT TCC GAG GCC CCT TT and *Eco*RI-p57[429—] reverse, GGG GAA TTC CCT CCT CCA GCC GAG ACA CG for p57[297—429].

The pGEX-p57[63—299] was generated from pGEX-p57[63—461] by introducing stop codons. The primers used were: E300Z, sense primer 5′-GGT ACT TTG AGA TCA CTT CCT AGG CCC CTT TCC TGC ACT ATC-3′ and antisense primer 5′-GAT AGT GCA GGA AAG GGG CCT AGG AAG TGA TCT CAA AGT ACC-3′.

Expression and Purification of GST Fusion Proteins
E. coli JM109 cells, transformed with each plasmid described above, were cultured overnight in 6 ml of Luria-Bertani (LB) medium (10 g/l of tryptone, 5 g/l of yeast extract, 10 g/l of NaCl, pH 7.0) at 37 °C, diluted to 500 ml with fresh Terrific Broth (TB; 12 g/l of tryptone, 24 g/l of yeast extract, 0.8% of glycerol and 1.25 mM of potassium phosphate buffer, pH 6.5), and grown for 48 h at 20 °C. Fusion proteins
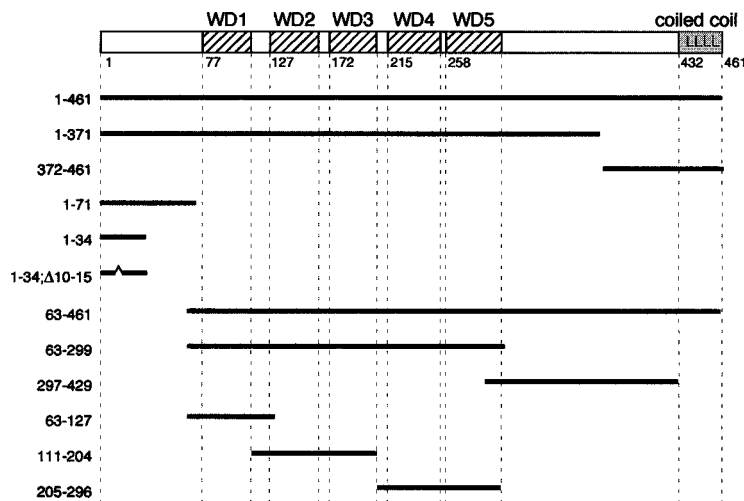

Fig. 1.   Structures of p57 and its Deletions

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

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 $10000 \times g$ 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, 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 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 $198000 \times g$ 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 (SDS)-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[4] or anti-p57 monoclonal antibody[9] 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 AGA ATG AGC CGG CAG GTG G-3′ and 5′-GGG GAA TTC AAG CTT GGG GCT CTA CTT GGC CTG G-3′. The amplified DNA fragments were digested with *Eco*RI and cloned into pEF1/Myc-His A (Invitrogen, Carlsbad, CA, U.S.A.). Then, TAG stop codon was 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′-GGA GAC AGT CCA GGC CAA GGC GAG CCC CAA GCT TGA ATT CTG C-3′ and 5′-GCA GAA TTC AAG CTT GGG GCT CGC CTT GGC CTG GAC TGT CTC C-3′. To generate the expression plasmid pcDNA3.1/p57-V5-His for full-length p57, which expresses p57 fused with viral epitope V5 and 6xHis tag, the DNA fragments encoding p57 were subcloned into pcDNA3.1/V5-His A (Invitrogen).

For expression of p57$^{1—371}$ and p57$^{372—461}$, corresponding DNA fragments were subcloned into pcDNA3.1/V5His A (pcDNA3.1/p57$^{1—371}$ and pcDNA3.1/p57$^{372—461}$). Expression plasmids encoding p57$^{1—34}$ and p57$^{1—71}$ were generated from pcDNA3.1/p57-V5-His by introducing stop codons (pcDNA3.1/p57$^{1—34}$ and pcDNA3.1/p57$^{1—71}$, respectively).

The p57$^{111—204}$ was expressed as a fusion protein with an N-terminal Xpress™ tag (Invitrogen) in order to facilitate detection of the expressed protein. The cDNA fragment of p57$^{111—204}$ was prepared by digestion of pGEX-p57$^{111—204}$ with *Bam*HI and *Eco*RI, and subcloned into pcDNA3.1/His A (Invitrogen).

Cell Culture and Transfection COS-1 (monkey kidney cell line) cells (American Type Culture Collection) 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% CO_2 in air).

Plasmids encoding p57 and its deletions were introduced into COS-1 cells by electroporation. Briefly, COS-1 cells (4×10^6 cells/300 μl) in phosphate buffered saline (PBS) were mixed with 10 μg plasmid DNA and placed in a 0.4 cm electrode gap electroporation cuvette (Bio-Rad, Hercules, CA, U.S.A.). Cells were subjected to a single pulse of 750 V/cm at a capacitance setting of 975 microfarads, transferred to 10 ml of IMDM containing 10% FCS, and maintained for 48 h under standard cell culture conditions.

Immunofluorescence Microscopy The transfected cells were cultured on a Lab-Tek II Chamber Slide (Nalge Nunc, Rochester, NY, U.S.A.) for 10 min, fixed with 4% neutral buffered formaldehyde (Kanto Chemicals Co., Ltd., Tokyo, Japan), and then permeabilized by treatment with 0.2% Triton X-100 in PBS for 10 min at room temperature. Permeabilized cells were rinsed with PBS and incubated with a monoclonal antibody to p57, N7,[20] which recognizes the C-terminal region of p57, at 5 μg/ml in PBS containing 3% BSA for 1 h. For the detection of N-terminal fragments of p57, a rabbit polyclonal antibody, which recognizes N-terminal 20 amino acids, was used. For the detection of Xpress-tagged fusion protein, an anti-Xpress antibody (1 : 5000, Invitrogen) was used. After being washed with PBS, the cells were incubated with FITC-labeled goat anti-mouse IgG (KPL, Gaithersburg, MD, U.S.A.) or FITC-labeled goat anti-rabbit IgG (KPL) for 30 min. F-actin was detected by using rhodamine-conjugated phalloidin (Sigma). Fluorescently labeled cells were washed three times with PBS and then mounted with 2.3% 1,4-diazabicycle-2,2,2-octane (Sigma) containing glycerol on slide glass. Samples were analyzed by using a confocal laser microscope (Radiance 2100 laser scanning system, Bio-Rad, Hercules, CA, U.S.A.).

RESULTS

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-p57$^{1—371}$ (a fusion protein containing five WD repeats but lacking a C-terminal coiled-coil motif) (Fig. 1) co-precipitated with F-actin (Figs. 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-p57$^{372—461}$ (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-p57$^{1—71}$ and GST-p57$^{1—34}$ (Fig. 1). These deletion mutants
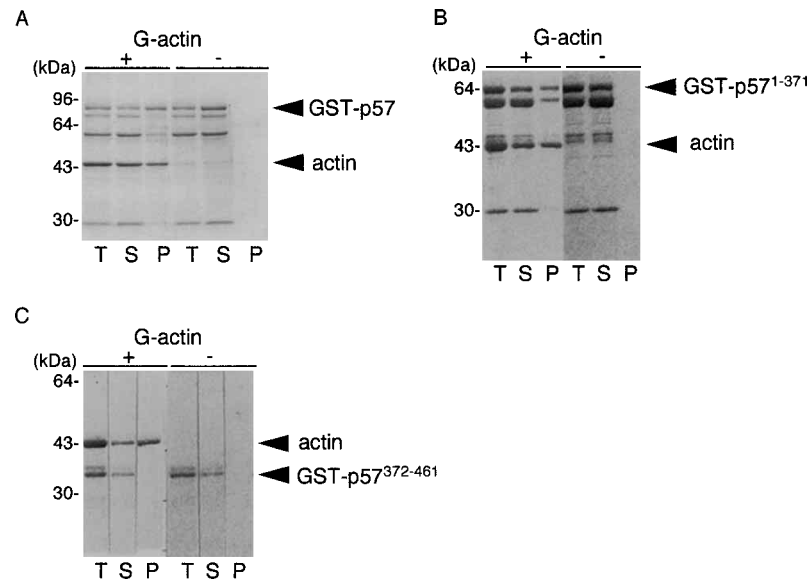
Fig. 2. Co-sedimentation Assay of GST-p57 (A), GST-p57$^{1—371}$ (B), and GST-p57$^{372—461}$ (C) Fusion Proteins with F-Actin

The supernanant (S) and the precipitate (P) after ultracentrifugation, as well as the total reaction mixture (T), were separated on SDS-polyacrylamide gel electrophoresis. Two sets of samples in the presence (+) or absence (−) of G-actin were assayed.
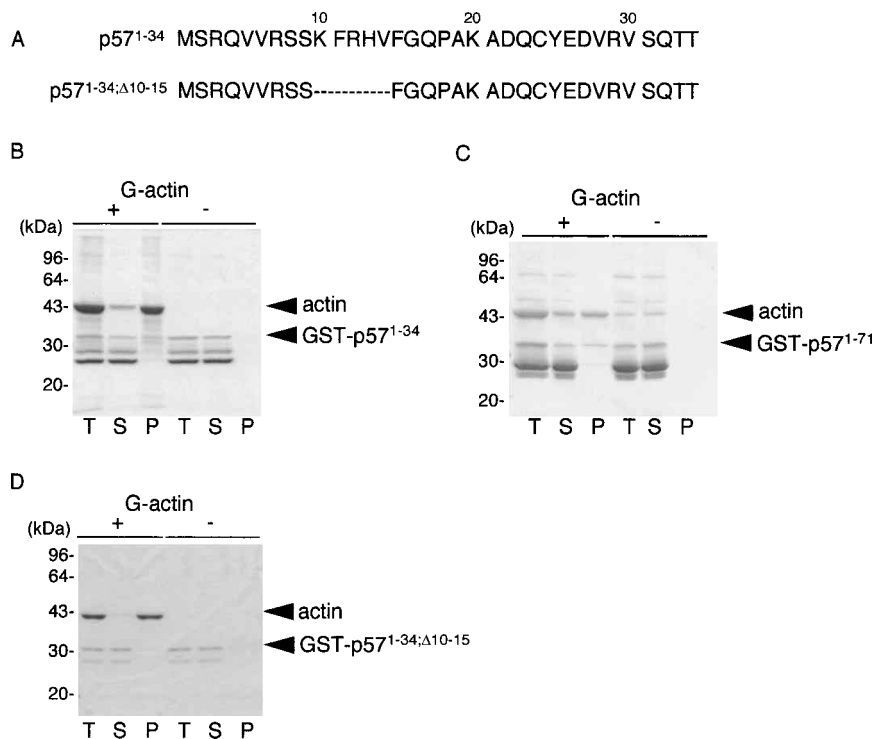

Fig. 3. Amino Acid Sequences of GST-p57$^{1—34}$ and GST-p57$^{1—34;Δ10—15}$ (A) and the Co-sedimentation Assay of GST-p57$^{1—34}$ (B), GST-p57$^{1—71}$ (C), and GST-p57$^{1—34;Δ10—15}$ (D) Fusion Proteins with F-Actin

After F-actin was sedimented by ultracentrifugation, each fraction was subjected to SDS-polyacrylamide gel electrophoresis; T, total reaction mixture; S, ultracentrifuged supernatant; P, ultracentrifuged precipitate. Each fusion protein, with (+) or without (−) G-actin, was incubated in F-actin buffer for co-sedimentation assay.

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}KFRHVF^{15}, from p57$^{1—34}$ (GST-p57$^{1—34;Δ10—15}$) 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-
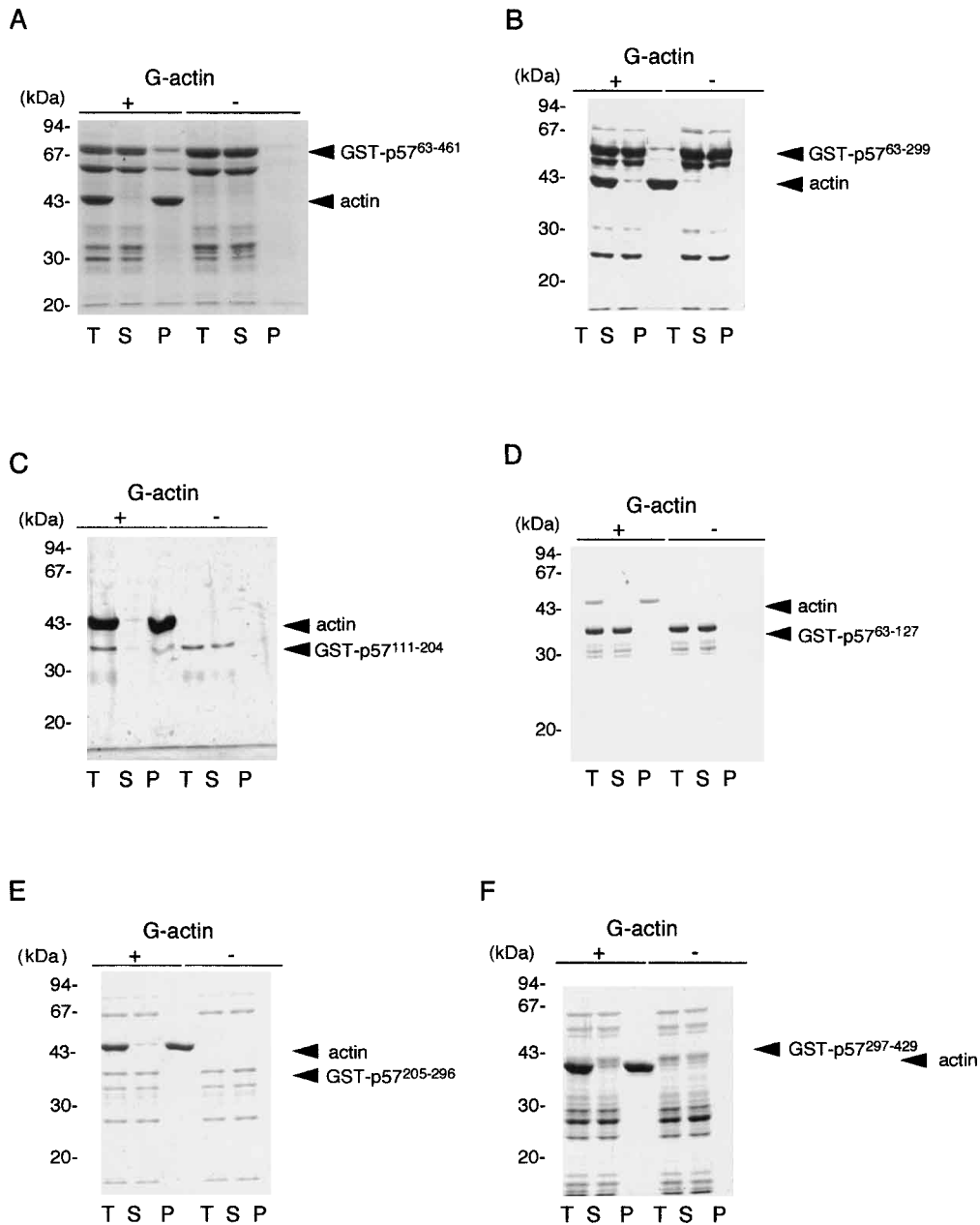
Fig. 4. Co-sedimentation Assay of GST-p57$^{63—461}$ (A), GST-p57$^{63—299}$ (B), GST-p57$^{111—204}$ (C), GST-p57$^{63—127}$ (D), GST-p57$^{205—296}$ (E), and GST-p57$^{297—429}$ (F) Fusion Proteins with F-Actin

After F-actin was sedimented by ultracentrifutgation, each fraction was subjected to SDS-polyacrylamide gel electrophoresis; T, total reaction mixture; S, ultracentrifuged supernatant, P, ultracentrifuged precipitate. Each fusion protein, with (+) or without (−) G-actin was incubated in F-actin buffer for co-sedimentation assay.

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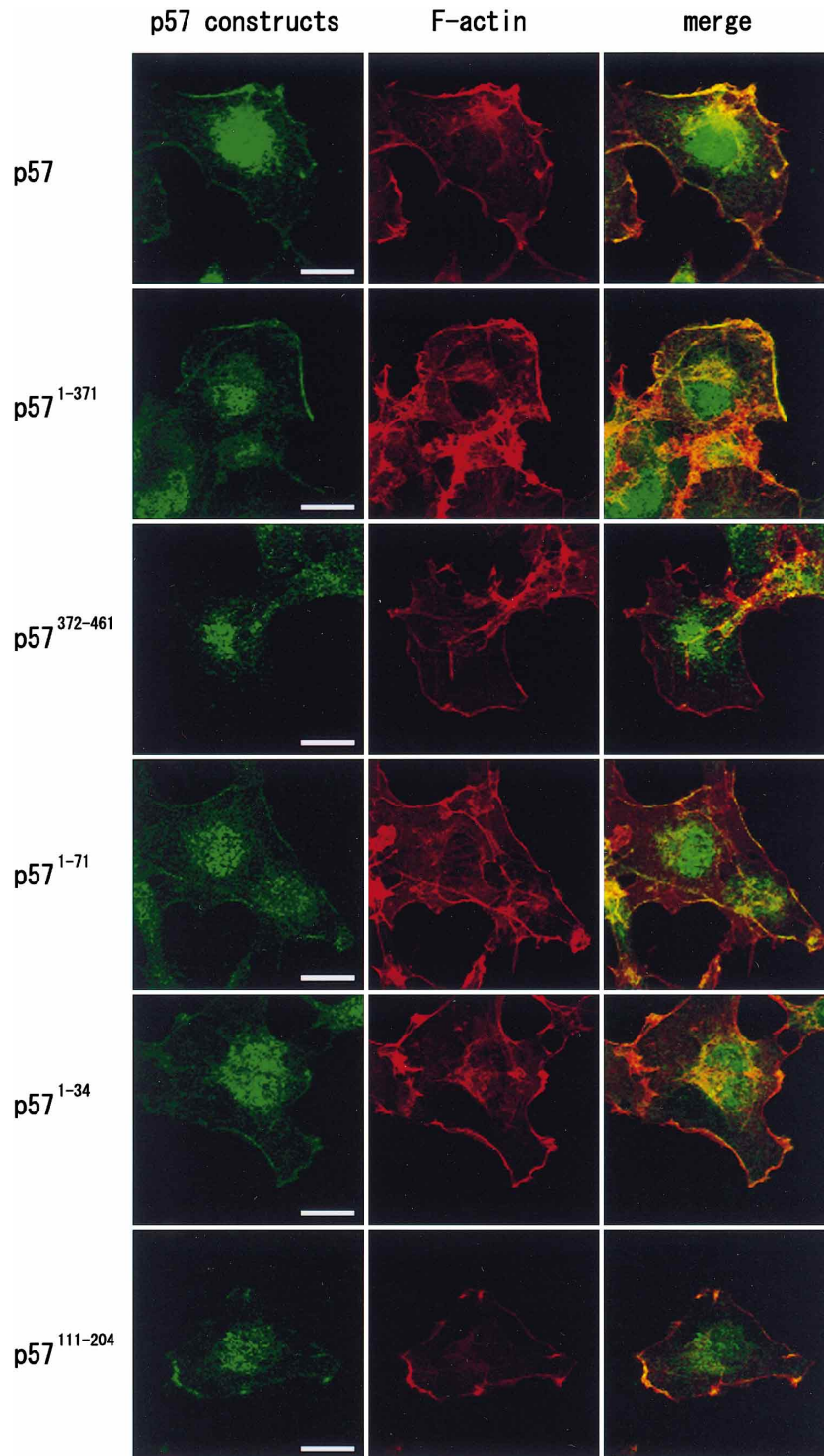
Fig. 5.   Intracellular Localization of p57 and Its Deletions Expressed in COS-1 Cells

Expression plasmids pcDNA3.1/p57V5His (p57), pcDNA3.1/p57[1—371] (p57[1—371]), cDNA3.1/p57[372—461] (p57[372—461]), pcDNA3.1/p57[1—71] (p57[1—71]), pcDNA3.1/p57[1—34] (p57[1—34]) and pcDNA3.1/p57[111—204] (p57[111—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.

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

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human p57 -MSRQVVRSSK FRHVFGQPAK ADQCYEDVRV SQTT
rabbit pp66 MSFRKVVRQSK FRHVFGQPVK NDQCYEDIRV SRVT
human clipin C MSWRPQYRSSK FRNVYGKVAN REHCFDGIPI TKNV
Xenopus laevis X coronin A ---MRRVVRQSK FRHVFGQALK NDQCYDDIRV SRVT
Dictyostelium coronin ---MSKVVRSSK YRHVFAAQPK KEECYQNLKV TKSA
S.cerevisiae Crn1p -MSGKFVRASK YRHVFGQAAK KELQYEKLKV TNNA
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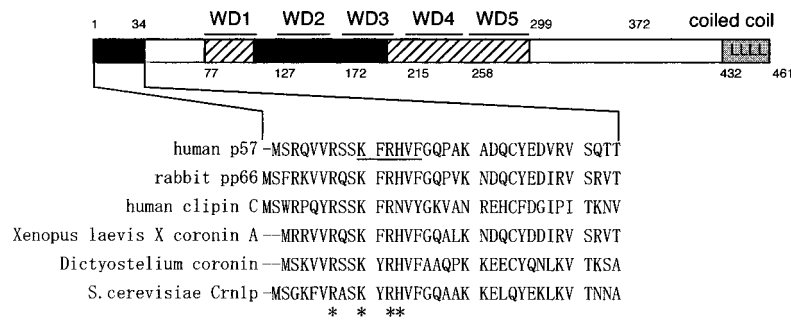
Fig. 6. Alignment of the Putative Actin-binding Region at the N-terminus of p57 and the Corresponding Regions of Other Coronin Family Actin-Binding Proteins

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

by Goode *et al.*[8] 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[13] 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 reduction 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 ($p57^{1—34}$) was bound to F-actin (Figs. 3, 5). Deletion of the sequence ^{10}KFRHVF^{15} abolished the actin-binding activity of $p57^{1—34}$, suggesting that the stretch of these six amino acids is essential for actin binding of the N-terminal fragment. Several known actin-binding proteins possess regions rich in basic amino acid residues that contribute to actin binding through their interaction with acidic amino acid clusters contained in actin.[25—28] This cluster of basic amino acid residues may be responsible not only for actin binding by p57, but may also represent a motif common to related proteins, as the KXRHXX (where X represents a hydrophobic amino acid) is conserved in many other coronin-family actin-binding proteins (Fig. 6).

In addition to $p57^{1—34}$, we identified a second region of p57 that exhibited actin-binding activity with Ile-111 to Glu-204 (Fig. 4). $p57^{111—204}$ includes two out of the five WD repeats present in p57. The WD motifs were found in a variety of proteins and are known to participate in various critical cell functions, including signal transduction, gene regulation, vesicular trafficking, regulation of the cytoskeleton and the cell cycle.[29] Regions in proteins with WD repeats are thought to organize into a β-propeller structure and to promote protein–protein interactions.[30,31] However, the WD repeats in p57 do not seem to mediate its actin-binding, but more likely serve another function, perhaps providing a scaffold for the responsible regions of p57 to bind actin and/or link actin to another protein. In the case of $p57^{111—204}$ there are WD repeats sufficient for only one blade of a β-propeller, thus it is inadequate to fold properly into a functional β-propeller structure.[30] Therefore, the actin binding of $p57^{111—204}$ more likely depends on a structure other than the WD repeats. The determination of the second sequence responsible for the binding of $p57^{111—204}$ to F-actin awaits further investigation.

The presence of two actin-binding regions in a 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).

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