

The identification of a new actin-binding region in p57

Chang Zhen Liu¹, Yong Chen¹, Sen Fang Sui¹

¹*Department of Biological Sciences and Biotechnology, State Key Laboratory of Biomembranes, Tsinghua University, Beijing 100084, China*

The actin-binding protein p57 is a member of mammalian coronin-like proteins. The roles of this protein in phagocytic processes conceivably depend on its interactions with F-actin. Two regions, p57¹⁻³⁴ and p57¹¹¹⁻²⁰⁴, were previously reported to be actin-binding sites. In this study, we found that the C-terminal region of p57, p57²⁹⁷⁻⁴⁶¹, also possessed F-actin binding activity. Furthermore, the leucine zipper domain at the C-terminus of p57²⁹⁷⁻⁴⁶¹ was essential for this actin-binding activity. The F-actin cross-linking assay revealed that the region contained in p57²⁹⁷⁻⁴⁶¹ was sufficient to cross-link actin filaments. Our results strongly suggested that there was a new actin-binding region at the C-terminus of p57.

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Introduction

Coronin-like proteins are a family of actin-binding proteins that are represented in a large variety of eukaryotic cells from yeast to human [1]. The first family member was characterized in *Dictyostelium discoideum* and was shown to play crucial roles in various cell functions, including cell locomotion, phagocytosis, and cytokinesis [2-4]. p57, also named coronin1, was the first coronin-like protein identified in mammals [1, 5]. The 57 kDa protein reveals an open reading frame of 1386 base pairs encoding 461 amino acids. Similar to the other coronin-like protein family members, p57 has a central region that comprises five WD repeats, which is speculated to create a stable platform that can interact reversibly with other proteins [6]. The N-terminal extension of p57 is composed of approximately 70 amino acid residues conserved in most mammalian coronin-like proteins. The C-terminal extension contains a

leucine zipper domain, which evolved from a coiled coil of the coronin-like protein family and was reported to mediate the homotypic dimerization or trimerization of p57 [7-9].

It has been reported that p57 plays essential roles in the generation of early phagosomes and maturation of phagolysosome in phagocytes [10, 11]. The transient accumulation of p57 on early phagosomes and the gradual disassociation of p57 from the phagosomal membrane after phagosomes maturation had the same kinetics as those of F-actin [11], which suggested that p57 might take part in the processes of phagocytosis by interacting with F-actin. However, its precise role associated with actin cytoskeleton remodeling during phagocytic processes and the underlying mechanisms of interaction between p57 and F-actin have yet to be defined.

Due to the importance of the interaction between p57 and actin, and the ambiguity of the underlying mechanisms, the actin-binding sites of p57 have been investigated. A previous study suggested that two regions within N-terminal and central part of p57 (Met-1 to Asp-34 and Ile-111 to Glu-204) are responsible for actin binding [12]. However, it has been reported that the deletion of the C-terminal 65 amino acids from Xcoronin, a *Xenopus* homologue of coronin, significantly reduces its actin-binding activity [13]. In another report, the F-actin interaction of human coronin 3 (Hcoronin3) is mediated by its C-terminus [14]. These studies suggest that the C-terminus of p57 may contribute to

Correspondence: Sen Fang Sui

Tel: +86-10-62784768;

E-mail: suisf@mail.tsinghua.edu.cn

Abbreviations: G-actin (globular actin); F-actin (filamentous actin); GST (glutathione *S*-transferase); RT-PCR (reverse transcription-polymerase chain reaction)

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its actin binding. In this study, we examined actin-binding activity of the C-terminus and confirmed that p57²⁹⁷⁻⁴⁶¹, a C-terminal region of p57, had the activity of actin binding and F-actin cross-linking. We also found that the leucine zipper domain might be essential for the actin-binding activity of p57²⁹⁷⁻⁴⁶¹.

Materials and methods

Construction of plasmids expressing full-length and deletion mutants of p57 in *E. coli* and mammalian cells

The full-length cDNA of p57 was prepared by RT-PCR amplification of mRNA from U937 cells. PCR primers (sense primer: 5'-GTC GGA TCC ATG AGC CGG CAG GTG G-3', and antisense primer: 5'-GTC GAA TTC CTA CTT GGC CTG GAC TGT CTC-3') were synthesized based on the sequence of the coding region of Homo sapiens coronin 1A (GenBank accession No. XM055133). The construction for expression of full-length p57 in *E. coli* was prepared by digestion of the PCR products with *Bam*HI and *Eco*RI, and then cloned into vector pGEX-6P-1 (Amersham Pharmacia). The constructions for expression of deletion mutants (p57²⁹⁷⁻⁴⁶¹ and p57³⁸⁶⁻⁴⁶¹) (Figure 1) in *E. coli* were prepared by the same procedure, and the primers were used as follows: a. (for p57²⁹⁷⁻⁴⁶¹): sense primer: 5'-GTA GGA TCC ATC ACT TCC GAG GCC CCT TTC-3', and antisense primer: 5'-GTC GAA TTC CTA CTT GGC CTG GAC TGT CTC-3'; b. (for p57³⁸⁶⁻⁴⁶¹): sense primer: 5'-GTC GGA TCC CCC CTC CTC ATC TCC CTC AAG-3', and antisense primer: 5'-GTC GAA TTC CTA CTT GGC CTG GAC TGT CTC-3'. The mutagenesis of Leu-441 and Leu-447 of p57²⁹⁷⁻⁴⁶¹ to alanine (p57^{297-461/Mut}) (Figure 1) was performed by PCR amplification with mutagenic primers: (sense primer: 5'-GTA GGA TCC ATC ACT TCC GAG GCC CCT TTC-3' and antisense primer: 5'-CTA CTT GGC CTG GAC TGT CTC CTC CAG CCT GTC CAA GCG CTT CTG GAG CTC CTG CAC CGT GGC CTG GAG CTT CCG-3'). Plasmids expressed in mammalian cells were prepared by corresponding digestion of constructed pGEX-6P-1 fusion plasmids with *Bam*HI and *Eco*RI, and subcloning the digested products into vector pcDNA 4.0/His-max (Invitrogen). Proteins encoded from these plasmids were expressed as fusion proteins with an N-terminal XpressTM tag (Invitrogen). Constructions described above were confirmed by DNA sequencing (Sangon Corp, China).

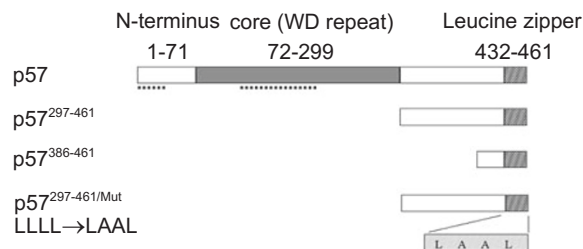


Figure 1 Schematic views of p57 and its deletion mutants. The WD repeat region (solid box) and the leucine zipper-containing region (hatched) are highlighted. The underlines indicate the two actin-binding regions reported in study by Oku *et al* [12].

Expression and purification of GST fusion proteins

E. coli BL21 transformed with constructed plasmids was used to express GST fusion proteins of full-length and deletion mutants of p57. The expression and purification of GST fusion proteins were performed according to the instruction of GST Gene Fusion System (Amersham). The GST moiety of GST-p57²⁹⁷⁻⁴⁶¹ was cut off by treatment of the washed fusion protein-bound Glutathione-Sepharose 4B beads with PreScissionTM Protease (Amersham).

F-actin co-sedimentation assay

Actin-binding assays *in vitro* were performed to investigate the activity of fusion proteins to bind actin [7]. G-actin (3 μ M, Sigma) was incubated in F-actin buffer containing 20 mM imidazole (pH 7.0), 50 mM KCl, 1 mM DTT, 5 mM ATP, 2 mM MgCl₂, and 5 mM CaCl₂ for 40 min at 25°C to be assembled into F-actin. Various fusion proteins were incubated with pre-assembled F-actin for 40 min at 25°C. After the incubation, the reaction mixtures were ultracentrifuged at 100,000 \times g for 30 min at 4°C (step 1). The pellets were resuspended in F-actin buffer and transferred to new tubes for another ultracentrifugation at 100,000 \times g for 30 min at 4°C (step 2). The supernatants of step 1 (S), the pellets of step 2 (P), and the total reaction mixtures (T) were resolved in SDS-PAGE loading buffer and subjected to SDS-PAGE (8% gel), followed by staining with Coomassie brilliant blue R-250. The procedures for conducting F-actin cross-linking experiments were similar to those for actin-binding assays with slight modifications that the mixture was centrifuged at 15,000 \times g for 10 min at 4°C [15] instead of 100,000 \times g for 30 min at 4°C, and the samples were subjected to SDS-PAGE (12% gel). Pre-assembled F-actin was used as a control.

Cell culture and transfection

U937 cells and COS-1 cells (American Type Culture Collection) were grown respectively in RPMI 1640 medium (Hyclone) and DMEM (Invitrogen) supplemented with 10% heat inactivated fetal calf serum (Hyclone), ampicillin (100 μ g/ml) and streptomycin (100 μ g/ml) at the standard cell culture condition (37°C, humidified 5% CO₂ in air). Plasmids pcDNA 4.0/p57, pcDNA 4.0/p57²⁹⁷⁻⁴⁶¹, pcDNA 4.0/p57³⁸⁶⁻⁴⁶¹, and pcDNA 4.0/p57^{297-461/Mut} were introduced into COS-1 cells (5 \times 10⁵ cells/well) by lipofectamineTM 2000 (Invitrogen) in 6-well plates according to the manufacturer's instructions. After that, cells were maintained for 48 h at standard cell culture conditions. To detect the expressed proteins, aliquots of the cells transfected with plasmids were resolved in SDS-PAGE loading buffer and subjected to SDS-PAGE (12% gel), followed by immunoblotting analysis with anti-XpressTM monoclonal antibody (Invitrogen). The blots were detected by enhanced chemiluminescence (ECL) (Amersham).

Cross-linking of p57²⁹⁷⁻⁴⁶¹ and p57^{297-461/Mut} expressed in COS-1 cell

COS-1 cells transfected with plasmids pcDNA 4.0/p57²⁹⁷⁻⁴⁶¹ or pcDNA 4.0/p57^{297-461/Mut} for 48 h were washed twice with ice-cold phosphate buffered saline pH 7.4 (PBS), and scraped into 0.5 ml PBS. Disuccinimidyl suberate (DSS) (1 mM) prepared as a 10 \times stock solution in dimethyl sulfoxide (DMSO) was used for cross-linking. The cross-linking reactions were allowed to proceed for 1 h on ice and terminated for 30 min with 50 mM Tris pH 7.5. Aliquots from the reaction mixtures were subjected to immunoblotting analysis described above.

Immunofluorescence microscopy

The transfected COS-1 cells were plated onto poly-L-lysine coated 12 mm glass cover slips. Actin skeletal frameworks were prepared according to the reported method [16]. After incubation for 30 min at room temperature with PBS containing 1% BSA, cells were co-incubated with mouse anti-Xpress™ antibody (1:1000, Invitrogen) which recognizes the N-terminal Xpress™ tag of expressed fusion proteins, and goat anti-actin antibody (I-19) (1:500, Santa Cruz) for 3 h at room temperature. After being washed with PBS, the cells were incubated with rhodamine-labeled rabbit anti-goat IgG (1:400, Santa Cruz) for 20 min in the dark at room temperature. After further washing steps, the cells were incubated with FITC-labeled goat anti-mouse IgG (1:400, Santa Cruz) for 20 min in the dark at room temperature. Fluorescently labeled cells were washed three times with PBS for 5 min and then mounted on slide glass with 90% glycerol. Samples were analyzed by using a Fluorescence Microscope (Nikon Eclipse E800).

Electron Microscopic Analysis

Actin filaments cross-linking was visualized by electron microscopy. Pre-assembled F-actin (2 μ M) or the mixture of F-actin and purified p57²⁹⁷⁻⁴⁶¹ at molar ratios of 3:1 were incubated in F-actin buffer for 30 min at 25°C. After the incubation, the reaction mixtures were centrifuged at low speed (15,000 \times g) for 10 min at 4°C, and the pellets were resuspended for electron microscopic analysis. Aliquots from both sets of reactions were spotted onto copper grids, negatively stained with 1% aqueous uranyl acetate, and examined by using a transmission electron microscope (Philips CM120).

Results and discussion

p57²⁹⁷⁻⁴⁶¹ possesses the activity of actin binding

To examine whether the C-terminus of p57 contains a putative actin-binding region, the actin-binding activities of full-length p57 and two C-terminal fragments, p57²⁹⁷⁻⁴⁶¹ and p57³⁸⁶⁻⁴⁶¹ (Figure 1), were analyzed by the F-actin co-sedimentation assay *in vitro*. If fusion proteins have the activity of actin binding, they would co-precipitate along with F-actin by ultracentrifugation [7, 17]. In this experiment, as a positive control, GST-p57 was detected in the pellet in the presence of F-actin, but not in the absence of F-actin (Figure 2A). Similarly, GST-p57²⁹⁷⁻⁴⁶¹ was detected in the pellet only in the presence of F-actin (Figure 2B). In contrast, in both the absence and presence of F-actin, GST-p57³⁸⁶⁻⁴⁶¹, a fusion protein previously reported having no actin-binding activity [12], was found only in the supernatant (Figure 2C). These results indicated that the purified recombinant protein GST-p57²⁹⁷⁻⁴⁶¹ possessed the capability to bind F-actin.

The actin-binding activities of full-length p57 and p57²⁹⁷⁻⁴⁶¹ were then compared by F-actin co-sedimentation assay *in vitro*. As shown in Figure 2D, in the GST-p57 group, after incubation with F-actin and ultracentrifugation, most

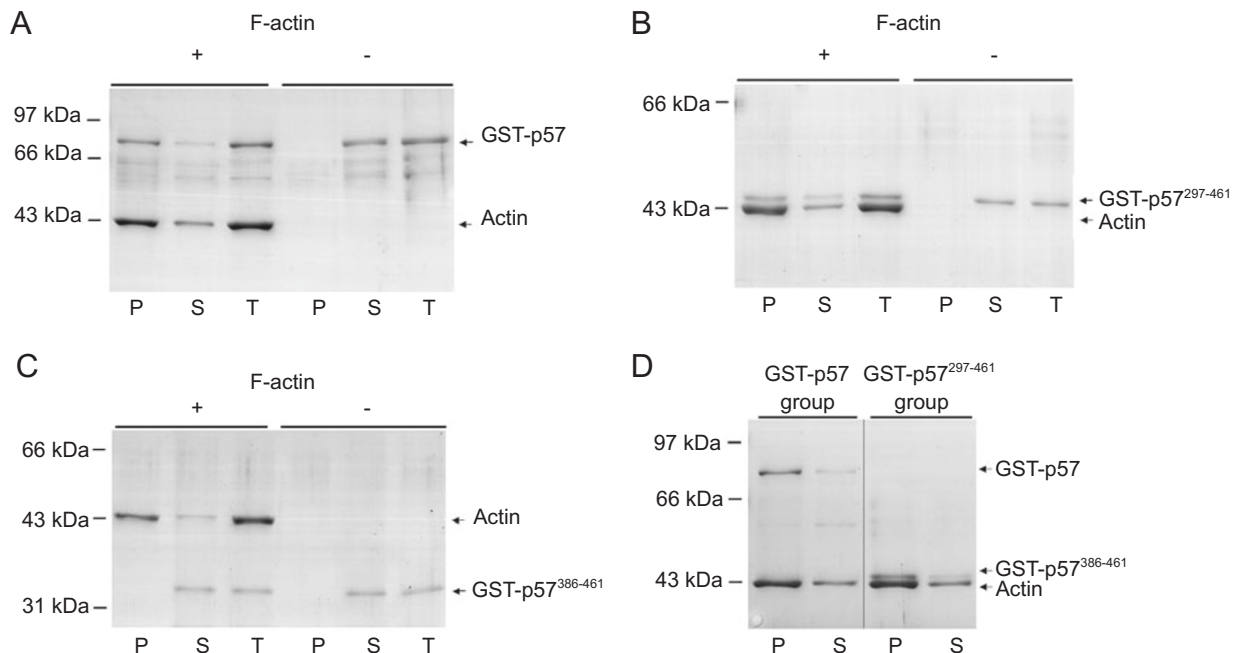


Figure 2 *In vitro* co-sedimentation assay of GST fusion proteins with F-actin. After mixtures were sedimented by ultracentrifugation, fractions were subjected to SDS-PAGE. T, total reaction mixture; S, ultracentrifuged supernatant; P, ultracentrifuged precipitate. Fusion proteins GST-p57 (A), GST-p57²⁹⁷⁻⁴⁶¹ (B) and GST-p57³⁸⁶⁻⁴⁶¹ (C) were incubated with (+) or without (-) F-actin for co-sedimentation assay. (D) Equal mole amounts of GST-p57 and GST-p57²⁹⁷⁻⁴⁶¹ were incubated with the same concentrations of F-actin for co-sedimentation assay.

of GST fusion protein was precipitated into pellet (P) with a small fraction remaining in the supernatant. In the GST-p57²⁹⁷⁻⁴⁶¹ group, a similar result was obtained. The results of comparison indicated that p57²⁹⁷⁻⁴⁶¹ possessed similar actin-binding activity as full-length p57, suggesting the potential important role of this region in actin binding of p57.

The binding activity of p57²⁹⁷⁻⁴⁶¹ to actin cytoskeleton was further analyzed *in vivo* by immunofluorescence co-localization studies. First, the expressions of full-length p57, p57²⁹⁷⁻⁴⁶¹ and p57³⁸⁶⁻⁴⁶¹ with Xpress-tag in COS-1 cells were identified by immunoblotting analysis. The results showed that these proteins were expressed as expected (57 kDa, 23 kDa and 13 kDa, respectively) and the expression levels of them were similar (Figure 3A). Then cells were treated with 0.5% Triton X-100 before fixation to release most cytosol protein [16], avoiding the interference of unbinding expressed proteins to the observation of immunofluorescence co-localization. The expressed Xpress-tag fusion proteins and actin cytoskeleton were stained by antibody conjugated with FITC and rhodamine respectively, and were observed by fluorescence microscopy. Full-length p57 and p57²⁹⁷⁻⁴⁶¹ (green color) were both demonstrated partial co-localization (*arrowheads*) with cortical actin cytoskeletons (red color) (Figure 3B). In contrast, p57³⁸⁶⁻⁴⁶¹ (green color), which did not exhibit actin-binding activity *in vitro*, was not co-localized with cortical actin cytoskeletons (red color) (Figure 3B). These results confirmed that p57²⁹⁷⁻⁴⁶¹ had the similar activity to bind actin cytoskeleton *in vivo* as full-length p57. The results from immunofluorescence co-localization analysis were in good agreement with those of the co-sedimentation assay *in vitro*.

Previous study demonstrated that a p57 molecule possesses two regions responsible for binding F-actin but neither exists at the C-terminus [12]. In this study, we found that the C-terminal region of p57, p57²⁹⁷⁻⁴⁶¹, had the similar actin-binding property as full-length p57, as demonstrated by co-sedimentation of expressed protein with F-actin *in vitro* and co-localization of expressed protein with actin cytoskeleton *in vivo*. These results revealed that the C-terminal region of p57 might also be responsible for binding F-actin which was consistent with actin-binding sites of other coronin-like proteins such as Xcoronin and Hcoronin3 [13, 14].

Leucine zipper domain is essential to actin-binding activity of p57²⁹⁷⁻⁴⁶¹

In study by Oku *et al* [12], two C-terminal regions, p57²⁹⁷⁻⁴²⁹ and p57³⁷²⁻⁴⁶¹, were examined for actin-binding activity while they were both found to have no actin-binding activity. In contrast, the whole length of these two C-terminal regions, p57²⁹⁷⁻⁴⁶¹, was shown to have the actin-binding activity in this study. The conflict between

the actin-binding activities of the separated fragments and that of the whole length region impelled us to explore further. It has been reported that the last 30 amino acids at the C-terminus of p57 (residues 432-461) form a leucine zipper domain [7] (Figure 1). Since p57²⁹⁷⁻⁴⁶¹ contains the leucine zipper domain, while p57²⁹⁷⁻⁴²⁹ does not, the effect of this domain on actin-binding activity of p57²⁹⁷⁻⁴⁶¹ was

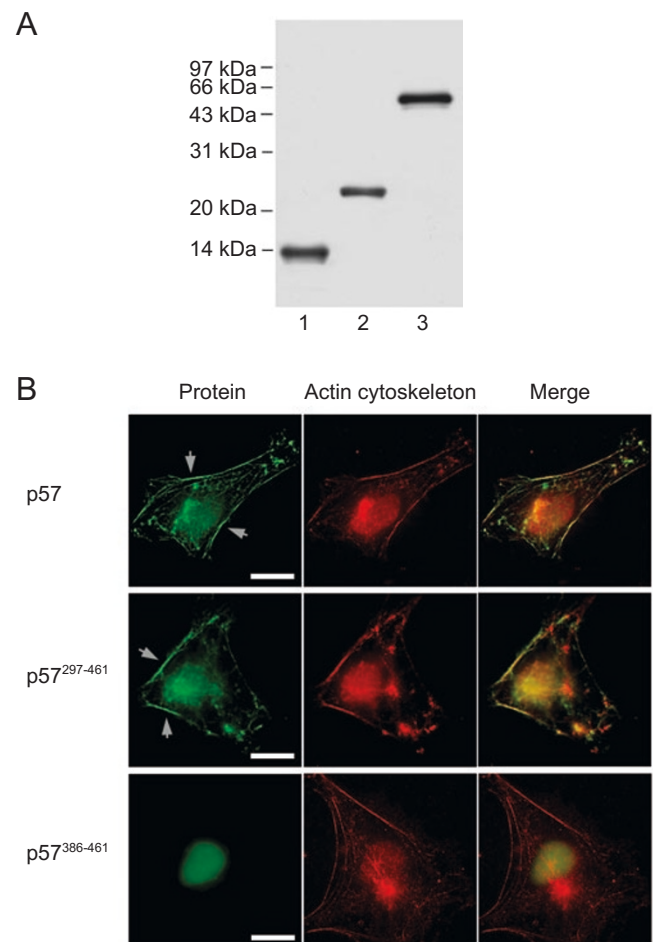


Figure 3 Co-localization analysis of p57 and its deletions with actin cytoskeleton in COS-1 cells. **(A)** Immunoblotting analysis. Expression plasmids pcDNA 4.0/p57, pcDNA 4.0/p57²⁹⁷⁻⁴⁶¹ and pcDNA 4.0/p57³⁸⁶⁻⁴⁶¹ were transiently expressed in COS-1 cells. Aliquots of cells were subjected to SDS-PAGE, followed by immunoblotting by anti-XpressTM monoclonal antibody; 1, p57³⁸⁶⁻⁴⁶¹ group; 2, p57²⁹⁷⁻⁴⁶¹ group; 3, p57 group. **(B)** Co-localization analysis. Expressed proteins and actin cytoskeleton were stained by anti-XpressTM antibody and anti-actin antibody, followed by secondary antibodies conjugated with FITC or rhodamine respectively. Images were acquired by fluorescence microscopy. *Arrowheads* marked areas of cortical localization of the expressed proteins. Scale bar=20 μ m. Experiments were performed more than three times with representative data shown.

investigated.

Firstly, two leucine residues (Leu-441 and Leu-447) out of the four that constitute the leucine zipper structure were replaced with alanine residues to destruct the leucine zipper domain. The destructive effect of the point mutation was then confirmed by a chemical cross-linking assay in cells. After p57²⁹⁷⁻⁴⁶¹ and p57^{297-461/Mut} were expressed in COS-1 cells, the cross-linking agent DSS was added to assess whether expressed proteins formed oligomers or not. The results from western blotting revealed that cross-linking of p57²⁹⁷⁻⁴⁶¹ by DSS led to the appearance of a dimer band (46 kDa, the monomer is 23 kDa) (Figure 4A), which was

consistent with the report of Oku *et al* [8] since no trimer or other higher-molecular-weight band was observed. However, in the p57^{297-461/Mut} sample, no dimer band was detected (Figure 4A). The results of the cross-linking experiment indicated that the point mutation destructed the leucine zipper domain effectively, which induced the dissociation of p57²⁹⁷⁻⁴⁶¹ dimer.

The actin-binding activity of p57^{297-461/Mut} was then examined. In co-sedimentation assay *in vitro*, most of GST-p57²⁹⁷⁻⁴⁶¹ protein co-sedimented with F-actin; however, little of GST-p57^{297-461/Mut} protein was detected in the pellet (Figure 4B). The results of immunoblotting analysis

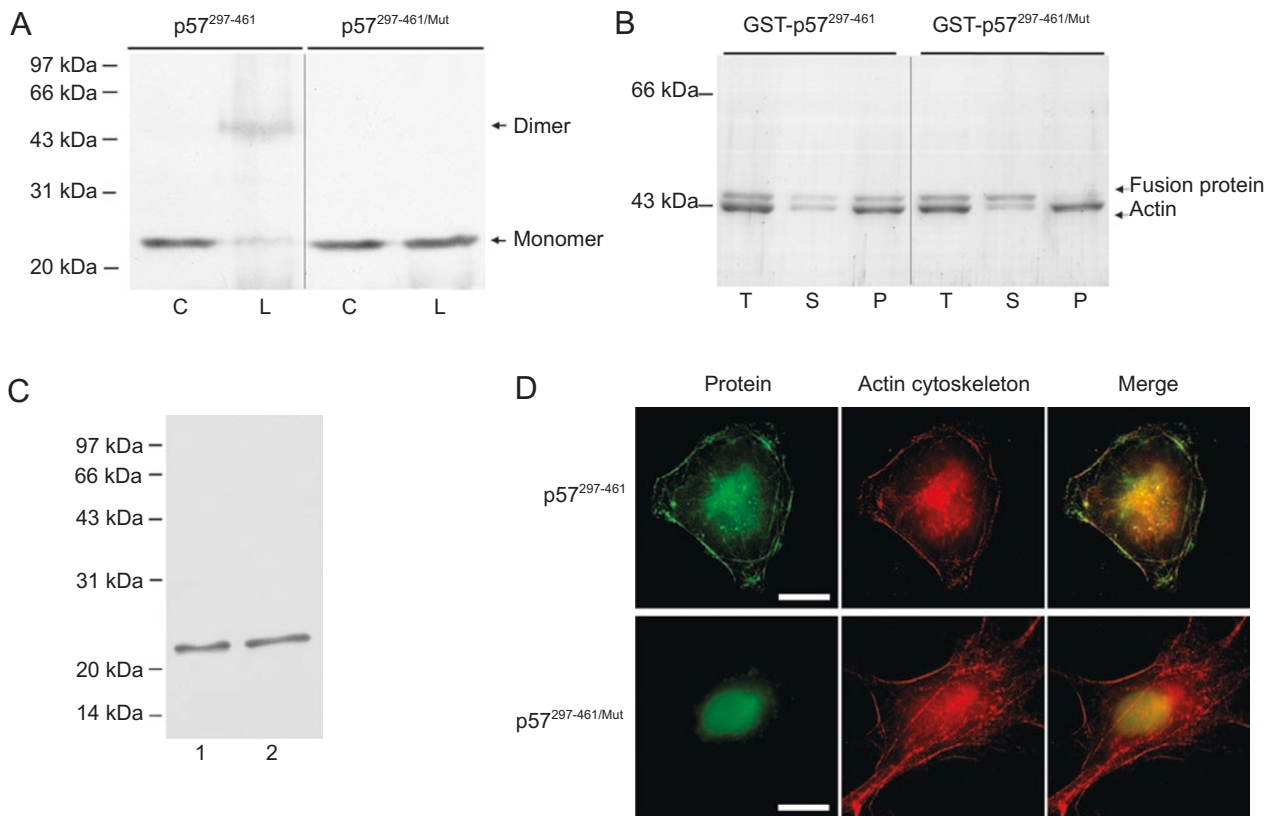


Figure 4 Effect of introduction of mutation into the leucine zipper domain of p57²⁹⁷⁻⁴⁶¹ on the actin-binding activity. **(A)** Cross-linking assay of p57²⁹⁷⁻⁴⁶¹ and p57^{297-461/Mut} expressed in COS-1 cell. Expression plasmids pcDNA 4.0/p57²⁹⁷⁻⁴⁶¹ and pcDNA 4.0/p57^{297-461/Mut} were introduced in COS-1 cells and expressed proteins were cross-linked by DSS. Cell fractions were subjected to SDS-PAGE and the monomer and dimer form of expressed p57²⁹⁷⁻⁴⁶¹ and p57^{297-461/Mut} were detected by immunoblotting analysis with anti-XpressTM monoclonal antibody; C, control group; L, cross-linking group. **(B)** *In vitro* co-sedimentation assay of GST-p57²⁹⁷⁻⁴⁶¹ and GST-p57^{297-461/Mut} with F-actin. After each fusion protein was incubated with F-actin in F-actin buffer, the mixtures were sedimented by ultracentrifugation, and fractions were subjected to SDS-PAGE. T, total reaction mixture; S, ultracentrifuged supernatant; P, ultracentrifuged precipitate. **(C)** Immunoblotting analysis. Expression plasmids pcDNA 4.0/p57²⁹⁷⁻⁴⁶¹ and pcDNA 4.0/p57^{297-461/Mut} were transiently expressed in COS-1 cells. Aliquots of cells were subjected to SDS-PAGE, followed by immunoblotted by anti-XpressTM monoclonal antibody; 1, p57²⁹⁷⁻⁴⁶¹ group; 2, p57^{297-461/Mut} group. **(D)** Co-localization analysis of p57²⁹⁷⁻⁴⁶¹ and p57^{297-461/Mut} with actin cytoskeleton in COS-1 cells. Expressed proteins and actin cytoskeleton were stained by anti-Xpress antibody and anti-actin antibody, followed by secondary antibody conjugated with FITC or rhodamine respectively, and images were acquired by fluorescence microscopy. Scale bars, 20 μm. Experiments were performed more than three times with representative data shown.

showed that p57²⁹⁷⁻⁴⁶¹ and p57^{297-461/Mut} in COS-1 cells were expressed as expected (both 23 kDa) and the expression levels of them were similar (Figure 4C). Then the results from immunofluorescence co-localization analysis indicated that the distribution of p57^{297-461/Mut} was different from that of p57²⁹⁷⁻⁴⁶¹ (Figure 4D), but similar to that of p57³⁸⁶⁻⁴⁶¹ (Figure 3B), which has no actin-binding activity. The above results suggested that the leucine zipper domain was essential for the actin-binding activity of p57²⁹⁷⁻⁴⁶¹.

The leucine zipper-containing region has no actin-binding activity, based on the actin-binding activity of p57³⁷²⁻⁴⁶¹ in report of Oku *et al* [12] and actin-binding activity of p57³⁸⁶⁻⁴⁶¹ in the present study (Figure 2C and 3B). However, this domain is important for actin-binding activity of p57²⁹⁷⁻⁴⁶¹. This conclusion is supported by studies of several proteins whose biological functions are affected by the leucine zipper domain [18-20]. Moreover, the N-terminal part of p57²⁹⁷⁻⁴⁶¹ (the region located among the residues 297-386) has no actin-binding activity, based on the actin-binding activity of p57²⁹⁷⁻⁴²⁹ in report of Oku *et al.* [12] and actin-binding activity of p57^{297-461/Mut} in the present study (Figure 4B and 4D), in both of which the contribution of the leucine zipper domain to actin binding was lacked. However, this region is also important for actin-binding activity of p57²⁹⁷⁻⁴⁶¹, judged from the difference of actin-binding activity between p57²⁹⁷⁻⁴⁶¹ and p57³⁸⁶⁻⁴⁶¹. Therefore, it is suggested that at least the leucine zipper-containing region and the N-terminal part of p57²⁹⁷⁻⁴⁶¹ are contained in the potential new actin-binding region at C-terminus of p57, since both the two regions are required for actin binding of p57²⁹⁷⁻⁴⁶¹.

Furthermore, the results of this study showed that the potential new actin-binding region in p57²⁹⁷⁻⁴⁶¹ was active only in the case of the integrity of the leucine zipper domain since the introduction of point mutation into the leucine zipper domain resulted in the complete loss of the actin-binding activity of p57²⁹⁷⁻⁴⁶¹ (Figure 4). The leucine zipper domain is closely related to the dimerization (or oligomerization) of p57 molecule [8, 9]. Therefore, we suppose that the dimerization (or oligomerization) of the p57²⁹⁷⁻⁴⁶¹ monomers may lead to the conformational change in the region surrounding the actin-binding site, which results in the presence of the actin-binding activity. This hypothesis seems reasonable for the similar contributions of the dimerizations to the conformational changes in relation to the biological functions reported in other proteins [21, 22], although these dimerizations are not mediated by the leucine zipper domain. Further structural analysis of p57 may examine this hypothesis and contribute to understand the underlying mechanism.

p57²⁹⁷⁻⁴⁶¹ can cross-link actin filaments

Since p57²⁹⁷⁻⁴⁶¹ has the actin-binding activity and is a homodimer through the leucine zipper domain [8], its activity to cross-link actin filaments was further investigated. p57²⁹⁷⁻⁴⁶¹ without GST tag was used to cross-link pre-assembled actin filaments and a low speed co-sedimentation assay was utilized to test the effect of cross-linking [23]. At low speed (15,000 × g), neither F-actin nor p57²⁹⁷⁻⁴⁶¹ alone was pelleted upon centrifugation (Figure 5). However, addition of p57²⁹⁷⁻⁴⁶¹ to pre-assembled actin filaments caused co-precipitation of the complex (Figure 5). Moreover, ex-

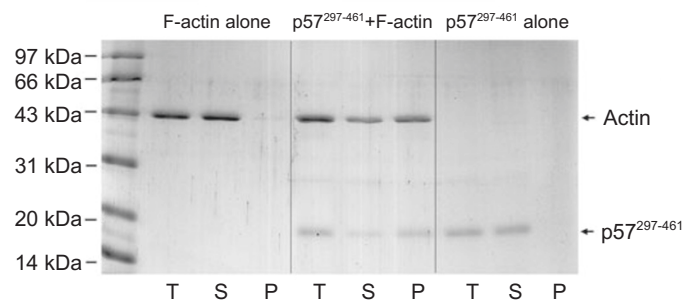


Figure 5 *In vitro* F-actin cross-linking assay of p57²⁹⁷⁻⁴⁶¹. Purified p57²⁹⁷⁻⁴⁶¹ without GST tag, F-actin or the mixture of them was sedimented at low speed (15,000 × g, 10 min). The fractions were subjected to SDS-PAGE. T, total reaction mixture; S, ultracentrifuged supernatant; P, ultracentrifuged precipitate.

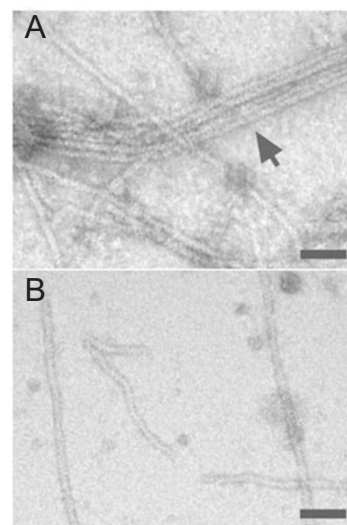


Figure 6 Visualization of actin filaments cross-linking by electron microscopy. F-actin or the mixture of p57²⁹⁷⁻⁴⁶¹ and F-actin was sedimented at low speed (15,000×g, 10 min). The pellets were resuspended, negatively stained and then examined by electron microscopy; (A) sample of the mixture of p57²⁹⁷⁻⁴⁶¹ and F-actin; (B) sample of F-actin alone. Arrowhead marks cross-linked actin filaments. Scale bar=50 nm.

amination of samples by electron microscopy showed that F-actin cross-linking networks formed only in the presence of p57²⁹⁷⁻⁴⁶¹ (Figure 6). The results from the co-sedimentation assays and electron microscopy indicate that p57²⁹⁷⁻⁴⁶¹ had the activity of cross-linking actin filaments *in vitro*.

It has been reported that the coronin-like proteins from budding yeast (*Saccharomyces cerevisiae*, Crn1p) can cross-link actin filaments into bundles and more complex networks [24]. The filament cross-linking activity of Crn1p is shown to depend on its C-terminal coiled-coil domain. Our study demonstrated that the F-actin cross-linking activity might be applicable to other coronin-like proteins including p57, since the C-terminal region of p57, p57²⁹⁷⁻⁴⁶¹, was efficient to cross-link actin filaments.

Based upon our works, as well as previous studies [12], we conclude that there are at least three regions responsible for actin binding of p57. The presence of multiple actin-binding regions on p57 may not only enhance the interaction between the protein and F-actin, but also contribute to the protein activity regulation (e.g. fixation of protein in a functional state when they bind with F-actin). These hypotheses await further investigation.

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