Homotypic dimerization of the actin-binding protein p57/coronin-1 mediated by a leucine zipper motif in the C-terminal region

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The actin-binding protein p57/coronin-1, a member of the coronin protein family, is selectively expressed in immune cells, and has been implicated in leucocyte migration and phagocytosis by virtue of its interaction with F-actin (filamentous actin). We previously identified two sites in the N-terminal region of p57/coronin-1 by which it binds actin, and in the present study we examine the role of the leucine zipper motif located in the C-terminal coiled-coil domain in mediating the homotypic association of p57/coronin-1. Recombinant p57/coronin-1 protein in solution formed a homodimer, as analysed by Superose 12 column chromatography and by sucrose density gradient centrifugation. *In vivo*, a truncated form consisting of the C-terminal coiled-coil domain co-precipitated with full-length p57/coronin-1 when both were co-expressed in COS-1 cells. A chimaeric construct composed of the C-terminal domain of p57/coronin-1 (which lacks the actin-binding sites) fused with green fluorescent protein co-localized

with cortical F-actin-rich regions in COS-1 cells only when full-length p57/coronin-1 was expressed simultaneously in the cells, suggesting that the C-terminal region is required for the homotypic association of p57/coronin-1. Furthermore, p57LZ, a polypeptide consisting of the C-terminal 90 amino acid residues of p57/coronin-1, was sufficient for dimerization. When two leucine residues out of the four that constitute the leucine zipper structure in p57LZ or full-length p57 were replaced with alanine residues, the mutants failed to form homodimers. Taken together, these results demonstrate that p57/coronin-1 forms homodimers, that the association is mediated by the leucine zipper structure in the C-terminal region, and that it plays a role in the cross-linking of F-actin in the cell.

Key words: actin-binding protein, coiled-coil domain, coronin, homodimer, leucine zipper.

INTRODUCTION

Actin-binding proteins participate in reorganization of the actin cytoskeleton during chemotaxis, phagocytosis, degranulation and cellular signalling [1–5] and thus are essential for normal leucocyte function. We previously identified p57/coronin-1, an actin-binding protein that is selectively expressed in immune cells [6] and shares sequence identity with coronin, an actin-binding protein purified from the actomyosin complex of *Dictyostelium discoideum* [7]. Coronin localizes at intracellular sites of active reorganization of the actin cytoskeleton, such as the lamellipodium, phagocytic cup and contractile ring in *Dictyostelium*, and has thus been assigned an important functional role in migration, phagocytosis and cell division [8–14]. Homologues of coronin have been identified in many eukaryotes from yeasts to mammals [15], consistent with its important function in cell biology. Whereas lower eukaryotes such as *Dictyostelium* and *Saccharomyces* possess only one isoform, at least five homologues have been identified in humans [15,16]; these are currently designated coronin-1 to -5, and constitute collectively the coronin protein family. The differential tissue distribution of specific family members implies a selective and tissue-specific function for each isotype. For example, immune cell-specific p57/coronin-1 plays a crucial role in phagolysosome formation in leucocytes [17]. Successful intracellular parasitism of murine macrophages by mycobacteria has been linked to organism-dependent modulation of p57/coronin-1 [18].

Most coronin protein family members, including p57/coronin-1, contain 450–500 amino acid residues and share common structural features, such as five WD (Trp-Asp) repeats located at the centre of the molecule and a coiled-coil domain at the C-terminus. Our previous report demonstrated that p57/coronin-1 possesses two actin-binding sites, one located in the N-terminal region and the other within the WD repeats, and that each also contains a cluster of basic amino acids [19]. The coiled-coil domain of p57/coronin-1 includes a so-called leucine zipper motif, with four leucine residues that appear every seven amino acid residues [6]. Because the leucine zipper motif is not found in other mammalian coronin members [16,20–24], we reasoned that it may serve a function unique to p57/coronin-1, and tested the hypothesis that the leucine zipper motif mediates homodimer formation.

MATERIALS AND METHODS

Reagents

Restriction endonucleases and modifying enzymes were purchased from TaKaRa (Osaka, Japan), Toyobo (Osaka, Japan) and Gibco BRL (Rockville, MD, U.S.A.). Glutathione–Sepharose 4B, Protein G–Sepharose 4 Fast Flow and Hybond-ECLTM nitrocellulose membranes were products of Amersham Biosciences (Piscataway, NJ, U.S.A.). Triton X-100, aprotinin, PMSF and rhodamine-conjugated phalloidin were purchased from Sigma

Abbreviations used: EGFP, enhanced green fluorescent protein; F-actin, filamentous actin; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; p57FL, full-length p57/coronin-1; p57LZ, truncated form of p57/coronin-1 comprising amino acid residues 372–461; p57WD, truncated form of p57/coronin-1 comprising amino acid residues 1–371.

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Figure 1 Analysis of the molecular size of recombinant p57/coronin-1 and its truncated forms by gel chromatography

(A) Schematic diagrams of structures of p57FL and its truncated forms (p57WD and p57LZ) are shown. (B) Recombinant p57FL, p57WD and p57LZ were applied to a column of Superose 12 10/300 GL and fractions (0.5 ml) were collected. Each fraction was analysed by SDS/PAGE followed by immunoblotting using anti-p57/coronin-1 antibody. The molecular mass markers used are thyroglobulin (670 kDa), IgG (158 kDa), BSA (67 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa).

(St. Louis, MO, U.S.A.). Nonidet P-40 was from Nacalai Tesque (Kyoto, Japan). Lysozyme and IPTG (isopropyl β -D-thiogalactoside) were purchased from Seikagaku Corp. (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan) respectively. Oligonucleotides were supplied by Amersham-Pharmacia Biotech (Tokyo, Japan).

Antibodies

Two antibodies against human p57/coronin-1 were used in the present study: a monoclonal antibody (N7) that recognizes the C-terminal region of human p57/coronin-1 [25], and a rabbit polyclonal antibody that recognizes the N-terminal 20 amino residues of human p57/coronin-1 [19]. Horseradish peroxidase-conjugated goat antibodies to mouse IgG and rabbit IgG were purchased from Kirkegaard & Perry Laboratories Inc. (Guildford, Surrey, U.K.) and Caltag Laboratories (Burlingame, CA, U.S.A.) respectively.

DNA constructs

The cDNAs for p57FL (full-length p57/coronin-1) and the truncated forms p57WD (amino acid residues 1–371, including five WD repeats) and p57LZ (amino acid residues 372–461, including a coiled-coil domain with a leucine zipper motif) (Figure 1A) in a pGEX-5X-1 vector (Amersham Biosciences) were prepared as described previously [19]. A His-tagged p57FL construct was also prepared by the insertion of the p57FL cDNA into the BamHI/SalI site of a pQE-32 vector (Qiagen, Hilden, Germany). Mutations were introduced into the leucine zipper motif of p57LZ and p57FL by using a QuickChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. pGEX-p57LZ[AALL] and pQE-p57FL[AALL] (in which Leu-433 and Leu-440 of p57/coronin-1 are replaced with Ala) were generated from pGEX-p57LZ and pQE-p57FL respectively, by using the primers 5'-CGTGTCTCGGG-CGGAGGAGGAGATGCGGAAGGCCAGGCCACGGTGCA-GG-3' (sense) and 5'-CCGTGGCCTGGGCCTTCCGCATCTC-CTCCTCCGCCGAGACACGGCATCC-3' (antisense). p57LZ-[AAAA] (in which Leu-433, Leu-440, Leu-447 and Leu-454 are replaced with Ala) was prepared using pGEX-p57LZ[AALL] as a

template and the primers 5'-CGGTGCAGGAGGCCAGAACG-GCTTGGACAGGGCGGAGGAGACAGTCCAG-3' (sense) and 5'-CTGTCTCTCCGCCCCTGTCCAAGCGCTTCTGGGCCT-CCTGCACCGTGGC-3' (antisense). The cDNAs for the full-length and truncated forms of p57/coronin-1 for expression in mammalian cells were constructed in pcDNA3.1-V5-His (Invitrogen, Carlsbad, CA, U.S.A.) as described in [19]. A construct for a fusion protein of p57LZ with EGFP (enhanced green fluorescent protein) (pEGFP-p57LZ) at the N-terminus was prepared by PCR using a set of primers (sense, 5'-GGGGAATCCCTTGCCCTCACGGCTGA-3'; antisense, 5'-GGGGATCCCTA-CTTGGCCTGGACTGTCT-3'), followed by digestion with EcoRI and BamHI and subcloning in a pEGFP-C2 vector (BD Bioscience Clontech, Palo Alto, CA, U.S.A.).

Expression and purification of recombinant proteins

The following recombinant proteins were produced in *Escherichia coli* (JM109 or DH5 α) as fusion proteins with GST (glutathione S-transferase): p57FL (Met-1 to Lys-461), p57WD (Met-1 to Asp-371), p57LZ (Pro-372 to Lys-461), p57LZ[AALL], and p57LZ[AALL]. The transformed *E. coli* grown to exponential phase was cultured further in TB medium [500 ml; 12 g/l tryptone, 24 g/l yeast extract, 0.4% (v/v) glycerol, 17 mM KH₂PO₄ and 72 mM K₂HPO₄] containing 0.5 mM IPTG at 20 °C for 16 h [19]. The cells were harvested by centrifugation, resuspended in 30 ml of sonication buffer (50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl and 1 mM EDTA), and lysozyme was added to the suspension to a final concentration of 1.0 mg/ml. After the mixture was incubated at 4 °C for 20 min, the cells were disrupted by sonication and centrifuged at 15 000 g for 30 min. The supernatant was mixed with glutathione-Sepharose 4B beads (0.5 ml of a 50% suspension), and the mixture was incubated at 4 °C for 30 min with occasional stirring. After washing the beads five times with sonication buffer to remove unbound proteins, recombinant proteins were recovered by treatment of the washed beads with Factor Xa under conditions as follows: fusion protein-bound glutathione-Sepharose 4B was suspended in 0.5 ml of 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 1 mM CaCl₂ and 40 units/ml Factor Xa protease (Qiagen) and incubated at 22 °C for 16 h.

The recombinant wild-type and mutated full-length p57/coronin-1 proteins were also purified from the culture medium of *E. coli* transformed with pQE-p57FL[LLLL] and pQE-p57FL[AALL] respectively. The *E. coli* cell pellet obtained from 300 ml of culture [LB (Luria-Bertani) medium containing 0.5 mM IPTG at 37 °C for 3 h was disrupted by sonication, and the supernatant was collected by centrifugation at 15 000 g for 30 min. The recombinant proteins were purified by affinity chromatography on a column of anti-p57/coronin-1 antibody (N7) conjugated on HiTrapTM NHS (*N*-hydroxysuccinimide)-activated HP (Amersham Biosciences). The purity of the recombinant proteins was found to be > 95%, as checked by SDS/PAGE followed by the silver staining method.

Gel chromatographic analysis

Recombinant p57/coronin-1 solutions (0.1–0.5 mg/ml; 0.1 ml) were separated by gel chromatography on a column of Superose 12 10/300 GL (Amersham Biosciences) equilibrated with 20 mM Tris/HCl, pH 7.5, containing 140 mM NaCl. The elution was performed with the same buffer at a flow rate of 0.5 ml/min at room temperature. Fractions of 0.5 ml were collected, and each fraction was analysed by immunoblotting using anti-p57/coronin-1 antibody [19]. The molecular mass markers used were: thyroglobulin (670 kDa), IgG (158 kDa), BSA (67 kDa),

ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa).

Electrophoresis and immunoblotting

SDS/PAGE was conducted in 10–20% (w/v) polyacrylamide gels in the presence of 0.1% SDS [26]. The separated proteins were electrotransferred to nitrocellulose membranes with a semi-dry blotting system (Bio-Craft, Tokyo, Japan) using transfer buffer [5.82 g/l Tris, 2.93 g/l glycine, 0.39 g/l SDS, 20% (v/v) methanol] at 1.6 mA/cm² for 45 min. After blocking with a milk protein-based reagent (BlockAce™; Dainippon Pharmaceutical Co., Osaka, Japan) for 20 min, the membranes were treated successively with anti-p57/coronin-1 monoclonal antibody (N7; 5 µg/ml) or polyclonal antibody (dilution of 1:100) for 1 h, and then with a horseradish peroxidase-conjugated secondary antibody (dilution of 1:1000 to 1:5000) for 30 min. The bands were visualized by enhanced chemiluminescence (ECL®; Amersham Biosciences).

Transient expression of p57/coronin-1 in COS-1 cells

COS-1 cells (A.T.C.C.) were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco BRL) at 37°C under humidified 5% CO₂.

The plasmids encoding p57/coronin-1 and its deletion mutants were introduced into COS-1 cells by electroporation. Briefly, COS-1 cells (3.5×10^6 cells/0.7 ml) in PBS were mixed with 40 µg of plasmid DNA. The mixture was transferred into a 0.4 cm electrode gap electroporation cuvette and subjected to a single pulse of 300 V at a capacitance of 975 µF (Gene Pulser Xcell™; Bio-Rad Laboratories, Hercules, CA, U.S.A.). The cell suspension was diluted with 10 ml of RPMI 1640 medium containing 10% (v/v) fetal calf serum and cultured at 37°C for 48 h.

Immunoprecipitation

COS-1 cells (3.5×10^6 cells) transiently expressing p57/coronin-1 were incubated with 1.0 ml of lysis buffer (50 mM Tris/HCl, pH 7.5, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 µg/ml aprotinin and 1 mM PMSF) at 4°C for 30 min, and the cell lysate was centrifuged at 15000 g for 10 min. The supernatant was precleared by incubation with 50 µl of Protein G-Sepharose (50% suspension) at 4°C for 1 h with gentle shaking. After the suspension was centrifuged at 3300 g for 3 min, the supernatant was mixed with 20 µl of Protein G-Sepharose and 20 µl of anti-p57/coronin-1 polyclonal antibody (N-terminal specific), and the mixture was incubated at 4°C for 16 h. Protein G-Sepharose beads were then washed five times with lysis buffer to remove unbound proteins. Proteins bound to the beads were recovered by treatment with a sample buffer for SDS/PAGE at 95°C for 5 min.

Immunofluorescence microscopy

COS-1 cells that had been transfected with plasmids containing a gene for EGFP were cultured on a Lab-Tek II Chamber Slide (Nalge Nunc, Rochester, NY, U.S.A.) at 37°C for 48 h and fixed with 3.8% neutral buffered formaldehyde (Wako Pure Chemicals). For the analysis of localization of F-actin (filamentous actin), the cells were then permeabilized by treatment with 0.2% Triton X-100 in PBS for 10 min at room temperature, washed with PBS, and incubated with rhodamine-conjugated phalloidin (15 units/ml) in PBS containing 3% (w/v) BSA for 30 min. Fluorescently labelled cells were washed three times with PBS and then mounted with 2.3% (v/v) 1,4-diazabicyclo-2,2,2-octane (Sigma) containing 90% (v/v) glycerol on glass slides. Fluor-

escent images were analysed with a confocal laser-scanning microscope (Radiance 2100; Bio-Rad). Fluorescence in the green and red channels was visualized using the 488 nm (argon laser) and 543 nm (helium/neon laser) laser lines respectively. Images were collected with non-saturating conditions set-up by the use of an output LUT (look-up table).

Sucrose density gradient centrifugation

Solutions of recombinant p57FL[LLLL] and p57FL[AALL] (approx. 0.5 mg/ml; 0.2 ml) were loaded on to the top of a 12 ml linear 5–20% (w/v) sucrose gradient in buffer comprising 40 mM Tris/HCl, pH 7.5, 1 M NaCl, 0.2 mM EDTA and 0.02% Nonidet P-40. Centrifugation was conducted at 150000 g with a Hitachi P40ST rotor (Hitachi, Ltd., Tokyo, Japan) for 18 h at 4°C, and fractions of 1.0 ml were collected. An aliquot of each fraction was analysed by SDS/PAGE followed by immunoblotting using polyclonal anti-p57/coronin-1 antibody. Native p57/coronin-1 endogenously expressed in human HL60 promyelocytic leukaemia cells was analysed in an identical fashion. HL60 cells were lysed as described above for immunoprecipitations and separated by centrifugation on a sucrose density gradient. Fractions were separated by SDS/PAGE and probed for p57/coronin-1 by immunoblotting.

RESULTS

Analysis of molecular size of recombinant p57/coronin-1 by gel chromatography

We separated human p57/coronin-1 produced in *E. coli* by gel chromatography on a Superose 12 column to assess its molecular size. p57FL eluted from the chromatography column in fractions corresponding to a molecular mass of 90–110 kDa (Figure 1B), rather than the predicted mass of 57 kDa, suggesting that recombinant p57/coronin-1 formed homodimers. The sequence of p57/coronin-1 contains both WD repeats and a coiled-coil domain containing a leucine zipper motif (Figure 1A), each of which can mediate protein–protein interactions and thus are potential participants in homodimer formation. To distinguish if these motifs are responsible for dimerization of the recombinant p57/coronin-1, we prepared two truncated forms, p57WD (amino acid residues 1–371, including five WD repeats) and p57LZ (amino acid residues 372–461, including a coiled-coil domain with a leucine zipper motif) (Figure 1A), and analysed their elution profiles after separation by gel chromatography. p57WD was eluted in a fraction that corresponded to a molecular mass of 35–45 kDa (Figure 1B), consistent with its being a monomer compactly folded due to the WD repeats. On the other hand, p57LZ was recovered in fractions corresponding to a molecular mass of 20–25 kDa, approximately twice the size predicted (~11 kDa) based on its sequence, consistent with it being a dimer. Taken together, these findings suggest that recombinant p57/coronin-1 formed a homodimer and that the C-terminal 90 amino acids were responsible for dimerization.

Homotypic association of p57/coronin-1 in COS-1 cells

To determine if p57FL and p57LZ associate physically in intact cells, we expressed the constructs in COS-1 cells and assessed their interactions immunochemically. For these studies we utilized two anti-p57/coronin-1 antibodies: an antibody recognizing the N-terminal 20 amino acids of p57/coronin-1 to perform immunoprecipitation, and an antibody recognizing the C-terminal region of p57/coronin-1 (N7) to probe p57FL and p57LZ on blotted membranes. The N-terminal-specific antibody precipitated p57FL, but not p57LZ, when COS-1 cells were transfected

Figure 2 Homotypic interaction of p57/coronin-1 mediated by its C-terminal region

COS-1 cells were transfected with cDNAs encoding p57FL alone, p57LZ alone, or both. Cell lysates were subjected to immunoblotting using an antibody that selectively recognizes the C-terminal region of p57/coronin-1 (right panel). Immunoprecipitates by antibody recognizing the N-terminal region of p57/coronin-1 were analysed by immunoblotting using an antibody specific for the C-terminal region of p57/coronin-1 (left panel).

separately with either p57FL or p57LZ cDNA (Figure 2, left panel), consistent with the absence from p57LZ of the N-terminal sequence. We detected no band corresponding to p57FL in immunoprecipitates from either p57LZ-transfected (Figure 2, left panel) or untransfected (results not shown) COS-1 cells, indicating that these cells do not constitutively express p57/coronin-1. By contrast, when the cells were co-transfected with p57FL and p57LZ cDNAs, p57LZ (molecular mass 11 kDa) was co-precipitated with p57FL (molecular mass 57 kDa) by the N-terminal-specific antibody (Figure 2, left panel). Failure to recover p57LZ in the singly transfected cells did not reflect the absence of protein, as the expression of p57FL and p57LZ after each cDNA transfection was confirmed by SDS/PAGE followed by immunoblotting (Figure 2, right panel). These results strongly suggest that p57LZ was physically associated with p57FL in COS-1 cells.

We next analysed the intracellular distribution of p57LZ after co-transfection of p57FL and p57LZ cDNAs into COS-1 cells. For this purpose, we prepared a construct in which p57LZ cDNA was ligated with a vector containing the EGFP gene. The chimaeric

fusion protein (EGFP-p57LZ) was expressed in COS-1 cells and the transfected cells were examined using confocal laser-scanning microscopy. EGFP-p57LZ was diffusely distributed in the cytoplasm after transfection with pEGFP-p57LZ alone (Figure 3, upper panels). In contrast, when EGFP-p57LZ and p57FL were expressed simultaneously in COS-1 cells, EGFP-p57LZ was, at least in part, localized in the F-actin-rich cortical region of the cell (Figure 3, lower panels). The intracellular distribution of EGFP-p57LZ after the co-transfection was similar to that of p57FL expressed in COS-1 cells as assessed by immunofluorescence in our previous study [19]. Co-localization of F-actin and EGFP-p57LZ, a construct devoid of actin-binding sites, suggests physical interaction between EGFP-p57LZ and p57FL. These results support the conclusion that the C-terminal region of p57/coronin-1 mediates its homotypic association. Furthermore, the failure of EGFP-p57LZ to associate with F-actin-rich subcellular compartments after the single transfection indicates that the coronin species endogenous to COS-1 cells did not associate with the tagged construct.

Dimerization of p57/coronin-1 via leucine zipper motifs

The sequence of the coiled-coil domain at the C-terminus of p57/coronin-1 contains four leucine residues that appear every seven amino acid residues, a structural unit known as a leucine zipper motif (Figure 4A) [27–29]. When the sequence is arranged in α -helical heptad repeats as shown in Figure 4(B), the leucine residues at position 'd' as well as valine, leucine and methionine residues at position 'a' form a hydrophobic surface. To examine a role of the leucine zipper motif in the dimerization of p57/coronin-1, we expressed in *E. coli* the mutant protein p57LZ with replacement of these specific leucine residues: p57LZ[AAAA] (Leu-433, Leu-440, Leu-447, and Leu-454 replaced with Ala) and p57LZ[AALL] (Leu-433 and Leu-440 replaced with Ala). Under chromatography conditions identical to

Figure 3 Intracellular localization of EGFP-p57LZ expressed in COS-1 cells

EGFP-p57LZ was transiently expressed in COS-1 cells after transfection with pEGFP-p57LZ alone (upper panels) or co-transfection of pEGFP-p57LZ and the cDNA for p57FL in pcDNA3.1-V5-His (lower panels). Fluorescent images were observed 48 h after the transfection with a confocal laser-scanning microscope. Co-localization of EGFP-p57LZ and F-actin (stained with phalloidin) is shown by arrows. Scale bars = 20 μ m.

Figure 4 Formation of a homodimer of p57LZ mediated by a leucine zipper motif

(A) The sequence of the seven-amino-acid repeats at the C-terminus of p57/coronin-1 [6]. (B) Schematic diagram of a dimeric parallel coiled-coil structure [27]. (C) Immunoblotting analysis of fractions from gel chromatography. Wild-type p57LZ or its mutants were applied to a Superose 12 10/300 GL column and fractions (0.5 ml) were collected. Each fraction was analysed by SDS/PAGE followed by immunoblotting with anti-p57/coronin-1 antibody (p57LZ[LLLL] and p57LZ[AALL]) or Coomassie Brilliant Blue staining (p57LZ[AAAA]).

those used previously, both p57LZ[AAAA] and p57LZ[AALL] were retarded in their elution pattern when compared with wild-type p57LZ (p57LZ[LLLL]) (Figure 4C). Whereas the wild-type construct eluted with a molecular mass of 22 kDa, consistent with it being dimeric, the elution positions of the mutants corresponded to a monomeric form of p57LZ (molecular mass 11 kDa), suggesting that mutation of the leucine zipper motif results in failure of homodimer formation of p57/coronin-1.

We next examined the effects of site-directed mutation of full-length p57/coronin-1 with regard to its capacity to form dimers. A mutant containing substitution of Ala residues for Leu-433 and Leu-440, p57FL[AALL], was chromatographed on a Superose 12 10/300 GL column and each fraction was analysed by SDS/PAGE and immunoblotting (Figure 5A). The mutant p57FL[AALL] showed a retarded elution time as compared with wild-type p57FL[LLLL]. When proteins were monitored by A_{280} , p57FL[LLLL] and p57FL[AALL] were eluted at 23.85 min and 25.58 min respectively. Based on the calibration curve with standard proteins, the molecular masses of these proteins were estimated to be approx. 110 kDa for p57FL[LLLL] and approx. 55 kDa for p57FL[AALL] (Figure 5B). The estimated molecular masses of these proteins strongly suggested that p57FL[LLLL] formed a homodimer, but that p57FL[AALL] remained as a monomer. The differential ability of p57FL[LLLL] and p57FL[AALL] to dimerize was confirmed by sucrose density gradient centrifugation followed by SDS/PAGE and immunoblotting (Figure 5C). The bulk of the mutant protein p57FL[AALL] sedimented in lower-density fractions as compared with wild-type p57FL[LLLL]. When the lysate of HL60 cells was applied to sucrose density centrifugation under the same conditions, p57/coronin-1 was recovered in identical fractions to the recombinant p57FL[LLLL]. Furthermore, we conducted a pull-down assay, using an approach similar to the experiment shown in Figure 2, by using the mutant p57FL[AALL] instead of p57FL[LLLL]. However, the N-terminal-specific antibody did not co-precipitate wild-type p57LZ[LLLL] after COS-1 cells had been transfected with both p57FL[AALL] and p57LZ[LLLL] cDNA (results not shown). These results suggest that p57/coronin-1 forms homo-

Figure 5 Effects of mutations in the leucine zipper motif of p57/coronin-1 on homodimer formation

(A) Wild-type p57FL[LLLL] and the mutant p57FL[AALL] were chromatographed on a Superose 12 10/300GL column, and an aliquot of each fraction (0.5 ml) was analysed by SDS/PAGE and immunoblotting using a polyclonal anti-p57/coronin-1 antibody. (B) The elution times of standard proteins from a Superose 12 10/300GL column and their molecular masses were plotted on a semi-logarithmic scale: IgG (158 kDa), BSA (67 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). (C) p57FL[LLLL] and p57FL[AALL] were subjected to 5–20% (w/v) sucrose density gradient centrifugation. Fractions (1.0 ml) were collected and an aliquot of each fraction was analysed by SDS/PAGE and immunoblotting using an anti-p57/coronin-1 antibody. Arrowheads indicate the fractions containing most of the recombinant proteins. G-actin (43 kDa) and p57/coronin-1 derived from HL60 cells were recovered in the fractions indicated by arrows.

dimers mediated by the leucine zipper motif in the C-terminal region of the molecule.

DISCUSSION

Since the expression of p57/coronin-1 is restricted to immune cells, we reasoned that this protein participates in cellular events that are integral for normal leucocyte responses, such as phagocytosis, chemotaxis and signal transduction via actin reorganization. During phagosome maturation, p57/coronin-1 associates transiently with nascent phagosomes, co-incident with F-actin and with the same kinetics [17,25]. Furthermore, translocation of p57/coronin-1 is regulated by protein kinase C-mediated phosphorylation [17], as inhibitors of protein kinase C prevent the dissociation of p57/coronin-1 and F-actin from the periphagosome and subsequent phagosome-lysosome fusion. Taken together, these observations suggest that p57/coronin-1 contributes to the reorganization of the periphagosomal F-actin network during phagocytosis.

In the present study, we assessed the multimerization of p57/coronin-1 both in solution and in cells by employing isolated recombinant proteins and cDNA transfection of COS-1 cells.

When separated by gel chromatography on a Superose 12 column and by sucrose density centrifugation, p57/coronin-1 behaved like a homodimer. Furthermore, the isolated C-terminal region, consisting almost exclusively of the coiled-coil domain (p57LZ), was sufficient to support dimer formation (Figure 1), implicating that domain in mediating homodimer formation by the holoprotein. Homodimer formation was not a property restricted to recombinant proteins in solution, but occurred *in vivo* as well. As assessed by co-immunoprecipitation and by fluorescence microscopy, the homotypic association of p57/coronin-1 mediated by the C-terminal region was also observed in COS-1 cells after expression of p57FL and its truncated forms (Figures 2 and 3). Moreover, mutation of at least two leucine residues in the leucine zipper motif of p57LZ and p57FL caused failure of dimer formation (Figures 4 and 5), thus indicating that the leucine zipper motif is essential for dimer formation. It is most likely that the homotypic association is mediated by hydrophobic interaction between the surface formed on one side of the C-terminal coiled-coil domain of each polypeptide, with four leucine residues that are present in position 'd' of the α -helical heptad structure and hydrophobic amino acid (valine, leucine and methionine) residues in position 'a' (Figure 4B).

Although most members of the coronin protein family contain coiled-coil domains in their C-terminal regions [15], and this domain has been suggested to be involved in homotypic multimer formation, p57/coronin-1 is the first mammalian coronin, to our knowledge, that has been demonstrated to form a homodimer. Human coronin-3 forms a homotypic oligomer [21], although gel chromatographic analysis indicates that it is a higher-ordered oligomer than that formed by p57/coronin-1, and its oligomerization seems to be heterogeneous, with the molecular mass of most of this protein estimated to be 150–200 kDa. Coronin-3 is therefore thought to form trimeric or tetrameric oligomers rather than dimers [21], an organization likely to reflect more complex interactions between the N-terminal and C-terminal regions. Differences in intermolecular associations among members of the coronin family of proteins may reflect in part structural polymorphisms within their C-terminal regions. Among mammalian coronins, only p57/coronin-1 possesses a leucine zipper motif in the coiled-coil domain, and this feature is highly conserved among several species, including human, bovine and mouse [6,18].

The homotypic dimer formation of p57/coronin-1 via a leucine zipper structure appears to be rather stable, as the dimer did not dissociate under various stringent buffer conditions (e.g. non-ionic detergents, chaotropic ions and high ionic strength) (T. Oku, S. Itoh, R. Ishii and T. Tsuji, unpublished work). Schematically, the overall structure of p57/coronin-1 can be represented as a V-shaped homodimeric structure, with each polypeptide chain with an interconnecting domain including a leucine zipper motif at the C-terminus and two actin-binding sites near the N-terminus [19] (Figure 6). Such a domain organization for p57/coronin-1 resembles that of filamin, another actin-binding protein [30]. These actin-binding proteins have a domain structure that is well suited for cross-linking or bundling of F-actin. Filamin is, in fact, known to promote the gelation of actin filament solutions [31]. Among the coronin family of proteins, for example, Crn1p from yeast promotes the assembly of actin filaments and cross-linking of filaments into bundles [32], although its oligomeric structure is not known. It is likely that each member of the coronin protein family adopts a specific oligomeric structure especially tailored to its function. Members of the coronin protein family have at least one method of homodimer formation that is independent of the leucine zipper. A non-mammalian coronin isolated from *Xenopus* oocytes (Xcoronin), which lacks a leucine zipper motif, also forms a homodimer [33]. It is likely that protein domains in

Figure 6 Schematic representation of the structure of p57/coronin-1

p57/coronin-1 forms a homodimer, which is supported by a leucine zipper motif (L) located at the C-terminus of each polypeptide chain. Actin-binding sites are located at the N-terminus and within WD repeats, which are organized into a β -propeller structure [19].

Xcoronin substitute functionally for the leucine zipper motif in supporting dimer formation [34].

In conclusion, our data demonstrate that p57/coronin-1 formed a homodimer, mediated by a leucine zipper motif in the C-terminal region, both in a cell-free system with recombinant proteins in solution and in COS-1 cells. Among members of the coronin protein family, dimer formation is a property unique to p57/coronin-1, and thus is likely to participate in specialized cellular functions, such as the reorganization of the actin cytoskeleton during phagocytosis by immune cells. Elucidation of the mechanism for the cross-linking or bundling of actin filaments by p57/coronin-1 homodimers and its regulation during phagocytosis will provide insight into the cell biology underlying a fundamental and essential activity of cells in the innate immune system.

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