Molecular cloning of a novel actin-binding protein, p57, with a WD repeat and a leucine zipper motif

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Abstract A 57 kDa protein (p57) was obtained during the study on phosphatidylinositol-specific phospholipase C. Its cDNA was isolated from calf spleen and human leukemia cell line HL60 libraries and cloned. In the primary structures of p57, they have two unique amino acid sequence motifs, a WD repeat and a leucine zipper motif. Furthermore, p57 shared sequence similarity (40%) with coronin, an actin-binding protein responsible for chemotaxis, cell motility, and cytokinesis of *Dictyostelium discoideum*, which has only the WD repeat. p57 also showed an actin-binding activity and was mainly expressed in immune tissues. From these results, we conclude that p57 is a coronin-like novel actin-binding protein in mammalian cells but may also have a different function from coronin.

Key words: cDNA cloning; Actin-binding protein; WD repeat; Leucine zipper motif; Coronin-homologue

1. Introduction

We have been interested in signal transduction processes at various receptor systems and studying signal transduction molecules involved in these processes, especially in processes involving those of leukocyte activation and chemotaxis. Phosphatidylinositol-specific phospholipase C (PI-PLC) is one of the key molecules in these signal transduction pathways and has been examined extensively [1–4]. At the process of studying the PLC with a smaller molecular mass, we obtained a protein with an apparent molecular mass of 57 kDa (p57) from the cytosol fraction of calf thymocyte. The primary structure of p57 shared sequence similarity with coronin [5], an actin-binding protein responsible for chemotaxis, cell motility, and cytokinesis [6] of *Dictyostelium discoideum*. From this result, we expected that p57 might have an actin-binding activity.

Many actin-binding proteins have been identified and studied extensively [7], because the interaction of actin and actin-binding proteins is considered to be very important for the cytoskeletal function. The cytoskeleton does not only regulate a cell shape, motility, and cytokinesis but also provides a place for various signal transduction pathways.

In the present study, cDNA of p57 was isolated and its sequence was determined. Sequence analysis showed that p57 has two unique amino acid motifs, a WD repeat and a leucine zipper motif, and that the protein shares significant similarity with coronin, an actin-binding protein of *Dictyostelium discoideum*. Therefore, an assay for actin-binding activity was also performed. Furthermore, its tissue distribution in mouse was investigated. The results of these studies suggest that p57 is a novel actin-binding protein that was mainly expressed in immune tissues.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following Accession Numbers D44496: bovine mRNA for bovine p57 and D44497: Human mRNA for human p57.

2. Materials and methods

2.1. Purification of p57 according to phosphoinositide hydrolysis activity

All steps were performed at 4°C. According to the phosphoinositide hydrolysis activity, purification steps were done with DEAE Sepharose CL-6B, Heparin Sepharose CL-6B, Sepharose CL-6B, Mono S and Superose 12 column chromatography, sequentially. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [10] of an active fraction from the final purification step gave a single band with molecular mass of 57 kDa. The phosphoinositide hydrolysis assay was performed as described previously [11].

2.2. Sequence analysis of the lysyl-C peptides of purified p57

Purified p57 was digested with endopeptidase lysyl-C (Boehringer Mannheim) as described previously [11]. p57-derived lysyl-C peptides were microsequenced using a PSQ-1 peptide sequencer (Shimadzu, Kyoto, Japan).

2.3. cDNA cloning of calf p57

For the plaque hybridization assay of calf p57 cDNA, a cDNA probe was produced by PCR. The template (5′-GATCAGTGCTATGAGG-ACGTCCGCGTCTCCAG-3′) was prepared from the results of peptide microsequencing, that was derived from a lysyl-C peptide of purified p57. The complement primer (5′-CTGGAGA-3′) was used for labeling. The produced probe was used for screening a λgt10 calf spleen cDNA library constructed with a cDNA synthesis kit (Pharmacia). Hybridization was carried out with standard procedures. Positive clones were subcloned in M13mp18 and sequenced by a DNA sequencer (ABI).

2.4. cDNA cloning of human p57

For the plaque hybridization assay of human p57 cDNA, a cDNA was produced by PCR. PCR primers (5′-ATGAGCCGGCAGGTGG-3′, and 5′-CTTGGCCTGGACTGTCTCCTCCAG-3′) were synthesized based on the sequence of the coding region of calf p57 cDNA (Fig. 2). PCR was performed on HL60 cDNA using these primers, the product of which product contained the entire sequence from starting codon to termination codon. The produced probe was used for screening a λgt 10 HL60 cDNA library constructed with a TimeSaver cDNA synthesis kit (Pharmacia). Hybridization was carried out with standard procedures. Positive clones were subcloned in pBluescriptII sk(−) (Stratagene) and sequenced with an A.L.F. DNA sequencer (Pharmacia). Homology alignments were done with a GeneWorks program (Intelligenetics).

2.5. Generation of polyclonal antiserum against p57

Anti-p57 antiserum was generated in rabbits immunized with BSA-

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conjugated synthetic peptide, KRLDRLEETVQA. This peptide corresponded to residues 449–460 of calf p57. Serum was prepared after three successive immunizations given in complete Freund's adjuvant and in incomplete Freund's adjuvant. The generated antiserum was termed 1440.

2.6. Immunoblotting of p57 in various tissues

The tissues of Balb/c mice were homogenized in lysis buffer (10 mM Tris, pH 8.0, 5 mM ethyleneglycol bis-(2-aminoethylether)tetraacetic acid (EGTA), 0.1 mM DTT, 100 μg/ml phenylmethyl-sulphonyl fluoride, and 5 μg/ml leupeptin). SDS was added to the homogenized samples at the final concentration of 1% and the samples were rotated at 4°C. After the centrifugation at 14,000 × *g* for 20 min at 4°C, the supernatants were recovered. Then, the supernatants were mixed with Tris-SDS-BME Seprasol (enprotech) and these mixtures were boiled for 5 min. The samples (15 μg protein/lane) were subjected to SDS-PAGE. The Western transferred membrane was blocked with 10% FCS/Block Ace (Dainippon Seiyaku) and immunoblotted with 1440, in

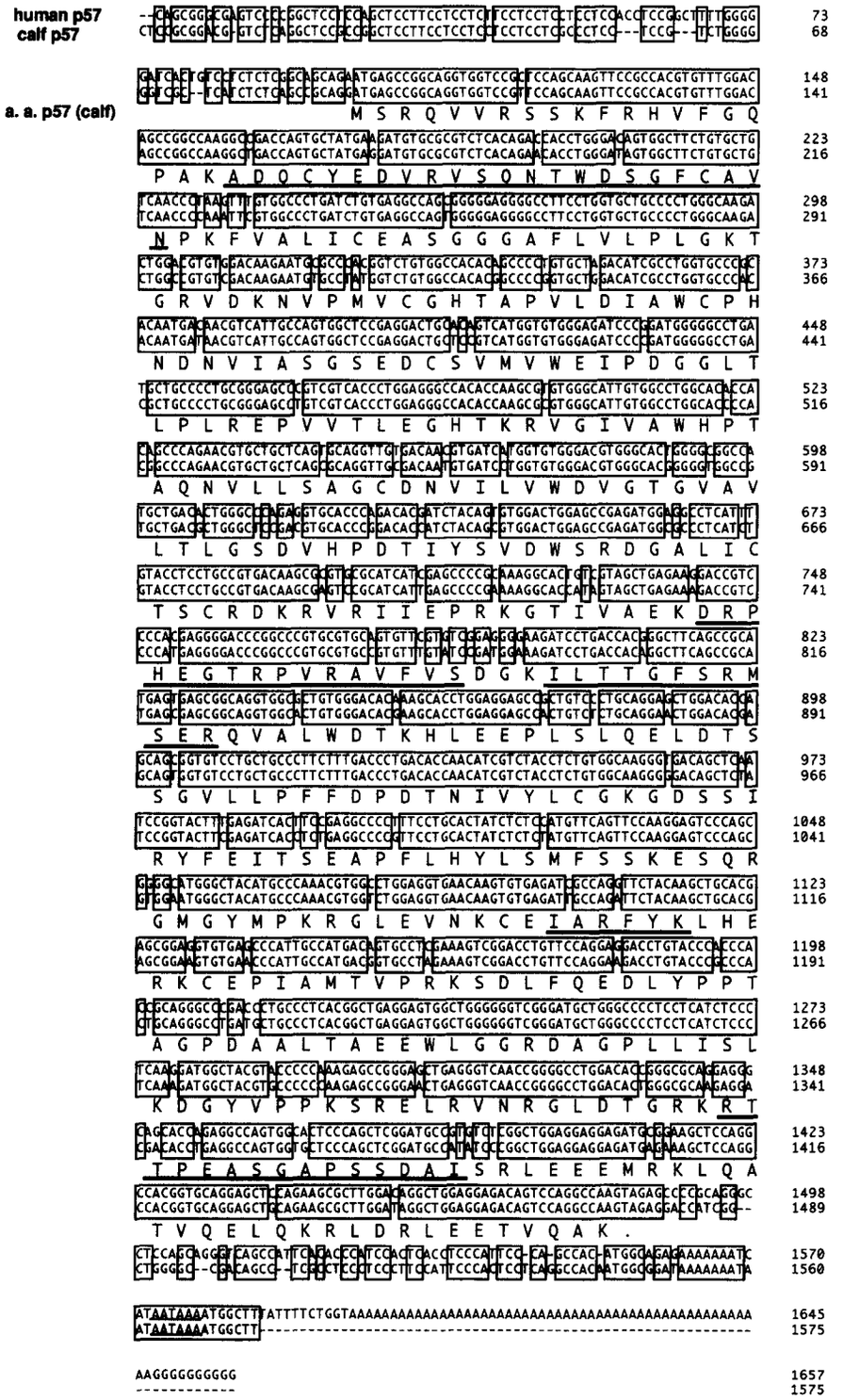

Fig. 1. Nucleotide sequences of calf and human p57 and a deduced amino acid sequence of calf p57. The underlines indicate a polyadenylation signal sequence (AATAAA). The bold underlines indicate the lysyl-C peptides (details in section 2) derived from calf p57.

10% FCS and Block Ace/PBS containing 0.05% Tween-20. This membrane was washed with 0.05% Tween-20/PBS three times and was immunoblotted with the secondary antibody, HRP-conjugated goat anti-rabbit IgG. The detection was performed using the ECL system (Amersham).

2.7. Expression of glutathione S-transferase (GST)-p57 fusion protein

The expression vector of p57 was prepared by restrictional endonuclease digestion and adaptor ligation. The fragment for expression of human p57 was prepared by digestion of full-length cDNA of p57 with *Pma*CI and *Sac*I and both adaptor I and adaptor II (adaptor I; upper strand 5′-GATCCGAATGAGCCGGCAGGTGGTCCGCTCCAGC-AAGTTCCGCCAC-3′ and lower strand 5′-GTGGCGGAACTTGC-TGGAGCGGACCACCTGCCGGCTCATTCG-3′, adaptor II; upper strand 5′-CCAGAAGCGCTTGGACAGGCTGGAGGAGACAGT-CCAGGCCAAGTAGG-3′ and lower strand 5′-TCGACCTACTTG-GCCTGGACTGTCTCCTCCAGCCTGTCCAAGCGCTTCTGGA-GCT-3′) were ligated with the *Pma*CI–*Sac*I fragment. The ligated fragments and adaptors were subcloned into pGEX-5X-1, GST fusion protein expression vector (Pharmacia) digested with *Bam*HI and *Sal*I. GST fusion protein was overexpressed in bacteria. Transformed *E. coli* was cultured overnight in 100 ml medium and diluted 10-fold with fresh LB medium, grown for 2 h, and then induced with 0.2 mM isopropylthio-β-D-galactoside. After 3 h the bacteria were pelleted, suspended in 100 ml of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA), and 0.5% Nonidet P-40), and incubated with 0.2 mg/ml lysozyme for 20 min on ice. The suspensions were then sonicated. The insoluble materials were pelleted at 10,000 × *g* for 10 min and the supernatants were incubated with 2.5 ml of glutathione-Sepharose beads (Pharmacia) for 30 min at 4°C. The beads were washed with washing buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) three times. The bound GST fusion proteins were eluted with 5 mM reduced-glutathione in 50 mM Tris, pH 8.0.

2.8. Co-sedimentation assay of GST-p57 fusion protein with F-actin

A co-sedimentation assay was performed under the following conditions. Both G-actin (15 μg/reaction) (Sigma) and fusion protein, and fusion protein alone were incubated in F-actin buffer (20 mM Tris, pH 8.0, 160 mM KCl, 0.2 mM adenosine 5′-triphosphates) for 1 h at 25°C. After the incubation, the reaction mixtures were ultracentrifuged at 100,000 × *g* for 1 h at 4°C. In the following, the reaction mixture before ultracentrifugation is termed the total reaction mixture. The supernatants, pellets and total reaction mixture were resolved by SDS-PAGE (4–20% gradient gel) and gels were stained with Coomassie brilliant blue (CBB).

3. Results

3.1. Purification of p57

p57 was purified according to phosphoinositide hydrolysis activity. At the final step of purifications, p57 was detected in single-band on SDS-PAGE stained with silver (data not shown). But the specific activity of the p57 fraction was lower than that in the partially purified fraction (data not shown). This suggested that PLC activity in the purified fraction was due to a co-purified protein, and then we attempted to determine the characteristics of p57. First, to examine the primary structure of this protein, the partial amino acid sequences of p57 were determined. From the results of lysyl-C peptide microsequencing and homology searching, two fragments of p57 had a weak homology with coronin (shown in Figs. 1a and 2) [5], an actin-binding protein. And we also found that other peptides were homologous to coronin (Figs. 1a and 2). Coronin has been shown to be an important functional molecule in *Dictyostelium discoideum* but coronin-like actin-binding protein has not been found in mammalian cells. Therefore, to examine whether p57 was a coronin-homologue in mammalian cells, we tried to isolate a cDNA of p57.

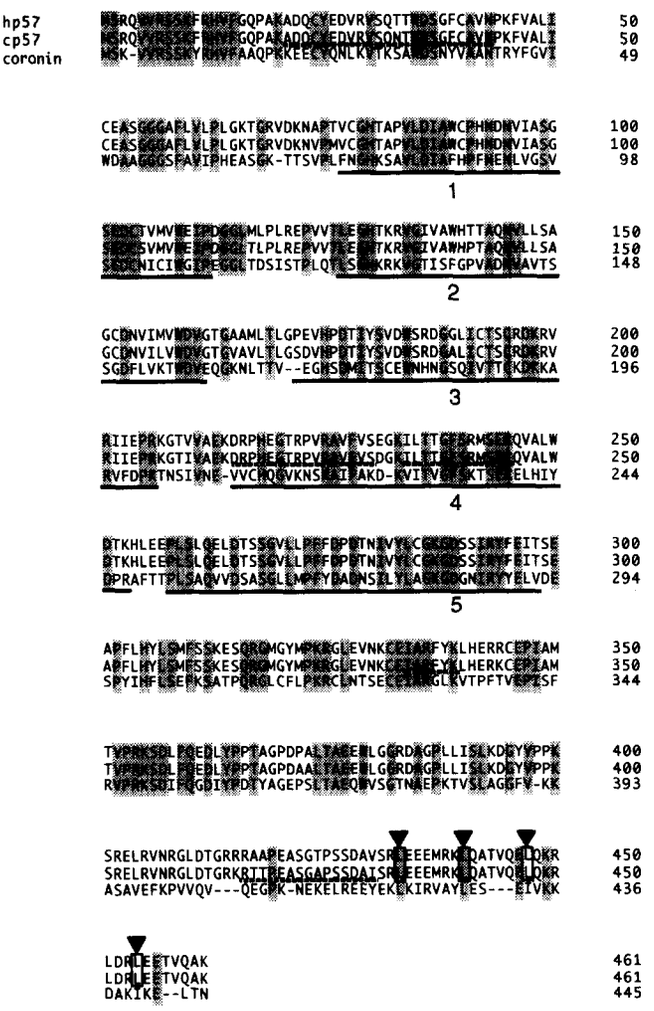

Fig. 2. Alignment of deduced amino acid sequence of human p57, calf p57 and coronin from *Dictyostelium discoideum*. Upper lanes, middle lanes and lower lanes show the amino acid sequence of human p57, calf p57 and coronin, respectively. The broken lines indicate the lysyl-C peptides (details in section 2) derived from calf p57. Underlines show the WD repeats in p57 and coronin. Amino acids indicated by arrow heads and boxes show the leucine zipper motif in the p57 sequence.

3.2. cDNA cloning of calf and human p57

A pair of oligonucleotides were designed corresponding to the determined amino acid sequences (Fig. 1) and a cDNA fragment probe was prepared. With the cDNA fragment, 1.0×10^5 independent clones of the calf spleen λgt10 cDNA library were screened. Two positive clones were isolated and were the same cDNA length. One of them was sequenced. This clone encoded a full-length of calf p57 and contained 5′ and 3′ non-coding regions, an ATG starting codon, a TAG termination codon, and a polyadenylation signal (Fig. 1). The results of peptide microsequencing were confirmed by the deduced amino acids sequence of calf p57.

For further analysis, we identified human p57 cDNA from HL60 cells. For plaque hybridization, a probe was prepared with PCR based on N-terminal and C-terminal sequence of calf p57. With the prepared cDNA fragment, 9.0×10^4 independent clones of the HL60 λgt10 cDNA library were screened. Of 5 positive clones, the longest positive clone was sequenced. This

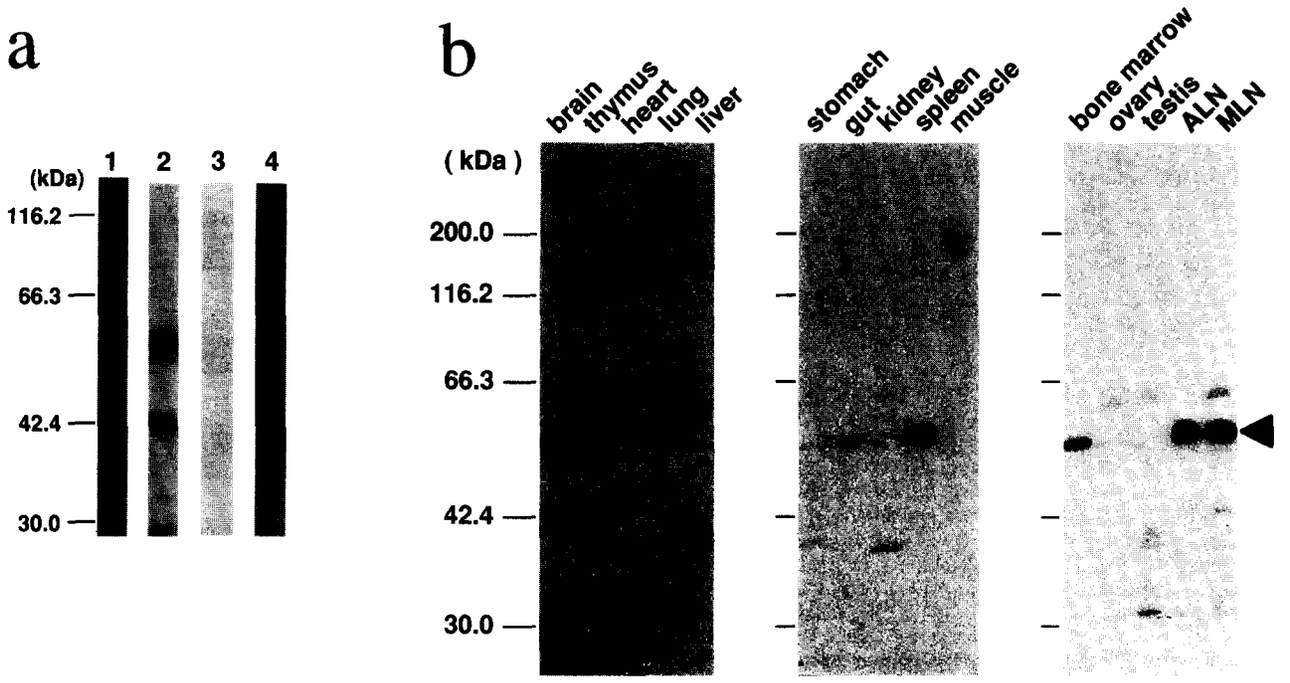

Fig. 3. Immunoblotting analysis of p57 with 1440. The bands of p57 are indicated with the arrow heads at the right side of lanes. In panel (a), lane 1, 2, and 4 show the calf thymus extract, human HL60 cell extract, and mouse thymus extract, respectively. And in lane 3, 1440 against HL60 cell extract is immuno-absorbed with the 1440 peptide for the immunization. Panel (b) indicates the tissue-distribution of p57 in mouse. Each lane reveals the tissue as shown in the figure. ALN and MLN is axiallary lymph node and mesenterium lymph node, respectively.

clone was shown to encode a full-length of human p57 and contained 5′ and 3′ non-coding regions, an ATG starting codon, a TAG termination codon, a polyadenylation signal, and a poly(A) tail (Fig. 1). The open reading frame of calf and human p57 encoded 461 amino acids. The primary structures of calf and human p57 were highly homologous to each other (Fig. 2). Furthermore, in the primary structure of calf and human p57, there were two unique amino acid sequence motifs, a WD repeat and a leucine zipper motif.

3.3. Homology search

The deduced amino acid sequences of calf and human p57 were compared with entries in the data base. These results indicated that p57 has homology with coronin, an actin-binding protein of *Dictyostelium discoideum*. No homology was detected with PLCs. As shown in Fig. 2, p57 exhibited significant sequence identities to coronin (40%). The WD repeat was completely conserved between p57 and coronin, but the leucine zipper motif was not conserved. Only p57 had the leucine zipper motif.

3.4. Immunoblotting analysis of tissue distribution of p57

The 57 kDa bands were observed in the lane of 1, 3, and 5 (Fig. 3a). And the specificity of polyclonal antiserum was tested with immuno-absorption experiment using the synthesized peptide, KRLDRLEETVQA. From the results of immunoblotting analysis against calf thymus, human HL60 cells, and mouse thymus with 1440, the generated polyclonal antiserum was proven to cross-react with calf, human, and mouse p57 (Fig. 3a). Since 1140 cross-reacts with mouse p57, we examined the tissue distribution of p57 in mouse by using 1440. We expected that the tissue distribution of p57 might suggest us the biological function of p57. The p57 was clearly detected in brain, thymus, spleen, bone marrow, and lymph node. And the weak bands were detected in lung and gut. Furthermore, p57 was not detected in liver, kidney, stomach, and skeletal muscle. The bands with lower molecular masses were considered to be non-specific (Fig. 3b). These data shows that p57 is mainly expressed in immune tissues, and interestingly, also expressed in brain.

3.5. Co-sedimentation assay with F-actin

The GST-fused p57 was expressed in *E. coli*. The expressed fusion protein was resolved by SDS-PAGE, and indicated the calculated molecular mass of GST-fused p57 (about 80 kDa) (data not shown). The similarity between p57 and coronin suggested that p57 is a novel actin-binding protein in mammalian cells. Therefore, a co-sedimentation assay of the GST-fused human p57 with F-actin was performed. When GST was used in co-sedimentation assay with F-actin or without F-actin, it was not able to bind to F-actin (Fig. 4). On the other hand, GST-fused p57 was detected in the lane of with actin, but not in the lane of without actin (Fig. 4). These data suggest that p57 is responsible for the actin-binding activity, and an actin-binding protein.

4. Discussion

In an attempt to isolate a PLC with a smaller molecular mass than those of known PLCs, we purified a protein from calf thymus that has phosphoinositide hydrolytic activity and a molecular mass of 57 kDa (p57) (data not shown). At the final step of purifications, p57 was detected in single-band on SDS-PAGE stained with silver (data not shown). But the specific activity of the purified p57 fraction was lower than that in the

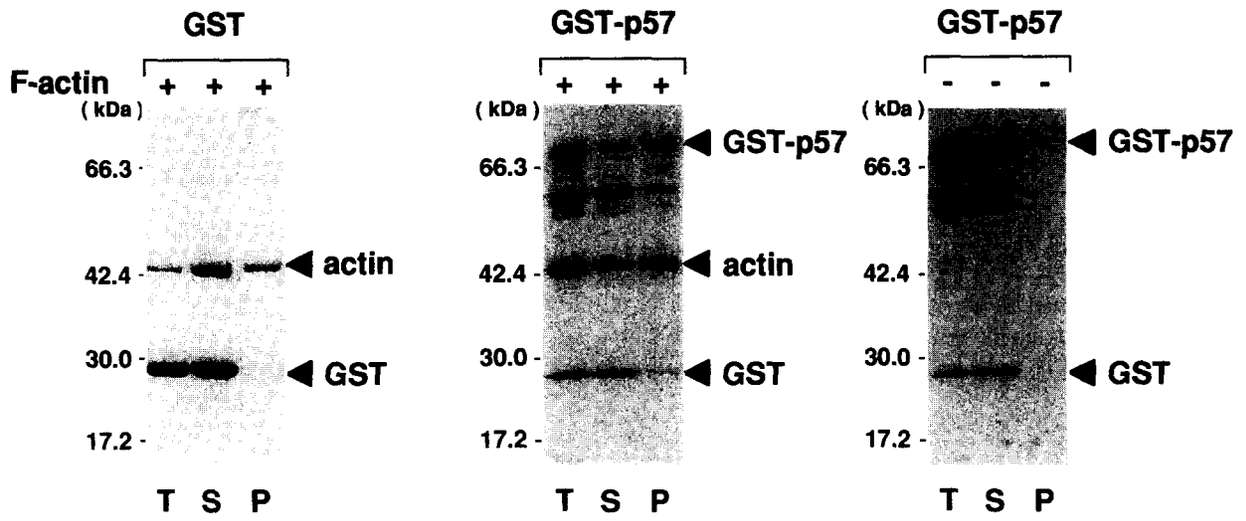

Fig. 4. Co-sedimentation assay of GST-p57 fusion protein with F-actin. T, total reaction mixture; S, ultracentrifuged supernatant; P, ultracentrifuged precipitate (details in section 2). Each protein is indicated with arrow heads.

partially purified fraction (data not shown). The results suggest that PLC activity in the purified p57 fraction was due to a co-purified protein but not due to p57 itself. Then, we were interested in what is p57. To examine the characteristics of this purified protein, first we determined the primary structure of this protein. From the results of lysyl-C peptide microsequencing and homology searching, two peptides of p57 had a weak homology with coronin (shown in Figs. 1a and 2) [5], an actin-binding protein responsible for chemotaxis, cell motility, and cytokinesis [6] of *Dictyostelium discoideum*. Furthermore, we found that other peptides were also homologous to coronin [5] (Figs. 1 and 2). From these results, we considered that p57 was a coronin-like novel actin-binding protein in mammalian. Then, we isolated cDNA clones of calf and human p57 (Fig. 1). The isolated cDNA of human p57 contained an open reading frame of 461 amino acids that had revealed a striking similarity (95%) to bovine p57. The WD repeat and leucine zipper motif are completely conserved in human and bovine p57 (Fig. 2). The expressed p57 in COS cells did not show significant PLC activity (data not shown).

p57 may have a role in signal transduction because its primary structure has two unique amino acid sequence motifs that are characteristic of signal transduction molecules: five WD repeats (Fig. 2) and a leucine zipper motif (Fig. 2) with a small basic region near the C terminus. The seven WD repeats have been found in the β subunits of heterotrimeric guanine nucleotide binding-proteins (G-proteins) [9]. More than 20 proteins are known to contain the similar repeating unit and to be essential components of large functional protein complexes, such as G-proteins, PRP4, Tup1, TAFII80, p55CDC and *LIS-1* protein [10]. Though the function of the WD repeat is not yet clear, one possible function has been reported recently [12]. The β subunit of heterotrimeric G-protein was shown to bind to the PH (pleckstrin homology) domain via the WD repeat. On the other hand, the proteins that contain a PH domain have been suggested to be involved in signal transduction pathways and in cytoskeletal function [13–16]. Based on these reports, we suggest that p57 may form a protein complex with proteins containing a PH domain. It is assumed that such a complex serves some function. Moreover, the leucine zipper motif in the C terminus of p57 may have a role in interacting with other proteins that also have a leucine zipper motif. The leucine zipper motif of p57, which has a small basic region, is similar to *fra-1* (fos-related antigen-1) [17]. Most of the basic leucine zipper (bZIP) proteins, such as transcriptional factors, contain a strikingly conserved sequence [18], but p57 does not have this sequence. Therefore, p57 may not be a transcriptional factor but may be able to bind to DNA indirectly as a heterodimer with other transcriptional factors containing bZIP and play a role in transcriptional regulation. Since another known function of the leucine zipper motif in some proteins is to participate in the formation of homopolymers [19], p57 may have a similar function. Another possible role of p57, in view of its two interesting motifs that suggest the formation of a large protein complex, is in intracellular signal transduction.

Although p57 was homologous (40% identical residues) to coronin, an actin-binding protein of *Dictyostelium discoideum* [5] that has been associated with chemotaxis, cell motility and cytokinesis [6] (Fig. 2), coronin does not have a leucine zipper motif [6]. The long α-helical structure that is a predicted secondary structure in C-terminal was conserved between coronin and p57. It has been discussed that the actin-binding activity of coronin is responsible for this α-helical structure [5]. The similarity between p57 and coronin suggests that p57 is a novel actin-binding protein in mammalian cells. Therefore, a co-sedimentation assay of GST-fused human p57 with F-actin was performed. As shown in Fig. 4, GST-fused human p57 co-precipitated with F-actin but GST did not. This result shows that although the conserved actin-binding motifs are not found in the primary structure of p57 (Figs. 1 and 2), this protein is an actin-binding protein. Furthermore, the tissue-distribution of p57 is very interesting. p57 was clearly expressed in immune tissues and brain but was weakly detected in lung and gut, that contain alveolar macrophages and mesenterium lymph node, respectively (Fig. 3). Although the mechanism of neuronal migration is not yet known, the cells that are included in the immune tissues are considered to be included in the chemotaxis. And the neuronal cells require the activity of the cell migration

(chemotaxis-like) and the neuronal extension. The similarities in the primary structure, actin-binding activity, and the tissue-distribution of p57 suggest that p57, like coronin, may take part in the signal transduction pathways of chemotaxis [7].

In order to understand the biological functions of p57 in more detail, it is important to identify the proteins associated with p57 via the WD repeat and leucine zipper motif. The isolation of these binding proteins may reveal a novel signal transduction pathway of chemotaxis in mammalian cells. And we also need to investigate whether p57 has a role in cytoskeletal function related to cell motility and migration. These studies will help us to understand the mechanism of cell migration and chemotaxis.

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