Constitutive Turnover of Phosphorylation at Thr-412 of Human p57/Coronin-1 Regulates the Interaction with Actin*  

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Background: Biological functions of actin-binding protein p57/coronin-1 may be regulated by phosphorylation.
Results: Ser-2 and Thr-412 were identified as major phosphorylation sites of p57/coronin-1, and the phosphorylation at Thr-412 reduced the binding affinity for actin.
Conclusion: Physical interaction between p57/coronin-1 and actin is regulated by phosphorylation at Thr-412.
Significance: The results provide mechanistic insight into the actin-related immunological processes.

The actin-binding protein p57/coronin-1, a member of the coronin protein family, is selectively expressed in hematopoietic cells and plays crucial roles in the immune response through reorganization of the actin cytoskeleton. We previously reported that p57/coronin-1 is phosphorylated by protein kinase C, and the phosphorylation down-regulates the association of this protein with actin. In this study we analyzed the phosphorylation sites of p57/coronin-1 derived from HL60 human leukemic cells by MALDI-TOF-MS, two-dimensional gel electrophoresis, and Phos-tag human leukemic cells by MALDI-TOF-MS, two-dimensional gel electrophoresis, and Phos-tag. We identified Ser-2 and Thr-412 as major phosphorylation sites. A major part of p57/coronin-1 was found as an unphosphorylated form in HL60 cells, but phosphorylation at Thr-412 of p57/coronin-1 was detected after the cells were treated with calyculin A, a Ser/Thr phosphatase inhibitor, suggesting that p57/coronin-1 undergoes constitutive turnover of phosphorylation/dephosphorylation at Thr-412. A diphosphorylated form of p57/coronin-1 was detected after the cells were treated with phorbol 12-myristate 13-acetate plus calcycin A. We then assessed the effects of phosphorylation at Thr-412 on the association of p57/coronin-1 with actin. A co-immunoprecipitation experiment with anti-p57/coronin-1 antibodies and HL60 cell lysates revealed that β-actin was co-precipitated with the unphosphorylated form but not with the phosphorylated form at Thr-412 of p57/coronin-1. Furthermore, the phosphorylation mimic (T412D) of p57/coronin-1 expressed in HEK293T cells exhibited lower affinity for actin than the wild-type or the unphosphorylated form but not with the phosphorylated form at Thr-412 of p57/coronin-1. These results indicate that the constitutive turnover of phosphorylation at Thr-412 of p57/coronin-1 regulates its interaction with actin.

Coronin, an actin-binding protein, was first purified from the actin-myosin complex in the amoeboid protozoan Dictyostelium discoideum and was so named because it was found to form a crown-like structure on the dorsal surface of the cell (1). This actin-binding protein is thought to play important roles in cytokinesis, cell motility, chemotaxis, phagocytosis, and macropinocytosis through the regulation of assembly/disassembly of actin filaments (2–6). Many proteins homologous to coronin have been successively identified in various eukaryotes from yeasts to mammals (7). In humans, the coronin family consists of seven proteins that can be categorized into three subtypes (8). All seven proteins are characterized by the presence of evolutionarily conserved structural domains that include tryptophan/aspartic acid repeats that are implicated in the association with actin filaments. We previously identified p57/coronin-1 as the first mammalian coronin and found that this protein was selectively expressed in immune cells (9). We have also reported that p57/coronin-1 possesses at least two actin binding regions (10) and forms a homodimer via a leucine zipper motif present in the C-terminal coiled-coil region (11).

Several studies, including ours, have indicated that p57/coronin-1 plays crucial roles in phagocytosis and chemotaxis of leukocytes (12–15). During the phagocytic process of bacterial infection, p57/coronin-1 was shown to transiently accumulate on the surface of phagosomes and control the subsequent phagosome-lysosome fusion. Recent studies have also demonstrated that p57/coronin-1 was enriched in immunological synapses and involved in various immune regulatory functions such as signal transduction via T cell receptors (16–18), survival of T cells (19, 20), and intracellular Ca2+ mobilization in T and B cells (19, 21). More recently, it was reported that the p57/coronin-1 gene was responsible for human and mouse severe combined immunodeficiency (22, 23). In mice deficient in the p57/coronin-1 gene, differentiation and chemokine-mediated trafficking of T cells were severely impaired (22, 24, 25). It was further demonstrated that the development of experimental autoimmune encephalomyelitis (26, 27) and lupus-like autoimmune disease (28) were suppressed in mice with genetic defects of p57/coronin-1. These studies suggested the potential...
relevance of p57/coronin-1 to allergic and autoimmune diseases.

Our previous reports indicated that p57/coronin-1 was phosphorylated by protein kinase C (PKC) during phagocytosis in neutrophil-like differentiated HL60 cells (14). The treatment of the cells with a PKC inhibitor, chelerythrine, prevented the phagosome-lysosome fusion in parallel with the inhibition of the dissociation of p57/coronin-1 from phagosomes. Thus, the phosphorylation of p57/coronin-1 seems to be an important process in the regulation of phagosome maturation. We also reported that the activation of PKC in p57/coronin-1-transfected HEK293 cells by phorbol 12-myristate 13-acetate (PMA)² treatment reduced the association of p57/coronin-1 with the actin-rich cytoskeleton and that p57/coronin-1 molecules associated with the actin cytoskeleton were phosphorylated at lower levels than those recovered in the cytosolic fraction (29).

Although we previously found that p57/coronin-1 molecules possess at least two phosphorylation sites, no biochemical information on the phosphorylation sites is thus far available. In this study, therefore, we attempted to identify the phosphorylation sites of p57/coronin-1 by MALDI-TOF-MS, two-dimensional gel electrophoresis, Phos-tag acrylamide gel electrophoresis, and site-directed mutagenesis and found two major phosphorylation sites, Ser-2 and Thr-412. In addition, we also examined the relevance between phosphorylation at Thr-412 of p57/coronin-1 and its interaction with actin and found that the phosphorylation at Thr-412 down-regulated the binding of p57/coronin-1 to actin.

**MATERIALS AND METHODS**

*Reagents—*Restriction endonucleases and modifying enzymes were purchased from Roche Diagnostics, Takara (Osaka, Japan), and Toyobo (Osaka, Japan). Hybond-ECL nitrocellulose membranes, protein G-Sepharose, Immobiline DryStrips, and IPG buffer were products of GE Healthcare. Coomasie Brilliant Blue R250 was from Merck. Calyculin A, chelerythrine chloride, Tween 20, hypoxanthine-aminopterin-thymidine medium, zymosan, and human serum (blood group AB) were purchased from Sigma. Nonidet P-40 and Phos-tag acrylamide were from NuPAGE (Rockford, IL), respectively. CHAPS, dithiothreitol (DTT) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Phos-tag acrylamide gel electrophoresis, and site-directed mutagenesis and found two major phosphorylation sites, Ser-2 and Thr-412. In addition, we also examined the relevance between phosphorylation at Thr-412 of p57/coronin-1 and its interaction with actin and found that the phosphorylation at Thr-412 down-regulated the binding of p57/coronin-1 to actin.

**DNA Constructs—**The human p57/coronin-1 construct (pcDNA6-p57) was prepared by insertion of the full-length p57/coronin-1 cDNA from pEGFP-p57FL (29) into the BamHI/XbaI sites of a pcDNA6/V5-HisA vector (Invitrogen) using ER2925, a strain of Escherichia coli that is deficient for both dam and dcm (New England Biolabs Inc., Ipswich, MA). Mutations were introduced into the phosphorylation site(s) of p57/coronin-1 by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. pcDNA6-p57/S2A (in which Ser-2 is replaced with Ala) and pcDNA6-p57/T412A (in which Thr-412 is replaced with Ala) were generated from pcDNA6-p57 by using the following sets of primers: 5’-AGC TCG GAT CCG AAA GGC GCA GGT GGT C-3’ (sense primer for S2A) and 5’-GCC GAC CAT CTG CCG GCC CAT TCAG GAC CGC AGG AGC-3’ (antisense primer for S2A) and 5’-AAC CGG GCC CTG GAC GCC CCC GGC CGC AGG AGG-3’ (sense primer for T412A) and 5’-CCC CCT CCG CCC GCC GTC CAG CCC CCG GTT-3’ (antisense primer for T412A). pcDNA6-p57/S2AT412A (a double mutant at Ser-2 and Thr-412) is generated from pcDNA6-p57/S2A by using primers for T412A. pcDNA6-p57/T412D (in which Thr-412 is replaced with Asp) was generated from pcDNA6-p57 by using the primers 5’-AAC CGG GCC CTG GAC GCC CCC GGC AGG AGG-3’ (sense primer for T412D) and 5’-CCT CCG CCC GGC CCC GTC GAG CCC GGT-3’ (antisense primer for T412D). pcDNA6-p57/T412D2 is generated from pcDNA6-p57 by using the primers 5’-ACE GGC TCG GAC GAG GCG GGC GCC AGG AGG-3’ (sense primer for T412D) and 5’-CCT CCG CCC GGC CCC GTC GAG CCC GGT-3’ (antisense primer for T412D). pcDNA6-p57/T412D2 was generated from pcDNA6-p57 by using the primers described above. The nucleotide sequences of these clones were confirmed with a DNA sequencer (Applied Biosystems, Foster City, CA) using the dye-terminator method.

**Cell Culture and Transfection—**HL60 and HEK293T cells were grown in RPMI1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT) at 37°C under humidified 5% CO₂. Transfection of HEK293T cells was performed by electroporation (Gene Pulsar Xcell; Bio-Rad) as described previously (29). Bacterialin-S (20 µg/ml) was used to select for the stable clones.

**Electrophoresis, Immunoblotting, and Gel Staining—**Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

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²The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; EGFP, enhanced GFP; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonic acid; Arp, actin-related protein.
(PAGE) and immunoblotting were conducted as described previously (11). The procedures for two-dimensional gel electrophoresis were also as described previously (29). Phosphate-affinity SDS-PAGE was performed with Phos-tag® gel containing 50 μM Phos-tag acrylamide and 0.1 mM MnCl₂, Gel staining with Flamingo™, SYPRO® Ruby, or Pro-Q® Diamond staining was conducted according to the manufacturer’s instructions. The fluorescently stained protein bands were visualized by using a Typhoon 9410 (GE Healthcare) or FluoroPhoreStar with Dynabeads®.

A protein band stained with Coomassie Brilliant Blue on a gel was excised and subjected to in-gel digestion with trypsin (sequencing grade; Promega Corp., Madison, WI). Phosphopeptides were isolated from total tryptic peptides using a TitanSphere Phos-TiO kit according to the manufacturer’s instructions (GL Sciences, Tokyo, Japan). The resulting phosphopeptides were analyzed by MALDI-TOF-MS with an AXIMA-CFRplus (Shimadzu/Kratos, Kyoto, Japan), and a subsequent database search was performed with MASCOT search engine (Matrix Science, Boston, MA).

**Immunoprecipitation Assay**—HL60 cells (5 × 10⁶ cells) were lysed with a buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 1% Nonidet P-40 at 4 °C, and the supernatant was recovered after centrifugation at 15,000 × g for 20 min. An immunoprecipitation assay was performed essentially as described previously (29), except for the use of Dynabeads® protein G in place of protein G-Sepharose in some of the experiments. Anti-p57/coronin-1 antibody (N7, 3 μg) was incubated with Dynabeads® protein G (20 μl) in a rotator for 1 h at room temperature. For the use of anti-Thr(−P)−412 of p57/coronin-1 antibody (pT412, IgM), Dynabeads® protein G was first incubated with anti-mouse IgG antibody (2 μl) and then with antibody pT412 (3 μg). The conjugate of Dynabeads® protein G and antibody thus obtained was resuspended with cell lysates, and incubated for 16 h at 4 °C. The beads were then washed three times with the same buffer to remove unbound proteins. Proteins bound to the beads were recovered by the treatment with a sample buffer for SDS-PAGE at 95 °C for 3 min.

**Binding of Phosphorylation Mimics to Actin**—HEK293T cells transfected with cDNAs for the wild-type or mutants (T412A and T412D) of p57/coronin-1 were lysed with F-actin buffer (25 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 1 mM ATP, pH 8.0) containing 1% Triton X-100. The cell lysates were mixed with F-actin (pre-formed filaments, 4 μg), and the mixtures were incubated for 60 min at 25 °C. After centrifugation at 100,000 × g for 60 min, the supernatant and precipitate were analyzed by SDS-PAGE/immunoblotting using anti-p57/coronin-1 (N7) and anti-β-actin antibodies. HEK293T cells were also transfected with cDNAs for the wild-type or mutants (T412A and T412D) of the C-terminal-truncated form of p57/coronin-1 fused with EGFP (EGFP-p57LZ). The cells were lysed with G-actin buffer (5 mM Tris-HCl, 0.2 mM CaCl₂, 0.5 mM DTT, 0.2 mM ATP, pH 8.0) containing 1% Triton X-100. The cell lysates were subjected to immunoprecipitation with a conjugate of Dynabeads® protein G (20 μl) and anti-p57/coronin-1 antibody (N7, 3 μg). The precipitated beads were suspended in G-actin buffer and mixed with G-actin (5 μg), and the mixtures were incubated for 2 h at 4 °C. The beads were then washed three times with the same buffer to remove unbound proteins. The proteins bound to the beads were analyzed by SDS-PAGE/immunoblotting using anti-p57/coronin-1 (N7) and anti-β-actin antibodies.

**Phagocytosis of Opsonized Zymosan by Neutrophils**—Neutrophils were isolated from human peripheral blood by a combination of dextran sedimentation and Ficoll-Paque gradient centrifugation (30, 31). Purified neutrophils (1 × 10⁷ cells) were mixed with opsonized zymosan (14) and incubated at 4 °C for 15 min. After centrifugation at 800 × g for 3 min, the cell pellet and opsonized zymosan were suspended in a small volume (0.03 ml) of phosphate-buffered saline and incubated at 37 °C for 0–30 min. The cells were then lysed with 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40. An immunoprecipitation and subsequent SDS-PAGE/immunoblotting were conducted as described above.

**RESULTS**

**Phosphorylation and Actin Binding Activity of p57/Coronin-1**—To examine the actin binding activity of p57/coronin-1, we conducted a co-immunoprecipitation assay using HL60 cell lysate and anti-p57/coronin-1 antibody. When the immunoprecipitate from the lysate of untreated HL60 cells was analyzed by SDS-PAGE followed by silver staining, we detected a band of ~43 kDa as well as p57/coronin-1 and IgG heavy and light chains (Fig. 1A, upper panel). The 43-kDa band was identified as β-actin by MALDI-TOF-MS and MASCOT database searching (data not shown). It was further confirmed by immunoblotting that the 43-kDa band was β-actin (Fig. 1A, lower panel). By contrast, the 43-kDa band was not detected in the immunoprecipitate from HL60 cells that had been treated with calyculin A (Ser/Thr phosphatase inhibitor) and PMA (PKC activator) by either silver-staining or immunoblotting. When we utilized Pro-Q® Diamond to visualize phosphorylated proteins on the gel, p57/coronin-1 precipitated from the lysate of untreated HL60 cells was only faintly stained, whereas that of calyculin A/PMA-treated HL60 cells was intensely stained (Fig. 1B). These results suggested a negative correlation between phosphorylation and actin binding activity of p57/coronin-1; i.e., the phosphorylated form of p57/coronin-1 present in calyculin A/PMA-treated HL60 cells possessed weaker affinity for actin than the unphosphorylated form present in untreated HL60 cells.

**Two Major Phosphorylation Sites of p57/Coronin-1**—We next analyzed changes in the phosphorylation status of p57/coronin-1 by two-dimensional gel electrophoresis and SDS-PAGE using Phos-tag® acrylamide (Phos-tag-PAGE) after the cells were treated with calyculin A and/or PMA. When the lysates of untreated HL60 cells were separated by these two procedures, p57/coronin-1 was detected as a single spot in two-dimensional gel electrophoresis (Fig. 2A) and as a single band in Phos-tag-PAGE (Fig. 2B), respectively. From the lysate of calyculin A-treated HL60 cells, in contrast, the p57/coronin-1 was detected as two spots in two-dimensional gel electrophoresis and as doublet bands in Phos-tag-PAGE. The results suggest that the calyculin A treatment resulted in the appearance of the phosphorylated form of p57/coronin-1. The HL60 cells treated with PMA, however, gave spot/band profiles similar to those of...
untreated cells. When the cells were treated with calyculin A and PMA, we observed a third spot on two-dimensional gel electrophoresis in a more acidic region and a third band with lower mobility on Phos-tag-PAGE. These results indicate that p57/coronin-1 undergoes constitutive turnover of phosphorylation/dephosphorylation in HL60 cells and that p57/coronin-1 possesses at least two major phosphorylation sites. When HL60 cells were treated with \( ^{3}H_9251 \)-bromo-4-hydroxyacetophenone (30–300 \( ^{3}H_9262 \)-M), a protein-tyrosine phosphatase inhibitor) instead of calyculin A, however, no phosphorylated spot/band was observed in two-dimensional gel electrophoresis or Phos-tag-PAGE (data not shown) as was observed in the case of untreated HL60 cells. This result is in good agreement with the previous observation that phosphorylation of p57/coronin-1 was catalyzed by PKC, a Ser/Thr kinase. We also analyzed the phosphorylation of p57/coronin-1 in other cell types by the same procedures and found that human neutrophils, human platelets, and three human leukemic cell lines (THP-1, U937, and Jurkat) exhibited similar profiles to HL60 cells in two-dimensional gel electrophoresis or Phos-tag-PAGE (Supplement 1).

To identify phosphorylation sites of p57/coronin-1, we performed peptide mass fingerprinting analysis with MALDI-TOF-MS and MASCOT database searching after tryptic digestion of p57/coronin-1 isolated from HL60 cells that had been stimulated with calyculin A plus PMA. By the MASCOT search, we detected two putative phospho-peptides, \( ^{1}MpSRQVVR7 \) and \( ^{409}GLDpTGRR415 \), suggesting that Ser-2 and Thr-412 are strong candidates for the major phosphorylation sites of p57/coronin-1. Ser-2 of p57/Coronin-1 Is a Phosphorylation Site—MALDI-TOF-MS/MASCOT analysis suggested that Ser-2 is one of the major phosphorylation sites of p57/coronin-1 isolated from HL60 cells that had been stimulated with calyculin A plus PMA. By the MASCOT search, we detected two putative phospho-peptides, \( ^{1}MpSRQVVR7 \) and \( ^{409}GLDpTGRR415 \), suggesting that Ser-2 and Thr-412 are strong candidates for the major phosphorylation sites of p57/coronin-1. Ser-2 of p57/CORONIN-1 Is a Phosphorylation Site—MALDI-TOF-MS/MASCOT analysis suggested that Ser-2 is one of the major phosphorylation sites of p57/coronin-1 isolated from HL60 cells that had been stimulated with calyculin A plus PMA. By the MASCOT search, we detected two putative phospho-peptides, \( ^{1}MpSRQVVR7 \) and \( ^{409}GLDpTGRR415 \), suggesting that Ser-2 and Thr-412 are strong candidates for the major phosphorylation sites of p57/coronin-1.
Phosphorylation of p57/Coronin-1

The position 412 of mouse p57/coronin-1 (also known as TACO) is a Ser residue. We examined whether Ser-412 of mouse p57/coronin-1 is a phosphorylation site by site-directed mutagenesis as described above (Supplement 3). The S412A mutant expressed in HEK293T cells exhibited no phosphorylated form on Phos-tag-PAGE and immunoblotting even after the cells were treated with calyculin A. This result suggests that Ser-412 of mouse p57/coronin-1 is a major phosphorylation site like Thr-412, the counterpart in human p57/coronin-1. We also prepared 17 mutants (S9A, S1A, S194A, T209A, S230A, S243A, S290A, S291A, S311A, Y338F, S356A, S391A, S401A, S422A, S426A, S427A, and S431A) of human p57/coronin-1. None of them exhibited a phosphorylation spot profile distinct from the wild-type in two-dimensional gel electrophoresis (data not shown), suggesting that these sites are not putative phosphorylation sites.

Constitutive Phosphorylation at Thr-412 of p57/Coronin-1—Next we analyzed the phosphorylation of p57/coronin-1 mutants at Ser-2 and Thr-412 expressed in HEK293T cells by using Pro-Q® Diamond, a dye selectively staining phosphoproteins. HEK293T cells transfected with the wild-type or mutant constructs (S2A, T412A, and S2A/T412A) were treated with calyculin A and PMA, and p57/coronin-1 was purified by immunoprecipitation with anti-p57/coronin-1 antibody (N7) after the cells were lysed with a buffer containing Nonidet P-40. The immunoprecipitates were separated by SDS-PAGE, and the gel was stained with Pro-Q® Diamond and Flamingo™ for phosphoproteins and total proteins, respectively. As shown in Fig. 5A, the intensity of the band stained with Pro-Q® Diamond for the S2A mutant was slightly lowered, and that for the T412A mutant was markedly lowered when compared with that for the wild type. The band for the double mutant (S2A/T412A) was no longer detected. The results indicate that p57/coronin-1 is phosphorylated exclusively at Ser-2 and Thr-412 under the conditions employed in this study.

In two-dimensional gel electrophoresis (Fig. 5B), the monophosphorylated form of the wild-type p57/coronin-1 appeared to consist of two closely associated (or almost overlapped) spots, but that of each single mutant (S2A and T412A) was composed of a single spot with a distinct isoelectric point (pI); i.e. the mono-phosphorylated form of T412A was slightly more acidic than that of S2A. Therefore, the result suggests that the phosphorylation of p57/coronin-1 at Ser-2 or Thr-412 resulted in a differential effect on the change in pI of the protein. Furthermore, the separation of the mono-phosphorylated isoforms at Ser-2 and Thr-412 was also achieved by Phos-tag-PAGE (Fig. 5C). The mono-phosphorylated form of the wild-type p57/coronin-1 was detected as a doublet; the band with higher mobility was intensely stained, and the band with lower mobility was faintly stained. From the phosphorylation profile of the two single mutants (S2A and T412A), the upper and lower bands were deduced to be phosphorylated forms at Ser-2 and at Thr-412, respectively. The phosphorylation at Thr-412 of p57/coronin-1 (i.e. phosphorylation of S2A) was observed when the transfected cells were treated with calyculin A. These results again indicate that Thr-412 of p57/coronin-1 is constitutively phosphorylated but is not detected unless the cells are
treated with calyculin A, probably due to the dominance of phosphatase activity in the cell.

We generated a novel monoclonal antibody specific for Thr(P)-412 of p57/coronin-1 (pT412) using a synthetic phosphorylated peptide as an immunogen. Immunoblotting analysis revealed that this monoclonal antibody reacted with p57/coronin-1 from HL60 cells treated with calyculin A but not with that from untreated HL60 cells (Fig. 6A). This result confirmed that constitutive phosphorylation/dephosphorylation turnover of p57/coronin-1 at Thr-412 took place in the HL60 cells.

**Phosphorylation at Thr-412 of p57/Coronin-1 Is Inhibited by a PKC Inhibitor**—To evaluate the role of PKC in the phosphorylation at Thr-412 of p57/coronin-1, we analyzed phosphorylated p57/coronin-1 after HL60 cells were treated with chelerythrine, an inhibitor of PKC. In Phos-tag-PAGE, phosphorylation of p57/coronin-1 was suppressed by chelerythrine in a dose-dependent manner (Fig. 6B). The proportion of the phosphorylated form of p57/coronin-1 in the absence of chelerythrine was estimated to be ~60% and decreased to less than 10% after treatment with the inhibitor at 30 μM. Immunoblotting analysis by using anti-Thr(P)-412 antibody also indicated that the phosphorylation at Thr-412 of p57/coronin-1 was dose-dependently inhibited by chelerythrine (Fig. 6C).

**The Minor Spots of p57/Coronin-1 Were Independent of Phosphorylation**—In two-dimensional gel electrophoresis, we detected two minor spots of p57/coronin-1 from HEK293T transfectants and HL60 cells in addition to three major spots (Figs. 3B, 4B, and 5B). However, the bands corresponding to these minor spots were not detected by Phos-tag-PAGE (Fig. 5C).
5C). To clarify this apparent inconsistency, we examined whether these minor spots would be generated by a particular level of phosphorylation. The lysate of calyculin A/PMA-treated HL60 cells was separated by the combination of isoelectric focusing in the first dimension and regular SDS-PAGE or Phos-tag-PAGE in the second dimension followed by immunoblotting using anti-p57/coronin-1 antibody (N7). B, the lysate was subjected to immunoprecipitation with protein G-Sepharose and anti-p57/coronin-1 antibody (N7), and the immunoprecipitates were separated by two-dimensional gel electrophoresis. The gel was stained with Pro-Q® Diamond for phosphoproteins (upper panel) or SYPRO® Ruby for total proteins (lower panel). The experiments were repeated twice, and similar results were obtained.

Phosphorylation of p57/Coronin-1 at Thr-412 Regulates the Interaction with Actin—We next examined the relation between phosphorylation at Thr-412 of p57/coronin-1 and the interaction of this protein with actin. We first searched for proteins co-immunoprecipitated with p57/coronin-1 from lysates of HL60 treated with calyculin A (100 nM) and/or PMA (150 nM) by SDS-PAGE and Flamingo™ staining (Fig. 8A). Bands of ~43 and 220 kDa were detected in the immunoprecipitates from untreated and PMA-treated HL60 cells, whereas no such bands were observed in those from calyculin A- or calyculin A/PMA-treated HL60 cells. These proteins of 43 and 220 kDa were identified as β-actin and myosin heavy chain 9, respectively, by MALDI-TOF-MS and peptide mass fingerprinting analyses (data not shown). Immunoblotting confirmed that the 43-kDa protein was β-actin (Fig. 8B). Then we evaluated the relationship between the amount of actin co-immunoprecipitated with p57/coronin-1 and phosphorylation at Thr-412 of p57/coronin-1 by immunoblotting. We detected β-actin clearly in the immunoprecipitate from untreated HL60 cells using anti-p57/coronin-1 antibody (N7), but no β-actin was observed in the immunoprecipitate with antibody specific for Thr-412-phosphorylated p57/coronin-1 (pT412), although comparable amounts of p57/coronin-1 were detected in both specimens (Fig. 8C).

We prepared two mutants of p57/coronin-1 at Thr-412, an Ala mutant (T412A) and an Asp mutant (T412D), that mimic the unphosphorylated and phosphorylated forms, respectively. Then the co-immunoprecipitation experiment was conducted with the lysates from the HEK293T transfectants and anti-p57/coronin-1 antibody (N7). When the immunoprecipitates were analyzed by immunoblotting, a larger amount of β-actin was co-immunoprecipitated with p57/coronin-1 from the T412A transfectant as compared with the wild-type transfectant. By contrast, almost no β-actin was co-immunoprecipitated from the lysate of the T412D transfectant (Fig. 8D). These results support the notion that the phosphorylation of p57/coronin-1 at Thr-412 down-regulates the association with actin.

It is interesting to consider whether the phosphorylation of p57/coronin-1 at Thr-412 affects the interaction with G- or F-actin. To examine whether Thr-412 phosphorylation affects the interaction of p57/coronin-1 with F-actin, we conducted co-sedimentation of p57/coronin-1 (wild-type and T412A and T412D mutants) with F-actin. The lysates of HEK293T cells that had been transfected with wild-type, T412A, or T412D cDNAs were ultracentrifuged after the addition of preformed F-actin, and the supernatants and precipitates were subjected to SDS-PAGE-immunoblotting using anti-p57/coronin-1 and anti-β-actin antibodies (Fig. 9A). However, we observed no significant difference among the amounts of the wild type and these mutants of p57/coronin-1 co-sedimented with F-actin, suggesting that the influence of phosphorylation at Thr-412 on the binding of p57/coronin-1 to F-actin is limited. We also performed a co-sedimentation assay using GST-tagged recombinant p57/coronin-1 expressed in E. coli and obtained similar results (data not shown).

We then assessed possible modulation of the binding activity of p57/coronin-1 to G-actin by Thr-412 phosphorylation. Because p57/coronin-1 is known to possess multiple binding sites for actin (10, 34, 35), we used the C-terminal fragment of p57/coronin-1 (p57LZ; amino acid residues 372–461), which exhibited almost no binding capacity to F-actin (10). The lysates of HEK293T cells transfected with EGFP-fused p57LZ
(EGFP-p57LZ) (11) or its mutants (T412A and T412D) were subjected to co-immunoprecipitation assay with anti-p57/co-coronin-1 antibody (N7), and the immunoprecipitates were separated by SDS-PAGE. A, the gel was stained with Flamingo™Fluorescent Gel Stain. An arrowhead and asterisks indicate β-actin (~43 kDa) and IgG heavy and light chains, respectively. B, p57/co-coronin-1 (upper panel) and β-actin (lower panel) on the gel were detected by immunoblotting with anti-p57/co-coronin-1 antibody (N7) and anti-β-actin antibody, respectively. An asterisk indicates IgG heavy chain. C, the immunoprecipitation (IP) was conducted with anti-p57/co-coronin-1 antibody (N7) or anti-phospho-Thr-412 of p57/co-coronin-1 antibody (pT412), and β-actin (upper panel) or p57/co-coronin-1 (lower panel) on the gel was detected by immunoblotting as described above. D, HEK293T cells that expressed the wild-type or mutants (T412A or T412D) of p57/co-coronin-1 were treated with calyculin A (100 nM) and PMA (150 nM). The cells were then lysed with a buffer containing 1% Nonidet P-40, and the lysates were subjected to immunoprecipitation with anti-p57/co-coronin-1 antibody (N7). The immunoprecipitates were separated by SDS-PAGE and immunoblotting with anti-p57/co-coronin-1 antibody (N7) and anti-β-actin antibody. An asterisk indicates IgG heavy chain. The experiments were repeated five times, and representative results are shown.

**FIGURE 8. Effects of phosphorylation at Thr-412 of p57/co-coronin-1 on the interaction with actin.** HL60 cells were treated with calyculin A (100 nM) and/or PMA (150 nM) at 37 °C for 30 min and lysed with a buffer containing 1% Nonidet P-40. The lysates were subjected to immunoprecipitation with Dynabeads® protein G and anti-p57/co-coronin-1 antibody (N7), and the immunoprecipitates were separated by SDS-PAGE. A, the gel was stained with Flamingo™Fluorescent Gel Stain. An arrowhead and asterisks indicate β-actin (~43 kDa) and IgG heavy and light chains, respectively. B, p57/co-coronin-1 (upper panel) and β-actin (lower panel) on the gel were detected by immunoblotting with anti-p57/co-coronin-1 antibody (N7) and anti-β-actin antibody, respectively. An asterisk indicates IgG heavy chain. C, the immunoprecipitation (IP) was conducted with anti-p57/co-coronin-1 antibody (N7) or anti-phospho-Thr-412 of p57/co-coronin-1 antibody (pT412), and β-actin (upper panel) or p57/co-coronin-1 (lower panel) on the gel was detected by immunoblotting as described above. D, HEK293T cells that expressed the wild-type or mutants (T412A or T412D) of p57/co-coronin-1 were treated with calyculin A (100 nM) and PMA (150 nM). The cells were then lysed with a buffer containing 1% Nonidet P-40, and the lysates were subjected to immunoprecipitation with anti-p57/co-coronin-1 antibody (N7). The immunoprecipitates were separated by SDS-PAGE and immunoblotting with anti-p57/co-coronin-1 antibody (N7) and anti-β-actin antibody. An asterisk indicates IgG heavy chain. The experiments were repeated five times, and representative results are shown.

*Phosphorylation of p57/Coronin-1* (11) or its mutants (T412A and T412D) were subjected to co-immunoprecipitation assay with anti-p57/co-coronin-1 antibody (N7). In the SDS-PAGE/immunoblotting analysis, substantial amounts of β-actin were observed in the immunoprecipitates from the cell lysates of the wild type and the Ala mutant (T412A) of EGFP-p57LZ, but no β-actin was detected in those of the Asp mutant (T412D) (Fig. 9B). Thus, phosphorylation at Thr-412 is likely to reduce the binding affinity of p57/co-coronin-1 for G-actin.

**Transient Phosphorylation of p57/Coronin-1 in Phagocytosing Cells**—Finally, we analyzed the phosphorylation at Thr-412 of p57/co-coronin-1 during phagocytosis. For this purpose, human neutrophils were incubated with opsonized zymosan at 37 °C for 0–30 min. The cell lysates were then subjected to immunoprecipitation with anti-p57/co-coronin-1 antibody followed by SDS-PAGE/immunoblotting with anti-p57/co-coronin-1 antibody (N7) or anti-Thr(P)-412 of p57/co-coronin-1 antibody (pT412). The phosphorylated form of p57/co-coronin-1 at Thr-412 was increased at 5–15 min and then decreased to the basal level at 30 min (Fig. 10), suggesting that Thr-412 of p57/co-coronin-1 was transiently phosphorylated during phagocytosis.

**DISCUSSION**

In this study, we identified Ser-2 and Thr-412 as major phosphorylation sites of p57/co-coronin-1 in HL60 cells. Because the phosphorylation at Thr-412 was found in calyculin A-treated HL60 cells, Thr-412 seems to be constitutively phosphorylated in these cells. The enhanced phosphorylation was likely caused by inhibition of a phosphatase involved in the dephosphorylation of p57/co-coronin-1 and/or continuous activation of a Ser/
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Thr kinase involved in the phosphorylation of p57/coronin-1. On the other hand, Ser-2 phosphorylation was observed when the cells were treated with a PKC activator (PMA). However, the phosphorylation at either Ser-2 or Thr-412 was only found in the presence of calcineurin A, suggesting that phosphatase activity is dominant in the cells. The phosphorylation of both sites is probably catalyzed by PKC, because phosphorylation was completely inhibited by the treatment with the PKC inhibitor chelerythrine. Although it is not concluded that the inhibitory effects of chelerythrine were due to the direct action toward PKC responsible for the phosphorylation of p57/coronin-1 in the cell, we previously observed that p57/coronin-1 was phosphorylated by purified PKC in vitro (14). The amino acid sequences flanking Ser-2 (Ser-QXV) and Thr-412 (Thr-PGRR) are in good agreement with the consensus sequence for PKC substrates (X-S/T) C-X_{-3/2}(K/R) (33). In the case of another family member, coronin-2, Cai et al. (32) reported that Ser-2 was the single major phosphorylation site for PKC. Because both coronins share homologous N-terminal amino acid sequences (Fig. 3A), PKC is likely to catalyze phosphorylation of Ser-2 of p57/coronin-1. It would be of considerable interest to determine which subtype of PKC is responsible for the phosphorylation of p57/coronin-1 and whether or not phosphorylation at Ser-2 and Thr-412 is catalyzed by a distinct subtype of PKC.

We also analyzed phosphorylation of p57/coronin-1 from other cell types and found that human neutrophils and platelets and three human leukemic cell lines yielded two-dimensional gel electrophoresis profiles quite similar to that of HL60 cells (Supplement 1). It is, therefore, likely that the major phosphorylation sites are common to various cell types. Recently, Pareek et al. (36) reported that Thr-418 of mouse p57/coronin-1 was phosphorylated by cyclin-dependent kinase 5 in lymphocytes. However, human p57/coronin-1 has no Ser or Thr residues corresponding to Thr-418 of mouse p57/coronin-1 (Supplement 3A). We also found that the mutation of Thr-418 of mouse p57/coronin-1 caused no substantial changes in the Phos-tag-PAGE profile, although the mutation of Ser-412 suppressed the phosphorylation (Supplement 3B). We have so far no evidence showing that Thr-418 is a major phosphorylation site of mouse p57/coronin-1 under the conditions employed in this study. One possibility is that phosphorylation of p57/coronin-1 is differentially regulated by various protein kinases depending on cell types.

In two-dimensional gel electrophoresis, we observed minor spots in addition to the three major spots (Figs. 3B, 4B, and 5B). We originally assumed that these minor spots were attributable to a particular level of phosphorylation but eventually concluded that the appearance of the minor spots is independent on phosphorylation, based on the results of combined analyses with Phos-tag-PAGE and isoelectric focusing as well as staining with a phosphoprotein-specific dye (Fig. 7). Possible reasons for the appearance of the minor spots include 1) posttranslational modification other than phosphorylation, 2) incomplete denaturation of proteins by urea before isoelectric focusing, and/or 3) complex formation with other proteins.

This study indicates that the phosphorylation at Thr-412 of p57/coronin-1 modulates the interaction between p57/coronin-1 and actin (Figs. 1 and 8). Specifically, the results indicated that phosphorylation at Thr-412 reduced the affinity of p57/coronin-1 for actin. We assume that the molecular interaction is a direct rather than an indirect association mediated by other proteins such as the Arp 2/3 complex, because we did not detect any subunits of the Arp 2/3 complex in the SDS-PAGE of immunoprecipitate with anti-p57/coronin-1 antibody after protein staining with FlamingoTM (Fig. 8A). Indeed, it has been suggested that p57/coronin-1 physically interacts with the Arp 2/3 complex and that the interaction is weakened by the mutation at Ser-2 of p57/coronin-1 in lymphocytes (24, 32; i.e. the wild-type and S2A mutant (in which Ser-2 was replaced with Ala) of p57/coronin-1 bound to the Arp 2/3 complex, whereas the phosphorylation mimic S2D (in which Ser-2 was replaced with Asp) exhibited decreased binding activity, suggesting the possible involvement of Ser-2 phosphorylation in the interaction between p57/coronin-1 and the Arp 2/3 complex. On the other hand, the present study suggests that Thr-412 phosphorylation preferentially affects the interaction with G-actin. Co-sedimentation of the wild-type p57/coronin-1 and T412A and T412D mutants with F-actin revealed that both mutants possessed binding activity comparable to that of the wild type (Fig. 9A). By contrast, a similar experiment using the C-terminal-
truncated form of p57/coronin-1 (p57LZ, amino acid residues 372–461), which lacks binding capacity to F-actin (10), indicated that p57LZ/T412A had higher binding affinity for G-actin than p57LZ/T412D (Fig. 9B). The previous studies, including ours, suggested that p57/coronin-1 possessed at least two binding sites for actin, i.e. the 34 N-terminal amino acid residues (10) and 17 amino acid residues in the C-terminal region (amino acid residues 400–416) (35). Considering the recent findings by Föger et al. (37) that Ser-2 phosphorylation regulated the interaction of p57/coronin-1 with an actin cytoskeleton, phosphorylation at Ser-2 and Thr-412 may independently regulate the affinity of the adjacent actin-binding regions.

Due to the selective distribution of p57/coronin-1 within hematopoietic cells, much attention has been focused on the immunoregulatory functions of p57/coronin-1, especially with respect to its roles in the phagocytosis of leukocytes. p57/coronin-1 was transiently associated with phagosomes in the early phase of phagocytosis and then dissociated before the phagosome-lysosome fusion (14). Therefore, the dissociation of p57/coronin-1 from phagosomes is thought to be an essential step for the phagosome-lysosome fusion. During mycobacterial infection to macrophages, however, the dissociation of p57/coronin-1 from phagosomes and subsequent phagosome-lysosome fusion were impaired (38). The relationship between these phenomena and intracellular parasitism of mycobacteria has been discussed. Recent studies using p57/coronin-1-deficient macrophages showed that lysosomes were efficiently fused with phagosomes containing Mycobacterium bovis BCG (39, 40). Thus, p57/coronin-1 is regarded as a molecule blocking lysosomal delivery. Our previous studies also indicated that a PKC inhibitor suppressed the dissociation of p57/coronin-1 from phagosomes and the subsequent phagosome-lysosome fusion (14) and that phosphorylation of p57/coronin-1 was increased in phagocytosing cells (14, 29). In the present study, we used a newly developed anti-phospho-p57/coronin-1 antibody for immunoblotting and found that Thr-412 of p57/coronin-1 was transiently phosphorylated during phagocytosis of opsonized zymosan by neutrophils (Fig. 10). These results strongly suggest that the phosphorylation/deshphorylation turnover of p57/coronin-1 regulates the phagocytic process, including phagosome maturation, through reorganization of the actin-containing cytoskeleton. The regulatory mechanism underlying the phosphorylation of p57/coronin-1 is expected to provide insight into the phagocytosis of immunocytes and also a variety of immunological processes that involve p57/coronin-1, such as leukocyte chemotaxis, cytokine production, and immunological synapse formation.

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