Oligomerization, F-actin Interaction, and Membrane Association of the Ubiquitous Mammalian Coronin 3 Are Mediated by Its Carboxyl Terminus*

Zhiqiang Spoerl, Maria Stumpf, Angelika A. Noegel, and Andreas Hasse

From the Institute of Biochemistry 1, Medical Faculty, University of Cologne, Joseph-Stelzmann-Strasse 52, D-50931 Köln, Germany

Coronin 3 is a ubiquitously expressed member of the coronin protein family in mammals. In fibroblasts and HEK 293 cells, it is localized both in the cytosol and in the submembranous cytoskeleton, especially at lamellipodia and membrane ruffles. The carboxyl terminus of all coronins contains a coiled coil suggested to mediate dimerization. We show here that in contrast to other coronin homologues, the recombinant human coronin 3 carboxyl terminus forms oligomers rather than dimers, and that this part is sufficient to bind to and cross-link F-actin in vitro. The carboxyl terminus alone also conferred membrane association in vivo, and removal of the coiled coil abolished membrane localization but not in vitro F-actin binding. Coronin 3 is exclusively extracted as an oligomer from both the cytosol and the membrane fraction. Because oligomerization was not reported for other coronins, it might be a key feature governing coronin 3-specific functions. Cytosolic coronin 3 showed a high degree of phosphorylation, which is likely to regulate the subcellular localization of the protein.

Coronins are a family of F-actin-associated proteins expressed in a large variety of eukaryotes from yeast to man (1). The coronin prototype was isolated from actomyosin complexes of Dictyostelium discoideum (2). Mutant analysis and use of GFP fusion proteins in Dictyostelium showed that the protein is involved in phagocytosis, locomotion, and cytokinesis (3–5). Members of the family were found in lower (Saccharomyces, Entamoeba, and Trichomonas) and higher eukaryotes (Xenopus, Caenorhabditis, Drosophila, and mammals). Whereas lower eukaryotes contain unique coronins, metazoa express several homologues. Two genes were identified in the Drosophila and Caenorhabditis genomes, whereas in the human data base, five different coronin-like proteins were found (own data base search, GIs 7290756 and 7302313) (1, 6). According to a suggestion by Okumura (7), extended by de Hostos (1), these can be classified as coronins 1–5. Coronins 1–3 have also been grouped as coronins 1A–1C and coronins 4 and 5 as 2A and 2B, based on their relative homology. All coronins share a central sequence, the WD40 repeat elements, and an additional 70–40 amino acids are predicted to form a coiled coil structure in all homologues, which could mediate self-association of the protein. Recently, dimerization via the coiled coil has been demonstrated for a Xenopus coronin (9), but the role of this module in mammalian homologues has not been investigated. Most coronins are expressed in a strictly tissue-specific manner indicative of tissue-specific functions. Coronin 1 (p57, TACO) is mainly expressed in hematopoietic tissues and is involved in the assembly of the NADPH oxidase complex in neutrophils, and it also localizes to phagosomes in macrophages (10–12). Coronin 2, a variant of coronin 2 expressed in secretory cells of the gastric mucosa and the kidney, is found at secretory canaliculi (13), coronin 4 (“IR10”) is expressed in testis and, to a lesser extent, in brain (14). Coronin 5 (“Clipin C”) is restricted to neuronal tissue (15), where it accumulates at growth cones and colocalizes with focal adhesions. The most widely expressed mammalian isoform, coronin 3, has not been extensively characterized yet. Its mRNA is found in all tissues examined (7, 16). F-actin colocalization was found for transfected hemagglutinin-tagged coronin 3 (16). Because of its ubiquitous expression, the role of coronin 3 may be a general instead of a tissue-specific one. In this study, we have examined the properties of the unique COOH-terminal region of human coronin 3 (Hcoronin). Using in vitro F-actin binding studies, subcellular fractionation, and stable expression of transfected EGFP1-tagged proteins in HEK 293 cells, we found a role for the COOH terminus in F-actin association and cross-linking as well as in localization of the protein to plasma membranes. Whereas the COOH-terminal coiled coil was not necessary for F-actin binding in vitro, it was essential for cortical localization as well as for the formation of homo-oligomers in vitro. Gel filtration of high salt-treated cell fractions suggests that coronin 3 is present in the form of stable oligomers in vivo as well, which contrasts with the suggested coronin dimerization and may be specific for coronin 3, because this function is exerted by the unique region not shared by other homologues.

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† To whom correspondence should be addressed. Tel.: 49-221-478-6980; Fax: 49-221-478-6979; E-mail: noegel@uni-koeln.de.

‡ The abbreviations used are: EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; PMA, phorbol 12-myristate 13-acetate; WD40 repeat, tryptophan and aspartate containing repeat; mAAb, monoclonal antibody; TRITC, tetramethylrhodamine isothiocyanate; aa, amino acid(s); BSA, bovine serum albumin; ER, endoplasmic reticulum.
EXPERIMENTAL PROCEDURES

DNA Constructs—A complete Hcoronin 3 open reading frame was constructed by completing an EST clone (accession number W40565, obtained from the IBRC Centre, Cambridge, United Kingdom). Roche lacked the 5′ 140 bp of the putative coding sequence with an overlapping PCR fragment covering the 5′ 554 bp. Primers used for PCR were 5′-TGTAGCACGAGAAGTTTCCG-3′ (forward) and 5′-CTGCTTCTGGAAGCTTGGCAG-3′ (reverse), based on overlapping clones in the human EST data base. The PCR fragment was constructed with the EST cloning vector an Nsi I site in the overlapping region to generate a cDNA. The sequence was verified and inserted in-frame into pEGFP-C1 (Clontech) to yield an EGF fusion protein. Constructs coding for truncated EGF-Hcoronin 3 fusion proteins were cloned by insertion of the respective sequences generated by PCR into the pEGFP-C-series of vectors. COOH-terminal fragments of Hcoronin 3 generated by PCR were inserted into a pcDNA3.1mycHis vector (Invitrogen) to express proteins carrying a C-terminal Myc tag. All constructs were verified by sequencing. The plasmids pEXV mycV12Rac1 and pEXVmyc-V12N17Rac1 were a generous gift of Dr. M. F. Olson (Cheresty Beaty Laboratories, Institute of Cancer Research, London, UK) and have been published (17, 18). Plasmid DNA used for transfection was prepared and purified using NucleoBond AX plasmid mini and maxi kits (Macherey & Nagel).

Antibodies and Immunoblotting—A 500 bp fragment coding for the COOH-terminal 160 amino acids of Hcoronin 3 was inserted into pQE30 (Qiagen) for expression as a His+-tagged polypeptide. Expression in E. coli was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Purification of the His-tagged protein by nickel nitriiotriacetate-agarose affinity chromatography was according to the manufacturer’s instructions (Qiagen). Immunization of female Balb/c mice was done using the immunochemical method (Qiagen). Antibodies were generated according to standard protocols. Monoclonal antibody K6-444 was used in this study. It specifically recognized coronin 3 in human, mouse, and COS-1 cells and did not react with other recombinant mammalian coronins. MAb K3-184 was used for detection of EGFPP, mAb 203–217 recognized annexin A7 (19), and monoclonal antibody mAB 444 recognized β-COP (20). Anti-58K Golgi protein, and polyclonal anti-pan cadherin were obtained from the manufacturer. The primary antibody K6-444 (hybridoma culture supernatant) and secondary IgG (goat anti-mouse coupled to Alexa 488, 1:2,000, Molecular Probes) was used for 60 min each. For coimmunoprecipitation experiments, Swiss 3T3 cells were fixed in 3% paraformaldehyde followed by permeabilization with 0.2% Triton-X100 in PBS. F-actin was labeled by incubation for 60 min with 200 ng/ml TRITC-phalloidin (Sigma). Cells coexpressing EGFPP-Hcoronin 3 fusion protein and Mys- teganin were described above. Confocal microscopy, with a polyclonal rabbit anti-Myc antibody diluted 1:1,000 (Santa Cruz) followed by detection with polyclonal goat anti-rabbit IgG conjugated with Alexa 568 (1:2,000, Molecular Probes). Alternatively, methanol fixation was done as described (20). Images were taken with a Leica DMR microscope and SensiCam camera and Software (PCO), or (for colocalization studies) a Leica DM IBBE (inverted) microscope and TCS SP confocal laser scanning technology with TCSNT software. Image processing was done with Adobe Photoshop or TCSNT software, respectively.

Subcellular Fractionation, Gel Filtration, and Two-dimensional Gel Electrophoresis—Differential centrifugation was done according to standard protocols. Briefly, confluent cell monolayers (cloned HEK 293) were detached and scraped off in HES buffer (20 mM Hepes, pH 7.2, 1 mM EDTA, 0.25 M sucrose) containing Roche complete protease inhibitor mixture. Cells were disrupted with the help of a tight fitting Dounce homogenizer and nuclei and intact cells were removed by centrifugation at 500 × g for 10 min. The subsequent pelleting steps were performed at 2,000 × g (10 min), 10,000 × g (30 min), and 100,000 × g (60 min), respectively. All pellet fractions were resuspended in HES buffer and recentrifuged to purify the pellets. The pellets were finally suspended in equal volumes of HES and subjected to Western blot analysis. For Triton X-100 extraction, 10,000 × g pellets were incubated for 1 h at 4 °C in HES containing 1% Triton X-100 and recentrifuged at 10,000 × g for 60 min. For detergent solubilization of the COOH terminus of Hcoronin 3 protein was done with mAb K6-444 followed by incubation with goat anti-mouse IgG antiserum coupled to horsedarsh peroxidase (Sigma), followed by enhanced chemiluminescence and exposure to x-ray films (Kodak).

Mammalian Cell Culture, Stimulation, and Transfection—HER 293 human embryonic kidney cells were grown in Dulbecco’s modified Eagle’s medium with 4 g/liter glucose (Invitrogen) supplemented with 10% fetal calf serum (Biochrom), 2 mM L-glutamine (Biochrom), 1 mM sodium pyruvate (Biochrom), 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen). Swiss 3T3 murine fibroblasts were obtained from the DSMZ (Braunschweig, Germany) and cultured under standard conditions in Dulbecco’s modified Eagle’s medium with 1 g/liter glucose, 10% fetal calf serum, penicillin/streptomycin, and L-glutamine. Primary human skin fibroblasts were kindly provided by Dr. Th. Krieg (Depament of Dermatology and Venerology, University Hospital, Cologne, Germany) and passed up to 10 times in Dulbecco’s modified Eagle’s medium with 4 g/liter glucose, 10% fetal calf serum, 50 μg/ml l-ascorbic acid, penicillin/streptomycin, and L-glutamine. For induction of membrane ruffling, confluent Swiss 3T3 cells seeded on 12-mm glass coverslips were serum-starved overnight (16 h) and subsequently treated with 20 ng/ml FGF-2 (Sigma) for 15 min or 30 ng/ml bovine insulin (Biochemica) for 30 min. For actin depolymerization studies, cells were treated with cytochalasin D (10 μM, 30 min, Sigma) or latrunculin A (5 μM, 60 min, Sigma) prior to fixation. Transfection of HEK 293 cells was done by electroporation, stably transfected clonal cells were selected in culture medium containing 500 μg/ml gene-
was done according to the manufacturer's instructions (Qiagen). The identity of the recombinant proteins was confirmed by matrix-assisted laser desorption/ionization, and the α-helical structure was determined for the COOH-terminal fragment by CD spectra. Recombinant Hcoronin 3 proteins were analyzed by gel filtration using the SMART system (Amersham Biosciences), 2 g of protein were loaded on a Superdex 200 column. For actin binding experiments, -actin was prepared from rabbit muscle (21) and in vitro F-actin cosedimentation assays were performed essentially as described (20). For determination of F-actin bundling activity, the recombinant polypeptides were incubated with F-actin under the same conditions and samples were pelleted at 12,000 g for 30 min. To assay the binding of different recombinant Hcoronin 3 fragments to each other, Maxisorp 96-well plates (Nunc) were coated with Hcoronin 3 (aa 1-71, 0.25 nmol/well) or BSA as a control. After washing and blocking with BSA, wells were incubated with PBS containing different concentrations of recombinant Hcoronin 3 (aa 315-444) and 1% BSA. Bound Hcoronin 3 (aa 315-444) was detected by anti-Hcoronin 3 monoclonal antibody and secondary alkaline phosphatase-conjugated anti-mouse antibody (Sigma), followed by a colorimetric assay (Sigma Fast p-nitrophenyl phosphate substrate).

RESULTS

Generation of a Coronin 3-specific Antibody and Analysis of Tissue Distribution—We have cloned a complete cDNA for Hcoronin 3 by reverse transcriptase-PCR using information from the EST data base. At the DNA level, the sequence shows 97% identity to mouse coronin 3 and contains an open reading frame coding for a 474-amino acid protein. At the amino acid level, the Hcoronin 3 sequence is 73% homologous to mouse coronin 2 and 65% to Hcoronin 1 (Fig. 1). We have raised monoclonal antibodies specific for coronin 3 by immunizing mice with a recombinant His6-tagged peptide containing the carboxyl-terminal 164 amino acids of Hcoronin 3 that are outside of the WD repeats. The antibody specifically reacted with EGFP-tagged Hcoronin 3 and did not bind to EGFP fusions of the closely related human coronins Hcoronin 1 and 2 in transfected HEK cell lines. Similar results are shown in Table 1.
293 cells, whereas a monoclonal antibody specific for GFP detected proteins of the correct size in all extracts (Fig. 2A). We also tested the expression of the Hcoronin 3 protein in several tissues by probing total homogenates from murine organs. Brain, lung, liver, kidney, spleen, ovary, and thymus harbored a 57-kDa protein. In skeletal muscle and heart, a single protein of a slightly higher molecular mass was detected, and both forms were present in brain, with the 57-kDa protein being more abundant (Fig. 2B).

Subcellular Localization of Endogenous and EGFP-Hcoronin 3—We used primary human skin fibroblasts to determine the subcellular localization of endogenous Hcoronin 3. In methanol-fixed, fully adherent cells, coronin 3 antibodies labeled filamentous as well as punctate structures in the cytoplasm (Fig. 3A). The latter were most prominent around the nucleus. Cells forming lamellipodia and the typical shape of migrating cells revealed staining of lamellipodia and leading edges, respectively (Fig. 3B). Spreading cells having adhered to coverslips showed a prominent cortical rim of Hcoronin 3 in addition to the punctate perinuclear staining (Fig. 3C). Because formation of lamellipodia and membrane ruffles is strongly enhanced by activators of the small GTPase Rac1 in Swiss 3T3 fibroblasts (22, 23), we treated confluent, serum-starved Swiss 3T3 cells with PMA or insulin to detect a possible translocation of coronin 3 during remodeling of the cortical cytoskeleton. Pronounced membrane ruffling was verified by F-actin staining (not shown). In fact, virtually all PMA-treated and a significant number of insulin-stimulated cells exhibited accumulation of coronin 3 at membrane ruffles (Fig. 3, E and F) in addition to the punctate pattern of unstimulated cells (Fig. 3D). To test whether the subcellular localization of endogenous Hcoronin 3 is dependent on an intact F-actin structure, Swiss 3T3 cells were treated with the actin depolymerizing drug cytochalasin D. Nonconfluent Swiss 3T3 cells left untreated (G) or incubated with 10 μM cytochalasin D (CyD, 30 min, H and I). Cells were fixed in paraformaldehyde and stained for coronin 3 (G and H) and F-actin (TRITC-phalloidin, I). Arrowheads, localization of coronin 3 (H) and actin accumulation (I). J and K, Swiss 3T3 cells transfected with the plasmids indicated and paraformaldehyde-fixed 16 h post-transfection. Only cells expressing the Myc-tagged GFP fusions as identified by staining with rabbit anti-Myc antibody followed by Alexa 568-coupled goat anti-rabbit IgG are shown. Bars, 50 μm.

In Vitro Interaction with F-actin—The results reported by Mishima and Nishida (25) suggest that NH2- and COOH-terminal parts of Xenopus coronin are required for F-actin colocalization in vivo. The COOH terminus of Hcoronin 3 contains regions homologous to Xenopus coronin as well as unique sequences, and might thus play a similar role in F-actin interaction. Sequence analysis of amino acids 315–474 containing the unique region of Hcoronin 3 revealed homology to other F-actin-binding proteins with coiled coil structures such as myosins, tropomyosins, and the COOH terminus of VASP. Recombinant NH2- and COOH-terminal Hcoronin 3 polypeptides were tested in an in vitro F-actin cosedimentation assay. A considerable amount of the COOH-terminal protein (residues 315 to 474) pelleted in a high speed spin with F-actin (Fig. 4A). Moreover, under low speed spin conditions (12,000 × g) that do not pellet single F-actin filaments, this fragment interacted...
with F-actin in such a way that it could be pelleted (Fig. 4B). The COOH-terminal coiled coil was suggested to mediate self-interaction of coronins. This might be necessary for F-actin cross-linking and bundling activity of proteins carrying a single F-actin binding site per molecule. Thus, the role of the coiled coil for F-actin interaction was tested with a recombinant protein lacking the COOH-terminal 30 amino acids (residues 315–444). Cosedimentation at low and high speed conditions were essentially the same as for the complete COOH terminus (Fig. 444). Cosedimentation at low and high speed conditions were indicated. Aliquots of eluted fractions were analyzed by Western blots with mAb K6-444.

Subcellular Distribution of Hcoronin 3—To determine the subcellular distribution of Hcoronin 3, we have performed differential centrifugation experiments with homogenates of HEK 293 cells that endogenously express high amounts of the protein. A significant part of Hcoronin 3 was found in the 2,000 × g postnuclear pellets, about 60% remained in the 10,000 × g cytosolic supernatant. No Hcoronin 3 was detected in the microsomal 100,000 × g pellet (Fig. 5A). We further analyzed the cellular distribution by isopycnic sucrose step gradient centrifugation of total cell homogenates and the 10,000 × g pellets of HEK 293 cells. When total homogenates were separated, Hcoronin 3 was present in two positions in the gradient. About half of the material was present in fractions representing soluble material (Fig. 5B, lanes 6–8), the other half cofractionated with the plasma membrane marker E-cadherin. These fractions also contained the ER marker BiP/GRP78. D, 10,000 × g pellets were extracted with 1% Triton X-100 in PBS or PBS (C, control), the presence of Hcoronin 3 and annexin A7 in the pellets after treatment and recentrifugation was determined by Western blotting. E, partial colocalization of EGFP-Hcoronin 3 with E-cadherin at the plasma membrane in paraformaldehyde-fixed HEK 293 cells.
Coronin 3 could not be extracted from the 10,000 × g pellet by Triton X-100 treatment (Fig. 5D). For control, the samples were also probed for the partially membrane-associated protein annexin A7, which was removed from the pellet to a larger extent (26). Coronin 3 could therefore represent a component of the Triton-insoluble submembranous cytoskeleton or of membranes that are not solubilized by this treatment. In colocalization studies with EGFP-Hcoronin 3-transfected cells stained with antibodies against E-cadherin and the ER, lysosome, and Golgi markers, only E-cadherin partially colocalized with EGFP-Hcoronin 3, especially at cell-cell junctions. In addition, EGFP-Hcoronin 3 was also found at E-cadherin-negative areas of the plasma membrane (Fig. 5E, arrowheads).

Both NH2 and COOH Termini Are Required for Membrane Localization—Next, we examined the role of different Coronin 3 domains for membrane localization by expressing EGFP-fused deletion proteins in clonal HEK 293 lines. Expression levels of the fusion proteins were similar to that of endogenous Coronin 3, as determined by Western blots. Full-length EGFP-Hcoronin 3 (aa 1–474), NH2-terminal (aa 72–474) and COOH-terminal (aa 1–444) deleted proteins as well as the NH2-terminal domain (aa 1–71), the conserved “core” part containing the WD repeats (aa 72–404), and the COOH terminus alone (aa 315–474) were tested. Extracts from stably transfected cells were prepared and separated by differential centrifugation. The 10,000 × g pellet and the cytosolic 100,000 × g supernatants were subjected to Western blot analysis and only a minor fraction was in the 10,000 × g supernatants (P) as well as 100,000 × g supernatants (S) were analyzed in Western blots detecting EGFP fusions (upper panel), endogenous Coronin 3 (middle panel), and β-actin (lower panel). B, cell lysates from cells expressing EGFP-fused Coronin 3 versions indicated by the amino acid numbers were lysed and total cell homogenates as well as 10,000 × g pellets extracted with Triton X-100 were analyzed using anti-EGFP antibody in Western blots.

Localization of EGFP-Hcoronin 3 Domains—The distribution of the EGFP fusions was also visualized by fluorescence microscopy. Paraffin-embedded fixed cells were stained with TRITC-labeled phalloidin to visualize F-actin (Fig. 7). Full-length EGFP-Hcoronin 3 localized to cortical F-actin-rich regions and to punctate actin-positive structures in the center of the cells. The proteins lacking the NH2 terminus (72–474) or the major part of the coiled coil (1–444) were diffusely distributed and showed neither colocalization with fibers nor the F-actin-positive spots in the cell center, although EGFP-Hcoronin 3-(1–474) behaved like the endogenous protein and were present in the insoluble fraction (Fig. 6B).

Coronin 3 Forms Oligomers in Vivo That Are Phosphorylated in the Cytosol but Not in Particulate Fractions—Because deletion of the most COOH-terminal 30 amino acids led to a redistribution of EGFP-Hcoronin 3-(1–444) to the cytosol (Fig. 6A) and these residues were responsible for oligomerization in vitro (Fig. 4C), it seemed plausible that the failure to oligomerize led to a relocation to the cytosol. We therefore tested the oligomerization state of soluble and particle-bound Coronin 3.
by gel filtration of 10,000 × g pellets and 100,000 × g supernatants of HEK 293 cells. To reduce interactions with other proteins and to extract Hcoronin 3 from insoluble compartments, the subcellular fractions were treated with 0.6 mM KCl and the supernatants were subjected to gel filtration, followed by Western blot analysis with anti-coronin 3 monoclonal antibodies. KCl treatment allows disintegration of cytoskeletal complexes (27). The majority of Hcoronin 3 eluted with an apparent molecular weight of about 150,000–200,000 when derived from the cytosol as well as from the particulate fractions under high salt conditions (Fig. 8A). The fractions were also probed for β-actin as an endogenous control that eluted exclusively as a monomer from the columns. This suggests that the oligomerization state of Hcoronin 3 is the same in both cellular fractions.

As PKC-dependent phosphorylation occurs with other coronins (15, 28), we have also analyzed this feature as a possible determinant of subcellular localization. 10,000 × g pellets and 100,000 × g supernatants were analyzed by two-dimensional gel electrophoresis, followed by Western blotting. Fig. 8B shows that Hcoronin 3 from cytosolic supernatants exhibits different PI values. A smaller amount has the predicted PI of 6.65, and a high amount of cytosolic Hcoronin 3 was present in considerably more acidic spots. Similar acidic spots were also observed for stably expressed EGFP-Hcoronin 3 (not shown). In contrast to this, nearly all of the particle-associated Hcoronin 3 had a PI of 6.65 as calculated from the amino acid sequence. The acidic PI values are most likely because of phosphorylation, because Hcoronin 3 as well as other coronins contain several potential serine and threonine phosphorylation sites.

**Interactions of the NH2-terminal and COOH-terminal Domains of Hcoronin 3**—In a further attempt to confirm Hcoronin 3 self-association via the coiled coil, Myc-tagged full-length and COOH-terminal (aa 315–474) Hcoronin fusion proteins were expressed transiently in HEK 293 cells that stably expressed EGFP-Hcoronin 3 full-length and truncated proteins. Myc-tagged proteins were immunoprecipitated with an anti-c-Myc antibody, and the precipitates were analyzed by Western blots for the presence of the EGFP-tagged proteins. In these experiments, EGFP-Hcoronin 3-(1–444) that lacks the coiled coil was coprecipitated both with full-length Hcoronin 3-Myc and Hcoronin 3-(315–474)-Myc (data not shown). Thus, the Hcoronin 3 COOH terminus interacted with the full-length protein also via regions distinct from the coiled coil. To determine an additional interaction region(s), a Myc-tagged Hcoronin 3 C-terminal protein lacking the coiled coil (residues 315–444) was used for coimmunoprecipitation. The full-length EGFP-Hcoronin 3, the COOH-terminal deleted EGFP-Hcoronin 3-(1–444) (not shown), and EGFP-Hcoronin 3-(1–71) efficiently coprecipitated with this truncated protein, whereas the EGFP fusions containing amino acids 72–474 and 315–474 did not (Fig. 9A). Interaction of recombinant amino acids 315–344, comprising the COOH-terminal noncoiled coil part, with amino acids 1–71 was also found in vitro. NH2 terminus or BSA as a control were immobilized on enzyme-linked immunosorbsorbent assay plates and incubated with different amounts of the COOH-terminal fragment that was detected semiquantitatively by mAb K6-444 specifically reactive with the COOH terminus. The binding of the COOH terminus was concentration-dependent and reached saturation. Nonspecific binding to BSA-coated wells was con-

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**Fig. 7.** Localization of full-length and truncated EGFP-Hcoronin 3 in HEK 293 cells. Cells expressing the EGFP-tagged regions of Hcoronin 3 containing the amino acids indicated were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with TRITC-phalloidin. Arrowheads mark areas of cortical localization of the EGFP-Hcoronin 3 fusion proteins.

**Fig. 8.** Oligomerization and two-dimensional gel electrophoresis of particle-associated and cytosolic Hcoronin 3. A, gel filtration of HEK 293 cells 100,000 × g cytaiosolic supernatants and 10,000 × g membrane pellets were incubated with 0.6 mM KCl and subjected to gel filtration using a Superdex 200 column. Fractions were collected and analyzed by Western blots with anti-coronin 3 and anti-β-actin antibodies. The elution of molecular weight standards used for calibration is indicated. B, the 10,000 × g pellet and cytosolic fraction of HEK 293 cells were separated on two-dimensional gels and Hcoronin 3 was visualized by Western blots using coronin 3-specific mAb. The blots shown are representative of five independent experiments. Arrowhead, predicted PI of Hcoronin 3 (6.65).
Fig. 9. Interaction of Hcoronin 3 NH2- and COOH-terminal regions. A, coimmunoprecipitation of EGFP-tagged Hcoronin 3 polypeptides containing the amino acids indicated with Myc-tagged Hcoronin 3-(315–444) from cell extracts prepared from cotransfected HEK 293 cells. Left panel, total lysates were probed for the presence of EGFP fusion proteins with mAb K12-184. Right panel, the immunoprecipitates obtained with a Myc-specific polyclonal antibody were probed for the presence of EGFP fusion proteins with mAb K12-184; IgG, IgG heavy chain. B, binding of Hcoronin 3-(315–444) to immobilized Hcoronin 3-(1–71). Recombinant NH2-terminal Hcoronin 3 fragment (filled symbols) and BSA (open symbols) were immobilized on 96-well enzyme-linked immunosorbent assay plates (0.25 nmol/well, filled symbols) and incubated with the indicated amounts of Hcoronin 3-(315–444). A bound COOH-terminal fragment was quantitated by enzyme-linked immunosorbent assay using mAb K6-444 reactive with the COOH terminus of coronin 3 and alkaline phosphatase-conjugated anti-mouse antibody. Measurements were performed in quadruplicate. C, cosedimentation of recombinant polypeptides with F-actin. D, F-actin cosedimentation of constant amounts of recombinant Hcoronin 3-(315–444) in the presence of different amounts of Hcoronin 3-(1–71). S, supernatant; P, pellet.

DISCUSSION

Coronin 3 expression is ubiquitous and overlaps with other coronin homologues (7, 16). This protein may thus be involved in non-tissue-specific processes or play an accessory role in tissue-specific events. We found endogenous coronin 3 at punctate and filamentous cytoplasmic structures and lamellipodia in different cell lines. Coronin 3 was shuttling between an intracellular pool and the cell cortex during remodeling of the cortical cytoskeleton.

Hcoronin 3 Localizes to the Plasma Membrane in an Actin-independent Manner—In addition to its presence in the cytosol, Hcoronin 3 is also associated with membranes in a form that is not extracted by Triton X-100. Because the Hcoronin 3 sequence contains no putative transmembrane domains or prenylation motifs, a direct association with membranes seems unlikely. This view is also supported by the fact that localization of Hcoronin 3 both to the membrane and to punctate cytoplasmic structures in immunofluorescence experiments are abolished by treatment of cells with actin-depolymerizing drugs. In flotation experiments, Hcoronin 3, but not β-actin or E-cadherin, was also found at the bottom of the gradient. This might be because of dissociation of Hcoronin 3-containing protein complexes from the membranes during the assay. Together with the localization to membrane ruffles, this suggests a likely role of coronin 3 in organization of the submembranous cytoskeleton during spreading, adhesion, and cell migration. Stably expressed truncated versions of EGFP-Hcoronin 3 that lacked the COOH-terminal or NH2-terminal non-WD part were not found in the membrane fraction and showed no clear colocalization with cortical F-actin in fluorescence images, although EGFP-Hcoronin 3-(1–444) retained a weak enrichment at lamellipodia. The COOH terminus alone, which harbors both F-actin binding and oligomerization sites, was sufficient for Triton-resistant membrane association. EGFP fluorescence, but not membrane association, has also been studied for truncated versions of Xenopus coronin with similar results (25).

A further interesting outcome is that the truncated Hcoronin 3 only containing the core region and thus lacking all regions implied in F-actin colocalization, not only failed to localize to membranes, but also affected the shape of the cells (Fig. 7). These cells showed impaired spreading and adhesion to solid supports, whereas cell-cell adhesion was obviously unaffected. This led to a rounded or spindle-like cell shape. Because similarly truncated Xenopus coronin led to impaired Rac-mediated spreading and lamellipodia formation, Mishima and Nishida (25) suggested that the coronin core might directly interact with this small GTPase and might block signal transmission to downstream effectors.

In Vitro F-actin Interaction of the Hcoronin 3 COOH Terminus—The COOH-terminal part of Hcoronin 3 is a region most likely harboring both homologue-specific and conserved func-
Properties of the Mammalian Coronin 3 COOH Terminus

Fig. 10. Schematic view of functions assigned to different regions of coronin 3. Upper panel, summary of the functions assigned to coronin 3; lower panel, activities of different truncated constructs. The WD repeat region (solid box) and the coiled coil (hatched) are highlighted. *, indicates data from Ref. 1.

Interactions of NH2- and COOH-terminal Regions—The NH2-terminus of Coronin 3 bound to the COOH terminus both in vitro and in vivo in coimmunoprecipitation experiments (Fig. 9). The regions of the COOH terminus required for the binding of the NH2-terminus and of F-actin are close to each other and may even overlap (Fig. 10). Thus, the NH2-terminus might interfere with F-actin binding. In fact, addition of the NH2-terminus to the COOH-terminal fragment Hcoronin 3-(315–444) markedly reduced F-actin cosedimentation by the NH2-terminus in the full-length protein. The other possibility is that the WD repeat region actively causes cytosolic localization and is “antagonized” by the NH2-terminus in the full-length protein.

Oligomerization of the COOH Terminus—A further feature revealed by the COOH terminus was its ability to form oligomers. Although the coiled coil of coronins is generally regarded to be a dimerization domain (1), dimerization was only proven for Xenopus coronin (9). Our gel filtration data for the recombinant Hcoronin 3 COOH terminus show the formation of higher order oligomers, most likely trimers, instead of dimers. Computer prediction (multicore program, Ref. 30) suggests a high probability of trimer formation for the Hcoronin 3 coiled coil (0.61 trimer versus 0.15 dimer) in contrast to Xenopus coronin (0.19 trimer versus 0.5 dimer) and human coronin 1 (0.09 trimer versus 0.9 dimer). The latter two contain a leucine zipper motif that is absent in coronin 3. We therefore suggest that different coronins might form different homomers, which may be related to their specific functions, e.g. by extending the number of multiple protein-protein interactions. Oligomerization, F-actin binding, and cross-linking properties of the Hcoronin 3 COOH terminus are reminiscent of the Ena-VASP homology domain 2 of mammalian VASP protein (31). The COOH terminus of VASP contains a putative coiled coil as well, is similar in length, and contains one F-actin binding site per molecule. The coiled coil mediates VASP tetramerization, which is a prerequisite for F-actin bundling. The Ena-VASP homology 2 domain represents a novel type of F-actin-binding module and the coronin 3 COOH terminus shows a weak homology to Ena-VASP homology domain 2. Both sequences contain heptad repeats forming a “mixed charge cluster,” which is not found in other coronins. This might point to a common structure for both COOH termini, and the coronin 3 COOH terminus could represent a functionally related module for F-actin interaction. It is not known whether mammalian coronins exhibit F-actin bundling or cross-linking activity also in vivo, but we found that overexpression of EGFP-Hcoronin 3 in cell types other than HEK 293, like COS-7 and Jurkat cells, caused the formation of thick bundle-like structures containing F-actin and EGFP-Hcoronin 3 (not shown). The yeast coronin homologue Cnp1 leads to the formation of similar rod-like F-actin bundles upon overexpression and mediates F-actin cross-linking in vitro (32). We also detected the presence of oligomers in vivo. Hcoronin 3 was extracted by 0.6 M KCl both from the cytosolic and particulate fractions of HEK 293 cells nearly completely as an oligomer with an apparent mass of about 150–200 kDa, as shown by gel filtration. This treatment dissolves cytoskeletal complexes (27), and it is likely that Hcoronin 3 exists exclusively in the form of oligomers that require hydrophobic interactions. Dimers formed by Xenopus coronin have been reported to be stable even in 2 M KCl and 2 M urea (9). Together with the fact that deletion of the coiled coil decreases cortical localization (Fig. 7), our results suggest that
oligomerization seems to be a prerequisite, but not sufficient, for membrane association, and other factors may determine translocation of the protein between the cortex and the cytosol.

One likely mechanism directing Hcoronin 3 localization is its phosphorylation, because this modification occurs with other coronins (11, 13, 28). In agreement with this, we found that the majority of Hcoronin 3 from cytosolic fractions had a pI different from the predicted one, whereas Hcoronin 3 from the particulate fraction was not modified. This suggests that association with and dissociation from the membrane-associated cytoskeleton requires phosphorylation and dephosphorylation events. Similarly, the dissociation of coronin 1 from phagosomes requires phosphorylation by protein kinase C (33). The Hcoronin 3 sequence contains, among potential target sites for other Ser/Thr kinases, eight possible target sites for PKC (34), allowing for comparable processes in the dissociation of coronin 1 from phagosomes and in the dissociation of coronin 3 from the cortical cytoskeleton.

Acknowledgments—We thank Dr. Michael F. Olson for the generous gift of Rac1 expression plasmids, Dr. Th. Krieg for providing primary cortical cytoskeleton, and Drs. M. Schleicher and E. Korenbaum for providing rabbit skeletal muscle actin.

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