Palladin Is an Actin Cross-linking Protein That Uses Immunoglobulin-like Domains to Bind Filamentous Actin

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Palladin is a recently described phosphoprotein that plays an important role in cell adhesion and motility. Previous studies have shown that palladin overexpression results in profound changes in actin organization in cultured cells. Palladin binds to the actin-associated proteins α-actinin, vasodilator-stimulated phosphoprotein, profilin, Eps8, and ezrin, suggesting that it may affect actin organization indirectly. To determine its molecular function in generating actin arrays, we purified palladin and asked if it is also capable of binding to F-actin directly. In co-sedimentation and differential sedimentation assays, palladin was found to both bind and cross-link actin filaments. This bundling activity was confirmed by fluorescence and electron microscopy. Palladin fragments were then purified and used to determine the sequences necessary to bind and bundle F-actin. The Ig3 domain of palladin bound to F-actin, and a palladin fragment containing Ig3, Ig4, and the region linking these domains was identified as a fragment that was able to bundle F-actin. Because palladin has multiple Ig domains, and only one of them binds to F-actin, this suggests that different Ig domains may be specialized for distinct biological functions. In addition, our results suggest a potential role for palladin in generating specialized, actin-based cell morphologies via both direct actin cross-linking activity and indirect scaffolding activity.

The actin cytoskeleton is a dynamic assembly that provides the cell with mechanical support and elasticity (1, 2), participates in cell locomotion through the formation and disassembly of protrusions (3), and provides a scaffold for the trafficking of cellular components (4, 5) and organization of signaling complexes (6). The organization of actin networks within the cell is tightly controlled by a variety of regulatory proteins that either cross-link actin filaments into robust bundles or regulate the assembly and disassembly of actin filaments by: 1) capping/uncapping or severing the filament ends or 2) promoting the polymerization of actin at specific sites (7). To date, more than 23 classes of proteins have been shown to cross-link actin filaments into tight parallel bundles, loosely spaced bundles, or flexible networks (8). Actin cross-linking proteins such as filamin (9, 10), α-actinin (11), and fascin (12) play important roles in maintaining cell shape and allowing cells to move and adhere to a substrate. In addition, in differentiated cells in vivo, actin cross-linking proteins have a critical function in generating specialized actin-based structures such as sarcomeres, microvilli, and stereocilia (reviewed in Refs. 13 and 14). Thus, the extraordinary diversity of actin-binding proteins provides cells with a wide variety of molecular tools for constructing both dynamic and stable arrays of actin filaments.

Palladin is a recently described phosphoprotein that is widely expressed in vertebrate cells and tissues (15–17). Palladin exists as three major isoforms, which display apparent molecular masses of 90, 140, and 200 kDa by SDS-PAGE (15, 16). Palladin is the most widely expressed member of a novel subfamily of actin-associated proteins (18). The two other members of this family of proteins, myotilin (19) and myopalladin (20), are primarily expressed in striated muscle. Palladin has been detected in structures that contain contractile bundles of actin filaments, such as stress fibers and sarcomeres (15, 17). Palladin also localizes to anchoring structures such as focal adhesions and podosomes, and motile structures such as neuronal growth cones and dorsal ruffles (15, 16, 21, 22). Palladin is ubiquitously expressed in embryonic organs but is down-regulated in some adult tissues (15). Within smooth muscle and non-muscle cells, palladin localizes to actin filaments in regularly spaced puncta that have also been found to contain α-actinin (23) and vasodilator-stimulated phosphoprotein (VASP)4 (24). Palladin possesses a large number of molecular partners, including a cohort of proteins that bind directly to actin: α-actinin (23), VASP (24), profilin (25), ezrin (17), and Eps8 (22). Palladin also binds to a second cohort of proteins that indirectly influence actin organization: ArgBP2 (26), LPP (27), and SPIN90 (28). The larger isoforms of palladin also bind to the actin-binding protein Lasp-1 (16). The observation that palladin binds to an unusually large number of actin-binding proteins suggests that it may function as an actin-associated scaffolding molecule.

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4 The abbreviations used are: VASP, vasodilator-stimulated phosphoprotein; Ig, immunoglobulin; DTT, dithiothreitol; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; SEC-MALS, size exclusion chromatography-multangle light scattering.
Although the precise cellular function and mode of subcellular localization for palladin is still under investigation, results to date indicate that palladin plays an important role in organizing the actin arrays needed for normal cell adhesion, motility, and changes in morphology during cell development. Palladin expression has been explored in a variety of cell types and a mouse model organism. With antisense and small interfering RNA knockdown approaches, loss of palladin expression is associated with a failure of cells to assemble stress fibers, focal adhesions, dorsal ruffles, and podosomes (15, 22). Up-regulation of palladin correlates with changes in cell morphology and the assembly of the actin cytoskeleton in maturing peripheral blood monocytes (17). Decreased palladin expression correlates with the loss of filamentous actin as polygonal astrocytes become stellate (29). However, up-regulation of palladin and increased stress fibers are both seen in stellate astrocytes in response to injury (29). The function of palladin has also been explored in a knock-out mouse model. The palladin null mice die at about embryonic day 15, demonstrating that palladin is essential for normal mammalian embryonic development (30). Palladin null embryos exhibit striking defects in body wall closure, both dorsally and ventrally, which supports the view that cell motility is impaired in the absence of palladin expression. In addition, palladin null mouse embryo fibroblasts are defective in their ability to move, adhere, and assemble stress fibers (30, 31). Together, these results suggest that palladin is required for normal assembly and remodeling of the actin cytoskeleton.

A common feature of the palladin family of proteins is the presence of multiple immunoglobulin-like (Ig) domains. The three isoforms of palladin are transcribed from a series of nested promoters within a single gene; consequently, the three C-terminal Ig domains (Ig3, Ig4, and Ig5) are present in all three isoforms (16). The 140- and 200-kDa isoforms contain an additional fourth Ig domain (Ig2) near the N terminus, whereas the 200-kDa isoform contains a total of 5 Ig domains (Ig1–Ig5) (16). Myotilin has two Ig domains that are homologous to the Ig4 and Ig5 domains of palladin (20), whereas myopalladin has five Ig domains that are in the same relative positions and homologous to the five Ig domains of the 200-kDa isoform of palladin (16, 20). The Ig-fold is a modular domain that appears in both extracellular and intracellular proteins and is often involved in protein–protein interactions (32, 33). Ig domains typically contain about 100 amino acids and consist of 7–9 β strands that adopt a sandwiched β sheet fold (34). In addition to being a signature domain of this protein family, Ig domains have been described in a small number of other intracellular proteins that are associated with actin and myosin in vertebrates, including myosin light chain kinase, myomesin, titin, MyBP-C, and MyBP-H (35–38). The majority of these Ig-containing proteins are specifically expressed in striated muscle, suggesting that this particular type of Ig domain may play a special role in creating the highly ordered cytoskeleton of the sarcomere (37, 39). It is interesting to note that inherited forms of heart disease are associated with mutations affecting the Ig domains of either titin or MyBP-C, suggesting that the Ig domains have a key role in maintaining sarcomere integrity (40–43).

Currently, the precise molecular function of palladin family Ig domains is a matter of debate. The binding site of ezrin has been mapped to the Ig4 and Ig5 domains of palladin (17). However, a number of recent reports suggest the interesting possibility that certain Ig domains can function as actin-binding modules. Isolated protein fragments containing Ig domains and flanking sequence derived from the palladin relative to myotilin have been shown to bind directly to F-actin (44, 45). Myotilin even appears to function as an actin-bundling protein in vitro, as purified myotilin promotes the formation of large, multifilament aggregates that have been imaged by electron microscopy and also detected in differential sedimentation assays (44). In certain invertebrate species, skeletal muscle also contains a protein called kettin, which has 31 copies of a similar Ig domain (46). Recently, a fragment containing four of the Ig domains of kettin were shown to bind directly to F-actin (47). This suggests, first, that binding of actin by Ig domains may be a highly conserved molecular mechanism shared by both vertebrate and invertebrate proteins, and second, that Ig domains within the same molecule may be specialized for different functions. Yet, although these results strongly suggest that certain Ig domains could function as F-actin binding sites, no previous study has shown this conclusively using an isolated Ig domain.

The high degree of homology between palladin and myotilin in their Ig domains raises the possibility that palladin may also function as a direct binding partner for F-actin, and we undertook to test this idea using a combination of biochemical and microscopy-based assays. In this report, we show that purified 90-kDa palladin generates actin bundles directly in vitro and that one of its Ig domains possesses actin-binding activity. These results support the view that Ig domains can function as conserved actin-binding modules, and add further support to accumulating evidence that multiple Ig domains within the same protein can have specialized functions.

**EXPERIMENTAL PROCEDURES**

**Identification and Cloning of Palladin Immunoglobulin Domains**—The three tandem Ig domains of the 90-kDa palladin isoform were identified using a BLAST search and were initially characterized as C2-type Ig domains. However, secondary structure prediction using PSIPredict (48, 49) and ClustalX (50) alignment with telokin (1TLK), titin I1 (1G1C), and twitchin (1WIT) indicated that the three Ig domains of the palladin 90-kDa isoform are members of the I-type immunoglobulin-like domains (51). The Ig domains of palladin were originally described as the C2-type, based on their high homology to other Ig domains that had been classified as C2-type Ig domains, including titin, myosin–light chain kinase, and myotilin (15). The reclassification of Ig domains into four sets (V, C1, C2, and I) was initiated by a structural analysis of telokin (51) and subsequently, the Ig domains of titin and many other muscle proteins have been reclassified as members of the I-set (52).

Domain boundaries of the palladin Ig domains were determined from the secondary structure prediction using PSIPredict (48, 49). The DNA sequences and the translated protein sequences for the palladin Ig domains are included in the supplementary section.

**Expression and Purification of Palladin Fragments**—Full-length 90-kDa palladin was expressed as His6 fusion protein in Sf9 insect cells using a commercially generated baculovirus (BD
Biosciences), and purified using a commercial affinity purification kit (BD Biosciences) (25).

DNA sequences encoding: 1) the individual Ig3, Ig4, and Ig5 domains; 2) a fragment containing the linker sequence between the Ig3 and Ig4 domain; and 3) the tandem Ig3-Ig4 and Ig4-Ig5 domains of palladin were inserted into a modified pMAL-c2x (New England Biolabs) expression vector with the sequence ENLYFQG encoding a tobacco etch virus protease cleavage site inserted between the maltose-binding protein affinity tag and the palladin inserts. The plasmids were transformed into BL21(DE3) Codon-Plus-RIPL *Escherichia coli* (Stratagene) and colonies were selected from agar plates containing 75 mg/ml ampicillin. Cell cultures were grown in Luria broth supplemented with 10 g/liter of glucose at 37 °C to *A*<sub>600</sub> = 0.6 – 0.8 and induced with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside. Following induction, cells were grown at 30 °C for 4 h, then harvested by centrifugation for 20 min at 3,000 × g. Cells were resuspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1× BAL (1000× BAL = 10 mg/ml benzamidine, 2 mg/ml antipain, and 1 mg/ml leupeptin)) and the cell membranes were lysed by sonication using a Fisher Scientific model 550 Sonic Dismembrator. The cell lysate was centrifuged at 17,000 × g for 1 h to pellet any insoluble contents. The soluble contents of the cell lysate were loaded at ~2 ml/min on a column of amylose-functionalized agarose resin (New England Biolabs). The column was washed with 50 ml of column buffer (50 mM Tris, pH 7.4, 200 mM NaCl, 2 mM DTT, 1 mM EDTA) after which the fusion protein was eluted with an elution buffer (column buffer + 10 mM maltose) and collected in 3-ml fractions. Fractions containing protein were identified by Bradford assay (Bio-Rad) and checked by SDS-PAGE. The fractions containing the fusion protein were combined, the affinity tag was removed overnight at room temperature in the elution buffer containing the fusion protein were combined, the affinity tag was removed overnight at room temperature in the elution buffer (25 mM KH<sub>2</sub>PO<sub>4</sub>, pH 5.5, 2 mM DTT, and 0.1% NaN<sub>3</sub>) to lower the pH and salt concentration. The diluted protein sample was purified by ion-exchange on an ÄKTA FPlc (GE Healthcare) with a HiPrep<sup>TM</sup> SP XL column. The protein was loaded on the column with Buffer A (25 mM KH<sub>2</sub>PO<sub>4</sub>, pH 5.5, 25 mM NaCl, 2 mM DTT, and 0.1% NaN<sub>3</sub>) with a flow rate of 0.5 ml/min. The column was washed with 2 column volumes and then the protein was eluted using a gradient with a target concentration of 100% Buffer B (25 mM KH<sub>2</sub>PO<sub>4</sub>, pH 5.5, 1 mM NaCl, 2 mM DTT, and 0.1% NaN<sub>3</sub>) over 10 column volumes. Fractions containing protein were determined by UV absorbance at 280 nm and confirmed by SDS-PAGE. Reagents were purchased from Sigma, unless otherwise indicated.

**Actin Co-sedimentation Assay**—A solution of 6.4 mg/ml actin purified from rabbit muscle acetone powder was diluted 2-fold with an equal volume of 2× F-actin buffer (20 mM Tris pH 8.0, 200 mM KCl, 5 mM MgCl<sub>2</sub>, and 4 mM DTT) and allowed to polymerize at room temperature for 30 min. Purified palladin was centrifuged at 150,000 × g for 20 min to pellet any insoluble protein immediately before the co-sedimentation samples were prepared. Samples were prepared that contained 10 μM of the polymerized actin and 10 μM of the palladin proteins in a total volume of 200 μl. A control sample was prepared substituting the palladin protein solution with an equal volume of buffer. The samples were incubated for 1 h at room temperature and centrifuged at 150,000 × g for 30 min. Supernatants were collected and boiled in Laemmli sample buffer. The pellets were washed quickly with 1× F-actin buffer and re-suspended in 200 μl of water, which was also boiled in Laemmli sample buffer. Twenty-five micro liters of each sample was loaded onto a BisTris 4–12% gradient polyacrylamide gel and separated by electrophoresis in MES running buffer. The gels were stained with Simply Blue Safe Stain (Invitrogen) and destained with water. Gel images were acquired by scanning the gels with Licor’s Odyssey infrared scanner, which allows for quantitative measurements.

For the differential sedimentation assay of actin bundle formation, one additional centrifugation step was added. The samples were first centrifuged at 5,000 × g for 10 min. The 5,000 × g pellets were washed quickly with 1× M buffer and re-suspended in 100 μl of water before being boiled with Laemmli buffer. The supernatants were collected and centrifuged at 150,000 × g for 30 min and treated as described above. To determine actin bundling ratios, increasing concentrations of the Ig3-Ig4 fragments of palladin were combined with 10 μM F-actin and samples were subjected to differential sedimentation as described above. For each sample, the ratio of actin appearing in bundles, supernatant, and pellet were measured by densitometry of SDS-PAGE bands.

**Electron Microscopy of Actin Filaments**—A solution of 6.4 mg/ml actin purified from rabbit muscle acetone powder was polymerized at 74 μM concentration by adding an equal volume of 2× F-actin buffer (20 mM Tris, pH 8.0, 200 mM KCl, and 5 mM MgCl<sub>2</sub>). After polymerizing for 1 h, the F-actin gel was diluted to 10 μM actin in 1× F-actin buffer. Baculovirus-purified palladin was added to 1 μM in the actin gel. Palladin buffer was added to the control. The samples were allowed to incubate for 30 min before being diluted, first to 1 μM actin with 2.0 μM Alexa Fluor-488 phallloidin (Molecular Probes) and then to 100 nM actin. Samples were pipetted onto a carbon-coated, copper-mesh grid and stained with 1% uranyl acetate for 30 s, blotted, and then allowed to dry. Negative stain images were acquired using an FEI-Philips Tecnai 12 (FEI Company, Hillsboro, OR) transmission electron microscope. Images were collected at 80 kV with a 1k × 1k CCD camera (Gatan, Pleasanton, CA).

**Fluorescence Microscopy of Actin Filament Bundles**—A solution of 6.4 mg/ml actin purified from rabbit muscle acetone powder was polymerized at 74 μM concentration by adding an equal volume of 2× F-actin buffer (20 mM Tris, pH 8.0, 200 mM KCl, and 5 mM MgCl<sub>2</sub>). After polymerizing for 30 min, the F-actin gel was diluted to 10 μM actin in 1× F-actin buffer. Baculovirus-purified palladin was added to 1 μM in the actin gel. Palladin buffer was added to the control. The samples were allowed to incubate for 30 min before being diluted, first to 1 μM actin with 2.0 μM Alexa Fluor-488 phallloidin (Molecular Probes) and then to 100 nM actin. Samples were pipetted onto a carbon-coated, copper-mesh grid and stained with 1% uranyl acetate for 30 s, blotted, and then allowed to dry. Negative stain images were acquired using an FEI-Philips Tecnai 12 (FEI Company, Hillsboro, OR) transmission electron microscope. Images were collected at 80 kV with a 1k × 1k CCD camera (Gatan, Pleasanton, CA).

**Actin-Covariance Scatter**—A solution of 6.4 mg/ml actin purified from rabbit muscle acetone powder was polymerized at 74 μM concentration by adding an equal volume of 2× F-actin buffer (20 mM Tris, pH 8.0, 200 mM KCl, and 5 mM MgCl<sub>2</sub>). After polymerizing for 30 min, the F-actin gel was diluted to 10 μM actin in 1× F-actin buffer. Baculovirus-purified palladin was added to 1 μM in the actin gel. Palladin buffer was added to the control. The samples were allowed to incubate for 30 min before being diluted, first to 1 μM actin with 2.0 μM Alexa Fluor-488 phallloidin (Molecular Probes) and then to 100 nM actin. Samples were pipetted onto a carbon-coated, copper-mesh grid and stained with 1% uranyl acetate for 30 s, blotted, and then allowed to dry. Negative stain images were acquired using an FEI-Philips Tecnai 12 (FEI Company, Hillsboro, OR) transmission electron microscope. Images were collected at 80 kV with a 1k × 1k CCD camera (Gatan, Pleasanton, CA).
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Size Exclusion Chromatography-Multiple Angle Light Scattering (SEC-MALS)—The purified 90-kDa palladin isoform and Ig3-Ig4 fragment were buffer exchanged into a solution of 20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM DTT, and 0.01% NaN₃. Samples were also repeated in a similar buffer containing 100 KCl in place of the 150 mM NaCl. The samples were loaded onto a Superdex 200 column (GE Healthcare) with a flow rate of 0.5 ml/s and the elution was sampled every 0.5 s. The laser wavelength was set at 690 nm and data were analyzed using ASTRA 4.90.08 (Wyatt Technology).

Homology Modeling of the Palladin Ig3 Domain—Suitable templates for the homology modeling of the palladin Ig3 domain were chosen using a BLAST search (53) and the Inub server at the University of Buffalo Center of Excellence in Bioinformatics (54). Both search queries identified Ig domains of titin and the C-terminal domain of myosin light chain kinase, also called telokin, as good candidates. After examining a sequence alignment with the palladin Ig3 domain using Clustal X (50), x-ray structures of the titin I1 domain (Protein Data Bank 1G1C) and telokin (1F1G) were chosen as templates for homology modeling of the palladin Ig3 domain. The homology model was built for the Ig3 construct that we have been able to express and purify, namely residues 277–381 of the murine 90-kDa palladin sequence. The protein sequence for the 90-kDa isoform of mouse palladin is given in the supplemental section 1 of Rachlin and Otey (16). Both templates required the addition of a residue at the position occupied by Pro-323 of the Ig3-Ig4 fragment were buffer exchanged into a solution of 20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM DTT, and 0.01% NaN₃. After centrifugation at 14,000 g, 50% of the filamentous actin, causing it to sediment in the low speed spin. C, actin bundles visible by EM. Purified 90-kDa palladin was added to F-actin and prepared for imaging by negative stain transmission electron microscopy. Micrographs show thick actin bundles in the presence of palladin, whereas only individual filaments appear in control samples (not shown).

Palladin Binds Directly to F-actin and Cross-links Filaments into Bundles—The recruitment of palladin to stress fibers and other filamentous actin arrays may be achieved in one of two ways: 1) palladin may bind directly to actin filaments or 2) palladin may be associating with actin indirectly through one of its binding partners (e.g. α-actinin or VASP). To determine whether palladin binds to F-actin directly, we performed an actin co-sedimentation assay using purified actin and purified 90-kDa palladin. After incubating palladin with filamentous actin, the samples were spun at high speed to pellet the insoluble actin fraction. Approximately 40% of the palladin co-sedimented with the F-actin fraction, indicating that palladin can bind directly to actin (Fig. 1A). In a control sample containing palladin alone, the protein remained soluble and did not sediment by itself (data not shown).

Previous studies have shown that modifying palladin expression in COS-7 cells results in dramatic changes in cytoskeletal architecture (16, 23). We reasoned that if palladin is binding to actin directly, it could also be acting as a filament cross-linking protein, which could give rise to the observed hyperbundling phenotype when palladin is overexpressed. To test this, we repeated the co-sedimentation assay, this time adding an initial low speed (5,000 × g) spin to pellet the actin bundles before using a high speed spin (150,000 × g) to pellet the remaining actin filaments. Only a small fraction of the actin filaments sedimented at low speed in the absence of palladin, whereas about half of the actin pelleted in the presence of palladin (Fig. 1B).
This indicates that palladin is cross-linking individual filaments into actin bundles.

To confirm these results, actin bundles were visualized using electron microscopy. When actin is incubated with full-length palladin, robust multifilament bundles were seen (Fig. 1 C). The bundles were closely spaced and often slightly curved, suggestive of flexible cross-linking.

**Ig3 Domain of Palladin Binds to Actin**—We next sought to identify the specific region(s) of palladin that is responsible for actin binding. Analysis of the sequence of palladin did not reveal any of the canonical actin-binding domains described for other actin-modifying proteins (7). However, the C-terminal half of palladin contains three immunoglobulin-like domains, similar to those that have been implicated in F-actin binding in palladin’s relatives myotilin and kettin (45, 47), so we undertook to test the ability of isolated Ig domains derived from palladin to bind directly to F-actin. As shown in Fig. 2B, the Ig3 domain was the minimum fragment necessary for binding to filamentous actin. This is a novel result, because in no previous case has a single Ig domain been shown to be sufficient for actin binding.

The Ig4 and Ig5 domains showed no actin-binding activity, demonstrating that although the domains appear to be structurally similar on the basis of secondary structure prediction and sequence alignments, the actin binding ability of the Ig3 domain of palladin is unique. The tandem Ig4-Ig5 fragment also did not bind to F-actin. Although the isolated Ig4 domain did not bind to F-actin in this assay, a palladin fragment containing both Ig3 and Ig4 exhibited much greater affinity for actin than the Ig3 domain alone. The residues in the Ig3/Ig4 linker region that are near the C terminus of Ig3 contain a high incidence of basic residues (eight of the first 18 residues are basic). Because basic residues have been implicated in a number of actin-binding interfaces (55–62), it is reasonable that the linker region may enhance an electrostatic interaction between the Ig3 domain and F-actin. However, neither the Ig4 domain nor the Ig3/Ig4 linker region alone are able to bind to actin.

**Determination of the Binding Affinity of the 90-kDa Isoform and Palladin Fragments with F-actin**—The concentration of F-actin was held constant and increasing amounts of palladin protein were used in co-sedimentation assays to determine the binding affinity for the Ig3 and Ig3-Ig4 fragments as well as the full-length 90-kDa isoform. We could not determine a value for the Ig3 fragment, as the binding did not completely saturate within the concentration range we could achieve with the purified fragment. We estimate that the $K_d$ is between 60 and 80 μM, based on repeated attempts to determine this binding affinity. The data were fit to a hyperbolic curve, assuming a 1:1 stoichiometry and specific binding only. We determined $K_d$ val-
ues to be 2.1 ± 0.5 μM for the full-length 90-kDa isoform (Fig. 2C) and 9 ± 2 μM for the Ig3-Ig4 fragment (Fig. 2D).

**The Interaction between the Ig3 Domain and F-Actin of Palladin Is Salt-dependent** — We determined the dependence of F-actin binding to palladin Ig fragments as a function of salt concentration, as a number of other actin-binding proteins have been shown to bind actin in a salt-dependent manner (55, 57, 59–61). An increase in the ability to bind actin as the salt concentration is lowered is usually an indication that the binding is driven by electrostatic interactions. To test this idea, we performed F-actin co-sedimentation assays at KCl concentrations of 25, 50, 100, and 200 mM. We found that the interaction between F-actin and the palladin Ig3 domain was strongly salt-dependent and that binding greatly increased as the salt concentration was lowered (supplemental Fig. 1). Additionally, we confirmed that the Ig4 domain of palladin exhibited no interaction with actin at lower salt concentrations (data not shown).

**The Tandem Ig3 and Ig4 Domains of Palladin Are Required to Bundle Actin** — The palladin fragments were then used in a differential sedimentation assay to identify the smallest fragment of palladin that is capable of bundling actin filaments. Whereas Ig3 continued to sediment with actin in the high speed centrifugation, we did not detect a significant amount of actin bundle formation when F-actin was incubated with the Ig3 domain (Fig. 3A). The previous co-sedimentation experiments indicated that the fragments Ig4, Ig5, Ig4-Ig5, and the Ig3/Ig4 linker sequence do not bind F-actin so it is not unexpected that they also fail to bundle F-actin. We did, however, detect actin bundles when F-actin was incubated with a fragment containing both Ig3 and Ig4 (Fig. 3, A and B). We used fluorescence microscopy to visualize the bundles formed by the Ig3-Ig4 construct (Fig. 4). As expected, bundles were not observed when F-actin was incubated with Ig3 or the tandem Ig4-Ig5 fragment. Actin bundles generated by Ig3-Ig4 were also imaged at high resolution by electron microscopy, as shown in supplemental Fig. S2.

We also attempted to quantify the ability of the Ig3-Ig4 fragment to bundle F-actin by incubating varying amounts of Ig3-Ig4 with 10 μM actin. The differential sedimentation assay combined with densitometry was used to determine the amount of F-actin that was bundled in response to the amount of Ig3-Ig4 fragment that was added. As shown in Fig. 5A, in the absence of the Ig3-Ig4 fragment F-actin does not sediment following a low
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Our results show that the widely expressed, actin-associated protein palladin functions as an actin cross-linking protein in vitro, and that the Ig3 domain of palladin is involved in F-actin binding. These results indicate that palladin occupies an unusual functional niche: because the proline-rich domains in the N-terminal half of palladin have been shown previously to be docking sites for multiple actin-binding proteins, it appears that palladin is essentially a cytoskeletal-scaffolding protein fused to an actin cross-linking protein. This distinctive molecular function is likely to underlie the dramatic effects on actin organization that result from palladin overexpression (16, 23). It is interesting to note that palladin binds to three other proteins that have been shown to cross-link actin filaments in vitro: α-actinin, VASP, and Eps8 (22–24). This observation raises some interesting questions about the evolution of actin-bundling proteins in vertebrate cells: do these multiple actin cross-linking proteins function synergistically or redundantly, or do they possess subtle functional differences in their ability to generate multifilament arrays? In the case of Drosophila proteins forked and fascin, both cross-linkers are required for the formation of robust, mature actin bundles, and their functions seem to be temporally regulated (63). Fascin and α-actinin in mammalian cells can individually cross-link actin, although together they function synergistically to enhance cell stiffness and alter the geometry of actin filament bundles (64). Recently, Disanza et al. (65) describe their work on the actin cross-linking pair Eps8 and IRS-p53, which, when bound to one another, releases autoinhibition of IRS-p53 and unmasks its actin-binding domain. Lasp-2 forms a complex with α-actinin and Lasp-2 is also able to bind and bundle F-actin directly (66). When Lasp-2 and α-actinin are simultaneously incubated with F-actin the resulting actin filaments are more densely packed than the actin bundles generated by either α-actinin or Lasp-2 alone, which suggests that Lasp-2 and α-actinin are able to act in concert to generate actin assemblies with a distinct organization (66). Similar to these examples, palladin and α-actinin may co-regulate their activities, cross-link synergistically, or contribute to different phases of stress fiber formation.

Whereas it is not clear whether palladin and α-actinin may be functioning cooperatively as actin-bundling proteins, there is evidence to suggest that the region of palladin that is responsible for its binding interaction with α-actinin is particularly important for the cellular function of palladin. A recent report has linked a point mutation in palladin to a form of familial pancreatic cancer, and has also shown that palladin RNA levels are increased in familial and sporadic precancerous and cancerous pancreatic tissues (67). The disease-causing mutation occurs in the α-actinin-binding region of palladin, and HeLa cells transfected with the mutated form of palladin showed cytoskeletal abnormalities, altered localization of palladin, and increased motility (67). In addition to pancreatic cancer, other human diseases and conditions in which palladin has been implicated include pre-eclampsia (68, 69), invasive breast cancer (70), and increased risk of heart attack (71). Clearly, additional studies will be necessary to determine the relative importance of bundling activity, actinin-binding activity, and other molecular activities, to its cellular function of palladin and its precise role in these diverse pathologies.

The results of the F-actin co-sedimentation experiments (Fig. 3A) indicate that the Ig3 domain is an actin-binding domain that is not capable of bundling actin. The isolated Ig3 binding affinity of the domains constant for actin is outside of the physiological range (Kd > 60 μM), suggesting that additional palladin sequences may contribute to create a stable binding interaction when the intact palladin protein binds to F-actin. The Ig3-Ig4 fragment binds to F-actin almost as well as the full-length protein (see Fig. 2, C and D) and is also capable of bundling actin filaments (see Figs. 3A and 4). For palladin to cross-link actin, it must bind two actin filaments, and the majority of the F-actin can be pelleted by centrifugation at high speed. However, following the incubation with 0.5 μM Ig3-Ig4, bundled F-actin was detected in the low speed step. As we increased the amount of Ig3-Ig4, increasing amounts of F-actin were found in the bundled pellet. A plot of the densitometry of the bands corresponding to the bundled F-actin were plotted as a function of the concentration of the Ig3-Ig4 fragment to quantify the ability of the palladin Ig3-Ig4 fragment to bundle F-actin. The measured points are shown as open boxes and a dotted line corresponds to a hyperbolic fit of the data, assuming specific binding only with a 1:1 stoichiometry. From the fitted curve, an apparent Kd of 0.5 ± 0.3 μM was determined.

**DISCUSSION**

**FIGURE 5. Differential sedimentation assay of the palladin Ig3-Ig4 fragment.** A, samples containing 10 μM F-actin, alone, or with varying amounts of the palladin Ig3-Ig4 fragment, were first subjected to a low speed (5000 × g) centrifugation step and the pellets, which contains bundled actin filaments, were removed. Subsequently, the soluble fraction was subjected to a high speed centrifugation step (150,000 × g) and the low speed pellet (B), soluble fraction (S), and high speed pellet (P) were analyzed by SDS-PAGE. Addition of the palladin Ig3-Ig4 fragment induces the formation of F-actin bundles, which sediment during the low speed centrifugation step. B, densitometric analyses of the bands corresponding to the bundled F-actin were plotted as a function of the concentration of the Ig3-Ig4 fragment to quantify the ability of the palladin Ig3-Ig4 fragment to bundle F-actin. The measured points are shown as open boxes and a dotted line corresponds to a hyperbolic fit of the data, assuming specific binding only with a 1:1 stoichiometry. From the fitted curve, an apparent Kd of 0.5 ± 0.3 μM was determined.
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filaments simultaneously (72). This can be achieved if palladin has one actin-binding site and forms dimers, or if palladin contains two actin-binding sites per monomer. We have not been able to find a second actin-binding site within the Ig3-Ig4 construct; neither the Ig4 domain nor the region linking the two Ig domains appears to bind actin in the co-sedimentation assays (see Fig. 2). We have also been unable to detect the presence of a dimer in either the full-length molecule or the Ig3-Ig4 fragment using SEC-MALS (see supplemental data). One explanation is that filament cross-linking by palladin could be a two-step process, similar to the mechanism that has been demonstrated previously for vinculin, in which F-actin binding activates a cryptic dimerization site within the vinculin tail domain (58, 73). Given our data, we cannot exclude the possibility that binding of the palladin Ig3 domain to F-actin plays a role in promoting either dimerization or a second binding site within the Ig3-Ig4 fragment. Future work will focus on the structural basis for the interaction of Ig3 with F-actin and elucidation of mechanisms by which paladin promotes F-actin bundling through its Ig domains.

Our results agree with a general pattern that has been reported previously: Ig domains often occur as multiple copies within one protein and different Ig domains often have specialized functions within the same molecule. For example, MyBP-C possesses three fibronectin type III (Fn3) domains and seven or eight Ig domains, depending on the isoform (74). Only four of the Ig domains bind to myosin, two at the C terminus (36, 75) and two near the N terminus (76). Additionally, interactions between domains of MyBP-C have been found for two of the Ig domains and one Ig domain with a Fn3 domain (77), whereas the functions of three of the Ig domains have not been clearly defined. Similarly, the functions of the N-terminal Ig domains found in the 140- and 200-kDa isoforms of palladin remain to be determined, and these could turn out to be the same or different from the C-terminal domains. Based on our results and others, the Ig domains found within cytoskeleton-associated proteins may fall into the following functional groups: actin-binding (45, 47), myosin-binding (36, 75), ezrin-binding (17), dimer-forming (78), or regulators of molecular spacing (78) and elasticity (79–82). As more structural information on different Ig domains becomes available, it may be possible to determine structural specializations that correspond to each of these functional categories.

To begin structural analysis of the palladin Ig domains, we have constructed a homology model of the Ig3 domain based on the II domain of titin (1G1C) (Fig. 6A). We predict that the Ig domains of palladin are I-type immunoglobulin-like domains and will have the common features of the I-frame (Fig. 6B).

We expect that the actin-binding ability of the Ig3 domain is due to the interaction surface created by the amino acid sequence, rather than a large scale difference in the protein fold compared with other I-set Ig domains. We are in the process of determining the solution structure of Ig3 by NMR spectroscopy. The homology model will aid in both structure determination efforts and in guiding mutagenesis efforts to identify the site of F-actin binding. Although Ig domains are present in a number of actin/myosin-associated proteins and have been suggested to be involved in actin-binding, the palladin Ig3 domain is the first isolated Ig domain shown to bind F-actin. It will be interesting to determine the structural basis for how, in palladin, the Ig fold has been adapted for actin-binding and other functions, e.g. dimer formation and interaction with ezrin. These questions will be the focus of additional future experimental efforts.

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