Characterization of Palladin, a Novel Protein Localized to Stress Fibers and Cell Adhesions

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Abstract. Here, we describe the identification of a novel phosphoprotein named palladin, which colocalizes with α-actinin in the stress fibers, focal adhesions, cell–cell junctions, and embryonic Z-lines. Palladin is expressed as a 90–92-kD doublet in fibroblasts and coimmunoprecipitates in a complex with α-actinin in fibroblast lysates. A cDNA encoding palladin was isolated by screening a mouse embryo library with mAbs. Palladin has a proline-rich region in the NH$_2$-terminal half of the molecule and three tandem Ig C2 domains in the COOH-terminal half. In Northern and Western blots of chick and mouse tissues, multiple isoforms of palladin were detected. Palladin expression is ubiquitous in embryonic tissues, and is downregulated in certain adult tissues in the mouse. To probe the function of palladin in cultured cells, the R cho-1 trophoblast model was used. Palladin expression was observed to increase in R cho-1 cells when they began to assemble stress fibers. Antisense constructs were used to attenuate expression of palladin in R cho-1 cells and fibroblasts, and disruption of the cytoskeleton was observed in both cell types. At longer times after antisense treatment, fibroblasts became fully rounded. These results suggest that palladin is required for the normal organization of the actin cytoskeleton and focal adhesions.

Key words: focal adhesion • adherens junction • microfilament • α-actinin • trophoblast

Introduction

The actin cytoskeleton is intimately involved in cell adhesion and maintenance of cell shape. In cultured cells, actin filaments are associated with two types of junctional sites: the cell–cell adherens junctions, and the cell–matrix focal adhesions. Each type of junction possesses its own specialized transmembrane protein: integrins in focal adhesions and cadherins in adherens junctions. In the focal adhesions, the array of cytoplasmic proteins that colocalize with integrins is complex, and includes both structural proteins that bind directly to actin (such as talin, vinculin, tensin, and α-actinin), and low-abundance adapter proteins and signaling molecules (including FAK, paxillin, zyxin, and p130Cas; Craig and Johnson, 1996; Gilmore and Burridge, 1996). A subset of these proteins is found also in the cell–cell junctions, where they associate with the cadherin–catenin complex (Aberle et al., 1996; Provost and Rimm, 1999; Gumbiner, 2000).

α-A-actinin is an actin–cross-linking protein that is common to both cell–cell and cell–matrix junctions. M onomers of α-actinin form head-to-tail dimers, which are capable of organizing actin microfilaments into stable parallel bundles (Flood et al., 1995; Djinovic-Carugo et al., 1999). In organized tissues, α-actinin has been localized to a variety of junctional sites, including the dense bodies of smooth muscle and the intercalated discs of cardiac muscle. This localization pattern is consistent with the idea that α-actinin plays a highly conserved role in the stable attachment of actin filaments to the plasma membrane. α-A-actinin appears to serve this role in part by binding directly to transmembrane proteins. Binding to α-actinin, either in vitro or in vivo, has been detected with a diverse group of transmembrane receptor proteins, including several different β integrin subunits (Otey et al., 1990; Pavalko and LaRoche, 1993; Sampath et al., 1998), L-selectin (Pavalko et al., 1995), ICAM-1 (Carpen et al., 1992), and the NMDA neurotransmitter receptor (Wyszynski et al., 1997).

Within the sarcomeres of striated muscle, α-actinin is concentrated in the Z-disc, where actin thin filaments are anchored. The skeletal muscle isoform of α-actinin, actinin-2, has an especially large number of binding partners, including the giant protein, titin (Ohtsuka et al., 1997; Sorimachi et al., 1998), and the recently described myotilin (Salmikangas et al., 1999), both of which contain an Ig-like domain called Ig C2. A actinin-2 also binds to three PDZ...
domain proteins, ALP, ZASP, and Cypher, all of which colocalize with α-actinin in the Z-line (Xia et al., 1997; Faulkner et al., 1999; Pomies et al., 1999; Zhou et al., 1999). Binding of actinin-2 to dystrophin (Hance et al., 1999) and CapZ (Papa et al., 1999) has also been reported.

In fibroblasts and many other nonmuscle cells, α-actinin is not restricted only to junctional sites, but is also found in a distinctive beads-on-a-string punctate pattern along stress fibers, which resembles the striated pattern of myofibrils (Lazarides and Burridge, 1975). In addition to α-actinin, these striations have been reported to contain vasodilator-stimulated phosphoprotein (VASP), promyelocytic leukemia (PML), and multiple LIM domain proteins, all of which have multiple LIM domains, some of which are expressed in a developmentally regulated manner. Palladin exists as multiple isoforms, some of which are specific to skeletal muscle, palladin is detected in both muscle and nonmuscle tissues and cells. Palladin interacts with profilin (Reinhard et al., 1995a; Gertler and Ctiter et al., 1996). Taken together, these results suggest that α-actinin may have an important role in addition to its ability to cross-link actin and to bind transmembrane proteins: it may act as an adapter molecule to organize LIM proteins, PDZ proteins, and Ig C2 proteins into functional complexes closely associated with actin filaments. The recent report that α-actinin binds to a serine-threonine kinase in the MAP kinase pathway, MEKK1, further supports the idea that α-actinin serves to integrate signaling pathways with the actin cytoskeleton (Christerson et al., 1999).

In this report, we describe the identification of a novel protein that colocalizes with α-actinin in focal adhesions, cell–cell junctions, and stress fiber striations. We propose the name palladin for this protein, in honor of the Renaissance architect Palladio, to reflect the localization of the protein to architectural elements of the cell. Palladin contains three tandem Ig C2 domains. Unlike most intracellular Ig C2-containing proteins, which are specific to skeletal muscle, palladin is detected in both muscle and nonmuscle tissues and cells. Palladin exists as multiple isoforms, some of which are expressed in a developmentally regulated pattern. Most importantly, palladin appears to play a critical role in the organization of the actin cytoskeleton and focal adhesions in cultured trophoblast and fibroblast cells.

### Materials and Methods

#### Cell Culture

Unless otherwise stated, all cells were cultured in DMEM (GIBCO BRL) containing 100 U/ml penicillin G, and 100 μg/ml streptomycin (pen/strep; GIBCO BRL), and supplemented with 10% FBS (GIBCO BRL). Day-10 chick embryos were used as a source for all avian cells and tissues. Chick embryo fibroblasts (CEF s) were prepared as described previously (Hungerford et al., 1996). Cardiac myocytes were prepared from embryo hearts following the protocol of Dabiri et al. (1999). To obtain pigmented epithelium, eyes were removed from the embryo, the pigmented epithelium was teased off and placed on sterile gelatin-covered coverslips, and was cultured for 24 h. Mouse embryo fibroblasts (MEFs) were prepared from day-13–14 embryos. Both CEF s and MEFs were used in early passage (pass 3-10). CHO cells were grown in α-MEM with 10% FBS and pen/strep, and HeLa cells were grown in DME with 5% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 mM Hepes, pH 7.2. Rat choriocarcinoma (Rcho-1) stem cells were grown as described by Kamei et al. (1997). To maintain the Rcho-1 as proliferative stem cells, they were fed growth medium: NCTC-133 (Sigma-Aldrich) with 20% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and pen/strep. To induce differentiation of Rcho-1 cells, the stem cells were lightly trypsinized for 1 min to remove the most undifferentiated cells, and the remaining cells were fed differentiation medium, which is the same as the growth medium except with 10% horse serum instead of FBS.

#### mAbs and Immunofluorescence

Palladin immunoprecipitated from CEF s was used as an immunogen to obtain additional mAbs. Mice were immunized with excised SD-PAGE bands of 90-KD palladin, splenic lymphocytes were isolated, and hybridomas were obtained as described in Chang et al. (1995). HybriDoma supernatants were screened initially by ELISA for reactivity with fixed CEF s. A second screen was performed using immunofluorescence staining and Western blots of CEF s and Swiss 3T3 cells. Eight mAbs were generated, two of which were chick-specific (2D12 and 8H11) and six of which reacted with mouse (1E6, 7C6, 3C9, 4D10, 8B10, and 9C12). All the mAbs produced the same staining patterns as the original C10 mAb in fixed cells and detected the same band by Western blot.

Immunofluorescence staining was performed on cells fixed in 3.7% formaldehyde and permeabilized in 0.2% Triton X-100. A rabbit polyclonal antibody was the gift of Dr. Michael Schaller (U. of North Carolina, Chapel Hill, NC). α-actinin polyclonal antibody was the gift of Dr. K. eith Burridge (U. of North Carolina, Chapel Hill, NC), and antimyc mAb was the gift of Dr. Doug D. Esmone (U. of Virginia, Charlottesville, VA). α-Actinin monoclonal (A5044) and FITC-conjugated phalloidin were from Sigma-Aldrich. Texas red-conjugated antimouse antibodies were from Jackson Immunochemicals.

#### Triton Extraction Experiments

To fractionate cellular proteins into a soluble pool and a cytoskeleton-associated pool, the Triton extraction method was used, as previously described (A dams et al., 1996; Wulffhule et al., 1999). In brief, MEF s were scraped on ice in 0.5% Triton X-100 in cytoskeleton-stabilizing buffer (50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 30 mM sucrose, 0.7 mM pepstatin, 4 mM pefabloc, 5 μg/ml leptin, 2 μg/ml apronin, and 7 μM sodium ortho-ovanadate; all protease inhibitors from Boehringer). Then the centrifuged at 14,000 rpm at 4°C. The pellet was resuspended in cytoskeleton-stabilizing buffer plus 0.5% SD-P. Protein concentration was determined using the Coomassie reagent (Pierce Chemical Co.), and 20 μg each of the supernatant and pellet were analyzed by SD-PAGE and Western blot.

#### Immunoprecipitation and Western Blots

Whole-cell lysates were prepared by scraping adherent cultured cells into lysis buffer (1% Triton X-100, 1% deoxycholate in TBS, pH 7.5, with 10 mM EDTA, pH 8.0, 0.7 μg/ml pepstatin, 4 μM pefabloc, 5 μM leptin, 2 μg/ml apronin, and 7 μM sodium ortho-ovanadate). Tissue lysates were prepared the same way, except that they were donece-homogenized in lysis buffer. The crude lysate was centrifuged at 14,000 rpm for 20 min in a microcentrifuge at 4°C to remove large particulates. The supernatant was passed through a 26 gauge needle to shear the DNA. Coomassie reagent (Pierce Chemical Co.) was used to determine the protein concentration of the cell and tissue lysates.

For immunoprecipitation studies, the above protocol was followed, and then primary antibody was added to the lysis supernatant and incubated for 1 h at 4°C, and precipitated by addition of Gamma- Bind Plus Sepharose beads (A mersham Pharmacal Biotech). The beads were washed five times with 110 diluted lysis buffer, and were then eluted by boiling in Laemmli sample buffer. A ntitoxides were used for immunoprecipitating palladin were mAb C10 (for immunoprecipitations [IPS] from CEF s) and mAb 1E6 (for IPS from Swiss 3T3 cells). The antibody used for immunoprecipitating α-actinin was rabbit polyclonal A 2543 (Sigma-Aldrich).

For large-scale, preparative IPS of palladin (to obtain gel bands for microsequencing or immunization purposes), a clean precipitation was de-
sired. In this case, best results were obtained if the actin cytoskeleton was first disassembled by trypsinizing the cells before lysis. Trypsinized cells were centrifuged, the cell pellet was resuspended in the above lysis buffer on ice for 15 min, and then centrifuged and incubated with antibody, as described for immunoprecipitation studies.

For Western blot analysis, samples resolved on SDS-5%AGE gels were transferred to either nitrocellulose (Fisher Scientific) or PVDF (NEN Life Science Products) membranes. Membranes were blocked in 5% nonfat dried milk in TBS (PBS with 0.5% Tween 20), and were then incu-
bated with primary antibody for 1 h. After multiple changes of wash buffer, membranes were incubated with HRP-conjugated secondary anti-
body (Jackson Immunonochemicals), and were then washed again before applying SuperSignal chemiluminescent substrate (Pierce Chemical Co.) and exposing to autoradiographic film (Eastman Kodak Co.).

Transfection and Infection of Cells with Antisense Construct

The partial mouse cDNA, 7a-1, was cloned in the antisense orientation into the EcoRI site of the adenovirus shuttle vector, pA dlox. This con-
struct was used to transient transfections, along with a GFP vector (pEGFP-N2 from CLONTECH Laboratories, Inc.) as a transfection marker. For infecting cells, the partial antisense construct in pA dlox was packaged into viral particles by the Cre-lox recombination method of H ardy et al. (1997). Three control viruses were also made. Empty viral particles were generated using pA dlox without an insert, and GFP-express-
ing virus was made using a pA dlox-GFP construct. The third control virus expressed a mutant form of cdc42 that has been shown to be inactive (B ourne et al., 1991; Ridley et al., 1992). This virus was made by cloning A 35-cdc42 (cdc42 with a Thr 11 Ala mutation at position 35) into pA dlox and was a kind gift of Sean A eder and Dr. A n Sutherland (University of Virginia, Charlottesville, VA.). We confirmed the presence of the correct construct by restriction digest and sequencing of DNA from all recombi-
nant viruses. De-ollution of the viral prep was done using PD-10 columns prepacked with Sephadex G-25M (A mersham Pharmacia Biotech). Vi-

eral stocks were titered using transformed human embryonic kidney cells (293 cells) in a plaque assay, according to previous protocols (T ollelfson et al., 1999). In brief, cells were infected in duplicate using eight different dilu-
tions of each viral stock, and were then fed with agar overlay me-

Results

The C10 mAb Recognizes a Novel Protein that Colocalizes with α-Actinin

A n mAb designated C10 was made over a decade ago by immunizing mice with partially purified vinculin and screening the hybridoma supernatants by immunofluorescent labeling of fibroblasts. A t that time, C10 was misiden-
tified as an α-actinin antibody. U pon further charac-
terization, we realized that C10 recognizes an antigen distinct from α-actinin, although the C10 antigen colocal-
ized closely with α-actinin in many types of cells. A s shown in Fig. 1 A , C10 stained regularly spaced puncta along actin stress fibers, and also stained the ends of the stress fi-
bers intensely, a pattern that closely resembles the labeling that is typical of α-actinin. L ike α-actinin, the C10 antigen also localized to the cell–cell junctions of epithelial cells (Fig. 1 C ) and the Z-lines of embryonic cardiac myocytes (Fig. 1 D ). When fibroblasts were double-labeled with C10 and a polyclonal antibody to α-actinin, the staining pat-
terns were remarkably similar, as shown in Fig. 2. B oth the C10 antibody (Fig. 2 A and D, in green) and the α-actinin antibody (Fig. 2 C and F, in red) strongly labeled the ends of actin stress fibers, and both antibodies also stained many of the same stress fiber puncta.

The C10 antibody did not perform well in the mononuc-
however, it did recognize native protein in detergent lysates of cultured chick fibroblasts. In IPs from 35S-labeled, trypsinized CEFs, the major band resolved as a blurry doublet with an apparent molecular weight of 90–92 kD (Fig. 3 A), which is significantly smaller than the monomer molecular mass of α-actinin at 105 kD. This provided the first clue that the C10 antigen was distinct from α-actinin. The 90–92-kD band immunoprecipitated from CEFs was excised from Coomassie blue-stained gels and subjected to tryptic digestion, and a search of the GenBank/EMBL/DDBJ database revealed the tryptic map to be novel. Subsequently, three of the tryptic peptides, 9–15 residues in length, were sequenced by mass spectrometry, and analysis of the existing database showed that all three were novel (data not shown), thus providing additional evidence that the C10 antigen was a novel protein with no overall sequence similarity to α-actinin.

Not only did the C10 antibody fail to perform in Western blots, it also did not cross-react with species other than chicken, which severely limited its usefulness as a probe. To obtain better antibodies to the same antigen, the major band immunoprecipitated by the C10 mAb was excised from gels and used to immunize mice. mA bs were subsequently obtained and characterized: eight of these performed well in Western blots, and six of those blotting antibodies cross-reacted with all vertebrate species tested (including frog, mouse, rat, dog, and human). All mA bs recognized the same size protein in CEF lysate as the original C10 antibody. Furthermore, they also cross-reacted only with the protein precipitated by the C10 mAb on Western blot and not with purified α-actinin (Fig. 3 B).

The mA bs were used in Western blots to characterize the association of the C10 antigen with the actin cytoskeleton. To determine if the C10 antigen was tightly bound to the cytoskeleton, Triton extraction experiments were performed. MEFs were extracted in 0.5% Triton in a cytoskeleton stabilizing buffer (see Materials and Methods) and equal amounts of protein from the pellet and the supernatant were analyzed for palladin by Western blot. As shown in Fig. 3 C, most of the C10 antigen was found to be in the Triton-insoluble pellet, with only a small fraction in the soluble pool, indicating that the C10 antigen is tightly associated with the actin cytoskeleton.

The colocalization of the C10 antigen with α-actinin suggested that the two proteins might form a stable complex in vivo. To address this question, we immunoprecipitated the C10 antigen from adherent Swiss 3T3 cells and blotted for α-actinin. Fig. 3 D shows that α-actinin coimmunoprecipitates specifically with the C10 antigen (C10 IP lane) and not with Sepharose beads alone (preclear lane). This association depends on an intact cytoskeleton, because if the cells are first trypsinized, then lysed and immunoprecipitated for the C10 antigen, α-actinin does not coimmunoprecipitate (data not shown). The interaction appears to be specific, as only a trace amount of actin was detected in both the preclar lane and the IP lane (data not shown),

Figure 1. Immunofluorescent labeling with the C10 mAb. Cells were immunolabeled with C10 (A, C, and D) or fluorescent phalloidin (B). A and B, Cultured CEFs. C, Chick pigmented epithelium, in an en face preparation on gelatin-coated coverslips. D, Embryonic cardiac myocytes isolated from a day-10 chick heart. Bars: (B) 8 μm; (C) 20 μm; (D) 25 μm.

Figure 2. Colocalization of the C10 antigen and α-actinin in stress fibers. Cultured fibroblasts were stained with C10 mAb (A and D, green fluorescence) or polyclonal anti-α-actinin (C and F, red fluorescence). The merged images (B and E) show that the C10 antigen largely colocalizes with α-actinin in a punctate pattern along stress fibers and in a concentration at the ends of stress fibers. Bars, 5 μm.
and the immunoprecipitates did not contain talin, vinculin, or zyxin as determined by Western blot (Fig. 3 D). The C10 antigen was also detected in α-actinin immunoprecipitates (Fig. 3 D), which adds further support to the idea that these two proteins are found in a complex in vivo. The relative intensity of the C10 and α-actinin band suggests that they do not coimmunoprecipitate in a 1:1 ratio, so that further experimentation will be required to determine if these two proteins bind directly to each other or if another unknown molecule contributes to the formation of an α-actinin-C10 antigen complex.

The blurred appearance of the C10 band in both IPs and Western blots suggests that the C10 antigen might be associated with the C10 antigen in the middle lane. C, A analysis of the fractionation of the C10 antigen. MEF lysates were extracted with 0.5% Triton X-100 in cytoskeleton-stabilizing buffer (see Materials and Methods) and the insoluble pellet was collected by centrifugation at 14,000 rpm. 20 μg of protein from pellets and supernatants was loaded and blotted with α-actinin, vinculin, and zyxin. The Western blots were precleared with Gamma-bind beads and used to immunoprecipitate the C10 antigen. Both sets of beads were eluted and blotted with either α-actinin, vinculin, and zyxin (all negative; center). In the reverse experiment, α-actinin was immunoprecipitated using a rabbit polyclonal antibody, and the IP was blotted for α-actinin and for the C10 antigen (right).

Molecular Cloning and Characterization of Palladin cDNA

The mAbs were pooled and used to screen both chick and mouse embryo cDNA libraries. Partial overlapping clones were obtained, which encoded the COOH-terminal two-thirds of the open reading frame, including the stop codon and 100 bp of the 3′ untranslated region (Fig. 4 A). Re-screening of the respective libraries with these partial clones as probes did not yield longer cDNA sequences. Using the sequence of the partial clones, we searched the EST database and found several matching sequences, most of which were also partial COOH-terminal fragments. One EST, a 3-kb long mouse embryo heart cDNA (GenBank/EMBL/DBJ accession #A 671190), spanned our clones and extended them at the 5′ end. Using the partial sequence of this EST, we designed primers and performed RT-PCR using mouse embryonic RNA. The 1,433-bp PCR product was sequenced, and a methionine with a Kozak consensus sequence (Kozak, 1991) was identified as the start site, the predicted molecular weight of the full-length clone would be 85.7 kD, close to the 90-kD molecular weight that had been estimated for the C10 antigen based on SDS-PAGE. To confirm that this was indeed the sequence of the 90-kD protein, we repeated the RT-PCR using RNA from Swiss 3T3 fibroblasts. The PCR product was sequenced and found to be identical to that obtained from mouse embryo heart.

The full-length clone (Fig. 4 D) contained all three of the tryptic peptides that had been obtained by microsequencing, increasing our confidence that we had successfully cloned the C10 antigen. The partial chick clone, 18b-1, was found to be 72% identical to the mouse clone over the region of the protein (amino acids 61-330, data not shown). To further verify that we had cloned the correct cDNA, we expressed the full-length clone with an epitope tag. The myc-tagged construct was transfected into Swiss 3T3 fibroblasts and stained with an antimyc antibody. A as shown in Fig. 4 B, the myc-tagged construct was localized in precisely the same striated pattern that had been observed for the endogenous C10 antigen. By Western blot, both antimyc antibodies and anti-C10 monoclonals detected the same band (Fig. 4 B). Together, these results confirm that we have succeeded in cloning the same protein that was detected by the original C10 antibody and this novel protein was named palladin.

Analysis of Protein Motifs in Palladin

The sequence of the full-length clone was analyzed by BLAST search of the nonredundant GenBank/EMBL/DBJ database to identify proteins with homology to palladin, and by SMART search (Schultz et al., 1998) to identify conserved domains. The results of this analysis are summarized diagramatically in Fig. 4 C. In the NH2-terminal half of palladin, a proline-rich region was observed (amino acids 99-289, 30% proline overall). This region contains three polyproline motifs (FPFP, LPPPPP, and SPPPPP, underlined in Fig. 4 D) homologous to those contained in zyxin, vinculin, and VASP/Mena family mem-

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Figure 3. The C10 mAb immunoprecipitates a cytoskeleton-associated protein in lysates of fibroblasts. A, Immunoprecipitation from 35S-labeled CEFs. Metabolically labeled fibroblasts were trypsinized and lysed on ice, and were then centrifuged. The supernatant was subjected to IP with the C10 mAb. The major band resolves as a broad doublet at 90–92 kD. B, The C10 antigen was also detected in the insoluble pellet was collected by centrifugation at 14,000 rpm. 20 μg of protein from pellets and supernatants was loaded and blotted with the anti-C10 mAb, 7C6. Note that 7C6 failed to detect purified α-actinin in the middle lane. C, Analysis of the fractionation of the C10 antigen. MEF lysates were extracted with 0.5% Triton X-100 in cytoskeleton-stabilizing buffer (see Materials and Methods) and the insoluble pellet was collected by centrifugation at 14,000 rpm. 20 μg of protein from pellets and supernatants was loaded and blotted with α-actinin, vinculin, and zyxin. D, C10 antigen coimmunoprecipitates with α-actinin, talin, vinculin, and zyxin as determined by Western blot (Fig. 3 D). The C10 antigen might be associated with the C10 antigen in the middle lane. C, A analysis of the fractionation of the C10 antigen. MEF lysates were extracted with 0.5% Triton X-100 in cytoskeleton-stabilizing buffer (see Materials and Methods) and the insoluble pellet was collected by centrifugation at 14,000 rpm. 20 μg of protein from pellets and supernatants was loaded and blotted with α-actinin, vinculin, and zyxin. The Western blots were precleared with Gamma-bind beads and used to immunoprecipitate the C10 antigen. Both sets of beads were eluted and blotted with either α-actinin, vinculin, and zyxin (all negative; center). In the reverse experiment, α-actinin was immunoprecipitated using a rabbit polyclonal antibody, and the IP was blotted for α-actinin and for the C10 antigen (right).
bers. The proline-rich sequences are flanked by numerous serine residues. A iso of interest are the three tandem repeats of an Ig-like sequence called the Ig C2 domain. Ig C2 domains have been identified previously in a group of intracellular proteins that are associated with the actin cytoskeleton in skeletal muscle, including the giant protein, titin (Labelt et al., 1990); the muscle-specific C protein (Einheber and Fischman, 1990); H protein (Vaughan et al., 1993), and M protein (Noguchi et al., 1992); myosin light chain kinase (Olson et al., 1990); and myotilin (Salminen et al., 1999). The Ig C2 domains in palladin share varying degrees of homology with previously identified proteins. The most NH₂-terminal Ig C2 domain of palladin has the highest homology to the NH₂-terminal, Z-line-associated, Ig C2 domains of titin (44% at the protein level), and the COOH-terminal Ig C2 domains of myosin light chain kinase (42%). Palladin’s middle and COOH-terminal Ig C2 domains are most homologous to myotilin’s NH₂-terminal Ig C2 (60% and 55%, respectively) and titin’s COOH-terminal, M-line–associated, Ig C2 domains (48% and 37%, respectively).

Palladin Exists as Multiple Isoforms and Is Widely Expressed in Embryonic Tissues

Northern blot analysis of three tissues (brain, heart, and gizzard) from a day-10 embryonic chick were probed with the chick cDNA, 18b-1. A shown in Fig. 5 A, the major band in all three tissues was 4.4 kb. In addition, a larger transcript (6 kb) was detected in brain and gizzard, and faintly detected in heart. The largest band seen by Northern blot was a transcript of 7.4 kb, which was only observed in heart. The existence of multiple isoforms of palladin was confirmed by Western blot. A shown in Fig. 5 A, the protein bands detected by antipalladin mAb 1E6 corresponded precisely to the sizes that were predicted from the mRNA transcripts seen in the Northern blot. A doublet of 90–92 kD was detected in all three chick tissues. In brain and gizzard, a less intense band of 140 kD was stained, and a band of 200 kd was observed specifically in heart. A unusual band of 99 kD was also detected specifically in the Western blot of heart (Fig. 5 A). A corresponding size of message was not detected in the Northern blot, suggesting that the 99-kD band may result from post-translational modification of the 92-kD isoform.

Kozak sequence is shown. B. The full-length clone localizes properly and is recognized by anti-C10 antibodies. The myc-tagged construct was transfected into Swiss 3T3 cells, which were fixed and stained with an antimyc antibody. Note that the construct localized in focal adhesions, at the ends of stress fibers, and in a punctate pattern along the stress fibers. The transfected cells were also lysed and analyzed by Western blot. The same band is recognized by both antimyc and anti-C10 mAb b 1E6 corresponded precisely to the sizes that were predicted from the mRNA transcripts seen in the Northern blot. A doublet of 90–92 kD was detected in all three chick tissues. In brain and gizzard, a less intense band of 140 kD was stained, and a band of 200 kd was observed specifically in heart. A unusual band of 99 kD was also detected specifically in the Western blot of heart (Fig. 5 A). A corresponding size of message was not detected in the Northern blot, suggesting that the 99-kD band may result from post-translational modification of the 92-kD isoform.
To compare the pattern of palladin expression in embryonic versus adult tissues, immunoblot analysis was performed on six tissues obtained either from a day-15 mouse embryo or from an adult mouse. As shown in Fig. 5 B, the 90–92-kD form of palladin was detected in all embryonic tissues tested. The larger 140-kD isoform was present in embryonic kidney, spleen, gut, and skeletal muscle, but not in liver or heart. Embryonic mouse heart also expressed the 200-kD isoform. In the adult tissues, however, the expression of palladin was more variable (Fig. 5 B). Only the 90–92-kD isoform was detected and only in adult spleen and gut; it was almost undetectable in adult kidney, liver, heart, and skeletal muscle.

**Palladin Isoforms Are Expressed in Cultured Cells**

Since organized tissues contain a variety of cell types, we used cultured cell lines to determine if the palladin size variants seen in developing tissues could be assigned to specific types of cells. Palladin expression was compared in primary cultures of fibroblasts and vascular smooth muscle cells, as well as in the epithelial cell lines MDCK, CHO, and HeLa. As shown in Fig. 5 C, the 90–92-kD isoform of palladin was detected in Swiss 3T3 fibroblasts and in smooth muscle cells, which have a fibroblast-like morphology in culture; a faint band at 140 kD was sometimes detected in fibroblasts (see Fig. 3 A). However, all epithelial cells tested expressed both the 90–92-kD and the 140-kD isoforms, and an additional band of 110 kD was detected in HeLa cells. While preliminary, these results suggest the possibility that the 90–92-kD and 140-kD forms of palladin may be specifically associated with fibroblast-type and epithelial-type cytoarchitecture, respectively.

**Palladin Expression Correlates with Cytoskeletal Organization in Differentiating Trophoblasts**

The ubiquitous presence of palladin in developing tissues suggests that this protein may have a special role in organizing the actin cytoskeleton in cells that are undergoing the process of structural and functional differentiation. To investigate the function of palladin in cultured cells, we sought a cell line that undergoes cytoskeletal reorganization in response to specific growth conditions. We chose the rat choriocarcinoma (Rcho-1) cell line, which has been used as an in vitro model for rodent trophoblast giant cell differentiation (Faria and Soares, 1991). These cells grow as proliferative stem cells in medium containing 20% FBS, and the stem cells have a generally round morphology (Kamei et al., 1997). As shown in Fig. 6 A, top, the Rcho-1 stem cells have a poorly organized cytoskeleton: actin is localized in rings or patches, and no stress fibers are visible. Rcho-1 stem cells also have only small, peripheral focal complexes (Fig. 6 A, top).

Rcho-1 cells can be induced to differentiate into trophoblast giant cells by switching the medium from 20% FBS to 10% horse serum for three to seven days. Upon differentiation, Rcho-1 cells undergo a dramatic change in morphology and cytoskeletal organization (Fig. 6 A, bottom): the cells form abundant stress fibers and large, numerous focal adhesions. Western blot analyses were performed on Rcho-1 cells to determine if proteins associated with stress fibers and focal adhesions were upregulated in differentiated cells. As shown in Fig. 6 B, the expression of actin, α-actinin, and vinculin did not change in Rcho-1 stem cells versus differentiated cells; however, palladin expression was undetectable in the stem cell population, and increased dramatically by three to seven days after differentiation (Fig. 6 C). This correlation suggested that palladin could play an important role in cytoskeletal organization and remodeling in this cell model. It should
be noted that Rcho-1 cells express only the 90–92-kD form of palladin.

The role of palladin in Rcho-1 cells was explored further by using antisense technology. A partial antisense construct, corresponding to the COOH-terminal two-thirds of palladin, was made in the adenoviral vector, pAdlox. This construct was cotransfected with a GFP construct (as a transfection marker) into Rcho-1 stem cells. Whole cell lysates were prepared by scraping cells in boiling Laemmli sample buffer; equal amounts of lysates were loaded in each lane and blotted for the respective protein. C, Palladin expression increases in differentiating cells. Whole cell lysates were prepared as in B and equal amounts loaded in each lane and blotted with antipalladin mAb 7C6. Only the 90–92-kD isoform was detected. D3 and D7 refer to 3 and 7 d after differentiation.

Figure 6. Rcho-1 cells upregulate the expression of palladin, concomitant with the formation of stress fibers and focal adhesions. A, Proliferative Rcho-1 stem cells have no organized actin stress fibers; when induced to differentiate, the cells assemble numerous stress fibers and large focal adhesions. Bar, 10 μm. B, Expression of actin, α-actinin, and vinculin does not change when R cho-1 cells differentiate. Whole cell lysates were prepared by scraping cells in boiling Laemmli sample buffer; equal amounts of lysates were loaded in each lane and blotted for the respective protein. C, Palladin expression increases in differentiating cells. Whole cell lysates were prepared as in B and equal amounts loaded in each lane and blotted with antipalladin mAb 7C6. Only the 90–92-kD isoform was detected. D3 and D7 refer to 3 and 7 d after differentiation.

Figure 7. Rcho-1 cells fail to make stress fibers when treated with palladin antisense. A, GFP vector was cotransfected either with an empty pA dlox vector (top) or with pA dlox–palladin antisense construct (bottom) into Rcho-1 stem cells. 2 d later, the cells were induced to differentiate. Three days after differentiation, the cells were fixed and stained with Texas red-conjugated phalloidin. Representative transfected cells are shown. Note that in the presence of the antisense construct, the cells have no stress fibers. Bar, 10 μm.

Palladin Antisense Treatment Alters the Cytoskeleton and Causes Cell Rounding in Fibroblasts

In initial antisense experiments on fibroblasts, both Swiss 3T3 cells and primary MEFs were transfected with the same antisense construct described above, and the effect on the actin cytoskeleton was monitored at three days after transfection by staining with rhodamine phalloidin. In both types of fibroblasts, a loss of stress fibers was observed (data not shown); however, the transfection efficiency was much lower than in Rcho-1 cells (~10% in fibroblasts, as compared with 30% in Rcho-1), resulting in a small sample of antisense-transfected cells. To obtain larger populations of antisense-treated cells, we decided to make a recombinant adenovirus to deliver the palladin antisense construct. Four viruses were made: one expressing the partial palladin antisense; one expressing no construct, to use as a control; and two containing irrelevant inserts (GFP and inactive cdc42) as additional specificity controls. MEFs were infected separately with equal concentrations of these four viruses, and the effect on palladin expression was analyzed by Western blot. As shown in Fig. 8 A, palladin expression decreased in antisense-transfected cells, indicating that the antisense construct was effective.
expression in the antisense-treated cells was dramatically decreased, and this decrease was specific, as it correlated with increasing concentrations of antisense virus. Empty virus and the two other control viruses did not significantly affect palladin expression (data not shown).

At three days after infection, phalloidin staining of infected cells was performed to assay the effect on the actin cytoskeleton. Cells infected with the empty virus remained well-spread and had numerous stress fibers (Fig. 8 B), as did cells infected with virus expressing GFP (Fig. 8 B) or inactive cdc42 (data not shown). In cells infected with increasing concentration of the antisense virus, an increasing proportion of the cells was observed to have assumed a rounded morphology (Fig. 8 B). At the highest concentration of virus, where >90% of the cells were infected (according to parallel infections with the GFP virus), virtually all cells were rounded.

The appearance of the actin cytoskeleton was also assayed in some cells at earlier times after antisense treatment. As shown in Fig. 9, the cells with a lowest level of palladin expression (as determined by immunofluorescent labeling) lacked robust stress fibers and instead had wispy arrays of actin (Fig. 9, B and D). In addition, cells with reduced palladin expression displayed focal adhesions only at the periphery and showed a striking absence of focal adhesions in the interior of the cell (Fig. 10, B and D). Taken together, these results suggest that the expression of palladin is required for cultured fibroblasts to maintain a normal organization of actin and focal adhesions.
Discussion

Here, we describe a novel protein that was found in an unusual way, by recognizing that an older mAb had been mischaracterized and by identifying the correct antigen. Palladin is a new member of a small group of cytoskeletal proteins that contain Ig C2. The Ig C2 repeat was first identified in the extracellular domain of cell surface molecules involved in cell adhesion (Williams and Barclay, 1988). Subsequently, the Ig C2 repeat was found in intracellular proteins, including the giant protein titin, which contains up to 166 copies of this motif (Labeit et al., 1990). Ig C2 domains are characteristic of many members of the myosin-binding superfamily, such as C protein, H protein, and M protein (Einheber and Fischman, 1990; Noguchi et al., 1992; Vaughan et al., 1993). The majority of the intracellular Ig C2 domain proteins are found specifically in striated muscle (Furst and Gautel, 1995), suggesting that Ig C2 domains may have a special role in achieving the highly ordered cytoskeletal structure of the sarcomere. It is somewhat surprising, then, that palladin has been detected in every embryonic tissue tested to date, and its expression is actually downregulated in striated muscle of mature animals.

The function of the Ig C2 domain has been widely debated. There appears to be the potential for functional specialization of the Ig C2 repeats found within a single molecule. In titin, for example, two Ig C2 repeats at the extreme NH$_2$-terminal end bind to a novel Z-line protein called the T-cap (Gregorio et al., 1998). In the C protein, a high-affinity binding site for myosin has been mapped specifically to the COOH-terminal Ig C2 domain, and this activity is not shared with the remaining six Ig C2 domains found in the same protein (Okagaki et al., 1993). The ability to bind myosin is not a universal feature of the intracellular Ig C2 domain proteins, as the Z-line protein myotilin does not appear to bind myosin; instead, the Ig C2 do-

Figure 9. Loss of palladin expression results in a loss of stress fibers. At 2 d after infection, at the lowest virus concentration ($2 \times 10^7$ pfu/ml), cells were fixed and stained with a 1:1 mixture of antipalladin mAbs 7C6 and 1E6 (A and C), and phalloidin (B and D). In each field examined, the cells with the lowest level of palladin staining exhibited a more round morphology and a lack of robust stress fibers. Bars: (A and C) 10 µm; (B and D) 5 µm.

Figure 10. Cells with attenuated palladin expression have only peripheral focal adhesions. At 2 d after infection (as in Fig. 9), cells were fixed and stained for palladin with a 1:1 mixture of mAbs 7C6 and 1E6 (A and C) and double-labeled for paxillin (B and D), as a focal adhesion marker. Cells with the lowest level of palladin staining consistently exhibited a clearing of focal adhesions from the center of the cell, such that only peripheral focal adhesions remained. Bars: (A and C) 10 µm; (B and D) 5 µm.
mains of myotilin have been implicated in the formation of homodimers (Salmikangas et al., 1999). A dothial exper-
iments will be needed to determine the function of palla-
din’s Ig C2 domains; however, careful sequence compari-
sion has shown that the three Ig C2 domains of palladin
are, overall, more highly homologous to those of myotilin
and titin, rather than to those of C protein or M protein.

A nother striking feature of palladin is the proline-rich
region in the NH2-terminal half of the molecule. Proline-
rich sequences have been shown to play an important role
in the reorganization of the actin cytoskeleton, based on
analysis of the Listeria monocytogenes protein, A cta . It is
thought that the intracellular pathogen, Listeria, is able to
usurp the host cell’s cytoskeleton because of A cta -
proteins mimics host cell proteins that normally regulate actin-
based cell motility (Kocks et al., 1992). A cta contains four
proline-rich repeats that have been shown to serve as dock-
ing sites for members of the Ena/VASP family of proteins
(Smith et al., 1996; Niebuhr et al., 1997). A sequence
within the proline-rich repeats, FPPPP, is minimally suffi-
cient to bind the EVH1 domain of Ena/VASP family
members in vitro (Niebuhr et al., 1997; Prehoda et al.,
1999). This sequence has been found in two eukaryotic
proteins, zyxin and vinculin (Reinhard et al., 1995b; Brin-
dle et al., 1996; Gertz et al., 1996; Huttelmaier et al.,
1998). Zyxin and vinculin may share limited functional ho-

mology with A cta : both proteins bind to VASP in vitro
and both are concentrated in subcellular sites that are en-
riched in actin filaments. Zyxin is of particular interest, be-
cause it contains three FPPPPP sequences and colocalizes
with VASP, both in focal adhesions and along the stress fi-
bers (Bekerle, 1998).

In addition to the FPPPPP motif, a second polyproline se-
quence has been implicated in actin-based motility. The sequen-
x X PPPPP (where X = A, G, L, or S) is shared be-

 tween a group of actin regulatory proteins including zyxin,
VASP, its Drosophila relative E na, and the human Wis-
cott-Aldrich syndrome protein (Symons et al., 1996; Pur-
rich and Southwick, 1997; Zeile et al., 1998). In VASP, this
sequence has been shown to bind to the G actin-binding
protein, profilin (R einhard et al., 1995a; K ang et al., 1997).
While the precise role of the FPPPPP and X PP PPPP motifs
in eukaryotic proteins is not yet clear, it is nevertheless in-
triguing that palladin contains copies of both consensus se-
quencies, and thus shares limited homology with a group of
molecules that have been implicated in modulating the as-
sembly of actin filaments. Future experiments will focus on
identifying the in vivo binding partners that interact with
the polyproline sequences of palladin.

The existence of multiple size variants of palladin has
been demonstrated by Northern and Western blot, and the
pattern of isoform expression appears to be determined by
both cell type and developmental status. This suggests the
interesting possibility that certain isoforms of palladin may
be adapted for participation in specialized cytoskeletal or-
ganizations. The 90-92-kD form, which is the one we have
cloned and sequenced, is the most abundant and ubiqui-
tous in tissues of both the embryonic and adult mouse.
Whereas it appears that one or more isoforms of palladin
are expressed in every tissue of a developing mouse or chick,
palladin expression was greatly reduced in a number of
adult tissues, including heart, skeletal muscle, liver, and
kidney. One explanation for this pattern could be that pal-
adin is involved in establishing the cytoskeletal organiza-
tion of cells as they differentiate, and that palladin is then
 replaced by another protein in certain fully differentiated
cells. For example, the palladin detected in embryonic
muscle may be replaced in differentiated sarcomeres by
the related molecule myotilin, which is specific to striated
muscle (Salmikangas et al., 1999).

Although much remains to be learned about the regula-
tion of palladin and its in vivo protein—protein interac-
tions, two lines of evidence suggest that palladin plays a
role in the organization of the actin cytoskeleton in cul-
tured cells. First, in the Rcho-1 cell line, endogenous pal-
din expression is specifically upregulated when the cells
began to assemble stress fibers and focal adhesions in re-
sponse to a change in serum concentration. A though this
result is only correlative, it is compelling to note that
Rcho-1 stem cells, which do not assemble stress fibers,
nonetheless express the same amount of actin as the dif-
f erentiated cells that form abundant stress fibers. To date,
the only cytoskeletal protein found to be upregulated in
the differentiated Rcho-1 cells is palladin, suggesting that
this protein may be key to the dramatic cytoskeletal reor-
ganization observed in these cells. The second line of
evidence was obtained by attenuating the expression of
palladin with antisense constructs, introduced either by
transfection or by delivery via adenovirus. Using either
method, in primary fibroblasts, the result was the same:
cells with low levels of palladin expression displayed few,
wispy stress fibers and few, peripheral focal adhesions, and
eventually rounded up from the substrate. Together, these
data indicate that palladin is important for the mainte-
nance of organized arrays of actin and associated cell ad-
hesions. Future experiments will focus on understanding
the mechanism by which palladin has this effect. It will be
interesting to determine if palladin has its primary action
on the assembly of actin microfilaments or, like α-actinin,
on the bundling of microfilaments to form stable stress fi-
bers, or on the anchorage of microfilaments to the focal
adhesions.

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