Identification of palladin isoforms and characterization of an isoform-specific interaction between Lasp-1 and palladin

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Summary

Palladin is a recently described phosphoprotein with an important role in cytoskeletal organization. The major palladin isoform (90-92 kDa) binds to three actin-associated proteins (ezrin, VASP and α-actinin), suggesting that palladin functions as a cytoskeletal scaffold. Here, we describe the organization of the palladin gene, which encodes multiple isoforms, including one (140 kDa) with a similar localization pattern to 90 kDa palladin. Overexpression of the 90 kDa or 140 kDa isoforms in COS-7 cells results in rearrangements of the actin cytoskeleton into super-robust bundles and star-like arrays, respectively. Sequence analysis of 140 kDa palladin revealed a conserved binding site for SH3 domains, suggesting that it binds directly to the SH3-domain protein Lasp-1. Binding of 140 kDa palladin, but not 90 kDa palladin, to Lasp-1 was confirmed by yeast two-hybrid and GST-pull-down assays. Isoform-specific siRNA experiments suggested that 140 kDa palladin plays a role in recruiting Lasp-1 to stress fibers. These results add Lasp-1, an actin-binding protein with a crucial role in cell motility, to the growing list of palladin's binding partners, and suggest that 140 kDa palladin has a specialized function in organizing the actin arrays that participate in cell migration and/or cellular contractility.

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Introduction

The ability to move in response to a stimulus is a fundamental property of animal cells. Cell motility is essential for embryological development and is also required for normal physiology in the adult, as it contributes to wound healing, axonal extension and the immune response. Although the precise molecular mechanisms that control cell motility are not completely understood, it appears to be a complex process that requires the simultaneous assembly of branched, rapidly growing actin arrays to move the leading edge forward by protrusion, and of contractile actin arrays in the body of cell to allow the rest of the cell to follow the leading edge (reviewed in Pollard and Borisy, 2003; Krause et al., 2003; Raftopoulou and Hall, 2004). In addition, the cell must coordinate the assembly of new focal adhesions at the front of the cell and detachment of focal adhesions at the rear of the cell. Thus, in order for a cell to move normally, the organization of functionally distinct actin-based structures must be coordinated both spatially and temporally with remarkable precision. Multiple actin-binding proteins contribute to this process, and the task of recruiting actin-binding proteins to specific subcellular locations is performed by cytoskeletal scaffolds.

Lasp-1 is a multi-domain, actin-associated protein that possesses the key features of a cytoskeletal scaffold. Lasp-1 contains a variety of conserved domains that function as protein-interaction sites: an N-terminal LIM domain, followed by an actin-binding nebulin repeat and a C-terminal SH3 domain. Several lines of evidence suggest that Lasp-1 plays an important role in the cytoskeletal changes that drive cell migration. First, Lasp-1 was originally cloned from a cDNA library derived from metastatic breast cancer and is overexpressed in many breast tumors, suggesting that it may be involved in the abnormal motility that is characteristic of metastatic cells (Tomasetto et al., 1995; Bieche et al., 1996). Second, Lasp-1 binds directly to actin and is concentrated in actin-rich structures that are required for motility, including focal adhesions and lamellipodia (Schreiber et al., 1998; Chew et al., 2002; Lin et al., 2004), and in the motile growth cones of cultured neurons (Phillips et al., 2004). Third, a recent study demonstrated that either depletion of Lasp-1 by RNAi technology or overexpression of Lasp-1 by transient transfection disrupts the ability of cultured cells to migrate in a chemotaxis assay (Lin et al., 2004). It appears that in order for a cell to migrate normally, the amount of Lasp-1 within the cell must be maintained within precise limits.

Palladin is a recently described, multi-domain protein that also appears to function as a potent cytoskeletal scaffold. Similar to Lasp-1, palladin is a phosphoprotein that localizes to actin-rich structures and focal adhesions (Parast and Otey, 2000). Depletion of palladin from a variety of cultured cells (including fibroblasts, neurons and trophoblasts) results in a dramatic loss of actin organization and gross disruptions in cell

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morphology, suggesting that palladin plays an important role in the maintenance of a normal actin cytoskeleton (Parast and Otey, 2000; Boukhelifa et al., 2001). Palladin expression is also required for normal mammalian embryogenesis, because a palladin knockout mouse was shown to have an embryonic lethal phenotype (Luo et al., 2005). Palladin belongs to a family of proteins, all of which bind to α-actinin (reviewed in Otey and Carpen, 2004). The two other family members, myotilin and myopalladin, are both expressed in striated muscle and localize to the Z-disc (Salmikangas et al., 1999; Bang et al., 2001). Mutations in the myotilin gene have been implicated in a human limb girdle muscular dystrophy, indicating that this family member is required for the maintenance of a normal, functional sarcomeric cytoskeleton (Hauser et al., 2000; Hauser et al., 2002; Salmikangas et al., 2003). The third family member, myopalladin, binds to nebulin (in skeletal muscle) and nebulette (in cardiac muscle), and might have a crucial function in linking these cytoskeletal proteins to the Z-line (Bang et al., 2001; Ma and Wang, 2002). Thus, all three family members appear to play important roles in the assembly of specialized actin arrays, and in the maintenance of normal cell morphology.

Unlike myopalladin and myotilin, palladin is widely expressed in developing mouse tissues. It has been reported that a single palladin gene gives rise to multiple size variants, some of which are expressed in tissue-specific patterns (Parast and Otey, 2000; Mykkanen, 2001). The most common palladin isoform in mouse runs as a closely spaced doublet at 90-92 kDa (Parast and Otey, 2000). A 140 kDa isoform is also widely expressed and was observed to be particularly abundant in epithelial-derived cell lines, whereas a 200 kDa isoform was detected in heart (Parast and Otey, 2000). The tissue-specific expression pattern of palladin isoforms suggests that they contribute to the acquisition of distinct cell morphologies. In the current study, we set out to identify and characterize the larger isoforms of palladin in the mouse, and to understand how their molecular structure differs from that of the ubiquitous 90-92 kDa isoform. In addition, we report here that the 140 kDa isoform of palladin binds directly to Lasp-1 and, thus, might be involved in recruiting Lasp-1 to specific subcellular locations. In support of this, we observe that knockdown of the 140 kDa isoform of palladin results in diminished localization of Lasp-1 to bundled F-actin in HeLa cells.

Results

Palladin isoforms were identified in silico

Subsequent to the publication of the original papers describing palladin (Parast and Otey, 2000; Mykkanen et al., 2001), a large amount of nucleotide sequence was added to genome and transcriptome databases (Lennon et al., 1996; Okazaki et al., 2002). Initially, palladin was identified from λ-phage and yeast two-hybrid clones of the predominant 90-92 kDa isoform and as a partial clone of the 140 kDa isoform. Since we were aware of the expression of additional isoforms, we searched NCBI/GenBank nucleotide databases for new transcripts and identified multiple cDNA entries. These nucleotide sequences made it possible to determine the peptide sequences of the 140 kDa and 200 kDa isoforms that had previously been identified by western blot. The coding sequences of the 140 kDa and 200 kDa isoforms are best represented by GenBank cDNA accession numbers AK173081 and AK052489, respectively. As shown in Fig. 1D, these isoforms are essentially N-terminal extensions of the 90 kDa protein. The extended N-terminal sequence of 140 kDa palladin is proline-rich (~14% proline) and contains two additional putative binding sites for members of the Ena/Mena/VASP protein family. It also encodes a fourth IgC2 domain. The 200 kDa isoform contains this same N-terminal sequence, but is extended by a further 389 amino acids. This additional sequence includes a fifth IgC2 domain and a large span of sequence that does not appear to fall into any recognizable domain class by primary sequence analysis. The IgC2-domain organization and sequence of the 200 kDa palladin isoform shares very strong homology with the recently described protein myopalladin, suggesting that divergence of myopalladin and palladin arose from genetic duplication. Sequence alignment of myopalladin and 200 kDa palladin is shown in supplementary material Fig. S1. Two smaller mouse isoforms were also identified in the transcriptome databases.

The coding sequence of 90 kDa palladin was originally identified by using BLAST to search the GenBank database with a partial λ-phage clone (Parast and Otey, 2000). This first partial clone had been originally identified by virtue of its reactivity with palladin antibodies. Its partial sequence was extended 5′ by using BLAST to compare it against the databases to reveal a putative start codon. This revealed the transcription of an apparent full-length sequence in murine heart (GenBank accession number AA671190) that was expected to result in expression of a protein with molecular mass that approximates the apparent SDS-PAGE mass of the 90 kDa isoform. However, during the genetic analysis of palladin isoforms, it became apparent that the initial discovery of murine palladin was based on the identification of a partial transcript of the 140 kDa or 200 kDa isoforms (GenBank accession number AF205079). Since the 90 kDa coding sequence is wholly contained within these larger transcripts, it was initially believed that this sequence corresponded to the predominant 90 kDa isoform. However, we now realize that the 90 kDa isoform begins with the peptide sequence MSALA, 126 amino acids downstream of the start site originally identified. The 90 kDa transcript was identified in the human transcriptome (GenBank accession number AB023209) and a partial transcript was identified in the murine transcriptome (GenBank accession number BQ927960). Despite this amended understanding of the 90 kDa palladin sequence, it is not necessary to revise past characterizations of 90 kDa isoform's activities, because all protein interactions that had been previously characterized are retained in this amended sequence, as shown in Fig. 1D. However, rather than containing two Ena/VASP-binding motifs, it is now recognized that the 90 kDa isoform contains only one.

Transcription is initiated from one of three sites within the palladin gene

The murine palladin gene (*Palld*) spans approximately 390 kbp on chromosome 8 and comprises at least 24 exons. Three of these exons, respectively the first, third and twelfth are 5′ noncoding exons that are specific for one of three possible transcription start sites. Analysis of the 5′ untranslated regions of 90 kDa, 140 kDa and 200 kDa transcripts reveals that although these mRNAs are transcribed from a single locus, their expression is under the control of three independent promoters nested within the murine gene, as shown in Fig. 1A.

The 200 kDa transcript is transcribed from a distal 5′ promoter, whereas the 140 kDa transcript is initiated at a promoter 140 kb downstream. The predominant 90 kDa transcript is initiated at the most 3′ promoter, which lies approximately 340 kb downstream of the 200 kDa promoter. The sequences of the palladin exons and their splice sites are provided in the supplementary material Table S1.

Full-length murine cDNA sequences have also provided strong evidence for the existence of other palladin isoforms that utilize alternative splicing or termination mechanisms. A fourth isoform, 'C-terminus', is putatively expressed as the C-terminal sequence common to the 90 kDa, 140 kDa and 200 kDa isoforms. This transcript of this isoform results from initiation at the 90 kDa promoter and the utilization of an alternative splice site within this transcript's second exon and the gene's thirteenth (represented by GenBank accession number AK087270). A recent paper describing a paladin-knockout mouse provides evidence for the expression of this C-terminal isoform and shows it to migrate with an apparent molecular mass of about 50 kDa, which approximates its predicted size (Luo et al., 2005). This splicing event appears to be more common in human cells, in which this exon is skipped in transcripts initiated at all three promoters (accession numbers NM016081, AK095512 and AF151909). A fifth isoform is putatively expressed as the N-terminal extension of the 140 kDa isoform. This transcript of this isoform results from initiation at the 140 kDa promoter and the ostensible utilization of alternative termination and polyadenylation signals upstream of the 90 kDa promoter (represented by GenBank accession number AK087573). The expression of the N-terminus isoform remains yet unconfirmed. Because these smaller isoforms lack the epitopes detected by monoclonal antibodies used in the initial characterization of palladin, they were not previously detected in cells or tissues.

The 140 kDa isoform is widely expressed in developing tissues

Earlier studies of palladin have focused on the 90 kDa isoform. We undertook to explore the tissue distribution of the 140 kDa and 200 kDa isoforms more carefully. A new polyclonal antibody was generated, using a fragment encoding the N-terminal extension of the 140 kDa isoform as an immunogen.

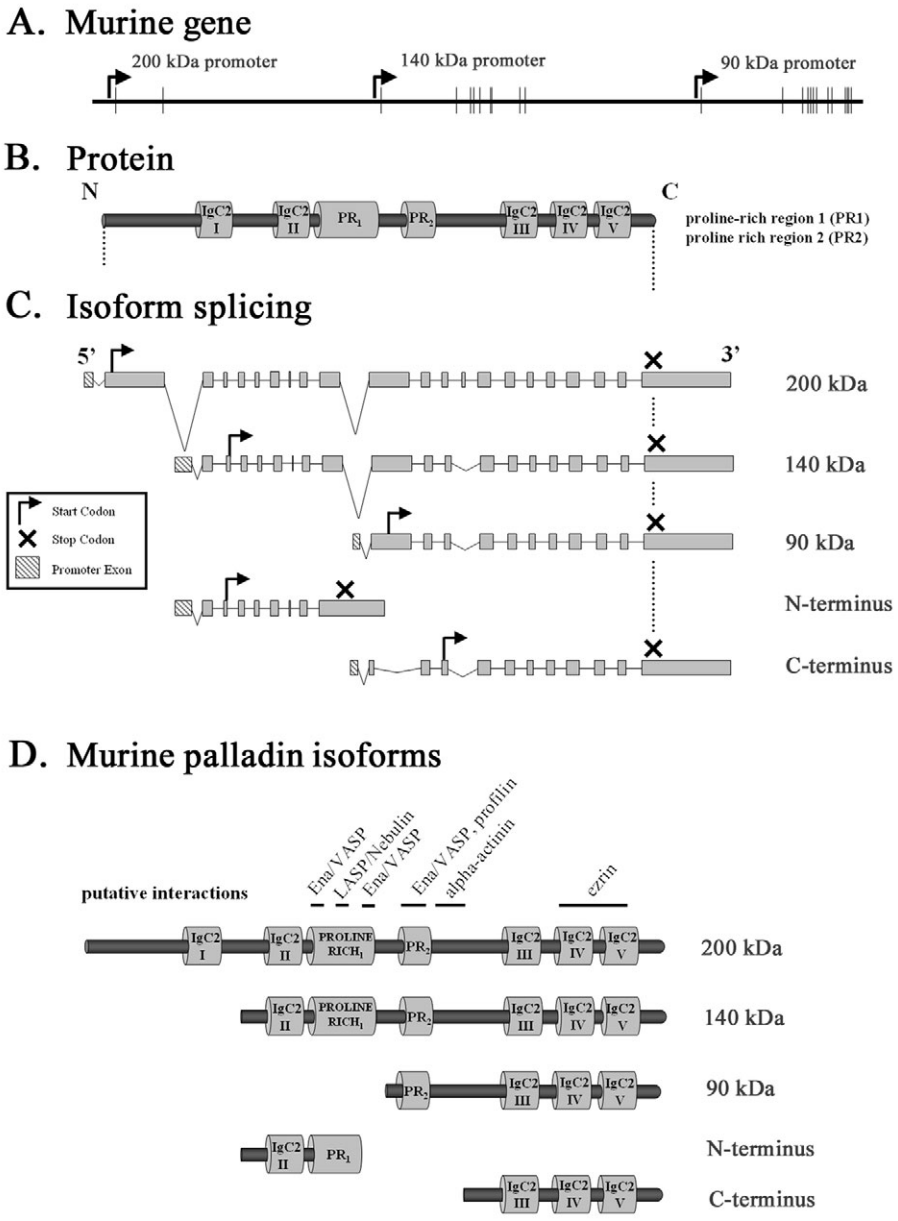

Fig. 1. Multiple palladin isoforms are transcribed from one gene. (A) Schematic representation of the gene structure of palladin . The murine palladin gene comprises at least 24 exons over ~390 kb on chromosome 8 (*Palld*; cytoband B3.1). Transcripts are initiated from one of three nested promoters, which are indicated with arrowheads. (B) Schematic representation of the largest palladin isoform (200 kDa), which is transcribed from the most 5′ promoter. The two proline-rich regions have been designated PR1 and PR2.
(C) Schematic representation of mRNA splicing patterns for observed isoforms. Translation initiation and termination sites have been aligned with the above peptide sequence for the 200 kDa isoform.
(D) Schematic representation of palladin isoforms. The 200 kDa, 140 kDa and 90 kDa isoforms are the primary products of the palladin gene and have been detected by immunoblotting. They share binding sequences for ezrin, α-actinin, Ena/VASP family members and profilin. The 140 kDa and 200 kDa isoforms also encode two additional FPPPP motifs predicted to interact with Ena/VASP family members. Two other murine isoforms, designated N-terminus and C-terminus, have been reported at the cDNA level. Expression of the N-terminus isoform has not yet been directly confirmed.

This antibody recognizes the 140 kDa and 200 kDa palladin isoforms but not the 90 kDa isoform. Although sequence homology between palladin and myopalladin is strong in this region, no crossreactivity to myopalladin was observed (data are shown in supplementary material, Fig. S2). As shown in Fig. 2A, immunoblot analysis shows that the 140 kDa isoform is widely expressed in neonatal tissues (postnatal day 1), because it was detected in brain, heart, lung, stomach, intestine, kidney, bone and skin. In late development, the expression of the 140 kDa isoform is downregulated in many tissues. By adulthood, expression of the 140 kDa isoform is restricted to cardiac muscle and to organs rich in smooth muscle (Fig. 2B). The 90 kDa isoform is a phosphoprotein (Parast and Otey, 2000), and the observation of the 140 kDa isoform as a doublet suggests that it is also phosphorylated. The 200 kDa isoform, unlike the 90 kDa and 140 kDa isoforms, is highly restricted in its expression. In neonatal tissues, the 200 kDa isoform is detected only in striated muscle and bone. The 200 kDa isoform remains highly expressed in skeletal and cardiac muscle of adult mice, where it might play a role similar to myopalladin in the organization of thin filaments in the Z-line of sarcomeres. The detection of a band slightly below the predominant 200 kDa band in heart (Fig. 2A, lane 2) may represent post translational modification of this isoform, or alternatively, may be a yet unidentified splice variant.

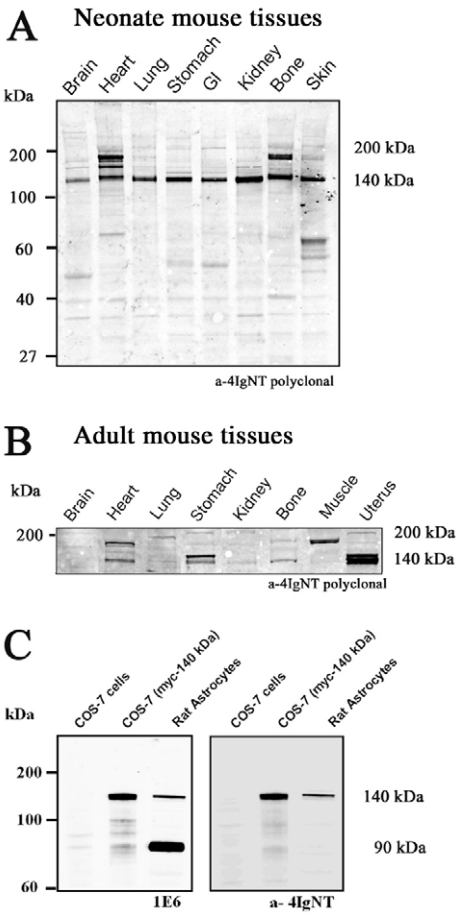

Fig. 2. Immunoblot of larger palladin isoforms. (A) Tissues were dissected from a neonatal mouse and immunoblotted with the polyclonal antibody a-4IgNT that detects the N-terminal extension of the 200 kDa and 140 kDa palladin isoforms. This antibody does not crossreact with the 90 kDa isoform. Similar to the reported expression of the 90 kDa isoform, the 140 kDa isoform of palladin is expressed in most neonatal mouse tissues, whereas the 200 kDa isoform is predominantly restricted to heart, muscle and bone. (B) The 140 kDa isoform is considerately downregulated in many adult tissues, but is abundant in tissues rich in smooth muscle, such as stomach and uterus. The 200 kDa isoform is expressed in striated muscle of the adult mouse. (C) Vector-driven expression of the putative 140 kDa palladin nucleotide sequence in COS-7 cells results in the translation of an immunoreactive band with the same apparent molecular mass observed for endogenous protein in astrocytes. Astrocytes express relatively high levels of the 140 kDa isoform of palladin. Immunodetection with a previously characterized monoclonal (left blot) that reacts with the 90 kDa, 140 kDa and 200 kDa isoforms demonstrates that the 90 kDa and 140 kDa isoforms are expressed in cultured astrocytes. The polyclonal antibody a-4IgNT (right blot) specifically detects the 140 kDa isoform in these cells.

The 90 kDa and 140 kDa isoform colocalize

To study the 140 kDa isoform of palladin in more detail, the full-length coding sequence was cloned from a primary culture of mouse astrocytes. Vector-driven expression of this myc-tagged sequence in mammalian cells confirmed that in SDS-PAGE it migrates with the same apparent molecular mass as the endogenous protein (Fig. 2C). In addition, sequencing of the 140 kDa palladin cDNA confirmed published database entries. Since many tissues express both the 140 kDa and 90 kDa isoforms of palladin, it was of interest to determine whether these isoforms are specialized for different functions. To explore this possibility, we first asked whether these two isoforms differ in their subcellular localization patterns. For this purpose we chose to use primary cultures of rat astrocytes because both the 140 kDa and 90 kDa isoforms are highly expressed in these cells (Fig. 2C). The a-4IgNT polyclonal antibody was used to specifically detect the 140 kDa isoform by immunocytochemistry (Fig. 3A-C). To specifically localize the 90 kDa isoform in these cells, it was necessary to express myc-tagged palladin, because it is not possible to use an antibody to detect exclusively the endogenous 90 kDa isoform. Accordingly, cells were infected with adenovirus expressing the 90 kDa sequence with an N-terminal myc-epitope tag, incubated for 12 hours, fixed and stained with a monoclonal anti-myc antibody to detect the exogenous 90 kDa palladin. Immunostaining of subconfluent cultures demonstrates that the 90 kDa and the 140 kDa isoforms colocalize with an extremely high coincidence (Fig. 3). Both isoforms mainly localize in a punctate pattern along stress fibers (Fig. 3B,E) and in nodes between bundled F-actin arranged in a geodesic pattern (Fig. 3A,D). An interesting feature of astrocytic cultures is that the morphology of the cells changes from fibroblastic to epithelial when the cultures are maintained at high-density for two weeks or more. In these 'long-term' cultures, both the 90 kDa and 140 kDa isoforms localize to cell-to-cell junctions (Fig. 3C,F).

Overexpression of palladin isoforms results in distinctive cytoskeletal phenotypes

We went on to ask whether the 90 kDa and 140 kDa isoforms of palladin exhibit functional differences at the molecular level. Previous results have shown that overexpression of 90 kDa palladin in cultured astrocytes or COS-7 cells results in the

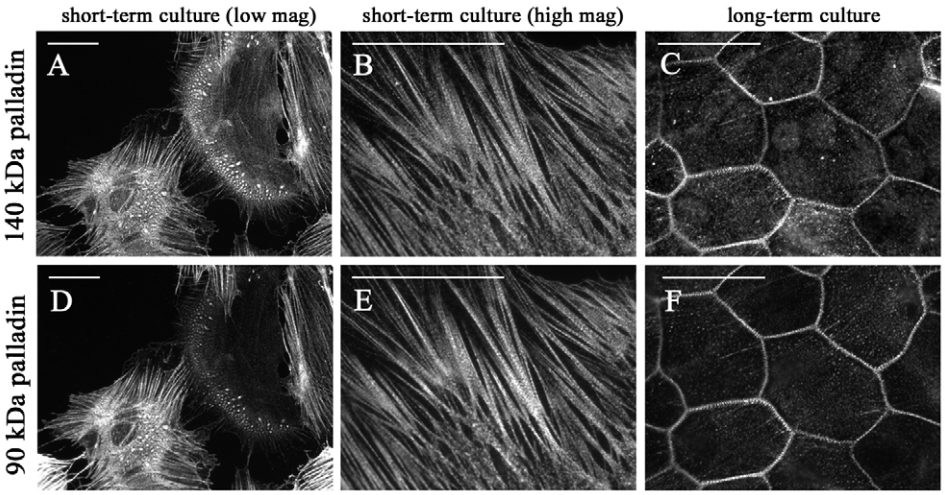

Fig. 3. Immunolocalization of palladin isoforms in cultured astrocytes. The myc-tagged, 90 kDa isoform of palladin was transfected into embryonic rat astrocytes with adenovirus. (D-F) Expressed 90 kDa palladin was detected 12 hours later with a monoclonal antibody directed against the N-terminal myc-epitope tag. (A-C) The endogenous 140 kDa isoform of palladin was detected with the polyclonal antibody a-4IgNT. The two isoforms strongly colocalize. Both isoforms are detected periodically along stress fibers (B and E) and both localize to cell-cell junctions (C and F). Bars, 50 μm.

formation of unusual, extra-thick actin bundles (Boukhelifa et al., 2003; Rönty et al., 2004). To compare the overexpression phenotypes of the 90 kDa and 140 kDa isoforms of palladin, myc-tagged versions were transfected into COS-7 cells. Twenty-four hours after transfection, expression of the 90 kDa isoform induced dramatic bundling of F-actin. In most of these cells, the actin bundles were arranged in a parallel fashion (Fig. 4Ai,Bi) and appeared to be anchored at focal adhesions (data not shown). Expression of the 140 kDa isoform resulted in dramatic actin reorganization in all transfected cells, but by contrast, F-actin was frequently organized in a star-like pattern exhibiting a pronounced radial symmetry and significant compaction (Fig. 4Aiv,Bii). To quantify the distinct phenotypes resulting from overexpression of 90 kDa and 140 kDa palladin, 100 cells were scored blindly for each transfection. All cells that expressed exogenous protein at detectable levels exhibited significant rearrangements of actin, and these were grouped into one of three categories: star-like, large bundles and small bundles. Star-like assemblies were classified by both radial symmetry and a diameter that did not exceed 20 μm. Large and small bundles were more arbitrarily distinguished by the extent of bundling within the cell and the reduction of background fluorescence from nonbundled filaments. Examples of large and small bundles are provided in supplementary material Fig. 2. Out of 100 cells transfected with the 140 kDa isoform, 46 formed star-like patterns, 46 formed large bundles and eight formed small bundles (Fig. 4C). Most cells with star-like assemblies contained two of them, but a range of 1-6 star-like arrangements per cell was observed. Out of 100 cells counted that were transfected with the 90 kDa

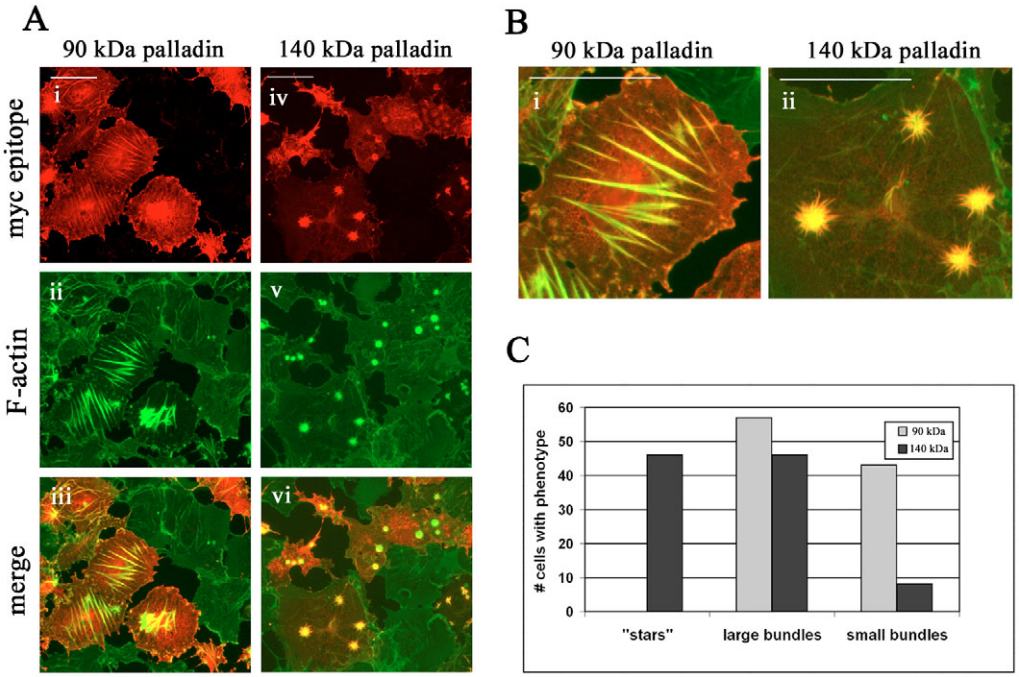

Fig. 4. (A) Overexpression of palladin isoforms in COS-7 cells results in striking, but distinct, actin organization phenotypes. Low-magnification images of COS-7 cells stained for F-actin and exogenous palladin 24 hours after transfection of the 90 kDa or 140 kDa isoforms. The overexpression of the 90 kDa isoform results in the formation of unusually robust actin-cables, whereas overexpression of the 140 kDa isoform results in the assembly of compacted star-like F-actin arrays. In the merged images, phalloidin staining is shown in green and myc-epitope staining is in red. Bars, 50 μm. (B) High-magnification images of single cells from the same fields. Bars, 50 μm. (C) Bar graph, indicating the differences between phenotypes overexpressing the 90 kDa and 140 kDa isoforms. For each transfection, 100 cells were scored blindly and F-actin phenotypes were grouped into one of three classes: stars-like assemblies, large bundles and small bundles (examples are given in supplementary material, Fig. S3). Overexpression of the 140 kDa isoform leads to more pronounced, compacted F-actin phenotype than overexpression of the 90 kDa isoform.

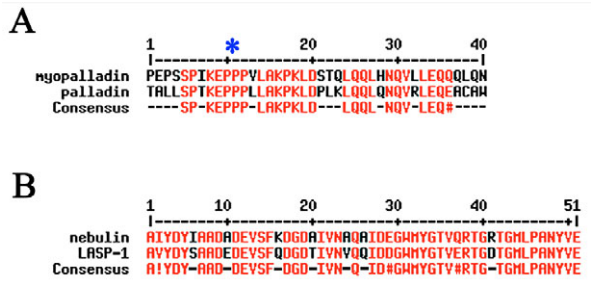

Fig. 5. (A) Alignment of the sequence conserved between myopalladin and palladin that mediates binding of the SH3 domain of nebulin. The poly-proline motif responsible for the nebulin and Lasp-1 interactions is indicated by an asterisk. (B) Alignment of the SH3 domain of nebulin with the SH3 domain of Lasp-1. The core SH3-domain sequence is 80% identical.

isoform, none formed compacted star-like assemblies, 57 formed large bundles and 43 formed small bundles (Fig. 4C). These observations demonstrate first that both isoforms of palladin have potent effects on F-actin organization when expressed exogenously and, second that these two isoforms possess functional differences that result in distinct overexpression phenotypes.

A conserved poly-proline motif mediates the interaction of 4IgC palladin with Lasp-1

Since the 140 kDa isoform of palladin contains an additional 339 amino acids at its N-terminus relative to the 90 kDa isoform, we reasoned that it was likely to have additional binding partners not shared with the smaller isoform. As shown in Fig. 5, the 140 kDa and 200 kDa isoforms of palladin contain a short sequence motif that is shared with myopalladin. A proline triplet within this sequence has previously been shown to mediate a direct interaction between myopalladin and the SH3 domain of the sarcomeric protein nebulin (Bang et al., 2001; Ma and Wang, 2002). To identify new proteins that might interact with this motif, we undertook a database and literature search to identify proteins with similar tissue-expression patterns that contain an SH3 domain with a high degree of homology to that of nebulin. We identified Lasp-1 as a protein that met these criteria. The SH3 domain of Lasp-1 is 80% identical and 86% conserved to that of nebulin (Fig. 5B). Since Lasp-1 and the 140 kDa isoform of palladin are both widely expressed, it seemed probable that the two proteins could interact in vivo. To determine whether 140 kDa palladin binds directly to Lasp-1, a targeted yeast two-hybrid assay was used. The coding sequences of 140 kDa and 90 kDa isoforms of palladin were cloned into GAL4-binding-domain vectors (bait). In this assay, 90 kDa palladin was not predicted to bind to Lasp-1 and functioned as a negative control. The Lasp-1 coding sequence and the coding sequence of the Lasp-1 SH3 domain were cloned into GAL4-activation-domain vectors (prey). The ability of bait-and-prey fusion proteins to interact with one another was assayed by observing growth and galactosidase-dependent color change in co-transfected colonies. As shown in Fig. 6A, yeast two-hybrid results indicate that, as expected, Lasp-1 binds directly to the 140 kDa isoform of palladin but not the 90 kDa isoform. Furthermore, the SH3 domain of Lasp-1 is sufficient for this interaction. To confirm the exact binding site for Lasp-1 on the 140 kDa isoform, we mutagenized two proline residues to alanines in the putative SH3-binding motif. A similar mutation was shown to ablate the interaction between myopalladin and nebulin (Bang et al., 2001). In 140 kDa paladin, this mutation similarly disrupts its interaction with Lasp-1. As expected, this mutation did not affect the interaction of palladin with VASP, which binds to the distinct proline-rich (FPPPP) motif. To confirm these results, we cloned the Lasp-1 and SH3-domain sequences into a GST-fusion expression construct. These two proteins were expressed, purified from bacteria and used to pull-down endogenous proteins from lysates of HeLa cells, a cell line that expresses both the 90 kDa and 140 kDa palladin isoforms. The SH3 domain of Lasp-1 specifically pulled-down the 140 kDa isoform but not the 90 kDa isoform of palladin from these cell lysates (Fig. 6B), as does the full length Lasp-1 protein (data not shown). Thus, we have identified Lasp-1 as an isoform-specific binding partner for palladin.

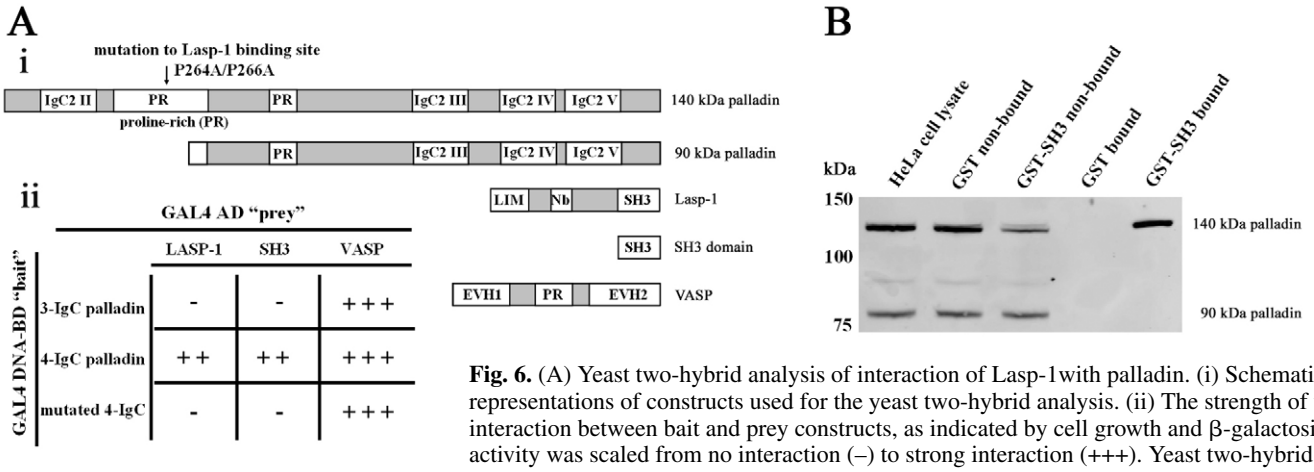

Fig. 6. (A) Yeast two-hybrid analysis of interaction of Lasp-1with palladin. (i) Schematic representations of constructs used for the yeast two-hybrid analysis. (ii) The strength of the interaction between bait and prey constructs, as indicated by cell growth and β-galactosidase activity was scaled from no interaction (–) to strong interaction (+++). Yeast two-hybrid analysis indicates that Lasp-1 interacts through its SH3 domain with the poly-proline motif in the 140 kDa isoform, but not that in the 90 kDa isoform. A mutation to the putative poly-proline motif abrogates this interaction in the 140 kDa isoform. (B) HeLa cells express both the 140 kDa and 90 kDa isoforms of palladin. The SH3 domain of Lasp-1 co-precipitates the 140 kDa palladin. GST protein or the GST domain fused to the SH3 domain of Lasp-1 was incubated with HeLa-cell lysate and precipitated with glutathione Sepharose. Western blot analysis indicates that the SH3 domain of Lasp-1 specifically co-precipitates the 140 kDa isoform but not the 90 kDa isoform of palladin (lane, GST-SH3 bound).

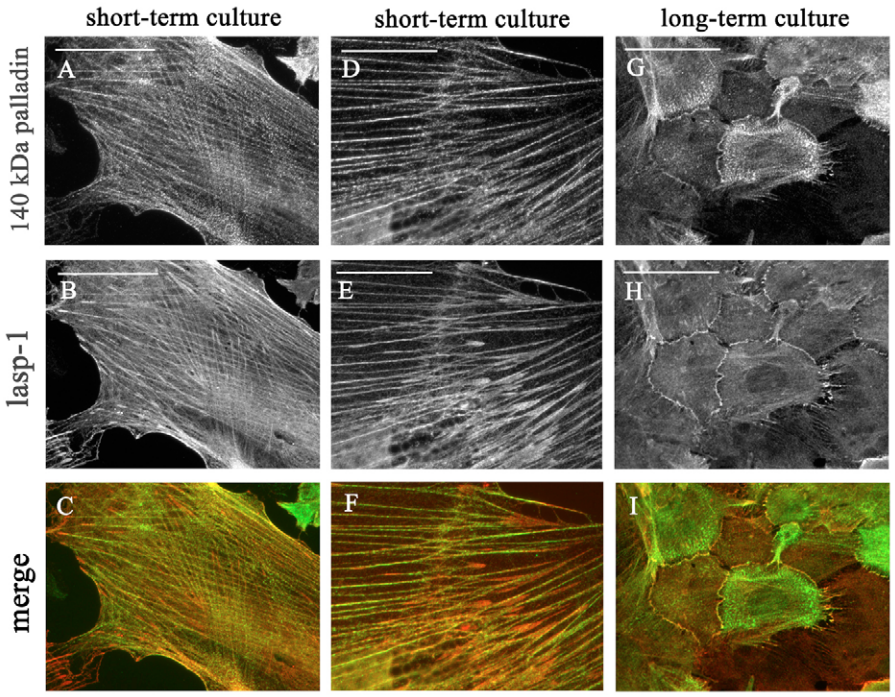

Fig. 7. Localization of Lasp-1 and 140 kDa palladin in astrocytes. The 140 kDa isoform of palladin was detected with the polyclonal 4IgNT antibody characterized in Fig. 2, and Lasp-1 was detected with the 3H8 monoclonal antibody. In the merged images, palladin staining is shown in green and Lasp-1 staining is shown in red. Both Lasp-1 and the 140 kDa isoform of palladin colocalize in stress fibers of astrocytes (A-F), although Lasp-1 exhibits a more pronounced localization to focal adhesions. In longer-term cultures of astrocytes that have formed monolayers of epithelial-like cells, both palladin and Lasp-1 are detected within cell-cell-contacts (G-I). Bars, 50 μm.

Lasp-1 and 140 kDa palladin colocalize in stress fibers and cell-cell-contacts of astrocytes

Lasp-1 has been shown to localize to F-actin and focal adhesions in a variety of cell types, but its localization pattern in astrocytes has not been explored previously. To determine the degree of colocalization of Lasp-1 with endogenous 140 kDa palladin, double-label immunofluorescence staining of cultured astrocytes was performed. Staining with a previously characterized Lasp-1 antibody (Chew et al., 2000) indicates that Lasp-1 is expressed in astrocytes and significantly colocalizes with 140 kDa palladin. In short-term cultures, the cells mainly assume a fibroblastic morphology, and Lasp-1 primarily localizes to stress fibers in a manner similar to paladin (Fig. 7A-F). In cultures incubated for longer, both the 140 kDa isoform of palladin and Lasp-1 were observed to colocalize in cell-cell-contacts of epithelial-like cells (Fig. 7G-I). These colocalization results support the idea that 140 kDa palladin and Lasp-1 form a stable molecular complex in vivo.

Silencing 140 kDa palladin results in the loss of Lasp-1 from stress-fibers in HeLa cells

Lasp-1 exhibits significant variability in its cellular distribution. In different cell types, Lasp-1 may localize to focal adhesions, stress fibers, dorsal ruffles and/or membrane protrusions. In HeLa cells, Lasp-1 was detected in focal adhesions and stress fibers (Fig. 8A), and 140 kDa palladin was concentrated in stress fibers. To determine whether this palladin isoform plays a role in recruiting Lasp-1 to stress fibers, HeLa cells were transfected with small interference RNA (siRNA) that silences the 140 kDa isoform but not the 90 kDa isoform of palladin (western blots are shown in supplementary material Fig. S4). In cells where the 140 kDa isoform has been silenced, Lasp-1 localizes to peripheral focal

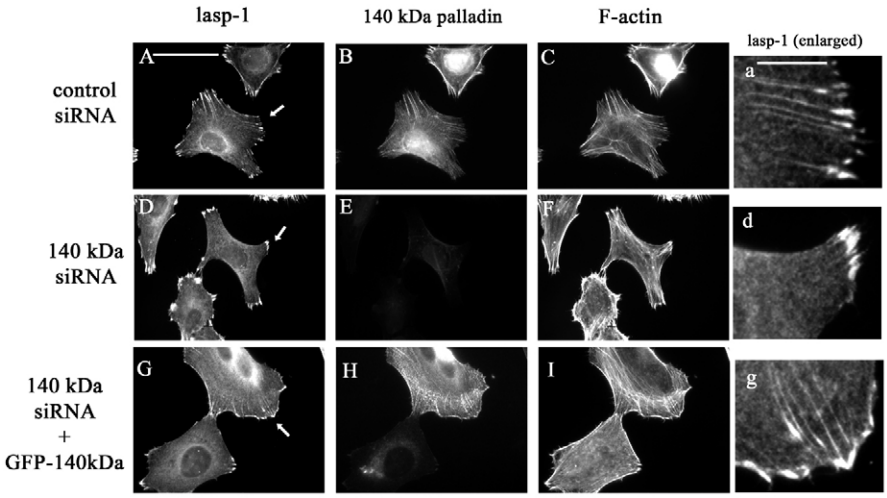

Fig. 8. siRNA targeting of 140 kDa palladin results in loss of Lasp-1 from stress fibers. HeLa cells were transfected with either control siRNA or palladin siRNA specific for 140 kDa palladin. (A-C) Both 140 kDa palladin and Lasp-1 are detected in stress fibers (controls). Lasp-1 is also detected in peripheral adhesions. (D-F) Loss of 140 kDa palladin from these cells results in the loss of Lasp-1 recruitment to stress fibers, although it is still strongly detected in peripheral adhesions. When 140 kDa palladin is re-expressed in these cells by transfection of a construct in which GFP is conjugated to the 140 kDa isoform, Lasp-1 is observed to regain its localization to stress fibers (G-I). Higher magnification of Lasp-1 staining in these cells is provided in a, d, and g. Bars, 50 μm (A-I) and 10 μm (a, d and g).

adhesions, but not to stress-fibers (Fig. 8D). This difference in localization does not appear to be a secondary effect of actin disruption because stress fibers remain relatively intact under these conditions (Fig. 8F). Re-expression of GFP-140 kDa palladin into the siRNA-treated cells resulted in the restoration of Lasp-1 to stress fibers. These experiments demonstrate that the expression of 140 kDa palladin modulates the localization of Lasp-1 and, thus, may serve a role in regulating the function of Lasp-1 within the cell.

Discussion

The actin-associated protein palladin has been shown previously to exist as multiple-size variants in different tissues (Parast and Otey, 2000; Hwang et al., 2001; Boukhelifa et al., 2001; Mykkanen et al., 2001), but these earlier studies provided no insight into the sequence of palladin isoforms and how they are related to the common 90 kDa form. In this report, we demonstrate that the palladin gene has a nested structure, such that expression of each of the three major isoforms (200 kDa, 140 kDa and 90 kDa) is controlled by one of three independent promoters. This new information on the palladin gene suggests that control of isoform expression is likely to be tightly regulated within cells, supporting the view that palladin isoforms might contribute to cell morphology in unique ways. Previously, palladin was shown to have a crucial role in establishing specialized cell morphologies in neurons, astrocytes, trophoblasts and dendritic cells (Parast and Otey, 2000; Mykkanen et al., 2001; Boukhelifa et al., 2001; Boukhelifa et al., 2003). More recently, the creation of a palladin-knockout mouse demonstrated the requirement for palladin during mammalian embryogenesis. Loss of palladin expression resulted in neural-tube-closure defects, ventral herniation and embryonic lethality (Luo et al., 2005). Palladin might also serve an important function in the human vascular system, because a single nucleotide polymorphism in the human palladin gene has been found to be associated with increased risk for myocardial infarction (Shiffman et al., 2005).

The importance of palladin for cell morphology, motility and contractility is supported by the observation that the two most common palladin isoforms uniquely organize F-actin into bundled arrays. It remains to be determined whether this effect is due to an actin-crosslinking activity, enhanced actin-myosin contractility and/or effects on actin polymerization. In this report, we also show that palladin isoforms participate in distinct biochemical pathways because the 140 kDa isoform of palladin binds to Lasp-1, a molecular partner that is not shared with the more abundant 90 kDa form of palladin. The functional significance of this interaction remains to be determined because the precise molecular function of Lasp-1 is still unknown.

Lasp-1 is a protein with complex localization patterns. In general, Lasp-1 is associated with sites of dynamic cytoskeletal reorganization. In hippocampal neurons, Lasp-1 is concentrated in growth cones and dendritic spines, which are motile structures (Phillips et al., 2004). In cultured fibroblasts, Lasp-1 is detected in focal adhesions and at the leading edge of lamellipodia and the tips of filopodia (Chew et al., 2002). Its localization pattern is variable and depends, in part, upon growth factor stimulation and the activation of specific protein kinase pathways. In gastric mucosal fibroblasts, Lasp-1 is recruited from focal adhesions to the leading edge of lamellipodial protrusions by treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) (Chew et al., 2002). In fibroblasts, Lasp-1 translocates from the cell periphery to focal adhesions following treatment with growth factors that stimulate cell motility; downregulation of Lasp-1 results in cells failing to migrate (Lin et al., 2004). In cultured epithelial cells, phosphorylation of Lasp-1 appears to trigger its redistribution from focal contacts to the cytosol, with a concomitant reduction in cell migration (Keicher et al., 2004). Together, these results demonstrate the importance of identifying the molecular binding partners that interact with Lasp-1 in different subcellular locations, in order to gain insight into the molecular pathways that regulate cell motility and adhesion. The effects of Lasp-1 on actin organization might be mediated directly, because Lasp-1 binds to filamentous actin (Chew et al., 2002). However, the multi-domain-organization of Lasp-1 suggests that it also functions as a scaffold to recruit components of signaling pathways to the actin cytoskeleton. The binding of Lasp-1 to palladin, another multi-domain protein, is suggestive of a hierarchy of scaffolds binding to scaffolds, thus, highlighting the complexity of the pathways that control actin dynamics.

Analysis of the molecular partners of Lasp-1 and palladin reveals an interesting degree of apparent redundancy in these pathways. Lasp-1 binds to another actin-associated scaffold, zyxin, which binds to both VASP family members and α-actinin (Crawford et al., 1992; Reinhard et al., 1995; Drees et al., 2000; Li et al., 2004). Since palladin also binds to Lasp-1, VASP and α-actinin (Boukhelifa et al., 2004; Ronty et al., 2004), it appears that palladin and zyxin probably share some degree of overlap in their cellular functions. However, because zyxin and palladin both interact with the SH3 domain of Lasp-1, it is possible that these two proteins compete for binding to Lasp-1 and this might also play a role in regulating the localization and function of Lasp-1.

Lasp-1 was identified as a binding partner for palladin by virtue of its homology with the SH3 domain of nebulin. Bang and co-workers demonstrated that myopalladin, a palladin homologue, binds directly to nebulin through its SH3 domain (Bang et al., 2001). This strongly suggests that, the 140 kDa and 200 kDa isoforms of palladin can also bind to nebulin in skeletal muscle and nebulette in cardiac muscle through the conserved polyproline sequence that is shared with myopalladin. Therefore, probably a significant functional overlap exists between myopalladin and palladin in these tissues. Palladin might also interact with newly characterized protein Lasp-2 and Lim-nebulette (Terasaki et al., 2004; Li et al., 2004), which share the conserved SH3 domain.

To date, all of the molecular partners identified for palladin bind to either monomeric or filamentous actin. Thus, these latest results support the hypothesis that palladin functions as a highly potent scaffolding molecule, with the potential to influence both actin polymerization and the assembly of existing actin filaments into bundles and other higher-order arrays.

Materials and Methods

Identification and cloning of palladin isoforms

The mouse cDNA databases (GenBank) were compared by using BLAST with previously published palladin sequences using the 'nonredundant' program of the National Center for Biotechnology Information (NCBI). This initial search for palladin cDNAs revealed the existence of several new clones that could code for alternative isoforms. Full-length sequences were corroborated in BLAST by using the expressed sequence tag (EST) databases with isoform specific sequences, namely the 5′ untranslated regions (5′ UTRs). The organization of the murine

palladin gene was determined by blasting all palladin cDNA sequences against the genomic database. The three putative promoters of paladin were identified by comparing the 5′ UTR of identified transcripts with the genomic sequence.

Several full-length clones encoding the 140 kDa four-IgC2-domain isoform were identified (GenBank accession numbers AK03196, AK173081 and 4947562). First-strand cDNA corresponding to the coding sequence of the 140 kDa isoform was polymerized from cultured embryonic astrocyte mRNA with the gene-specific primer TCAAAATCCTAAATTGGGGCTGTT and Superscript III reverse transcriptase (Invitrogen). The coding sequence of 140 kDa isoform was PCR-amplified with Pfu-Ultra (Stratagene), the above primer and the forward primer ATGCCACAAGCTCAGAAGAAA. This sequence was cloned into the mammalian expression vector pRK5-MYC for myc-epitope-tagged protein expression.

Isoform-selective palladin polyclonal antibody

The N-terminal sequence of the 140 kDa isoform not found within the 90 kDa isoform was cloned into a GST-expression vector (pGEX-4T-1 ″4IgNT, Amersham). cDNA was amplified with the forward primer GTCGACCACAAGCTCAGAAG-AAAACAACGT and the reverse primer GCGGCCGCAAATCCTAAATTGGG-GCT. The PCR product was cloned into *Sal*I and *Not*I sites of pGEX-4T-1. BL21(DE3)pLysS bacteria (Stratagene) were transformed and the protein was expressed following induction with isopropyl-beta-D-thiogalactopyranoside (IPTG). The fusion protein was purified with glutathione-Sepharose (Amersham) and eluted with bovine thrombin (Sigma). Two rabbits were immunized with the purified eluate. The antibody was affinity-purified with the GST-4IgNT protein crosslinked to cyanogen-bromide-activated Sepharose. This antibody has been designated a-4IgNT.

Western blots

Tissues from neonatal mice (postnatal day one) were freshly dissected, immediately frozen in liquid nitrogen, ground to a fine power with mortar and pestle and solubilized in lysis buffer (50 mM Tris-buffered saline pH 7.0, with 4 M urea, 2% SDS, 10 mM EDTA and protease inhibitor cocktail for mammalian tissue extracts, Sigma Aldrich). Lysates were boiled with 2× Laemmli buffer. Per lane, 10 μm of protein were resolved by SDS-PAGE on a 4-12% polyacrylamide gel, then immunoblotted. The larger 140 kDa and 200 kDa isoforms were detected with the a-4IgNT polyclonal antibody. For immunoblotting of astrocytes, cultures were prepared by the method previously described (Boukhelifa et al., 2003). Astrocytes were lysed after a 1 week incubation by scraping cells into 50 mM Tris pH 7.0 with 150 mM NaCl, 10 mM EDTA, 1% deoxycholate, 1% NP-40 and a protease inhibitor cocktail for mammalian tissues (Sigma). The 90 kDa isoform was detected with the previously characterized 1E6 monoclonal antibody (Parast and Otey, 2000). For imaging, secondary antibodies conjugated to either IRdye700 or 800 (Rockland) were used with the Odyssey infrared imaging system (Licor).

Yeast two-hybrid assay

The coding sequence of 140 kDa palladin isoform was cloned into the GAL4 DNA-binding domain (bait) vector (pGBKT7; Clontech) with forward and reverse primers CATATGCCACAAGCTCAGAAGAA and GTCGACTCACAGGTCTT-CACTTTCTAC, respectively. The Lasp-1-binding poly-proline motif was mutated (P264A; P266A) with the Quickchange site-directed mutagenesis kit (Stratagene), with primers CACCCACTAAGGAAGCACCGGCACTCCTTGCCAAAC and GTTTGGCAAGGAGTGCCGGTGCTTCCTTAGTGGGTG. The full-length Lasp-1-coding sequence and the sequence corresponding to the SH3 domain of Lasp-1 were PCR-amplified from a Lasp-1 cDNA clone (GenBank accession number BC010840) and ligated into the GAL4 activation-domain (prey) vector (pGADT7; Clontech). The primer pairs CATATGAACCCTAACTGTGCCCGGT and GAATTCTCAGATGGCCTCCACGTAGTT, and CATATGTCCATACAGCGC-AGTGCC and GAATTCTCAGATGGCCTCCACGTAGTT, respectively, were used for the Lasp-1 constructs. A GAL4-AD fusion of VASP in the pACT2 vector was used as a positive control (provided by Frank Gertler, M.I.T. Boston, MA). The AH109 yeast strain was transfected with the bait and prey vectors using the Li/PEG transfection protocol (Clontech). The transfected yeast cells were first grown on minimal synthetic defined (SD)-Leu-Trp medium (Clontech) agar plates to select for the pGBKT7 and pGADT7 vectors. After 3 days of growth at 30°C, colonies were picked from each plate, resuspended and re-plated on minimal SD-Leu-Trp-His-Ade medium (Clontech) agar plates supplemented with 20 μg/ml X-Gal (Clontech) and 7.5 mM 3-amino-1,2,4-triazole (3AT; Sigma). Yeast cells were incubated for 7 days, at which time β-galactosidase reporter activity had peaked. The strength of the interaction was gauged by comparing changes in growth and color with the provided positive control.

GST-pull-down assay

The Lasp-1-coding sequence was PCR-amplified with primers GAATTCATGA-ACCCTAACTGTGCCCGGT and CTCGAGTCAGATGGCCTCCACGTAGTT. The SH3 domain coding sequence was PCR-amplified with primers GAATTC-TCCATACAGCGCAGTGCC and CTCGAGTCAGATGGCCTCCACGTAGTT. Both sequences were cloned into the *Eco*RI and *Xho*I sites of GST-fusion expression vector pGEX-4T-1 (Amersham). BL21 pLysS DE3 cells were transformed with these plasmids. Expression was induced with IPTG. The proteins were purified with glutathione-Sepharose beads (Amersham).

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin-amphotecerin B. Confluent (70%) cultures were lysed with 1% Triton X-100 in 50 mM Tris pH 7.4 containing 10 mM EDTA and a protease inhibitor cocktail for mammalian tissues (Sigma). Lysates were centrifuged at 100,000 ***g*** for 30 minutes. GST or GST-SH3 protein (10 μg), which had been crosslinked to cyanogen bromide activated sepharose (Sigma) was added to the supernatant and allowed to incubate for 1 hour. The beads were centrifuged and washed four times in lysis buffer. The bound fraction was eluted by boiling in 2× Laemmli buffer. Samples were resolved by SDS-PAGE on a 4-12% gel, transferred to nitrocellulose and blotted for palladin with the previously described monoclonal antibody 1E6 (Parast and Otey, 2000). The blot was developed with IRdye700-conjugated anti-mouse IgG secondary antibody (Rockland) and imaged with the Odyssey infrared imaging system (Licor).

Cell culture, transfection and immunofluorescence

Astrocytes were prepared from embryonic day 18 rats as previously described (Boukhelifa et al., 2003). Cells were cultured for 1 week before fixing and immunofluorescence staining. Extended cultures of astrocytes that form epithelial-like monolayers were prepared by allowing the cells to grow for an additional 2 weeks in DMEM supplemented with 10% FBS. These cells stained positively for the intermediate filament protein GFAP, indicating that they were astrocytes and not of vascular origin. For colocalization studies of the 90 kDa and 140 kDa isoforms, astrocytes were infected for 12 hours with adenovirus expressing the 90 kDa sequence with an N-terminal myc-epitope tag (10^7 PFU/ml). This virus was engineered by cloning the 90 kDa sequence into the pVQ CMV K-NpA shuttle vector (Viraquest). Virus was produced as previously described (Parast and Otey, 2000). Cells were fixed in 3.7% formaldehyde in PBS with 5 mM $CaCl_2$ and 5 mM $MgCl_2$ for 5 minutes. Cells were permeabilized with 0.2 % Triton X-100 for 2 minutes and stained for the myc-epitope with the 9E10 monoclonal antibody (Sigma), for Lasp-1 with the previously characterized 3H8 monoclonal antibody (provided by Catherine Chew, Medical College of Augusta, Augusta, CA), and/or for the 140 kDa isoform of palladin with the a-4IgNT rabbit polyclonal antibody. Primary antibodies were detected with Alexa Fluor 488 and Alexa Fluor 568 anti-mouse IgG and anti-rabbit IgG conjugates (Molecular Probes). Coverslips were examined with a Nikon TE200-U microscope with 20× and 60× objectives, an optional 1.5× tube lens and a Hamamatsu Orca-ER camera. Images were processed using Adobe Photoshop 7.0 (Adobe Systems Inc).

For overexpression experiments in COS-7 cells, cells were grown to 70% confluency on glass coverslips in DMEM with 10% FBS and transfected with pRK5-myc 90 kDa and 140 kDa vectors using Effectene reagent (Qiagen). Cells were fixed 24 hours after transfection and stained for F-actin with Alexa-Fluor-488-phallodin (Molecular Probes) and for exogenous palladin with 9E10 anti-myc and Alexa Fluor 568 anti-mouse IgG.

For HeLa siRNA experiments, a 70% confluent culture of cells grown in DMEM with 10% FBS was transfected with a final concentration of 50 nM 140 kDa palladin specific siRNA duplex (GGCAAAGCUAACAGUAAUAdTdT, dTdTCCGUUU-CGAUUGUCAUUAU; Dharmacon) or a control siRNA duplex (control X; Dharmacon), which was transfected using TransIT siQuest (Mirus Bio). The cells were trypsinized and seeded onto fibronectin-coated coverslips 8 hours after transfection (bovine, 25ug/ml; Sigma Aldrich). The cells were fixed 72 hours after transfection. For the rescue experiments, cells were seeded onto fibronectin-coated coverslips and transfected with nontargeted murine pEGFP-140 kDa palladin using TransIT-LT1 (Mirus Bio) 2 days after the initial siRNA transfection. The cells were allowed to incubate for 16 hours before they were fixed in paraformaldehyde. All coverslips were stained for Lasp-1 and 140 kDa palladin as described above. F-actin was visualized with Alexa-Fluor-350-phalloidin (Molecular Probes). Images were processed as described above.

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