The role of palladin in actin organization and cell motility

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Abstract

Palladin is a widely expressed protein found in stress fibers, focal adhesions, growth cones, Z-discs, and other actin-based subcellular structures. It belongs to a small gene family that includes the Z-disc proteins myopalladin and myotilin, all of which share similar Ig-like domains. Recent advances have shown that palladin shares with myotilin the ability to bind directly to F-actin, and to crosslink actin filaments into bundles, in vitro. Studies in a variety of cultured cells suggest that the actin-organizing activity of palladin plays a central role in promoting cell motility. Correlative evidence also supports this hypothesis, as palladin levels are typically upregulated in cells that are actively migrating: in developing vertebrate embryos, in cells along a wound edge, and in metastatic cancer cells. Recently, a mutation in the human palladin gene was implicated in an unusually penetrant form of inherited pancreatic cancer, which has stimulated new ideas about the role of palladin in invasive cancer.

Keywords

Introduction

Cell motility is critically dependent on the actin cytoskeleton. Understanding the dynamics of disassembly, relocation, and reassembly of cytoskeletal structures within the cell is essential to understand the progression of many diseases, including cancer invasion and metastasis (Fritz and Kaina, 2006; Lambrechts et al., 2004). Palladin is a recently discovered protein that co-localizes with actin-rich structures in a wide variety of cell types (Mykkanen et al., 2001; Parast and Otey, 2000). There is now accumulating evidence, obtained from both cultured cell studies and microarray analysis of gene expression, to support the hypothesis that palladin plays a key role in the migration of invasive cells. Below, we describe results indicating that palladin has a critical function in generating the actin-based podosomes that contribute to invasive motility. In addition, our recent results suggest that palladin occupies an unusual functional niche, as it is both a molecular scaffold for multiple actin-binding proteins and also an actin-crosslinking protein itself. Thus, it appears that palladin plays an important role in organizing actin arrays within migrating cells, through both direct and indirect molecular mechanisms.

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Palladin and its relatives: a small family of Ig-domain proteins

Palladin was cloned independently in the Otey lab and in the lab of Olli Carpen, and was found to be very widely expressed in vertebrates (Mykkanen et al., 2001; Parast and Otey, 2000). In fact, 90-kDa palladin is ubiquitous in tissues of developing mammals, although it is downregulated in many adult tissues (Parast and Otey, 2000; Rachlin and Otey, 2006). Palladin exists as multiple isoforms that arise from a single gene, and the complexity of palladin isoform expression is still a subject of active investigation. In mice, there are three major isoforms that arise from alternative start sites (resolving at 90, 140 and 200 kDa), but many other isoforms may be generated in specific cell types via alternative splicing. Like the 90-kDa isoform, 140-kDa palladin is widely expressed in developing organs, while the 200-kDa isoform has been detected only in heart and bone (Rachlin and Otey, 2006). The domain structures of these isoforms are illustrated in Figure 1. In addition, a palladin isoform of ~115 kDa was reported in HeLa cells (Parast and Otey, 2000), while 50-kDa and 75-kDa isoforms have been observed in mouse embryo fibroblasts and human tumor-associated fibroblasts, respectively (Luo et al., 2005; Salaria et al., 2007). These isoforms have not yet been fully characterized, so their domain structures are unknown.

Palladin has two close relatives that are expressed in a more restricted pattern: myopalladin is found only in heart and skeletal muscle (Bang et al., 2001), and myotilin is expressed mostly in skeletal muscle (Salmikangas et al., 1999). It appears that myotilin and palladin may share many functions, as myotilin also plays an important role in generating actin-based arrays. Mutations in human myotilin are associated with two distinct inherited muscular disorders, limb-girdle muscular dystrophy 1A and myofibrillar myopathy (Hauser et al., 2000; Selcen and Engel, 2004). In both disorders, affected individuals exhibit ultrastructural changes of the sarcomere, such as Z-disc streaming, and also weakness of the extremities and cardiomyopathy. These disease phenotypes support the hypothesis that proteins of the palladin family are essential for normal actin organization and anchoring of actin filaments.

Palladin, myotilin and myopalladin all contain multiple copies of a distinctive Ig-like domain (immunoglobulin-like domain). Ig domains typically contain ~100 amino acids and consist of seven to nine folded strands. In addition to being a signature domain of this protein family, similar Ig domains have been described in a small number of other intracellular proteins in vertebrates, including myomesin, titin, MyBP-C and MyBP-H (Okagaki et al., 1993; Vaughan et al., 1992). The majority of these intracellular Ig-like domain-containing proteins are specifically expressed in striated muscle, suggesting that this particular type of Ig domain may play a special role in creating the highly ordered cytoskeleton of the sarcomere (Gilbert et al., 1999; Vaughan et al., 1992). Certain inherited forms of heart disease are associated with mutations affecting the Ig domains of either titin or MyBP-C, which supports the idea that Ig domains have a key role in maintaining sarcomere integrity (Gerull et al., 2002, 2006; Watkins et al., 1995).

Currently, the precise molecular function of palladin family Ig domains is a matter of debate. Myotilin has been shown to bind directly to F-actin, to promote the bundling of actin in vitro, and to have an overexpression phenotype similar to that of palladin, generating unusual, super-robust actin bundles in transfected cells (Salmikangas et al., 2003; von Nandelstadh et al., 2005). An analysis of myotilin fragments suggests that the Ig domains of myotilin are required for its actin-binding activity. In addition, the skeletal muscles of certain invertebrate species express a protein called kettin, which has 31 copies of an Ig domain (Hakeda et al., 2000). Recently, a kettin fragment containing only the four C-terminal Ig domains was shown to bind directly to F-actin (Ono et al., 2006), suggesting that binding of actin by Ig domains may be a highly conserved molecular mechanism shared by both vertebrate and invertebrate proteins.

The fact that purified myotilin crosslinks actin filaments in vitro suggests the interesting possibility that palladin might also function as an F-actin-binding protein. To test this idea, it was necessary to express and purify both full-length palladin and multiple palladin fragments, and assay for their ability to bind directly to F-actin and to crosslink F-actin into bundles. In actin co-sedimentation assays, full-length 90-kDa palladin was shown to bind F-actin with a K_d of about 2 µM, consistent with the affinity measurements made for other actin-binding proteins, such as α-actinin (Dixon et al., 2008). Furthermore, purified 90-kDa palladin crosslinked actin filaments into bundles, indicating that monomeric palladin may either possess two actin-binding sites, or that palladin monomers with single actin-binding sites may dimerize.

Because the actin-binding region of myotilin was located near its C-terminal Ig domains, fragments of the palladin C-terminus were purified to test for actin binding. Ig3, but not Ig4 or Ig5, bound to F-actin in a salt-dependant manner, suggesting an electrostatic interaction. A tandem construct consisting of Ig3, a linker region, and Ig4 bound actin with a K_d of about 8 µM, and was capable of crosslinking actin filaments in vitro. The mechanism of actin bundling by the Ig3-Ig4 fragment is still under investigation, and may involve homodimer formation, although that is not yet clear (Dixon et al., 2008).

Palladin possesses the characteristics of a molecular scaffold

In addition to its role as an actin-crosslinking protein, palladin appears to function as a cytoskeletal scaffolding molecule, as it interacts with a large number of actin-binding proteins with important roles in organizing actin filament arrays. All palladin isoforms contain one or two proline-rich regions that function as protein interaction sites. One proline-rich region (PR2) is found in all three of the major palladin isoforms, and this domain binds directly to the actin-regulating proteins VASP (and its relatives Mena and EVL), profilin and Eps8 (Boukhelifa et al., 2004, 2006; Goicoechea et al., 2006). The 140- and 200-kDa isoforms of palladin possess another proline-rich region (PR1), which contains two more binding sites for members of the VASP protein family. This proline-rich region also binds to Lasp-1, an actin-binding protein from the nebulin/nebulette family (Rachlin and Otey, 2006). It is noteworthy that Lasp-1 expression is required for normal cell migration, and that mis-regulated Lasp-1 has been implicated in the motility of ovarian cancer and breast cancer cells (Grunewald et al., 2006, 2007a, b; Lin et al., 2004).

In addition, palladin binds to α-actinin, a widely expressed actin-crosslinking protein, which docks in the region between PR2 and Ig-3 (Ronty et al., 2004). Alpha-actinin is a member of the spectrin/dystrophin family, and it is ubiquitously expressed in vertebrate cells. In all non-muscle cells, it exists as two isoforms: actinin-1 and actinin-4. α-Actinin plays an important role in both cell-matrix and cell-cell adhesion (Otey and Carpen, 2004). A potential role for α-actinin overexpression in tumorigenicity was first demonstrated over a decade ago in cultured cells (Gluck and Ben-Ze'ev, 1994), and, both actinin-1 and actinin-4 are overexpressed in metastatic sub-populations of cancer cells (Hatakeyama et al., 2006; Honda et al., 1998; Suehara et al., 2006). The strong binding interaction between α-actinin and palladin, and their high degree of co-localization in cells and tissues, suggest that these proteins may have a shared function in motility and adhesion that may be dis-regulated in cancer cells.

Palladin also binds to ezrin, a member of the ERM family of actin-associated scaffolds (Mykkanen et al., 2001). A large body of literature implicates ezrin in the metastasis of many different types of human tumors (Curto and McClatchey, 2004), which suggests that multiple components of the palladin pathway may be coordinately upregulated in invasive cancer cells.

In addition to the diverse set of actin-binding proteins described above, palladin also binds to signaling intermediaries such as ArgBP-2 and SPIN-90 (Ronty et al., 2005, 2007). Palladin binds to Src, a key player in podosome formation, and expression of palladin is required for

the actin-cytoskeleton rearrangements that occur downstream of active Src (Ronty et al., 2007). Taken together, the diversity of palladin binding partners suggests that palladin regulates the organization of the actin cytoskeleton via multiple molecular pathways.

The role of palladin in cell motility

Many lines of evidence indicate that palladin plays a critical role in the process of cell motility. Palladin is required for one specific type of actin-based motility, the outgrowth of neuronal extensions that occurs through the migration of growth cones: when palladin expression was knocked down in primary neurons and neuroblastoma cells, neurite extension failed to occur (Boukhelifa et al., 2001). The role of palladin in cell motility is likely to be the direct result of its role in organizing actin filaments into functional arrays, as both knockdown and overexpression of palladin results in gross reorganization of the actin cytoskeleton. For example, when palladin expression was down-regulated in cultured fibroblasts using antisense approaches, stress fibers and focal adhesions were lost, and the cells rounded up (Parast and Otey, 2000). The converse is also true: when palladin was overexpressed in Cos7 cells and astrocytes, a dramatic increase in the number and size of actin bundles was observed (Boukhelifa et al., 2003; Rachlin and Otey, 2006). These findings suggest a molecular function of palladin in promoting the assembly of subcellular arrays of actin. These results were confirmed recently through the use of palladin null fibroblasts. Fibroblasts cultured from palladin knockout embryos display defects in cell motility, adhesion, integrin expression, and actin organization (Liu et al., 2007; Luo et al., 2005). Together, these published results show that palladin plays a central role in organizing the actin arrays required for normal cell adhesion and motility.

Recently, we obtained results that shed light on one specific role that palladin may play in invasive motility. Invadopodia are actin-rich structures found in invasive cells and in cells that cross tissue boundaries (Buccione et al., 2004; Chen and Kelly, 2003; Weaver, 2006). They provide an attractive model to study the mechanics of the actin cytoskeleton at sites of cell-matrix interaction. Invadopodia are membrane protrusions that form on the ventral surfaces of cells, and they are thought to be closely related to two other types of actin-rich structures: podosomes and dorsal ruffles. Podosomes are highly dynamic structures involved in the adhesion of cells to solid substrates, and they also play a role in tissue invasion and matrix remodeling. Podosomes contain a densely-bundled core of actin filaments, surrounded by a ring that contains adhesion and scaffolding proteins (Calle et al., 2004; Chellaiah et al., 2000; Destaing et al., 2003; Pfaff and Jurdic, 2001; Buccione et al., 2004). Podosomes are classically described in monocyte-derived cells such as macrophages and osteoclasts, but have recently been discovered in many other cell types, where they are found in specific patterns such as clusters, bands, belts or rosettes (Buccione et al., 2004; Linder and Kopp, 2005). Dorsal ruffles (also called waves or ring ruffles) are also highly dynamic, and form transiently on the dorsal plasma membrane (Buccione et al., 2004; Orth and McNiven, 2006). They form in response to stimulation by a variety of receptor-tyrosine-kinase growth factors and are important for cytoplasmic remodeling, the establishment of polarity in motile cells and macropinocytosis (Dowrick et al., 1993; Krueger et al., 2003; Orth and McNiven, 2003; Swanson and Watts, 1995; Warn et al., 1993).

The palladin-binding protein Eps8 is critically involved in the formation of dorsal ruffles and podosomes, and Eps8 also plays a role in breast and thyroid cancer, and in metastatic invasion (Griffith et al., 2006; Matoskova et al., 1995; Yao et al., 2006). To determine if palladin shares a role with Eps8 in the formation of both dorsal ruffles and podosomes, RNA$_i$ techniques were used to knock down palladin expression in A7r5 cells, a cultured cell line that is highly responsive to stimulation with platelet-derived growth factor and phorbol-12,13-dibutyrate. These experiments revealed that *(1)* palladin is strongly recruited to protrusive dorsal ruffles

that form transiently in response to growth factor stimulation, as shown in Figure 2, *(2)* palladin also localizes to podosomes on the ventral cell surface after phorbol ester stimulation (Fig. 2), *(3)* knockdown of palladin results in greatly impaired formation of podosomes and dorsal ruffles, and *(4)* palladin plays a role in Rac activation (Goicoechea et al., 2006). These results suggest that palladin and Eps8 act together in remodeling the actin cytoskeleton in response to growth factor and phorbol ester treatment. As we discuss above, palladin plays a role as an actin-crosslinking protein and also as a cytoskeletal scaffolding molecule, leading to the hypothesis that palladin may be involved in any of the following steps of ruffle and podosome formation: *1)* by being part of an intracellular signaling complex induced by growth factor or phorbol ester stimulation; *2)* by organizing the actin networks at specific subcellular sites by crosslinking actin filaments into bundles; or *3)* by functioning as a scaffolding molecule to recruit other actin-binding proteins that are required for podosome and ruffle formation.

The role of palladin in embryonic development and wound healing

Using a microarray approach, the Bronner-Fraser lab showed that the palladin gene is highly upregulated in migratory neural crest cells, a cell type that plays a complex and essential role in vertebrate development (Gammill and Bronner-Fraser, 2002). Neural crest cells are well known to display a very aggressive form of invasive motility, suggesting that palladin upregulation might be required in order for neural crest cells to achieve their final destination during embryonic development. This idea gained support when a palladin knockout mouse was generated that was embryonic lethal (Luo et al., 2005). The embryos die by day E15 and display multiple defects before dying, including exencephaly (anterior neural tube closure defect), and herniation of abdominal organs due to failure of the body wall to close ventrally, which supports the hypothesis that palladin is critically required for normal cell motility during embryogenesis.

In addition to its role in mammalian development, palladin also appears to have a conserved function in tissue remodeling in response to injury. In wounded tissue, a strong correlation exists between high levels of palladin expression and increased cell migration. The first observation of this type was made by Boukhelifa and colleagues, who reported that palladin is rapidly up-regulated in the reactive astrocytes that develop after injury to the central nervous system. Quiescent astrocytes in the adult central nervous system do not express detectable levels of palladin, nor do they contain appreciable levels of F-actin. After injury, these cells become hypertrophic and migratory, increasing palladin expression and assembling filamentous actin arrays (Boukhelifa et al., 2003).

Similarly, palladin was shown to be rapidly up-regulated when fibroblasts differentiate into the highly-contractile myofibroblasts, either in response to skin injury or in response to TGF-β1 treatment. TGF-β1 increased the expression of the 90-kDa palladin isoform, and led to the de novo expression of the 140-kDa form (Ronty et al., 2006). The investigators of this study utilized both human patient samples of skin lesions and a rat model of experimental dermal wounds, and in each case, palladin was observed in myofibroblasts near the wound site, and its upregulation preceded the expression of the classic myofibroblast marker α-smooth muscle actin.

Finally, palladin was found to be up-regulated downstream of angiotensin II treatment in vascular smooth muscle cells (SMCs). SMCs become highly migratory after vascular injury, and participate in the vessel remodeling that occurs throughout chronic hypertension. Palladin was detected in SMCs of the tunica media and neointima of injured rat aorta, and experimental overexpression of palladin in SMCs was shown to increase their migration rate in vitro (Jin et al., 2007).

The role of palladin in invasive cancers

Multiple recent studies suggest that palladin may also play a role in the invasive cell motility that characterizes metastatic cancer cells. For example, the Kern lab used serial analysis of gene expression to identify a cluster of invasion-specific genes in pancreatic and colorectal cancers, and the palladin gene was found within this cluster (Ryu et al., 2001). More recently, the Condeelis lab applied an innovative approach to address the same question in human breast cancer. They coated a microneedle with growth factors and used it to capture invasive breast cancer cells that were migrating out from a human tumor implanted in a mouse host. Microarray analysis was then utilized to identify specific genes that were upregulated in this captured population of aggressively motile cells, as compared to the non-motile cells in the primary tumor, to provide information on the "gene expression signature of invasion". In this study, the human palladin gene was highly upregulated (~3-fold) in the invasive cells (Wang et al., 2004). These correlative studies point to the need for additional mechanistic approaches to test directly the hypothesis that palladin overexpression contributes to metastasis.

Perhaps the strongest evidence for a role of palladin in invasive cancer comes from a recent study of the genes involved in pancreatic cancer. Pancreatic adenocarcinoma is uniformly lethal, usually in less than a year after diagnosis, and highly invasive. It is believed that approximately 10% of all cases of pancreatic cancer result from an inherited susceptibility, but very few candidate genes have been identified (Banke et al., 2000; Rulyak and Brentnall, 2001). In the search for novel pancreatic cancer genes, much attention has been focused on a large North American family, called "Family X", who has an exceptionally high incidence of this disease: in four generations, nine members of Family X have been diagnosed with pancreatic cancer, and nine more with pre-cancerous lesions of the pancreas. In 2002, the Brentnall lab at the University of Washington mapped the susceptibility locus in Family X to a region of chromosome 4q (Eberle et al., 2002), and subsequently, an exonic point mutation was identified in the palladin gene in Family X (Pogue-Geile et al., 2006). Although this discovery is exciting, it is not yet possible to state with certainty that the palladin mutation causes pancreatic cancer in Family X, as not all of the genes within the susceptibility locus have been fully sequenced; however, the evidence in support of this idea is compelling. First, the palladin mutation occurs in all of the affected family members, but none of the unaffected members in Family X. In addition, it is important to note that genes that are mutated in inherited forms of cancer are frequently mis-regulated through other mechanisms in sporadic forms of the disease. In the case of pancreatic cancer, palladin is overexpressed in sporadic pancreatic tumors, as shown by both microarray analysis and immunohistochemical staining of tumor sections (Pogue-Geile et al., 2006; Salaria et al., 2007). Finally, it is interesting to note that two of the palladin-binding proteins, ezrin and Eps8, are also upregulated in pancreatic tumors and metastatic tumor-derived cell lines (Akisawa et al., 1999; Welsch et al., 2007), which supports the idea that multiple members of an actin-organizing molecular pathway may be coordinately upregulated in highly invasive cancer cells.

An integrated model: palladin as a TGF-β1-induced actin organizer

When considering the potential role of palladin in embryonic development, wound healing and cancer metastasis, a unifying theme emerges, as illustrated in Figure 3. In each of these processes, two types of signals are involved: chemical signals such as cytokines and growth factors, and mechanical signals such as changes in matrix stiffness and cytoskeletal tension. For example, recent reviews have highlighted the fact that TGF-β1 stimulation acts in concert with increased matrix rigidity to trigger the differentiation of the myofibroblasts associated with wound healing (Hinz, 2007). Myofibroblast differentiation includes an associated increase in stress fiber formation and cellular contractility, suggesting that upregulation of actin-bundling proteins is a key part of the process. Interestingly, very similar stimuli appear to

activate the carcinoma-associated fibroblasts (sometimes referred to as "tumor-associated myofibroblasts") that contribute to the tumor microenvironment and promote the invasive motility of adenocarcinoma cells (reviewed in (Kalluri and Zeisberg, 2006)). Mechanical forces are also involved in regulating stem cell differentiation: human mesenchymal stem cells will differentiate along a neuronal lineage when cultured on soft matrices, or along an osteoid linage when grown on more rigid substrates (Engler et al., 2006). The stiffness of the matrix, in turn, determines the organizational state of the actin cytoskeleton, which also plays a role in establishing the lineage of stem cells (McBeath et al., 2004). There is also a connection between TGF-β1 and podosome formation, as rosettes of podosomes form in aortic endothelial cells in response to TGF-β1 treatment (Varon et al., 2006). In this context, it is interesting to note that palladin protein levels increase significantly in human dermal fibroblasts in response to TGF-β1 treatment (Ronty et al., 2006), and palladin is upregulated in human adipose-derived stem cells in response to both chemical signals and mechanical stimulation (Wall et al., 2007). Thus, in the future, it will be important to determine if changes in palladin expression, stimulated by growth factors or mechanical signals or both, represent an essential step in determining the behavior, morphology and actin organization of embryonic cells, myofibroblasts, osteoclasts, endothelial cells, tumor cells, and other cell types that possess the ability to actively remodel the extracellular matrix.

Acknowledgements

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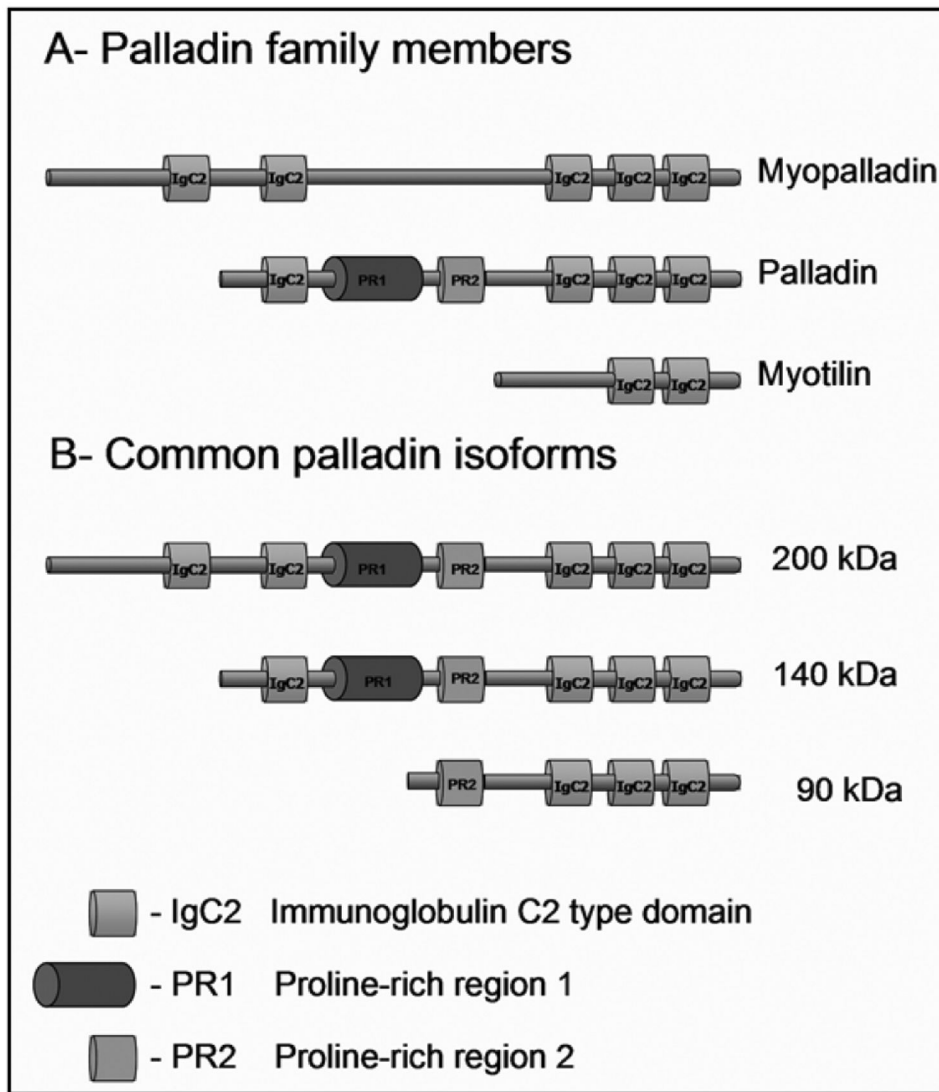
Fig. 1.
Schematic representation of major palladin isoforms, palladin family members, and their domain organization.
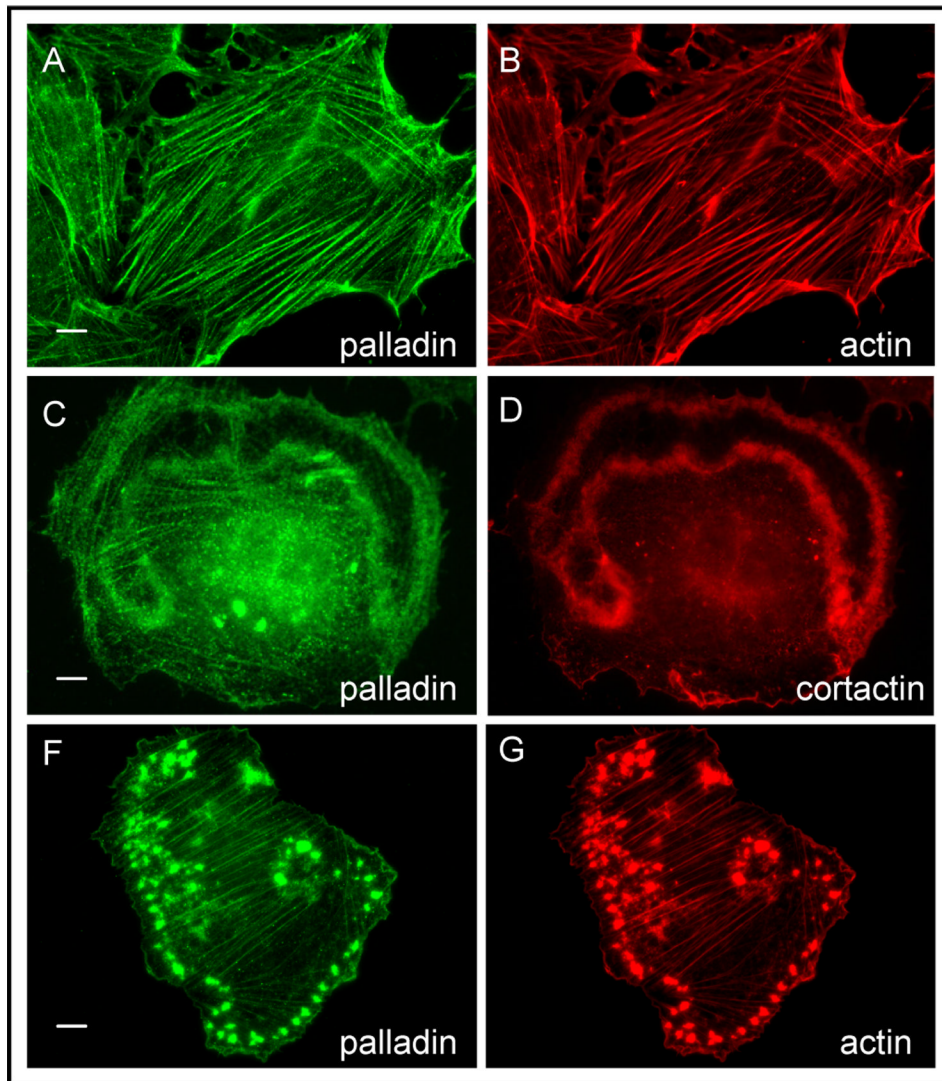
Fig. 2.
Subcellular localization of palladin in A7r5 vascular smooth muscle cells. Cells grown under
normal conditions (control) (A, B), treated with PDGF (C, D) or with phorbol ester PDBu (F,
G) were stained with anti-palladin antibody and phalloidin. Control cells show pronounced
localization of palladin to stress fibers. In PDGF-treated cells, palladin and cortactin co-localize
in dorsal ruffles. In cells treated with PDBu, palladin and actin co-localize in podosomes. For
experimental details, see (Goicoechea et al., 2006).
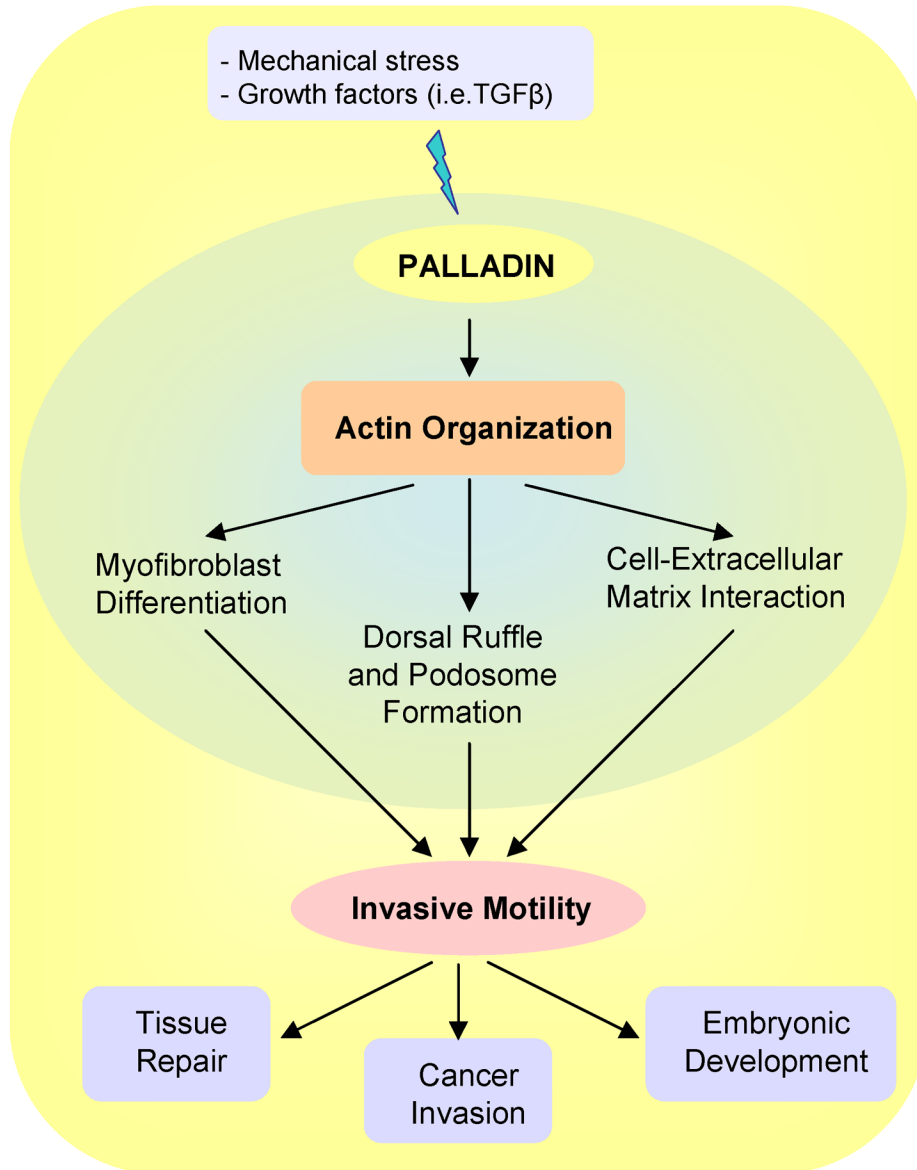
Fig. 3.
A working model for the role of palladin in multiple processes required for cell migration across tissue boundaries.