

Palladin binds to Eps8 and enhances the formation of dorsal ruffles and podosomes in vascular smooth muscle cells

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Summary

Palladin is a widely expressed phosphoprotein that plays an important role in organizing the actin cytoskeleton. Palladin is concentrated in multiple actin-based structures involved in cell motility and adhesion, including stress fibers, focal adhesions, cell-cell junctions, growth cones and Z-discs. Here, we show that palladin also localizes to the dorsal, circular ruffles that form transiently in response to growth factor stimulation. More importantly, palladin knockdown results in decreased ruffle formation and decreased Rac activation following PDGF treatment. In addition, we describe a novel interaction between palladin and Eps8, a receptor tyrosine kinase (RTK) substrate that participates in the activation of the Rac-specific guanine nucleotide-exchange function of Sos-1. Eps8 was identified as a molecular partner for palladin in a yeast two-hybrid screen, and the interaction was confirmed biochemically in

co-immunoprecipitation assays. The two proteins were found to colocalize extensively in dorsal ruffles. Palladin also localizes to podosomes after phorbol ester stimulation, and palladin knockdown results in decreased podosome formation in response to PDBu. Together, these data provide strong evidence for a direct and specific interaction between palladin and Eps8, and suggest that they act together in the rapid and transient remodeling of the actin cytoskeleton, which promotes the formation of highly dynamic membrane protrusions in response to PDGF and phorbol ester treatment.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/119/16/3316/DC1>

Key words: PDGF, Phorbol ester, Stress fibers, Rac

Introduction

The actin cytoskeleton participates in many fundamental processes including the regulation of cell shape, motility and adhesion. Cell motility is dependent on the dynamic remodeling of the actin cytoskeleton, a process that is reliant on actin-binding proteins that organize actin filaments into functionally specialized arrays. These arrays support the well-studied surface specializations including lamellipodia, filopodia and the phagocytic cup. In addition to these structures, many motile cells frequently form membrane surface structures such as dorsal ruffles and podosomes. Essential for the motility and invasion of both normal, highly differentiated cells and neoplastic cells, these structures also use an actin-based machinery to distort the plasma membrane. Dorsal ruffles and podosomes share common architectural features and functions but, depending on the cell types, vary in their molecular components and regulation (Buccione et al., 2004; Linder and Aepfelbacher, 2003).

It is now well established that activation of receptor tyrosine kinases (RTKs) by growth factors often results in the formation of peripheral membrane ruffles or circular dorsal ruffles (Buccione et al., 2004). Circular dorsal ruffles (also called waves or ring ruffles) are highly dynamic, and form transiently

on the dorsal plasma membrane. Although the precise function of dorsal ruffles is a matter of debate, these structures are believed to be important in 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). Active RTKs induce the formation of dorsal ruffles through the activation of the small GTPases Ras and Rac; however, the detailed molecular events leading to the formation of circular ruffles are not clear (Eriksson et al., 1992; Hall, 1998). Podosomes are also highly dynamic actin-based structures first described for Rous sarcoma virus-transformed fibroblasts (Gavazzi et al., 1989). Podosomes are adhesive structures that form transiently in the ventral surface of the membrane in response to Src and phorbol ester stimulation (Fultz et al., 2000; Gimona et al., 2003; Hai et al., 2002; Moreau et al., 2003; Osiak et al., 2005). A core of actin filaments and actin-associated proteins is surrounded by a ring of vinculin, talin and paxillin (Gavazzi et al., 1989), together with proteins associated with the actin polymerization machinery such as gelsolin, cortactin, dynamin, WASP/NWASP and Arp2/3 (Buccione et al., 2004; Linder and Aepfelbacher, 2003). Podosomes also contain metalloproteases (Sato et al., 1997),

supporting the concept that podosomes may serve to spatially restrict sites of matrix degradation.

Eps8 is a signaling molecule that was originally identified as a substrate for the epidermal growth factor receptor (EGFR) (Fazioli et al., 1993; Provenzano et al., 1998). Eps8 belongs to a family of proteins that link growth factor stimulation to actin dynamics, participating in the transduction of signals from Ras to Rac (Offenhauser et al., 2004). It has been reported that Eps8 binds directly to several proteins, including F-actin, EGFR, IRSp53, RN-tre and Dvl1 (Castagnino et al., 1995; Funato et al., 2004; Inobe et al., 1999; Matoskova et al., 1996). Eps8 participates in the formation of a trimeric complex that also includes the scaffold protein Abi-1 and the guanine nucleotide exchange factor (GEF) Sos-1. This macromolecular complex is one of the signaling pathways that activates the small GTPase Rac, which regulates actin assembly and promotes lamellipodia and dorsal ruffle formation (Innocenti et al., 2003; Scita et al., 1999; Scita et al., 2001). Eps8 participation in this complex is essential, as demonstrated by the lack of Sos-1-dependent Rac-GEF activity, Rac activation and remodeling of actin cytoskeleton that occurs in Eps8-null fibroblasts (Scita et al., 1999).

Palladin, a recently discovered phosphoprotein, appears to be a unique molecular scaffold that interacts with a variety of proteins involved in actin polymerization and crosslinking. Palladin localizes to many actin-containing structures, including stress fibers, focal adhesions, cell-cell junctions, and embryonic Z-lines (Mykkanen et al., 2001; Parast and Otey, 2000). Analysis of the palladin sequence revealed a number of consensus motifs that function as binding sites for known actin-regulating proteins. The N-terminal half of palladin contains polyproline stretches that bind to members of the Ena/Mena/VASP family of proteins (Boukhelifa et al., 2004). Within its N-terminal half, palladin also contains a binding site for the filament crosslinking protein, α -actinin (Ronty et al., 2004). It has recently been shown that palladin also binds via its N-terminal polyproline sequences to ArgBP2 and profilin, two proteins that are involved in the regulation of cytoskeletal dynamics (Ronty et al., 2005; Boukhelifa et al., 2006). Palladin is required for normal actin organization, as demonstrated by knockdown studies in cultured cells (Parast and Otey, 2000) and in cells cultured from a palladin-knockout mouse, both of which displayed reduced actin organization (Luo et al., 2005). In the present study, we show that palladin localizes not only to the highly dynamic dorsal ruffles that form transiently in response to growth factor stimulation, but also to podosomes. Palladin expression enhances the formation of both the dorsal ruffles and podosomes and plays a role in Rac activation. In addition, we show that palladin's N-terminal polyproline domain interacts with the receptor tyrosine kinase substrate Eps8.

Results

Palladin localizes to membrane ruffles after PDGF stimulation

To date, palladin has been detected in many actin-containing structures, such as stress fibers, cell-cell junctions and focal adhesions (Parast and Otey, 2000). In recent years, much attention has been paid to another type of dynamic structure implicated in cell motility: dorsal membrane ruffles. Because dorsal ruffles are also actin-rich structures, we asked whether

palladin is a component of these structures. Growth factor stimulation of quiescent cells typically results in a transient increase in membrane ruffling that precedes motility and mitogenic effects. To determine whether palladin is recruited to these dynamic actin-based protrusions, immunostaining was used to visualize endogenous palladin in A7r5 cells after stimulation with PDGF. Interestingly, although some palladin is still detected in its characteristic punctuate pattern along actin stress fibers in PDGF-stimulated cells, palladin is also recruited to circular membrane protrusions or ruffles along with filamentous actin (Fig. 1). It is worthwhile to note that palladin localizes to dorsal ruffles with a variety of shapes, ranging from small circular to larger elongated ruffles. To analyze the dynamics of palladin recruitment to dorsal ruffles, GFP-tagged palladin was transiently transfected into A7r5 cells, which were then serum-starved for 2 hours prior to imaging. The addition of PDGF induced the extension of dynamic ruffles and revealed that GFP-palladin is rapidly recruited to these structures on a similar time scale to that reported for other proteins (Anton et al., 2003; Dharmawardhane et al., 1997; Hedberg et al., 1993) (supplementary material Movies 1 and 2).

Palladin knockdown decreases ruffle formation induced by PDGF

To determine the role of palladin in PDGF induction of ruffles, we examined the effect of downregulation of palladin expression on the cellular response to PDGF. Short hairpin RNAi (shRNAi) constructs were used to knockdown the expression of palladin. Fig. S1 shows that palladin expression was suppressed in shRNAi-transfected cells (supplementary material Fig. S1). When transfected cells were treated with PDGF, dorsal ruffle formation was found to be inhibited in the palladin-knockdown cells (Fig. 2A). Quantification of these results showed that palladin knockdown reduces the percentage of cells with ruffles from 50% to 10% (Fig. 2B). These results suggest that palladin plays an important role increasing the efficiency of dorsal ruffle formation induced by PDGF.

Palladin localizes to PDBu-induced podosomes and enhances podosome formation after phorbol ester stimulation

Treatment of the rat vascular smooth muscle A7r5 cells with the phorbol ester phorbol-12,13-dibutyrate (PDBu) induces podosome formation (Hai et al., 2002). When cultured in serum, A7r5 cells displayed a robust actin cytoskeleton, highlighted by contractile actin stress fibers. Upon stimulation with the phorbol ester PDBu, the actin cytoskeleton of A7r5 cells undergoes the dissolution of stress fiber and focal adhesions, with the concomitant formation of dynamic podosomes (Hai et al., 2002). To determine whether palladin is recruited to these actin-based structures, immunostaining was used to visualize endogenous palladin in A7r5 cells after stimulation with PDBu. Fig. 3 shows that palladin was clearly enriched in the podosomes and co-localized with actin in cells doubly stained with anti-palladin antibodies and phalloidin.

To determine the role of palladin in PDBu induction of podosomes, we examined the effect of palladin-knockdown on the cellular response to PDBu. Short hairpin RNAi (shRNAi) constructs were used to knockdown the expression of palladin. Fig. 4A shows that when palladin shRNAi transfected cells

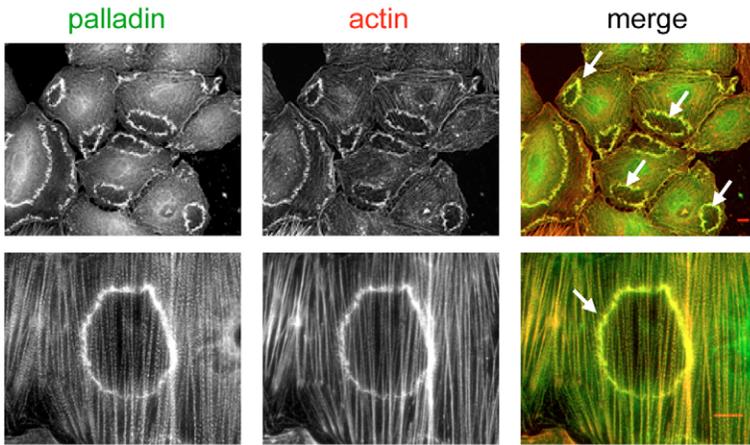


Fig. 1. Palladin localizes to PDGF-induced membrane ruffles. A7r5 cells were plated on fibronectin overnight and serum-starved for 2 hours prior to growth factor treatment. The addition of PDGF led to the induction of dynamic, actin-based membrane protrusions (indicated by arrows). After fixation, endogenous palladin was detected in these structures by immunofluorescence. Co-labeling with TRITC-phalloidin and polyclonal anti-palladin antibody reveals that palladin co-localizes with filamentous actin in ruffles and along stress fibers. Top: low magnification image. Bottom: high magnification image to show detail. Bar, 10 μ m.

were treated with PDBu, a significant percentage of the siRNA-transfected cells were unable to form podosomes. The number of cells that formed podosomes after phorbol ester stimulation was determined, showing that palladin knockdown reduces the percentage of cells that form podosomes from 42% to 20% (Fig. 4B). These results suggest that, similarly to what we observed for dorsal ruffles, palladin plays an important role increasing the efficiency of podosome formation induced by PDBu.

Yeast two-hybrid screen to identify potential palladin interacting proteins

In an effort to understand palladin participation in membrane ruffles and podosomes, we set out to identify novel molecular partners for palladin that might contribute to ruffle and podosome formation. Previous studies have found that members of the VASP, ezrin, ArgBP2, profilin and α -actinin

protein families all bind directly to palladin (Boukhelifa et al., 2004; Boukhelifa et al., 2006; Mykkanen et al., 2001; Ronty et al., 2004; Ronty et al., 2005); however, these binding sites account for only a small fraction of palladin's total sequence. We reasoned that palladin was likely to have additional binding partners, and a yeast two-hybrid approach was employed to identify them. Full length-palladin cDNA was used as a bait to screen a mouse embryonic fibroblast cDNA library (Fig. 5A). Colonies that grew on nutritionally restrictive medium (i.e. medium lacking histidine, tryptophan, adenine and leucine) and expressed α -galactosidase as detected by the addition of X- α -gal were isolated. Redundant clones were identified by PCR and unique clones were purified, sequenced, and compared to sequences in GenBank with BLAST. Several previously identified palladin-binding proteins, such as VASP and α -actinin, were identified from this screen. One of the novel clones identified in this screen corresponded to Eps8 (Fig. 5B). The isolated Eps8 cDNA clone was truncated at the 5' end and encoded amino acids 67-821. Purified pGADT7-Eps8 was reintroduced into yeast along with pGBKT7-palladin, pGBKT7-lamin C or pGBKT7 vector and re-tested for two-hybrid interaction. These data confirmed the results obtained in the two-hybrid screen and showed that Eps8 interacts specifically with palladin and not with control constructs or empty vector.

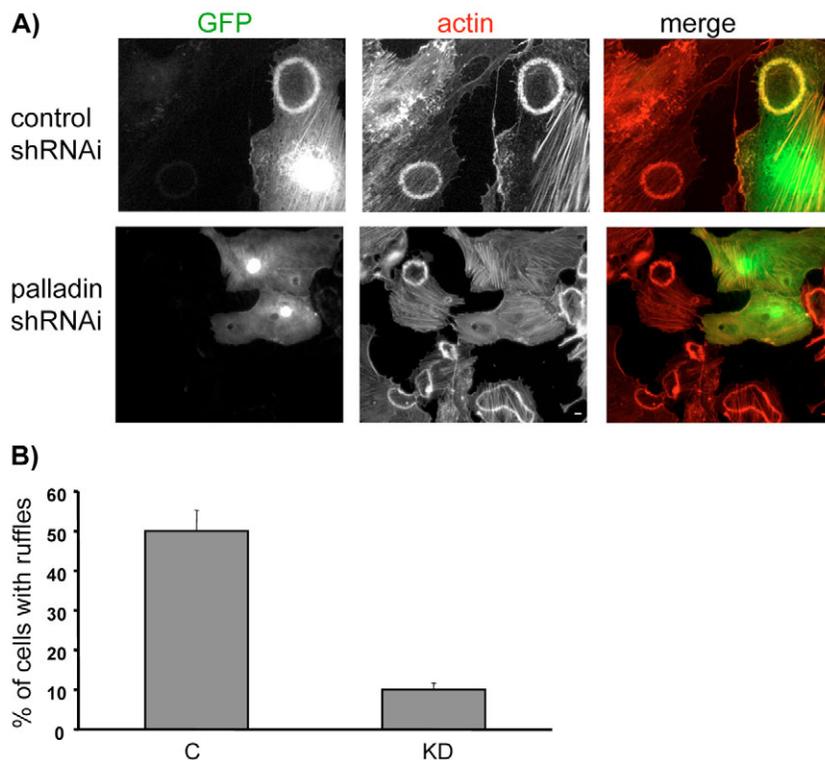


Fig. 2. Palladin knockdown decreases PDGF-induced ruffle formation. (A) A7r5 cells transfected with control pSuper RNAi and pSuper RNAi targeting palladin were plated on fibronectin overnight and serum-starved for 2 hours prior to PDGF treatment. Cells were fixed, permeabilized and stained with TRITC-phalloidin. Transfected cells (green fluorescence) were detected by the presence of GFP encoded in the pSuper vector. (B) The proportion of cells developing ruffles after PDGF stimulation is shown for cells transfected with palladin siRNA (knockdown, KD, 10 \pm 2%) and transfected with control siRNA (C, 50 \pm 5%). Results are representative of three independent experiments in which at least 100 transfected cells were counted. Bar, 10 μ m.

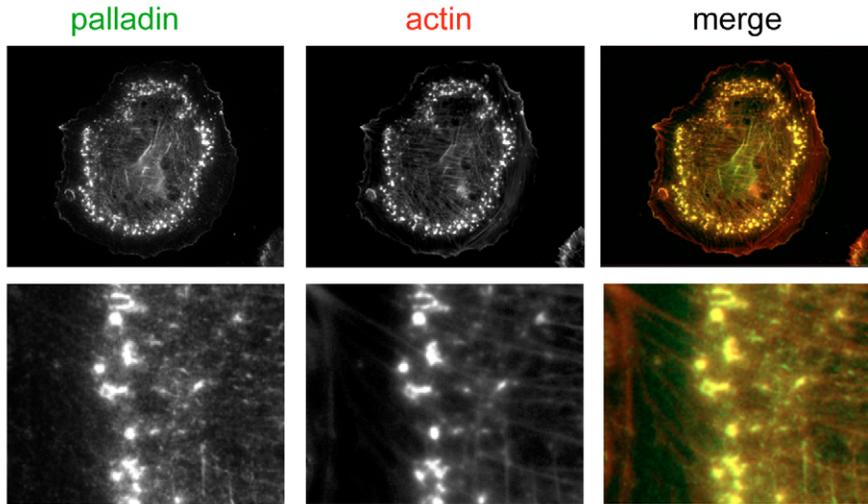


Fig. 3. Palladin localizes to PDBu-induced podosomes. A7r5 cells were plated on fibronectin and treated with the phorbol ester PDBu. After fixation, endogenous palladin was detected by immunofluorescence. Co-labeling with TRITC-phalloidin and polyclonal anti-palladin antibody reveals that palladin co-localizes with actin. Top: low magnification image. Bottom: high magnification image to show detail.

Palladin interacts with Eps8 in vivo

Next, a co-immunoprecipitation assay was carried out to determine whether a physical complex of endogenous palladin and Eps8 is detectable in cell lysates. Palladin was immunoprecipitated from Swiss 3T3 cells with monoclonal antibodies, and the immunoprecipitates were probed with monoclonal anti-Eps8 antibodies by immunoblotting. A sample of whole cell lysate was run as a positive control, and a sample that contained no primary antibody (beads alone) was run as a negative control. Fig. 5 shows that Eps8 was detected in anti-palladin immunoprecipitates, but not in control immunoprecipitates (Fig. 5C, left panel). These experiments

were also performed in reverse, and palladin was detected in the anti-Eps8 immunoprecipitates but not in control immunoprecipitates (Fig. 5C, right panel).

Since Eps8 is required for the formation of dorsal ruffles that occurs after growth factor stimulation (Scita et al., 1999), we wanted to determine whether its interaction with palladin was affected by growth factor treatment. As shown in Fig. 5D, there is a shift in the molecular weight of palladin in cells stimulated with PDGF for 10 or 30 minutes, which suggests a post-translational modification of palladin in response to growth factor stimulation. However, this had no effect on the interaction between palladin and Eps8, as judged by the amount of palladin that can be co-immunoprecipitated with Eps8 (Fig. 5E, left panel). Control experiments showed that Eps8 antibodies failed to co-immunoprecipitate palladin in Eps8 null cells (Fig. 5E, right panel). Fig. S2 shows that actin and tubulin levels remained the same in $MEF^{-/-}$ and $MEF^{-/-}$ + Eps8 (supplementary material Fig. S2). Taken together, these experiments validate the yeast two-hybrid results and demonstrate that palladin associates with Eps8 in vivo.

It has been reported that Eps8 forms a trimeric complex together with Sos-1 and Abi-1, in which Abi-1 holds together Eps8 and Sos-1 (Scita et al., 1999). To investigate whether palladin participates

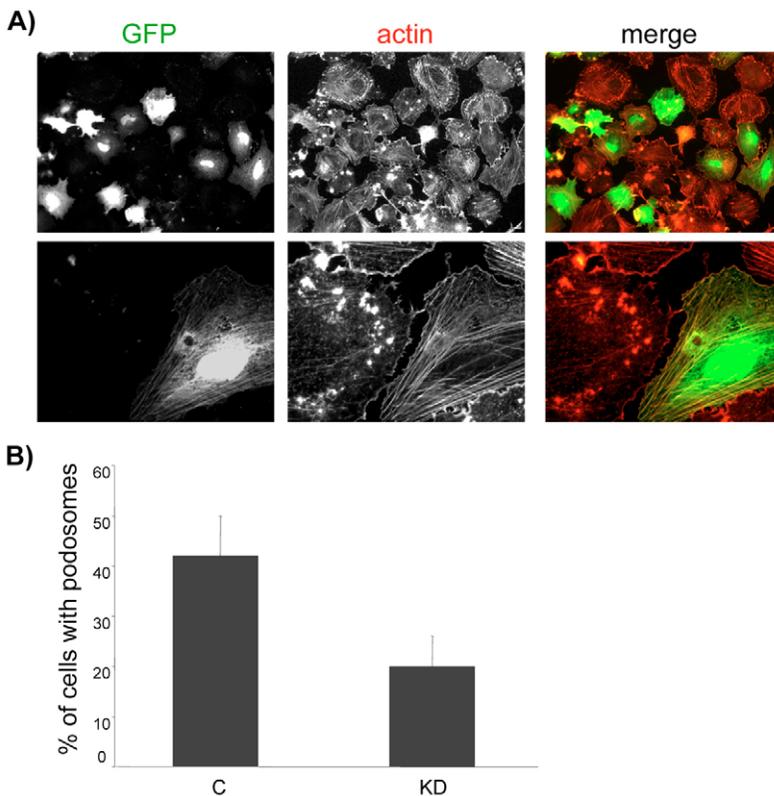


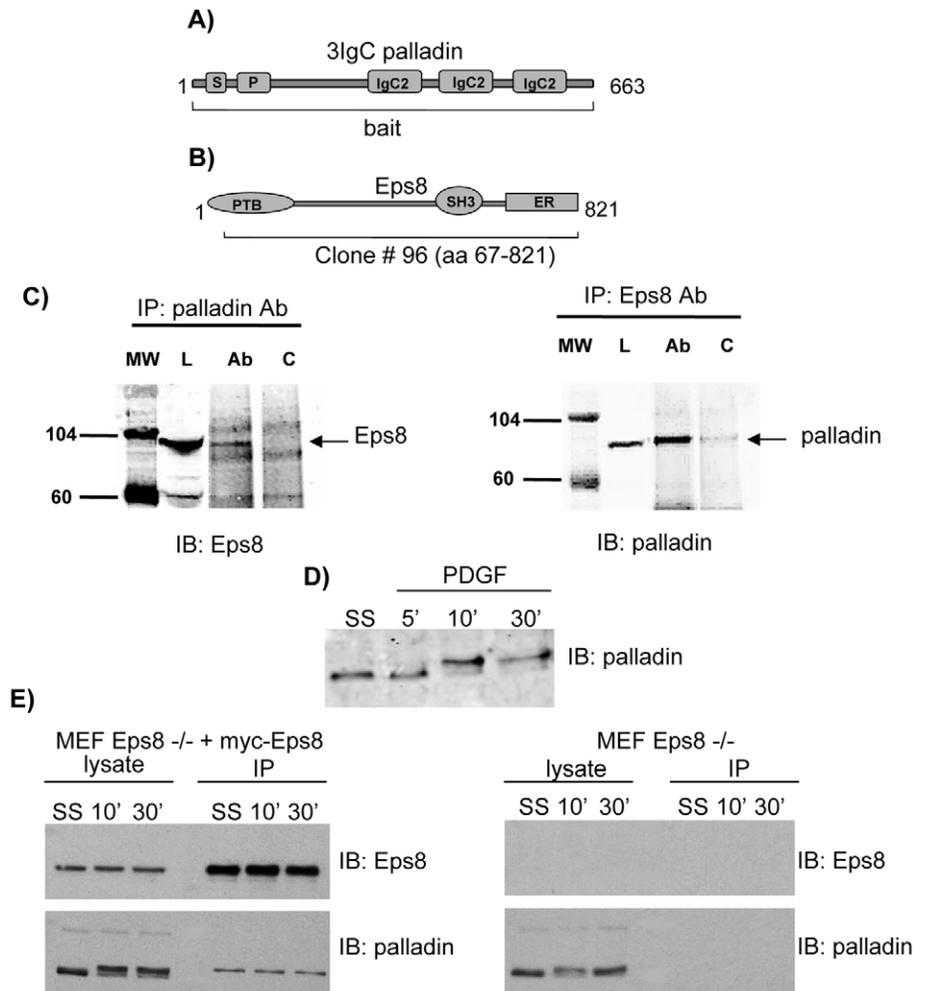
Fig. 4. Palladin knockdown decreases PDBu-induced podosome formation. (A) A7r5 cells transfected with control pSuper RNAi and pSuper RNAi targeting palladin were plated on fibronectin overnight and treated with PDBu. Cells were fixed, permeabilized and stained with TRITC-phalloidin. Transfected cells (green fluorescence) were detected by the presence of GFP encoded in pSuper. Top: low magnification image. Bottom: high magnification image to show detail. (B) The proportion of cells developing podosomes after PDBu stimulation is shown for cells transfected with palladin siRNA (knockdown, KD, 20±6%) and transfected with control siRNA (C, 42±8%). Results are representative of three independent experiments in which at least 100 transfected cells were counted.

Fig. 5. Palladin specifically interacts with Eps8. (A) Schematic representation of the palladin protein used as bait. The amino acid positions are indicated by the flanking numbers. The structural domains are shown as boxes: the N-terminal half of the molecule contains a serine-rich (S) and a proline-rich (P) domain, and the C-terminal half of the molecule contains three tandem repeats of a domain called 'IgC2'.

(B) Schematic representation of full-length Eps8 and the truncated form found in the yeast two-hybrid screen. The amino acid positions are indicated as in A. The structural domains are shown as boxes: a phosphotyrosine-binding domain (PTB), an SH3 domain and an effector region (ER).

(C) Palladin and Eps8 form complexes in cultures cells. Left panel: palladin was immunoprecipitated with 1E6 monoclonal antibody from lysed Swiss 3T3 fibroblasts, and the samples were probed with anti-Eps8 monoclonal antibodies to determine whether Eps8 co-precipitates with palladin. Whole cell lysate was run as positive control, and a sample that contained no primary antibody (beads alone) was run as negative control. Right panel: Eps8 was immunoprecipitated with anti-Eps8

antibody and blotted for palladin, also with whole cell lysate and beads alone as positive and negative controls, respectively. Note that Eps8 was detected in the palladin immunoprecipitate and palladin was detected in the Eps8 immunoprecipitate. L, whole cell lysate; C, beads alone; Ab, antibody used for IP. (D) Western blot analysis from cytosolic extracts of A7r5 cells treated with PDGF. Cells were untreated (SS, serum starvation) or treated with PDGF for 5, 10 and 30 minutes (5', 10' and 30'). Blots were probed with anti-palladin 1E6 monoclonal antibody. (E) Co-immunoprecipitation of palladin and Eps8 from cytosolic extracts of Eps8 null mouse embryonic fibroblasts (MEF Eps8^{-/-}) and rescued MEF (MEF Eps8^{-/-} + myc-Eps8). Cells were untreated (SS) or treated with PDGF for 10 and 30 minutes (10' and 30'). Eps8 antibody was used for immunoprecipitation. Blots were probed with anti-Eps8 Abs or anti-palladin 1E6 monoclonal antibody.



in the formation of this complex, we performed pulldown assays from palladin overexpressing and palladin-knockdown cells, using GST-E2 (aa 331-480), which is the region of Abi-1 that binds to both Eps8 and Sos-1. HeLa cells were transfected with empty vector or myc-palladin, or treated with control siRNA or palladin siRNA. Cell lysates were subjected to pull down assays using GST-E2 or GST as a control. As shown in Fig. S3 neither the overexpression of palladin nor its knockdown interfere with the ability of Abi-1 to bind to Eps8 or to Sos-1 (supplementary material Fig. S3).

Mapping of binding sites in palladin and in Eps8

To map the binding sites on palladin and Eps8 in detail, a series of deletion mutants of both interacting partners was generated and their interaction was analyzed using the yeast two-hybrid system (Fig. 6). Structurally, Eps8 contains (from N- to C-terminus): a phosphotyrosine binding (PTB) domain, an SH3 domain and an effector region (Di Fiore and Scita, 2002). Fig. 6 shows the schematic representation of the palladin and Eps8 constructs used in these experiments and a summary of the

two-hybrid results. When different fragments of Eps8 were used as prey and full length palladin as bait, a positive two-hybrid interaction was observed only in the constructs containing amino acids 350-532 of Eps8 (Fig. 6A). These results demonstrate that the fragment constituted by amino acids 350-532 of Eps8 mediates the interaction with full-length palladin.

To map the binding site of palladin responsible for the interaction with Eps8, a series of palladin constructs truncated at their N- or C-terminus were tested in yeast. As shown in Fig. 6B, the N-terminal fragment of palladin (aa 1-198) was able to interact with full-length Eps8. No interaction was detected when the C-terminal fragment of palladin (aa 287-663) was used as bait (Fig. 6B). Fig. 6B also shows that the proline-rich domain (aa 54-114) is the region of palladin's N-terminal half that is required to interact with full-length Eps8.

Palladin co-localizes with Eps8

In previous reports, palladin has been localized to regularly-spaced puncta along the stress fibers of well-spread fibroblasts

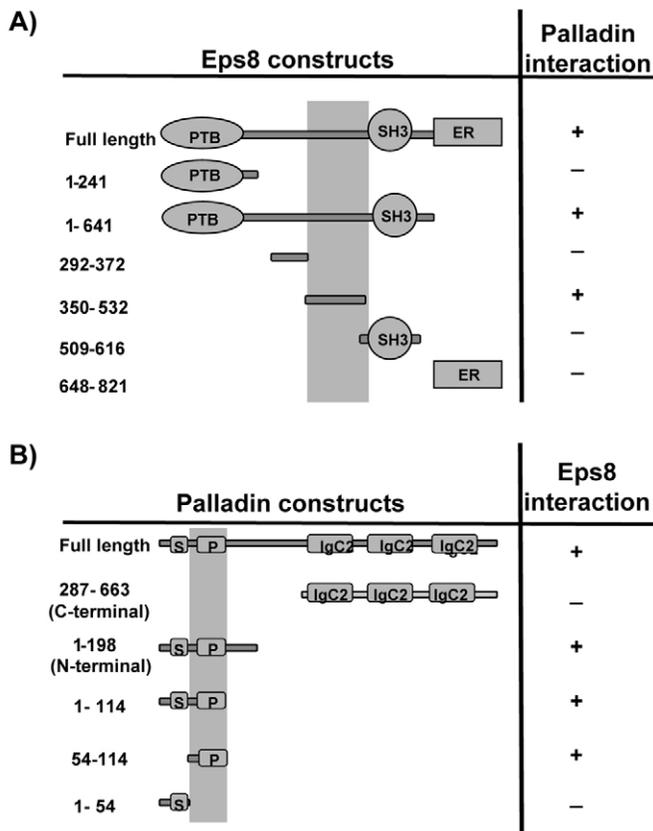


Fig. 6. Yeast two-hybrid analysis of palladin-Eps8 interaction. (A) Mapping of the palladin-binding site in Eps8. Yeast cells were co-transformed with various Eps8 fragments in prey vector and full-length palladin in bait vector. (B) Mapping of the Eps8 binding site in palladin. Palladin C-terminal, N-terminal and overlapping N-terminal constructs were tested for interaction with Eps8. Growth was estimated after 5 to 7 days of incubation at 30°C. The formation of colonies secreting X-alpha-gal was taken as an indication of an interaction between the expressed proteins and is noted by '+'. No interaction between expressed proteins is noted with '-'. Boxes shown are the regions mapped to be essential for the interaction in each analysis. Controls were systematically performed for each construct with the opposite vectors without insert. Each experiment was repeated at least twice.

and in cell-cell junctions (Parast and Otey, 2000). The observations described above demonstrate that antibodies to palladin also label dorsal ruffles in cultured A7r5 cells (Fig. 1). In addition to its localization in phagocytic cups, comet tails and cell-cell contacts, Eps8 has been detected in circular, dorsal ruffles (Disanza et al., 2004; Provenzano et al., 1998), and so we next investigated the degree of co-localization of palladin and Eps8 in ruffles. A7r5 vascular smooth muscle cells were serum-starved for 2 hours and then treated with PDGF for 5 minutes. Immunofluorescence staining shows a high degree of overlap of palladin and Eps8 staining in PDGF-induced dorsal ruffles (Fig. 7A). These results demonstrate that both palladin and Eps8 are recruited to circular ruffles in response to PDGF stimulation.

Eps8 has also been detected in podosomes (Provenzano et al., 1998), and so we also explored whether palladin and Eps8

co-localized to these actin-based structures. A7r5 vascular smooth muscle cells were treated with 1 μ M PDBu for 30 minutes. Immunofluorescence staining shows a high degree of overlap of palladin and Eps8 staining in PDBu-induced podosomes (Fig. 7B).

Palladin knockdown decreases Rac activation

Eps8 has been reported to play a critical role in the activation of Rac (Scita et al., 1999). To determine whether palladin participates in this pathway, we measured the levels of active GTP-loaded Rac in control cells and in cells in which palladin levels have been knocked down using palladin-specific siRNA oligos. The levels of active Rac were measured using a previously developed GST pull-down assay that takes advantage of the ability of Rac effectors to bind selectively to the active GTP-bound form of the GTPase (Ren et al., 1999). The Cdc42/Rac-interactive binding region (CRIB) of α -Pak, which binds specifically to activated GTP-bound Rac and Cdc42, was used to precipitate active Rac. These results show that reducing the expression levels of palladin in cells using siRNA causes a significant decrease in the amount of active Rac when compared with control cells (Fig. 8). Taken together, these results suggest that palladin, through its interaction with Eps8, plays a role in the regulation of Rac activity *in vivo*.

Discussion

Previously, it was shown that palladin is required for the maintenance of normal stress fibers and focal adhesions in cultured fibroblasts and trophoblasts (Parast and Otey, 2000). More recently, palladin was shown to play a critical role in embryonic development, as the palladin-knockout mouse had an embryonic lethal phenotype and exhibited defects in body wall closure (Luo et al., 2005). Fibroblasts cultured from the palladin null embryos showed impaired stress fiber formation, reduced adhesion to fibronectin, and reduced cell migration (Luo et al., 2005). These results are consistent with the hypothesis that palladin functions as a key regulator of actin organization in a wide variety of cell types. In the current study, we identified one new molecular partner for palladin and two additional actin-based structures that contain palladin: podosomes formed on the ventral surface of the cell after exposure to phorbol esters and the highly dynamic dorsal membrane ruffles that form in response to PDGF.

Membrane ruffling is significantly decreased in palladin-knockdown cells, which suggests an important role for palladin in the life cycle of these transient structures. These observations led us to hypothesize that palladin may be directly or indirectly involved in any of the following stages of ruffle formation: (1) intracellular signaling pathways after growth factor stimulation; (2) actin filament nucleation or stability at sites of protrusion; or (3) structural organization of actin filaments leading to the formation of higher-order actin arrays that support membrane protrusion.

In support of hypotheses 2 and 3, palladin has been shown to bind to a host of actin-associated proteins that may alter nucleation rates, stability and bundling. Notably, binding interactions have been described between palladin and α -actinin, ezrin, ENA/VASP proteins and ArgBP2, each of which plays a role in actin organization (Boukhelifa et al., 2004; Mykkanen et al., 2001; Ronty et al., 2005; Ronty et al., 2004). In this report, we provide the first evidence that palladin may

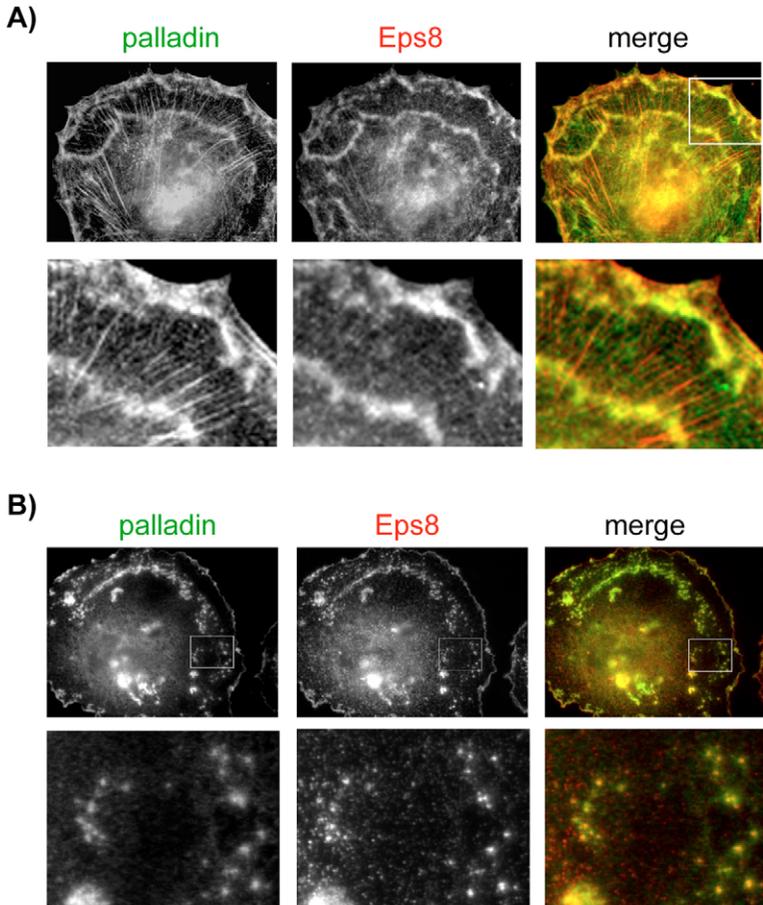


Fig. 7. Palladin co-localizes with Eps8. A7r5 cells were plated on fibronectin-coated coverslips and were either incubated for 2 hours in serum-free media prior to treatment with PDGF (A) or treated with the phorbol ester PDBu (B). Cells were fixed and immunolabeled with polyclonal anti-palladin antibody and monoclonal anti-Eps8 antibody. The two images were merged (overlay) to show the relative localization of palladin (green) and Eps8 (red). Top: low magnification image. Bottom: high magnification image to show detail.

also play a role in the signaling pathways leading to ruffling through its interaction with a novel binding partner, Eps8. It is interesting to note that Eps8, like all the other binding partners for palladin identified to date, is a protein that regulates the actin cytoskeleton; thus, these results place palladin within a known biochemical pathway that links growth factor stimulation to dynamic actin changes that are involved in cell motility and morphological plasticity. Moreover, these and previous results support the hypothesis that palladin might function 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 involved in adhesion and migration.

The mechanism by which palladin alters Rac activity and ruffling will require further study, but interaction with Eps8 may prove to be an essential link. Eps8 integrates different signaling pathways by participating in: (1) actin remodeling through Rac, forming a complex with Abi-1 and Sos-1; (2) receptor endocytosis modulating Rab5 activity, forming a complex with RN-tre; and (3) actin-based motility processes by capping the barbed ends of actin filaments (Disanza et al., 2004; Innocenti et al., 2003; Lanzetti et al., 2000). Our results show that neither the overexpression of palladin nor the knockdown interfere with the ability of Abi-1 to bind to Eps8 or Sos-1, which suggests that palladin binding to Eps8 does not trigger a ligand-dependent association with Abi-1 and Sos-1. It remains to be determined whether palladin forms part of the

Eps8/Abi-1/Sos-1 complex; however, our results suggest that palladin might be stabilizing the Eps8/Abi-1/Sos-1 complex, thus promoting Rac activation and actin reorganization. Alternatively, palladin could be involved in regulating the actin capping activity of Eps8. It has been recently reported that full-length Eps8 is auto-inhibited and does not cap barbed ends, while the binding of Abi-1 alters the conformation of Eps8 and releases its barbed-end actin capping activity (Disanza et al., 2004). The binding of palladin to Eps8 could be involved in a similar mechanism, modulating Eps8 barbed-end capping activity. These possibilities will be explored in future studies.

Our results suggest that palladin and Eps8 participate together in a pathway that leads to the formation of dorsal ruffles. Thus, our future efforts will focus on exploring how the interactions of these two proteins are regulated by cellular signaling pathways. In the present study we investigated whether palladin/Eps8 interaction is regulated by growth factors. We performed coimmunoprecipitation analysis with lysates from growth factor-stimulated or nonstimulated cells (Fig. 5E); however, we did not see any significant difference in the amount of immunoprecipitated proteins. If the palladin/Eps8 interaction is being regulated dynamically in vivo, it is possible that an intact cytoskeleton may be necessary to maintain the interaction. Additional experiments will be needed to address this possibility. Eps8 is tyrosine-phosphorylated by a variety of tyrosine kinases, of both the receptor (RTKs) and non-receptor type (Fazioli et al., 1993).

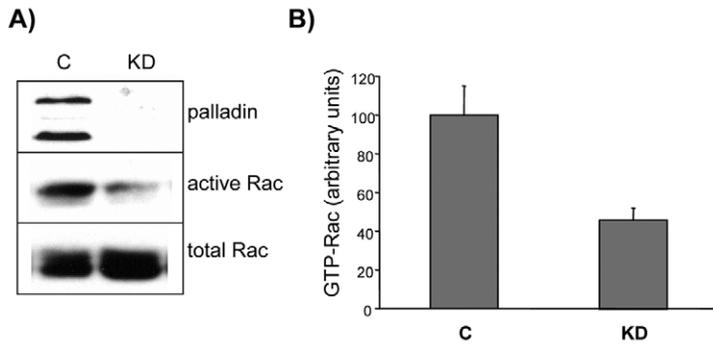


Fig. 8. Rac activity is decreased in palladin knockdown cells. (A) Western blot analysis of whole cell lysates of HeLa cells transfected with control siRNA (C) or siRNA targeting palladin (knockdown, KD). Active Rac was specifically pulled down from cell lysates containing equal amounts of proteins with immobilized recombinant PAK-CRIB and analyzed by western blotting using anti-Rac antibody. A representative experiment is shown. (B) The level of active Rac was quantified by densitometric analysis of western blots and the amount of active Rac was normalized to the amount of total Rac. Rac activation was quantified from three independent assays.

Palladin is also a phosphoprotein and it contains clusters of serine-rich sequences next to the Eps8-binding site, which suggests that serine phosphorylation could play a role in regulating palladin's binding to Eps8. It remains to be determined whether palladin and Eps8 are phosphorylated downstream of the same kinase pathways, and whether their binding interactions are regulated by their phosphorylation state.

Our observation of palladin in membrane ruffles led us to examine another actin-based structure involved in motility, the podosome. These structures have been described in several human cancer cell lines, particularly invasive breast carcinomas and melanomas, and their presence has been correlated with invasiveness *in vitro* (Bowden et al., 1999; Kelly et al., 1998; Monsky et al., 1994). It has been reported that palladin levels are increased in cancer cell lines, including invasive breast carcinomas (Wang et al., 2004). Localization of palladin to structures resembling podosomes in immature dendritic cells was reported earlier by Carpen and collaborators (Mykkanen et al., 2001). In this report, we show not only that palladin localizes to podosomes in A7r5 cells but also that palladin expression enhances the formation of podosomes after phorbol ester stimulation. One possible role for palladin in podosome formation may be that it functions as a scaffolding molecule to recruit proteins known to be required for podosome formation. For example, palladin binds to α -actinin, which is localized to podosomes (Fultz et al., 2000; Linder and Aepfelbacher, 2003). Here, we show that palladin associates with Eps8, which has also been reported to localize to podosomes (Provenzano et al., 1998). Future studies will examine the role of palladin in the signaling pathways leading to podosome formation.

Materials and Methods

Materials

The following antibodies were used: Eps8 (BD Biosciences); Rac (Transduction Laboratories) and palladin (polyclonal antibody and monoclonal 1E6 antibody previously characterized by Parast and Otey (Parast and Otey, 2000)). Human recombinant platelet-derived growth factor BB (PDGF-BB) and protease inhibitor cocktail for mammalian tissues were from Sigma. MATCHMAKER GAL4 Two-Hybrid System 3 and MATCHMAKER mouse embryonic pACT2 cDNA library were from BD Biosciences. Secondary antibodies conjugated to either IRdye700 or IRdye 800 were from Rockland Immunochemicals. TransIT siQuest transfection reagent was from Mirus, and the Fugene6 transfection reagent was from Roche. Alexafluor-488- and Alexafluor-568 anti-mouse IgG and anti-rabbit IgG-conjugated secondary antibodies were from Molecular Probes.

Stimulation and immunofluorescence staining

Rat vascular smooth muscle cells A7r5 cells were grown in DMEM, containing 10% fetal bovine serum (FBS) and supplemented with 1% penicillin/streptomycin (all from Gibco BRL). Cells were grown on glass coverslips and fixed in 4% paraformaldehyde in PBS, then permeabilized in 0.2% Triton X-100 and incubated

with the specific primary antibodies for 1 h. Primary antibodies were detected with Alexafluor-488 and Alexafluor-568 anti-mouse IgG and anti-rabbit IgG conjugates. Coverslips were examined with a Nikon TE200-U microscope with 20 \times and 60 \times objective lenses, an optional 1.5 \times tube lens and a Hamamatsu Orca-ER camera. Images were processed using Adobe Photoshop 7.0 (Adobe Systems). Where indicated, cells were treated with PDGF (20 ng/ml) for 3 minutes. Podosome formation was induced by the addition of 1 μ M phorbol-12,13-dibutyrate (PDBu; Sigma-Aldrich), as previously described (Gimona et al., 2003; Hai et al., 2002).

siRNA experiments

To knock down the expression of the 90-92 kDa palladin isoform by RNA interference, two 21-base oligonucleotides were purchased from Dharmacon Research. The RNA sequences were as follows: sense, 5'-CUACUC-CGUGUCACAUUU-3' and antisense, 5'-UAAUGUGACAGCGGAGUA-GUU-3'. As a control we used siCONTROL Non-Targeting siRNA #1 from Dharmacon. HeLa cells were transfected using the TransIT siQuest transfection reagent following manufacturer's instructions. Cells were assayed 86 hours after transfection.

In some experiments, the pSuper RNAi system (Oligoengine) was used to knock-down expression of palladin in A7r5 cells. Generation of the RNAi vector followed manufacturer's protocols. Forward and reverse oligos containing the anti-palladin short hairpin RNAi sequence were generated, and were the following: forward, GATCCCCAAACGCTCTCAACATCCATTCAAGAGATGGATGTTGAAGAC-GTTTGTTTT; reverse, AGCTTAAAAACAACGTC TTCAACATCCATCTC-TTGAATGGATGTTGAAGACGTTTGGGG. A7r5 cells were passaged the day before the experiment. Cells were transfected using Fugene6 transfection reagent following manufacturer's instructions. Cells were assayed 72 hours after transfection.

Cell lysis and co-immunoprecipitation and immunoblot

A7r5 cells were briefly rinsed with PBS and then scraped into a lysis buffer containing 50 mM Tris (pH 7.0), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail. The supernatant was collected after centrifugation at 10,000 *g* for 15 minutes. Eps8-KO mouse embryonic fibroblasts (MEFs) or rescued Eps8-KO MEFs were scraped into a lysis buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 5 mM EGTA, 1 mM DTT and protease inhibitors. The cell lysates were analyzed by immunoblot, processed for co-immunoprecipitation or frozen with liquid nitrogen and stored at -80°C for future use. For the immunoblot, lysates were boiled in Laemmli buffer and proteins were resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and immunoblotted. For imaging, IRdye700 or 800-conjugated secondary antibodies were used with an Odyssey infrared imaging system (Licor). For immunoprecipitation, primary antibodies were added to the lysate, incubated for 1 hour at 4°C, and precipitated by addition of 50 μ l of 50% slurry Gamma-Bind Plus Sepharose beads. The beads were washed with lysis buffer and then analyzed by immunoblot as described above.

Yeast two-hybrid analysis

MATCHMAKER GAL4 Two-Hybrid System 3 was used for cDNA library screening. The full-length 90-92 kDa palladin isoform was cloned into pGBKT7 bait vector and expressed as a fusion protein to the GAL4 DNA-binding domain in the yeast strain AH109. Palladin bait was used to screen a MATCHMAKER mouse embryonic pACT2 cDNA library (>1 \times 10⁶ independent clones). Positive clones were selected on synthetic dropout plates lacking leucine, tryptophan, histidine and adenine (SD/-his/-leu/-trp/-ade). To test the interaction between two known proteins in the yeast two-hybrid assay, the two corresponding cDNAs were separately inserted into pGBKT7 and pGADT7 (BD Biosciences), respectively, and were co-transformed into the host strain AH109. Positive clones were selected as described above. Both pGBKT7 and pGADT7 without inserts were used as negative control.

Rac activity assay

HeLa cells were grown in DMEM, containing 10% fetal bovine serum and supplemented with 1% penicillin/streptomycin. For the Rac activation assay, cells were lysed in 300 µl of 50 mM Tris, pH 7.4, 10 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate and protease inhibitors. Lysates were cleared at 16,000 g for 5 minutes. Supernatants were rotated with 50 µg GST-PBD (GST fusion protein containing the Rac/Cdc42 binding domain of PAK1) prebound to glutathione-Sepharose beads (Amersham). Beads were washed, resuspended in SDS sample buffer and bound material was immunoblotted for Rac.

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