Published in final edited form as:

Biochem J. 2016 February 15; 473(4): 383–396. doi:10.1042/BJ20151050.

Actin Polymerization is Stimulated by Actin Crosslinking Protein Palladin

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Abstract

The actin scaffold protein palladin regulates both normal cell migration and invasive cell motility, processes that require the coordinated regulation of actin dynamics. However, the potential effect of palladin on actin dynamics has remained elusive. Here we show that the actin binding immunoglobulin-like domain of palladin, which is directly responsible for both actin binding and bundling, also stimulates actin polymerization *in vitro*. Palladin eliminated the lag phase that is characteristic of the slow nucleation step of actin polymerization. Furthermore, palladin dramatically reduced depolymerization, slightly enhanced the elongation rate, and did not alter the critical concentration. Microscopy and *in vitro* crosslinking assays reveal differences in actin bundle architecture when palladin is incubated with actin before or after polymerization. These results suggest a model whereby palladin stimulates a polymerization-competent form of G-actin, akin to metal ions, either through charge neutralization or conformational changes.

Keywords

actin; polymerization; nucleation; crosslinking; kinetics

Introduction

Palladin is an actin filament (F-actin) binding protein that is involved in both normal and invasive cell motility. Palladin is upregulated in cells that are actively migrating such as in developing vertebrate embryos [1], along a wound edge [2], during metastatic invasion [3] and in the development of cardiovascular diseases [4, 5] [6] [7]. Conversely cells that are depleted of palladin have defects in cell motility [8], display disorganized actin cytoskeleton architecture [9] and a significant decrease in the amount of polymerized actin [10]. In particular, the correlation between the loss of palladin and decreased levels of actin polymer

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Author Contribution: MRB conceived and coordinated the study and wrote the paper. JGB performed and analyzed the experiment shown in Figures 1 and 9. AO and EHE performed the experiments shown in Figure 10 A-B. RY designed and performed the experiment in Figure 10 C-D, provided technical assistance and edited the paper. RG designed, performed and analyzed all other experiments as well as wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

suggests that palladin may have a direct role in stabilizing F-actin and/or enhancing actin polymerization. While palladin has been causally linked to the invasive cell motility associated with metastasis, the mechanistic roles of palladin in organizing cellular actin networks and governing actin filament dynamics have remained unclear.

Palladin directly crosslinks actin filaments, and this activity is mediated by its Ig3 domain [11]. In addition, palladin binds a number of actin regulating proteins (VASP [2], profiling [12], and Eps8 [13]), actin cross-linking proteins (α -actinin [14], Lasp-1 [15], and Ezrin [16]), matrix degrading proteinase (MT1-MMP14 [3] and signaling intermediaries (Src, Abl/Arg kinase binding protein (ArgBP2), and SPIN-90 [17, 18]). The direct interaction of palladin with actin filaments is mediated by two basic patches on the Ig3 domain, which are critical for both F-actin binding and bundling [19]. Other than its recognition as an F-actin binding and bundling protein, little is known about how palladin regulates actin filament dynamics or structure. Here we show that palladin Ig3 domain (designated as Palld-Ig3 from here on) increases the rate and extent of actin polymerization *in vitro* via a mechanism that involves enhanced nucleation and diminished depolymerization. While Palld-Ig3 does not alter actin critical concentration, it does modestly enhance the rate of filament elongation. The major effect of Palld-Ig3 in stimulating actin filament formation was an increase in the rate of nucleation. The filaments nucleated by Palld-Ig3 domain also are highly crosslinked. Our results suggest dual roles for Palld-Ig3 that includes alteration of both actin polymerization kinetics and the organization of resulting filaments. These roles provide a possible mechanistic explanation for palladin's critical *in vivo* functions in generating actin filament structures required for normal cell adhesion as well as cell motility associated with cancer metastasis.

Experimental

Protein preparation and purification

The Palld-Ig3 domain was sub-cloned from the pMAL-Ig3 construct [11] into the pTBSG expression vector [20]. The Palld-Ig3 domain was overexpressed in BL21 (DE3)-RIL *E. coli* cells (Agilent Technologies) and purified using HisPur Ni-NTA resin (Thermo Scientific) followed by cation exchange chromatography (SP sepharose, GE Healthcare Life Sciences) [11]. Purified protein was stored in HEPES buffer at 4 °C (20 mM HEPES, pH7.5, 5 mM DTT, 50 mM NaCl) and used within 2-4 weeks.

Actin was purified from rabbit muscle acetone powder (Pel-Freez Biologicals) by using the method of Spudich and Watt [21] and gel-filtered on 16/60 Sephacryl™ S-200 column (GE Healthcare Life Sciences). Purified monomeric actin was stored at 4 °C in G-buffer (5 mM Tris-HCl, pH 8, 0.1 mM CaCl₂, 0.2 mM DTT, 0.2 mM ATP, 0.02% sodium azide) and used within 2-4 weeks. Pyrene-labeled actin was prepared by the reaction of N-(1-pyrenyl) iodoacetamide (Sigma-Aldrich) with gel-filtered G-actin as described previously [22].

Actin binding and crosslinking assay

The actin co-sedimentation assay was adapted to quantitate binding that occurs during polymerization of actin as opposed to crosslinking of preformed, mature actin filaments

[11]. In these assays Ca-G-actin (5 μ M) was incubated with various amount of Palld-Ig3 (0-25 μ M) in non-polymerizing conditions (G-buffer) for 1 hour. To isolate bound Palld-Ig3, the reaction mixture was centrifuged at 100K $\times g$ for 30 min (Beckman TL-100 ultracentrifuge). The supernatant was removed, the pellet was resuspended in 100 μ l of 0.1% SDS buffer (25 mM Tris, pH 8.3, 25 mM glycine, and 0.1% SDS) and the proteins in pellet and supernatant were separated using 16% SDS-PAGE gels. The amount of actin and Palld-Ig3 present in each fraction was quantified by using ImageJ software [23]. At least 3 data sets were averaged and standard deviation calculated.

To quantitate the effect of Palld-Ig3 on actin crosslinking that occurs during co-polymerization versus mature filaments, 10 μ M Ca-G-actin was incubated with various amount of Palld-Ig3 (0-20 μ M) in non-polymerizing conditions (G-buffer) and polymerizing conditions of F-buffer (5 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mM $MgCl_2$), respectively. The reaction mixtures were incubated for 1 hour and then centrifuged at 5,000 $\times g$ for 10 min. To pellet all actin filaments, the supernatant was then centrifuged at 100K $\times g$ for 30 minutes. Supernatant and pellet fractions were separated by SDS-PAGE and quantified as stated for binding assay.

Effect of Palld-Ig3 on spontaneous actin polymerization

Polymerization of G-actin was quantified by the increase in fluorescence intensity of 5% pyrenyl F-actin, which is 7-10 times greater than the fluorescence intensity of monomeric actin as described [22]. Pyrenyl actin and unlabeled G-actin were mixed together to make 10 μ M, 5% pyrene labeled G-actin stock. Right before the experiment, 5 μ M of this stock was incubated for 2 minutes upon addition of 10 $\times$ priming solution (10 mM EGTA and 1 mM $MgCl_2$) to convert Ca-G-actin into Mg-G-actin. Polymerization was induced by adding 25 mM KCl (polymerizing condition) or without KCl (G-buffer condition) and all the pyrene fluorescence was measured with excitation at 365 nm and emission at 385 nm in fluorescence spectrophotometer (PTI). Until otherwise stated, we added equal amounts of storage buffer in the entire reaction sample by taking the measurement from highest amount of Palld-Ig3 used to insure that no contributions from Palld-Ig3 storage buffer affected polymerization. The experiments were repeated at least twice with similar results. Raw data were normalized first by subtracting the baseline fluorescence and dividing by the steady-state plateau fluorescence. The overall polymerization rate of each polymerization curve was determined by plotting the slope of linear region of curve and converting relative fluorescence units/s into nM actin/s. We can assume that at equilibrium, the total amount of polymer is equal to the total concentration of actin minus the critical concentration, as Palld-Ig3 does not alter the critical concentration [24, 25].

Critical concentration determination of barbed-ends and pointed-ends

The critical concentrations of barbed and pointed-ends of actin filaments were determined by serially diluting polymerized actin or gelsolin-seed polymerized actin to a range of concentrations (0-3 μ M) with and without the addition of Palld-Ig3. For the barbed-end critical concentration, 10 μ M 5% pyrene actin was polymerized and serially diluted; however, for pointed-end critical concentration, gelsolin-actin seeds were made first [26] and then added in 1:5 ratio (gelsolin-seed: pyrene actin) to make filaments [24]. In both

circumstances, MKEI polymerization buffer (2 mM MgCl_2 , 50 mM KCl, 1 mM EGTA, 20 mM imidazole, pH 7), was added to polymerize actin. Upon dilution of polymerized actin, 1 mM DTT and 0.2 mM ATP were supplemented in polymerizing condition reactions. The reaction mixtures were incubated at room temperature for 4 hours and the steady-state pyrene fluorescence was then measured. The critical concentration in the presence or absence of Palld-Ig3 is determined by the intersection between the fluorescence intensity measurements for monomeric actin and either serially diluted F-actin or gelsolin-seeded F-actin.

Barbed-end actin assembly

Barbed-end assembly was measured in the presence and absence of Palld-Ig3 as described [24]. Briefly, G-actin was polymerized at a high concentration (20 μM) by adding MKEI polymerization buffer, the mixture was incubated at room temperature for 1 hour and subsequently vortexed for 30 s to use fragments as a seeds for elongation. The concentration of vortexed F-actin seeds relative to the total actin concentration was below 5% [27]. The spontaneous barbed-end assembly was performed using 5 μM , 5% pyrene actin, 20 μM Palld-Ig3 and 0.5 μM F-actin seeds. Next, the rate of the barbed-end elongation assay was determined using 5 μM , 5% pyrene actin, Palld-Ig3 (10 or 20 μM) with varying concentrations of F-actin seeds (0.1- 0.5 μM) until saturation was achieved.

Pointed-end actin assembly

Gelsolin-actin seeds were formed by mixing plasma gelsolin (Cytoskeleton, Inc.) with a two-fold molar excess of actin in G-buffer in the presence of 500 μM CaCl_2 and 10 mM Tris, pH 8.0. The reaction mixture was incubated for 2 hours at room temperature and then incubated at 4 °C overnight before adding 10% of the total volume of 10 $\times$ KME (10 mM EGTA, 10 mM MgCl_2 , 250 mM KCl). This mixture was employed with five-fold excess 5% pyrene actin either with or without 20 μM Palld-Ig3. Gelsolin-actin seeds were warmed to room temperature before mixing [24, 26].

Actin filament depolymerization

Pyrene-labeled F-actin (10 μM , 5% pyrene) was prepared by incubating G-actin for one hour at room temperature in MKEI polymerization buffer as described previously [28, 29]. In 200 μl reactions, 2 μM , 5% pyrene pre-assembled filaments and variable concentrations of Palld-Ig3 (1-20 μM) were mixed along with 1 mM DTT and 0.2 mM ATP. After 30 minutes of incubation, 2 μl of 1 mM Latrunculin A (Calbiochem) prepared in DMSO was added to the mixture and actin filament disassembly was immediately monitored by pyrene fluorescence for 600 s. At steady state, the fluorescence signal of each sample was normalized to 1 (AU) by dividing the first point of fluorescence intensity to the rest of the polymerization curve.

Mathematical modeling of actin polymerization with and without Palld-Ig3

Actin polymerization kinetics were simulated in the presence and absence of Palld-Ig3 using the MLAB modeling program (Civilized Software). Fluorescence values were converted to actin filament concentrations by assuming that 0.1 μM actin (C_c) was unpolymerized at equilibrium. We used a model based on the previous work of Wegner and Engel [30], as

modified by Cooper *et al.* [31] and further refined by Beall and Chalovich [27]. In this model it was assumed that nucleation consisted of a two-step addition of activated actin monomers: $A_1 + A_1 = A_2$ and $A_1 + A_2 = A_3$. The rate equations for these two steps of nucleation are then identical, resulting in rate constants for the forward k_3 ($M^{-1}s^{-1}$) and reverse k_4 (s^{-1}) reactions. To solve the differential equations describing the time course of A_3 , it was necessary to make the assumption that the value of $A_n = A_{n+1}$ so that A_4 can be set equal to A_3 . Elongation was then assumed to occur by addition of actin monomers to the nucleus (A_3), with the forward rate constant k_5 ($M^{-1}s^{-1}$) and the reverse rate constant k_6 (s^{-1}) as described elsewhere [27, 32, 33]. The equations for the simulations and data fitting are given below and have been slightly modified from [27]:

$$dA_1/dt = k_1(A_{total} - A_1 - 2*A_2 - 3*A_3 - 4*P) + 2*k_4*A_2 - k_2*A_1 - k_3*A_1^2 \quad (1)$$

$$dA_2/dt = k_3*A_1^2 + k_4*A_3 - k_4*A_2 - k_3*A_2*A_1 \quad (2)$$

$$dA_3/dt = k_3*A_2*A_1 + k_6*P - k_5*A_1*A_3 - k_4*A \quad (3)$$

$$dP/dt = k_5*A_3*A_1 - k_6*A_3 \quad (4)$$

MLAB was first used to simulate an individual data set by adjusting the rate constants so that the time course of polymerization (P) matched that of experimental curves. Then the data was fit globally by varying k_3 and/or k_5 , while the values of the other rate constants were held constant at $k_1 = 1 \times 10^8 s^{-1}$, $k_2 = 1 \times 10^{-4} s^{-1}$, $k_4 = 500 s^{-1}$, and $k_6 = 0.1 s^{-1}$. As noted in Table 1, it was also necessary to float k_6 to obtain some of these fits.

Sample preparation for electron and confocal microscopy

For EM, 2 μM of G-actin was mixed with 20 μM of Palld-Ig3 and incubated for 7-15 min before loading on carbon-covered discharged copper grids. The specimens were negatively stained with 2% uranyl acetate [34]. Images were collected on CCD-digital camera using Tecnai-12 electron microscope (FEI, Inc.) with an accelerating voltage of 80 kV and 30,000 $\times$ magnification.

Oregon Green 488 iodoacetamide (Molecular Probes) labeled G-actin was prepared as described previously [35]. In G-imaging buffer condition (5 mM Tris-HCl, pH 8, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP, 0.040 mg/mL catalase, 0.2 mg/mL glucose oxidase), 1 μM G-actin (5% labeled) was mixed with Palld-Ig3 (1-2 μM) and incubated for 50 minutes before mounting on microscope. Similarly, 1 μM actin was polymerized in F-imaging buffer (5 mM Tris-HCl, pH 8, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM ATP, 0.040

mg/mL catalase, 0.2 mg/mL glucose oxidase) for 50 minutes then Palld-Ig3 was mixed and further incubated for 50 minutes before mounting on microscope. All images were collected on Leica TCS SP5 (Leica Microsystems) confocal microscope with Oregon Green setting for excitation and emission with 63x oil immersion lens objective.

Results

Palld-Ig3 promotes actin polymerization

Previous studies show that the Ig3 domain of palladin (Palld-Ig3) is sufficient for binding and bundling of F-actin [11, 19]; however, whether or not Palld-Ig3 directly influences *de novo* assembly of actin monomers is not known. Liu *et al.* revealed that fibroblasts cultured from the palladin knockout mouse display a reduction in stress fiber density and a decrease in the amount of polymerized actin [10], therefore we hypothesized that Palld-Ig3 regulates actin assembly and/or disassembly.

To directly test the effects of Palld-Ig3 on actin polymerization, we first relied on the well-established actin cosedimentation assay. Typically this assay is used to assess binding and/or bundling of F-actin under conditions where actin polymerizes, however we assessed the amount of polymerized actin and bound Palld-Ig3 by quantifying Palld-Ig3 in the pellet fraction and virtually all of the actin sediments upon high-speed centrifugation (Fig. 1A). In this case polymerization of actin was induced by Palld-Ig3 in non-polymerizing condition (G-buffer). The polymerization of actin is dependent on the concentration of Palld-Ig3 and reaches a plateau at a 2:1 ratio (Palld-Ig3:actin) (Figure 1B). Care was taken to ensure that ionic contributions from the buffer containing palladin did not contribute to the polymerization by maintaining a constant volume of the protein storage buffer in all samples. These data suggest that Palld-Ig3 promotes polymerization of actin filaments under conditions where actin monomers are typically refractory to assembly.

To monitor the effect of Palld-Ig3 on the kinetics of F-actin assembly, we quantified the rate of polymerization in bulk solution using pyrene-actin [22]. When pyrenyl-G-actin polymerizes, the fluorescence intensity increases 7-10 fold and this increase is directly proportional to the amount of G-actin incorporated into F-actin [22]. To evaluate how Palld-Ig3 affects the rate and extent of actin polymerization, we measured the complete time course of polymerization, using 5 μ M G-actin in the presence of varying concentrations of Palld-Ig3 (0-30 μ M) under both non-polymerizing conditions (Figure 2A) and polymerizing conditions (Figure 2B). Palld-Ig3 increased the rate of F-actin polymerization in a dose-dependent manner (Figure 2C, D), with a four-fold increase in the polymerization rate in G-buffer and two-fold increase in F-buffer compared to control (actin alone) reactions. The rate of actin polymerization reached a plateau around 4 nM/s in both buffers, indicating that Palld-Ig3 stimulates actin polymerization even in the absence of KCl. It is striking that the lag phase, corresponding to a nucleation step, was not observed for Palld-Ig3 induced polymerization under either buffer condition. This suggests that Palld-Ig3 either stabilizes actin nuclei or lowers the critical concentration for polymer formation.

Effect of Palld-Ig3 on critical concentration

The observed increase in the rate of F-actin polymerization by Palld-Ig3 could result from several different mechanisms, including nucleus stabilization or a decrease in the critical concentration for actin assembly at barbed-ends. We first considered whether Palld-Ig3-actin interactions alter the critical concentration for assembly at filament barbed-ends, which is the lowest concentration of actin (0.1 μM) above which filaments are in steady state equilibrium with G-actin and below which actin cannot exist as filaments. Critical concentration measurements were made by observing the steady-state fluorescence of varying concentrations of pyrenyl-F-actin in the presence and absence of 1 μM Palld-Ig3 (Figure 3A). Palld-Ig3 did not alter the critical concentration, which is 0.07 μM in the presence and absence of 1 μM Palld-Ig3.

Effect of Palld-Ig3 on elongation from the barbed-ends

Our spontaneous polymerization data (Figure 2) show that Palld-Ig3 reduces the lag phase for polymerization, indicating again that palld-Ig3 promotes nucleation of actin monomers. To test the possible effects of Palld-Ig3 on filament elongation, as opposed to nucleation, we measured actin assembly from sonicated actin filaments seeds. Our data show that assembly of G-actin stimulated by seeds resembles the spontaneous assembly of G-actin in the presence of Palld-Ig3 (Figure 4A), where the nucleation phase is considerably diminished. Moreover, the extent of actin polymerization in Palld-Ig3 with actin seeds was higher in comparison to either Palld-Ig3 or actin seeds alone, suggesting that Palld-Ig3 may also affect filament stability.

Proteins that alter nucleation can also influence the rate of barbed-end elongation [36]. Therefore we determined the elongation rate constant by initiating polymerization from short actin filament nuclei, or seeds, in the presence or absence Palld-Ig3. The slope of the plot was used to find the rate constant for elongation, which was plotted against the sonicated actin concentration ($m_{1/2}$) [37] in the absence and presence of Palld-Ig3 (10 or 20 μM) (Fig 4B). The concentration of both G-actin and Palld-Ig3 was held constant and the sonicated F-actin seeds did not contribute more than 5% of total actin. Our results reveal that the rate of elongation increased linearly with increasing concentrations of F-actin seeds for samples with 10 μM Palld-Ig3 (Figure 4B), and did not reach a plateau. However, a plateau was reached at 0.3 μM F-actin seeds with higher concentrations of Palld-Ig3 (20 μM) and these results show that the addition of more F-actin seeds had no effect on the rate of elongation. The nucleation rate with 10 μM Ig3 increased by a factor of ~ 2 over that of actin seeds alone, as highlighted by the gap between the two sets of data that occurs even in the absence of any seeds. Yet, the slopes for the change in elongation rate as a function of actin or Palld-Ig3 concentration are not parallel (Fig. 4B). There is an additional increase in actin polymerization in the presence of both seeds and Palld-Ig3, which indicates that Palld-Ig3 also effects filament elongation. Yet, this apparent increase in the elongation rate may arise from the fact that the assembly rate in these experiments is a combination of the rate of elongation from the added seeds along with newly formed nuclei that arise from G-actin.

Palld-Ig3 promotes assembly of actin from the pointed-end

Since Palld-Ig3 has some effect on barbed-end polymerization, we next sought to determine whether Palld-Ig3 might also regulate elongation from the pointed-ends. To examine the influence of Palld-Ig3 on the pointed-end of actin filaments, gelsolin-actin seeds (1 μM) were diluted five-fold with 5 μM 5% pyrene actin in presence and absence of 20 μM Palld-Ig3 before measuring the assembly. Remarkably, our results show that Palld-Ig3 promotes the pointed-end assembly of actin filaments (Figure 5A). Actin assembly initiated in the presence of Palld-Ig3 and gelsolin-actin seeds was accelerated 2-fold by Palld-Ig3, suggesting Palld-Ig3 influences actin polymerization at pointed-ends. The plateau fluorescence values of actin polymerization initiated with gelsolin-capped seeds only (control, yellow squares) were lower than without gelsolin as expected given the pointed-end critical concentration is 0.6 μM , whereas the barbed-end critical concentration is 0.12 μM [38]. Actin polymerization initiated by gelsolin-actin seeds in the presence of 20 μM Palld-Ig3 did not result in lower steady-state fluorescence values and remained identical to actin polymerization with 20 μM Palld-Ig3 in the absence of gelsolin (where both ends are free) suggesting that Palld-Ig3 affects the rate of pointed-end assembly.

We next examined whether Palld-Ig3 alters the critical concentration for monomer addition at pointed-ends. This critical concentration was determined under polymerizing conditions by serially diluting gelsolin capped actin filaments in the presence of 1 μM Palld-Ig3. There was no change in the critical concentration of pointed-ends, which remained at 0.6 μM in the presence and absence of Palld-Ig3 (Fig.5B).

Effect of Palld-Ig3 on nucleation rate

To determine the effect of Palld-Ig3 on the various rate constants associated with actin polymerization, we simulated our pyrene-actin polymerization time courses using a mathematical model similar to that of Beall and Chalovich [27]. This model assumes that nucleation consists of a two-step addition of activated actin monomers, followed by elongation [27]. The nucleation step was optimized by globally fitting the kinetic data at a range of concentrations, while fixing all other parameters (Fig.6 A and B; Tables 1 and 2). This model fit the experimental data well, yielding a good visual fit to the experimental data and a low best weighted sum of squares (Fig.6). Optimization of the nucleation rate showed that k_3 approaches a minimum threshold ($1.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$), beyond which increases in the on rate do not improve the fit (Fig.6 C and D). The nucleation rate constant (k_3) increased six-fold (in G-buffer) and three-fold (in F-buffer) in the presence of Palld-Ig3, while the elongation rate (k_5) only increased slightly for Palld-Ig3 in F-buffer. This marginal difference in elongation rate reiterates the distinction noted for the seeded polymerization assays (Fig. 4B), where the slope for the elongation rate in the presence of Palld-Ig3 only varied slightly from that of actin alone.

Role of electrostatics in Palld-Ig3 induced actin polymerization

Charge neutralization can be a determining factor in the function of actin binding and polymerizing proteins that are predominately basic by nature [27] [39, 40]. Previous research has shown that interactions between palladin and F-actin are electrostatically driven [11, 19]. Specifically, basic patches in the Palld-Ig3 (lysine residues 15, 18 and 51) are

necessary for F-actin binding [19]. In general, an increase in ionic strength correlates with a decrease in the affinity of a cationic protein ligand to an anionic filamentous substrate, such as actin [40]. To understand how electrostatics may modulate Palld-Ig3 interactions with actin, we measured the effect of ionic strength in Palld-Ig3-induced actin polymerization. As expected, the nucleation activity of Palld-Ig3 was greatly enhanced when the concentration of KCl was decreased (Fig. 7A); as opposed to the higher polymerization of actin reported previously with increasing salt concentration (0-100 mM KCl) [41].

To determine whether the basic patches on the Ig3 domain of palladin are also involved in Palld-Ig3 induced polymerization of actin, we compared the polymerization of actin in the presence of wild type Palld-Ig3 and a mutant Palld-Ig3 with three lysine residues mutated to alanine (K15/18/51A) (Fig. 7B). As predicted, the rate of actin polymerization in the presence of Palld-Ig3 was significantly reduced in the presence of the lysine triple mutant compared to that obtained with wild type Palld-Ig3. This suggests that disruption of the Palld-Ig3 actin interaction likewise interferes with actin polymerization. However, other residues are likely involved in the interaction, as these lysine mutations do not completely abolish actin-binding or enhanced actin polymerization.

G-actin requires bound metals to polymerize [42] and has one high affinity site for divalent cations Mg^{+2} or Ca^{+2} that bind with nanomolar affinity [43, 44]. Furthermore, there are approximately nine low affinity binding sites for metals (Mg^{+2} , Ca^{+2} and K^{+}); and these must be sufficiently occupied to induce conformational changes required to initiate polymerization [43, 45]. Actin polymerization can be initiated *in vitro* by adding physiological concentrations of neutral salts (1 mM Mg^{+2} and/or 10-100 mM K^{+}) [46]. Here, we reveal that Palld-Ig3 induced actin polymerization at a total Mg^{+2} concentration that was much lower than that required to saturate the low affinity binding sites essential for polymerization (12.5 μM $MgCl_2$ for 5 μM G-actin) (Fig. 7C). This suggests that Palld-Ig3 produces an effect on G-actin that is similar to that of metal ions and that yields polymerization-competent G-actin. This promotion of $G \rightarrow G^*$ transition by palladin likely occurs through charge neutralization or conformational changes.

Palld-Ig3 prevents depolymerization

To determine whether Palld-Ig3 has any role in stabilizing actin filaments, we measured the disassembly rate of pyrene-labeled actin filaments in the presence and absence of various concentrations of Palld-Ig3 (0-20 μM) and 10 μM Latrunculin A (Lat A), an actin monomer sequestering agent [28]. As expected, the fluorescence intensity decreased in a time-dependent manner in the presence of Lat A, indicating net actin filament depolymerization (Fig. 8). We measured the rate of disassembly of filaments from the initial rate of the curves. In the presence of Lat A, the rate of disassembly was increased by 12-fold when compared to actin filaments alone. Addition of Palld-Ig3 decreased the rate of disassembly significantly in a dose-dependent fashion. For the highest concentration of Palld-Ig3 (20 μM), the rate of Lat A-induced disassembly was decreased by 10-fold compared to actin alone. These results indicate that binding of Palld-Ig3 to F-actin protects the filaments from depolymerization and decreases the dissociation of monomers from filaments.

Palld-Ig3 enhances crosslinking during polymerization

Crosslinking of actin filaments is a dynamic process that may also be altered by palladin, however we were unable to detect this change in our bulk pyrene kinetic assays that only measure filament formation. While previous crosslinking experiments have been carried out with pre-polymerized actin to reveal that the Palld-Ig3 domain bundles F-actin [19], we wanted to determine whether Palld-Ig3 alters the extent of actin filament crosslinking that occurs during polymerization. Therefore we revisited the cosedimentation assay to compare the amount of bundled F-actin formed in the presence of Palld-Ig3 when incubated with either F-actin or G-actin. We show that the crosslinking that occurs during co-polymerization with G-actin far exceeds that resulting from addition of Palld-Ig3 to preformed, mature filaments (Fig. 9). This suggests that Palld-Ig3 induced polymerization of G-actin involves both nucleation and crosslinking activities. Furthermore, electron micrographs and confocal images of Palld-Ig3 added to mature F-actin reveal crosslinked actin filaments arrayed in linear bundles; whereas, co-polymerization with G-actin reveals a relatively more crosslinked network (Fig. 10). These results also indicate that actin crosslinking occurs early in the polymerization process, and suggest that Palld-Ig3 binds to actin by forming or stabilizing a nucleus from which genuine actin filament bundles could assemble.

Discussion

Previous research has established the importance of palladin in diverse actin based structures including podosomes [4], stress fibers [47], dorsal ruffles [48], cell adhesions [10], growth cones [9], and lamellipodia [49]. Furthermore, elevated expression of palladin has been linked to the invasive cell motility of malignant cells [6, 50, 51]. In this study we reveal an important facet of Palld-Ig3's function with our discovery of robust actin polymerization in the presence of Palld-Ig3, even in non-polymerizing condition (no Mg^{2+} or KCl). Previous studies revealed that the Palld-Ig3 domain directly interacts with actin and represents the minimal domain for its actin binding and bundling activity [11, 19]. Here we show that Palld-Ig3 also regulates polymerization dynamics directly. First, we observed dose-dependent effects of Palld-Ig3 on spontaneous actin assembly that eliminated the initial lag phase observed in solutions of actin monomer alone. Furthermore, Palld-Ig3 increased the initial rate of actin polymerization by 4-fold. Based on our results, we rule out actin filament severing activity by Palld-Ig3 because addition of the Palld-Ig3 decreased the rate of disassembly, whereas severing factors are expected to increase disassembly (Fig. 9). Palld-Ig3 also stabilizes filaments, preventing depolymerization. Despite the observed filament stabilization, we were unable to observe any alteration to the critical concentration from either the pointed- or barbed-ends brought about by Palld-Ig3, which may be too small to accurately measure with our assays. Furthermore, we observed only a slight increase in the rate of elongation by Palld-Ig3 in F-actin seeded assembly assays, suggesting that Palld-Ig3 has a minor effect on elongation. Therefore, our data as a whole suggests that the considerable increase in actin polymerization brought about by Palld-Ig3 results from enhanced filament nucleation that likely arises from palladin's ability to promote the $G \rightarrow G^*$ transition of monomeric actin.

Actin nucleators are known to use one of three different processes to promote nucleation: 1) by structurally mimicking the filament barbed-end, like Arp2/3 [52]; 2) by stabilizing the spontaneously formed intermediates, like formins [53]; or 3) by aligning and/or recruiting the actin monomers to form polymerization seeds, like WH2 domain proteins such as Spire [54], Cobl [55], Lmod [56], and JMY [57]. Unlike the Arp2/3 complex, Palld-Ig3 does not require any cofactors, and also lacks recognizable WH2 sequences. Yet, Palld-Ig3 may share some formin-like properties, such as stabilization of polymerization intermediates via dimerization. This mechanism is supported by our recent results showing that the Palld-Ig3 domain forms homodimers upon binding to actin and may therefore stabilize nuclei by providing multiple binding sites for actin [58]. In contrast to formins, Palld-Ig3 requires saturating concentrations to significantly enhance polymerization and likely decorates actin filaments, as evident from the significant co-localization with actin stress fibers in cells [59]. This interaction with actin is electrostatic as Palld-Ig3 induced actin polymerization was dramatically reduced by increasing salt concentrations. This indicates that Palld-Ig3 may overcome the kinetic barrier leading to actin polymerization through specific and/or non-specific charge neutralization of actin's surface. Additional support for this mechanism comes from the fact that the Palld-Ig3 is highly basic in nature ($pI > 9$) and specific, surface-exposed basic residues are critical for the interaction with actin [19]. Therefore charge neutralization may also account for the actin polymerization we observed in the presence Palld-Ig3 in non-polymerizing condition (G-buffer).

To understand mechanistically how Palld-Ig3 influences actin polymerization kinetics, we used simulations and subsequent fitting routines to model the polymerization kinetics as a function of Palld-Ig3 concentration. This analysis allowed us to explicitly model how Palld-Ig3 affects both the rate of nucleation (k_3) and rate of elongation (k_5) for actin polymerization. Our results reveal that the rate of nucleation (k_3) increased six-fold at the highest concentration of Palld-Ig3 used compared to actin alone in non-polymerizing buffer. In contrast we found no significant difference in the rate of elongation (k_5) for Palld-Ig3 induced actin polymerization as compared to actin alone in our seeded elongation assays (Table 1). Overall the kinetic simulations and non-linear least squares fitting provided a more detailed understanding of the specific steps in actin polymerization that were affected by Palld-Ig3 and emphasize that Palld-Ig3 has pronounced effect on the nucleation process compared to filament elongation.

Numerous studies have shown that actin filaments primarily elongate at filament barbed-ends in cells [60-64]. However, we observed only a modest effect on barbed-end assembly by Palld-Ig3. Our biochemical studies suggest that Palld-Ig3 promotes filament growth from filament pointed-ends, without altering the critical concentration for assembly. Recently, pointed-end actin assembly by Lmod and SALS was shown to play important roles in maintaining the highly organized sarcomeric structure of muscle cells [65-68]. Thus, it is intriguing that Palld-Ig3 appears to function at pointed-ends and leads us to speculate that palladin could be involved in the regulation of pointed-end filament initiation in muscle and non-muscle cells, where it is also abundant.

Palld-Ig3 also stabilizes actin filaments as revealed from assays of filament depolymerization. This supports a model in which palladin decorates the sides of actin

filaments for stability. Palld-Ig3 produces long, linear bundles of actin when added to preformed filaments; but co-polymerization of actin and Palld-Ig3 results in shorter, orthogonally crosslinked filaments, as observed from confocal and electron microscopy (Fig. 10). This difference in actin structures is also supported by our bundling assays that revealed a significant increase in crosslinking observed when palladin was added prior to polymerization versus after filaments formed (Fig. 9). These observed differences in actin filament structure may be indicative of the plasticity of palladin's activity in actin organization, consistent with its involvement in formation of distinct protrusive structures that execute both normal and metastatic cell motility. As mentioned previously, expression of the major isoform of palladin (a.k.a. 90 kDa or isoform 4) is dramatically increased in actively migrating cells, whether that be in wound healing or metastatic cancer [4, 51, 59]. This would obviously result in a higher local concentration of palladin. Moreover, when we consider the higher apparent binding affinity for actin of full-length palladin ($K_d \sim 2 \mu\text{M}$) versus the isolated Palld-Ig3 domain ($K_d \sim 60 \mu\text{M}$), it is likely that full-length palladin may have a more dramatic effect on actin polymerization [11]. Unfortunately this has been difficult to establish given the instability of the recombinant, full-length palladin.

The presence of palladin in several different types of actin-rich structures (dorsal ruffles, stress fibers, podosomes, invadopodia, etc.) raises important questions about how palladin regulates cytoskeletal organization in healthy and diseased conditions. The overall cellular function of palladin is likely tied to two roles, one that involves direct manipulation of actin filament structures/networks via nucleating, crosslinking, and stabilizing activities and the other in which palladin acts as a scaffold for other actin binding proteins (ABPs) and signaling partners. A scaffolding function for palladin has been the focus of most work to date and reveals that palladin either activates and/or recruits several important ABPs such as profilin, α -actinin, and ENA/VASP [2, 12, 69]. Additionally, as we show here, palladin may also organize assembly of actin filament networks by directly manipulating actin filament dynamics. An intriguing possibility is that increasing the pool of filamentous actin may concomitantly influence the localization of palladin and its recruitment of other actin regulatory proteins. Palladin has been shown to translocate to the nucleus and also interacts with the transcription factor MRTF, which accumulates in the nucleus in response to decreased cytoplasmic G-actin levels [7, 70]. Moreover, mutations in the Ig3 domain of palladin that interrupt actin binding result in disruption of the actin cytoskeleton and nuclear localization of palladin [19]. By extension, control of actin nucleation, bundling, and monomer availability by palladin in the cytoplasm may also influence transcriptional regulation by nuclear actin. Future research will address this possibility more directly. Our work here provides the first connection between palladin and the dynamic regulation of cytoplasmic actin filaments.

Palladin is recognized for its key role in invasive protrusions, which was previously associated with the activation of cdc42, ENA/VASP, Eps8, and matrix metalloproteases (MT1-MMP14) [3, 48, 51]. Previous studies showed that palladin overexpression promotes invadopodia formation; however, a direct link between palladin and the alterations to actin assembly required for protrusions had not yet been established. Our results suggest that palladin decorates filaments heavily, which helps guide monomers onto the ends of the filaments in order to stimulate polymerization. This function of palladin could then promote

nucleation and/or stabilize actin filaments at invadopodia, perhaps in collaboration with Arp2/3 complex [57, 71]. Therefore, we suggest a mechanism for invasive motility that links changes in filament dynamics and cytoskeletal rearrangements with the enhanced expression of palladin.

While the majority of this previous research has focused on the scaffolding activity of palladin, our results here add a new dimension to the relationship between palladin and actin by highlighting the direct role in actin assembly. This study was limited to assays involving only one of the immunoglobulin-like domains of palladin due to stability issues involved with the full-length protein. Yet previous work demonstrated that the Ig3 domain is both the minimal domain necessary for actin binding and is the only domain present in the 90 kDa isoform of palladin that displays any detectable interaction with actin [11]. Here we show that the Ig3 domain of palladin, also stimulates actin polymerization *in vitro*. Additionally the actin binding domain of palladin eliminates the lag phase that is characteristic of the slow nucleation step of actin polymerization and dramatically reduced depolymerization. Actin filament growth was not inhibited by barbed-end blockers, thus palladin appears to stimulate filament growth from the pointed-end. Microscopy and *in vitro* crosslinking assays reveal differences in actin bundle architecture when palladin is incubated with actin before as opposed to after polymerization. Similar to metal ions, palladin also appears to stimulate a polymerization-competent form of G-actin, either through charge neutralization or conformational changes. Together with the localization of palladin in Z-disks, podosomes, and stress fibers, our results demonstrate that palladin stimulates actin polymerization and indicate that palladin is part of an actin nucleation complex involved in both actin dynamics and organization. Given the high structural similarity of palladin, myotilin, and myopalladin, we suggest similar effects on filament dynamics by other members of this distinct actin binding protein family. Further research will integrate the complex network of interactions involving palladin to understand its role in linking actin dynamics to gene expression and the cytoskeletal rearrangements associated with normal development and during invasive metastasis of tumors.

Acknowledgments

We thank Bruce Goode and Dorothy Schafer for their critical review and suggestions for our manuscript. We are also grateful for technical assistance and helpful discussions with Joseph Chalovich, John A. Cooper, Peter Rubenstein, and Carol Otey.

Funding: This project was supported by grants from the National Center for Research Resources (5P20RR017708), both a COBRE grant (8P20GM103420) and an Institutional Development Award (IDeA) (P20GM103418) from the National Institute of General Medical Sciences at the National Institutes of Health, Burroughs-Wellcome Trust Collaborative Grant, the Flossie E. West Memorial Foundation Trust, and by funds from Wichita State University.

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Abbreviations

Ig	immunoglobulin
G-actin	globular or monomeric actin
F-actin	filamentous actin
Palld-Ig3	actin binding domain of palladin
Lat A	Latrunculin A
MKEI	polymerization buffer containing MgCl ₂ , KCl, EGTA, imidazole
T_{5%}	polymerization time for 5% completion
K_d	dissociation constant
k₃	nucleation rate constant
k₅	elongation rate constant

Summary

Our results indicate palladin stimulates actin polymerization by accelerating the nucleation step, while also enhancing filament stability and altering filament architecture. Palladin regulates a distinct actin nucleation mechanism that may underlie the assembly of actin in invasive cell motility.

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Figure 1. Co-sedimentation assays show that the Palld-Ig3 induces polymerization of G-actin
(A) Representative SDS-PAGE gels of co-sedimentation assay in G-buffer conditions with supernatant fractions (top) and pelleted fractions (bottom) at varying concentrations of Palld-Ig3 (0-25 μM). **(B)** G-actin (5 μM) was mixed with various concentrations of Palld-Ig3 and allowed to incubate for 1 hour. The amount of actin in the supernatant and pellet was quantified from the relative band intensity on the gel and the molar fraction of actin in the pellet was calculated.

Figure 2. *In vitro* actin polymerization assays reveals that the Pallid-Ig3 accelerates actin polymerization

Spontaneous assembly reactions were performed by simultaneous addition of actin (5% pyrene labeled, primed with 1 mM EGTA and 0.1 mM MgCl_2) and increasing concentrations of the Pallid-Ig3 in G-buffer (A) or F-buffer (B). Polymerization was monitored by measuring an increase in fluorescence intensity. At all concentrations examined, palladin increased the rate and extent of actin polymerization. Plots of overall polymerization rate, nM/s versus Pallid-Ig3 concentration in G- (C) and F-buffer conditions (D).

Figure 3. Barbed-end critical concentration measurements made by the net depolymerization of pyrene-labeled F-actin

Pyrene fluorescence (AU) is plotted against actin concentration for samples incubated in G-buffer (circles), MKEI (open squares) and actin incubated with 1 μM Palld-Ig3 (closed squares). The intersection between the plots of G-actin and F-actin indicates the critical concentration.

Figure 4. Effect of Palld-Ig3 in the elongation from barbed-end in the presence of F-actin nuclei (A) Time course of polymerization in the absence (pink and black) and in the presence (yellow and green) of F-actin seeds was followed in the presence of 20 μ M Palld-Ig3. (B) The elongation rate was measured by using varying amount of F-actin sonicated seeds (0-0.5 μ M) with 5 μ M G-actin in the absence (circles) or in presence of 10 μ M (triangles) or 20 μ M Palld-Ig3 (open squares).

Figure 5. Effect of Pallid-Ig3 at the pointed-end of actin filament

We measured the spontaneous actin assembly and critical concentration in presence of gelsolin seeds. (A) Time course of polymerization in the absence (pink and black) and in the presence (yellow and green) of 1 μM gelsolin-actin seeds with or without 20 μM Pallid-Ig3. (B) Pyrene fluorescence (AU) is plotted against actin concentration for samples incubated in G-buffer (circles) for samples containing actin with gelsolin in MKEI (open squares) and gelsolin with Pallid-Ig3 in MKEI (closed squares). The intersection between the plots of G-actin and F-actin indicates the critical concentration.

Figure 6. Simulated fits of Palld-Ig3 enhanced polymerization of actin

Representative plots of pyrene-actin polymerization (symbols) with simulated fit (solid lines) at various concentrations of Palld-Ig3 (0-30 μM) in G-buffer conditions (A) and F-buffer conditions (B). The theoretical curves were generated by using equations 1-4 and a series of rate constants. The data were globally fitted by varying k_3 and/or k_5 while fixing other rate constants ($k_1 = 1 \times 10^8 \text{ s}^{-1}$, $k_2 = 1 \times 10^{-4} \text{ s}^{-1}$, $k_4 = 500 \text{ s}^{-1}$, and $k_6 = 0.1 \text{ s}^{-1}$). The rate of nucleation (k_3) and rate of elongation (k_5) were determined from least-squares fitting and plotted as a function of the concentration of Palld-Ig3 for G-buffer (C) or F-buffer (D) conditions.

Figure 7. Effect of electrostatics (A and C) and triple mutation (B) on Palld-Ig3-induced actin polymerization

(A) The salt dependence of palladin was monitored by measuring the time course of actin polymerization at different concentrations of salt, KCl (25 mM- 100 mM) with 5 μM, 5 % pyrene G-actin and in the absence (circles) or presence (triangles) of Palld-Ig3. Prior to the polymerization, Ca-actin was pre-exchanged to Mg-actin by adding priming solution and the polymerization was initiated by adding KCl of different concentrations. (B) Lysine triple mutant (K15/18/51A) of Palld-Ig3 was made previously [19]. Triple mutant protein was expressed and purified as discussed for wild type Ig3. The polymerization of 5 μM, 5% pyrene actin with 1 μM and 20 μM of Palld-Ig3 and triple mutant Palld-Ig3 were initiated by 25 mM KCl. Prior to the polymerization, Ca-actin was pre-exchanged to Mg-actin by adding priming solution and the data were normalized by subtracting the baseline fluorescence and also dividing by plateau fluorescence. (C) Palld-Ig3 induced actin polymerization at very low and high ionic strengths. Conditions: 5 μM, 5% pyrene actin was used in G-buffer condition (2 mM Tris, pH 8, 0.2 mM ATP) with low (12.5 μM) or high (0.1 mM) MgCl₂.

Figure 8. Palld-Ig3 stabilizes actin filaments

G-actin (2 μ M, 5% pyrene labeled) was preassembled to filaments in F-buffer with various concentrations of Palld-Ig3 (1-20 μ M). Depolymerization was induced by adding Latrunculin A after 30 minute to each reaction mixture at time point 0, and fluorescence measurement were taken immediately by transferring reaction mixture to a quartz cuvette. The fluorescence signal was adjusted so that fluorescence of G-actin was 0 and F-actin was 100.

Figure 9. Differences in actin crosslinking when Palld-Ig3 is added before or after polymerization of actin

Co-sedimentation studies were used to quantitate the amount of F-actin bundles formed during co-polymerization of Palld-Ig3 and G-actin versus bundles formed when Palld-Ig3 is added to pre-polymerized actin. The amount of actin in bundles (black), monomers (white) and filaments (gray) recovered from incubation of either F- or G-actin with Palld-Ig3 was quantitated with ImageJ.

Figure 10. Different structures form in the presence of Palld-Ig3 when added to monomeric versus filamentous actin

Negatively stained electron micrograph images of actin filaments formed after copolymerization of Palld-Ig3 (20 μ M) with G-actin (2 μ M) in G-buffer condition (A) and Palld-Ig3 incubated with preassembled actin filaments in F-buffer condition (B). Samples were loaded on carbon-covered discharged copper grids after 7-15 min. of incubation. The scale bars in panels (A) and (B) represent 100 nm. Confocal images of crosslinked actin filament formed after copolymerization of actin (1 μ M) with Palld-Ig3 (2-4 μ M) (C) and bundled actin filaments formed by incubating Palld-Ig3 with preassembled actin filaments in F-buffer condition (D). The scale bars in panels (C) and (D) represent 25 μ m.

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Table 1

G-actin fitting parameters

[Pald-Ig3], μ M	k_3^*	k_3	k_5^{**}	k_3	k_5	k_6
0	0.291	0.241	0.562	0.284	0.377	0.178
1	0.519	0.862	0.381	0.893	0.374	0.374
5	0.981	1.016	0.489	N.A.	N.A.	N.A.
10	1.378	1.099	0.589	0.944	0.618	0.622
20	1.425	1.571	0.466	N.A.	N.A.	N.A.
30	1.554	1.647	0.479	N.A.	N.A.	N.A.

* $k_3=0.4$
and $k_5=0.5$

** $k_3=0.4$

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Table 2

F-actin fitting parameters

[Pald-Ig3], μM	k_3^*	k_3	k_5^{**}	k_3	k_5	k_6
0	0.422	0.639	0.409	0.666	0.406	0.359
1	0.568	0.613	0.460	0.587	0.483	0.439
5	0.888	0.606	0.627	0.588	0.596	0.510
10	1.117	0.747	0.645	0.716	0.614	0.549
20	1.185	1.054	0.539	0.958	0.552	0.528
30	1.300	1.295	0.501	1.155	0.531	0.526

* $\chi^2 = 0.029$ and $\chi^2 = 0.057$