Dimerization of profilin II upon binding the $(GP_5)_3$ peptide from VASP overcomes the inhibition of actin nucleation by profilin II and thymosin β4

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Abstract Profilin II dimers bind the $(GP_5)_3$ peptide derived from VASP with an affinity of approximately 0.5 μM. The resulting profilin II-peptide complex overcomes the combined capacity of thymosin β4 and profilin II to inhibit actin nucleation and restores the extent of filament formation. We do not observe such an effect when barbed filament ends are capped. Neither can profilin I, in the presence of the peptide, promote actin polymerization during its early phase consistent with a lower affinity. Since a Pro_{17} peptide-profilin II complex only partially restores actin polymerization, the glycine residues in the VASP peptide appear important.

Key words: Actin nucleation; Biacore; Polyproline; Profilin isoform

1. Introduction

Certain types of cell movement are characterized by a rapid spatially defined turnover of the microfilament system upon stimulation of cells. Actin desequestration and F-actin polymerization occur close to the cell membrane [1–3] or for instance in the comet tails formed by *Listeria* [4]. A variety of proteins are involved in this assembly and disassembly mechanism of actin, two of these key proteins are thymosin β4 and profilin.

Profilin is a 12–15 kDa protein present in all eukaryotic cells at concentrations in a range of 5×10^{-6} to 5×10^{-5} M. Several ligands for profilin were identified and can be classified into four different categories: actin [5], poly(L-proline) and poly(L-proline) containing sequences [6,7], L-α-phosphatidylinositol 4,5-bisphosphate (PIP_2) [8,9] and a complex of proteins containing actin related proteins [10,11]. Families of proteins containing proline rich sequences are known. Representatives of the Ena/VASP family are the focal adhesion proteins VASP [12,13] and the mammalian homologue of Ena, known as Mena [14,15]. The formin homology protein group consists of *Saccharomyces cerevisiae* Bni1p and Bnr1p [16,17], *Schizosaccharomyces pombe* cdc12p [18], cappuccino from *Drosophila melanogaster* [19,20], the mammalian homologue of the *Drosophila* protein diaphanous, p140mDia, [21–23], the *Schizosaccharomyces pombe* gene fus [20,24], the *Aspergillus nidulans* gene figA [25] and the members of the vertebrate limb deformity complex [26].

Profilin I was first identified as a G-actin sequestering agent [5]. However its function is more complex, it also lowers the steady state concentration of G-actin by the addition of profilin I-ATP-G-actin complexes onto the barbed end of the F-actin filament [27–29]. It is believed this association is followed by ATP hydrolysis resulting in the release of profilin I from the barbed end caused by a reduction in affinity of profilin for the terminal subunits [30]. In the presence of thymosin β4, the main G-actin sequestering agent in cells, profilin I promotes desequestration of G-actin from the thymosin β4-G-actin pool [29]. When equilibrium between G- and F-actin is reached in vitro and thymosin β4 is present the desequestration activity of profilin I has a much greater impact on actin polymerization than its sequestration activity. Under non-equilibrium conditions in vitro, profilin I decelerates the nucleation step of actin polymerization. The phenomena described above obviously only take place when the barbed filament ends are free. If the barbed ends of the F-actin filaments are capped (for instance by gelsolin), profilin I displays a pure G-actin sequestering activity [28–30].

Mammalians have two profilin isoforms, profilin I and II [31–34]. Both form a 1:1 complex with G-actin and have a similar K_d value of approximately 0.5 μM [29,34]. In contrast, their affinity for their other ligands is quite different. Whereas profilin I is preferentially associated with PIP_2, profilin II displays a higher affinity for poly(L-proline) rich sequences [34,35].

In this paper we studied the interaction of the profilin isoforms with proline peptides derived from VASP using Biacore experiments and the effect of profilin II complexed to this peptide on the polymerization of actin using fluorimetric assays. The results of our Biacore experiments indicate that profilin II dimerizes upon binding to the peptVASPwt peptide and that the affinity of this profilin isoform for this peptide is much higher in comparison to the affinity of profilin I. With our fluorimetric measurements we prove that the interaction of profilin II with the peptVASPwt peptide overcomes the combined capacity of profilin II and thymosin β4 to inhibit actin nucleation. Under the conditions used, this positive effect on actin polymerization is only observed for profilin II and not for profilin I. It is neither observed when the barbed

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Abbreviations: ENA, enabled; F-actin, filamentous actin; G-actin, globular or monomeric actin; HBS, HEPES buffer saline; peptVASPwt, peptide composed of residues 169–188 of VASP; peptVASP(G/P), mutant of the previous peptide in which the glycine residues are substituted to prolines; peptVASPs, peptide composed of residues 116–124 of VASP; PIP_2, L-α-phosphatidylinositol 4,5-bisphosphate; RU, response units; RUmax, the experimentally observed maximal response; $RUmax^t$, the theoretical maximal response expected; VASP, vasodilator stimulated phosphoprotein; WASP, Wiskott-Aldrich syndrome protein

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ends of the actin filament are capped by gelsolin, in this case the profilin II-peptVASPwt complex merely sequesters G-actin.

2. Materials and methods

2.1. Protein preparation and peptide synthesis

We purified skeletal muscle actin from rabbit muscle [36] and isolated it as calcium G-actin by Sephadex G200 chromatography [37] in G-buffer (5 mM Tris-HCl pH 7.7, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.01% sodium azide). Cys-375 pyrene labeled actin was prepared as described previously by Brenner and Korn [38]. We isolated profilin I and II from bovine spleen and brain respectively, as described previously [34,39,40]. We dialyzed profilin against HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20, Pharmacia) for use in the Biacore experiments or against G-buffer for the fluorimetric assays. We prepared human plasma gelsolin following the procedure of Bryan [41].

We chemically synthesized thymosin β4 and poly(L-proline) peptides on a model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA, USA), and purified them using reversed phase HPLC. We used electrospray mass spectrometry to assess the mass and purity of the synthesized peptides. The proline peptides used in the fluorimetric assays had the following sequences: Ac.$GP_5GP_5GP_5$GL (peptVASPwt) and Ac.GGP_5GL (peptVASPs), both derived from the VASP sequence, and a mutant peptide, Ac.CGP_{17}GL (peptVASP(G/P)), in which the glycines are substituted by prolines. The analogous peptides used in the Biacore experiments, have an amino terminal cysteine. The latter amino acid was biotinylated with EZ-LinkIodoacetyl-LC-Biotin (Pierce). The biotinylated peptide was purified by reversed phase HPLC.

2.2. BIACORE experiments

We determined the affinity and stoichiometry of the profilin-peptide interaction using a BIACORE X (Pharmacia) with two flow cells. We immobilized, by non-covalent capture, biotinylated peptide (the ligand) on a streptavidin coated sensor chip (Pharmacia) in the first flow cell using a flow of 5 μl/min HBS buffer (Pharmacia). Different concentrations of profilin (the analyte) were passed over the sensor chip at 20 μl/min HBS buffer. We chose this flow rate to exclude concentration effects due to mass transport limitations and to minimize the buffer jump. The second flow cell was used as the reference cell. The interaction between analyte and ligand was monitored as a kinetic curve (being the subtraction of the curves in flow cells one and two) with an association, an equilibrium and a dissociation phase.

We used the following formula to interpret the results of the Biacore kinetic curves

$$\mathrm{RUmax} = M_a/M_l \cdot \mathrm{RU}_l \cdot S$$

RUmax is the maximum net increase of response units (RU), which can be obtained at equilibrium when monitoring the interaction of analyte with immobilized ligand on the sensor chip. RU_1 is the net increase of RUs in flow cell one obtained at the end of the immobilization procedure. M_a is the molecular mass of the analyte (profilin I or II, 14 970 Da and 14 943 Da, respectively), M_l is the molecular mass of the ligand (biotinylated forms of peptide, peptVASPwt: 2343 Da and peptVASPs: 1313 Da and peptVASP(G/P): 2424 Da), S is the number of binding sites for the interaction between analyte and ligand.

From the Biacore kinetic curves, one can deduce the equilibrium dissociation constant for the interaction between analyte and ligand, K_d. It was proven theoretically that the K_d is that concentration of analyte needed to cause a half maximum net RU increase at equilibrium (RUmax/2).

2.3. Spectrofluorimetry

In our fluorimetric assays, we studied the polymerization of actin (5.5% of the total actin concentration was pyrene labeled actin) in combination with various proteins as a function of time using a Hitachi F-4500 spectrophotometer (excitation and emission wavelength were set at 365 nm and 388 nm respectively). Each time we started the polymerization by adding F-buffer (G-buffer+0.1 M KCl+1 mM $MgCl_2$) to the reaction mixture (see Figs. 2 and 4 for compositions). G-actin and thymosin β4 were pre-incubated at room temperature before inducing polymerization. When present together, poly(L-proline) peptides and profilin were pre-incubated (at least 15 min) at room temperature before incubating with G-actin or with the G-actin-thymosin β4 mixture. The actin used in all the measurements was always pre-incubated at room temperature for an equal time so as to exclude temperature effects between the different assays. To obtain capped actin nuclei we prepared gelsolin capped actin filaments which

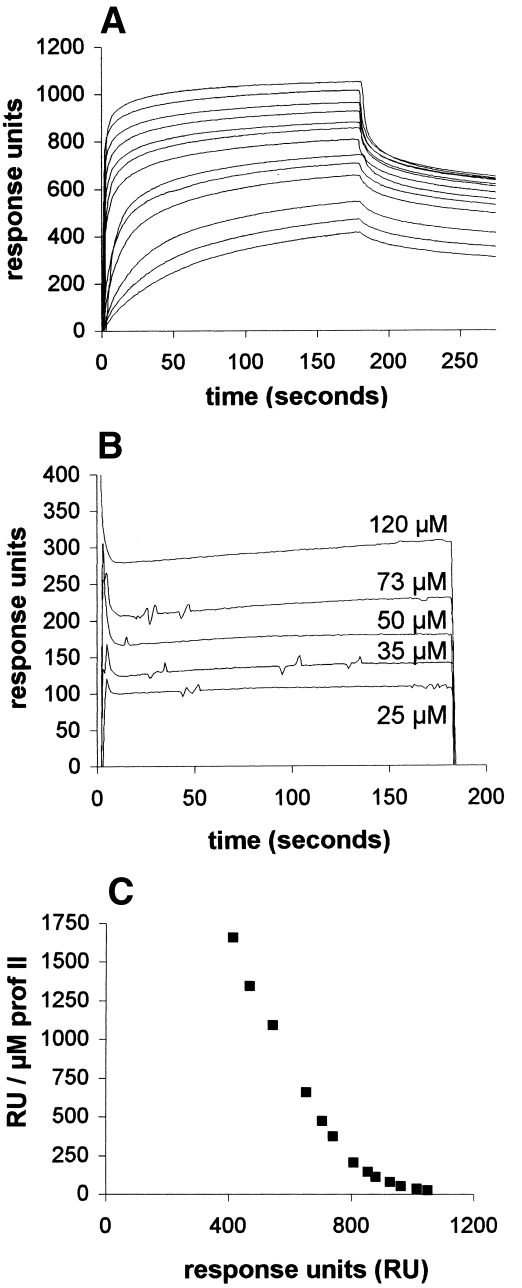

Fig. 1. Biacore binding curves indicate profilin II binds as a dimer to peptVASPwt. A: Profilin II in HBS buffer was sent over the immobilized peptide. The concentrations of profilin II are in descending order, 44 μM, 30 μM, 20 μM, 12 μM, 8 μM, 6 μM, 4 μM, 2 μM, 1.5 μM, 1 μM, 0.5 μM, 0.35 μM and 0.25 μM. Association starts at 0 s, dissociation at time 180 s. B: A similar experiment for profilin I, using concentrations of 120 μM, 73 μM, 50 μM, 35 μM and 25 μM. Note the difference in reaction rates between the two profilin isoforms for their interaction with the immobilized peptide. C: Plot of RU/profilin II concentration versus RU at time 175 s, from the data in A, suggesting the existence of multiple binding events (for details see [65]).

Table 1
Summary of the Biacore experiments with the different proline rich peptides and profilins

	PeptVASPwt Ac.CGP$_5$GP$_5$GP$_5$GL		PeptVASPs Ac.CGGP$_5$GL	PeptVASP(G/P) Ac.CGP$_{17}$GL
RUcoupled	87 RU	60 RU	67 RU	60 RU
	Profilin II	Profilin I	Profilin II	Profilin II
RUmax^t ($S=1$)	555 RU	384 RU	763 RU	370 RU
RUmax^t ($S=2$)	1110 RU	767 RU		740 RU
RU at [prof]	1052 RU at 44 μM	307 RU at 120 μM	52 RU at 35 μM	545 RU at 19 μM
RU at [prof]	545 RU at 0.5 μM	182 RU at 50 μM		183 RU at 1.9 μM (did not reach equilibrium)

Peptides were immobilized on the sensor chip to the indicated response units level (RUcoupled). RUmax^t is the calculated maximal response at a given stoichiometry (S) of profilin molecules. RU is the experimentally obtained value by sending profilin (I or II), at the indicated concentration, over the peptide on the chip.

were diluted into G-actin (5.5% pyrene labeled) to a final concentration of 10 μM actin and 40 nM gelsolin for each sample. Various proteins, with or without peptVASPwt, were then incubated with the actin sample in the same way as described above (see Fig. 5). Polymerization was started by adding F-buffer.

Additionally, we carried out fluorimetric measurements at steady state. F-actin (7% pyrene labeled) was capped with gelsolin (1:330) and diluted into F-buffer alone or into F-buffer with 3 μM profilin or into F-buffer with 3 μM profilin and 3 μM of the peptVASPwt, to obtain a concentration range of actin from 0 μM to 6 μM. The samples were incubated overnight at room temperature in the dark to reach steady state. By measuring the pyrene fluorescence, the amount of unpolymerized actin was determined [29]. For another experiment at steady state we incubated 4 μM F-actin (5.5% pyrene labeled) capped with gelsolin (1:330), with a concentration range of the VASP peptide (0–100 μM final concentration) in the absence and in the presence of 3 μM profilin II.

3. Results

3.1. Stoichiometry and equilibrium dissociation constant of the interaction between the poly(L-proline) peptide of VASP and profilin

We immobilized biotinylated peptVASPwt on the sensor chip to a level of 87 response units (RU). We monitored the kinetics of the interactions with this peptide using a concentration range of profilin II (0.25 μM–44 μM) in HBS buffer (Fig. 1A). The binding curves at the higher concentrations converge between 1000 and 1100 RU indicating the maximum level of response (RUmax) is approached and suggesting a stoichiometry of two profilin II molecules for one peptVASPwt (RUmax^t = 1110 for $S=2$, see Table 1). A plot of RU/profilin concentration versus RU (at time 175 s) (Fig. 1C) also indicates the existence of multiple binding events as no linear curve can be drawn through the data points. The curve approaches the X-axis at 1051 RU, close to the theoretically expected value of RUmax^t for a stoichiometry of two to one. At present we cannot exclude there is a third binding event with lower affinity but we note that in that case RUmax^t would have to be 1664 RU. The global K_d value derived from the Biacore binding curves, i.e. that concentration yielding RUmax/2, is 0.5 μM.

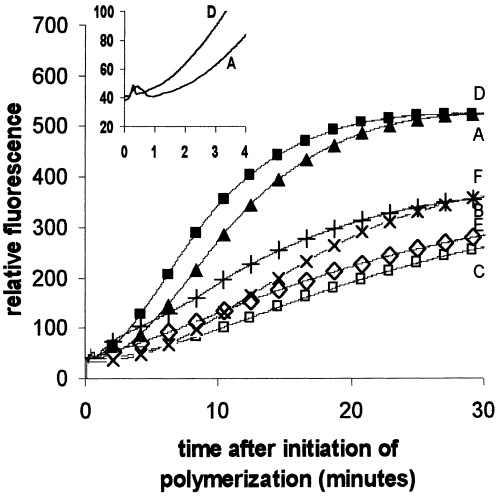

Fig. 2. The profilin II-peptVASPwt complex restores actin polymerization in the presence of thymosin β4 when barbed ends are free. We monitored the polymerization of either 10 μM actin alone (5.5% pyrene labeled) (curve A, ▲), with 10 μM thymosin β4 (curve B, ×), with 10 μM thymosin β4 and 5 μM profilin II (curve C, □), with 10 μM thymosin β4, 5 μM profilin II and 5 μM peptVASPwt (curve D, ■), with 10 μM thymosin β4, 5 μM profilin II and 5 μM of the short peptVASPs (curve E, ◇), or with 10 μM thymosin β4, 5 μM profilin II and 5 μM of the mutant peptVASP(G/P) (curve F, +). The inset shows the early nucleation phase for curves A and D only.

We performed the same analysis for profilin I, we immobilized 60 RU of biotinylated peptVASPwt and we used a concentration range of 25–120 μM profilin I (Fig. 1B). In this case the results are less unambiguous because of a rather low response even at high profilin concentrations. Two scenarios are possible for interpreting the interaction curves (Table 1). If only a 1:1 complex can be formed, the theoretical RUmax is 384 RU. RUmax/2 is then reached at about 50 μM profilin I, and this would be approximately the equilibrium dissociation constant. In contrast, if a 2:1 complex is formed, RUmax^t is 767 RU and RUmax/2 is not reached at 120 μM profilin I. Consequently the K_d must then be higher and this result is more consistent with those of Petrella et al. [42], who determined a K_d of 500 μM for profilin I when studying the interaction of profilin I with a slightly shorter poly(L-proline) peptide, and with the crystal structure of profilin I in complex with a proline peptide [43]. Thus, we can expect dimerization is also taking place but with a much higher equilibrium dissociation constant compared to profilin II.

The steepness of the curves is an indication of the fundamentally different reaction rates for the two profilin isoforms. The association and dissociation rate constants of the interaction of peptVASPwt with profilin I are much higher in comparison to the interaction with profilin II. From the curves, one can in theory also derive the value of the association and dissociation rate of the interaction. However, in the current Biacore software (version 3.0), a model for describing the multiple or co-operative binding of two identical analytes to one ligand is not available.

We also performed analogous Biacore experiments using other forms of biotinylated proline rich peptides and profilin

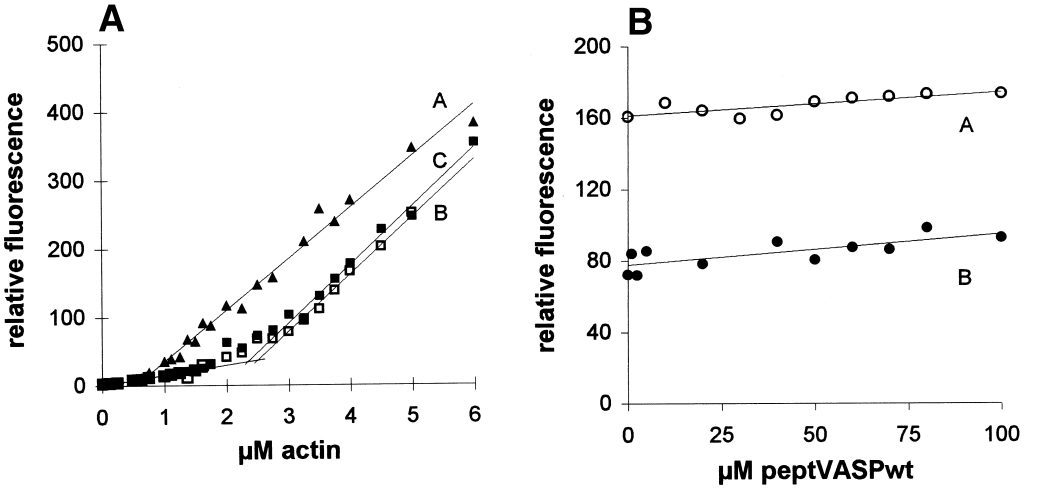

Fig. 3. peptVASPwt does not alter the affinity of profilin II for actin. A: F-actin was capped with gelsolin (gelsolin:actin ratio 1:330) and diluted into F-buffer to the concentration indicated on the *X*-axis. Curve A (▲) represents the actin control curve, in curve B (□) actin was incubated with 3 μM profilin II and in curve C (■) with 3 μM profilin II and 3 μM peptVASPwt. B: 4 μM F-actin (5.5% pyrene labeled) was capped with 12 nM gelsolin (1:330) and incubated with peptVASPwt, in a concentration range from 0 μM to 100 μM, in the absence (curve A, ○) or in the presence of 3 μM profilin II (curve B, ●).

II. We immobilized either a shorter peptide, peptVASPs, having only one GP_5 sequence instead of three repeats, or the peptVASP(G/P) peptide with the same length as the peptVASPwt but in which the glycine residues were substituted by prolines. We found a much lower affinity of profilin II for the short peptide (Table 1). When a relatively high concentration of profilin II was sent over the immobilized peptide only a small signal in comparison to the maximal possible response, was monitored. The peptide without glycine residues has a reasonably high affinity for profilin II because 19 μM of profilin II causes a signal close to the theoretical maximal response.

3.2. *The interaction between profilin II and the poly(L-proline) peptide of VASP overcomes the sequestering activity of thymosin β4*

The observation that profilin II dimerizes upon binding peptVASPwt combined with the effect of profilin I on the polymerization of actin with free barbed ends [29] urged us to study the effect of the peptVASPwt-profilin II complex on the polymerization of actin. First we focused on the polymerization of actin with free barbed ends. In Fig. 2, we followed the polymerization of 10 μM actin, either alone (curve A), in the presence of 10 μM thymosin β4 (curve B), with 5 μM profilin II and 10 μM thymosin β4 (curve C) or with the pre-incubated mixture of 5 μM profilin II and 5 μM peptVASPwt added to the G-actin-thymosin β4 mixture (curve D).

As expected, we observed a reduced and decelerated actin polymerization in the presence of thymosin β4 due to the inhibition of both actin nucleation and polymerization by thymosin β4 (curve B). When profilin II was added to this reaction mixture (curve C), a stronger inhibitory effect was displayed because of the sequestering of G-actin by both proteins, consistent with the findings of Pantaloni and Carlier [28] who found similar effects for profilin I and thymosin β4. Surprisingly, profilin II pre-incubated with peptVASPwt, at concentrations where a significant amount of complex is formed, fully restores actin polymerization in the presence of thymosin β4 (compare curves D and A with C). Remarkably also the nucleation period seems to be a little bit shorter (Fig. 2, inset). Both these effects cannot be attributed to an interaction between actin and the peptide because the polymerization curve of actin pre-incubated with peptVASPwt alone (curve not shown) coincides with the polymerization curve of actin alone.

One could argue that the binding of the peptVASPwt to profilin II prevents the latter from interacting with G-actin, hence profilin II could no longer sequester G-actin and consequently actin polymerization would be less inhibited (compared to the reaction without peptide). Three lines of evidence indicate this is not the case. First, we observed that the polymerization curve of actin in the presence of profilin II, thymosin β4 and the peptide (Fig. 2, curve D) reaches equilibrium at a higher value than the polymerization curve of

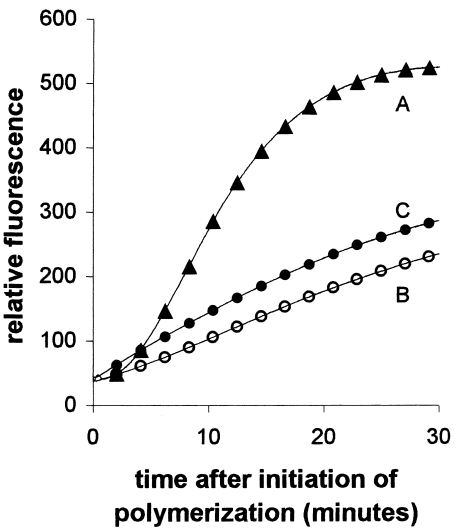

Fig. 4. Profilin I, in the presence of peptVASPwt, does not restore on actin polymerization. We monitored the polymerization of either 10 μM actin alone (curve A, ▲), in the presence of 10 μM thymosin β4 and 5 μM profilin I (curve B, ○) or in the presence of 10 μM thymosin β4, 5 μM profilin I and 5 μM peptVASPwt (curve C, ●).

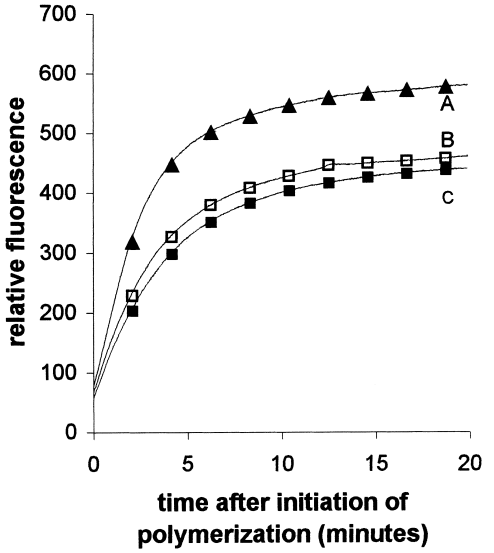

Fig. 5. The peptVASPwt-profilin II complex does not promote polymerization when barbed ends are capped. We followed the polymerization of 10 μM actin capped with 40 nM gelsolin (1:250) (curve A, ▲), in the presence of 10 μM thymosin β4 and 5 μM profilin II (curve B, □), and in the presence of 10 μM thymosin β4, 5 μM profilin II and 5 μM peptVASPwt (curve C, ■).

actin and thymosin β4 alone (Fig. 2, curve B). Second, we routinely observe a ternary complex between poly(L-proline), profilin and actin during purification (see for instance Fig. 5 in [34]). Third, we performed a profilin II sequestration assay (3 μM profilin II, Fig. 3A) using capped actin filaments in the presence of 3 μM VASP peptide, a concentration where significant amounts of peptide-profilin II complexes will be formed. If this results in less profilin for sequestration of actin, or if the profilin II in complex with the VASP peptide has a much reduced affinity for actin, this would shift the actin profilin II peptide curve (curve C) towards the curve for actin alone (curve A). This is not what we observe, adding the VASP peptide to the reaction hardly shifts the curve, suggesting the various profilin species in the different possible reactions have a similar affinity for actin. Moreover, we carried out a control experiment (Fig. 3B) in which a constant amount of capped F-actin filaments, 4 μM, with a constant amount of profilin II, 3 μM, but with varying concentrations of the VASP peptide from 0 to 100 μM, were incubated to reach equilibrium. We observe a more or less constant value for the pyrene fluorescence of the different samples, suggesting that the affinity of profilin II for actin does not change to a great extent when profilin is in complex with the VASP peptide.

We also investigated variants of peptVASPwt (Fig. 2). As already mentioned above, actin polymerization inhibited by thymosin β4 and profilin II is restored when this peptide is present. When we performed a similar experiment with a shorter variant, with only one GP_5 repeat, peptVASPs (curve E), we observed no restoration of actin polymerization, likely because of the lower affinity and the lack of dimerization of profilin II on this peptide (Table 1). In the case of the peptide with the glycine residues substituted by prolines, peptVASP(G/P) (curve F) we observe only partial restoration of actin polymerization, suggesting that the presence of glycine residues between the three proline stretches in the peptVASPwt may be important to mediate the effect of profilin II on actin polymerization.

3.3. Actin polymerization is not restored by profilin I in the presence of peptVASPwt

We performed a similar experiment using profilin I (Fig. 4). As expected, polymerization of 10 μM actin in the presence of 5 μM profilin I and 10 μM thymosin β4 is nearly completely abolished due to combined inhibition of nucleation of polymerization by profilin I and thymosin β4 (curve B). However, in contrast to the results for profilin II, we observe no restoration of actin polymerization in the presence of 5 μM peptVASPwt (curve C). We explain this by the lower affinity of this profilin isoform for the peptide (as evidenced by the Biacore experiments, Fig. 1B and Table 1), under the experimental conditions used almost no peptide-profilin I complexes are formed.

3.4. Polymerization is not restored by the profilin II-peptVASPwt complex when barbed filament ends are capped

In the model proposed by Pantaloni and Carlier [29], profilin I-G-ATP-actin complexes participate in the polymerization by adding to free barbed ends of actin filaments. If profilin II in complex with peptVASPwt follows a similar mechanism restoration of polymerization should not happen when barbed ends are not available. Therefore, we incubated gelsolin capped F-actin nuclei (10 μM actin, 40 nM gelsolin) and followed the polymerization of actin alone (Fig. 5, curve A), and of actin in the presence of 10 μM thymosin β4 and 5 μM of profilin II without (curve B) or with (curve C) 5 μM peptVASPwt. We observe no difference between the curve

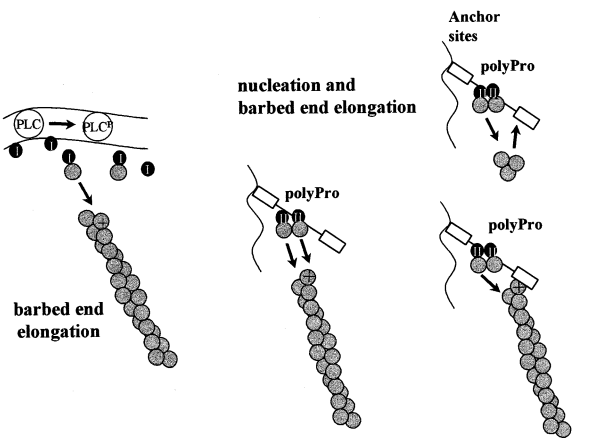

Fig. 6. Model for the differential regulation of actin polymerization by profilin I and II. The activity of profilin I (left) is under control of phospholipase C (PLC) [54]. Upon phosphorylation of the latter, profilin I is released from the membrane and can bind G-actin. This profilin-actin complex adds onto the barbed end of uncapped filaments, hence promoting the elongation step in the actin polymerization cycle. Profilin II (right) dimerizes with a high affinity onto the proline rich stretches of proteins. These proteins may be located in anchor sites and linked to proteins in focal adhesion sites or in *Listeria* tails. The dimer of profilin II on these proline stretches binds two actin monomers and promotes actin nucleation and actin elongation. Some of these proline rich proteins are known to bind directly to actin [60–62]. The barbed end of the actin filaments is indicated with (+).

with and without peptide, indicating profilin II acts in both cases as a pure G-actin sequestering agent (in analogy with profilin I [29]) and consequently the interaction no longer restores actin polymerization when the barbed ends are capped.

4. Discussion

In this paper we studied the kinetic properties of the interaction of profilin with a proline rich peptide derived from VASP and the effect of this interaction on actin polymerization. Using Biacore experiments, we showed that profilin II (and likely also profilin I) forms dimers upon binding the wild-type VASP peptide, peptVASPwt. For the equilibrium dissociation constant of the interaction between profilin II and peptVASPwt we derived a value of approximately 0.5 μM. For the interaction of profilin I with this peptide, we estimate a K_d that will be higher than 120 μM, in agreement with previous reported values for the interaction of this profilin isoform with slightly different peptides [42,44]. The observation that stable profilin II-peptVASPwt complexes can be made, prompted us to investigate their effect on actin polymerization. At high concentrations, profilins are strong inhibitors of nucleation [29,45,46], an effect that is augmented by thymosin β4. Consequently, little actin polymerization occurs (in the time frame monitored), when both these actin binding proteins are present. However, in the presence of peptVASPwt, the combined capacity of profilin II and thymosin β4 to inhibit nucleation is completely abolished. This positive effect depends on the formation of profilin II-peptVASPwt complexes, because profilin I, which has low affinity for this peptide, cannot restore the polymerization. Also, free barbed ends are required, as capping of filaments results in normal sequestering in the presence of profilin II-peptVASPwt complexes. The latter experiment also indicates that the peptide does not change the affinity of profilin for actin significantly, in agreement with data presented in Fig. 3 and with data that demonstrate formation of ternary complexes between actin, profilin and poly(L-proline), [7,34,44,47]. From our polymerization data, it appears that the nucleation and perhaps also the elongation phases are affected when peptVASPwt is present. The latter is consistent with the known role of profilin in barbed end elongation as reported previously [27,29,46,48]. Thus, it appears that actin bound to profilin II-peptVASPwt complexes can add more efficiently to plus ends of the filament than actin-profilin II complexes. In theory, the state of the nucleotide bound to G-actin could also influence the reaction, as profilin is known to catalyze the exchange of ADP for ATP, [49,50]. We expect this effect will be of minor influence in our experiments as we start with ATP-actin and no significant treadmilling occurs at the initial stages of polymerization.

We sought to explain this enhanced polymerization mediated by the profilin II-peptVASPwt complexes in a structural way. Therefore, we modeled a profilin II dimer on the peptide using the published crystal structure of the profilin I-L-Pro_{10} complex [43]. We then fitted this model to the ribbon [51] or filament model of actin [52], using the profilin I-actin structure as a template [51]. In neither case do the structural models allow one to explain why the profilin II-peptide complex results in more efficient polymerization. However, we note that the profilin molecules in the profilin I-L-Pro_{10} complex do not interact [43]. In contrast, our Biacore data are suggestive of co-operative binding of profilin II to the peptVASPwt, necessitating profilin II molecules to interact with each other. In addition, peptVASPwt may have a less rigid structure than L-Pro_{10} due to the presence of the glycine residues. These appear to play a role in the beneficial effect on actin polymerization because the peptide composed of 17 proline residues, peptVASP(G/P), only partially restores actin polymerization (Fig. 2A, curve F). However, the affinity of profilin II for this peptide is slightly reduced. In contrast, profilin I binds better to polyproline peptides of defined length than to their counterparts containing glycines [42]. The importance of the glycine residues also follows from an observation by Kang et al. [53], who studied *Listeria* motility. They reported an inhibition of motility, due to competitive blocking of VASP-binding to profilin, when they injected a $GP_5GP_5GP_5$ peptide into *Listeria* infected PtK2 cells but not when a pure poly(L-proline) peptide was used.

The results presented in this paper combined with the already known different affinities of the different profilin isoforms for PIP_2 and proline rich proteins [35] allow us to present a model (Fig. 6) that provides insight into the relation between actin based motility and the profilin isoforms. Profilin I is known to be preferentially associated with PIP_2 [35] and under this condition cannot bind actin nor promote actin polymerization [8]. Upon activation, phospholipase C is phosphorylated and hydrolyzes PIP_2 causing the release of profilin I from membranes [54]. This profilin isoform can then modulate the actin polymerization cycle [54] depending on the status of the plus end of the filament. In contrast, profilin II can be recruited by proteins containing proline stretches of sufficient length [35,42,44] such as VASP and Mena, localized in focal adhesions [13,15] or in the tail of *Listeria* [15,55,56]. Since VASP and presumably also the other Ena/VASP family proteins are oligomeric [12,57], this may result locally in high concentrations of the dimeric form of profilin II. This form may then help in more efficient nucleation of actin polymerization and elongation because our data show that rapid polymerization via the barbed end can be initiated by profilin bound to proline rich sequences. This may co-operate with the modest nucleation activity of the Arp 2/3 complex as this complex operates at the opposite end of the filament [58,59]. The effect we observe may be more complex with the intact proteins as several of these, like VASP and WASP related proteins, have been shown also to bind directly to actin [60–62] and this will complicate kinetics. Proline rich proteins are under control of regulation, VASP is a target of cAMP and cGMP dependent kinases [63] and Mena binds SH3 domains [15]. WASP [64], Diaphanous [23] and Bni [16,17] are under control of small GTPases. Future experiments will show if any of these types of ligands regulate dimer formation of profilin II coupled to enhanced polymerization.

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