FEBS Letters 397 (1996) 316–320 FEBS 17809

Probing phosphatidylinositolphosphates and adenosinenucleotides on talin nucleated actin polymerization

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Received 4 October 1996

Abstract We have investigated the binding of PI, PIP and PIP_2 to talin and the effect of phosphoinositides and adenosinenucleotides on talin-induced actin polymerization. At physiological salt concentrations, talin coprecipitates with liposomes when containing phosphoinositides but not when containing PI. The nucleating effect of talin as reflected by a twofold increase of fluorescence during the polymerization of actin labelled with NBD is not inhibited by phosphoinositides. The polymerization of ADP-actin versus ATP-actin was investigated in the presence and absence of talin by NBD fluorescence. ADP-actin nucleation induced by talin is comparably efficient as with ATP-actin. These experimental findings in summary have implications when evaluating the role of talin during cell activation.

Key words: Actin; Talin; Phosphoinositide; ADP-actin polymerization

1. Introduction

Investigations over the past few years have lent support to the idea that talin serves as a key protein for cytoskeleton/membrane interactions (for review see [1]). Evidence for this hypothesis comes from the findings that (1) talin binds actin [2,3], (2) talin binds to negatively charged phospholipids [4–6], (3) talin can be reconstituted into lipid mono- and bilayers [7,3] and (4) talin in its membrane bound state is able to promote actin filament assembly at the lipid interface [8,9,11]. Structural studies have revealed that all these functions are based on talin operating as a homodimer, a dumbbell-shaped elongated molecule of 51 nm in length with three characteristic areas of condensed mass [10]. Fluorescence assays with NBD-labelled actin polymerized in the presence of talin do not show any lag-phase but instead exhibit a rapid increase of fluorescence reflecting an increased amount of F-actin in comparison with controls. Conversely, when viewed by electron microscopy, the early polymerization time course ($\leq$5 min) is characterized by the existence of only short actin filaments ($\leq$1 μm) in length. Hence, it is deduced, that talin increases actin filament number concentration over filament length.

Recent reports point to the importance of the balance between ADP-actin and ATP-actin in cells, especially when actin monomer sequestering proteins such as profilin and thymosin β_4 are present [12]. Some proteins, such as profilin, will accelerate nucleotide exchange on actin [12,13], while others, such as cofilin and thymosin β_4, inhibit this exchange. Gelsolin on the other hand reacts much faster with ADP-actin than with ATP-actin [14]. Based on the actual intracellular concentrations of various binding partners it is possible that the cellular source for actin filament elongation is ADP-actin rather than ATP-actin [12].

Against this background it seemed to us of interest to investigate the nucleating effect of talin using ADP-actin versus ATP-actin as nucleation source.

Since the signaling cascade during cell activation gives rise to elevated levels of phosphoinositolphosphatides near the plasma membrane and these may either inhibit or accelerate various actin binding proteins [15], we also tested binding and the effect of inositolphosphates on talin promoted actin filament polymerization.

2. Materials and methods

2.1. Proteins

Talin was purified from human platelets as described [8]. ATP-actin was extracted from skeletal muscle acetone powder [16] and further purified by gel filtration [17]. ADP-actin was obtained by passing conventional actin over a Sephadex G-25 SF (Pharmacia) gel filtration column (2.5×70 cm), equilibrated and eluted with ADP-buffer: 0.5 mM ADP, 0.01 mM $MgCl_2$, 3 mM NaN_3, 5 mM triethanolamine/HCl pH 7.5. 5 ml actin containing 5% NBD-actin was applied to the column and eluted with a flow rate of 20 ml/h. A complete exchange of nucleotides was checked by HPLC analysis. ADP-actin was immediately used for experiments, since it degrades rapidly.

Protein concentrations were determined according to Bradford [18] and the purity was analyzed on SDS-polyacrylamide gels [19]. Concentrations of ATP-actin and ADP-actin were determined photometrically at 290 nm using an absorption coefficient of 24 900 M^{-1} cm^{-1}.

2.2. Fluorescence

Actin polymerization was followed by a 2.2–2.5-fold greater fluorescence intensity of polymeric actin compared to that of monomeric actin [20]. 5% of fluorescently labeled actin was copolymerized with unmodified actin. This low proportion of labeled actin does not significantly alter the polymerization rate or extent of assembly of unmodified actin [21]. The excitation wavelength was 480 nm, and the fluorescence intensity was measured at 540 nm. The fluorescence intensity of monomeric and polymeric actin was calibrated by measuring the fluorescence intensities of monomeric and polymeric actin to evaluate the changes of fluorescence intensities in terms of concentrations of monomeric and polymeric actin.

2.3. Sedimentation assays with phosphoinositol containing liposomes

PC and PS were obtained from Lipid Products, South Nutfield, Surrey, England. PIP_2, PIP and PI were obtained from Sigma Chemicals. In some of the experiments, PIP_2 obtained from Fluka, Switzer-

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PIP_2, phosphatidyl-inositol-4,5-diphosphate; PIP, phosphatidylinositol-4-monophosphate; PI, phosphatidylinositol; NBD-actin, 7-chloro-4-nitro-benzeno-2-oxa-1,3-diazole actin; ADP-actin, adenosine 5′-diphosphate; ATP-actin, adenosine 5′-triphosphate

PII S0014-5793(96)01203-3

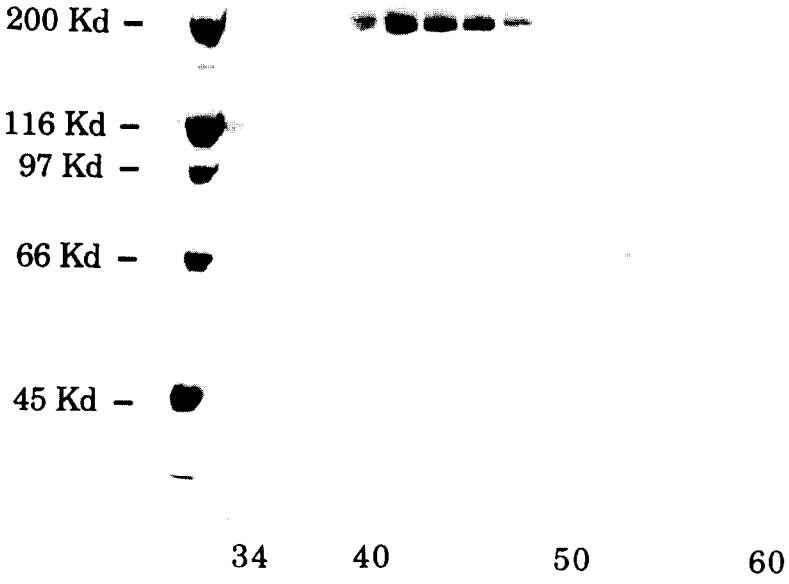

Fig. 1. Purity of talin preparations. SDS-polyacrylamide gel showing peak fractions of purified human platelet talin with 95–98% purity. 5 μg of protein was applied to each lane. Talin runs at approx. 230 kDa as judged by molecular masses of standard proteins (left lane).

land, was used. Large, multilamellar liposomes were prepared from PC, PS, and PI as described [11]. Briefly, lipids in chloroform/methanol were dried under N_2. PIP_2 or PIP was added as a stock solution in 20 mM HEPES pH 7.4, 0.2 mM EGTA to the dried lipids. Lipid mixtures were swollen in this buffer for 3 h at 42°C [22]. Vesicles were then centrifuged at 20 000 × *g* for 20 min at 4°C, and the pellets were resuspended in the same buffer at 5 mg/ml. Talin (dialyzed against 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 15 mM mercaptoethanol) was centrifuged prior to the experiments for 20 min at 20 000 × *g*, followed by protein determination [18]. Talin (0.1 mg/ml, final concentration) was subsequently incubated for 15 min at 22°C in the absence of liposomes, followed by a further incubation in the absence or presence of liposomes (1.25 mg total lipid/ml, final concentration) for 15 min. In some of the experiments, 130 mM KCl was included in the buffer. The samples were kept under nitrogen during incubation with lipids. The mixtures were subsequently centrifuged for 20 min at 20 000 × *g*, 4°C. The pellets were solubilized in 50–100 μl sample buffer [11]. The supernatants were mixed with the corresponding amount of the threefold concentrated sample buffer. After heating the samples for 5–10 min at 95°C, they were applied to 5–10% SDS-polyacrylamide gradient minigels [19]. The amount of protein present in pellets and supernatants was quantified by scanning the bands of the Coomassie blue stained gels. The amount of talin sedimented in the absence of liposomes was always subtracted from that sedimenting in the presence of lipid. The data are given as mean ± S.D. of *n* experiments. Differences between data were analyzed with the Student's *t*-test for paired data, with a *P* value of < 0.05 considered significant.

Preparation of phosphoinositol containing solutions for fluorescence assays was performed in talin buffer: 50 mM Tris-HCl pH 8.0; 3 mM EDTA, 0.1 mM DTT without sonication.

3. Results

3.1. Binding of talin to phosphoinositol containing liposomes

Talin is purified from human platelets with 95–98% purity as estimated by SDS-gel electrophoresis (Fig. 1). We have previously shown that this fraction of purified talin consists of a homogeneous population of molecules as judged by electron microscopy representing homodimeric talin in the native state [10]. We have now assayed the capacity of talin to interact with various phosphoinositol lipids (PI, PIP and PIP_2) in the absence of salt and by increasing the ionic strength up to 130 mM KCl (Fig. 2). In control experiments, talin, at low ionic strength, cosediments with mixed PS/PC liposomes, only when the PS concentration is higher than 50%. In contrast, talin cosedimented significantly ($P < 0.0125$) with PC liposomes containing 20% PIP_2 or 20% PIP in the presence of 130 mM KCl, but not at low ionic strength (Fig. 2). Talin showed selectivity for PIP_2 and PIP, as it did not significantly cosediment with liposomes containing 20% PI.

Cosedimentation with PC liposomes containing 20% PIP_2 or PIP was also markedly increased when compared to cosedimentation with liposomes containing 20% PS.

A control protein, bovine serum albumin (BSA), under identical conditions did not significantly cosediment with PS or PIP_2 containing liposomes: 5% of total BSA sedimented in

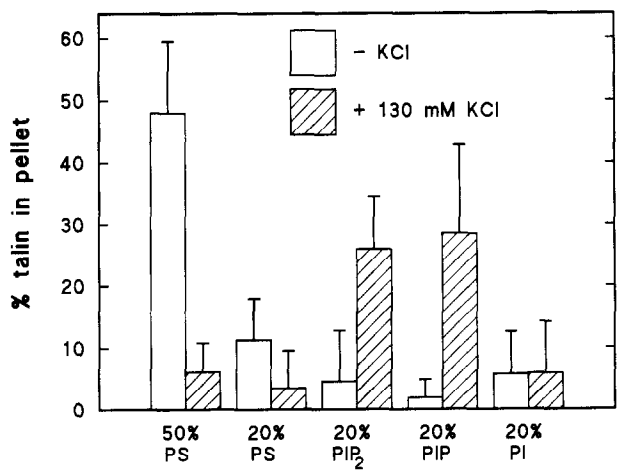

Fig. 2. Cosedimentation of talin with large liposomes containing different types of phospholipids. Talin (0.1 mg/ml) was incubated for 15 min at 22°C in the absence or presence of 130 mM KCl, and in the presence of large liposomes containing 50% PS, or 80% PC and 20% PS, or 20% PIP_2, 20% PIP, 20% PI, as indicated. After centrifugation at 20 000 × *g*, pellets and supernatants were analyzed by separation on 5–10% gradient gels and scanning of the Coomassie blue stained bands (mean ± S.D., $n = 3$–4).

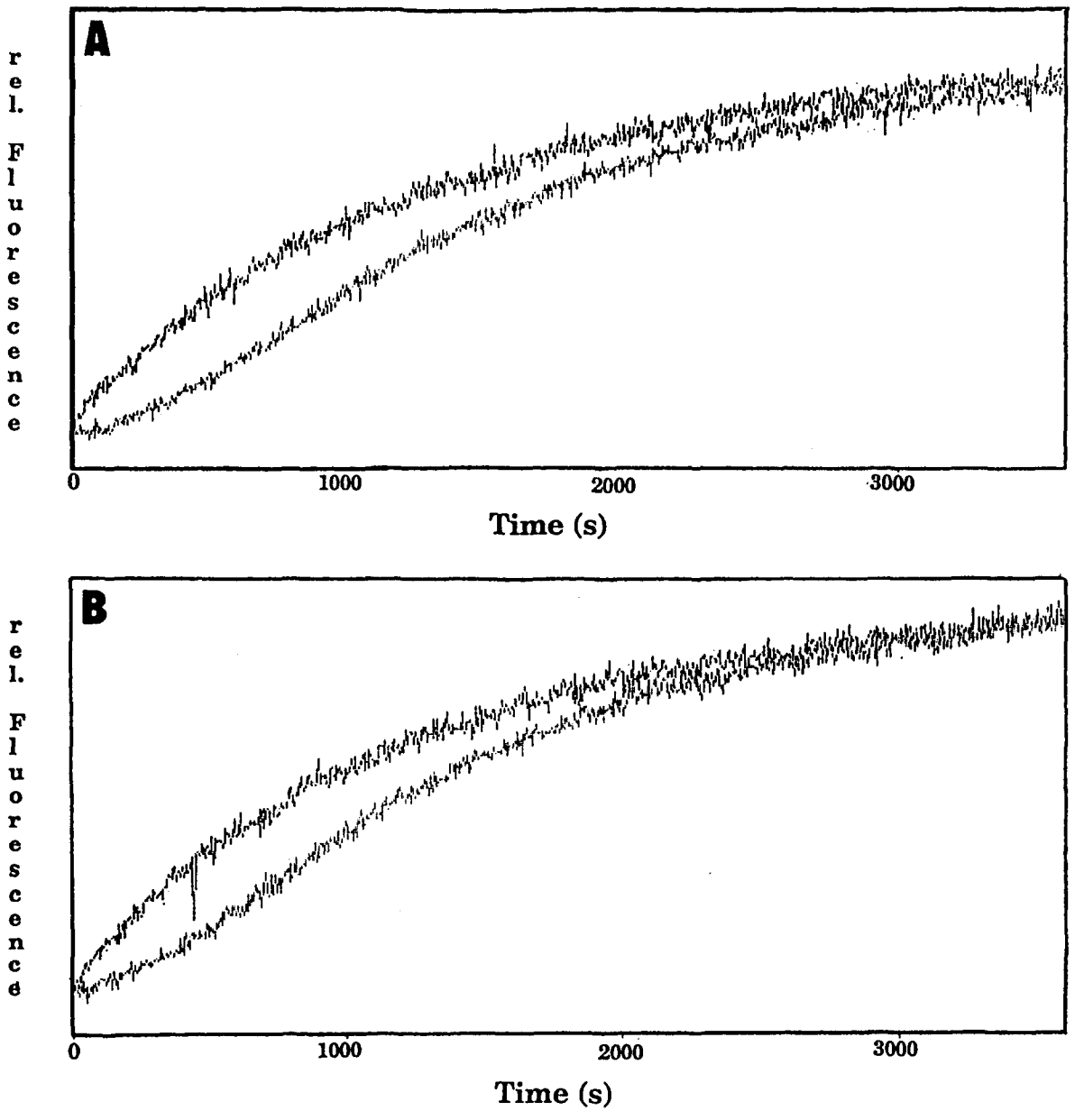

Fig. 3. Influence of PIP_2 on talin nucleated actin polymerization. A: Fluorescence traces of polymerizing NBD-labeled actin (total concentration 3 μM) in the absence (lower trace) and presence of 1.5 μM talin (upper trace). The actin concentration (95% actin plus 5% NBD-actin) was kept constant in a total volume of 0.75 ml. Polymerization was started by adding polymerization buffer: 2 mM Tris-HCl pH 8.0, 0.2 mM $MgCl_2$, 100 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT, 0.005% NaN_3. B: Conditions as in A, but with the addition of PIP_2. Talin was preincubated with 45 μM PIP_2 micelles (molar ratio PIP_2/talin: 30/1) for 30 min at 20°C (upper trace). As a control, actin alone was polymerized in the presence of 45 μM PIP_2 (lower trace).

the presence of PS liposomes as compared to 2% in its absence.

3.2. *Influence of phosphoinositols on talin nucleated actin polymerization*

The fluorescence increase of polymerizing actin containing 5% NBD-actin (total concentration 3 μM) was monitored in the presence (1.5 μM) and absence of talin and after the addition of various concentrations of phosphoinositols. In control samples (Fig. 3A), the addition of talin resulted in the previously reported increase in fluorescence during the early time course of polymerization. Polymerization of actin started immediately without an apparent lag-phase with an approximately twofold increase in fluorescence, which reflected the rapid formation of polymerization competent nuclei transiently stabilized by talin.

In Fig. 3B talin was preincubated with PIP_2 at a molar concentration PIP_2/talin of 30:1 for 30 min at 20°C. As with PI and PIP at various concentrations (data not shown) the incubation with PIP_2 did not have any detectable influence on talin promoted actin filament nucleation, since the fluorescent increase was comparable to controls without any added phosphoinositols.

3.3. *Talin nucleation of ADP-actin versus ATP-actin*

ADP-actin also polymerizes into filaments, but the critical

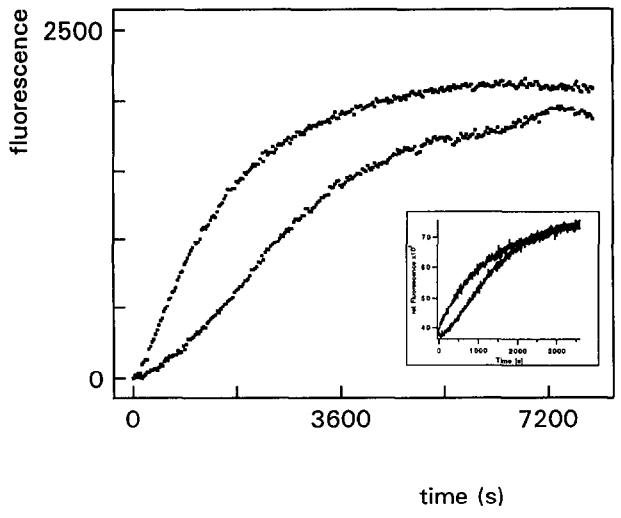

Fig. 4. Talin nucleated actin polymerization using ADP-actin versus ATP-actin as monomers. Fluorescence traces of NBD-labeled (5%) ADP-G-actin (total concentration 3 μM) polymerized in polymerization buffer: 2 mM Tris-HCl, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3 mM EDTA, 0.2 mM DTT in the absence (lower trace) and presence of 1.4 μM talin (upper trace). Under identical conditions ATP-G-actin is polymerized in the absence and presence of talin (inset).

concentration is 5–10-fold higher when compared with ATP-actin [23]. In addition, ADP-actin forms polymerization competent actin nuclei over a much extended time scale ([24], cf. Fig. 4) and monomers associated with a filament end 4–5-fold slower than ATP-actin subunits [25].

Fig. 4 shows the polymerization of ADP-actin with and without added talin. Controls exhibit the classical sigmoidal shape reflecting (i) an extended nucleation phase as the rate limiting reaction during the onset of filament formation, (ii) filament elongation and (iii) steady state in the plateau regime. In the presence of talin, an always hyperbolic shape is monitored. The fluorescence without any apparent lag-phase is increased by a factor of ≥2 as deduced from $t_{1/2}$ and the nucleation phase is decreased markedly.

In effectiveness, the talin enhanced filament formation of ADP-actin subunits is comparable with that of ATP-actin (Fig. 4, inset). Though, as expected, the time scales differ, due to the above mentioned kinetic differences, we measured a comparable increase of fluorescence induced by talin for both conditions, independent of whether ADP-actin or ATP-actin is used. Hence, it is suggested that talin promoted filament nucleation occurs independent of the available nucleotide source on actin subunits.

4. Discussion

4.1. The effect of phosphoinositolphosphatides

A transient increase of phosphorylated phosphoinositol lipids is a potential signal for regulating the cytoskeleton during cell activation [15,26]. As a consequence, actin binding proteins that inhibit polymerization, e.g. capping proteins [27], may be deactivated by PIP_2 [28,29]. On the other hand, PIP_2 enhanced binding of crosslinking proteins to actin, such as α-actinin, may stabilize the newly formed filament network [30]. The effect of phosphoinositolphosphates on talin, which is thought to be important in targeting and promoting actin filament assembly at the lipid interface, therefore seemed of special interest to us.

The cosedimentation assays have shown that talin binding to PIP_2 and PIP containing liposomes, but not PI containing liposomes, is markedly increased under physiological salt conditions. Interestingly, the talin related protein ezrin, which shares about 40% sequence homology in its N-terminal head domain with talin [31], has recently been demonstrated to interact selectively with PIP_2 containing liposomes via this N-terminal domain [32]. Hence, homologous lipid interacting domains may be involved. However, talin and ezrin also show some differences in phosphoinositol interaction: talin, but not ezrin, binding to both PIP_2 and PIP is elevated under physiological salt concentrations. On the other hand, ezrin, but not talin, is able to discriminate between PIP_2 and PIP as a substrate. Hence, there exists specificity. For both proteins, interaction with PIP_2 occurs under physiological ionic conditions, but in the absence of salt, talin, unlike ezrin, does not bind significantly to any of the phosphoinositol containing liposomes.

The elevated concentrations of PIP_2 near the plasma membrane during receptor mediated cell activation [26] may therefore be important to anchor talin in the lipid bilayer.

As expected from the various functions which have been attributed to different domains of the talin molecule [11], PIP_2 interaction within the 47 kDa N-terminal head domain of talin should not interfere with the nucleation promoting activity of talin, which resides in the very C-terminal end along the talin sequence. This seems to be the case, since under no conditions tested were phosphoinositolphosphates of any influence on talin nucleated actin polymerization (Fig. 3).

4.2. ADP-actin

As discussed by Goldschmidt-Clermont et al. [12], cells by the rapid turnover of actin filaments may produce a substantial amount of ADP-actin monomers. For these, assembly reactions in general are reduced and unfavorable. Rate limiting are the higher critical concentration and the much slower formation of actin nuclei of ADP-actin. Of advantage would be the binding of ADP-actin to a nucleation promoting protein. Since we have previously reported that talin exhibits such a nucleation promoting activity using ATP-actin [5,8], it was of interest to investigate the influence of talin on ADP-actin polymerization. Indeed, we found a comparable talin induced increase of the polymerization rate when comparing ADP-actin with ATP-actin, reflected by the ≥2-fold increase of NBD fluorescence during the early phase of polymerization. From this we conclude that the talin promoted nucleation of actin filament assembly is equally efficient for ATP-actin and ADP-actin.

In summary, the interaction of two essential cellular metabolites, phosphoinositolphosphates and ADP-actin, with talin and their effect on talin induced actin polymerization are in line with our interpretation of talin function hitherto: elevated concentrations of PIP_2 and PIP resulting from receptor mediated cell activation may help to target and concentrate talin in specific areas of the plasma membrane. These hot spots of accumulated talin, which by immunofluorescence microscopy were shown to closely overlap with nascent actin filament bundles in newly formed leading edges, may provide sufficient concentrated talin for the interaction with actin,

which is of relatively high stoichiometry in vitro when promoting actin filament nucleation. In support of the physiological relevance of talin nucleated actin polymerization, we have demonstrated that even under conditions where the normal ATP-actin pool would be depleted, ADP-actin could serve as an appropriate substrate for talin to exhibit its nucleation promoting activity.

Acknowledgements: The technical assistance of Mrs. H. Kirpal and K. Mujynya-Ludunge is greatly acknowledged. We thank A. Wegner for criticism and advice. This work was supported by Grants Is 25/7-2 and SFB 266 C-5 to G.I. and SFB 394 to A. Wegner from the Deutsche Forschungsgemeinschaft, the Swiss National Foundation for Scientific Research to V.N. and a NATO Collaborative Research grant to W.H.G.

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