The EMBO Journal vol.14 no.8 pp.1583–1589, 1995

The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins

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Communicated by B.M.Jockusch

Profilins are small proteins that form complexes with G-actin and phosphoinositides and are therefore considered to link the microfilament system to signal transduction pathways. In addition, they bind to poly-L-proline, but the biological significance of this interaction is not yet known. The recent molecular cloning of the vasodilator-stimulated phosphoprotein (VASP), an established *in vivo* substrate of cAMP- and cGMP-dependent protein kinases, revealed the presence of a proline-rich domain which prompted us to investigate a possible interaction with profilins. VASP is a microfilament and focal adhesion associated protein which is also concentrated in highly dynamic regions of the cell cortex. Here, we demonstrate that VASP is a natural proline-rich profilin ligand. Human platelet VASP bound directly to purified profilins from human platelets, calf thymus and birch pollen. Moreover, VASP and a novel protein were specifically extracted from total cell lysates by profilin affinity chromatography and subsequently eluted either with poly-L-proline or a peptide corresponding to a proline-rich VASP motif. Finally, the subcellular distributions of VASP and profilin suggest that both proteins also interact within living cells. Our data support the hypothesis that profilin and VASP act in concert to convey signal transduction to actin filament formation.
Key words: actin polymerization/cAMP/cGMP/poly-L-proline/protein kinase

Introduction

Profilins are ubiquitous, small (12–15 kDa) proteins that bind actin, phosphoinositides and poly-L-proline (PLP) and may thus be involved in growth factor-dependent regulation of actin polymerization and cell motility (Goldschmidt-Clermont and Janmey, 1991; Goldschmidt-Clermont *et al.*, 1991a; Machesky and Pollard, 1993; Theriot and Mitchison, 1993; Sohn and Goldschmidt-Clermont, 1994). *In vitro* binding of phosphatidylinositol 4,5-bisphosphate (PIP_2) to profilins interferes with the PIP_2 hydrolysis by soluble phospholipase Cγ (PLCγ), but this inhibition can be overcome by tyrosine phosphorylation of PLCγ (Goldschmidt-Clermont *et al.*, 1991a). These results offer an attractive model of profilins as mediators between signal transduction pathways and the microfilament organization in cellular regions of high dynamic activity (Machesky and Pollard, 1993). By this mechanism profilin may be released from its membrane-bound position, competent to interact with G-actin (Lassing and Lindberg, 1985) and the membrane-apposed ends of actin filaments (Pollard and Cooper, 1984; Kaiser *et al.*, 1986; Pring *et al.*, 1992). Moreover, as has been shown recently (Pantaloni and Carlier, 1993), low amounts of profilin are able to promote extensive F-actin assembly from actin-thymosin β_4 precursor pools, a mechanism that is thought to be a central component of rapid actin filament growth at the plasma membrane in animal cells. The localization of profilin in highly dynamic lamellipodia of fibroblasts (Buß *et al*, 1992) and at the backpole of *Listeria monocytogenes*, a microfilament-recruiting cytopathogenic bacterium (Theriot *et al.*, 1994), is consistent with such a view. However, the underlying molecular details of profilin's role in these processes are largely unknown, and it has been repeatedly suggested that additional regulatory proteins are required (Theriot *et al.*, 1994; Sohn and Goldschmidt-Clermont, 1994). Thus, the identification of natural profilin ligands should contribute to the understanding of the regulation of cytoskeletal dynamics.

In this context, interest has focussed on the PLP binding property shared by profilins from a wide variety of species, including animals, plants, slime moulds, protozoa and yeast (Machesky and Pollard, 1993; Sohn and Goldschmidt-Clermont, 1994), despite a rather limited sequence similarity (Staiger *et al.*, 1993). *In vitro*, peptides containing eight to ten consecutive prolines have been found to be sufficient for profilin binding (Machesky and Pollard, 1993). However, while significant progress has been made with respect to the molecular characterization of the PLP binding site (Björkegren *et al.*, 1993; Haarer *et al.*, 1993; Archer *et al.*, 1994; Metzler *et al.*, 1994), no natural proline-rich ligand has been identified as yet.

Here, we report on the interaction of various profilins with the vasodilator-stimulated phosphoprotein (VASP). VASP was originally discovered, purified and characterized as a protein which is phosphorylated in response to both cAMP- and cGMP-elevating vasodilators and platelet inhibitors mediated by cAMP- and cGMP-dependent protein kinases (cAK, cGK) (Waldmann *et al.*, 1987; Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990; Nolte *et al.*, 1991; Eigenthaler *et al.*, 1992; Butt *et al.*, 1994). Three distinct phosphorylation sites were identified in VASP which are used *in vitro* and in human platelets by both cAK and cGK (Butt *et al.*, 1994). Phosphorylation of VASP in human platelets closely correlates with the

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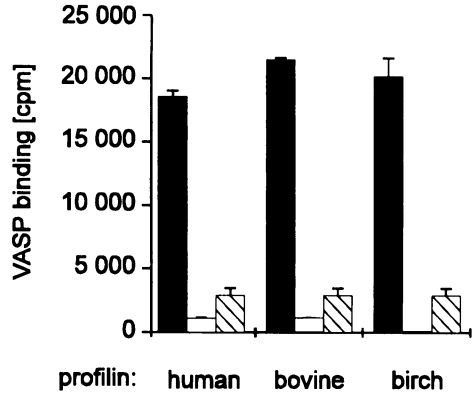

Fig. 1. Solid phase binding assay demonstrating the *in vitro* interaction of VASP purified from human platelets and profilins from different species. Binding of VASP to surface coated human platelets, calf thymus and recombinant birch profilin was detected using a VASP-specific antiserum and [^{125}I]protein A (black columns). Wells without profilin coat (hatched columns) or without VASP incubation (white columns) served as controls. The means and standard deviations of triplicates are indicated. The results shown are a representative of two to four independent experiments using different profilin and VASP preparations.

inhibition of both platelet aggregation and PLC activation (Waldmann *et al.*, 1987; Waldmann and Walter, 1989; Halbrügge *et al.*, 1990; Geiger *et al.*, 1992). Recently, we reported that VASP is associated with microfilaments, focal adhesions and highly dynamic membranes not only in human platelets but also in a wide variety of other cells and tissues, suggesting that VASP, like profilin, is involved in both signal transduction and cellular motility (Reinhard *et al.*, 1992). The biochemical, cell biological and structural properties of VASP (in particular its unusual proline-rich central domain; Haffner *et al.*, 1995) prompted us to investigate a direct interaction of VASP with profilins. Our data identify VASP and an additional protein as natural proline-rich ligands of profilin. Our present results suggest that VASP, like profilin, is a mediator between signal transduction pathways and cell motility, and that these proteins act in concert to confer signal perception to the microfilament system.

Results

VASP binds to both mammalian and plant profilins

A direct interaction between VASP and profilins was initially tested in solid phase binding assays with purified proteins. For this purpose, various profilins were coated to the plastic surface of removable microtiter wells. After blocking of non-specific binding sites, VASP was applied as a soluble ligand. Detection of profilin-bound VASP by a specific polyclonal antiserum (Halbrügge *et al.*, 1990; Reinhard *et al.*, 1992) and [^{125}I]protein A revealed significant VASP binding to immobilized profilins from human platelets and calf thymus (Figure 1). In addition, recombinant birch pollen profilin expressed in and purified from *Escherichia coli* also bound VASP (Figure 1), suggesting that VASP binding, as well as PLP binding, is a common feature of profilins from a wide range of species with only moderate sequence similarity. Moreover, the results indicate that non-specific electrostatic binding is not the cause of this VASP–profilin interaction, since calf

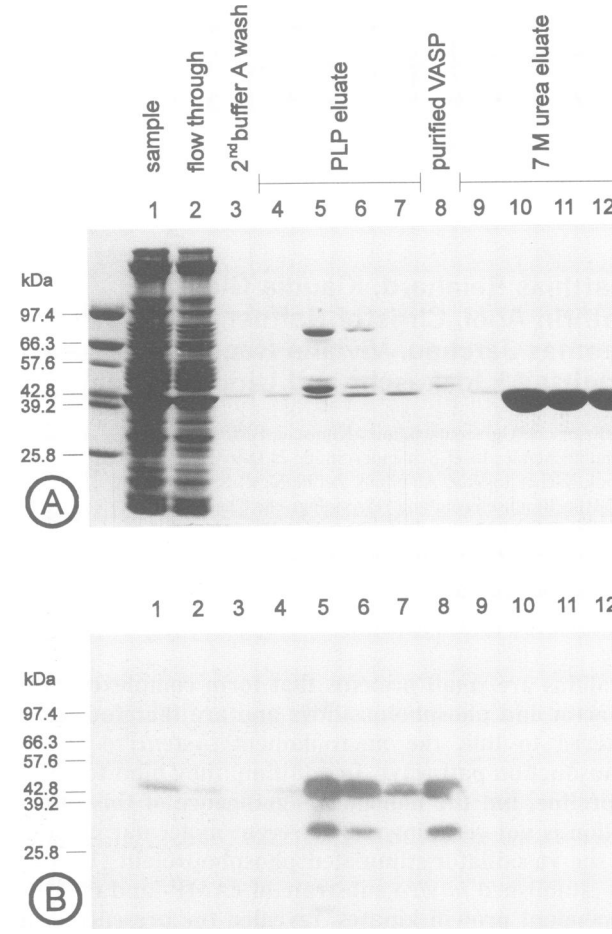

Fig. 2. Profilin affinity chromatography of an NaCl extract of a human platelet lysate revealing the specific elution of 46/50 kDa VASP and a 81 kDa protein by PLP. A profilin affinity matrix was loaded with platelet extract, washed with high- and low-salt buffer (lanes 3) and eluted with PLP (lanes 4–7) and 7 M urea (lanes 9–12). Aliquots of the platelet extract (lanes 1), flow through (lanes 2), TCA-precipitated buffer washes (lanes 3) and column fractions (0.5 ml) eluted with PLP (lanes 4–7) and subsequently, with urea (lanes 9–12), as well as a VASP standard (500 ng, lane 8) were separated by SDS–PAGE and analyzed subsequently by Coomassie Blue staining (**A**) and immunoblotting using affinity-purified anti-VASP antibodies and [^{125}I]protein A (**B**, autoradiograph).

and birch profilins differ vastly in their isoelectric points, being 9.1 (Malm *et al.*, 1983) and 5.6 (Giehl *et al.*, 1994), respectively.

VASP also bound to microtiter wells coated with a 1:1 complex of G-actin and profilin (profilactin; data not shown) suggesting that profilin can interact simultaneously with VASP and G-actin. However, it should be pointed out that there is an equilibrium between the profilactin complex and its individual constituents. Nevertheless, our data are in agreement with the previous observation that profilin simultaneously binds PLP and G-actin (Tanaka and Shibata, 1985).

Profilin affinity chromatography yields VASP and an 81 kDa polypeptide

The specificity and physiological relevance of a direct VASP–profilin interaction were investigated by probing this interaction in complex protein mixtures. Therefore, an NaCl extract of a total platelet lysate was applied to a profilin affinity matrix. Platelets were chosen because

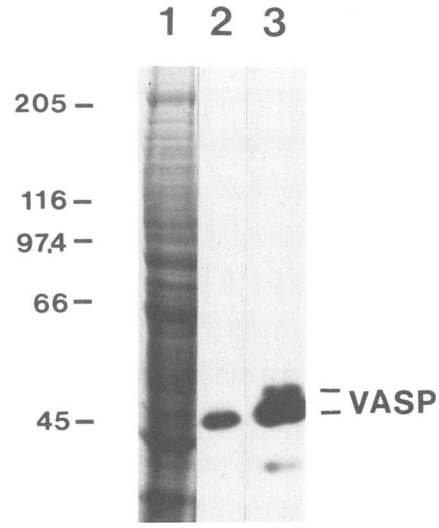

Fig. 3. Immunoblots demonstrating VASP as a profilin-binding protein present in calf thymus. Experimental conditions were as described in Material and methods, and in the legend of Figure 2. A calf thymus extract was separated by SDS–PAGE, and analyzed by Coomassie Blue staining (lane 1) or immunoblotting with VASP antiserum (lane 2). Identification of the VASP doublet in the PLP eluate of a calf thymus profilin column loaded with calf thymus extract is shown in the immunoblot, lane 3.

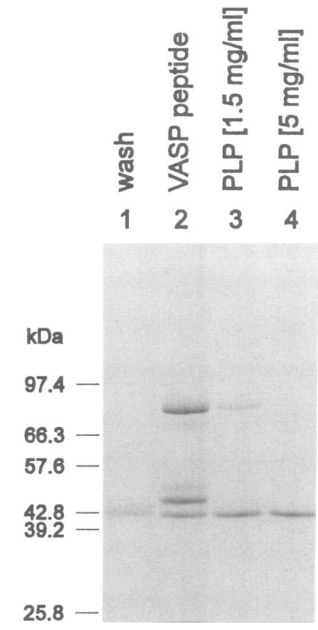

Fig. 4. Profilin affinity chromatography of an NaCl extract of a human platelet lysate followed by sequential elution (1.2 ml each) with a proline-rich VASP peptide [acetyl-$(GlyPro_5)_3Gly\text{-}NH_2$; 1.5 mg/ml; lane 2] and PLP (5.6 kDa) at 1.5 mg/ml (lane 3) or 5 mg/ml (lane 4). Aliquots (1/12) of the TCA-precipitated eluates and an equivalent aliquot of the buffer wash (lane 1) immediately preceding the elution were separated by SDS–PAGE and analyzed by Coomassie Blue staining.

these anucleate cells are highly specialized to respond rapidly to extracellular signals by remodelling their actin cytoskeleton (Furman *et al.*, 1993). Proteins involved in this process should thus be found in great abundance in these cells. Bona fide proline-rich ligands were eluted with PLP and analysed by SDS–PAGE. Coomassie Blue staining revealed two major protein bands of 81 and 46 kDa, respectively (Figure 2A, lanes 4–7). Minor protein bands of 43 and 50 kDa were also visible. Immunoblotting (not shown) identified the 43 kDa protein as actin which was also continuously eluted from the column during the preceding buffer washes (Figure 2A, lane 3). Using an affinity-purified VASP antibody, the 46 and 50 kDa bands were identified as the 46/50 kDa VASP doublet (Figure 2B) known to represent different phosphorylated forms of VASP (cf. Figure 5) (Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990; Eigenthaler *et al.*, 1992; Butt *et al.*, 1994). Subsequent regeneration of the column with a buffer containing 7 M urea yielded large amounts of actin (which probably dissociated from profilin–actin complexes) (Figure 2, lanes 9–12), but no substantial quantities of other proteins. These data indicate that both VASP and the 81 kDa polypeptide (p81) were completely eluted by PLP (Figure 2). Neither VASP nor p81 were retained when either BSA or no ligand had been coupled to the column matrix (not shown). As is evident from Figure 2B, VASP and a putative VASP degradation product were enriched in the PLP eluate relative to the sample applied, and the flow through fraction was partially VASP-depleted. In order to decide whether the specific retention of VASP and p81 is a peculiarity of platelets or is more widespread, we also extended this experiment to calf thymus profilin and thymus extracts. Again, VASP and a 81 kDa polypeptide present in calf thymus could be extracted by profilin affinity chromatography. Figure 3 shows the identification of VASP by immunoblotting in total calf thymus extracts and in the PLP-eluted fractions obtained from the profilin column.

A proline-rich VASP peptide releases VASP and p81 from a profilin affinity column

PLP elution of VASP from the profilin affinity column demonstrated that the PLP binding domain of profilin is involved in the interaction with VASP. Vice versa, the proline-rich central domain is the candidate profilin binding site of VASP. The longest proline stretches present in the human VASP cDNA sequence comprise five sequential prolines preceded by a glycine residue (Haffner *et al.*, 1995). This motif occurs once as a single copy and once as a 3-fold tandem repeat. Eight to ten consecutive prolines, however, are thought to be required for profilin binding (Machesky and Pollard, 1993). Therefore we were interested in whether or not an acetyl-$(GlyPro_5)_3Gly\text{-}NH_2$ peptide, corresponding to the VASP tandem repeat, could compete with VASP for profilin binding. As is shown in Figure 4 (lane 2), at a concentration of 1.5 mg/ml this peptide eluted all of the bound VASP and most of the 81 kDa polypeptide from the profilin affinity column. Subsequent elution with a similar concentration of PLP released the remainder of p81 (Figure 4, lane 3). A further elution step with 5 mg/ml PLP yielded neither additional VASP nor p81 (Figure 4, lane 4).

Characterization of p81

In contrast to VASP, which was easily identified by a specific antibody, there were no data revealing the identity of p81. Therefore, p81 containing gel pieces were excised from Coomassie Blue-stained gels and subjected to BrCN cleavage. Microsequencing and further analysis of two of

Table I. P81 microsequences

	5 10 15 20 25
Peptide A	F/WL/AQAVKENQKRAETEEKMRRAK/DLAK
Peptide B	XPPPPPFGFGVPAAPVLPFGLTPKKLYKPEVQLRRPNXXK
	5 10 15 20 25 30 35

the resulting peptides, including a proline-rich one, showed no significant homology of these p81 peptides to any protein sequences currently available in protein and nucleic acid databases (Table I).

For additional characterization of p81, a rabbit antiserum was raised against the electroeluted polypeptide and affinity purified using p81 blotted onto nitrocellulose. In immunoblots of total cell proteins, this affinity purified antibody specifically detected a ~160 kDa protein either as the only immunoreactive protein (HEK 293 cells) or in addition to variable amounts of the 81 kDa polypeptide (human platelets). Other experiments (data not shown) indicated that the 160 kDa protein is gradually degraded during platelet lysis and extraction leading to the concomitant accumulation of lower molecular weight immunoreactive polypeptides, with a 81 kDa species as a relatively stable intermediate. Thus, it seems reasonable to assume that p81 is a degradation product of this 160 kDa protein.

Fractionation experiments revealed that part of the 160 kDa protein separated with the supernatant of cell lysates (centrifuged at 10^5 *g*) in the absence of any detergent, suggesting that the protein is not an integral membrane protein.

Association of profilin with differently phosphorylated VASP

VASP is a major substrate for cyclic nucleotide-dependent protein kinases and is stoichiometrically phosphorylated and dephosphorylated in a variety of cells (Halbrügge *et al.*, 1990; Reinhard *et al.*, 1992). Each of three different VASP phosphorylation sites is phosphorylated by both cAK and cGK. However, the phosphorylation kinetics and preferences of both kinases with respect to these three sites differ (Butt *et al.*, 1994). A possible phosphorylation-dependent regulation of profilin activities by VASP could be achieved either through phosphorylation-evoked conformational changes of profilin-bound VASP or, alternatively, VASP–profilin binding *per se* could be affected by VASP phosphorylation. Therefore, we investigated whether the *in vitro* VASP–profilin interaction is dependent on the VASP phosphorylation status. VASP purified from human platelets is partially phosphorylated, but can be phosphorylated further by either cAK or cGK. VASP was either extensively phosphorylated by cGK or the catalytic subunit of cAK or dephosphorylated by protein phosphatase 2A (PP2A) (which essentially removes all phosphates incorporated *in vitro* by the aforementioned kinases; K.Abel and U.Walter, unpublished results). The different VASP preparations [partially (basal) and extensively (cAK,cGK) phosphorylated, or extensively dephosphorylated (PP2A); see Figure 5] were incubated with profilin covalently coupled to agarose beads. Beads were pelleted by centrifugation, washed with high and low salt buffer, and specifically bound VASP was then

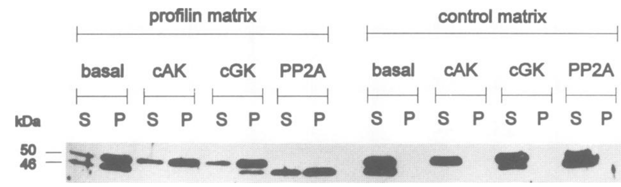

Fig. 5. Immunoblot showing profilin–agarose binding of various purified VASP forms which differed in their state of phosphorylation. Purified VASP (basal, partially phosphorylated) was extensively phosphorylated by either cAK or cGK or dephosphorylated by PP2A. After co-incubation of the different VASP preparations with profilin–agarose (profilin matrix), beads were sedimented by centrifugation, supernatants (S) were removed and the pellets were washed and then eluted with PLP. Equivalent aliquots of supernatants (S) and PLP pellet eluates (P) were analyzed by SDS–PAGE and immunoblotting with anti-VASP serum. Agarose without coupled ligand (control matrix) was used as a control. The positions of the 46 and 50 kDa VASP forms are indicated.

eluted with PLP. Comparison of the supernatants and PLP eluates revealed that the major part of VASP bound to the profilin beads, irrespective of its phosphorylation status (Figure 5; profilin matrix). In control experiments using agarose without coupled profilin, VASP remained in the supernatant (Figure 5; control matrix). These results indicate that the binding of purified VASP to a profilin matrix is not significantly affected by either cAK- or cGK-dependent phosphorylation or PP2A-catalyzed dephosphorylation. However, we cannot exclude the possibility that subtle differences may exist which are not detectable by the method employed.

VASP and profilin co-localize in highly dynamic membrane regions

The data reported so far demonstrate *in vitro* interactions of profilin with VASP and p81 which involve the PLP-binding domain of profilin. Additional evidence suggests that VASP–profilin interactions can indeed occur in vertebrate cells. VASP (Reinhard *et al.*, 1992) and profilin (Buß *et al.*, 1992) have been demonstrated in separate studies to be concentrated in the cortical regions of the leading lamellae of locomoting fibroblasts. Here we show by double label immunofluorescence of spreading fibroblasts that VASP and profilin display a closely matching co-localization in the periphery of protruding lamellae, in particular in ruffling areas (Figure 6). These observations are consistent with a direct VASP–profilin interaction in these highly dynamic regions characterized by rapid actin polymerization.

Discussion

The results presented here demonstrate that VASP and a variety of profilins interact directly without mediator proteins. VASP elution from the profilin affinity column by PLP strongly suggests that the proline-rich domain of VASP is involved in its interaction with profilin. The proline-rich domain of VASP is characterized by the occurrence of a Gly(Pro)$_5$ motif as a single copy and a 3-fold tandem repeat (Haffner *et al.*, 1995), which are the longest contiguous proline stretches present in human VASP. The Gly(Pro)$_5$ motifs consequently represent the candidate profilin binding site. As we have shown here, a peptide corresponding to the tandemly repeated VASP sequence indeed releases profilin-bound VASP and p81.

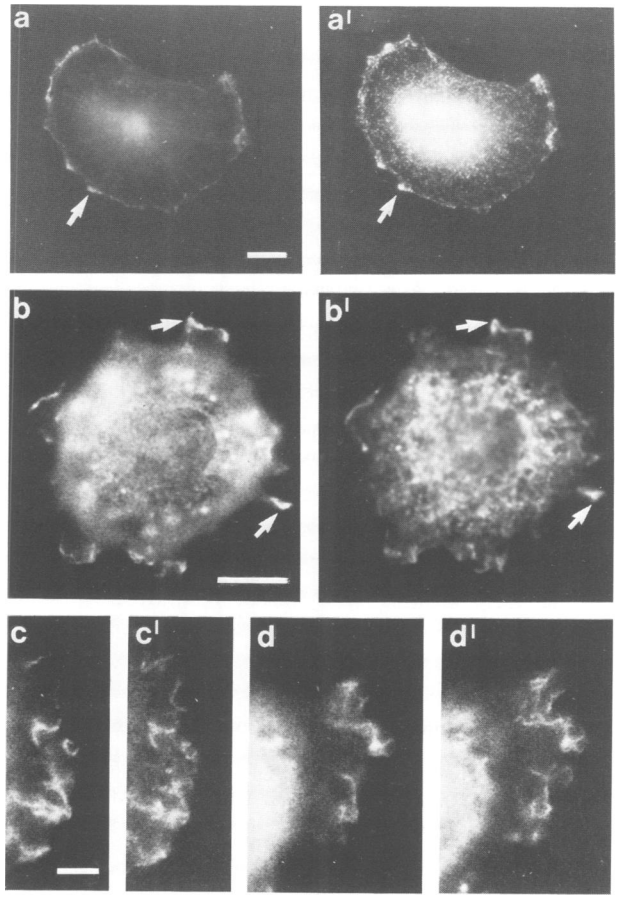

Fig. 6. Comparison of the subcellular localization of VASP (**a**, **b**, **c** and **d**) and profilin (**a'**, **b'**, **c'** and **d'**) in spreading human skin fibroblasts (a and a') and rat muscle fibroblasts (b and b'–d and d') as revealed by double label indirect immunofluorescence. VASP and profilin co-localize at the periphery of protruding lamellae (a and a'; arrow) and in ruffling areas developed by spreading fibroblasts (b and b'; arrows). A more detailed view is given in panels c and c' and d and d'. The bar shown in a and b represents 10 μm; the bar in c (valid for d also) represents 5 μm.

Hitherto, eight to ten sequential prolines were thought to be required for profilin binding (Machesky and Pollard, 1993), whereas Metzler *et al.* (1994) suggested that six residues would be sufficient to cover the entire PLP binding site of profilin, which matches exactly the length of one repetitive unit ($GlyPro_5$) of the VASP motif. Interestingly, a similar tandemly repeated $Gly(Pro)_{5-6}$ sequence found in the human homologue of yeast adenylyl cyclase associated protein (CAP) (Matviw *et al.*, 1992) may be involved in profilin binding. Although a direct CAP–profilin interaction has not been demonstrated, this would be consistent with genetic evidence suggesting a functional link between profilin and yeast CAP (Goldschmidt-Clermont and Janmey, 1991; Vojtek *et al.*, 1991; Sohn and Goldschmidt-Clermont, 1994).

Although the proline-rich VASP peptide eluted VASP and most of the p81 from profilin affinity columns, residual profilin-bound p81 required similar concentrations of PLP for elution. Selective elution of VASP was even more obvious when lower concentrations of the VASP peptide (~120 μg/ml) were used (data not shown). This possibly indicates different profilin binding affinities for VASP and p81. Also, p81 binding to the profilin column even after complete elution of VASP indicates that the p81–profilin interaction is direct rather than mediated by VASP.

Microsequence analysis of p81-derived peptides revealed no significant sequence homology to known proteins. In particular, p81 is not identical with the 80–85 kDa cell cortex or cytoskeletal proteins ezrin (Gould *et al.*, 1989), radixin (Funayama *et al.*, 1991), zyxin (Sadler *et al.*, 1992) and cortactin (Wu *et al.*, 1991), which all contain proline-rich sequence stretches. Instead, immunological data indicate that p81 may be a fragment of a ~160 kDa protein. Studies are now in progress to obtain more information on the primary structure of this protein.

Using profilin affinity chromatography, as well as conventional column chromatography and gel filtration with *Acanthamoeba* extracts, Machesky *et al.* (1994) succeeded in isolating seven different proteins with molecular masses between 13 and 47 kDa which appeared to constitute a profilin binding complex. However, there is no evidence that these proteins contain oligo-proline stretches (Machesky and Pollard, 1993; Machesky *et al.*, 1994). Thus, our profilin affinity chromatography experiments in combination with PLP elution identified VASP and p81 as the first biological ligands for the profilin PLP binding domain.

Since both VASP (Halbrügge *et al.*, 1990; Reinhard *et al.*, 1992; Halbrügge and Walter, 1993; Walter *et al.*, 1993) and profilin (Goldschmidt-Clermont and Janmey, 1991; Goldschmidt-Clermont *et al.*, 1991a; Machesky and Pollard, 1993; Sohn and Goldschmidt-Clermont, 1994) are members of signal transduction pathways and bind directly to actin, they may both be involved in the modulation of microfilament organization by external signals. This concept is further supported by recent studies on the actin filament recruitment by the intracellularly motile bacterial pathogen *Listeria monocytogenes* which is considered to be a model for the regulation of cellular microfilament organization (Sanger and Sanger, 1992; Tilney and Tilney, 1993; Kocks, 1994). As demonstrated recently (Chakraborty *et al.*, 1995), VASP binds directly to the *Listeria* surface protein ActA, the only bacterial factor needed for F-actin recruitment (Pistor *et al.*, 1994). Both ActA (Domann *et al.*, 1992; Kocks *et al.*, 1992) and host profilin (Theriot *et al.*, 1994) are essential for actin-based listerial motility. Our present results strongly suggest that VASP represents a long sought host factor (Sohn and Goldschmidt-Clermont, 1994; Theriot *et al.*, 1994) which recruits profilin to ActA or a host cell ActA analogue and thereby contributes to F-actin nucleation and/or elongation. The 81/160 kDa protein may either participate in this reaction, or, alternatively, exert a similar function at a different cellular location. The proposed function of VASP and profilin in regulation of microfilament organization is consistent with the localization of both proteins to highly dynamic membrane regions.

Furthermore, beyond its proposed role as physical link between profilin and ActA or its cellular analogues, VASP may modulate profilin's function(s) in a phosphorylation-dependent manner, although the VASP–profilin interaction *per se* is apparently not affected by VASP phosphorylation. It is conceivable that VASP phosphorylation may alter the interaction of profilin with other ligands which in turn then affect the profilin–actin interaction

(Lassing and Lindberg, 1985) as well as the PLC pathway. Since both PLC and the actin based cytoskeleton are thought to be involved in the regulation of the adhesive properties of integrins such as the platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ (Fox, 1993; Halbrügge and Walter, 1993; Hynes, 1994), a VASP–profilin interaction could account for the close correlation observed between VASP phosphorylation and concomitant PLC and $\alpha_{IIb}\beta_3$ inhibition (Waldmann and Walter, 1989; Halbrügge and Walter, 1993; Horstrup *et al.*, 1994). This may be especially relevant in human platelets which contain approximately equimolar concentrations of profilin and VASP (Goldschmidt-Clermont *et al.*, 1991b; Eigenthaler *et al.*, 1992). Molecular analysis of the interaction between VASP and profilin, actin and additional proteins, as well as its role in the regulation of signal transduction pathways, actin polymerization and cytoskeleton–membrane interactions should soon be possible due to the recent cloning and functional expression of VASP.

Materials and methods

Protein purification

Human platelet VASP was purified essentially as described (Halbrügge and Walter, 1989) with the modifications indicated (Reinhard *et al.*, 1992) and an additional hydroxyl apatite step (Bio-gel HT, Bio-Rad, Richmond, CA). Human platelet profilin (Janmey, 1991), calf thymus and recombinant birch pollen profilin (Giehl *et al.*, 1994) were purified essentially as decribed.

The native profilactin complex was isolated from calf thymus as described for profilin, except that the elution was performed with 2 M urea in 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.5 mM ATP. Profilactin containing fractions were pooled and dialysed against 10 mM imidazole (pH 7.0), 0.1 mM $CaCl_2$, 0.5 mM DTE, 0.01 mM EDTA. The complex was then separated from free actin and profilin by gel filtration (25 ml/h) on a Sephadex G-150 column (2.6×100 cm, Pharmacia) which had been equilibrated in the same buffer. Fractions (3.5 ml) were collected and analyzed by SDS–PAGE and Coomassie Blue staining. The protein concentration was determined spectrophotometrically at 280 nm (Larsson and Lindberg, 1988).

Proteins were judged by SDS–PAGE and Coomassie Blue staining to be >95% pure.

Solid phase binding assays

Microtiter wells (removawell, Dynatech Laboratories) were coated with profilins (20–25 μg/ml in PBS) for 1.5 h at room temperature and overnight at 4°C, blocked with 3% BSA in PBS (1 h; BSA blocking and all subsequent steps were carried out at room temperature), and incubated with the ligand solution (0.6 μg/ml in PBS/BSA) for 2 h. Both profilins and VASP were directly added to the wells and then diluted to a final volume of 400 μl. Binding of VASP was detected by incubating for 1 h with the VASP specific antiserum M4, used at a 1:1000 dilution in PBS/BSA (Halbrügge *et al.*, 1990; Reinhard *et al.*, 1992) followed by a 1 h incubation step with [^{125}I]protein A (3.7 kBq/ml in PBS/BSA) (ICN Radiochemicals, Irvine, CA). After each incubation step the wells were rinsed three times with PBS. Individual wells were removed, and bound radioactivity was measured in a γ-counter.

Peptide synthesis and purification

Synthesis (Dr D.Palm, Würzburg, Germany) and purification of the VASP peptide [acetyl-$(GlyPro_5)_3$Gly-NH_2] were essentially as described (Butt *et al.*, 1994). The peptide composition was verified by mass spectrometry (Dr R.Frank, Braunschweig, Germany) and the peptide content was estimated to be 70% (w/w). PLP (assumed peptide content 100%) was obtained commercially (Sigma).

Profilin affinity chromatography

Human platelet profilin (1.5 ml; 0.74 mg/ml) was coupled to a 1 ml NHS-activated HiTrap-column (Pharmacia) following the manufacturer's instructions. All subsequent steps were performed at 4°C. Platelet lysates were prepared essentially as described (Halbrügge and Walter, 1989), extracted with 250 mM NaCl for 30 min, and cleared by centrifugation for 1 h at 10^5 *g*. The supernatant was diluted with 0.67 vol of a buffer containing 30 mM NaP_i (pH 6.8), 2 mM EDTA, 0.5 mM DTT. The profilin HiTrap-column was loaded (0.4 ml/min) with an extract obtained from 1.8×10^{10} human platelets, washed with 15 ml of buffer A (20 mM NaP_i, pH 6.8, 2 mM EDTA, 150 mM NaCl, 0.5 mM DTT), 10 ml buffer A containing 500 mM NaCl, and finally with 6 ml of buffer A.

Calf thymus (100 g) was homogenized in a Waring blender in 1 vol of 20 mM NaP_i, pH 6.8, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF and 100 U/ml aprotinin (Bayer). After 30 min at 4°C, the extract was cleared by ultracentrifugation and filtration of the supernatant through glass wool. After addition of fresh protease inhibitors, the solution was loaded onto a 5 ml NHS-activated HiTrap column (Pharmacia) preloaded with calf thymus profilin (14 ml; 0.42 mg/ml). In both cases, proteins were eluted with 2 ml of 5 mg/ml PLP (10–30 kDa; Sigma) in buffer A. After PLP elution columns were again washed with buffer A (10 column volumes) and were then regenerated with 7 M urea in buffer A. Fractions of the PLP and urea eluates were collected (0.5 and 0.8 ml, respectively), TCA-precipitated, and aliquots equivalent to one quarter of the original fractions were analyzed by SDS–PAGE and immunoblotting.

Modified profilin affinity chromatography with an NaCl extract of human platelets followed by fractionated elution was performed as described above, except that the 1 ml column was sequentially eluted (1.2 ml each) with the VASP peptide acetyl-$(GlyPro_5)_3$Gly-NH_2 (1.5 mg/ml in buffer A), 1.5 mg/ml PLP (5.6 kDa), and 5 mg/ml PLP.

Binding assays with phosphorylated and dephosphorylated VASP

Human platelet profilin (1 ml; 2.6 mg/ml) was coupled to a 1 ml NHS-activated HiTrap column (Pharmacia) following the manufacturer's instructions. The matrix was resuspended in 1 vol of buffer B [50 mM Tris–HCl (pH 7.2), 1 mM DTE, 5 mM $MgCl_2$, 150 mM NaCl, 0.01% BSA]. Porcine platelet VASP (1 μg in a final volume of 40 μl) was incubated for 30 min at 30°C in buffer B containing 100 μM ATP and 20 μM cGMP either alone (basal), with the catalytic subunit of cAK (50 ng; Halbrügge and Walter, 1989), with cGK (100 ng; Halbrügge and Walter, 1989) or with the catalytic subunit of PP2A (500 ng; from rabbit muscle; generous gift from Dr G.Mieskes, University of Göttingen, Germany). Okadaic acid (7 μM; LC Laboratories) was then added in order to inhibit PP2A, followed by 100 μl of the profilin–agarose suspension (50%, v/v) or mock coupled agarose suspension (control matrix), and the incubation was continued for 1 h at 30°C under constant agitation. After centrifugation, the supernatants were removed and the pellet fractions were washed with 2×1 ml of 50 mM NaP_i (pH 7.2) containing 500 mM NaCl and 0.5 mM DTT, and subsequently with 1×1 ml of buffer B. VASP was eluted by incubation with PLP (5 mg/ml; 1–10 kDa; Sigma), and equivalent amounts of supernatant and PLP-eluate of the pellet fractions were analyzed by SDS–PAGE and immunoblotting with an anti-VASP serum.

Microsequencing

The PLP-eluate of a profilin affinity column was TCA-precipitated and separated by SDS–PAGE. The gel piece containing the p81 band was excised from the Coomassie Blue-stained gel and incubated with BrCN (35 mg/ml in 70% formic acid). After overnight incubation under vigorous agitation at room temperature, the supernatant containing the BrCN cleavage products was removed and the solvent was evaporated. Resulting peptides were separated on 16.5% urea-free tricine gels (Schägger and von Jagow, 1987) and blotted onto Selex 20 polypropylene membranes (Schleicher and Schuell, Dassel, Germany). Coomassie Blue-stained bands were excised and sequenced using a pulsed-liquid sequencer (Applied Biosystems 476A).

Indirect immunofluorescence

Human fibroblasts (3 h after plating) were processed for indirect immunofluorescence essentially as described (Reinhard *et al.*, 1992) using an affinity-purified rabbit VASP-antiserum M4 and a mouse monoclonal antibody 2D8 directed against bovine profilin (Buß *et al.*, 1992). Primary antibodies were detected by DTAF-conjugated donkey anti-rabbit antibodies (pre-adsorbed to mouse and other serum proteins; Dianova, Hamburg, Germany) and Cy3-conjugated goat anti-mouse IgG + IgM (H + L) antibodies (Dianova, Hamburg, Germany) which had been pre-adsorbed to immobilized rabbit γ-globulins (Serva, Heidelberg, Germany). Indirect immunofluorescence analysis of rat fibroblasts (6 h after plating) was done essentially as described (Buβ *et al.*, 1992).

Acknowledgements

The authors are grateful for the generous gift of protein phosphatase 2A from Dr G.Mieskes (Göttingen, Germany) and the birch profilin clone from Dr R.Valenta (Vienna, Austria). The peptide synthesis by Dr D.Palm (Würzburg, Germany) and mass spectrometry analysis by Dr R.Frank (Braunschweig, Germany) are gratefully acknowledged. We also thank Drs M.Rothkegel (Braunschweig), T.Chakraborty (Gießen, Germany), J.Wehland (Braunschweig) and our colleagues of the Klinische Forschergruppe for constructive criticism and stimulating discussions. This work was supported by the Deutsche Forschungsgemeinschaft (Ko 210/11-3; SFB 176 and 355; Jo 55/12-3).

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Received on October 13, 1994; revised on December 30, 1994