Cell motility: Proline-rich proteins promote protrusions

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Cell motility: proline-rich proteins promote protrusions

Mark R. Holt and Anna Koffer

Many proline-rich proteins participate in delivering actin monomers to specific cellular locations where actin-rich membrane protrusions, such as ruffles, filopodia and microspikes, are formed. These protrusions are necessary for cell motility. Actin monomer is usually delivered to the site of polymerization in the form of profilactin – a complex of G-actin with a polyproline-binding protein, profilin. Here, we describe proline-rich proteins involved in regulating actin polymerization and classify them according to their role in recruiting profilin to the membrane.

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Cells move in response to chemotactic signals. Polymerization of actin alone might be sufficient to drive some types of motility independently of myosins – for example, the formation of membrane protrusions or the propulsion of bacteria inside host cells. In cells, a dynamic equilibrium exists between G-actin (monomer) and F-actin (polymer) that is affected by actin-binding proteins such as profilin and cofilin. These proteins distinguish between ATP- and ADP-bound actin (Fig. 1). Generally, polymer elongation proceeds by the addition of ATP–G-actin–profilin complexes to the barbed (plus- or fast-polymerizing) ends of F-actin. After incorporation into the filament, ATP is hydrolysed and the ADP–actin subunits within the filament then bind to cofilin. Cofilin facilitates the disassembly of filaments and thus, together with profilin, increases actin turnover[1]. Proteins that participate in initiating actin polymerization, in particular capping proteins such as the Arp2/3 complex or gelsolin, have been described in several recent reviews[2–4,5]. Such proteins are usually associated with specific submembranous compartments within the cell; filament elongation requires continuous delivery of actin monomers to these sites. Here, we focus on two groups of proteins that are important for delivering ('funnelling') actin monomers to the sites of polymerization: those that bind to and those that contain proline-rich motifs (PRMs).

Proteins that bind to PRMs

Profilin[5–7] binds to poly-L-proline and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] as well as to G-actin and facilitates the funneling of actin monomers to membrane-associated sites of actin polymerization. The interaction with polyproline appears to be unique to profilin (see Fig. 2a and Box 1) and does not inhibit profilin binding to G-actin. On the other hand, owing to the overlap of the PtdIns(4,5)P_2- and G-actin-binding sites on profilin, PtdIns(4,5)P_2 participates in a mechanism releasing G-actin from profilin. After formation of profilactin (i.e., profilin–G-actin complex), nucleotide exchange on actin is accelerated and nucleation of new filaments suppressed. ATP-bound profilactin is recruited to the barbed ends of F-actin adjacent to the plasma membrane, and it is at this stage that the interaction of profilin with PRM-containing proteins plays an

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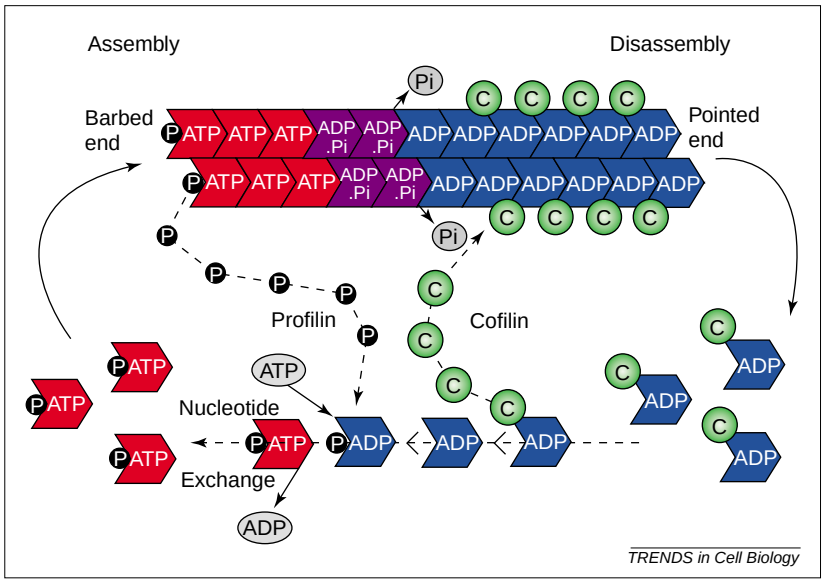

Fig. 1. Actin turnover. Actin filaments have polarity owing to the presence of barbed and pointed ends, fast- and slow-polymerizing, respectively. Profilin facilitates exchange of actin-bound ADP for ATP, forming a profilin–actin–ATP complex, which is recruited to and incorporated into the barbed end of F-actin. After hydrolysis of ATP, actin–ADP subunits within the filament can bind to cofilin (C), which increases their propensity to dissociate from the pointed ends. The cycle is completed by binding of profilin to ADP–actin, converting it to the ATP–actin form that is primed for polymerization.

important role. The involvement of profilin in the formation of actin-rich structures was clearly demonstrated by the effects of a profilin I mutant (H119E) that was defective in actin binding but able to interact with other proline-rich proteins; cells expressing this mutant were deficient in actin polymerization associated with microspike formation[8].

Other proteins that bind to PRMs usually do so through so-called EVH1, Src-homology 3 (SH3) and WW domains. Fig. 2 shows structures of these domains in complex with proline-rich peptides. Unlike profilin, EVH1, SH3 and WW domains do not bind to polyproline itself but to distinct classes of PRMs that also contain non-proline residues. The positioning and chemistry of the non-proline residues within and surrounding PRMs define these classes. Although SH3 and WW domains participate in the regulation of actin polymerization, the mechanisms are still unclear and too speculative for this review. There are other examples of PRM-binding domains, such as LIM domains in paxillin or a region in α-actinin, but the function of these interactions is still not clear. We have focused on the PRMs that interact with profilin and with the EVH1 domains because these provide a direct mechanism for recruitment of G-actin. We have classified the profilin-binding motif as PRM1 and the EVH1-binding motif as PRM2. Table 1 shows consensus sequences for these two motifs, as well as for SH3-binding (PRM3) and WW-binding motifs (PRM4, PRM5, and PRM6).

Profilin-binding proteins contain PRM1

Proteins that contain PRM1, which binds to the hydrophobic (polyproline-binding) cleft on profilin, can recruit profilactin. Aromatic residues on profilin intercalate with specific prolines of the polyproline helix (Fig. 2). So far, all proteins that associate with profilin have been found to contain the specific PRM1 motif XPPPPP, where X = G, L, I, S or A (Table 1)[9,10]. Fig. 3a shows a general model for a 'profilactin-recruiting chain', visualized as a chain connecting profilactin to specific sites on the plasma membrane. The first link from profilactin is thus to a PRM1-containing protein 'X'. X has a site that links it to a protein 'M' (an integral membrane protein or a protein inserted into the membrane through a lipid moiety). This link can be direct (Fig. 3c,e) or indirect, via proteins 'Y' (a PRM2-containing protein) and/or 'Z' (Fig. 3b,d). The nature of the linking site on X determines the nature of the next component, leading to a variety of 'profilactin-recruiting chains'. Specific examples of these interactions are discussed below.

There are several classes of PRM1-containing proteins (Table 2). One class is the Ena/VASP (*Drosophila* Enabled/Vasodilator-stimulated phosphoprotein) family of proteins[11], which contain an Ena/VASP homology 1 (EVH1) domain[12]. The EVH1 domain interacts with a specific PRM2 motif FPPPP (Table 1). Since PRM2-containing proteins are recruited to the plasma membrane after cell activation, one possible sequence of interactions leading to profilactin recruitment to the membrane is: a membrane-associated protein(s) binds to a PRM2-containing protein, which in turn binds to a (PRM1 + EVH1)-containing protein, which binds to profilactin (see Fig. 3b,e).

The second class of PRM1-containing proteins includes proteins that act downstream of small Rho-related GTPases. These proteins interact, either directly, through their respective GTPase-binding domains (GBDs), or indirectly, with active, GTP-bound forms of Cdc42, Rho or Rac. Active GTPases are associated with the membrane, providing an alternative mode of profilactin recruitment: a Rho-related GTPase binds to a PRM1-containing downstream effector, which binds to profilactin (Fig. 3c). This class includes the following families: WASP/N-WASP, SCAR/WAVE and formin homology (FH) proteins such as diaphanous and formin.

The third class of PRM1-containing proteins contains some members of the ezrin–radixin–moesin (ERM) family of membrane–actin cytoskeleton linkers (Fig. 3d). The role of PRM1 in this class of proteins is still unclear. Finally, there are several PRM1-containing proteins such as WIP, drebrin, gephyrin, zyxin and Fyb/SLAP that do not fit easily into any of the above classes.

PRM1-containing proteins with EVH1 domain

Proteins containing the EVH1 domain (Fig. 2b) include VASP, Ena (and its homologues Mena and Avena from mouse and chick, respectively), Evl (Ena/VASP-like protein) and the Wiscott–Aldrich

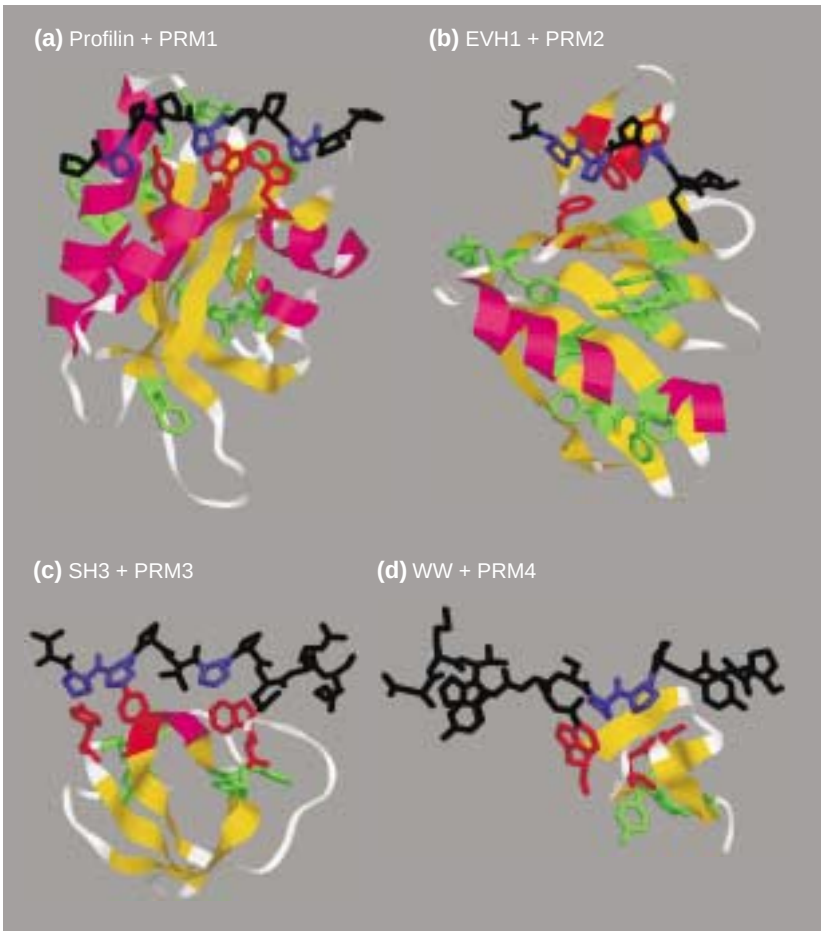

Fig. 2. Three-dimensional representations of proline-rich motif (PRM)-binding domains bound to their respective ligands. Tertiary structures of (a) profilin bound to poly L-proline (PRM1); (b) EVH1 domain (from Evl) bound to a peptide (from ActA), FPPPPT (PRM2); (c) Src-homology 3 (SH3) domain (from Grb2) bound to a peptide (from a GDP–GTP exchange factor, Sos), VPPPVPPRRR (PRM3); (d) WW domain (from dystrophin) bound to a peptide (from β-dystroglycan), NMTPYRSPPPYVP (PRM4). Three-dimensional coordinates were obtained from the Protein Data Bank (PDB) and were visualized with Rasmol 2.6. Accession codes are (a) 1AWI, (b) 1QC6, (c) 1GBQ and (d) 1EG4. In the structures, magenta ribbons represent α-helices, and gold ribbons represent β-strands. These are connected by white loops. Bound proline-rich ligands are depicted in black, except for those residues in blue that are directly involved in binding. Aromatic residues within the PRM-binding domains are shown in green, except those directly involved in the binding of the ligand (shown in red).

syndrome protein (WASP). Mutations in the gene encoding WASP cause severe immunodeficiency[13]. Owing to its presence in the N-terminal region of WASP, the EVH1 domain is also referred to as the WH1 domain (WASP-homology 1). Initially, it was thought that the WH1 domain in WASP was a PtdIns(4,5)P_2-binding pleckstrin homology (PH) domain[14]. Structural and sequence analyses of PH and EVH1 domains revealed unrelated sequences that have remarkable structural similarity[15]. Thus, PH- and EVH1-domains form a structural superfamily including domains that bind to the GTPase Ran (RanBD), phosphotyrosine-binding (PTB) domains and a subdomain in ERM-family proteins[16,17].

VASP is the prototypic member of the Ena/VASP family; it is widely expressed, although it was originally characterized as a substrate for cyclic AMP- and GMP-dependent kinases in platelets. It is localized to dynamic F-actin structures, focal adhesions, cell–cell contacts and stress fibres. The N-terminal EVH1 domains of VASP and Mena (Fig. 4) bind to the PRM2-containing focal adhesion proteins vinculin and zyxin (Fig. 3b), as well as to ActA, a bacterial protein of the intracellular pathogen *Listeria monocytogenes* (Fig. 3e)[11]. Microinjection of PRM2-like peptides displaces Ena/VASP proteins from focal adhesions. Furthermore, deletion of PRM2 repeats from ActA prevents VASP and Mena from binding to *Listeria* and reduces intracellular motility by 70%[18]. This demonstrates the importance of the EVH1–PRM2 interaction in Ena/VASP recruitment and motility. The C-terminal EVH2 domain binds to F-actin and mediates tetramerization[19]. The central proline-rich domain, separating EVH1 and EVH2, contains a number of proline-rich motifs that include PRM1, PRM3 and PRM4 (Table 1).

PRM1-containing proteins that act downstream of Rho GTPases

WASP family

One particular family that acts downstream of the Rho GTPase Cdc42 contains the proteins WASP (specific to hematopoietic cells) and N-WASP ('neural WASP', named thus – but actually ubiquitous; Fig. 3c). These proteins contain the EVH1 domain and, therefore, also belong to the group of proteins (described above) that contain EVH1 and PRM1. Potentially, membrane recruitment of WASP family members could occur through interactions with PRM2-containing proteins – for example, WIP (WASP-interacting protein, see below). They have, however, been characterized as proteins acting downstream of Cdc42, inducing formation of actin-rich microspikes[2,3,13]. In addition to the EVH1 domain, there are other functional domains present in the N-terminal region of WASP/N-WASP: a basic region (BR) and the GBD, termed CRIB (Cdc42/Rac interactive binding; Fig. 4). N-WASP co-immunoprecipitates with calmodulin through a putative IQ (calmodulin-binding) motif, adjacent to EVH1[20]. Our analyses using BLAST and PFAM databases did not, however, reveal any significant homologies with known IQ motifs.

A long central proline-rich domain links the N- and C-terminal regions of WASP proteins and includes several PRM1 and PRM3 sequences. The conserved C-terminal domain, VCA, consists of a verprolin-homology segment (V, identical to WH2), and a cofilin-homology segment (C) adjacent to an acidic region (A). Functional interpretation of cofilin homology is controversial because it does not overlap with the actin-binding region on cofilin[20,21]. VCA binds both to actin monomers (through V) and to Arp2/3 complex (through CA), thus facilitating Arp2/3-induced nucleation of actin polymerization. The N-terminal region has a regulatory role, controlled by binding of WIP to EVH1 and/or of Cdc42 to GBD and/or of PtdIns(4,5)P_2 to BR [PtdIns(4,5)P_2 might also bind to EVH1].

Box 1. Polyproline geometry

Proline is an imino acid, which, along with polyproline helices, is present in two different geometric structures. Type I polyproline helices (PPI) consist of *cis*-peptide bonds and are right-handed (not known to be present in cells and not shown), whereas type II helices (PPII) contain *trans*-peptide bonds and are left-handed (see the stereo image). Proline isomerases (e.g. PIN1) catalyse the *cis–trans* conversions: this could be a mechanism for regulating interactions of proline-rich motifs (PRMs) with their binding partners[a]. The PRMs discussed here adopt the extended type II polyproline conformation (PPII).

PPII-helices have three-fold rotational symmetry (see the end-on view shown in Fig. I). They are often found at the surface of globular proteins in stretches of 4–12 residues. PPII-helices might also form flexible links between domains, sharp bends within domains, and short stretches of *trans*-prolines allow turns, facilitating complete reversal of the direction of a polypeptide chain. This flexibility is particularly important in enabling intramolecular interactions, which allow regulation of PRM-protein function (Fig. 4b). Incorporation of a limited number of non-proline residues into the type II helices does not interfere with the topography but provides a basis for binding specificity.

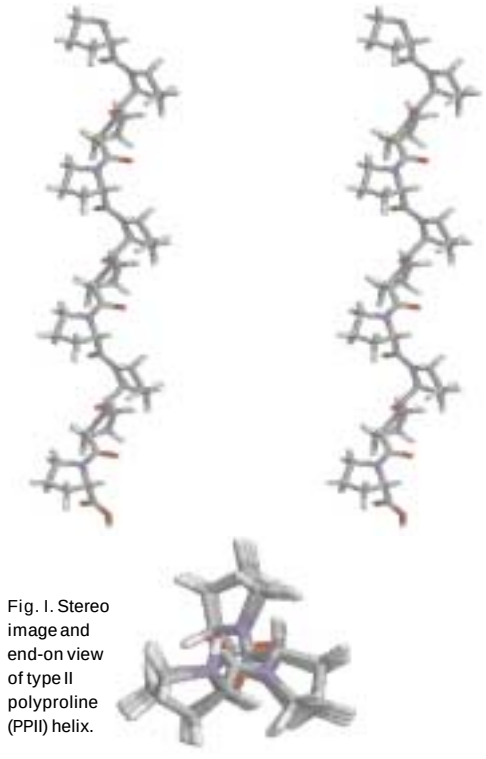

Fig. I. Stereo image and end-on view of type II polyproline (PPII) helix.

Reference

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Recent work has shown that N-WASP exists as a monomer in a closed, inhibited state (Fig. 4b). The N-terminal portion interacts directly with CA in the VCA domain, blocking Arp2/3 complex recruitment. Cdc42 binding to GBD interferes with this intramolecular interaction, releasing the auto-inhibition. The SH3-containing adaptor, Grb2, which binds to a PRM3 on N-WASP, exerts similar effects; Grb2 and Cdc42 act synergistically[22]. Synergy also exists between Cdc42 and PtdIns(4,5)P_2. The BR–PtdIns(4,5)P_2 interaction apparently destabilizes the auto-inhibitory fold of the adjacent GBD, facilitating the binding of Cdc42[23]. The physiological significance of the phospholipid-mediated effects remains to be established.

WAVE/SCAR family

Members of another WASP-like family of PRM1-containing proteins act downstream of the GTPase Rac. These proteins are termed WAVE because of their homologies with WASP and verprolin. They are also called SCAR due to their ability to suppress the effects of a deletion in the cAMP receptor cAR in *Dictyostelium*. WAVE proteins function downstream of Rac in membrane-ruffle formation. A GTPase-binding domain is not apparent, and therefore they might not bind to Rac directly. There are at least three isoforms of WAVE in humans[24]. They all have a Scar-homology domain (SHD) at their N-termini (Fig. 4a), the function of which is unknown. A basic region in the N-terminus is similar to that present in WASP and might therefore bind to acidic phospholipids. The similarity of WAVEs to WASPs is apparent particularly in the C-terminal VCA region, which is essential for their ability to associate with Arp2/3 and to regulate actin[24]. Significantly, some actin regulation can proceed independently of Arp2/3 as expression of a WAVE1 mutant, unable to associate with Arp2/3, still induces actin polymerization and clustering[25].

FH proteins

One effector of Rho GTPase has been found to contain PRM1 motifs and is called diaphanous (Dia; Fig. 3c). Dia was identified originally in *Drosophila* and is a member of the family of Formin-homology proteins. These proteins contain two conserved FH domains, FH1 and FH2, separated by ~160 residues[26] (Fig. 4a). FH1 is a very proline-rich domain, containing six PRM1s that bind to profilin, as well as PRM3- and PRM4-like motifs. Coiled-coil regions are present, N-terminal to FH1 and C-terminal to FH2, providing sites for possible interactions with other coiled-coil-containing proteins. Dia is an example of an FH protein that contains a Rho-GBD in the N-terminal region[10]. Two mouse homologues of *Drosophila* Dia exist, mDia1 and mDia2[27]. Mutation in the human Dia homologue leads to a hearing disorder, most probably arising from a dysfunctional cytoskeleton of the hair cells of the cochlea[28]. Dia and profilin are recruited to the membrane in a Rho-dependent manner:

Table 1. Consensus sequences for, and examples of, proline-rich motifs

Protein[a]	Species[b]	Sequence(s)[c]	Accession No.[d]
PRM1 consensus		**XPPPPP**	
VASP	Hs	**GPPPPP (X4)**	S51797
Mena	Mm	**APPPPP**P	U72520
		GP PPPPP	
		GPPPPPPPPPP	
		APPPPPPPP	
EVL	Mm	**GPPPPP**PPP	U72519
WASP	Hs	**LPPPPP**P	A55197
		GPPPPPPP	
		LPPPPP	
hDia1	Hs	**IPPPPP (X5)**	O60610
Formin	Mm	**IPPPPP**	Q05860
		SPPPPPPPPPP	
		GPPPPPPPPPP **(X2)**	
		APPPPP	
Ezrin	Hs	**APPPPP**PP	A34400
WIP	Hs	**APPPPP (X2)**	NP_003378
		LPPPPP	
Zyxin	Hs	**IPPPPP**	G02845
Drebrin 1	Hs	**LPPPPP**P	NP_004386
Gephyrin	Hs	**LPSPPP**P	CAC10537
PRM2 consensus		XXXX**FPPPP**XXXX	
ActA	Lm	NASD**FPPPP**TDEE	B43868
		SSFE**FPPPP**TDEE	
		SSFE**FPPPP**TEDE	
		NFSD**FPPIP**TEEE	
Zyxin	Hs	PPED**FPLPP**PPLA	G02845
		LGGA**FPPPP**PPIE	
		EEEI**FPSPP**PPPE	
Vinculin	Hs	QEPD**FPPPP**PDLE	M33308
Fyb	Hs	SGGI**FPPPP**DDDI	AAF62400
		EGSS**FPAPP**KQLD	
		SKPT**FPWPP**GNKP	
WIP	Hs	PISD**LPPPE**PYVQ	NP_003378
PRM3 consensus		****PXXP****	
PRM4 consensus		**PPLP**	
PRM5 consensus		**PPPXY**	
PRM6 consensus		***P*SP**	

[a]PRM1, -2, -3, -4, -5 and -6 are designated as consensus motifs binding to profilin (PRM1), EVH1 (PRM2), SH3 (PRM3) and WW domains (PRM4, -5, -6). Proteins containing PRM1 and PRM2 are listed together with the specific sequences, and number, of their respective PRMs.

[b]Indicated sequences are from the species shown as follows: Hs, *Homo sapiens*; Mm, *Mus musculus*; Lm, *Listeria monocytogenes*.

[c]PRM1 = XP5, where X = G, L, I, S or A; PRM2 = FP4, generally sandwiched between acidic residues (X); PRM3 = PXXP (the core motif), '**' refers to residues outside of this core, often basic and/or hydrophobic, that confer specificity; PRM6 = different consensus sequences exist; '*P*S' refers to phosphoserine.

[d]Accession numbers (for GenBank or SwissProt) of appropriate cDNAs were obtained through www.ncbi.nlm.nih.gov/.

the multitude of profilactins bound to this protein might form the basis for Rho-induced actin polymerization[10]. In permeabilized mast cells, addition of a constitutively active mutant of Rho, V14RhoA, is sufficient to induce *de novo* actin polymerization[29], and this effect is inhibited by polyproline (M.R. Holt and A. Koffer, unpublished).

The functional unit promoting F-actin assembly consists of both FH1 and FH2 domains and is masked by binding of the C-terminus to the N-terminal Rho-binding domain (Fig. 4b). Binding of GTP–Rho to mDia1 disrupts this intramolecular association, activating the protein[30]. This is similar to the Cdc42-induced activation of WASP. Active mDia1 cooperates with ROCK kinase, another Rho effector, to induce and organize formation of stress fibres[30,31].

PRM1-containing members of the ERM family

Ezrin, radixin and moesin are members of the ERM family of proteins that control membrane plasticity[32–34]. The N-terminal domain of these proteins is homologous with a domain in erythrocyte protein band 4.1 and is also referred to as the 'FERM' domain (Four-one, ERM homology). FERM domains bind directly to the integral membrane proteins CD44, CD43 or ICAMs or interact with the plasma membrane indirectly by binding to PDZ-domain-containing proteins such as EBP50 (ERM-binding phosphoprotein, 50 kDa; Fig. 3d). These, in turn, interact (through PDZ domains) with the cytosolic tails of integral membrane proteins such as the cystic fibrosis transmembrane-conductance regulator (CFTR). Like other PRM1-containing proteins, the conserved C-termini of ezrin and radixin interact with and anchor F-actin (Fig. 4a). An additional G-actin-binding site resides in the N-terminal domain (residues 280–309 on ezrin). An extended helical coiled-coil region separates the N- and C-terminal domains. PRM1 lies C-terminal to this helical domain; whether it interacts with profilin is not known. Moesin does not contain any PRM1 motif.

The N- and C-terminal regions of ERM proteins associate with each other both intra- and intermolecularly, thus masking several functional sites. Again, the flexibility of the PRM might be important for this interaction (Fig. 4b). The conformational changes that lead to the opening of the folded molecule involve phosphorylation of a C-terminal Thr (residue 566 in ezrin and 564 in radixin) by Rho kinase and/or PKC-theta. The site lies within the C-terminal F-actin-binding site, but its phosphorylation does not affect F-actin binding. Rho might be both upstream and downstream of ERM proteins: direct binding of a guanine nucleotide dissociation inhibitor, Rho-GDI, and of a guanine nucleotide exchange factor, Rho-GEF (Dbl), to the N-terminal domain have been reported. The unfolding of the molecule is facilitated by PtdIns(4,5)P_2 binding to subdomain C of the FERM domain. This subdomain is a member of the PH domain structural superfamily discussed above[16].

Other PRM1-containing proteins

Drebrin and gephyrin can be isolated from rat brain cytosol on a GST–profilin affinity column[35]. They both contain a PRM1-like motif (Table 1) that most probably mediates profilin binding. This interaction might be crucial for their function. Gephyrin interacts with and probably promotes the clustering of glycine

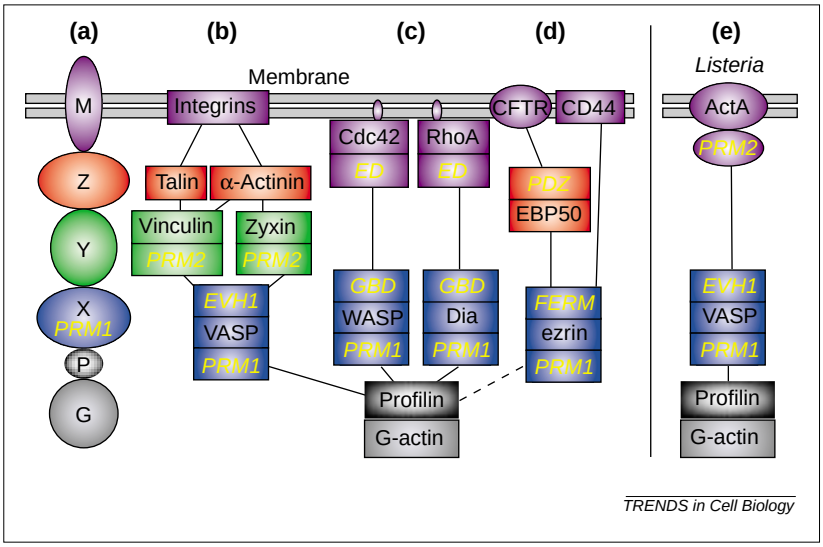

Fig. 3. Profilactin-recruiting chains. (a) A generic scheme depicting recruitment of profilactin to the membrane: G (G-actin), P (profilin), X (a PRM1-containing protein, blue), Y (a PRM2-containing protein, green), Z (a protein associated with an integral membrane protein, red), M (an integral membrane protein, purple). (b–e) Alternative mechanisms of profilactin recruitment: black lines indicate known direct interactions, the dotted line hypothesized interaction; black text refers to protein names, yellow text to the names of the interacting domains/motifs. Note that, in (e), ActA is a bacterial (*Listeria monocytogenes*) PRM2-containing integral membrane protein.

Table 2. Classification of proteins according to the presence of particular proline-rich motifs[a]

PRM1 (bind to profilin)	PRM2 (bind to EVH1)	PRM3 (bind to SH3)	PRM4 (bind WW)
Ena/VASP/Evl	ActA	Ena (Mena)	Ena (Mena)
WASP	Zyxin	WASP	Formin
WAVE/SCAR	Vinculin	WAVE	Dia
Dia	Fyb/SLAP	Dia	
Formin	WIP	Zyxin	
WIP		Vinculin	
Drebrin		Fyb/SLAP	
Gephyrin		WIP	
Ezrin/radixin			
Zyxin			
Fyb			

[a]Categories of proteins based on possession of the indicated proline-rich motifs (PRMs). For instance, Dia contains PRM1, PRM3 and PRM4 motifs. Proteins in italics possess PRM1 but have not been shown to bind to profilin.

receptors. Drebrin is a widespread actin-associating protein, and, in epithelial cells, it is enriched at specific junctional plaques that lack vinculin[36]. Fyb and WIP are PRM1-containing proteins that also possess a PRM2 and are described below.

PRM2-containing proteins bind to EVH1

EVH1 domains bind motifs containing the core sequence FPPPP designated as PRM2 (Table 1). The first and fourth proline residues are particularly important, whereas substitution of the second (with most other amino acids) or third proline (with hydrophobic residues) is acceptable, resulting in binding activity being retained[37]. The basis for this can be seen in Fig. 2b. Pro1, Pro3, and Pro4 intercalate with aromatic residues within the binding cleft of the EVH1 domain, whereas Pro2 points away from the binding cleft[38]. We discuss below five PRM2-containing proteins: ActA, zyxin, vinculin, Fyn-binding/SLP76-associated protein (Fyb/SLAP) and WIP.

ActA

Listeria ActA was the first protein in which PRM2 was identified. ActA contains four separate PRM2 motifs (Fig. 4a). *Listeria* evades the host immune system by entering cells, including epithelial cells and macrophages, where it moves around the cytoplasm by inducing actin polymerization. It does this by recruiting host proteins (including Ena/VASP homologues and profilactin) to one end of the bacterium, from where it induces formation of a 'comet tail' of F-actin[39,54]. Ena/VASP proteins bind to at least one of the four PRM2s present in ActA (Fig. 3e). Profilin, in turn, binds to at least one of the four PRM1s present in the Ena/VASP proteins (Table 1 and Fig. 4a). The stoichiometry of these interactions is interesting. VASP itself is a tetramer[19]. Thus each ActA molecule could recruit up to 64 profilins ($4 \times 4 \times 4$) and therefore 64 G-actins. This is the basis for the highly localized concentration of actin monomers that are primed for polymerization. An effective molarity of ~1.5 mM profilin binding sites could exist within a spatially defined patch on the surface of *Listeria*[9]. This would be a very efficient mechanism for the funnelling of monomers to F-actin barbed ends, resulting in a massive protrusive force.

When peptides that block interactions between profilin and VASP, or between VASP and ActA, are microinjected into infected host cells, actin polymerization is prevented and bacterial motility impaired[40,41]. There is also a retraction of the host cell's lamellipodia/filopodia. It is now clear that ActA analogues that contain PRM2s exist in normal cells, and we now describe four such analogues: zyxin, vinculin, Fyb and WIP.

Zyxin

Zyxin was the first ActA analogue identified. It is a low-abundance protein found in focal adhesions, cell–cell contacts and in a periodic pattern along F-actin stress fibres[42]. The C-terminus consists of three LIM domains, and the N-terminal proline-rich region contains three PRM2s, one PRM1 and a number of PRM3 motifs (bound by SH3-domain-containing proteins, for instance the Rho family exchange factor Vav; Fig. 4a and Table 1). The proline-rich region of zyxin also binds to α-actinin, and this interaction is essential for recruitment of zyxin to focal adhesions and stress fibres[43]. Since α-actinin is an integrin-binding protein, it is possible to envisage a role for zyxin in recruiting profilactin to sites of activated integrins. Integrins bind to α-actinin, which recruits the PRM2-containing zyxin; PRM2 binds to the EVH1 domain of a PRM1-containing Ena/VASP protein, which recruits profilactin through the PRM1 motifs (Fig. 3b).

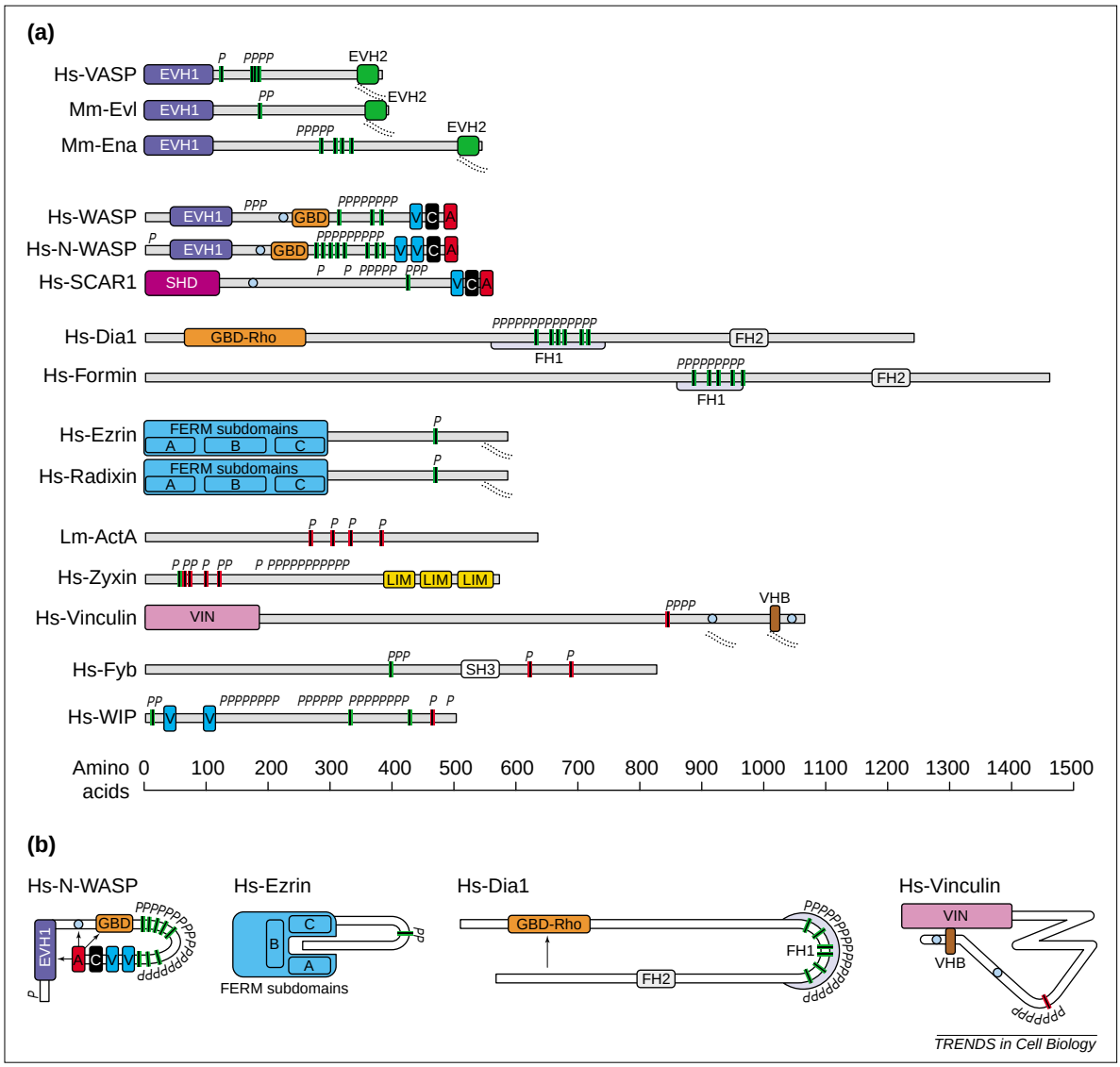

Fig. 4. Domain structures of proline-rich motif PRM1 and PRM2-containing proteins. (a) The size of the proteins and the position of regions of interest are indicated by the bottom scale. PRM1 and PRM2 are shown as green and red vertical stripes, respectively, within proline-rich domains. The extent of these domains is indicated by the length of 'PPPPP'. Proteins are grouped in the order: Ena/VASP family, WASP/WAVE family, FH family, ERM family, PRM2-containing proteins. Domains are indicated by coloured boxes: EVH1, -2 (Ena/VASP-homology 1 and -2), GBD (GTPase-binding domain specific for Cdc42 and Rac), GBD-Rho (GBD specific for Rho), V (verprolin homology, sometimes called WH2, for WASP homology 2), C (cofilin homology), A (acidic region), SHD (SCAR homology domain), FH1, -2 (formin homology 1 and -2), FERM (4.1-ERM homology domain with the indicated subdomains A, B, C), LIM (zinc-binding domain, rich in Cys and His, called 'zinc fingers'), VIN (vinculin-specific domain that binds to talin, α-actinin, and with a region in the vinculin C-terminus, VHB), VHB (VIN-binding region), SH3 (Src-homology 3). Blue circles represent acidic phospholipid-binding regions; referred to as BR (basic region) in WASP/WAVE family proteins. F-actin-binding sites are indicated by dotted lines. (b) Representation of four proteins in a folded auto-inhibitory conformation. The flexible proline-rich regions facilitate the intramolecular interactions. Interactions are indicated by arrows or close apposition. Regulation of these interactions plays an important part in the control of profilactin recruitment (see text).

Vinculin

Vinculin contains one PRM2 domain that binds to Ena/VASP family members[44] (Table 1 and Fig. 4a). This PRM2 is part of a proline-rich domain separating the N- and C-termini that are able to interact with one another (Fig. 4b). Relief from this 'closed' conformation is achieved by PtdIns(4,5)P_2, which exposes PRM2, allowing binding of VASP (through its EVH1 domain) to vinculin[45]. Such conformational regulation controls binding of other vinculin ligands, including the integrin-binding proteins talin and α-actinin[46]. Thus, we have another series of interactions that could recruit profilactin to active integrins. Integrins recruit talin and/or α-actinin, both of which bind to vinculin. Similarly to zyxin, the PRM2 motif in vinculin binds to the EVH1 domain of a PRM1-containing Ena/VASP protein, which again recruits profilactin (Fig. 3b).

Further evidence for the role of vinculin in profilactin recruitment is provided by studies with another intracellular pathogen, *Shigella flexneri*. Like *Listeria*, this bacterium hijacks the host cytoskeleton. It uses a bacterial protein, IcsA, to recruit Ena/VASP proteins and thus profilactin. Unlike ActA on *Listeria*, IcsA does not contain PRM2 – but it does bind to vinculin. *Shigella*

produces a protease that cleaves the C-terminal domain from vinculin, leaving PRM2 exposed and able to bind to Ena/VASP proteins. *Shigella* also associates with the PRM1-containing N-WASP as an alternative mechanism for recruiting profilactin[47]. There have been suggestions that *Shigella* does not require vinculin for bacterial motility[48], but subsequent studies have shown that assumptions about the absence of vinculin were flawed[49].

Fyb/SLAP

Another ligand for EVH1 domains has recently been identified. Fyb (or SLAP) is associated with the tyrosine kinase Fyn and the adaptor SLP-76 (Ref. 50). Fyb/SLAP has one PRM1 and two PRM2-like motifs (Fig. 4a); so far, only one of these has been shown to mediate EVH1-binding *in vitro*. Fyb/SLAP, together with Ena/VASP, WASP and the Arp2/3 complex, is recruited to the interface between beads coated with antibodies to CD3 and T-cells. This T-cell activation [by T-cell receptor (TCR) crosslinking] causes extensive actin rearrangements that result in polarization of the T-cell cytoskeleton towards the site of the crosslinking (physiologically, this would be an antigen-presenting cell). Inhibition of binding between Fyb/SLAP and Ena/VASP impairs TCR-dependent actin reorganization.

WIP

Finally, WASP-interacting protein, WIP, was identified using a two-hybrid screen for proteins interacting with WASP[13,51]. WIP is a very proline-rich protein that contains several profilin-binding motifs – that is, PRM1. In addition, residues 461–467 (DLPPPEP) constitute a potential EVH1-binding motif – that is, PRM2 (Fig. 4a). WIP might interact with EVH1 on WASP through this potential PRM2[52]. Indeed, deletion mutant analysis reveals that WASP residues 101–151 (within the EVH1 domain) are necessary for the interaction with WIP. This interaction is impaired in the three WASP mutants known to cause Wiskott–Aldrich syndrome[53]. Examples of PRM3 are also present in WIP, one of which binds to the SH3-containing adaptor Nck. Two verprolin-homology domains present in the WIP N-terminus are important to the actin-regulatory function.

Concluding remarks

Recruitment (funnelling) of profilactin to a particular location on the membrane is essential for actin-based motility. We have focused on the role of proline-rich motifs that bind to profilin and/or EVH1 domains, and designated them as PRM1 and PRM2, respectively. We have extended the classification to include proline-rich motifs that bind to SH3 domains (PRM3) and WW domains (PRM4, -5 and -6). Various other names have previously been used for these motifs with no apparent consistency.

We have classified proline-rich proteins according to their position in a schematic 'profilactin-recruiting chain' shown in Fig. 3a. In this chain, polymerization-competent, ATP-primed G-actin is funnelled to the plasma membrane in the form of profilactin by a series of protein–protein interactions. In all cases, profilactin binds to a PRM1-containing protein 'X'. Protein X often contains additional sites that recruit actin and actin-regulatory proteins – for example, the nucleating complex Arp2/3. Thus, X acts as a 'conveyor belt', where the proline-rich region (with PRM1s) serves to deliver building blocks to the 'construction site' at the C-terminus, overlooked by a 'supervisor' from the N-terminal site. X is linked to a membrane protein 'M'. This link can be a direct one – for example, binding of WASP to Cdc42, Dia to Rho (Fig. 3c), ezrin to CD44 (Fig. 3d) or VASP to ActA (Fig. 3e). Indirect links also exist and include a PRM2-containing protein 'Y' and/or a protein 'Z', which is associated with the membrane protein M. Examples include protein assemblies associated with activated integrins (integrin–talin/α-actinin–vinculin–VASP; Fig. 3b) or with other integral membrane proteins (CFTR–EBP50–ezrin; Fig. 3d).

The linking of the components in the chain is a highly regulated process. Auto-inhibitory intramolecular interactions often result in masking of functional sites. Release of this inhibition is achieved by a conformational change, induced by one of the following mechanisms: binding of phospholipids (vinculin, WASP), phosphorylation (ezrin) or binding of other proteins (WASP, Dia). The flexibility of the central proline-rich domain must play a crucial role in allowing the folding of these molecules (Fig. 4b). Thus, proline-rich domains act as hinges and at the same time provide the binding sites for the appropriate partners.

Future developments will shed light on the interplay between proteins that recruit profilactin and those that nucleate or organize actin. The upstream signal-transduction pathways that trigger the formation of profilactin-recruiting chains will be characterized further. Specific features of such pathways and of the resulting profilactin-recruiting chains in different cell types will be of particular interest. Characterization of such individual attributes will constitute relevant pharmaceutical targets since abnormal protrusive activity forms a basis for a wide rage of diseases –for example, inappropriate extravasation of immune system cells or metastasis of tumour cells.

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