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Minireview

Structure/function studies on cytoskeletal proteins in *Dictyostelium* amoebae as a paradigm

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Abstract **The actin cytoskeleton in motile non-muscle cells is being regulated by a large number of actin-binding proteins. A deeper insight into the complex nature of the dynamic rearrangements of the microfilament system during cell movement requires an experimental system that allows the combined application of biochemical, biophysical, cell biological and molecular methods. *Dictyostelium* amoebae are well suited especially for a genetic approach because they are amenable to gene disruption, antisense and gene tagging techniques. The actin-binding proteins profilin, hisactophilin and protovillin are being described in this context as typical examples that either bind to G-actin, or anchor the actin cytoskeleton to the plasma membrane, or are structurally similar to vertebrate proteins but distinct in their functions.**

Key words: Actin-binding protein; Cytoskeleton; Cell motility; *Dictyostelium*; Molecular genetics

1. Key proteins in the actin cytoskeleton of motile non-muscle cells

Compared to the extremely well organized and stably structured actin cytoskeleton in a striated muscle cell, the cytoskeleton is very dynamic in motile cells like leukocytes, platelets, satellite cells, fibroblasts and other cell types that migrate individually on a surface or through tissues. It is absolutely essential for these cells to be able to reorganize the cytoskeleton efficiently and fast, otherwise it would not be possible to fight against bacterial and viral infections, to undergo chemotaxis during muscle regeneration, or even to perform normal cytokinesis.

Among the three major classes of filaments (microfilaments, microtubules, intermediate filaments) the microfilament system in most cases seems to be responsible for whole cell movement, cell shape and distinct intracellular motile reactions. Microfilaments are about 6 nm wide and consist of actin. For the dynamics in the actin cytoskeleton it is important that actin filaments can quickly polymerize and depolymerize, which is triggered by the ionic conditions in the cytoplasm and by a large number of actin-binding proteins (for reviews see [1–3]). The sometimes confusing multitude of actin-binding proteins is easier to overlook if one keeps in mind that the modes of interaction with the target protein actin are limited and can be subdivided into functional classes. In a somewhat simplified scheme and with emphasis on the structure of the cytoskeleton, there are only three major reactions of actin-binding proteins with actin (Fig. 1):

Group (**a**) contains proteins that bind to monomeric, globular actin ('G-actin') and thus reversibly remove polymerizable actin from the equilibrium with filamentous actin ('F-actin'). This lowers the viscosity in the cytoplasm by decreasing the number and lengths of filaments. Typical members of this protein family are profilin, cofilin, thymosin [4–6].

All other proteins bind to filamentous actin and the type of interaction dramatically influences the viscoelasticity in the cytoplasm and consequently the ability of a cell to move. If one looks just at the geometry of an actin filament there are only three places to bind, namely at the two ends which are functionally distinct, and along the side of the filament.

Group (**b**) contains the end-binding proteins, appropriately called 'capping proteins'. They inhibit further addition of monomers, thus keeping filaments short and viscosity low. The best investigated examples of this group are heterodimeric capping proteins like cap32/34 [7], protovillin [8], gelsolin [9], villin [10], fragmin [11], severin [12,13]. Several of these capping proteins are able to cleave the filaments first which greatly enhances the disappearance of a filamentous network.

Group (**c**) contains proteins that bind along the side of filaments. can either stabilize the filament itself (e.g. tropomyosin [14]), they can crosslink filaments and form bundles as well as three-dimensional networks (e.g. α-actinin [15], filamin [16], fimbrin[17]), anchor filaments at membranes (e.g. spectrin [18], ponticulin [19], hisactophilin [20,21], work as motor molecules that either slide filaments against each other causing contraction, or move cargo along actin filaments (e.g. myosin I, myosin II [22]).

2. Genetic approaches to study cytoskeletal proteins in *Dictyostelium*

Single cell movement has been studied using very elegant tools and suited cell types from either tissue culture systems or higher organisms. A most attractive and versatile system however seems to be *Dictyostelium discoideum* because it combines the possibilities to investigate cell motility at the single cell and

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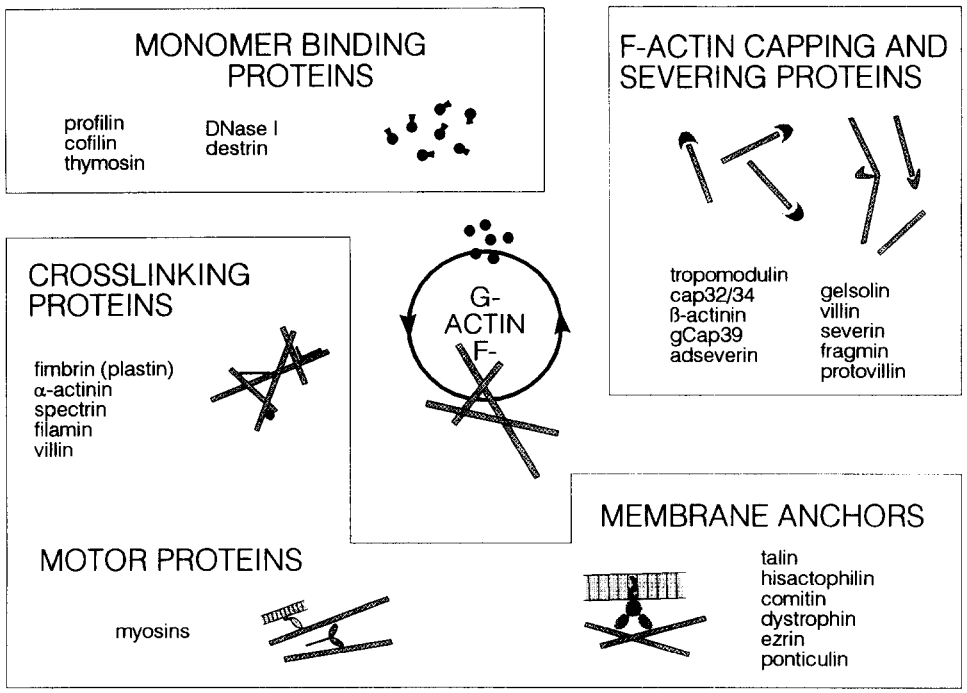


Fig. 1. Actin-binding proteins and their function. The equilibrium between monomeric G-actin and filamentous F-actin as well as the structural organization of the network of microfilaments are being influenced by actin-binding proteins. They either bind to monomeric actin thus inhibiting polymerization, or cap, sever, anchor, crosslink or move actin filaments via binding to the ends or along filaments.

multicellular stage with biochemical and genetic methods. There are no difficulties to obtain 500 g of packed cells over a period of three days and to characterize purified proteins in vitro. Moreover the system is amenable to molecular genetics which allows studies on cell motility and development after either knocking out distinct cytoskeletal proteins by gene disruption and antisense techniques, or overexpressing these proteins, point-mutated isoforms, or protein chimerae. Fig. 2 shows a schematic overview over the genetic approaches successfully used so far to generate mutants with defects in cytoskeletal components. The data that can be obtained with these approaches will shed light also onto the molecular principles during cell movement of leukocytes, and satellite cells, for instance.

In the following a more detailed description of studies on three representative actin-binding proteins will show how the system can be used to understand the function of cytoskeletal proteins.

2.1. Profilin

Profilin is a well known ubiquitous actin-binding protein and was discovered many years ago, but there is still a controversial discussion about its actual function in a living cell. The profilins are small proteins (about 12 kDa) and show in vitro a number of activities which include rather structural functions like the physical sequestration of monomeric actin or regulatory activities that influence the kinetics of actin polymerization or signal transduction via binding to lipids and poly-proline stretches of target proteins [6,23–25].

Dictyostelium contains two profilin isoforms (profilin I and II) which are encoded by single genes [26]. Like the profilins from other organisms, also *Dictyostelium* profilin is able to bind in vitro to monomeric actin in a 1:1 molar complex. To study the in vivo function of profilin we constructed *Dictyostelium* mutants by using antisense and gene disruption techniques [27]. The expression of profilin I was turned off by introducing an expression vector that contained the profilin I cDNA in antisense orientation and selection of transformants for G418 resistance; a similar approach for profilin II resulted in a complete deletion of the gene. Finally, the double mutant which did not contain any of the two profilin isoforms had the profilin I gene disrupted and the profilin II gene deleted.

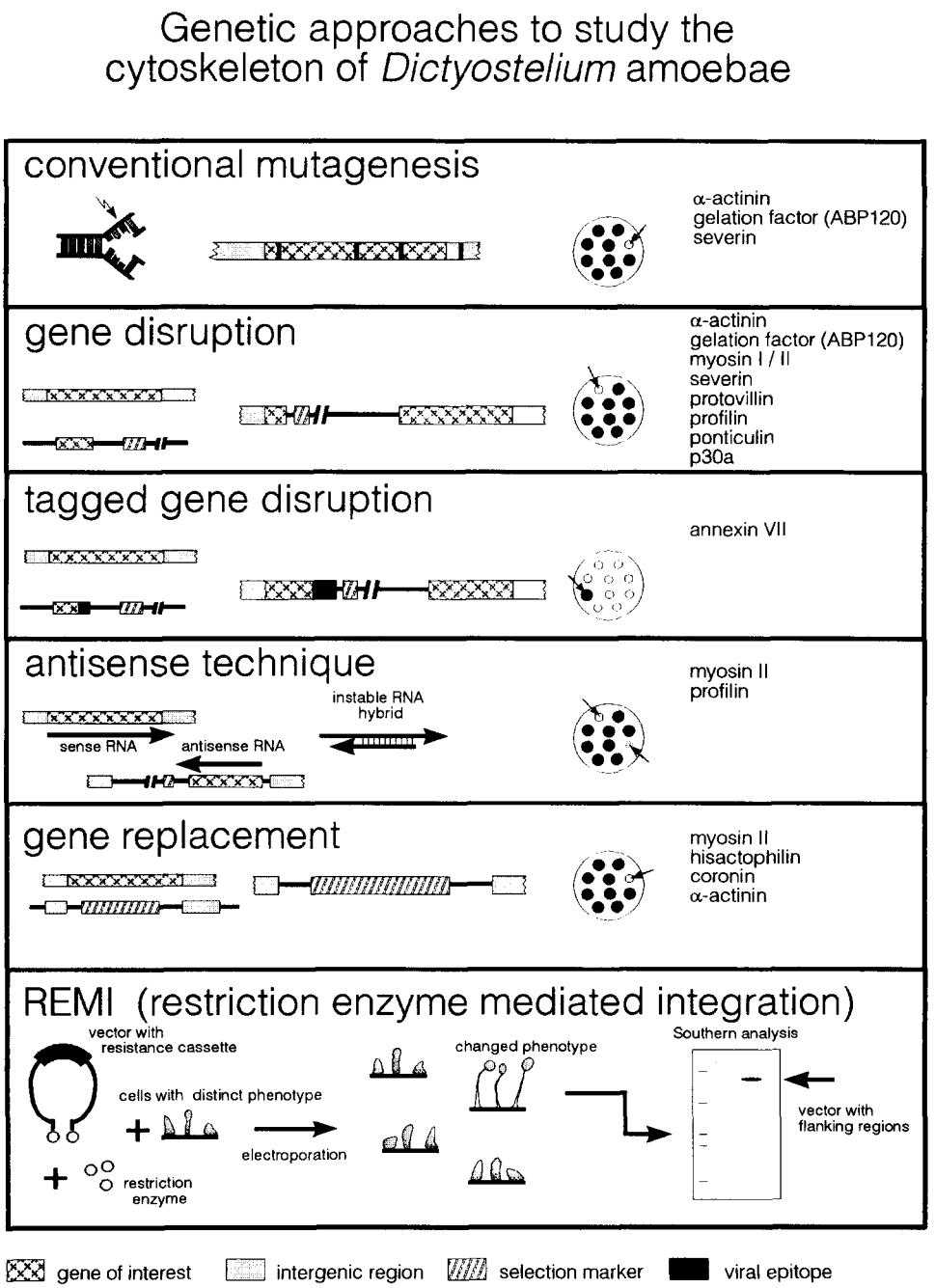
In contrast to the double mutant the loss of one of the two profilin isoforms did not lead to a drastic change in cellular behaviour. This agrees with the findings on other actin-binding proteins like the F-actin crosslinking proteins α-actinin and 120 kDa gelation factor. Sufficient viscoelasticity of the cytoskeletal network is apparently guaranteed by many cytoskeletal components ('functional redundancy') and a small change might not result in obvious aberrant phenotypes [28]. *Dictyostelium* amoebae lacking both profilins showed us what the major functions of profilin in a living cell might be. The cells did survive, i.e. for living in a laboratory profilin is not essential. However, using an image analysis system for evaluation of motility and chemotactic orientation we found that speed of migration was reduced by about 40%. The wild type cells in chemotaxis experiments with cAMP moved with an average speed of 10.2 μm/min, whereas the profilin-minus cells were significantly slower (6.4 μm/min). The same experiments showed, however, that chemotactic orientation was not impaired. This suggested that profilins are not involved in this way of signal transduction which connects the β-adrenergic type cAMP receptor via G-proteins finally with cytoskeletal responses. Further studies with these mutants indicated that the G-actin sequestering function of profilins plays the most important role. Amount and distribution of filamentous actin were

obviously disturbed and led to drastically enlarged cells with a broad rim of F-actin, to defects in cytokinesis and to a block during the developmental cycle at the stage of fruiting body formation. The ratio of F- to G-actin was increased from roughly 1:1 in wild type cells to about 3:1 in profilin-minus mutants [27].

The opportunity to manipulate *Dictyostelium* cells genetically enables us to dissect the functional activities of profilins in this system. Preliminary data show that profilins of distant origins (e.g. *Zea mays* pollen profilins) can rescue aberrant phenotypic behaviour thus proving general principles of action (Karakesisoglou, unpublished). Furthermore, we started to mutate functional sites within the molecule by deleting poly-proline-binding, actin-binding, or lipid-binding activities [29].

2.2. *Hisactophilin*

Among the proteins that bind to the side of an actin filament and anchor the cytoskeleton to the plasma membrane the two hisactophilin isoforms are especially interesting, due to their unusual amino acid composition. Out of 118 amino acids there are 31 and 35 histidine residues for hisactophilin I and II, respectively. This high content of histidine residues results in a pH-dependent activity in such a way that at a pH below 7.2 hisactophilin binds at a molar ratio of up to 1:1 to actin filaments whereas binding is completely abolished at pH values above 7.2 [30]. Because reversible binding occurs at physiological pH values, it was suggested that hisactophilin might function as a pH sensor at the plasma membrane. In collaboration with T. Holak (MPI f. Biochemistry, Martinsried, Germany)



we determined the three-dimensional structure of hisactophilin I which contains only β-strands and loops [20,31]. In the asymmetric molecule the loops contain almost all histidine residues and are clustered at one side of the protein. The opposite region harbours the N- and C-termini and forms a tight β-barrel structure which points towards the membrane and inserts via a covalently bound myristic acid into the inner leaflet of the plasma membrane [21,32].

We used a combined biochemical and genetic approach to investigate whether hisactophilin is a cytoskeletal protein that functions also in vivo in a pH-dependent fashion. With a gene replacement vector we inactivated the hisactophilin I gene and obtained cells that lacked hisactophilin I and overexpressed hisactophilin II by tandem insertion of the hisactophilin II gene with its own promoter. The overexpression was stable and very strong, the molar ratio of actin to hisactophilin increased from about 10:1 to 1:2. First studies on cellular behaviour showed that the overexpressing mutants behaved like wild-type cells in all assays performed, including growth in axenic medium, growth on *Klebsiella aerogenes*, chemotaxis and development. All these were unaltered even if one changed the pH in the surrounding medium from pH 5.5 to 8.5, most likely because the cells have very powerful abilities to keep the intracellular pH at a physiological level of about 7.2, conditions at which hisactophilin does not bind to actin in vitro. After application of proton pump inhibitors, however, the acidification of the cytoplasm caused in wild type cells the formation of balloon-like blebs at the plasma membrane, and the cells eventually burst. In hisactophilin overexpressing mutants the cells remained intact over a wide range of inhibitor concentrations which suggested that in fact upon acidification of the cytoplasm hisactophilin anchors the actin cytoskeleton at the membrane (Stöckelhuber, unpublished). In future studies, knock out transformants lacking both hisactophilins will be used; these cells might tell us whether specific responses of the cytoskeleton to extracellular signals are being disabled because small pH changes below the plasma membrane cannot be sensed anymore.

2.3. Protovillin

One of the difficulties to understand the dynamics of the microfilament system is the sheer and still increasing number of actin-binding proteins that can be present at the same time and same location in a cell. In comparison with specialized cells like nerve or striated muscle cells which can fulfil their function with a limited set of proteins, a rather omnipotent amoeba like a *Dictyostelium* cell needs apparently the whole spectrum of actin-binding proteins. No more than three years ago one believed that the evolution of actin-binding proteins can be thought of as a sequence of gene duplications. According to this hypothesis gene duplications starting from a profilin precursor led to F-actin severing and capping proteins like the three-domain proteins severin from *Dictyostelium* and fragmin from *Physarum*, followed by six-domain proteins like gelsolin and villin in vertebrates. Villin was a special case in point because it acquired with the headpiece at the C-terminal end of the six gelsolin-like domains a peculiar function which rendered the protein as specific for brush border microvilli [10].

In our search for severin-like proteins in *Dictyostelium*, we isolated and characterized a 100 kDa protein that capped but did not fragment actin filaments [8]. Cloning and sequencing of the cDNA revealed that it contained six domains and a C-terminal headpiece like villin; the similarity to the vertebrate protein villin was higher than to the homologous three-domain protein severin in the same cell [33]. This suggests that gene duplications might have happened but that they occurred long before *Dictyostelium* branched off in evolution. The protein in *Dictyostelium* which we designated 'protovillin' seems to be another example that many actin-binding proteins with similar functions exist in parallel, but that there are distinct differences which forced the cell to keep the protein during evolution. Also in this case, we aim at a genetic approach by knocking out the gene and by construction of double and triple mutants.

Taken together, the complex nature of the microfilament system in motile cells and the highly dynamic rearrangements that seem to be regulated by a large number of actin-binding proteins make it necessary to use a genetic approach to understand the cascades of reactions. Only by taking out one protein after another and by re-expressing engineered genes, we will obtain cells that can be investigated at the cell biological, biochemical and biophysical level.

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Fig. 2. Molecular techniques that have been successfully used to study the actin cytoskeleton in *Dictyostelium*. (Top panel) The first approach for isolation of cytoskeletal mutants was by conventional mutagenesis with nitrosoguanidine which sets mutations randomly in the replication fork. Besides many other defects in the genome, also the gene of interest can be disrupted several times. Screening was done with specific antibodies to select mutants that were deficient for α-actinin [34,35], severin [36] and ABP-120 gelation factor [37]. (Second panel) Since the discovery of homologous recombination in *Dictyostelium* [38,39] a large number of genes that encode cytoskeletal proteins was disrupted ([19,27,28,40–42], unpublished data). A piece of homologous DNA guides the vector carrying a resistance marker for selection to the gene of interest. The vector inserts and disrupts the endogenous gene; deficient mutants can be isolated with antibodies or by analyzing the chromosomal DNA for the disruption event. (Third panel) Tagged gene disruption can be used if there is no specific antibody available. For the disruption of annexin VII (synexin) a viral epitope was fused in frame to the homologous DNA piece in the vector. After transformation positive colonies can only occur if the vector inserted into the corresponding region of the target gene, and the viral epitope is expressed under the control of the endogenous promoter [43]. (Fourth panel) For deleting cytoskeletal proteins with the antisense technique, a promoter located at the 3'-end transcribes a homologous DNA region in the vector in antisense orientation. The presence of sense and antisense RNAs leads to hybrids which are being degraded. Analysis of the mutants with antibodies often shows that the concentration of the corresponding protein is only reduced. This can help to study in vivo functions of proteins whose complete deletion might be lethal. Antisense approaches were successfully used for isolation of myosin [44,45] and profilin mutants [27]. (Fifth panel) The complete loss of DNA from the endogenous gene can be achieved with a vector that carries two homologous DNA regions with the resistance cassette in between. A double crossover replaces endogenous DNA with the resistance cassette, reversion of normal gene expression is impossible. Replacement approaches for the deletion of cytoskeletal proteins were used for knocking out myosin II [46], hisactophilin (Stöckelhuber, unpublished), coronin [47]. (Bottom panel) Restriction enzyme mediated integration allows selection for altered phenotypes and concurrent tagging of the disrupted gene with the vector [48]. For selection of genes that are able to suppress the profilin-minus phenotype (no fruiting body formation) we inserted vector together with restriction enzyme into profilin-minus cells, selected for normal fruiting body formation, and currently try to re-isolate the vector together with flanking regions from the yet unidentified suppressor gene (Karakesisoglou, unpublished).

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