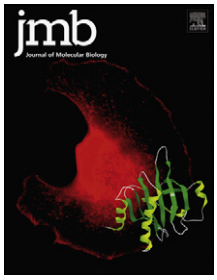

A Fluorophore Fusion Construct of Human Profilin I with Non-Compromised Poly(L-Proline) Binding Capacity Suitable for Imaging

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Abstract

Profilin is vital for actin organisation in eukaryotic cells. It controls actin filament formation by binding monomeric actin and numerous proteins involved in polarised actin assembly. Important for the latter is the interaction surface formed by the N- and C-terminal helices, which pack close to each other on one side of the molecule at a distance from the actin site and mediate binding to poly-proline sequences present in many of the targeted proteins. Via these interactions, profilin contributes to the spatiotemporal control of actin filament growth. Studies of profilin dynamics in living cells by imaging techniques have been hampered by problems to generate fusion constructs with fluorophore proteins without negatively impacting on its poly-proline binding. With the object to circumvent this problem, we have generated an internal fusion of profilin with the green fluorescent variant citrine, here referred to as citrine–profilin. The characterisation of citrine–profilin (CIT-Pfn) demonstrates that it has full capacity to interact with poly-proline and also binds phosphatidylinositol lipids and actin, albeit with 10 times reduced affinity for the latter. Imaging of living cells expressing CIT-Pfn showed a distribution of the fusion protein similar to endogenous profilin. Furthermore, CIT-Pfn rescued the phenotypes observed after the Crispr/Cas9 knockout of the profilin 1 gene, including the lost migratory capacity characterising the knockout cells. Based on this, we conclude that the CIT-Pfn construct will be useful as a tool for displaying profilin localisation in living cells and obtaining information on its dynamic organisation under different conditions and activations of the actin microfilament and microtubule systems.

Introduction

Cell migration and contact establishment with the extracellular matrix and neighbouring cells are a consequence of coordinated activities exerted by the dynamic actin microfilament and microtubule systems, respectively, in response to different signalling cues. A huge number of regulatory components are involved in this machinery. Profilin is one well-known actin monomer binding protein that impacts on actin nucleotide exchange and filament formation. Furthermore, through its ability to bind to actin nucleation and elongation promoting factors (NEPFs) such as formins, Ena/VASP, and WASP/WAVE family members independent of its interaction with actin, it is tightly linked to the spatiotemporal control of actin remodelling [1–3]. Moreover, profilin was recently found to influence microtubule organisation in cultured cells [4], making it a strong candidate for direct involvement in coordination of the organisation of the actin microfilament and microtubule systems. Therefore, it is important to map profilin distribution in living cells under different conditions to gain further mechanistic insights into how different actin and microtubule linked processes are coordinately regulated during cell growth and migration.

Despite its importance and as one of the first interaction partners to non-muscle actin to be isolated [5] and consequently studied for several decades, as reviewed in Refs. [6,7], it has so far not been possible to perform advanced imaging studies of profilin. A major reason to this is the difficulty to generate a fully functional fusion protein with any of the commonly used fluorophore proteins such as the green fluorescent protein (GFP) derivatives. Profilin consists of a central core of seven beta sheets with two α-helices on each side. The actin-binding site includes the most N-terminal residue of the C-terminal α-helix and residues in one of the α-helices on the opposite side of the molecule and in the central β sheets between them [8]. The poly-proline binding site is formed by residues in the N- and C-terminal helices, which pack close together against one of the β-strands with the aromatic interaction between W3 and W31 as an important structure-stabilising factor [8–11]. The binding of phosphatidylinositol lipids (PIPs) partly overlaps with this interaction surface and with the actin-binding surface in a second binding region [12–14]. In accordance with the importance of the structural motif formed by the profilin N- and C-terminal helices for its ability to bind poly-proline, manipulation of either end of the protein chain such as the expression of fusion constructs is known to disturb this function. Either this is a consequence of the fused molecule affecting the protein fold at this site or the construct sterically interferes with the binding of different interaction partners [15]. The ability to interact with proline-rich sequences is central to profilin function. By recruitment of profilin–actin via this interaction, a range of actin NEPFs spatiotemporally controls the supply of monomeric actin for filament formation and elongation. Together with the interaction with PIPs, poly-proline binding is therefore one reason that profilin antibodies, when employed for fluorescence microscopy, detect the protein with a particularly prominent fluorescence underneath the plasma membrane in cell edge protrusions typically involved in actin polymerisation such as lamellipodia and filopodia [16,17].

The importance of the poly-proline binding surface for profilin function and the difficulties to generate fusion constructs without interfering with this property have been a serious drawback for attempts to follow its distribution in living cells by imaging techniques. Here, we present a solution to the problem by having successfully inserted the GFP variant citrine internally in the profilin molecule at a distance from the poly-proline and actin-binding sites. Although this approach is not new, for instance, see Refs. [18,19], it has, to our knowledge, not been successfully employed before with such a small, single domain and multi-ligand binding protein as profilin. Our observations therefore suggest that this is a solution for proteins where a similar problem to functionally accept fusion molecules at either end of their polypeptide chain has been encountered.

Results and Discussion

The cellular localisation of profilin (unless otherwise stated, "profilin" refers to profilin 1) [4,16,17] is the collective result of interactions with its three principal binding partners, that is, monomeric actin (G-actin), other proteins with the typical profilin-binding poly-proline sequence motif [20] such as many NEPFs, and PIPs. With the exception of profilin-binding partners in the nucleus [21] and microtubule-associated formins [4], these are primarily contributing to accumulate profilin near the plasma membrane in regions where actin remodelling is particularly high. Of these, we consider the poly-proline interaction to be the principal factor determining the cellular localisation of profilin. Although the binding to actin is of key functional importance and, at every instance, is reported to engage a major fraction of profilin molecules in the cells [22,23], it largely reflects profilin:actin recruitment by different poly-proline containing NEPFs to sites where actin polymerisation occurs [17,24–27]. With respect to the PIPs, finally, this interaction involves two regions on the molecule that partially overlap with the poly-proline and actin sites, respectively [12,14] and causes the dissociation of profilin:actin [13]. Possibly, this actin-freed profilin remains transiently associated with the membrane as has been reported for cofilin [28,29], where it may influence other PIP-interacting components at the inner leaflet of the membrane [30]. However, we favour the poly-proline and actin interactions as the major localisation determinants, and based on the above reasoning, poly-proline binding to be the most decisive for profilin distribution.

The poly-proline interaction and the overlapping PIP-interacting region depend on a proper folding of the profilin N- and C-terminal termini [9,14]. Therefore, we aimed to generate a fluorophore fusion construct where the fusion protein did not affect the structure of the poly-proline binding surface nor was compromised in its ability to bind actin and PIPs. For this reason, the GFP-related fluorophore citrine was placed in a loop, which extends away from both the poly-proline and actin interacting surfaces to generate citrine–profilin (CIT-Pfn), where profilin Q79 is connected via a hexa-peptide (SGSSGS) to the citrine N-terminal and its C-terminal to profilin D80 via a linker peptide consisting of GGSLQ (Fig. 1a).

Bacterially expressed CIT-Pfn was isolated and compared to wild-type profilin expressed and isolated in parallel. The isolated fusion protein was homogeneous, as demonstrated by SDS-PAGE and Western blot after the detection of either the profilin or citrine parts of the chimeric molecule