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Mutagenesis of human profilin locates its poly(L-proline)-binding site to a hydrophobic patch of aromatic amino acids

Camilla Björkegren[a], Mike Rozycki[b], Clarence E. Schutt[b], Uno Lindberg[a], Roger Karlsson[a,*]

[a]*Department of Zoological Cell Biology, W-GI, Stockholm University, S-106 91 Stockholm, Sweden*
[b]*Department of Chemistry, Henry H. Hoyt Laboratory, Princeton University, Princeton, NJ 08544, USA*

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The actin-binding protein, profilin, contains a src-homology (SH) 3-like fold (Schutt, C.E. et al., submitted), and its tight interaction with poly(L-proline) is reminiscent of the binding activity exhibited by SH3-domains. Here we demonstrate that replacements of aromatic amino acids in a hydrophobic patch on the surface of the profilin molecule abolish its poly(L-proline)-binding capacity. However, the location of this hydrophobic patch is found in another region of the molecule than that displaying structural similarities with SH3 domains.

Profilin; Microfilament system; Mutagenesis; Poly(L-proline); SH3 domain

1. INTRODUCTION

The actin-binding protein, profilin, is implicated in the regulation of actin polymerization [1] and the hydrolysis of phosphatidylinositol-(4,5)-bisphosphate (PIP2) [2,3]. These functions of profilin link the dynamics of the microfilament system to processes involved in transmembrane signalling and may be part of the mechanisms behind the immediate mobilization of the microfilament system upon stimulation of cells with growth factors like epidermal growth factor and platelet-derived growth factor [4]. Direct evidence for the involvement of profilin in the early phase of cell signalling comes from studies of platelets where the dramatic morphological change caused by thrombin stimulation is initiated by the dissociation of the profilin–actin complex with extensive formation of actin filaments as a consequence [5]. Similar observations have also been made with polymorphonuclear leukocytes after stimulation with a chemo-attractant [6]. In addition to actin and PIP2, profilin interacts tightly with poly(L-proline) [7], and this feature is often exploited in the isolation of profilin from various tissue extracts by affinity chromatography on poly(L-proline)-coupled matrices [8].

Bovine profilin exposes an aromatic patch of hydrophobic amino acid residues on its surface that is formed by 6 highly conserved amino acid residues (W3, Y6, W31, H133, L134, Y139) brought together by the folding of the polypeptide chain (Schutt, C.E. et al., submitted). The fluorescence of the two tryptophans, W3 and W31, in this patch is quenched when profilin binds PIP2 [9], supporting the proposal of a PIP2-binding sequence in the closely positioned C-terminus [10].

Src-homology (SH)2 and 3 domains function as linker domains in proteins involved in receptor tyrosine kinase-mediated cell signalling, and play a decisive role in signal transmittance from the activated receptor to Ras by establishing specific protein–protein interactions (for refs. see [11]). The SH2 domain binds phosphotyrosine, and SH3 recognizes proline-rich stretches via a surface formed by aromatic amino acid residues and has been proposed to be involved in contacts with protein components of the microfilament system [12–16]. This led us to whether the poly(L-proline)–profilin binding occurs via an interaction analogous with that between proline-rich polypeptide sequences and SH3 domains by introducing the mutations, W3N and H133S, in the profile molecule. In addition, the glycine residue, G14, which is located outside this region, was mutated and included in the analysis. Previously we have shown that human profilin expressed in *Saccharomyces cerevisiae* and purified to homogeneity behaves as an authentic mammalian profilin with respect to its actin-, PIP2- and poly(L-proline)-binding properties [17]. This expression system and poly(L-proline)–Sepharose was used to express the mutant profilins and to analyze their poly(L-proline)-binding capacity.

2. MATERIALS AND METHODS

CNBr-activated Sepharose (LKB-Pharmacia, Sweden) was coupled with poly(L-proline) and bovine pancreatic DNase I (EC 3.1.21.1) according to the instructions provided by the manufacturer and as described in [8]. Non-muscle actin was isolated from calf spleen profilin–actin essentially as described in [8] except that the ammonium sulphate-precipitated complex was suspended in 5 mM potassium

*Corresponding author. Fax: (46) (8) 15 98 37.

phosphate buffer, pH 7.6, containing 0.5 mM ATP, 0.05 mM $CaCl_2$ and 5 mM DTT; and its dissociation and the concomitant actin paracrystal formation was induced by the addition of ethylenebis-(oxonitrilo)tetraacetic acid (EGTA) to 0.5 mM and $MgCl_2$ to 5 mM.

The amino acid replacements in the human profilin gene were introduced by oligonucleotide-directed mutagenesis following standard protocols for DNA manipulation. The mutagenesis was confirmed by sequence determination of the full-length mutant genes using the dideoxy technique [18].

Saccharomyces cerevisiae strain K923 (*HMLα*, mat::*LEU2*$^+$, *hmr*::*TRP1*$^+$, *ura3*, *ade2*, *sir3*ts, MATa at 23°C, MATα at 34°C) was transformed with a temperature-inducible expression system carrying the different profilin genes and cultured in selective media as described in [17]. Cells were collected by centrifugation, suspended in a buffer containing 5 mM Tris-HCl, pH 7.6, 0.1 mM $CaCl_2$, 0.5 mM ATP, and 0.5 mM DTT (G buffer). Protease inhibitors were added as in [19]. After addition of approximately 1 vol. of glass beads, the cells were lysed, vortexed vigorously and centrifuged. The resulting supernatants were applied to 0.2 ml poly(L-proline) columns equilibrated with G buffer. The flow-throughs were collected and subsequently applied to 0.2 ml DNase I–Sepharose columns which were equilibrated with G buffer and saturated with non-muscle actin by passing 2.5 vols. of G buffer containing 0.5 mg/ml of β/γ-actin through the column. The columns were washed with 15 vols. of G buffer, the resins were then isolated, and samples for SDS-PAGE were prepared by boiling in sample buffer. To enable a comparison of the level of profilin expression in the different extracts, the OD_{600} value of each culture was monitored and the volumes of isolated cultures were adjusted to compensate for differences in cell density prior to collection of the cells. The SDS-PAGE on 12.5% acrylamide minigels was performed as described by Matsudaira and Burgess [20]. Western blot analysis was performed using rabbit anti-profilin antibodies elicited against bovine profilin, and Amersham's ECL Western blotting Kit (horseradish peroxidase-labeled secondary antibodies and detection reagents, RPN 2106).

3. RESULTS AND DISCUSSION

The hydrophobic surface on the profilin molecule targeted in this mutagenesis study is indicated in the display of the polypeptide fold of the molecule shown in Fig. 1. The residue W3 is located between W31 and Y6, while H133 flanks the surface towards the C-terminus where Y139 is also found (not shown). Together with L134, these residues form a continuous hydrophobic surface on the molecule that is separated from the actin-binding surface and from its SH3-like fold (Schutt, C.E. et al., submitted).

To analyze the poly(L-proline)-binding activity of the recombinant profilins, extracts of yeast cells grown under conditions that allowed expression of human profilin from the introduced plasmid [17], were passed through poly(L-proline) columns, and the retained material was analyzed by SDS-PAGE after thorough washing of the columns. As expected, with the wild-type human profilin gene present on the plasmid, both human and endogenous yeast profilin were retained on the poly(L-proline)–Sepharose (Fig. 2a). This was also the case with extracts made of cells expressing either a G14A, or a G14D mutant profilin (data shown for G14A). However, when the W3N or H133S mutant profilins had been expressed, no human profilin was found in the poly(L-proline)–Sepharose-bound material, suggesting either that the interaction was abolished by the mutations or that the expression of the corresponding profilin genes had been reduced dramatically.

The aromatic patch of amino acid residues, which contains W3 and H133, in profilin is separated from the major actin-binding site, and the major contact with DNase I on the actin molecule is distinct from the major profilin binding site ([5,21]; Schutt, C.E. et al., submitted). To verify this in solution we passed an extract of cells expressing human profilin through a DNase I–Sepharose column saturated with non-muscle actin with the result that the human profilin was retained (data not shown). Furthermore, this enabled us to check whether the poly(L-proline) flow-through material derived from cells expressing the H133S and W3N mutant profilins contained profilin that still bound to actin. As shown in Fig. 2a, an extra band with the molecular weight of profilin was indeed present in the H133S mutant material. Also, with the W3N mutant, an additional band was seen, but in this case it migrated to a position indicating a somewhat higher molecular weight.

Western blotting with profilin antibodies identified the extra bands seen with the H133S and W3N material as profilin (Fig. 2b). All profilins were expressed in approximately the same amounts, as shown by Western blot analysis of the cell extracts prior to their separation on poly(L-proline)–Sepharose (Fig. 2c). This analysis confirmed the identity of the mutant profilins and that the W3N and H133S mutations abolished the binding of profilin to poly(L-proline). Sequence analysis of the full-length W3N mutant profilin gene did not reveal any anomalies that could explain the change in electrophoretic mobility observed with this mutant. It is therefore concluded that this is caused by the mutation itself.

These observations demonstrate that the targeted, surface-exposed aromatic amino acids are part of the poly(L-proline) binding site on the profilin molecule. Through this site, profilin could establish contacts between actin and other protein molecules carrying appropriate proline-rich motifs. Such an adaptor mechanism would be analogous to that expressed by SH3 domains, even though the hydrophobic patch of profilin is distinct from the corresponding active site in SH3 domains [13,22,23].

Sequence alignment of bovine profilin with the SH3 domain of PLCγ-1 reveals 54% sequence similarity and 24% identity between the two structures. In addition, profilin has an SH3-like fold and a hydrophobic patch with four consensus SH3 residues corresponding to Y24, F59, L78 and F83 of profilin ([22,24,25]; Schutt, C.E. et al., submitted). However, these SH3-like features do not coincide with the poly(L-proline) site mapped in this study. The significance of these SH3-shared qualities therefore remains to be seen.

Activation of growth factor tyrosine kinase receptors causes extensive reorganization of the microfilament

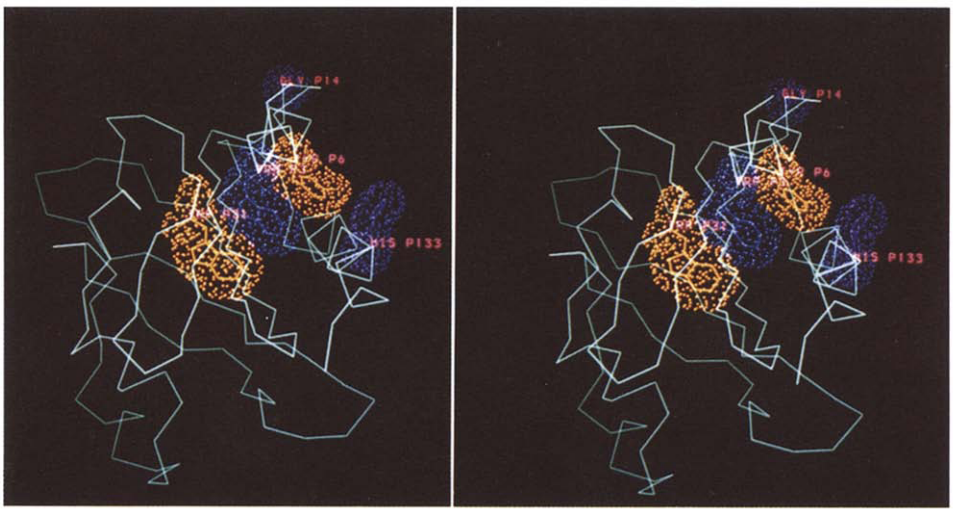

Fig. 1. The location of the SH3-like patch of aromatic amino acid residues in the profilin molecule. The main chain trace representing the 139 amino acid residues in profilin is displayed in light green. The residues shown with their side chains in blue are W3, H133 and G14 and were mutated in the current study. Those in yellow are additional residues that contribute to the aromatic patch on the surface of the molecule. The representation was generated with MRC.FRODO and is based on the crystallographic data presented in Schutt, C.E. et al., (submitted).

system implicating the Ras-like proteins, Rac and Rho [26,27]. Possibly the aromatic patch of amino acids on profilin functions in this signalling process. In addition, genetic studies in the yeast, *S. cerevisae*, functionally link profilin to the adenylyl cyclase-associated protein, *CAP*, which carries a poly(L-proline) stretch [28–30]. This may, therefore, represent another molecular target for profilin.

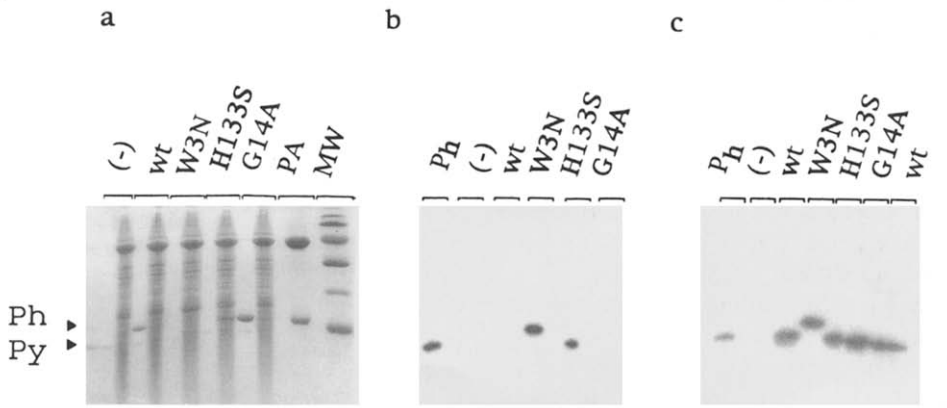

Fig. 2. Analysis of the poly(L-proline) and actin-binding activities displayed by the wild-type (wt) and the human profilin mutants (W3N, H133S, and G14A). Extracts of yeast cells expressing the different profilins were passed through Sepharose columns coupled with poly(L-proline). The flow-through materials from these columns were applied to DNase I–Sepharose columns saturated with non-muscle actin. After extensive washing, the different resins were isolated, boiled in SDS-PAGE sample buffer and centrifuged. The supernatants were then analyzed by SDS-PAGE as shown in (a) with the poly(L-proline) and actin–DNase I–Sepharose-bound material to the left and right, respectively. Each pair of lanes represents material from one profilin-expressing cell extract, as indicated. The lanes denoted (−) show the analysis of material derived from cells grown under non-expressing conditions. Arrowheads labeled Ph and Py indicate human and yeast profilin, respectively, and PA indicates a sample of bovine profilin–actin complex. The molecular weight markers (MW) indicate 94, 67, 43, 20.1 and 14.1 kDa. Note the absence of a protein corresponding to human profilin in the poly(L-proline)-bound material that was derived from cells expressing the W3N and H133S mutant profilins. Such a protein is instead present in the corresponding samples from the actin–DNase I columns (in the case of W3N it migrates to a position suggesting a higher molecular weight). (b) Identification of profilin in the actin–DNase I-bound material from the different poly(L-proline) flow-throughs by Western blot analysis using anti-profilin antibodies. Labeling is as in (a). (c) Western blot analysis as in (b) of the different cell extracts before affinity chromatography on poly(L-proline)–Sepharose using anti-profilin antibodies.

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