High Affinity, Paralog-Specific Recognition of the Mena EVH1 Domain by a Miniature Protein

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EVH1 domains are found within a large number of multidomain signaling proteins that regulate the dynamics of the actin cytoskeleton, including those where external stimuli regulate cellular motility, shape, and adhesion.[1] Examples include *Drosophila* Enabled (Ena)[2] and its mammalian counterparts Mena,[1a] vasodilator-stimulated phosphoprotein (VASP),[3] Enabled/VASP-like protein (Evl),[1a] and Wiskott–Aldrich syndrome protein (WASP).[4] EVH1 domains regulate actin filament dynamics through interactions with cytoskeleton-associated proteins including vinculin and zyxin, and are used by the ActA protein of *Listeria monocytogenes* during pathogenesis.[5] Like SH3 and WW domains, EVH1 domains recognize proline-rich sequences on target proteins[6] that are folded into type II polyproline (PPII) helices.[7] In the case of *L. monocytogenes*, the interaction of intracellular EVH1 domains with ActA contributes to the propulsion of the bacterium through the host cell cytoplasm and into neighboring cells.[8]

Previously we described a miniature protein design strategy in which the well-folded helix in avian pancreatic polypeptide (aPP) presents short α-helical recognition epitopes (Figure 1A).[10,11] The miniature proteins so designed recognize even shallow clefts on protein surfaces with nanomolar affinities and high specificity.[11] aPP consists of an eight-residue PPII helix linked through a type I β-turn to a 20-residue α-helix. Here we extend this protein design strategy to stabilize the functional epitope of ActA on the PPII helix of aPP. Like miniature proteins that use an α-helix for protein recognition, the miniature protein designed in this way displays high affinity for the Mena$_{1-112}$ EVH1 domain and achieves the elusive goal of paralogue specificity,[12] discriminating well between EVH1 domains of Mena$_{1-112}$, VASP$_{1-115}$, and Evl$_{1-115}$.

Our design began with the structure of Mena$_{1-112}$ in complex with the proline-rich peptide F$_1$P$_2$PP$_4$P$_5$ (FP$_4$).[13] The structure shows the pentapeptide bound as a type II polyproline helix, with residues P$_2$, P$_4$, and P$_5$ nestled into the concave, V-shaped, binding surface on Mena$_{1-112}$, and residue F$_1$ anchoring the peptide in the N-to-C direction.[13] Substitution of FP$_4$ residues F$_1$, P$_2$, and P$_5$ at positions S$_3$, Q$_4$, and Y$_7$ of aPP, and extension of this core sequence by two of three C-terminal acidic residues shown to improve affinity and specificity,[13,14,5c] led to the final sequence of pGolemi (Figure 1B).

pGolemi was synthesized using standard solid-phase methods[9] and examined by circular dichroism (CD) spectroscopy (Figure 1C). The CD spectrum of pGolemi at 25 °C exhibited minima at approximately 208 and 222 nm, as expected for a protein containing one or more α-helices, and was independent of concentration between 5 and 20 μM. The mean residue ellipticity (Θ_{MRE}) at 222 nm of −13 979 deg·cm^2·dmol^{-1} suggests that approximately 13

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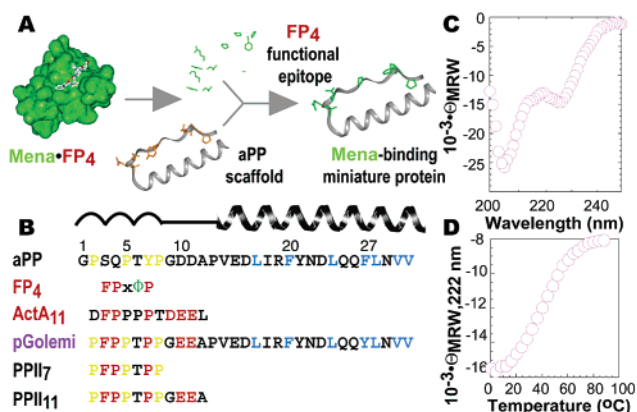[§] Department of Biochemistry, Stanford University School of Medicine.

Figure 1. (A) Strategy for display of the FP$_4$ epitope on a miniature protein scaffold. (B) Sequences of peptides and miniature proteins described in this work. Residues important for aPP folding are in blue or yellow, residues important for Mena$_{1-112}$ recognition are in red. (C and D) CD spectra showing (C) the mean residue ellipticity (Θ_{MRE}) of 5 μM pGolemi at 25 °C and (D) the temperature dependence of the Θ_{MRE} at 222 nm.[9]

residues of pGolemi possessed an α-helical conformation. The stability of pGolemi was examined further by monitoring the temperature-dependence of Θ_{MRE} at 222 nm. pGolemi underwent a reversible, moderately cooperative melting transition with $T_m =$ 40 °C (Figure 1D). These data suggest that pGolemi adopts a moderately stable, folded, aPP-like structure.

The affinity and specificity of pGolemi·EVH1 domain interactions were measured by tryptophan perturbation analysis (Figure 2A).[13] An 11-residue peptide comprising PPII repeat 1 of *L. monocytogenenes* ActA (ActA$_{11}$) and two peptides comprising the N-terminal 7 or 11 residues in pGolemi (PPII$_7$ and PPII$_{11}$) were prepared as controls. pGolemi bound Mena$_{1-112}$ with high affinity ($K_d = 700 \pm 30$ nM).[9] This affinity is 10-fold higher than that of ActA$_{11}$, the best previously known Mena ligand.[13] The interaction between pGolemi and Mena$_{1-112}$ was confirmed by fluorescence polarization experiments using a fluorescent pGolemi derivative (pGolemi^{Flu}) (Figure 2B); the K_d determined this way was 290 $\pm$ 50 nM. Furthermore, pGolemi and ActA$_{11}$ compete with pGolemi^{Flu} for binding to Mena$_{1-112}$ with IC$_{50}$ values of 542 $\pm$ 30 nM and 4.0 $\pm$ 0.2 μM, respectively.[9] Interestingly, PPII$_7$ and PPII$_{11}$ were poor Mena$_{1-112}$ ligands ($K_d = 480$ μM and > 1 mM, respectively), indicating that the pGolemi α-helix contributes at least 3.5 kcal·mol^{-1} to the Mena$_{1-112}$ affinity of pGolemi.

The folded structure of pGolemi also contributes to its ability to differentiate EVH1 domain paralogs in vitro (Figure 2C). The sequences of EVH1 domains Mena$_{1-112}$, VASP$_{1-115}$, and Evl$_{1-115}$ are 60% identical, and their structures are virtually superimposable.[14] Although ActA$_{11}$ binds equally to all EVH1 domains tested
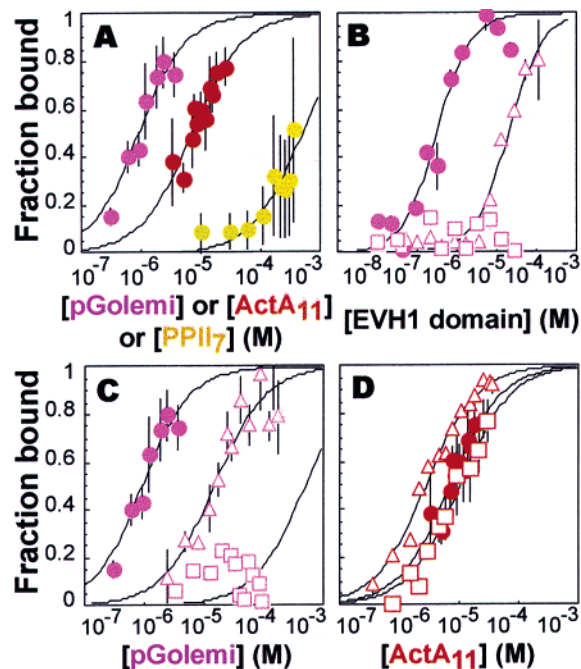
Figure 2. EVH1 domain binding interactions measured by tryptophan perturbation analysis (A, C, and D) or fluorescence polarization (B). (A) Binding of pGolemi (magenta), ActA$_{11}$ (red), or PPII$_7$ (yellow) to Mena$_{1-112}$ (500 nM). (B) Binding of pGolemi^{Flu} (25 nM) to Mena$_{1-112}$ (circle), VASP$_{1-115}$ (triangle) or Evl$_{1-115}$ (square). (C) Binding of pGolemi to Mena$_{1-112}$ (500 nM, circle), VASP$_{1-115}$ (500 nM, triangle), or Evl$_{1-115}$ (500 nM, square). (D) Binding of ActA$_{11}$ to Mena$_{1-112}$ (500 nM, circle), VASP$_{1-115}$ (500 nM, triangle), or Evl$_{1-115}$ (500 nM, square).[9] Fraction bound refers to the fraction of EVH1 domain (A, C, D) or pGolemi^{Flu} (B) bound.

(Figure 2D, $K_{rel} < 3$), pGolemi prefers Mena$_{1-112}$ to VASP$_{1-115}$ ($K_{rel} = 20$) and especially to Evl$_{1-115}$ ($K_{rel} > 120$) (Figure 2C). This level of specificity was confirmed by fluorescence polarization analysis (Figure 2B). pGolemi also discriminated well between Mena$_{1-112}$ and proteins that recognize proline-rich sequences or α-helices. The affinity of pGolemi for the KIX domain of CBP, which recognizes an α-helical ligand, was 15 ± 0.7 μM, and no interaction was detected between pGolemi and the N- or C-terminal SH3 domains of Grb-2.[9]

The properties of pGolemi were also examined in *Xenopus laevis* egg cytoplasmic extracts to reconstitute *L. monocytogenes* actin-based motility (Figure 3).[15] *L. monocytogenes* motility in mammalian cells and extracts is due to interactions between the 639-residue bacterial protein ActA and host proteins that recruit and activate actin polymerization. Addition of 10 μM ActA$_{11}$ decreased the median speed of *L. monocytogenes* by 89%, consistent with previous work.[16,5b] Addition of 10 or 27 μM pGolemi decreased the median speed of *L. monocytogenes* by 68% (Figure 3A) but, in addition, caused extreme speed variations and discontinuous tail formation at all times (Figure 3C). Discontinuous tails were not observed at any concentration of ActA$_{11}$ tested (Figure 3D). The differential effects of ActA$_{11}$ and pGolemi on *L. monocytogenes* motility may reflect their degree of specificity for EVH1 domain paralogs.

Many protein−protein interactions in cell signaling demand recognition of proline rich sequences,[6] and the design of molecules that perturb signaling pathways represents a foremost goal of chemical biology. Our results suggest that miniature proteins based
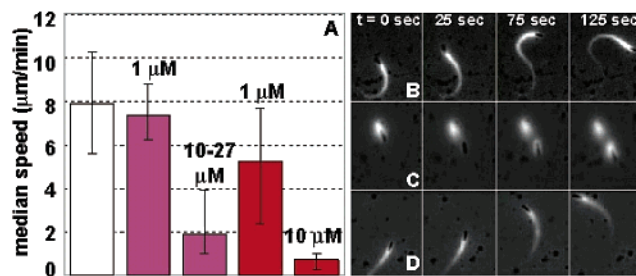

Figure 3. (A) Plot of median speed of *L. monocytogenes* observed in the absence (white) or presence (purple) of pGolemi and ActA$_{11}$ (red). Errors bars show the intraquartile range. (B−D) Time series of phase contrast and fluorescence micrographs of *L. monocytogenes* movement in a *Xenopus* egg cytoplasmic extract supplemented with rhodamine-labeled actin to mark the tails: (B) no added peptide, (C) 27 μM pGolemi, and (D) 1 μM ActA$_{11}$.[9]

on aPP may represent an excellent framework for the design of ligands that differentiate the roles of EVH1 domains in vitro and in vivo.

Supporting Information Available: Characterization of molecules described in this work; analysis of pGolemi^{Flu} affinity and specificity and effect on *L. monocytogenes* motility (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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