Structural insight into the interaction of proteins containing NPF, DPF, and GPF motifs with the C-terminal EH-domain of EHD1

Fabien Kieken,¹ Marko Jović,¹ Marco Tonelli,² Naava Naslavsky,¹ Steve Caplan,^{1*} and Paul L. Sorgen^{1*}

¹Department of Biochemistry and Molecular Biology and Eppley Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870

²National Magnetic Resonance Facility at Madison, Department of Biochemistry, University of Wisconsin-Madison, Wisconsin 53706-1544

Received 7 July 2009; Revised 26 August 2009; Accepted 17 September 2009

DOI: 10.1002/pro.258

Published online 1 October 2009 proteinscience.org

Abstract: Eps15 homology (EH)-domain containing proteins are regulators of endocytic membrane trafficking. EH-domain binding to proteins containing the tripeptide NPF has been well characterized, but recent studies have shown that EH-domains are also able to interact with ligands containing DPF or GPF motifs. We demonstrate that the three motifs interact in a similar way with the EH-domain of EHD1, with the NPF motif having the highest affinity due to the presence of an intermolecular hydrogen bond. The weaker affinity for the DPF and GPF motifs suggests that if complex formation occurs *in vivo*, they may require high ligand concentrations, the presence of successive motifs and/or specific flanking residues.

Keywords: EHD1; EH-domain; NPF motif; endocytic transport

Eps15 homology (EH)-domains are evolutionarily conserved regions of proteins containing ~100 residues and found in organisms as diverse as yeast and *Homo sapiens*.¹ The EH-domain was originally identified in three copies at the N-terminus of the mammalian protein Eps15,^{2,3} and serves as a protein interaction module, binding to proteins containing the tripeptide

asparagine–proline–phenylalanine (NPF).^{4,5} Although many EH-domain-containing proteins have other defined functional domains, most of these mammalian proteins have been characterized as regulators of endocytic membrane trafficking.^{6,7}

The majority of EH-domains are localized to the N-terminus of proteins that contain them, but interest has recently begun to shift to a group of proteins known as the C-terminal EHD/RME-1 family.^{8,9} Work in *C. elegans* and mammalian cells has led to the conclusion that these proteins are important regulators of endocytic trafficking for a wide variety of receptors internalized through different pathways^{10–19} (and reviewed in Ref. 8). Studies have demonstrated that C-terminal EH-domains also bind to an array of NPF-containing proteins involved in the regulation of endocytic transport.^{20–25} However, although the EH-domains of the four mammalian C-terminal EHDs remain highly homologous, they share lower identity with their N-terminal EH-domain counterparts.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: NIH; Grant numbers: GM072631, GM074876, P20RR018759, P41RR02301, P41GM66326; Grant sponsor: Nebraska Health and Human Services; Grant numbers: LB506, 2009-38; Grant sponsor: UNMC Eppley Cancer Center.

*Correspondence to: Steve Caplan, Department of Biochemistry and Molecular Biology, 985870 Nebraska Medical Center, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870. E-mail: scaplan@unmc.edu or Paul L. Sorgen, Department of Biochemistry and Molecular Biology, 985870 Nebraska Medical Center, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870. E-mail: psorgen@unmc.edu

The structure of several N-terminal EH-domains have been determined,²⁶ with the first being the second of three Eps15 EH-domains (Eps15 EH-2).²⁷ Recently, the NMR solution structure of the C-terminal EH-domain from EHD1 was solved,²⁸ as well as the crystal structure of the full-length mouse EHD2 protein dimer.²⁹ While the overall three-dimensional structures of the C-terminal EH-domains bear considerable similarity to the previously solved N-terminal EH-domains, several interesting differences were observed. First, the binding pocket of the C-terminal EHD1 EH-domain is narrower and not as deep as that of Eps15 EH-2.²⁸ Moreover, the electrostatic surface potential of the region around the EH-domain binding pocket of EHD1 is highly positively charged, as opposed to the neutral or negatively charged N-terminal EH-domains.²⁸ This suggested the possibility that C-terminal EH-domains may have an altered ligand binding preference, potentially facilitating interactions with the more negatively charged DPF motif.²⁸ Additionally, the EHD2 crystal structure indicated that the EH-domain interacts with an internal GPF motif in the linker between the central helical region of EHD2 and its EH-domain.²⁹ This study also suggested an interaction between the EHD2 EH-domain and a KPFxxxNPF sequence localized in the G-domain of the protein, with these interactions potentially regulating the function of the protein.²⁹

In this study, we provide the first quantitative data comparing the ability of a C-terminal EH-domain (that of EHD1) to bind to peptides containing NPF, DPF, and GPF motifs. The results indicate that NPF is the preferred binding motif for the EHD1 EH-domain, but both the DPF and GPF motifs are capable of binding with lower affinity. Although the NPF motif binds with a higher affinity, *in vivo*, it remains possible that the binding of different motifs may play a physiological role.

Results

Recognition of XPF motif ligands

Previous studies have demonstrated that N-terminal EH-domain containing proteins bind short peptides comprising either a NPF, DPF, or GPF motif.^{4,27,29,30–32} As the C-terminal EHD1 EH-domain has not been characterized in complex with peptides containing these motifs, and given its positively charged electrostatic surface potential adjacent to the EH-domain binding pocket (compared with N-terminal EH-domains), NMR was used to assess how these motifs interact with a C-terminal EH-domain. Accordingly, a peptide was used that included the second NPF motif of the protein Rab11-FIP2, which was previously shown to interact with EHD1.²² For comparison of DPF and GPF motifs, the same peptide was used, but replacing the Asn with Asp or Gly. Each peptide was able to interact with the EHD1 EH-domain (Fig. 1). All of

Figure 1. Interaction between the EHD1 EH-domain (residues D436-E534) and ligands containing NPF, DPF, and GPF motifs. A) The ¹⁵N-HSQC of the EH-domain alone (black) has been overlaid with the EH-domain in complex with 2 mM NPF (red), 3 mM DPF (green), and 4 mM GPF (blue) containing peptides. The resonance peaks, which exhibited the greatest change in chemical shift due to the ligands have been highlighted by circles. The residues colored red were used to calculate the binding affinities.

the ¹⁵N-HSQC resonance assignments for the EHD1 EH-domain have been previously determined²⁸ and highlighted in Figure 1 are those peaks that had the greatest change in chemical shift upon addition of the peptides. ¹⁵N-HSQC spectra showed smaller chemical shift variations upon binding with either the DPF or GPF peptides as compared to the NPF peptide at the same peptide concentration. The difference of chemical shift variation can be explained by the difference of affinity between these peptides and the C-terminal EH-domain. Analysis of the chemical shift variation versus the concentration of peptide showed that the peptides exhibited significantly different affinities for the EH-domain. The binding affinity was calculated by averaging the K_D values for each of the five residues highlighted red in Figure 1 (mean $\pm$ standard deviation over the five residues). The binding affinity for the NPF motif ($245 \mu\text{M} \pm 35$) was significantly higher than that of the DPF ($1.2 \text{ mM} \pm 0.1$) and GPF ($2.4 \text{ mM} \pm 0.1$) motifs (Fig. 2). Despite these differences in affinity, the presence of similar N-H resonances shifts upon titration of the three different peptides suggests that all three motifs bind with the EHD1 EH-domain by a similar molecular mechanism.

Figure 2. Binding analysis of the interaction between the EHD1 EH-domain (residues D436–E534) and ligands containing (A) NPF, (B) DPF, and (C) GPF motifs. The dissociation constants (K_D) were estimated by non-linear best fitting the chemical shift variation (in ppm) versus the concentration of peptide (K_D - NPF: $245 \mu\text{M} \pm 35$, DPF: $1.2 \text{ mM} \pm 0.1$, GPF $2.4 \text{ mM} \pm 0.1$). Chemical shift variation was calculated according to the formula $\Delta\sigma = \sqrt{(\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}/5})^2}$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Structure of the C-terminal EHD1 EH-domain with an XPF motif ligand

The structures of the different complexes were solved with the EH-domain ($600 \mu\text{M}$) in presence of 2 mM NPF, 3 mM DPF, and 4 mM GPF peptides. Despite the low binding affinities for the DPF and GPF peptides, a large number of intermolecular NOEs were identified using filtered NOESY experiments that were essential for the molecular modeling of these complexes (Supporting Fig. 1).

The backbone view of the final 10 structures of the EH-domain/XPF (X = Asn, Asp, or Gly) complex is displayed in Figure 3(A–C) and structural informa-

tion is presented in Table I. Overall, the interactions between the three different motifs and the EH-domain are similar. First, binding of each peptide to the EH-domain does not change the overall EH-domain structure. The EH-domain contains two associated helix–loop–helix motifs with the loops connected by a short antiparallel β -sheet. The XPF motif adopts a tight turn (type I β -turn for the NPF and DPF motifs) with the Phe buried within the groove between helix 2 and 3 in a pocket formed by EH-domain residues Lys⁴⁶⁸, Met⁴⁷¹, Val⁴⁷², Asn⁴⁷⁸, Leu⁴⁸¹, and Trp⁴⁸⁵ [Fig. 3(D–F)]. The β -turn is stabilized by hydrogen bonds between the carbonyl oxygen and the acyl oxygen of Asn or Asp with the amide protons of the Thr and Phe, respectively. The turn formed by the GPF motif in the complex is uniquely stabilized by the formation of a hydrogen bond between carbonyl oxygen of the Gly and the amide proton of the Thr. The proline adopts a trans conformation and is localized in a hydrophobic core formed by EH-domain residues Gly⁴⁶⁴, Ala⁴⁶⁷, Met⁴⁷¹, and Trp⁴⁸⁵. Two additional structural features were also noticed, which may help stabilize the complex: the γ -NH₂ of Asn⁴⁷⁸ and the ϵ -amino protons of Lys⁴⁶⁸ are in proximity of the Phe and the Pro carbonyl oxygen, respectively and may be engaged in hydrogen bonds.

The difference in affinities observed by NMR can be explained by the presence of an amide group on the side chain of the Asn residue. Analysis of the possible hydrogen bond network between the NPF motif and EH-domain revealed that Asn γ -NH₂ is in close proximity to the carbonyl oxygen of Gly⁴⁸². The presence of this intermolecular hydrogen bond between the Asn side chain and the backbone of the EH-domain may explain the binding affinity differences between the three motifs. Therefore, ligands lacking an amide group on the side chain (i.e. DPF or GPF) bind with lower affinity to the EH-domain. The difference in affinities between the DPF and GPF motifs can be attributed to the proximity of the side chains of Lys⁴⁸⁶ and Asp leading to the formation of an additional hydrogen bond, which may help to stabilize the complex [Fig. 3(E)]. Nevertheless, the key interaction for binding the C-terminal EH-domain is the hydrogen bond between the Asn side chain with the carbonyl oxygen of Gly⁴⁸².

Importance of the EHD1 EH-domain/GPF complex

The observation that the EHD1 EH-domain is able to bind a GPF motif is in agreement with the observation made by Daumke *et al.*²⁹ demonstrating that the C-terminal EH-domain of EHD2 interacts with an internal GPF motif found in the linker area between residues G420 and F422. Previously, we published the structure of the EHD1 EH-domain (residues E401–E534, 35 residues longer than the EH-domain construct used in this study), which contains a similar GPF motif (residues G420, P421, and F422).²⁸ The

Figure 3. Structure of EHD-1 EH-domain in complex with XPF peptides. A-C) Stereoview of the 10 lowest energy structures of the EHD-1 EH-domain in complex with XPF peptides. The structures of the EH-domain in complex with (A) NPF, (B) DPF, and (C) GPF peptides have been superpositioned according to the backbone atoms of the XPF residues (X = N, D, or G). The EH-domain residues involved in the interaction with the XPF residues are Gly⁴⁶⁴, Ala⁴⁶⁷, Lys⁴⁶⁸, Met⁴⁷¹, Asn⁴⁷⁸, Leu⁴⁸¹, Gly⁴⁸¹, Trp⁴⁸⁵. The side chains of these EH-domain residues have been labeled (red). (D-F) Close up view of the binding site between the EHD1 EH-domain and XPF peptides. The EH-domain is colored blue and the (D) NPF, (E) DPF, and (F) GPF peptides are colored orange. Residues involved in the direct interaction have been labeled in each panel.

data indicated that residues E401-D436 were mainly unstructured and that this GPF motif did not interact with the EH-domain. If a complex formed between the EHD1 EH-domain and this GPF motif, the Phe would be buried within the groove between helix 2 and 3.

This close proximity would be illustrated by the presence of a high number of NOEs between the H_δ of the Phe and the protons of the residues involved in the binding pocket in a manner similar to that observed with the EH-domain (residues D436-E534) in complex

Table I. Structural Statistics of the 10 Lowest Energy Structures of the EHD1 EH-Domain in Complex With Ligands Containing the NPF, DPF, and GPF Motifs

Conformational restraints	NPF	DPF	GPF
NOE distance restraints			
Total	3319	3098	2439
Intra-residue ($ i-j = 0$)	1200	1167	1035
Sequential ($ i-j = 1$)	758	707	527
Medium range ($2 \leq i-j < 5$)	502	453	324
Long range ($ i-j \geq 5$)	774	714	494
Intermolecular	85	57	59
Backbone hydrogen bonds	70	70	68
Residual violations	Average number per residue		
Distance restraints $> 0.5 \text{ \AA}$	0	0	0
Distance restraints $> 0.3 \text{ \AA}$	0.5	0.2	0.1
RMSD ^a from standard geometry			
Bond lengths ($\text{\AA}$)	0.0037 ± 0.0001	0.0037 ± 0.0001	0.0034 ± 0.0001
Bond angles (degrees)	0.52 ± 0.01	0.52 ± 0.02	0.50 ± 0.02
Impropers (degrees)	1.44 ± 0.07	1.43 ± 0.06	1.34 ± 0.12
Energies			
NOE	44 kcal/mol ± 7	39 kcal/mol ± 7	19 kcal/mol ± 7
Van der Waals	-1042 kcal/mol ± 8	-1030 kcal/mol ± 12	-1008 kcal/mol ± 17
Electrostatic	-5421 kcal/mol ± 34	-5345 kcal/mol ± 112	-5471 kcal/mol ± 54
Ramachandran maps			
Residues in most favored regions	84.0%	84.9%	85.6%
Residues in additional allowed regions	15.3%	14.4%	13.8%
Average RMSD ^a			
EH-domain (439-531)-(XPF)	$0.47^b/0.79^c$	0.58/0.86	0.50/0.83
EH-domain (439-531)	0.47/0.78	0.58/0.86	0.50/0.82
XPF motif	0.10/0.27	0.12/0.29	0.10/0.16
T-XPF-T motif	0.35/0.64	0.38/0.64	0.40/0.74

^a RMSD, root mean square deviation.^b RMSD, root mean square deviation for backbone/heavy atoms.^c RMSD, root mean square deviation for backbone/heavy atoms.

with the GPF ligand [Fig. 3(C,F)]. Such a network of connectivity was not observed with the longer C-terminal EH-domain construct (residues E401-E534).²⁸

An explanation as to why we did not observe these intermolecular NOEs emerges if we look at the concentration of the longer EH-domain used to previously solve its structure. The structure of the EHD1 EH-domain (residues E401-E534) was solved at a concentration of 600 μM .²⁸ With the binding affinity between the GPF motif and EH-domain calculated to be K_D of $2.4 \text{ mM} \pm 0.1$ [Fig. 2(C)], approximately 20% of the EH-domains would be occupied by a GPF motif (fractional occupancy = $[\text{ligand}]/([\text{ligand}] + K_D)$). This would make detection of the complex by NMR difficult. To investigate this hypothesis, ¹⁵N-HSQC were collected of the longer EHD1 EH-domain (residues E401-E534) at different concentrations (Fig. 4). The data showed that both residues in the GPF and binding pocket regions are affected if the concentration of the EH-domain was increased significantly suggesting that the GPF motif present in the linker region is effectively able to interact with the binding region of the EH-domain.

Next, we determined if the more favorable interaction was intra- or inter-molecular because the crystal structure of the EHD2 dimer illustrated that the GPF motif from one EHD2 molecule interacts with EH-do-

main from the other molecule.²⁹ ¹⁵N-HSQC demonstrated that the shorter EH-domain (residues D436-E534; no GPF motif) was able to interact with the longer EH-domain (residues E401-E534; contains the GPF motif) suggesting that the interaction between the GPF motif and EH-domain can be intermolecular (Supporting Fig. 2). Moreover, a perfect overlay was observed between the shorter EH-domain ¹⁵N-HSQC when titrated with either the longer EH-domain or the wild type GPF peptide (EHD1 residues F414-G425) at a similar concentration of ligand (1 mM) (Supporting Fig. 2). This data suggests that the interaction between GPF motif and EH-domain is predominately intermolecular, consistent with observations of the EHD2 dimer.²⁹ If this interaction was mainly intramolecular, two different ¹⁵N-HSQC would be observed, with a lower chemical shift variation in presence of the longer EH-domain (residues D436-E534) than the GPF wild type peptide (residues F414-G425) because the intramolecular interaction by the longer EH-domain would inhibit the binding to the shorter EH-domain. The binding affinity of the EHD1 GPF wild type peptide for the EH-domain was also determined [Fig. 5(A,B)]. This binding affinity ($2.3 \text{ mM} \pm 0.3$) is similar to the affinity previously measured with the other GPF containing peptide used in this study ($2.4 \text{ mM} \pm 0.1$) [Fig. 2(C)]. Thus, the data suggests that the EH-

Figure 4. Demonstration that the EHD1 EH-domain is capable of binding to its own GPF motif. Overlay of ^{15}N -HSQCs of the EH-domain (residues E401-E534) collected at different protein concentrations (black: 100 μM ; red: 500 μM ; and green: 1 mM).

domain is able to adopt a dimeric conformation by interacting via the GPF motif of the opposite monomer.

Discussion

Several studies have characterized the mechanism of NPF recognition by N-terminal EH-domain containing proteins. de Beer *et al.* initially showed that the NPF motif of Hrb binds the second EH-domain of Eps15 by adopting a type I β -turn.³⁰ A similar type of interaction has been observed for the C-terminal domain of EHD2 and a GPF motif.²⁹ No structural studies have yet investigated the interaction between an EH-domain and a DPF motif. However, Santonico *et al.*³² showed that the EH-domain of POB-1 binds Eps15, and that this interaction was initiated by binding a DPF motif.

In this study, we showed that the C-terminal EH-domain of EHD1 is capable of binding different proteins containing an NPF, DPF, or GPF motif. A similar mode of interaction was observed between the different motifs and the EH-domain. Each motif adopted a turn with the Phe buried within the groove between helix 2 and 3 and was stabilized by the proximity of the γ -NH₂ of Asn⁴⁷⁸ and the ϵ -amino protons of Lys⁴⁶⁸ with the Phe and the Pro carbonyl oxygen. The EH-domain preferred binding to the NPF motif as compared to the DPF or GPF motifs, due to the presence of an additional intermolecular hydrogen bond

involving Asn γ -NH₂, which is in agreement with de Beer *et al.*²⁷

Initially, upon identifying a cluster of positively-charged surface residues within the EH-domain binding pocket by our NMR solution structure,²⁸ we had proposed the hypothesis that C-terminal EH-domains may potentially have a higher affinity for proteins containing the negatively charged motif DPF rather than NPF. Whereas de Beer *et al.* suggested that the binding between Eps15 EH2 and a DPF motif ligand is unlikely due to the charge repulsion between the Asp of the DPF motif and Glu¹⁷⁰, our studies showed that the DPF motif can bind an EH-domain. The substitution of the Glu¹⁷⁰ (Eps15 EH2) by the Lys⁴⁸⁶ (EHD1) helps stabilize the complex between the EHD-1 EH-domain and ligands containing a DPF motif by the formation of hydrogen bonds between the side chains of these two residues (DPF and Lys⁴⁸⁶). Nevertheless, an NPF ligand remains the preferential interaction partner for binding to the EH-domain of EHD1.

The studies presented here also support the observation made by Daumke *et al.*,²⁹ which showed that a C-terminal EH-domain (from EHD2) is able to interact with a GPF motif. This interaction exists in solution for the EHD1 EH-domain; however, it can easily be displaced by other ligands on condition that their binding affinities are lower than 2 mM (such as, ligands containing an NPF or DPF motif). Future studies are needed to address the biological relevance of such a weak interaction *in vivo*.

Although ligands containing an NPF motif are greatly favored for binding EH-domains, the affinity between EH-domains and their ligand can change drastically by the presence of an additional NPF motif and adequate flanking regions. Rumpf *et al.*³³ showed that stonin2, a regulator of synaptic vesicle recycling, interacts with the second Eps15 EH-domain. They identified a two-site binding mode between the EH-domain of Eps15 and the NPF region of Stonin2. Similar to Epsin1, Stonin2 contains two NPF motifs separated by 13 residues but with different flanking regions. Whereas Epsin1 binds to the classical NPF binding site described for EH-domain, Stonin2 binds at both sites. The first NPF motif binds the classical site between helix 2 and 3, whereas the second motif is inserted into a hydrophobic groove formed between helices 1, 2, and 4. This specific binding creates a drastic change in affinity in Stonin2 so that its affinity for the second Eps15 EH-domain is 1000 fold higher than that of Epsin1. However, the first NPF is absolutely necessary for Stonin2 binding, whereas the second NPF acts as a modulator of affinity between the two binding partners.³³ While the EHD1 EH-domain can form complexes with NPF, DPF, or GPF motif ligands, their binding affinities illustrate that DPF and GPF are disfavored compared to the NPF motif. Nevertheless, the presence of multiple successive motifs may overcome this difference of affinity. For example, Eps15

Figure 5. Binding analysis of the interaction between the EHD1 EH-domain (residues D436-E534) and a ligand containing the native EHD1 GPF motif. (A) ^{15}N -HSQC of the EH-domain (residues 436–534) with different concentrations of GPF wild type (EHD1 residues F414–G425) (black: 0 mM; red: 1 mM; green: 2.5 mM; and blue: 5 mM). Resonances used to calculate the dissociation constant (K_D) have been circled. The K_D was estimated to be $2.3 \text{ mM} \pm 0.3$ by non-linear best fitting the chemical shift variation (in ppm) versus the concentration of peptide.

contains 15 consecutive DPF motifs, which could stabilize the complex between Eps15 and POB-1. Given that the EHD1 EH-domain presents a global positive surface potential compared to the second Eps15 EH-domain, the presence of charged residues flanking the XPF motif may help to the stabilization of the complex.

In summary, we report that the C-terminal EH-domain of EHD1 interacts with ligands containing an NPF, DPF, or GPF motif. We showed that globally the three motifs interact in a similar way with the EH-domain and that the NPF motif has the highest affinity due to the presence of an intermolecular hydrogen bond involving Asn γ -NH₂. The affinity of the EH-domain for the DPF and GPF motifs was weak and the formation of these complexes *in vivo* may require particular conditions, such as high concentrations of ligands, the presence of successive motifs, and/or specific flanking residues.

Material and Methods

Cloning and purification of the EHD1 EH-domain

The human EH-domain of EHD1 (residues D436-E534 and E401-E534) was subcloned into the bacterial expression vector pGEX-6P-2 (GE Healthcare, Piscataway, NJ) utilizing the restriction enzymes EcoRI and

XhoI (New England Biolabs, Beverly, MA). *Escherichia coli* strain BL21 (DE-3) (Novagen, Madison, WI) transformed with the cloned EHD1 EH-domain was grown in M63 minimum media using 1 g of $^{15}\text{NH}_4$ and 2 g of ^{13}C -glucose (Sigma, St. Louis, MO). Bacteria grown to 0.6 OD₆₀₀ were induced with 0.5 mM IPTG for 4 h at 37°C and pelleted at 3500 g for 20 min. Then, the bacteria were resuspended in 1 × phosphate buffer saline (PBS) containing 25 × stock of Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN) and 1 mM dithiothreitol. The cells were lysed by French Press (three times) and pelleted by centrifugation (17,000 g, 40 min) following the addition of 1 mM phenylmethylsulfonyl fluoride and 1% of nonyl phenoxypolyethoxylethanol. The supernatant was bound to glutathione Sepharose beads (Genscript, Piscataway, NJ) at 4°C for 2 h. The beads were then washed with 1 × PBS at pH 7.4, and the EH-domain was cleaved by incubating the beads overnight at 4°C with 80 units of PreScission Protease (GE Healthcare, Piscataway, NJ). The protein was separated from the beads by centrifugation (500 g for 5 min) and concentrated with an Amicon ultra 10K filter (Millipore, Billerica, MA). The buffer was exchanged into 20 mM Deuterated Tris, 100 mM KCl, and 2 mM CaCl₂ at pH 7.0 using the desalting column Econo Pac 10DG (Biorad, Hercules, CA). The purity of the protein was confirmed by SDS-

PAGE analysis and the concentration determined by absorbance reading at 280 nm using the molar extinction coefficient of $13,980 \text{ M}^{-1} \text{ cm}^{-1}$ as calculated by ExPASy ProtParam tool (<http://us.expasy.org/cgi-bin/protparam>).

Nuclear magnetic resonance (NMR) and molecular modeling

NMR data were acquired at 25°C using a Varian INOVA 600 and 800 spectrometers fitted with a cold probe at the University of Nebraska Medical Center's NMR Facility and the National Magnetic Resonance Facility at Madison. Peptides Phe-Asn-Tyr-Glu-Ser-Thr-X-Pro-Phe-Thr-Ala-Lys (X = Asn, Asp, or Gly) and Phe-Asp-Gly-Thr-Met-Asn-Gly-Pro-Phe-Gly-His-Gly (referred to as wild-type; same sequence as is found in EHD1, residues F414-G425) were purchased from Anaspec (Fremont, CA). Backbone assignments of the EHD1 EH-domain in complex with the XPF peptides were confirmed using HNCACB spectra. Peptide assignment was achieved using $\text{F}_1, \text{F}_2\text{-}^{13}\text{C}, ^{15}\text{N}$ filtered NOESY (mixing time of 150 and 300 ms). Distance constraints were derived from ^{15}N -NOESY-HSQC, ^{13}C -NOESY-HSQC (aliphatic and aromatic), $\text{F}_1, \text{F}_2\text{-}^{13}\text{C}, ^{15}\text{N}$ filtered NOESY, ^{13}C -edited- ^{13}C or ^{15}N filtered NOESY, and $\text{F}_2\text{-}^{13}\text{C}, ^{15}\text{N}$ filtered NOESY experiments (mixing time of 150 ms). Molecular modeling using ARIA 1.2³⁴ and evaluation of the lowest energy structures using PROCHECK-NMR³⁵ were performed as previously described.²⁸ The coordinates of the EHD1 EH-domain in the presence of XPF peptides have been deposited in the Protein Data Bank (2kff, 2kfg, 2kfh) and visualized using MOLMOL software.³⁶ The chemical shift assignments and NMR restraints used during the molecular modeling were deposited in the BioMagResBank (16179, 16180, 16181). Binding isotherms were obtained from gradient-enhanced two-dimensional $^1\text{H}\text{-}^{15}\text{N}$ HSQC experiments. Data were acquired with 1024 complex points in the direct dimension and 64 in the indirect dimension. Sweep widths were 8000 Hz in the proton dimension and 2100 Hz in the nitrogen dimension. Dissociation constants (K_D) were calculated by non-linear best fitting the H-N titration curves using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA), averaging over the five curves. Chemical shift variation was calculated according to the formula $\Delta\sigma = \nu((\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}/5})^2)$. The concentration of EH-domain was maintained constant at $100 \mu\text{M}$.

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