Published in final edited form as:

Biomol NMR Assign. 2014 October ; 8(2): 263–267. doi:10.1007/s12104-013-9497-z.

Chemical Shift Assignments of the C-terminal Eps15 Homology Domain-3 EH Domain*

Gaëlle Spagnol[#], Calliste Reiling[#], Fabien Kieken[#], Steve Caplan¹, and Paul L. Sorgen¹

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

[#] These authors contributed equally to this work.

Abstract

The C-terminal Eps15 homology (EH) domain 3 (EHD3) belongs to a eukaryotic family of endocytic regulatory proteins and is involved in the recycling of various receptors from the early endosome to the endocytic recycling compartment or in retrograde transport from the endosomes to the Golgi. EH domains are highly conserved in the EHD family and function as protein-protein interaction units that bind to Asn-Pro-Phe (NPF) motif-containing proteins. The EH domain of EHD1 was the first C-terminal EH domain from the EHD family to be solved by NMR. The differences observed between this domain and proteins with N-terminal EH domains helped describe a mechanism for the differential binding of NPF-containing proteins. Here, structural studies were expanded to include the EHD3 EH domain. While the EHD1 and EHD3 EH domains are highly homologous, they have different protein partners. A comparison of these structures will help determine the selectivity in protein binding between the EHD family members and lead to a better understanding of their unique roles in endocytic regulation.

Keywords

EHD3; EH domain; endocytic recycling; NPF-motif

Biological context

Endocytosis is a fundamental biological process that controls the composition of the plasma membrane and how cells respond to, and interact with their environment. Receptor-mediated endocytosis is crucial for a variety of integral processes, including nutrient uptake, cell signaling, proliferation, and migration. Aberrant endocytosis can lead to cancer, heart disease, diabetes, and a host of other afflictions. For example, the overexpression of endocytic regulatory protein Huntingtin-interacting protein 1 leads to oncogenic transformation, changes in total receptor levels on the plasma membrane, and altered trafficking of epidermal growth factor and transferrin receptors (1). In addition, mutant low

*This work was supported by United States Public Health Service Grant GM087455.

¹To whom correspondence should be addressed: Paul Sorgen Tel: 402-559-7557 psorgen@unmc.edu, Steve Caplan Tel: 402-559-7556 scaplan@unmc.edu, Fax: 402-559-6650 .

density lipoprotein receptors that fail to recycle to the plasma membrane cause hypercholesterolemia by not internalizing cholesterol from the serum into cells (2).

Upon ligand binding, receptors are internalized by the invagination of vesicles derived from the plasma membrane. These vesicles are commonly coated with clathrin and the Adaptor protein 2 complex and released from the membrane by dynamin-induced fission. Once internalized, the vesicles uncoat and fuse with an intracellular compartment known as the early or sorting endosome. While some receptors and/or ligands are transported to lysosomes and ultimately undergo degradation, other receptors are recycled back to the plasma membrane. A range of regulatory proteins controls the recycling steps to the plasma membrane, and key regulators in this process are the C-terminal Eps15 Homology Domain (EHD) family of proteins (reviewed in (3)). The best characterized family member, EHD1, acts primarily at the endocytic recycling compartment to regulate the exit of various receptors from this compartment, including the transferrin receptor, MHC class I molecules, and β 1 integrin receptors. All four highly homologous EHD mammalian paralogs contain an N-terminal helical region followed by a nucleotide-binding motif, a central helical domain, and an Eps15 Homology (EH) domain localized to the C-terminus of the protein. However, despite their high level of homology, each EHD protein appears to carry out distinct cellular functions, potentially through interactions with different binding partners.

The EH domain is conserved evolutionarily in species as diverse as yeast and homo sapiens, and serves as a protein-protein interaction module that facilitates binding to other endocytic regulators containing the Asn-Pro-Phe (NPF) motif (4). Despite the existence of hundreds of proteins that contain the tripeptide sequence motif NPF, the EHD proteins interact with only a small subset of these; specifically NPF-containing proteins that are implicated in the regulation of endocytic recycling events. For example, our lab has identified several NPF-containing interaction partners, including MICAL-L1, a membrane hub that recruits EHD1 to tubular recycling endosomes, and the Rab effector proteins Rabenosyn-5 and Rab11-FIP2 (reviewed in (3)). Rabenosyn-5 is an essential component of the early endosomal fusion machinery and binds Rab5 and Rab4, whereas Rab11-FIP2 binds Rab11 and is found on recycling endosomes. More recently we have identified Rabankyrin-5 as an additional dual EHD1 and Rab interaction partner (5).

While one layer of specificity between EHD proteins and binding partners might arise as a result of “compartmentalization” (i.e. the differential subcellular localization of these proteins within the cell), additional molecular mechanisms must be utilized to control this tight selectivity. We have shown in previous studies that NMR can provide important insights into the molecular mechanisms controlling this specificity. Based on the solution structure of the EHD1 EH domain in complex with a MICAL-L1 NPF peptide (NPFEEEEED), our data support a molecular mechanism by which NPF-containing proteins involved in the regulation of endocytic recycling events have co-evolved along with the EHD EH domains to arrive at higher affinity interactions. The acidic residues flanking the NPF motif mediate salt bridges with the positively charged residues on the EHD EH domain surface (6). While the EHD1 and EHD3 EH domains are highly homologous (82.5% identity), we recently identified a differential interaction partner that interacts with EHD1 but not EHD3. This protein, Rabankyrin-5, contains an NPF motif flanked by a shorter

acidic cluster (5). Therefore, we are now extending NMR studies to include the EHD3 EH domain to determine the selectivity in protein binding between the EHD family members; this will allow us to understand their unique roles in endocytic regulation. Reported here are the ^1H , ^{13}C , and ^{15}N assignments for the backbone and side chain resonances and the predicted secondary structure of the EHD3 EH domain.

Methods and experiments

The EH domain of EHD3 (residues G433-E535) was expressed and purified following the protocol previously developed for the EH domain of EHD1 (6). Briefly, the EHD3 EH domain was subcloned into the bacterial expression vector pGEX-6P-2 (GST-tagged; Amersham Biosciences) by standard PCR methods and overexpressed in BL21(DE3) competent cells (Agilent Technologies). Transformed bacteria were grown in 1 L of enriched minimum media (^{15}N -ammonium chloride and/or ^{13}C -glucose) and induced with 120 mg of IPTG at a cell density of 0.8 OD_{600} for 4 hrs. The bacterial cells were pelleted and resuspended in lysis buffer containing Complete Protease Inhibitor (Roche Molecular Biochemicals), 1% NP-40 and 1 mM PMSF, in 1x phosphate buffered saline (PBS) at pH 7.4. The cells were lysed by Emulsiflex and separation of the lysate from debris/intact cells was achieved by centrifugation (16500 g, 1 hr). The soluble GST-tagged EHD3 EH domain was incubated at 4°C for 2 hrs with 3 mL of glutathione resin (GenScript). The resin was then washed with 2x PBS to remove any non-specific-bound proteins, followed by digestion of the GST-tag by overnight incubation at 4°C with 10 units of Turbo3C Protease (Accelagen) in PBS buffer. The EHD3 EH domain was concentrated using a 10K Amicon® Ultra Centrifugal Filter (EMD Millipore Corporation) and the buffer exchanged into 20 mM Tris (pH 7.4), 100 mM KCl (Tris buffer) using a NAPTM-5 column (GE Healthcare). The final solution condition contained 1.2 mM of labeled protein in Tris buffer plus 3 mM CaCl_2 and 10% D_2O .

NMR data were acquired at 25°C using a Varian INOVA 600 MHz spectrometer fitted with a cryo-probe at the University of Nebraska Medical Center, processed using NMRPipe/NMRDraw (7), and analyzed using NMRView (8). Backbone assignments were obtained using the following 2D and 3D experiments: ^{15}N -HSQC, HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO. Side chain chemical shifts were obtained from 3D HCCH-TOCSY, ^{15}N -TOCSY-HSQC, ^{15}N -NOESY-HSQC, and ^{13}C -NOESY-aliphatic and -aromatic HSQC experiments.

Assignments and data deposition

The ^{15}N -HSQC spectrum and assignments for the EHD3 EH domain are displayed in Figure 1. All ^1H , ^{15}N , and ^{13}C backbone resonances were found except for the amide group of V441, N478 and S479, resulting in > 98% of the expected resonances, and most of the protons and carbons in the side chains and aromatic groups were assigned (> 97%). The chemical shift data of the EHD3 EH domain were deposited into the BioMag ResBank database, accession number 19109.

The secondary structure of the EHD3 EH domain was predicted using the chemical shift index (CSI) function in NMRView (8). The Wishart Database was used as reference to assess the $^1\text{H}\alpha$, $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ chemical shift deviations (Figure 2A). The result indicates that the EHD3 EH domain is expected to contain five α -helical and three β -strand domains. The following order $\alpha 1$ - $\beta 1$ - $\alpha 2$ - $\alpha 3$ - $\beta 2$ - $\alpha 4$ - $\beta 3$ - $\alpha 5$ suggests a series of two helix-loop-helix topologies containing a short β -strand in the loop, also known as an EF-hand. The CSI result was identical to the secondary structure prediction performed by TALOS+ (data not shown).

Several EH domain structures have been solved by NMR (6, 9), including the EH domain of EHD1 which shares 82.5% identity and 93.2% similarity with the EHD3 EH domain. The CSI for the $^1\text{H}\alpha$, $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ chemical shifts of the EHD1 EH domain (BioMag ResBank database, accession number 15279) also displays a series of helices and short β -strands (Figure 2A, bottom cartoon), which matches the structure obtained by NMR (6). This result indicates that the CSI is a reliable prediction method for EH domains and suggests structural similarity between the two domains. Other unique resemblances between the EHD1 and EHD3 EH domains are the same missing amide protons (V441, N478 and T479), conserved residues in the second EF-hand to bind Ca^{2+} (D489, D491, D493 and E500; green in Figure 2C), and the amide proton chemical shift of G494 at 10.55 ppm, which is a characteristic found in EF-hands (10). The abundance of structural similarities between the two EH domains would also imply comparable biophysical properties, such as Ca^{2+} -binding, which was unexpectedly different.

Helix-loop-helix structural motifs constitute EF-hands which mediate Ca^{2+} -binding of a large family of Ca^{2+} -modulated proteins such as calmodulin, troponin-C, and S100. The EH domain of EHD proteins contains two EF-hands but only the second one binds Ca^{2+} . Typically, upon Ca^{2+} -binding, EF-hands undergo a structural change that exposes a target binding surface. The EHD1 EH domain follows this model as its structure (both EF-hands) was affected in the presence of 1 mM of EGTA (Ca^{2+} -chelating agent) and stabilized upon addition of Ca^{2+} (Figure 2B, left panel; Figure 2C, top sequence). Surprisingly, under identical solution conditions to the EHD1 EH domain, EGTA had little-to-no effect on the EHD3 EH domain structure as observed in the ^{15}N -HSQC spectra (Figure 2B, right panel). The minor changes in signal intensity were mainly contained in the Ca^{2+} -binding pocket of the second EF-hand (Figure 2C, bottom sequence) indicating preservation of the overall structure integrity of the EHD3 EH domain. This is not unprecedented as the EF-hand containing protein calbindin D9k, whose apo- and Ca^{2+} -bound structures were solved by NMR (11), showed little-to-no significant change in conformation upon binding Ca^{2+} .

The difference in the structural role Ca^{2+} plays between these EH domains may lie in the presence of a Met (EHD3 EH domain) residue instead of a Leu (EHD1 EH domain) at position 495, located in the Ca^{2+} -binding pocket (Figure 2C, residues underlined purple). Based on the EHD1 EH domain structure, the side-chains of L495 and W485 directly face each other. The placement of M495 would ideally position the sulfur atom ~ 5 Å from the ring center, allowing for the formation of a sulfur-aromatic interaction with W485 (Figure 2C, brown) and stabilizing the EHD3 EH domain structure in the absence of Ca^{2+} (12). This explanation is supported by a bioinformatic search of the PDB which identified $\sim 1/3$ of all known protein structures contains an energetically stabilizing Met-aromatic motif (12).

Interestingly, M495 and W485 are also conserved in the EH domains of EHD2 and EHD4. Solving the EHD3 EH domain structure will be important towards supporting this hypothesis and a comparison of the structure and dynamics with the EHD1 EH domain in the presence and absence of Ca^{2+} could explain their differences in protein partner interactions.

Acknowledgments

This work is funded by the United States Public Health Service Grant GM087455 (Paul Sorgen and Steve Caplan).

References

1. Rao DS, Bradley SV, Kumar PD, Hyun TS, Saint-Dic D, Oravecz-Wilson K, et al. Altered receptor trafficking in Huntingtin Interacting Protein 1-transformed cells. *Cancer Cell*. 2003; 3(5):471–82. [PubMed: 12781365]
2. Ikonen E. Mechanisms for cellular cholesterol transport: defects and human disease. *Physiol Rev*. 2006; 86(4):1237–61. [PubMed: 17015489]
3. Naslavsky N, Caplan S. EHD proteins: key conductors of endocytic transport. *Trends Cell Biol*. 2011; 21(2):122–31. [PubMed: 21067929]
4. Whitehead B, Tessari M, Carotenuto A, van Bergen en Henegouwen PM, Vuister GW. The EH1 domain of Eps15 is structurally classified as a member of the S100 subclass of EF-hand-containing proteins. *Biochemistry*. 1999; 38(35):11271–7. [PubMed: 10471276]
5. Zhang J, Reiling C, Reinecke JB, Prislani I, Marky LA, Sorgen PL, et al. Rabankyrin-5 interacts with EHD1 and Vps26 to regulate endocytic trafficking and retromer function. *Traffic*. 2012; 13(5):745–57. [PubMed: 22284051]
6. Kieken F, Jovic M, Naslavsky N, Caplan S, Sorgen PL. EH domain of EHD1. *J Biomol NMR*. 2007; 39(4):323–9. [PubMed: 17899392]
7. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6(3):277–93. [PubMed: 8520220]
8. Johnson BAaB RA. NMRView: A computer program for the visualization and analysis of NMR data. *J Biomol NMR*. 1994; 4:603–14. [PubMed: 22911360]
9. Confalonieri S, Di Fiore PP. The Eps15 homology domain. *FEBS Lett*. 2002; 513(1):24–9. [PubMed: 11911876]
10. Kay LE, Forman-Kay JD, McCubbin WD, Kay CM. Solution structure of a polypeptide dimer comprising the fourth $\text{Ca}(2+)$ -binding site of troponin C by nuclear magnetic resonance spectroscopy. *Biochemistry*. 1991; 30(17):4323–33. [PubMed: 2021624]
11. Skelton NJ, Kordel J, Chazin WJ. Determination of the solution structure of Apo calbindin D9k by NMR spectroscopy. *J Mol Biol*. 1995; 249(2):441–62. [PubMed: 7783203]
12. Valley CC, Cembran A, Perlmutter JD, Lewis AK, Labello NP, Gao J, et al. The methionine-aromatic motif plays a unique role in stabilizing protein structure. *J Biol Chem*. 2012; 287(42):34979–91. [PubMed: 22859300]

Figure 1. Assigned ^{15}N -HSQC spectra of the EHD3 EH domain
 ^{15}N -HSQC spectrum of the EHD3 EH domain in 20 mM Tris (pH 7.4), 100 mM KCl, and 3 mM Ca^{2+} . The assignments for the backbone amide groups are labeled. Arrows depict the location of W485 and L503.

Figure 2. Comparison of the secondary structure analysis and Ca²⁺-binding properties of the EHD1 and EHD3 EH domains

A) Results from the chemical shift index (CSI) program using the Wishart Database as a reference. Threshold deviations from random coil ¹H, ¹³Cα, ¹³Cβ chemical shifts were plotted as a function of residue number. Sticks with a dot at the top or bottom are chemical shifts consistent with an α-helix or β-strand, respectively. Sticks with a dot in the middle are for non-deviated chemical shifts. The cartoons represent the portions of the EHD3 and EHD1 EH domains predicted to be α-helical (ribbon) or β-strand (arrow) according to the CSI consensus. B) Overlay of ¹⁵N-HSQC spectra of the EHD1 (left) and EHD3 (right) EH domains (Tris buffer, 100 μM) in absence (+ 1 mM EGTA, red) and presence (black) of 3 mM CaCl₂. Residues affected by the absence of Ca²⁺ have been labeled (bold font - broaden beyond detection; regular font - signal intensity decreased ~50%). C) Sequence alignment of the EHD3 and EHD1 EH domains. Symbols' significance is as follows: "*" - identical; ":" - conserved substitutions; "." - semi-conserved substitution. Residues affected by the absence of Ca²⁺ have been highlighted (orange highlight - broaden beyond detection; grey highlight - signal intensity decreased ~50%). The Ca²⁺-binding residues are colored in green. The substituted residues (L495/M495) in the Ca²⁺-binding pocket are colored/underlined in purple and the EHD3 W485 is colored in brown. The Ca²⁺-binding pocket and the EF-hands are indicated by pink and blue lines, respectively.