J Biomol NMR (2007) 39:323–329
DOI 10.1007/s10858-007-9196-0

NMR STRUCTURE NOTE

EH domain of EHD1

Fabien Kieken · Marko Jović · Naava Naslavsky · Steve Caplan · Paul L. Sorgen

Received: 26 June 2007 / Accepted: 5 September 2007 / Published online: 26 September 2007

Abstract EHD1 is a member of the mammalian C-terminal Eps15 homology domain (EH) containing protein family, and regulates the recycling of various receptors from the endocytic recycling compartment to the plasma membrane. The EH domain of EHD1 binds to proteins containing either an Asn-Pro-Phe or Asp-Pro-Phe motif, and plays an important role in the subcellular localization and function of EHD1. Thus far, the structures of five N-terminal EH domains from other proteins have been solved, but to date, the structure of the EH domains from the four C-terminal EHD family paralogs remains unknown. In this study, we have assigned the 133 C-terminal residues of EHD1, which includes the EH domain, and solved its solution structure. While the overall structure resembles that of the second of the three N-terminal Eps15 EH domains, potentially significant differences in surface charge and the structure of the tripeptide-binding pocket are discussed.

Keywords EHD1 · EH domain

Biological context

Receptor internalization at the plasma membrane is an important event that results in delivery of the receptor-containing vesicles to a compartment known as the early endosome. Receptors are then transported to the endosome/lysosome pathway for degradation, or recycled back to the plasma membrane. While some receptors are delivered directly back to the plasma membrane in a "fast recycling" pathway, many receptors are recycled in a highly regulated manner through a series of tubular and vesicular membranes collectively known as the endocytic recycling compartment (ERC), in a process known as "slow recycling". Among the important regulators of endocytic recycling that have been well characterized are the Rab-family of small GTP-binding proteins and their effectors, and the soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins involved in membrane fusion. More recently, an additional group of proteins, known as the C-terminal Eps15 Homology Domain (EHD) proteins, has also been implicated in the regulation of endocytic recycling (reviewed in (Naslavsky and Caplan 2005). The best characterized family member, EHD1, acts primarily at the ERC to regulate the exit of various receptors from this compartment, including the transferrin receptor (Lin et al. 2001), MHC class I molecules (Caplan et al. 2002) and β1 integrin receptors (Jovic et al. 2007). All four highly homologous EHD mammalian paralogs contain an N-terminal nucleotide-binding motif, a central coiled-coil region, and an Eps15 Homology (EH) domain localized to the C-terminus of the protein.

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 asparagine-proline-phenylalanine (NPF) motif and potentially also to proteins containing aspartate-proline-phenylalanine (DPF) motif (Whitehead et al. 1999). Recent studies have revealed that EHD1 interacts with Rab effectors, including the divalent

F. Kieken · M. Jović · N. Naslavsky · S. Caplan (✉) · P. L. Sorgen (✉)
Department of Biochemistry and Molecular Biology and Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198, USA
e-mail: scaplan@unmc.edu

P. L. Sorgen
e-mail: psorgen@unmc.edu

Rab4/Rab5 effector Rabenosyn-5 (Naslavsky et al. 2004) and the Rab11 effector, Rab11-FIP2 (Naslavsky et al. 2006), as well as the SNARE protein SNAP29/GS32, Syndapins I and II and other proteins involved in endocytic regulation (reviewed in Naslavsky and Caplan 2005). These interactions are mediated through the binding of the EH domain to the NPF motifs of the interacting proteins. Several studies aimed at dissecting the nature of other EH domains interacting with NPF motifs have revealed the importance of a hydrophobic pocket within the EH domain, where the NPF motif is capable of tightly interacting with a conserved tryptophan residue within the EH domain (de Beer et al. 1998, 2000). Accordingly, truncation of the EH domain alters the distribution of EHD1 in cells (Caplan et al. 2002) suggesting that this domain contains important localization signals. Furthermore the EH domains of EHD1 and Eps15 were recently shown to be capable of phosphatidylinositol binding (Naslavsky et al. 2007), thus expanding the functional importance of this domain.

To date, structural analysis of the EH domain has been limited to the more common EH domain-containing proteins with an EH domain(s) localized to their N-terminus. For example, the solution structures of the three Eps15 N-terminal EH domains were solved, as well as the EH domain of POB1, and Reps1 (reviewed in Confalonieri and Di Fiore 2002). Here, we report the solution structure of the C-terminal EH domain (residues 401–534) of EHD1. This is an important step in progression towards a better understanding of the molecular processes guiding EHD1-mediated receptor recycling.

Methods and results

The EH domain of EHD1 (residues 401–534) was subcloned into the bacterial expression vector pGEX-6P-2 (GST-tagged; Amersham Biosciences, Piscataway, NJ) by standard PCR methods. Rosetta bacterial cells (Novagen, Madison, WI) transformed with the pGEX-6P-2 vector containing the EHD1 EH domain gene were grown in 1 L of minimum M63 media in the presence of ^{15}N-ammonium chloride and ^{13}C-glucose and induced with 120 mg of IPTG at a cell density of 0.8 OD_{600} for 4 h. The bacterial cells were pelleted and resuspended in 1× Phosphate buffered saline (PBS) at pH 7.4 containing the Complete Protease Inhibitor (Roche Molecular Biochemicals, Mannheim, Germany) and

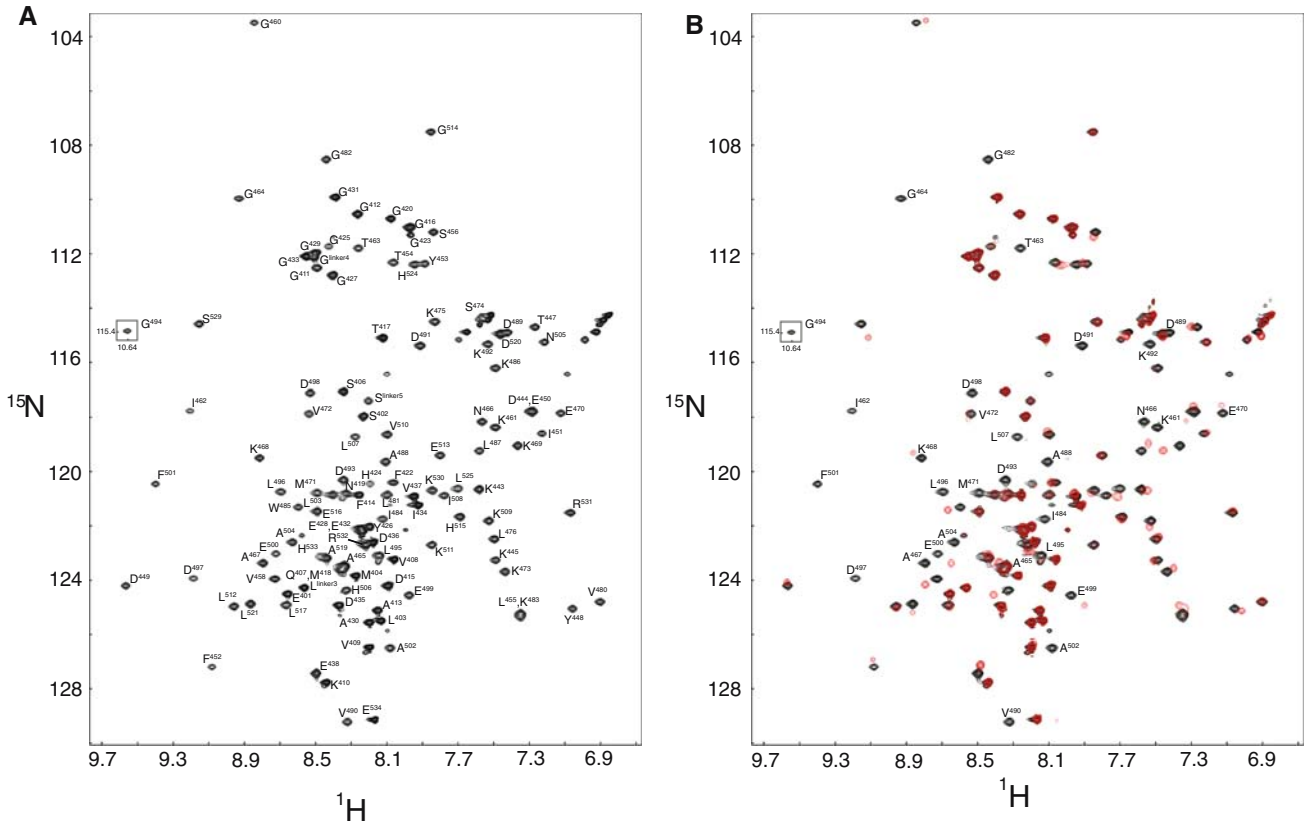

Fig. 1 ^{15}N-HSQC spectra of the EH domain of EHD1. (**A**) ^{15}N-HSQC spectrum of the EH domain of EHD1 in the presence of 3 mM calcium. The assignments for the backbone amide groups are labeled. (**B**) Effect of calcium on the EH domain structure. The ^{15}N-HSQC of the EHD1 EH domain in the presence of 3 mM calcium (black) has been overlaid with the EHD1 EH domain in the absence of calcium (red). The resonances which reappeared upon addition of calcium have been labeled

1 mM DTT. The cells were lysed by French Press and 1 mM PMSF and 1% NP-40 were added before separation of the lysate from the intact cells by centrifugation (12,000*g*, 20 min). The GST-tagged EHD1 EH domain was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) and the GST-tag was cleaved by incubation with 160 units of PreScission Protease (Amersham Biosciences, Piscataway, NJ) in PBS buffer overnight and concentrated using an Amicon Centriplus YM-10 column. Nuclear magnetic resonance (NMR) data were acquired at 25°C using a Varian INOVA 600 spectrometer fitted with a cold probe. Backbone sequential assignments were obtained using the following 3D experiments: HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, and HNHA spectra. The ^{15}N-HSQC spectrum of the EHD1 EH domain is shown in Fig. 1A. All backbone signals were assigned except for the amide proton of Val^{441}, Asn^{478} and Thr^{479}. The side chain chemical shifts were obtained from the 3D ^{13}C-HCCH-TOCSY and ^{15}N-TOCSY-HSQC experiments. Aromatic side chains were assigned using a 2D TOCSY and 3D ^{13}C-edited NOESY (aromatic). The chemical shift assignments were deposited in the BioMagResBank (accession code: 15279). All data were processed with the NMRPipe program (Delaglio et al. 1995) and analyzed with NMRView (Johnson 1994).

Distance constraints were derived from NOEs observed in the ^{15}N-NOESY-HSQC and ^{13}C-NOESY-HSQC spectra, each with mixing times of 150 ms. Model structures were calculated by using the program ARIA 1.2 (Linge et al. 2001), as implemented in the Crystallography & NMR System (CNS) 1.1 software (Brunger et al. 1998; Linge et al. 2001). The input data consisted of the chemical shifts obtained from the resonance assignments, cross-peak positions and volumes from the ^{15}N-NOESY-HSQC and ^{13}C-NOESY-HSQC spectra without assignment, $^{3}J_{HN\text{-}H\alpha}$ coupling constants from the HNHA spectrum, and hydrogen bonds based on deuterium exchange experiments. During the first ARIA run, an automated calibration was performed,

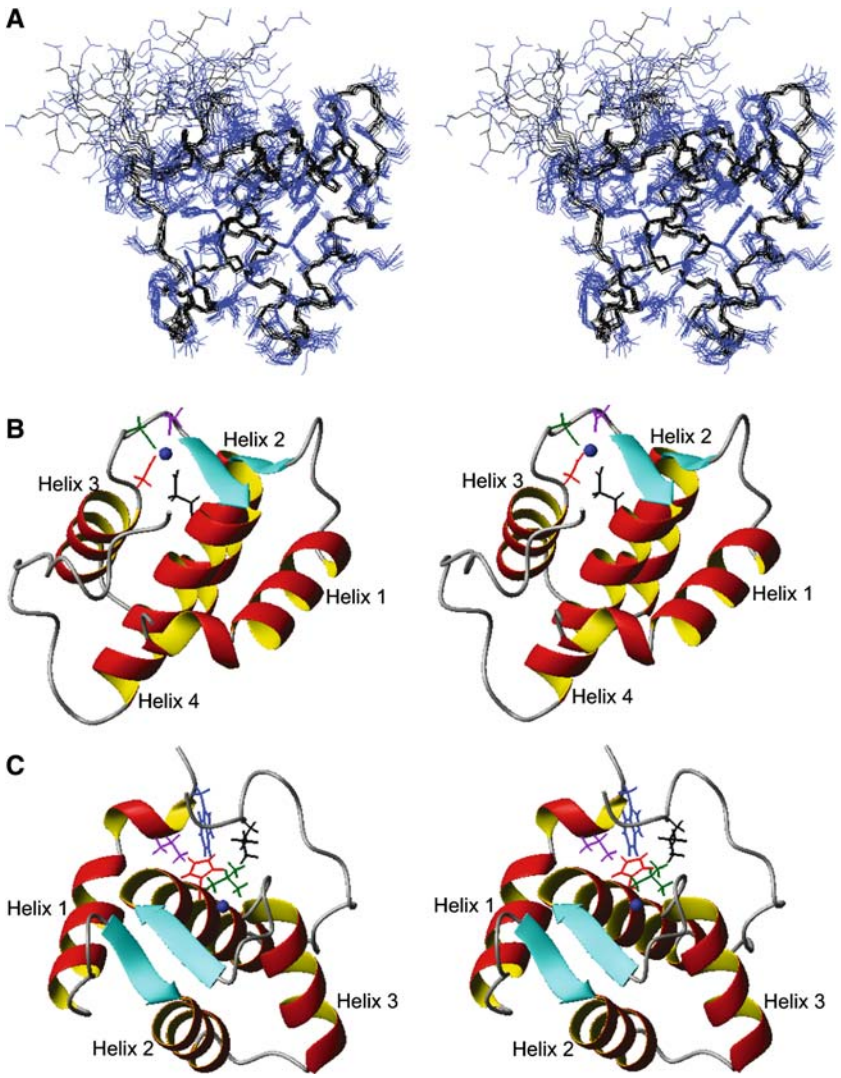

Fig. 2 Solution structure of the EH domain of EHD1. (**A**) Stereoview of the 10 lowest energy structures which have been superpositioned according to the backbone atoms (region Asp^{435}-Glu^{534}). (**B**) Stereoview of the ribbon diagram of the lowest energy EHD1 EH domain structure showing the location of the calcium binding site. Lateral chains of the residues involved in the chelating with calcium have been labeled (Asp^{489} in red, Asp^{491} in green, Asp^{493} in violet, and Glu^{500} in black.). (**C**) Stereoview of the ribbon diagram of the lowest energy EHD1 EH domain structure to highlight the first helical domain. Lateral chains of the residues involved in the kink of the first helical domain have been labeled (Trp^{439} in blue, Val^{441} in violet, Leu^{503} in green, His^{506} in red, and Leu^{525} in black)

unassigned NOE peaks were assigned, distance restraints were generated, and the 3D protein structures were calculated. At the end of each run, rejected restraints and residual NOE violations were analyzed. The new assignments were checked and introduced or omitted in the subsequent run. This procedure of assignment/refinement was repeated iteratively until the completion of the NOESY spectra assignments. In the final iteration, the 50 structures with the lowest restraint energy values were further refined by molecular dynamics simulation in water to remove artifacts (Linge et al. 2003). The final restraint file containing both ambiguous and unambiguous restraints was used as a reference for the next calculation.

Previous studies have shown that EH domains contains either 'canonical' or 'pseudo' EF-hands that bind calcium to stabilize the EH domain structure (de Beer et al. 1998; Strynadka and James 1989). This stabilization was evident when the ^{15}N-HSQC spectrum of the EHD1 EH domain in the presence of calcium was overlaid with the ^{15}N-HSQC spectrum in its absence. Calcium caused the reappearance of a number ($\sim$30) of resonances (Fig. 1B). These data suggest that in the absence of calcium, the residues comprising the EF-hand were in intermediate exchange with the solvent and calcium stabilized this region as evident by the slow exchange and reappearance of the peaks. The presence of the bonded calcium ion in the structure of the EHD1 EH domain was also confirmed by the unusual shift of the Gly^{494} amide proton at 10.64 ppm which is a characteristic signature for calcium binding in EF-hands (Fig. 1A) (Kay et al. 1991). Therefore, in another Aria run, calcium was modeled into the EH domain structure using eight distance restraints between calcium and the coordinating oxygen of the carboxyl group of Asp^{489}, Asp^{491}, Asp^{493}, and Glu^{500} based on the structure of the EH-2 domain of Eps15. The inclusion of the distance restraints did not violate the NOE restraints from the EH domain suggesting the calcium can be modeled into the structure. The 10 lowest restraint energy structures were evaluated using PROCHECK-NMR (Laskowski et al. 1996) and visualized using MOLMOL software (Koradi et al. 1996). The coordinates of the EHD1 EH domain (residues 401–534) in the presence of calcium have been deposited in the Protein Data Bank as entry 2JQ6.

The backbone view of the final 10 structures of the EH domain for residues Asp^{435}-Glu^{534} is displayed in Fig. 2A (residues 401–434 were unfolded and therefore not included) and structural information is presented in Table 1. The structure contains two associated helix-loop-helix motifs with the loops connected by a short antiparallel β-sheet (Fig. 2B, C), similar to the previously solved EH-2 domain structure of Eps15 (Confalonieri and Di Fiore 2002). The position of the first helix Val^{440}-Thr^{454} is perpendicular to the three other helical domains: Gly^{464}-Lys^{473}, Asn^{478}-Ala^{488} and Asp^{498}-Leu^{512}. The antiparallel β-sheet is comprised of loop residues Lys^{461}-Thr^{463} and Leu^{495}-Asp^{497}. The tight packing of the helices is reflected by the large number of long range distances (703). The residues C-terminal to the fourth helix, His^{515}-Glu^{534}, form a number of proline zigzags that position over the third and fourth helical domains to juxtapose both the amino- and carboxyl-termini. Additionally, residues Pro^{528}-Lys^{530} and Pro^{523}-Leu^{525} formed a residual helical domain.

Discussion and conclusions

We have characterized the structure of the C-terminus of EHD1 (Glu^{401}-Glu^{534}). The C-terminus contains an EH

Table 1 Structural statistics of the 10 lowest energy structures of the EH domain of EHD1

Conformational restraints		
NOE distance restraints		
Total		3127
Intra-residue (|i-j| = 0)		1050
Sequential (|i-j| = 1)		766
Medium range ($2 \le \|i-j\| < 5$)		608
Long range ($\|i-j\| \ge 5$)		703
Unambiguous restraints		3124
Ambiguous restraints		3
Backbone hydrogen bonds		62
Residual violations		Average number per residue
Distance restraints >0.3 Å		0
Distance restraints >0.5 Å		0
RMSD[a] from standard geometry		
Bond lengths (Å)		0.0045 ± 0.0001
Bond angles (degrees)		0.60 ± 0.01
Impropers (degrees)		1.66 ± 0.03
Energies		
NOE		83 kcal/mol ± 8
Van der Waals		–1,091 kcal/mol ± 22
Electrostatic		–6,033 kcal/mol ± 56
Ramachandran maps		
Residues in most favored regions		82.9%
Residues in additional allowed regions		16.7%
Average RMSD[a]	Backbone (Å)	All non-hydrogens (Å)
EH domain (439–528)	0.53 ± 0.10	0.92 ± 0.15

[a] RMSD, root mean square deviation

domain (residues Asp^{435}-Glu^{534}) which is preceded by an unfolded region (residues Glu^{401}-Ile^{434}). The C-terminal EH domain of EHD1 exhibits the same structural characteristics as the N-terminal EH domains previously solved, with two helix-loop-helix motifs connected by a short antiparallel β-sheet, between the loops.

EH domains have been identified in a large number of proteins involved in endocytosis or vesicle transport (Confalonieri and Di Fiore 2002). Among the EH domains which have been solved structurally, the EH-2 domain of Eps15 shares 49.5% identity with the EH domain of EHD1 (Fig. 3A) and presents a similar overall structural organization (Fig. 3B, C). The backbone RMSD between both lowest energy structures is 1.55 Å. One difference appears to be the organization of the first helical domain. The first helical domain of the EHD1 EH domain is composed of residues Val^{440}-The^{454} with a kink centered around Asp^{444} (Fig. 2C), whereas the first helix of the Eps15 EH-2 domain (Pro^{126}-Phe^{136}) is relatively straight. The structural difference is the result of a missing proline residue in the first helix of the EHD1 EH domain (Fig. 3A). The side chains of residues Trp^{122} and Val^{124} from the N-terminus of the Eps15

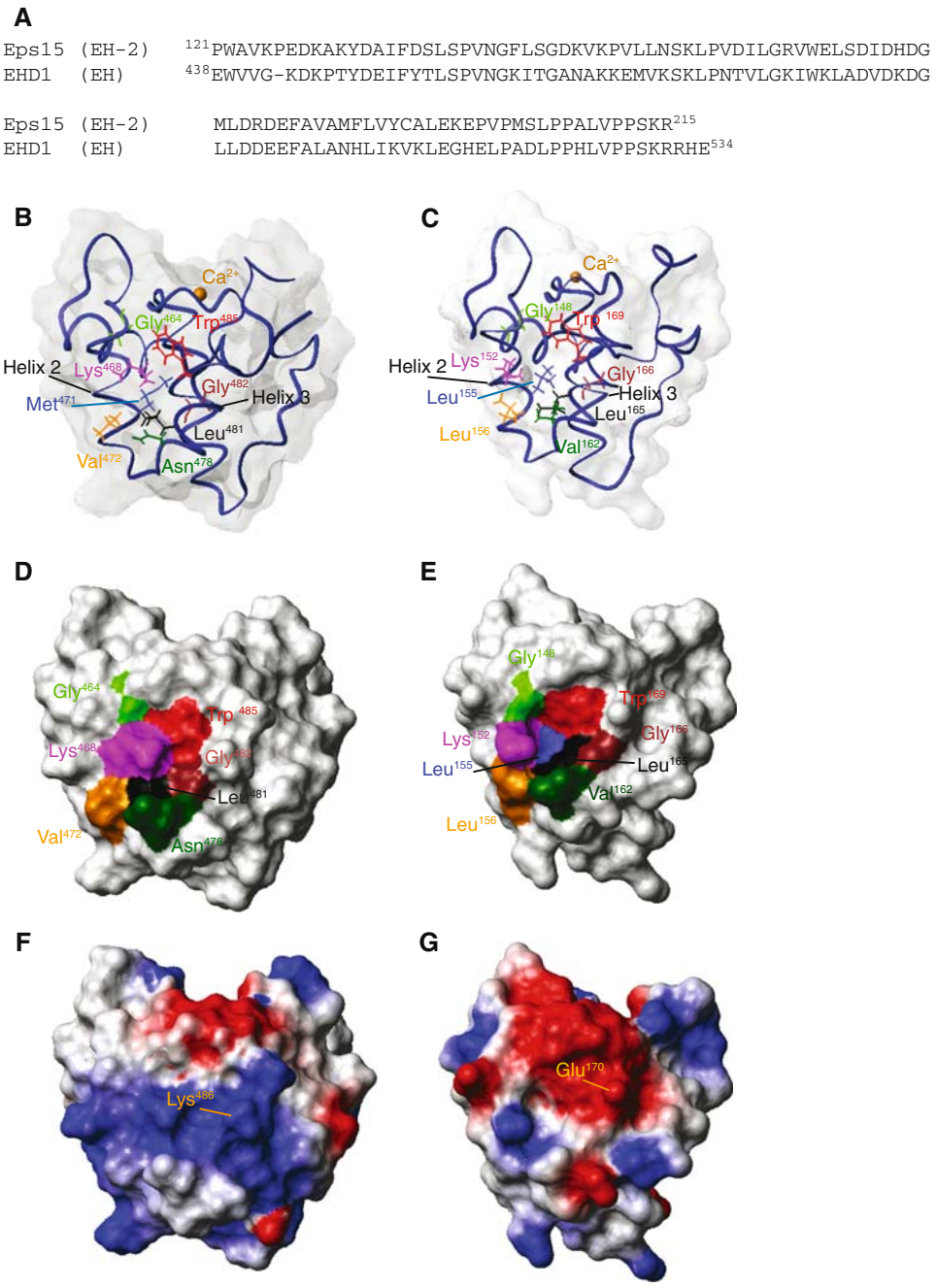

Fig. 3 Structural comparison of the EHD1 EH domain and the Eps15 EH-2 domain. (**A**) Sequence alignment between the EHD1 EH domain and Eps15 EH-2 domain. Ribbon diagram displaying the side chains of the EHD1 EH domain (**B**) and Eps15 EH-2 domain (**C**) residues involved in binding the NPF motif. Residues that form the binding pocket for both EH domains have been labeled. Molecular surface of the EHD1 EH domain (**D**) and Eps15 EH-2 domain (**E**) with the same orientation and residues colored as in (**A**) and (**B**). Electrostatic surface potential of the EHD1 EH domain (**F**) and Eps15 EH-2 domain (**G**). Red and blue colors represent regions of negative and positive electrostatic potential, respectively. Calculations were performed using MOLMOL (Koradi et al. 1996)

EH-2 domain form a pocket with the C-terminal residues His^{190}, Leu^{187}, and Leu^{209}. However, for the EHD1 EH domain residues Trp^{439} and Val^{441} to form a similar pocket with residues His^{506}, Leu^{503}, and Leu^{525}, the first helix needs to form a kink positioned at residue Asp^{444}.

Previous studies have solved the structure of the Eps15 EH-2 domain with an NPF motif peptide (de Beer et al. 2000). The NPF sequence lies in a hydrophobic pocket formed by the 2nd and 3rd α-helices and directly interacts with residues Gly^{166}, Arg^{167}, Trp^{169} and Glu^{170}. The Asn side chain from the NPF motif intercalates between the side chains of the Glu^{170} and Trp^{169}, the side chain of the phenylalanine lies on top of the binding pocket composed of Leu^{155} and Leu^{165}, and the proline carbonyl group forms a hydrogen bond with a Lys^{152} ε amino proton. The orientation of the residues forming the binding pocket between the EHD1 EH and Eps15 EH-2 domains are similar (e.g. $Trp^{485/169}$, $Leu^{481/165}$, and $Lys^{468/152}$, respectively), in agreement with their strong homology of sequence and structure. Nevertheless, comparison of molecular surfaces of both proteins indicates that the binding pocket of the EHD1 EH domain is narrower and not as deep as that of the Eps15 EH-2 domain (Fig. 3D, E). Moreover, Met^{471}, which corresponds to Leu^{155} of Eps15, is buried and not accessible to form the hydrophobic pocket.

Another difference between the two domains is the surface potential. Previous studies have established a high preference of the Eps15 EH-2 domain for binding NPF motifs as a result of the negative surface charge at the binding pocket that favors hydrogen bonding with the asparagine γNH_2 hydrogen atoms (de Beer et al. 2000). However, the EHD1 EH domain exhibits a more positively charged surface potential around the binding pocket as compared to the Eps15 EH-2 domain, due to the replacements of $Phe^{145} \rightarrow Lys^{461}$, $Asp^{149} \rightarrow Ala^{465}$, $Pro^{153} \rightarrow Lys^{469}$, $Asn^{157} \rightarrow Lys^{473}$, $Asp^{163} \rightarrow Thr^{479}$, and $Glu^{170} \rightarrow Lys^{486}$ (Fig. 3F, G). The charge differences, as well as the narrowing of the binding pocket, may change the specificity and nature of the ligand-binding preference for the EHD1 EH domain. The presence of a highly positive surface charge at the binding pocket suggests that the EHD1 EH domain may have a higher affinity for endocytic regulatory proteins containing the DPF motif, over those containing an NPF motif.

In summary, we provide the first structural characterization of a C-terminal EH domain. Given our recent discovery that this EH domain binds to phosphatidylinositol moieties, elucidation of the structure of the EHD1 EH domain will shed critical new light on the molecular mechanism by which it interacts with lipids and proteins to regulate endocytic trafficking.

Acknowledgements This work was supported by grants from the National Institutes of Health (GM072631, P.L.S.; GM074876, S.C.), the UNMC Eppley Cancer Center Collaborative Research Award (P.L.S. and S.C.), and the Nebraska Center for Cell Signaling fellowship supported by the National Institutes of Health (P20 RR018759, M.J.).

References

Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL (1998) Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr D Biol Crystallogr 54(Pt 5):905–921

Caplan S, Naslavsky N, Hartnell LM, Lodge R, Polishchuk RS, Donaldson JG, Bonifacino JS (2002) A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. EMBO J 21:2557–2567

Confalonieri S, Di Fiore PP (2002) The Eps15 homology (EH) domain. FEBS Lett 513:24–29

de Beer T, Carter RE, Lobel-Rice KE, Sorkin A, Overduin M (1998) Structure and Asn-Pro-Phe binding pocket of the Eps15 homology domain. Science 281:1357–1360

de Beer T, Hoofnagle AN, Enmon JL, Bowers RC, Yamabhai M, Kay BK, Overduin M (2000) Molecular mechanism of NPF recognition by EH domains. Nat Struct Biol 7:1018–1022

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR 6:277–293

Johnson BAaB R A. (1994) NMRView: a computer program for the visualization and analysis of NMR data. J Biomol NMR 4: 603–614

Jovic M, Naslavsky N, Rapaport D, Horowitz M, Caplan S (2007) EHD1 regulates beta1 integrin endosomal transport: effects on focal adhesions, cell spreading and migration. J Cell Sci 120:802–814

Kay LE, Forman-Kay JD, McCubbin WD, Kay CM (1991) Solution structure of a polypeptide dimer comprising the fourth Ca(2+)-binding site of troponin C by nuclear magnetic resonance spectroscopy. Biochemistry 30:4323–4333

Koradi R, Billeter M, Wuthrich K (1996) MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graph 14:51–55, 29–32

Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, Thornton JM (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR 8:477–486

Lin SX, Grant B, Hirsh D, Maxfield FR (2001) Rme-1 regulates the distribution and function of the endocytic recycling compartment in mammalian cells. Nat Cell Biol 3:567–572

Linge JP, O'Donoghue SI, Nilges M (2001) Automated assignment of ambiguous nuclear overhauser effects with ARIA. Methods Enzymol 339:71–90

Linge JP, Williams MA, Spronk CA, Bonvin AM, Nilges M (2003) Refinement of protein structures in explicit solvent. Proteins 50:496–506

Naslavsky N, Boehm M, Backlund PS Jr, Caplan S (2004) Rabenosyn-5 and EHD1 interact and sequentially regulate protein recycling to the plasma membrane. Mol Biol Cell 15:2410–2422

Naslavsky N, Caplan S (2005) C-terminal EH-domain-containing proteins: consensus for a role in endocytic trafficking, EH? J Cell Sci 118:4093–4101

Naslavsky N, Rahajeng J, Sharma M, Jovic M, Caplan S (2006) Interactions between EHD proteins and Rab11-FIP2: a role for EHD3 in early endosomal transport. Mol Biol Cell 17:163–177

Naslavsky N, Rahajeng J, Chenavas S, Sorgen PL, Caplan S (2007) EHD1 and Eps15 Interact with phosphatidylinositols via their Eps15 homology domains. J Biol Chem 282:16612–16622

Strynadka NC, James MN (1989) Crystal structures of the helix-loop-helix calcium-binding proteins. Annu Rev Biochem 58:951–998

Whitehead B, Tessari M, Carotenuto A, van Bergen en Henegouwen PM, Vuister GW (1999) The EH1 domain of Eps15 is structurally classified as a member of the S100 subclass of EF-hand-containing proteins. Biochemistry 38:11271–11277