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Crystallographic characterization of the PDZ1 domain of the human Na^+/H^+ exchanger regulatory factor

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The Na^+/H^+ exchanger regulatory factor (NHERF) contains two PDZ domains that mediate the assembly of transmembrane and cytosolic proteins into functional signal transduction complexes. The human NHERF PDZ1 domain, which spans residues 11–99, interacts specifically with carboxy-terminal residues of the β_2 adrenergic receptor and the cystic fibrosis transmembrane conductance regulator. The NHERF PDZ1 was expressed in *Escherichia coli* as a soluble protein, purified and crystallized in the unbound form using the vapor-diffusion method with 2 *M* ammonium sulfate as the precipitant. Diffraction data were collected to 1.5 Å resolution using synchrotron radiation. The crystals belong to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 51.6$, $c = 58.9$ Å, and one molecule in the asymmetric unit.

1. Introduction

PDZ (PSD-95/Discs-large/ZO-1 homology) domains are conserved protein modules that mediate specific protein–protein interactions. PDZ-containing proteins function as scaffolds for assembling membrane receptors, ion channels and other cellular signaling molecules in the vicinity of their substrates. By organizing such signal-transduction pathways at specific intracellular locations, PDZ proteins increase the specificity and efficiency of signal transduction (reviewed by Garner *et al.*, 2000; Craven & Bredt, 1998; Ponting *et al.*, 1997). PDZ domains bind to short carboxy-terminal peptides and have been categorized into two classes on the basis of target-sequence specificity. Class I domains bind to peptides with the consensus sequence S/T-*X*-V/I/L-COOH, whereas class II domains recognize the motif F/Y-*X*-F/V/A-COOH (Songyang *et al.*, 1997). In addition to recognizing carboxy-terminal peptides, PDZ domains can also interact with internal peptide sequences that form a β-hairpin finger (Hillier *et al.*, 1999). PDZ domains share a common fold consisting of a six-stranded antiparallel β-barrel capped by two α-helices, with one β-strand of the barrel participating in both β-sheets (Doyle *et al.*, 1996; Morais Cabral *et al.*, 1996). Peptidic ligands interact with PDZ domains by a process of β-sheet augmentation in which the peptide forms an additional antiparallel strand in the PDZ β-sheet. Conserved sequences in the carboxylate-binding loop situated between the first two strands of the PDZ domain lie adjacent to and recognize the last four residues of the target peptide (Doyle *et al.*, 1996; Daniels *et al.*, 1998; Hillier *et al.*, 1999; Tochio *et al.*, 1999; Kozlov *et al.*, 2000).

The Na^+/H^+ exchanger regulatory factor (NHERF) was originally cloned as an essential cofactor for the protein kinase A mediated inhibition of the Na^+/H^+ exchanger NHE-3 (Weinman *et al.*, 1995). NHERF was also identified independently as EBP50 [ezrin–radixin–moesin (ERM) binding phosphoprotein-50], a membrane–cytoskeleton linking protein that binds to the carboxy-termini of integral membrane proteins through its two PDZ domains and to the cortical actin cytoskeleton through its ERM-binding domain (Reczek *et al.*, 1997). The human NHERF PDZ1 domain consists of residues 11–99 and mediates interactions with several transmembrane receptors and ion channels, including the β_2 adrenergic receptor (β_2AR) and the cystic fibrosis transmembrane conductance regulator (CFTR) (Murthy *et al.*, 1998; Hall, Premont *et al.*, 1998; Hall, Ostergaard *et al.*, 1998; Wang *et al.*, 1998). Through binding to the motif DSLL at the carboxy-terminus of β_2AR, the NHERF PDZ1 mediates the sorting of internalized β_2AR between degradative endocytic pathways and plasma-membrane recycling (Cao *et al.*, 1999). Similarly, the high-affinity interaction of the NHERF PDZ1 with the sequence DTRL at the carboxy-terminus of CFTR is essential for the functional expression of CFTR in the apical plasma membrane (Moyer *et al.*, 2000).

As a first step towards elucidating the structural basis of the NHERF interaction with β_2AR and CFTR, we report here the expres-

Table 1
Data-collection statistics.

Data in parentheses refer to the last resolution bin.

Crystal	Native	$HgCl_2$ derivative	Native	$HgCl_2$ derivative	$HgCl_2$ derivative	$HgCl_2$ derivative
X-ray source	Cu rotating anode	Cu rotating anode	F2, CHESS	F2, CHESS	F2, CHESS	F2, CHESS
Wavelength (Å)	1.5418	1.5418	0.98401	0.99315	1.00784	1.00932
Temperature (K)	293	293	100	100	100	100
Resolution (Å)	2.0	2.5	1.5	1.9	1.9	1.9
Total reflections	63196	34044	111479	98737	118137	96716
Unique reflections	6496	3547	14916	6926	6820	6780
Completeness (%)	95.7 (82.6)	99.9 (100.0)	99.7 (98.9)	92.4 (60.0)	90.0 (56.1)	89.9 (53.4)
Redundancy	9.73 (7.0)	9.60 (7.5)	7.5 (4.6)	14.3 (10.7)	17.3 (12.6)	14.3 (10.6)
R_{sym}† (%)	9.6 (30.4)	10.7 (31.3)	3.9 (15.2)	6.4 (12.8)	6.5 (14.2)	6.6 (13.8)

† $R_{sym} = \sum |(I - \langle I \rangle)| / \sum I$, where I is the observed integrated intensity, $\langle I \rangle$ is the average integrated intensity obtained from multiple measurements and the summation is over all observed reflections.

sion, purification, crystallization and preliminary X-ray characterization of the human NHERF PDZ1 domain.

2. Materials and methods

2.1. Protein expression and purification

A DNA fragment encoding the NHERF PDZ1 domain (residues 11–99) was amplified using the polymerase chain reaction (PCR) with the human NHERF cDNA as a template (Murthy *et al.*, 1998). The PCR product was cloned into pGEX-2TJL1, a pGEX-2T-based vector (Pharmacia) with a modified polylinker. The resulting construct was transformed into *E. coli* BL21 (DE3) cells (Novagen). Overexpression of the GST-PDZ1 fusion protein was achieved by growing the cell cultures at 310 K until they reached an OD_{600} of 1.0, followed by induction with 0.1 m*M* IPTG for 3 h. The cells were pelleted by centrifugation at 4400*g* for 10 min and were either used immediately or stored frozen at 193 K. The cell pellets were resuspended in 1× PBS buffer at 277 K with 5 m*M* DTT and a protease inhibitor cocktail (Boehringer Mannheim) and were lysed using a Stansted pressure-gradient cell disrupter (Energy Service Co.). Triton X-100 was added to the lysate to a final concentration of 1% and the sample was agitated on a rocking platform at room temperature for 30 min, followed by centrifugation at 31 000*g* for 10 min. 1 ml bed volume of GSH agarose (Pharmacia) per litre of the original cell culture was then added to the supernatant and allowed to bind the GST-PDZ1 fusion protein at room temperature for 30 min. After several washes with PBS buffer, the protein bound on the agarose was cleaved at room temperature for 2 h using 40 units of thrombin (Haematologic Technologies) per litre of the original cell culture. The supernatant containing the free NHERF PDZ1 was passed through a spin cup (VivaScience) and dialyzed against 25 m*M* Tris–HCl pH 8 at 277 K for 1 h. The protein was concentrated in a Centriprep concentrator (Amicon) and was further purified on a Q12 anion-exchange column (BioRad) using a linear gradient of 0–1 *M* NaCl in a buffer of 25 m*M* Tris–HCl pH 8. The purified PDZ1 protein was dialyzed against 10 m*M* NaCl, 10 m*M* HEPES, 0.5 m*M* DTT pH 7.5 and concentrated using a Centriprep concentrator to a final concentration of ~20 mg ml^{-1}. The final purified sample was >99% pure as judged by SDS–PAGE analysis. The resulting NHERF PDZ1 protein contains five additional vector-derived amino-acid residues (GSSRM) at its amino terminus.

2.2. Crystallization

The NHERF PDZ1 was crystallized using the sitting-drop vapor-diffusion method (Ducruix & Giegé, 1992) with 4 µl drops (2 µl protein solution at a concentration of 20 mg ml^{-1} plus 2 µl of crystallization reservoir solution) equilibrated against 1 ml reservoir solution at 293 K. Initial crystallization conditions were tested using the sparse-matrix screening method (Jancarik & Kim, 1991) with commercially available reagents (Hampton Research), followed by optimization of conditions using fractional factorial experiments (Ducruix & Giegé, 1992) and additive screenings. The final optimized conditions were 0.05 *M* potassium sodium tartrate, 0.06 *M* sodium citrate, 2 *M* ammonium sulfate pH 5.9 and 20 m*M* $MnCl_2$. The PDZ1 crystals generally grew as clusters in the initial phase before one or two directions of growth were favored, yielding crystals of the characteristic morphology shown in Fig. 1. Useable crystals with maximum dimensions of 0.6 × 0.3 × 0.2 mm grew in 24 h. Attempts to co-crystallize the NHERF PDZ1 protein with its high-affinity peptidic ligand EVQDTRL from the carboxy terminus of CFTR were unsuccessful.

3. Conclusions

Crystals were mounted in a glass capillary and room-temperature X-ray diffraction data were collected on an R-AXIS IV imaging-plate area detector with Cu $K\alpha$ radiation focused through mirror optics (Molecular Structure Corporation, The Woodlands, Texas, USA). X-rays were from an RU-300 rotating-anode generator (Rigaku, Tokyo, Japan). Because of the unusual morphology of these crystals (Fig. 1), they were aligned for data collection with the axis of the preferred direction of growth parallel to the oscillation axis φ so that the X-ray beam was never incident upon the polycrystalline clusters. The best crystals diffracted to 1.8 Å and complete data to 2.0 Å could be collected from a single crystal at room temperature using a crystal-to-detector distance of 150.0 mm and collecting 50 non-overlapping oscillation frames of 3° (see Table 1 for data-collection statistics). The data were indexed and processed using the programs *DENZO* and *SCALEPACK* as implemented in the *HKL* package version 1.96 (Otwinowski & Minor, 1997). The crystals belong to space group $P3_121$ or its enantiomorph $P3_221$, with unit-cell parameters $a = b = 52.6$, $c = 59.6$ Å. The V_M value was calculated to be 2.32 Å^3 Da^{-1} with $Z = 6$ (Matthews, 1968), corresponding to one molecule per asymmetric unit and a solvent content of approximately 47%. Attempts to solve the NHERF PDZ1 structure with molecular replacement using the program *AMoRe* from the *CCP*4 crystallographic software package (Collaborative Computational Project, Number 4, 1994) and other PDZ domains (Doyle *et al.*, 1996; Daniels *et al.*, 1998) as search models were unsuccessful.

In parallel, a series of experiments to produce heavy-atom derivatives for phasing with multiple isomorphous replacement yielded a single $HgCl_2$ derivative. Phases derived from this derivative were not sufficient to solve the structure using single isomorphous replacement. To generate additional heavy-atom or selenomethionine derivatives, cysteine or methionine residues, respectively, were introduced into the PDZ1 protein using site-directed mutagenesis, generating the mutants L14C, E18C, S46C,

A51C, S77C, Q98C, L14M, C15M, and L17M (data not shown). Because none of these mutants produced diffraction-quality crystals, it was decided to collect multi-wavelength anomalous diffraction (MAD) data of the $HgCl_2$-derivative crystal, using the anomalous signal near the L_{III} absorption edge of mercury (Hendrickson & Ogata, 1997; Hubbard *et al.*, 1994). Both native and $HgCl_2$-derivative PDZ1 crystals were cryoprotected in 40% 2-methyl-2,4-pentanediol and then flash-frozen at approximately 100 K in a stream of liquid nitrogen. Subsequently, monochromatic data of the native crystals diffracting to 1.5 Å, as well as MAD data of the $HgCl_2$ derivative, were collected using a QUANTUM-4 CCD detector (Area Detector Systems Corp.) on the F2 beamline at the Cornell High Energy Synchrotron Source, Ithaca, NY (Table 1). The frozen crystals have slightly reduced unit-cell parameters: $a = b = 51.6$, $c = 58.9$ Å. Structural analysis of the NHERF PDZ1 using MAD methods is currently in progress.

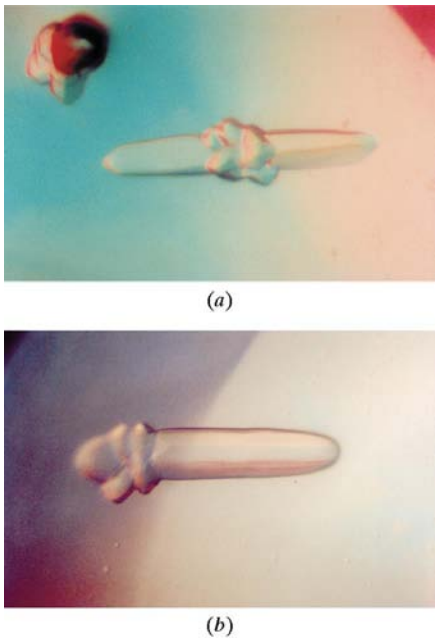

Figure 1
(*a*) Photograph of representative native crystals of the human NHERF PDZ1 domain. (*b*) Crystal showing a single preferred growth axis. Crystals of this morphology gave the best results in X-ray diffraction experiments and were used for the crystallographic analysis.

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