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Crystallization and initial crystal characterization of the N-terminal DH/PH domain of Trio

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Trio is a multidomain signaling protein that plays an important role in neurite outgrowth, axon guidance and skeletal muscle development. Trio contains two DH/PH tandem domains that respectively activate the small GTPases RhoG/Rac and RhoA. The N-terminal DH/PH domain, TrioN, crystallizes in space group $P3_121$, with one TrioN molecule in the asymmetric unit and diffracts to 1.7 Å resolution. The unit-cell parameters are $a = b = 99.5$, $c = 98.3$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. A greater than 90% complete native data set has been collected and structure determination using the multiple isomorphous replacement (MIR) method is ongoing.

1. Introduction

Members of the Rho-family of small GTP-binding proteins (Rho, Rac, Cdc42 and their isoforms) play an important role in the signal transduction pathways that regulate the re-organization of the actin cytoskeleton, gene transcription, cell-cycle progression, neurite extension and retraction and many other cellular processes (reviewed in Van Aelst & D'Souza-Schorey, 1997; Hall, 1998; Bishop & Hall, 2000; Etienne-Manneville & Hall, 2002). Like Ras, these proteins cycle between an active GTP-bound form and an inactive GDP-bound form. Cycling between the GTP- and GDP-bound forms is tightly regulated by accessory proteins. Guanine nucleotide-exchange factors (GEFs) catalyze the exchange of the bound GDP for GTP, while GTPase-activating proteins (GAPs) accelerate the rate of GTP hydrolysis. RhoGEFs form a family of more than 30 members that share a ~200 amino-acid domain called the Dbl-homology (DH) domain that was initially identified in a diffuse B-cell lymphoma cell line (reviewed in Cerione & Zheng, 1996; Whitehead *et al.*, 1997; Zheng, 2001; Hoffman & Cerione, 2002). A pleckstrin-homology (PH) domain, which has been implicated in protein targeting to the membrane, invariably follows the DH domain (reviewed in Lemmon & Ferguson, 2000; Lemmon *et al.*, 2002). The DH/PH tandems of various RhoGEFs have been shown both *in vivo* and *in vitro* to be sufficient for nucleotide exchange on Rho-family members.

Trio, a recently discovered member of the Dbl family, plays a crucial role in neuronal cell migration, neurite outgrowth and axon guidance (reviewed in Bateman & van Victor, 2001). Human Trio was initially identified by screening for binding partners of the leukocyte-antigen-related (LAR) protein, a receptor-like tyrosine phosphatase, using the two-hybrid system (Debant *et al.*, 1996). Trio is a large protein that contains four spectrin-like repeats, two DH/PH domains, two SH3 domains, one Ig-like domain and a C-terminal serine/threonine kinase. This multidomain structure of Trio suggests that it is at the crossroads of multiple signaling pathways and subject to tight regulation. Several Trio-like proteins have also been found in vertebrates (Trio and Kalirin), in *Caenorhabditis elegans* (UNC-73) and in *Drosophila* (DTrio). *In vitro* exchange experiments have shown that the N-terminal DH/PH domain, hereafter referred to as TrioN, activates RhoG and Rac1 specifically but not the homologous Cdc42, while the C-terminal DH/PH domain is specific for RhoA (Debant *et al.*, 1996; Blangy *et al.*, 2000).

Recently, the structures of DH/PH domains of a number of RhoGEFs have been determined alone or in complex with their cognate Rho-protein substrates (Soisson *et al.*, 1998; Worthylake *et al.*, 2000; Rossman *et al.*, 2002; Snyder *et al.*, 2002). These structures revealed differences in the spatial organization of the DH and PH domains, suggesting that unique characteristics exist for each exchange factor. In addition, they revealed that the PH domain might also participate in the interaction with RhoGTPases and assist in catalysis in certain cases.

As an important step toward understanding the molecular basis of substrate specificity and the mode of regulation of Trio, we have crystallized the TrioN structural module and report here the preliminary studies of TrioN crystals.

2. Materials and methods

2.1. Expression and purification

TrioN (residues 1225–1535) was cloned in the pET15b vector (Novagen) as an N-terminal

His_6-tagged protein for overexpression in *Escherichia coli* strain BL21. This cloning resulted in the insertion of the non-native sequence 'GSHMLE' after the thrombin cleavage site. Bacterial cultures were grown in Luria–Bertani broth supplemented with 50 mg l^{-1} ampicillin to a density of A_{600} = 0.5–0.7 OD, at which point protein expression was induced with 0.5 m*M* isopropyl thiogalactopyranoside (IPTG) at 310 K for 4 h. The cultures were pelleted and cells were washed with lysis buffer (50 m*M* sodium/potassium phosphate, 150 m*M* KCl, pH 7.5, 0.1 m*M* PMSF) and stored at 193 K. For purification, freshly thawed cells were suspended in lysis buffer supplemented with 20 m*M* imidazole and broken in a French press. Clarified cell lysates were passed over an Ni–NTA matrix (Qiagen) equilibrated with buffer *A* (20 m*M* Tris–HCl, 500 m*M* NaCl pH 7.5). Non-specifically bound proteins were eluted with buffer *A* supplemented with 40 m*M* imidazole. His_6-TrioN was eluted off the column by stepping the imidazole concentration up to 250 m*M* in buffer *A*. The His_6-tag cleaved protein (thrombin, Sigma) eluted with the flow-through of an ion-exchange Q-Sepharose column (Sigma). TrioN was additionally purified on a sizing column (Ultrogel Aca54, Biosepra) as a ~42 kDa monomer. The pure protein in 20 m*M* Tris–HCl pH 7.5, 0.1 *M* NaCl, 7 m*M* β-mercaptoethanol was concentrated by centrifugation using Amicon Centriprep concentrators (10 kDa molecular-weight cutoff).

2.2. Crystallization and data collection

Crystallization screens of TrioN were initially performed at 293 or 277 K using the hanging-drop vapor-diffusion method. Thin needle-like crystals were obtained within a few hours when TrioN (10 mg ml^{-1}) was mixed with equal amounts of a solution consisting of 8–10% polyethylene glycol (PEG 6000, Fluka), 0.2 *M* NaCl, 5 m*M* β-mercaptoethanol, 0.1 *M* HEPES pH 7.5 and equilibrated against 1 ml reservoirs of the same solution. The appearance of TrioN crystals was not systematic and showed strong dependence on the NaCl concentration. Attempts to improve the shape of these crystals by varying the protein concentration, the temperature, the PEG average molecular weight or the crystallization method (batch and sitting drop) failed. However, during these attempts we found that TrioN crystals can be obtained by simply incubating the protein at ~60 mg ml^{-1} in 0.1 *M* NaCl, 20 m*M* HEPES pH 7.5) with 10 m*M* dithioerythritol (DTE) on ice. These needle-like crystals were stable for several months on ice.

Manipulation of TrioN crystals was carried out in the cold room. One TrioN crystal was transferred into a solution consisting of 5% PEG 6000, 0.2 *M* NaCl and 0.1 *M* HEPES pH 7.5. Cryoprotection was achieved by adding glycerol to the previous solution to a final concentration of 30% in steps of 5% and equilibrating the crystal for few minutes. The crystal was mounted on a nylon loop (Hampton Research, Inc.) before freezing in liquid nitrogen. Native data to 1.7 Å resolution were collected on a 2k × 2k ADSC CCD detector at beamline A1 at the Cornell High Energy Synchrotron Source (CHESS). All intensities were indexed, processed and scaled with the *HKL*97 package (Otwinowski & Minor, 1997).

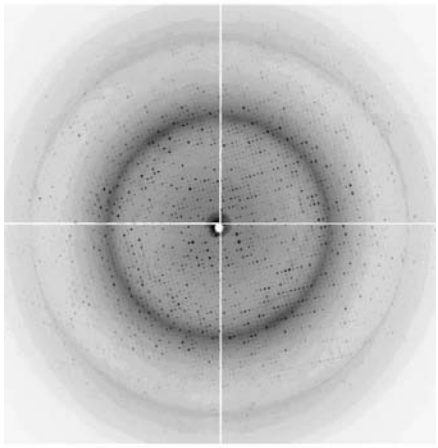
Table 1
Statistics of data collection and processing.

Values for the highest resolution shell are given in parentheses.

Beamline	A1 (CHESS)
Space group	$P3_121$
Unit-cell parameters (Å, °)	$a = b = 99.5$, $c = 98.3$, $\alpha = \beta = 90$, $\gamma = 120$
Wavelength (Å)	0.933
Resolution range (Å)	30–1.7 (1.73–1.70)
Total No. of observations	1391440
Unique reflections	56048
$\langle I/\sigma(I)\rangle$	18.8 (2.4)
Completeness (%)	90.4 (55.8)
R_{merge}† (%)	8.8 (41.2)

† $R_{merge}(I) = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$.

(*a*)



(*b*)

Figure 1
(*a*) Typical crystals (0.05 × 0.05 × 0.4 mm) of TrioN obtained after incubating the protein (60 mg ml^{-1}) on ice in the presence of 10 m*M* DTE. (*b*) Typical diffraction pattern of a TrioN crystal taken at beamline A1 (CHESS) at 100 K with 0.5° oscillation, a crystal-to-detector distance of 200 mm and 60 s exposure time.

3. Results and discussion

The purification of the N-terminal DH/PH domain of human Trio, TrioN, yielded approximately 50 mg of pure protein per litre of bacterial cell culture. Crystallization attempts using the hanging-drop, sitting-drop or under-oil batch methods resulted in crystals when a solution of 8–10% PEG 6000, 0.2 *M* NaCl, 5 m*M* β-mercaptoethanol, 0.1 *M* HEPES pH 7.5 was used as precipitant. However, shortly after appearance and in the majority of the setups, the TrioN crystals obtained with these techniques dissolved. Various attempts to stabilize the needle-like crystals failed. In one attempt, a ~60 mg ml^{-1} solution of TrioN was incubated with 10 m*M* DTE on ice. Long rod-shaped crystals appeared overnight that were stable for months as long as the protein was kept on ice (Fig. 1*a*).

Data extending to 1.7 Å resolution were collected at 100 K from one crystal at beamline A1 at CHESS and processed with *HKL*97. Fig. 1(*b*) represents a diffraction pattern obtained from a 0.5° oscillation. Statistics of data collection and processing are shown in Table 1. The crystal belongs to space group $P3_121$, with unit-cell parameters $a = b = 99.5$, $c = 98.3$ Å. Assuming one TrioN molecule in the asymmetric unit, the calculated V_M is 3.34 $Å^3$ Da^{-1}, which corresponds to a solvent content of 63.2%. Structure solution of TrioN by molecular replacement with *AMoRe* (Navaza, 1994) or *CNS* (Brünger *et al.*, 1998) using the DH/PH coordinates of Sos (Soisson *et al.*, 1998) or Tiam1 (Worthylake *et al.*, 2000) as search models did not yield a clear solution for the rotation or translation functions. Similar searches with the DH domains alone also failed to show a clear solution despite sharing ~25% sequence identity with TrioN. Structure solution of TrioN using multiple isomorphous replacement is in progress.

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