Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, purification, crystallization and preliminary crystallographic analysis of the diarrhoea-causing and virulence-determining region of rotaviral nonstructural protein NSP4

R. Deepa,[a] M. R. Jagannath,[a] M. M. Kesavulu,[b] C. Durga Rao[a] and K. Suguna[b]*

[a]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India, and [b]Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

Correspondence e-mail: suguna@mbu.iisc.ernet.in

Received 6 October 2003
Accepted 3 November 2003

The region spanning the tetrameric coiled-coil domain and the interspecies-variable virulence-determining region of the cytoplasmic tail of rotaviral nonstructural protein NSP4 has been crystallized. The crystals belong to space group $I222$, with unit-cell parameters $a = 30.70$, $b = 38.07$, $c = 181.62$ Å, and contain two molecules in the asymmetric unit. Diffraction data have been collected utilizing a MAR imaging plate to a resolution of 2.2 Å. The tetramer is generated by the crystallographic dyad along the c axis.

1. Introduction

The endoplasmic reticulum (ER) localized nonstructural glycoprotein NSP4 of rotavirus is 175 amino acids long and has been recently identified as the viral enterotoxin (Ball *et al.*, 1996; Estes, 2001). It plays a central role in viral morphogenesis and pathogenesis (Estes, 2001). While two of the three N-terminal hydrophobic domains facilitate ER localization, the cytoplasmic tail of approximately 131 residues, including the third hydrophobic domain, exhibits pleiotropic properties that include the induction of diarrhoea (Ball *et al.*, 1996; Horie *et al.*, 1999), membrane permeabilization (Tian *et al.*, 1996; Newton *et al.*, 1997), acting as an ER-resident receptor for immature double-layered particles (DLPs; Chan *et al.*, 1988; Meyer *et al.*, 1989), Ca^{2+} binding (Estes, 2001), intracellular calcium mobilization (Tian *et al.*, 1995; Dong *et al.*, 1997), inhibition of cellular secretory pathways (Xu *et al.*, 2000), interaction with the viral spike protein VP4 and viral pathogenesis (Estes, 2001). The extreme C-terminus binds to DLPs and is essential for virus maturation in the ER (Au *et al.*, 1993; O'Brien *et al.*, 2000). NSP4 has been observed to form homotetramers and the crystal structure of a synthetic peptide containing amino-acid residues 95–137 revealed a tetrameric coiled-coil structure (Bowman *et al.*, 2000). The Ca^{2+}- and VP4-binding sites are located within the coiled-coil region and a peptide spanning residues 112–135 of this domain has been shown to cause diarrhoea in the newborn mouse model system (Ball *et al.*, 1996; Estes, 2001). Furthermore, mutations in the region between residues 135–141, immediately downstream of the coiled-coil domain, were observed to alter the virulence phenotype of the virus (Zhang *et al.*, 1998).

Here, we report the expression, purification and crystallization of a region of NSP4 from residues 95–146 extending nine amino acids (TTGEIDMTK) downstream of the reported structure. The sequence from amino-acid residues 135–141 (TVQTTGEI) shows species-specific divergence and appears to influence the virulence phenotype of the virus (Mohan & Atreya, 2000; Zhang *et al.*, 1998). Using wild-type and attenuated pairs of two porcine strains and purified NSP4 proteins, mutations at residues 135 and 138 in the divergent region were observed to result in altered virus virulence (Zhang *et al.*, 1998). To determine the influence of individual amino acids in this region on diarrhoea-inducing ability, there is a need to evaluate a large number of NSP4 proteins containing amino-acid substitutions at each of the positions in the newborn mouse model system. Determining the three-dimensional structure of this divergent region located immediately downstream of the coiled-coil domain is important to understand the structural and molecular basis of the virulent/attenuated nature of rotaviruses.

2. Materials and methods

2.1. Expression and purification of NSP4 deletion mutant NSP4ΔN94ΔC29

The NSP4 gene fragment corresponding to residues 95–146 of the simian rotavirus strain SA11 was amplified by PCR using the 5′ and 3′ primers 5′-ATCCCCGGGATGATAGAAA-AACAAATGGAC-3′ and 5′-ATCTAAGCT-TATTTTGTCATA/GTCTATCTC-3′, respectively. The PCR product was digested with the restriction endonucleases *Sma*I and *Hin*dIII and was cloned in the pET22(b+) vector (Novagen) between *Nde*I and *Hin*dIII sites after blunting the *Nde*I site by a Klenow fill-in reaction. The deletion mutant protein NSP4ΔN94ΔC29 was expressed in *Escherichia coli* BL21(DE3) by induction with 1 m*M* IPTG at an OD_{600} of 0.4 for 3 h at 310 K. The cells were lysed in 10 m*M* Tris–HCl pH 8.0 by

Figure 1
Crystals of the NSP4 fragment (residues 95–146) from rotavirus.

sonication and the cell lysate was centrifuged at 30 000 rev min^{-1} in JA 30.50Ti rotor in a Beckman Coulter Avanti J-30I centrifuge for 2 h. The supernatant was adjusted successively to 10, 30 and 60% ammonium sulfate concentration and the pellet fraction from 60% ammonium sulfate concentration containing most of the recombinant protein was used for purification. The pellet was dissolved in Tris–HCl pH 8.0 buffer containing 50 m*M* NaCl, clarified by centrifugation and microfiltration and purified on a Sephacryl S300 column using Biorad Biologic HR chromatographic system. The purity and molecular weight (6.455 kDa) of the protein were determined by SDS–PAGE (Laemmli, 1970) and ESI mass spectrometry.

2.2. Crystallization

Initial screening of the crystallization conditions was carried out by the hanging-drop vapour-diffusion method using Hampton Crystal Screens I and II (Jancarik & Kim, 1991) at 293 K. 2 µl of protein sample at a concentration of 30 mg ml^{-1} in a buffer containing 100 m*M* NaCl and 10 m*M* Tris–HCl pH 8.0 was mixed with 2 µl reservoir solution (500 µl of each of the Hampton screening conditions). Crystals were obtained from a condition with reservoir solution consisting of 1.5 *M* lithium sulfate monohydrate in 0.1 *M* HEPES–Na pH 7.5. Crystals appeared in 5 d and maximum dimensions of 0.23 × 0.23 × 0.06 mm (Fig. 1) were attained within one week of their appearance. Further trials to optimize this crystallization condition did not improve the quality of the crystals.

Table 1
Details of data collection.

Statistics for the highest resolution shell (2.28–2.20 Å) are given in parentheses.

X-ray wavelength (Å)	1.5418
Temperature (K)	293
Space group	*I*222
Unit-cell parameters	
a (Å)	30.70
b (Å)	38.07
c (Å)	181.62
Resolution (Å)	2.2
No. of reflections	48068
No. of unique reflections	5785
Completeness (%)	96 (87)
Mean $I/\sigma(I)$	10.68 (8.39)
$R_{\rm merge}$ (%)	8.4 (18.8)

2.3. Data collection

A complete data set was collected to a resolution of 2.2 Å at 293 K using a MAR300 imaging-plate system. The Cu $K\alpha$ radiation used in the experiment was generated by a Rigaku RU-200 X-ray generator operating at 44 kV and 70 mA and focused with an Osmic mirror system. The crystal-to-detector distance was 120 mm. A total of 125 frames were collected with 1° oscillation range and of 1° frame width. The diffraction data were processed using program packages *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The crystals belong to space group *I*222, with unit-cell parameters a = 30.70, b = 38.07, c = 181.62 Å. Details of data collection and processing are presented in Table 1. Assuming the presence of a dimer in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 2.0 Å^3 Da^{-1}, with a solvent content of 39.3%. The phases of the reflections were determined by the molecular-replacement method with the program *AMoRe* (Navaza, 1994), using the reported structure of a synthetic molecule (PDB code 1g1j; Bowman *et al.*, 2000) as the search model. A clear solution was obtained with an R factor of 45.1% and a correlation coefficient of 64.3 using data in the 15–2.2 Å range. The search model and the asymmetric unit contain two polypeptide chains, the tetrameric four-helix coiled-coil being formed by the crystallographic twofold axis parallel to the c axis. Electron-density maps calculated at this stage did not show interpretable density for the extra nine-residue region. Further refinement and model building of the structure are in progress.

The diffraction data were collected at the X-ray Facility for Structural Biology at the Indian Institute of Science, supported by the Department of Science and Technology (DST) and the Department of Biotechnology (DBT), India. The mass-spectrometry facility was supported by the Drug and Molecular Design program of the DBT. This work was supported by the Structural Genomics programme under the Genomics Initiative at the Indian Institute of Science funded by the DBT.

References

Au, K.-S., Mattion, N. M. & Estes, M. K. (1993). *Virology*, **194**, 665–673.
Ball, J. M., Tian, P., Zeng, C. Q. Y., Morris, A. P. & Estes, M. K. (1996). *Science*, **272**, 101–104.
Bowman, G. D., Nodelman, I. M., Levy, O., Lin, S. L., Tian, P., Zamb, T. J., Udem, S. A., Venkataraghavan, B. & Schutt, C. E. (2000). *J. Mol. Biol.* **304**, 861–871.
Chan, W.-K., Au, K.-S. & Estes, M. K. (1988). *Virology*, **164**, 435–442.
Dong, Y., Zeng, C. Q. Y., Ball, J. M., Estes, M. K. & Morris, P. A. (1997). *Proc. Natl Acad. Sci. USA*, **94**, 3960–3965.
Estes, M. K. (2001). *Fields' Virology*, 4th ed., edited by D. M. Knipe & P. M. Howley, pp. 1747–1785. Philadelphia: Lippincott–Raven.
Horie, Y., Nakagomi, O., Koshimura, Y., Nakagomi, T., Suzuki, Y., Oka, T., Sasaki, S., Matsuda, Y. & Watanabe, S. (1999). *Virology*, **262**, 398–407.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Meyer, J. C., Bergman, C. C. & Bellamy, A. R. (1989). *Virology*, **171**, 98–107.
Mohan, K. V. & Atreya, C. D. (2000). *Arch. Virol.* **145**, 1789–1799.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Newton, K., Meyer, J. C., Bellamy, A. R. & Taylor, J. A. (1997). *J. Virol.* **71**, 9458–9465.
O'Brien, J. A., Taylor, J. A. & Bellamy, A. R. (2000). *J. Virol.* **74**, 5388–5394.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Tian, P., Ball, J. M., Zeng, C. Q. Y. & Estes, M. K. (1996). *J. Virol.* **70**, 6973–6981.
Tian, P., Estes, M. K., Hu, Y., Ball, J. M., Zeng, C. D.-Y. & Schilling, W. P. (1995). *J. Virol.* **69**, 5763–5772.
Xu, A., Bellamy, A. R. & Taylor, J. A. (2000). *EMBO J.* **19**, 6465–6474.
Zhang, M., Zeng, C. Q. Y., Dong, Y., Ball, J. M., Saif, L. J., Morris, A. P. & Estes, M. K. (1998). *J. Virol.* **72**, 3666–3672.