The importance of the Q motif in the ATPase activity of a viral helicase

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Abstract NS3 proteins of flaviviruses contain motifs which indicate that they possess protease and helicase activities. The helicases are members of the DExD/H box helicase superfamily and NS3 proteins from some flaviviruses have been shown to possess ATPase and helicase activities in vitro. The Q motif is a recently recognised cluster of nine amino acids common to most DExD/H box helicases which is proposed to regulate ATP binding and hydrolysis. In addition a conserved residue occurs 17 amino acids upstream of the Q motif ('+17'). We have analysed full-length and truncated NS3 proteins from Powassan virus (a tick-borne flavivirus) to investigate the role that the Q motif plays in the hydrolysis of ATP by a viral helicase. The Q motif appears to be essential for the activity of Powassan virus NS3 ATPase, however NS3 deletion mutants that contain the Q motif but lack the '+17' amino acid have ATPase activity albeit at a reduced level.

Key words: NS3; DExD/H box helicase; Q motif

1. Introduction

Powassan virus (POWV), like other members of the Flaviviridae family, is enveloped with a positive sense RNA genome [1]. It is a tick-borne flavivirus found primarily in Russia and North America. Sequence analysis indicates that the genome encodes a single polyprotein of ~3400 amino acids which contains the proteins in the order NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH [2]. C, prM and E are the structural proteins and are incorporated into virus particles, the remainder are non-structural (NS) proteins involved in virus replication and polyprotein processing.

The NS3 proteins of flaviviruses are multifunctional and are involved in polyprotein processing and replication of the viral RNA. The N-terminal third of NS3 contains a serine protease whereas the C-terminal two-thirds contains an ATPase/helicase. Both of these activities are essential for flavivirus replication [3]. Protease, ATPase and helicase activities have been demonstrated in vitro with recombinant NS3 derived from a number of flaviviruses [4–10].

Conserved motifs within the ATPase/helicase domain of NS3 proteins from flaviviruses identify them as RNA helicases that belong to the DExD/H box superfamily of proteins. DExD/H box proteins are involved in most, if not all, processes involving RNA molecules including transcription, editing, splicing, RNA export, RNA turnover and translation [11]. The motifs present in DExD/H box RNA helicases are responsible for binding of the RNA and ATP substrates and linking hydrolysis of the ATP to unwinding of the dsRNA.

Recently a new motif, the Q motif, has been identified in DExD/H box helicases and has been proposed to regulate ATP binding and hydrolysis [12]. This motif is composed of a cluster of nine amino acids (aa) about 21 aa upstream of the previously characterised motif I or Walker A motif, [13] and contains an invariant glutamine (Q). At a position approximately 17 aa upstream of the Q motif there is another residue ('+17'), usually F, W or Y in these RNA helicases, which is also thought to function in ATP binding and hydrolysis [12].

In this study we present the results obtained from analysis of the ATPase and helicase activities of full-length and truncated POWV NS3 expressed in *Escherichia coli*. The truncated NS3 proteins had progressively larger deletions to the protease domain and to residues in and around the Q motif (Fig. 1). The effects of these deletions on the ATPase activities of the proteins are detailed and a fuller analysis of the ATPase activities of the full-length and largest truncated NS3 proteins are described.

2. Materials and methods

2.1. Generation of POWV NS3 expression constructs

Viral RNA (vRNA) was extracted from 20% brain homogenate from a POWV-infected mouse following clarification of the serum by centrifugation for 5 min at 13 000×*g*. 100 μl of supernatant was mixed with 1 ml of Catrimox (Iowa Biotechnology) in an RNase-free Eppendorf tube, incubated at room temperature for 40 min and spun at 13 000×*g* for 10 min at room temperature. The supernatant was removed and the pellet was resuspended in 0.5 ml 2 M LiCl to dissolve DNA and dsRNA. The single-stranded (ss) RNA was then pelleted by centrifugation at 13 000×*g* for 5 min at room temperature, washed with 70% ethanol (ice-cold) and resuspended in 25 μl nuclease-free water.

Reverse transcription (RT) of vRNA was carried out using the RETROscript kit for RT-polymerase chain reaction (PCR) (Ambion) as described in the manual and the resulting cDNA was then used for PCR amplification of the NS3 gene.

Full-length POWV NS3 was amplified by PCR using the sense primer P3F (5′-GGTCGTCATATGACTGACCTGGTTTTTTCAGGG-3′) and the antisense primer P3R (5′-GCAGCCGGATCCTCACCTACGACCGGAAGCG-3′) which were designed to introduce a 5′ *Nde*I restriction site and a 3′ *Bam*HI restriction site (all primers were from Sigma-Genosys). PCR was carried out in a volume of 50 μl containing 10 units of Expand polymerase (Roche), 1×high fidelity buffer (supplied with the polymerase), 5 μl cDNA, 30 pmol each primer and 200 μM of each nucleotide (A, C, G and T) for 4 min

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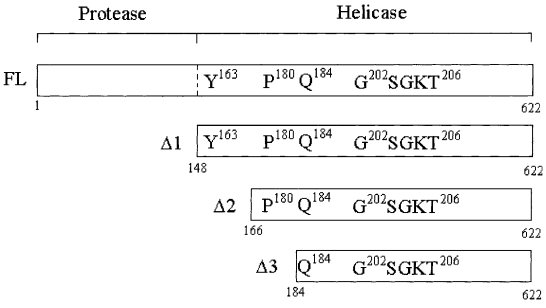

Fig. 1. Diagram of the full-length (FL) and truncated (Δ1, Δ2 and Δ3) POWV NS3 proteins used in this study. The positions of some of the important amino acids within the helicase domain are indicated.

at 94°C followed by 25 cycles of 1 min at 60°C, 2 min at 72°C and 1 min at 94°C. The 1.9 kb PCR product was then ligated into pGEM-T Easy vector using the enzyme provided with the vector (Promega) and the ligation was used to transform Top10 *E. coli* (Invitrogen). Insert-containing clones were selected by blue/white screening and the full-length POWV NS3-containing clones were confirmed by sequencing. A full-length clone of the correct sequence was then used for PCR amplification of truncated NS3 sequences (with deletions to the 5′ end of the NS3 coding sequence) using the same antisense primer as that used for amplification of the full-length sequence (P3R) and the following sense primers: P3TF1 (5′-GGTCGTCATATGGATGTG-CTGGGTCTGTAC-3′), P3TF2 (5′-GGTCGTCATATGGGTCGT-CATATGAGCATTGCTCAGGGCAAT-3′) and P3T3F (5′-GGT-CGTCATATGCAGGGTGGCAAATGGACA-3′). PCRs were carried out in exactly the same manner as the full-length amplification but 200 ng of template plasmid was used instead of 5 μl cDNA. PCR products were ligated into pGEM-T Easy, selected by blue/white screening and sequenced. The full-length and truncated NS3 sequences were excised from the vectors with *Nde*I and *Bam*HI and ligated into pET16b which had been similarly digested. To confirm that the correct inserts were present in the vector, sequence analysis of the insert/vector junction sequences was carried out.

2.2. Expression, isolation and refolding of recombinant proteins

Full-length and truncated POWV NS3 expression constructs and β-gal pET16b expression vector (Novagen) were used to transform JM109(DE3) *E. coli* (Promega). Induction of recombinant NS3 protein expression, inclusion body isolation, solubilisation and refolding were carried out as previously described [14].

2.3. ATPase, band shift and DNA helicase assays

Radioactive and malachite green ATPase assays were carried out as previously described [14,15]. Band shift assays were carried out using the helicase substrate and band shift conditions as previously described [14,15]. The double-stranded (ds) DNA unwinding assay was carried out using the substrates described in [16] under the conditions described in [17].

3. Results

3.1. Expression of full-length and truncated POWV NS3 in E. coli

Full-length and truncated NS3 proteins were expressed in *E. coli*, purified as inclusion bodies and solubilised. Expression of NS3 was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting (Fig. 2).

The observed size of the full-length NS3 protein was ~70 kDa and the truncated proteins (Δ1, Δ2 and Δ3) were 53, 51 and 49 kDa respectively which is consistent with the expected sizes. Smaller immunoreactive species were observed in each of the refolded NS3 samples. A similar pattern of degradation

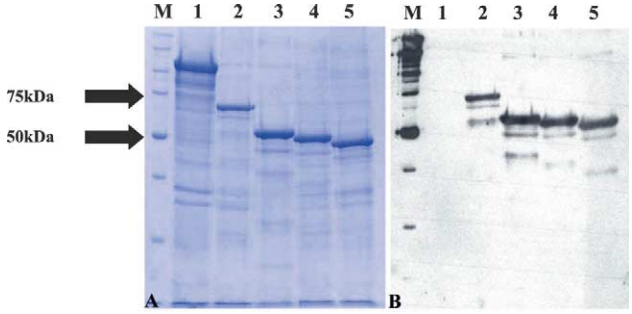

Fig. 2. Coomassie blue-stained 10% SDS–PAGE gel (A) and Western blot using Langat virus [1] specific serum (B) of different proteins expressed in JM109(DE3) and washed five times in phosphate-buffered saline. Lane M = molecular weight markers, 1 = β-galactosidase, 2 = full-length Powassan NS3, 3 = Δ1 Powassan NS3, 4 = Δ2 Powassan NS3, 5 = Δ3 Powassan NS3.

has also been observed following prokaryotic expression of NS3 proteins from other flaviviruses [3,4].

3.2. ATPase activity of full-length and truncated POWV NS3 proteins

NS3 proteins from flaviviruses have been shown to possess poly(U)-stimulated ATPase activity [3,4,10,18]. Full-length and truncated POWV NS3 proteins were analysed for ATPase activity in the absence and presence of poly(U) (Fig. 3). The full-length and Δ1 NS3 proteins demonstrated poly(U)-stimulated ATPase activity of similar magnitudes, the Δ2 NS3 protein had some poly(U)-stimulated ATPase activity and the Δ3 NS3 protein had no poly(U)-stimulated ATPase activity. In the absence of poly(U) the full-length NS3 showed some ATPase activity above that of the control but none of the truncated proteins showed ATPase activity above that of the control.

3.3. Nucleic acid binding and helicase activities of POWV NS3 proteins

The ability of the POWV NS3 proteins to bind to and unwind dsDNA substrates was investigated. All the POWV NS3 proteins were capable of binding to a partially dsDNA substrate (Fig. 4), however none of the proteins exhibited unwinding of the dsDNA substrate (results not shown). Hepatitis C virus (HCV) NS3, which had been expressed and refolded in the same manner, could unwind the substrate in the same assay (Fig. 4). No helicase activity was detected in

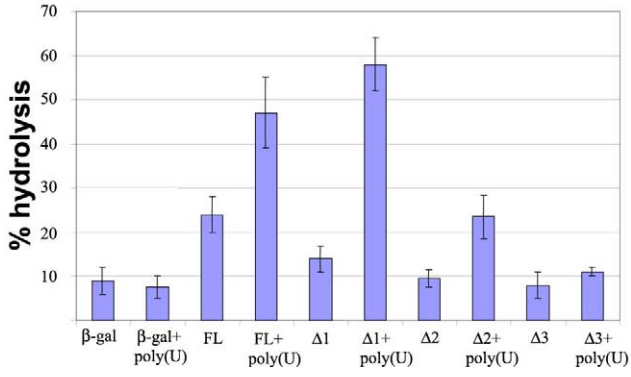

Fig. 3. ATP hydrolysis by recombinant proteins in the absence and presence of poly(U) as calculated from the radioactive ATPase assay.

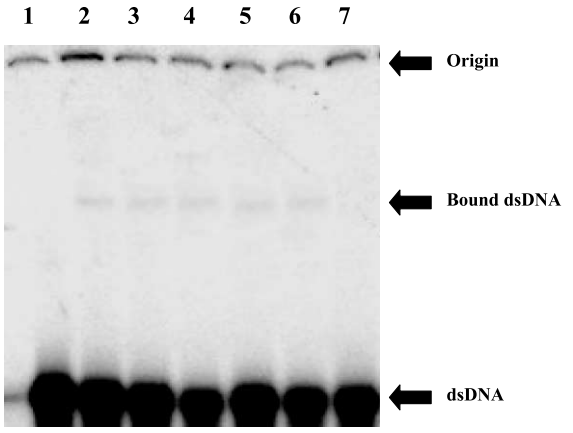

Fig. 4. Autoradiograph of a band shift assay (10% acrylamide-TBE gel) showing dsDNA (helicase substrate) binding by different proteins. Lane 1 = No protein, 2 = 15 pmol HCV NS3, 3 = 15 pmol full-length Powassan NS3, 4 = 15 pmol Δ1 Powassan NS3, 5 = 15 pmol Δ2 Powassan NS3, 6 = 15 pmol Δ3 Powassan NS3, 7 = 15 pmol β-galactosidase.

the POWV NS3 proteins over a range of pH conditions (pH 6.25–8.5) (results not shown).

4. Discussion

ATPase/helicase activities of NS3 proteins from a number of flaviviruses have been described [4–6,9,10]. The ATPase activities of all the NS3 proteins described were stimulated by the presence of poly(U). The ATPase activity of POWV NS3 was also stimulated by poly(U). In the absence of polynucleotide, full-length POWV NS3 had some residual ATPase activity whereas Δ1, Δ2 and Δ3 POWV NS3 had little or no ATPase activity above that of the control. These observations may reflect the fact that an interaction between the protease and helicase domains may alter the properties of the ATPase/helicase domain [3].

The ATPase activities of the full-length and Δ1 proteins in the presence of poly(U) were comparable which was an expected result considering the Δ1 NS3 protein is predicted to comprise the whole of the ATPase/helicase domain of POWV NS3. There were no significant differences in the observed biochemical characteristics of the two proteins apart from their sensitivities to poly(U) in the ATPase assays (results not shown). The full-length protein required a greater concentration of poly(U) to reach maximum ATPase activity, perhaps because the protease domain hinders access to the RNA binding sites within the full-length protein. Δ2 NS3 contained all of the Q motif but not the amino acid 17 aa upstream of it ('+17') which has a proposed role in the ATPase activity of DExD/H proteins [12]. The Δ2 NS3 protein showed ~15% hydrolysis of the ATP substrate above the level of the control in the presence of poly(U). Δ3 NS3 contained half of the Q motif but still retained the Q residue itself, however it showed no hydrolysis of the ATP substrate above that of the control even in the presence of poly(U). These observations indicate that the residues upstream of the Q motif, although important for ATPase activity, are not essential for ATPase activity but that the Q motif itself is essential for the ATPase activity of POWV NS3.

POWV NS3 was chosen to investigate the role of the Q motif in the ATPase activity of a viral enzyme since the Q residue in POWV NS3 is the same distance from the Walker A motif as it is in the majority of DExD/H box helicases [12]. Sequence analysis also indicates that POWV is more similar to other DExD/H box proteins in the region of the Q motif than NS3 proteins from Den-2 and HCV. In Den-2 NS3 the nearest Q residue is 29 aa upstream of the Walker A motif and in HCV NS3 there are two Q residues within a 10 aa portion upstream of the Walker A motif and none until about 100 aa upstream.

Site-directed mutagenesis of amino acids within the Q motif and of the '+17' residue in eIF4A indicated that the Q motif itself is more important to ATPase activity than the '+17' residue [12]. In addition to this, studies on truncated Den-2 NS3 proteins, which took place before the Q motif was described, indicate that the Q residue itself is more important than the upstream residues for enzyme activity. A truncated NS3 comprising the helicase domain including the Q motif and nine upstream amino acids possessed helicase activity whereas a similar protein, which was 20 aa shorter at the N-terminus, had much poorer ATPase activity [20].

The results presented here using POWV NS3 proteins are consistent with these observations. In the crystal structure of eIF4A, domain I (Walker A motif), the Q motif and the '+17' residue are in close proximity and form a pocket which can bind the phosphates of ATP [19]. If a similar structure is adopted by POWV NS3 then it is clear why the progressively truncated proteins display their respective activities: Δ1 possesses the whole pocket therefore binds and hydrolyses ATP like the full-length protein, Δ2 possesses part of the pocket and can bind and hydrolyse ATP but with less efficiency and Δ3 does not contain the pocket and cannot bind and hydrolyse ATP.

It is possible that these truncated proteins displayed different activities due to misfolding and/or deletion of other important unrecognised structures/motifs. Point mutations of various residues in the Q motif could also have been used to investigate the roles of different regions of the Q motif. However, induced point mutations can also have profound effects on the folding of proteins and can lead to ambiguous results in in vitro studies. For example mutation of certain residues in Den-2 NS3, which were expected to reduce enzyme activity, actually increased activity but abolished infectivity when introduced into an infectious clone [3].

None of the POWV NS3 proteins possessed helicase activity in dsDNA unwinding assays although HCV NS3 produced in the same way did. The reasons POWV NS3 failed to exhibit helicase activity when HCV NS3 did may be due to the fact that NS3 proteins from flaviviruses have lower helicase activity in vitro [4,20] than NS3 from HCV [4,20–22] and that the assay was not sensitive enough or that the POWV NS3 is more selective in the substrates that it unwinds.

In conclusion, we have cloned and expressed full-length and truncated POWV NS3 proteins and analysed their poly(U)-stimulated ATPase activities. Although the full-length, Δ1 and Δ2 proteins demonstrated poly(U)-stimulated ATPase and dsDNA binding activities, none of them exhibited helicase activity in dsDNA unwinding assays. Analysis of the ATPase activities of the truncated proteins indicates the importance of a recently described motif, the Q motif, in the ATPase activity of POWV NS3. To our knowledge, this is the first time the Q motif has been shown to be of importance for the ATPase activity of a viral helicase.

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References

[1] Gritsun, T.S., Lashkevich, V.A. and Gould, E.A. (2003) Antiviral Res. 57, 129–146.
[2] Mandl, C.W., Holzmann, H., Kunz, C. and Heinz, F.X. (1993) Virology 194, 173–184.
[3] Matusan, A.E., Pryor, M.J., Davidson, A.D. and Wright, P.J. (2001) J. Virol. 75, 9633–9643.
[4] Borowski, P., Niebuhr, A., Mueller, O., Bretner, M., Felczak, K., Kulikowski, T. and Schmitz, H. (2001) J. Virol. 75, 3220–3229.
[5] Cui, T., Sugrue, R.J., Xu, Q., Lee, A.K., Chan, Y.C. and Fu, J. (1998) Virology 246, 409–417.
[6] Kuo, M.D., Chin, C., Hsu, S.L., Shiao, J.Y., Wang, T.M. and Lin, J.H. (1996) J. Gen. Virol. 77, 2077–2084.
[7] Leung, D., Schroder, K., White, H., Fang, N.X., Stoermer, M.J., Abbenante, G., Martin, J.L., Young, P.R. and Fairlie, D.P. (2001) J. Biol. Chem. 276, 45762–45771.
[8] Scaramozzino, N., Crance, J.M., Drouet, C., Roebuck, J.P., Drouet, E., Jouan, A. and Garin, D. (2002) Biochem. Biophys. Res. Commun. 294, 16–22.
[9] Utama, A., Shimizu, H., Morikawa, S., Hasebe, F., Morita, K., Igarashi, A., Hatsu, M., Takamizawa, K. and Miyamura, T. (2000) FEBS Lett. 465, 74–78.
[10] Warrener, P., Tamura, J.K. and Collett, M.S. (1993) J. Virol. 67, 989–996.
[11] Tanner, N.K. and Linder, P. (2001) Mol. Cell 8, 251–262.
[12] Tanner, N.K., Cordin, O., Banroques, J., Doere, M. and Linder, P. (2003) Mol. Cell 11, 127–138.
[13] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.
[14] Wardell, A.D., Errington, W., Ciaramella, G., Merson, J. and McGarvey, M.J. (1999) J. Gen. Virol. 80, 701–709.
[15] Hess, H.H. and Derr, J.E. (1975) Anal. Biochem. 63, 607–613.
[16] Gwack, Y., Kim, D.W., Han, J.H. and Choe, J. (1997) Eur. J. Biochem. 250, 47–54.
[17] Henn, A., Medalia, O., Shi, S.P., Steinberg, M., Franceschi, F. and Sagi, I. (2001) Proc. Natl. Acad. Sci. USA 98, 5007–5012.
[18] Utama, A., Shimizu, H., Hasebe, F., Morita, K., Igarashi, A., Shoji, I., Matsuura, Y., Hatsu, M., Takamizawa, K. and Hagiwara, A. et al. (2000) Virology 273, 316–324.
[19] Benz, J., Trachsel, H. and Baumann, U. (1999) Struct. Fold. Des. 7, 671–679.
[20] Li, H., Clum, S., You, S., Ebner, K.E. and Padmanabhan, R. (1999) J. Virol. 73, 3108–3116.
[21] Preugschat, F., Averett, D.R., Clarke, B.E. and Porter, D.J. (1996) J. Biol. Chem. 271, 24449–24457.
[22] Tai, C.L., Chi, W.K., Chen, D.S. and Hwang, L.H. (1996) J. Virol. 70, 8477–8484.