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Echovirus 9 strain Barty non-structural protein 2C has NTPase activity

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Abstract

Non-structural protein 2C is known to play a fundamental role in the replication of picornaviruses. Sequence analyses revealed that 2C belongs to a rapidly expanding group of proteins containing a consensus sequence for nucleotide binding (NTB). We report that echovirus 9 polypeptide 2C displays NTPase activity in vitro. In our experiments, several P2 genes were expressed in *Escherichia coli* as fusion proteins linked to glutathione S-transferase (GST) prior to purification close to homogeneity. In contrast to GST-2B, both GST-2C and GST-2BC showed ATPase as well as GTPase activity indicating that the site for NTB binding and splitting is located in 2C. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Pircornaviridae; Enterovirus; Echovirus 9; Protein 2C; ATPase; GTPase; Nucleotide binding (NTB) motif

1. Introduction

Like polio- and coxsackieviruses, echovirus 9 strain Barty is a human pathogenic enterovirus within the large family *Picornaviridae* (Sabin et al., 1958; Eggers and Sabin, 1959). The replication cycle of picornaviruses has been extensively investigated (reviewed by Rueckert, 1996). After attachment, penetration, and uncoating of the virion, the positive-stranded RNA-genome is translated cap-independently into a single large polyprotein which is subsequently cleaved into four structural and seven non-structural mature proteins. A precise function could only be assigned to some members of the non-structural polypeptides (Wimmer et al., 1993).

Enterovirus non-structural protein 2C is a 329-amino acid polypeptide. Comparative sequence analysis indicates that it is related to a group of proteins containing a consensus sequence for nucleotide binding (NTB). The typical NTB-domain consists of at least two elements (Gorbalenya et al., 1990; Mirzayan and Wimmer, 1992). The 'A' element — directly involved in binding of the β- and γ-phosphates of NTP — is built up by a stretch of hydrophobic residues followed by the conserved sequence GxxxxGKS/T (amino acids 129–136). The 'B' element comprises a DD motif

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at amino acids 176–177 and is thought to be involved in the NTPase activity itself by chelating the magnesium of the Mg-NTP complex. An additional motif (element 'C') is an invariant asparagine at position 223 preceded by several hydrophobic residues. In combination with elements 'A' and 'B', there are similarities to virus-encoded RNA-helicases classified into superfamily 3 (SF3) (Gorbalenya et al., 1990; Kadaré and Haenni, 1997). An exact function of element 'C' is not yet known, but recently it has been shown that the mutation of the conserved asparagine of poliovirus 2C abolished ATPase activity (Pfister and Wimmer, 1999).

The main object of this study was to detect and to characterize a putative NTPase function of echovirus 9 polypeptide 2C in order to get more insight into the life cycle of echoviruses.

2. Materials and methods

2.1. Construction of expression plasmids

Construction of the fusion proteins 2B, 2C, and 2BC was carried out by standard molecular cloning techniques (Ausubel et al., 1994). In brief, each gene was amplified by polymerase chain reaction (PCR) using an infectious echovirus 9 strain Barty cDNA clone (Zimmermann et al., 1996) as template (Fig. 1). Within the PCR oligonucleotides, restriction sites were introduced immediately adjacent to the beginning or end, respectively, of the gene of interest (Fig. 1). After digestion with *Bgl*II and *Eco*RV, each PCR product was cloned into the procaryotic expression plasmid pGEX2T (Pharmacia) containing the coding region of glutathione S-transferase (GST) in front of a multiple cloning site. Prior to cloning, pGEX2T was digested with *Bam*HI and *Sma*I. The correctness of each construct (pGEX2T-2B, -2C and -2BC) was checked by sequence analysis using 3′- and 5′-pGEX sequencing primers (Pharmacia).

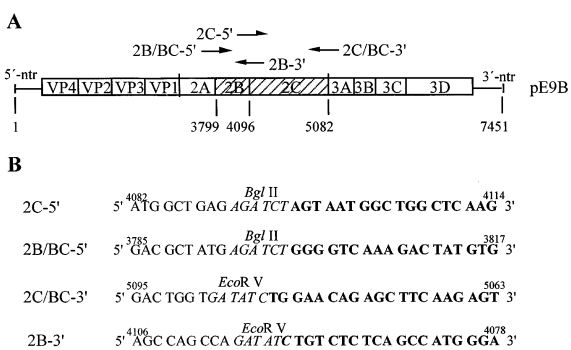

Fig. 1. Cloning of the expression plasmids pGEX-2B, pGEX-2C, and pGEX-2BC. (A) Schematic diagram of the echovirus genome. The coding box is represented by an open box, the four structural genes (VP1–VP4) as well as the seven non-structural genes (2A–3D) are indicated. Genes 2B, 2C and 2BC (hatched) were amplified by PCR using an echovirus 9 Barty full-length cDNA clone (pE9B) as template. Orientation and position of the four primers used for PCR are given by arrows above the diagram. (B) Listing of the oligonucleotides which serve as primers for PCR. The restriction sites (italics) are introduced by the primers to render the cloning procedure possible. They do not change the amino acid sequence of the investigated polyproteins and flank the start and end point, respectively, of the gene of interest (bold).

2.2. Expression and purification of fusion proteins

pGEX2T-2B, -2C and -2BC were transformed into an *Escherichia coli* BL21 (DE3) overexpression strain according to published protocols (Studier and Moffatt, 1986). As control, an empty pGEX2T vector was also transformed. Following incubation at 37°C in 2xYT-medium (Difco) under shaking at 250 rpm, the cells were induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). After having reached an optical density at 600 nm of ~ 0.8, the temperature was lowered to 30°C. Then 3 h later, cells were harvested by centrifugation and each pellet was resuspended in lysis buffer (20 mM HEPES/KOH pH 7.5, 0.14 M NaCl, 5 mM 2-mercaptoethanol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml pepstatin A and 1 μg/ml leupeptin). The following purification steps were carried out at 0–4°C. After three freeze-thaw cycles, the cells were lysed by mild sonication and centrifuged ($20\,000 \times g$, 1 h). Glutathione sepharose 4B beads (Pharmacia) were equilibrated three times with washing buffer (20 mM HEPES/KOH pH 7.5, 0.14 M NaCl, 5 mM dithiothreitol (DTT), 0.1% Triton X-100) according to manufacturer's instructions, and subsequently incubated for 30 min with supernatant. The fusion proteins were then eluted three times with 20 mM Tris/HCl pH 8.0 containing 10 mM reduced glutathione and 25% glycerol. Finally, all protein

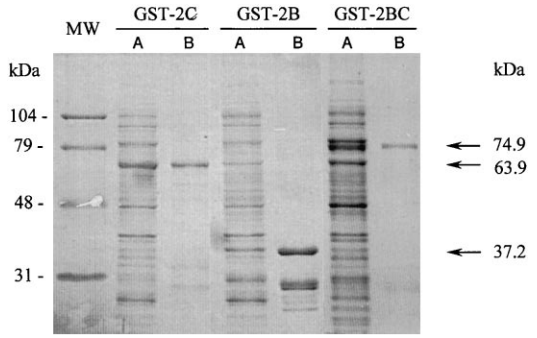

Fig. 2. Expression and purification of the fusion proteins GST-2C, -2B, and -2BC. *E. coli* BL21 cells transformed with the respective plasmid were grown in 2 × YT medium and induced with IPTG. The fusion proteins (arrows) were purified by affinity chromatography using glutathione sepharose 4B. For each reaction crude extract (lanes A) and purified fusion protein (lanes B) were separated on a 10%-SDS–PAGE. The expected protein sizes were calculated by computer. The purified GST protein, too, had the expected size of ~27 kDa (result not shown).

fractions were analyzed by SDS–PAGE followed by Coomassie blue staining.

2.3. NTPase activity assay

The ATPase reactions were performed according to Mirzayan and Wimmer (1994) with slight modifications: the buffer contained 20 mM HEPES/KOH pH 7.5, 5 mM $MgCl_2$, 2.5 mM DTT, 0.1% BSA, 0.05% Triton X-100, 50 μM ATP and 10 μCi/ml (γ-^{32}P)ATP (3.000 Ci/mmol, Amersham) with the indicated amount of purified fusion protein in a total volume of 20 μl. For GTPase assays, GTP was used instead of ATP. The proteins were preincubated in reaction buffer lacking NTP at 37°C for 10 min. Reactions were carried out at 37°C until they were stopped on ice by adding EDTA to a concentration of 50 mM. Then 2-μl aliquots were analyzed by polyethyleneimine-cellulose (Machery & Nagel) thin-layer chromatography (TLC) with 0.15 M LiCl, 0.15 M formic acid pH 3.0 as the liquid phase. In order to detect the cleavage of NTP, the dried TLC plates were analyzed by autoradiography or phosphorimager.

3. Results

The genome regions of the viral proteins 2B, 2C and 2BC were amplified by PCR using the full length echovirus 9 clone pE9-B (Zimmermann et al., 1996) as template. The PCR products were subsequently cloned into the expression vector pGEX2T (Fig. 1). Accuracy of the protein reading frame and the presence of an ATG codon were checked by sequencing, and the plasmids were transformed into competent *E. coli* BL21 cells. After overexpression in *E. coli* BL21 cells, purification of fusion proteins GST-2B, GST-2C, and GST-2BC was carried out by affinity chromatography. Size, yield, and purity of the obtained GST fusion proteins were checked by SDS–PAGE (Fig. 2). One discrete protein band indicates near homogeneity and correct size of each product. Proper induction and solubility of the respective protein was confirmed by co-electrophoresis of the corresponding cell extract supernatants. Only in the lane of GST-2B, an additional band of ~28 kDa was detectable, probably created by proteinase cleavage of the fusion protein.

The reaction conditions for the ATPase assay have been optimized with regard to reaction time and temperature. Both GST-2C and GST-2BC were found to cleave the γ-phosphate bond of ATP (Fig. 3A). In contrast, GST-2B and GST alone possessed no significant activity indicating a nucleotide splitting domain only in polypeptide 2C (Fig. 3A). The fact that GST-2C showed increasing ATPase activity for at least 45 min indicated well-defined in vitro reaction conditions and a proper folding of the fusion protein (Fig. 3B).

Besides ATP, the 2C fusion protein was also able to utilize GTP as substrate. Comparative measurements using a phosphorimager proved an approximately three times lower GTPase activity as compared to the ATPase activity (Fig. 4A). GST-2BC showed an identical behaviour (data not shown).

Competition experiments with high concentrations of non-radioactive ribonucleotides reveal that ATP possesses the highest affinity for binding, followed by GTP and CTP (Fig. 4B). On the

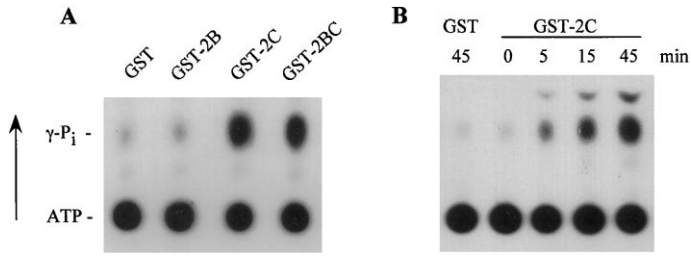

Fig. 3. ATPase assay of the GST fusion proteins by thin-layer chromatography. All reactions were carried out at 37°C in the presence of 1 μg of each purified protein. The direction of the migration of the split phosphates is given by an arrow. GST alone is used as a negative control. (A) ATPase activity of GST-2B, -2C, and -2BC, respectively, after incubation for 60 min. (B) Kinetics of ATPase activity of GST-2C. Reactions were stopped at indicated time points.

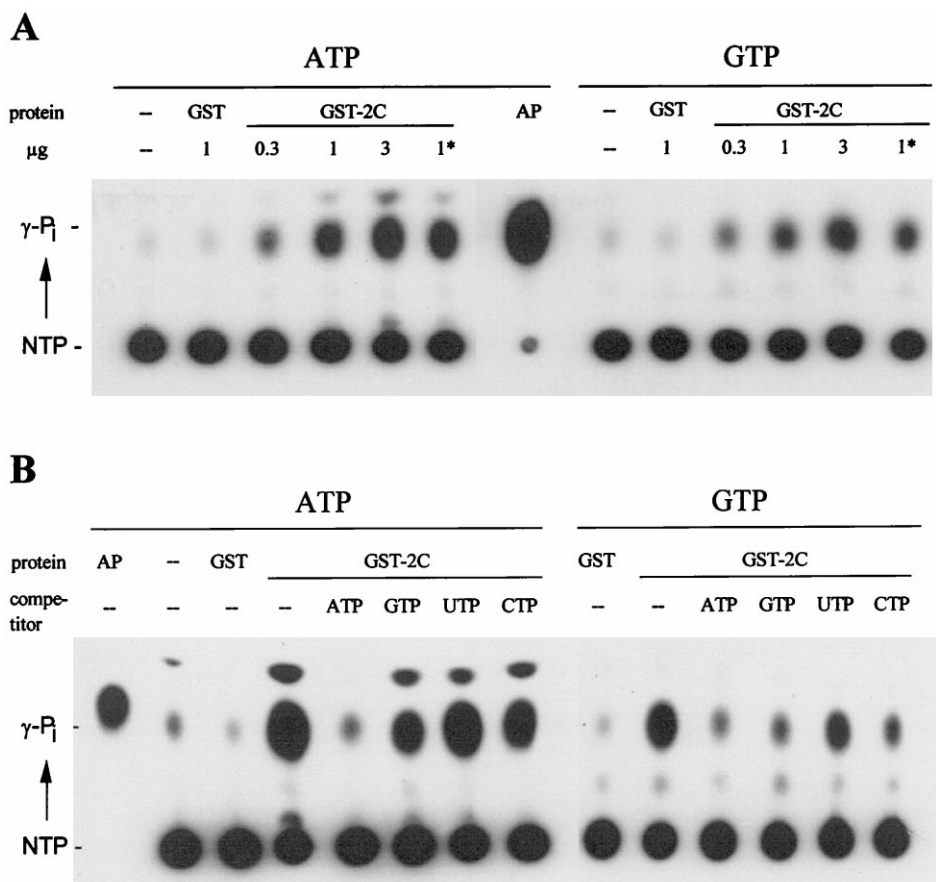

Fig. 4. ATPase and GTPase activity of GST-2C. Each reaction was carried out for 60 min. Alcaline phosphatase was used as positive control (lanes AP). The direction of migration of the split phosphates is given by an arrow. (A) Comparison of ATPase and GTPase activity with increasing amount of fusion protein GST-2C. 1*, 10 ng of poly(A)-RNA were added. (B) Competition experiments with unlabeled ribonucleotides (1 mM each). Labeled and unlabeled nucleotides were added simultaneously.

Table 1
Alignment of the NTB motifs of echovirus 9 and poliovirus

	Motif A aa 129–136	Motif B aa 172–177	Motif C aa 218–223
Echovirus 9	G S P G **A** G K S[a]	V V I M D D	V L A S T N
Poliovirus	G S P G **T** G K S[a]	V V I M D D	V L A S T N

[a] Divergent amino acids (aa) are indicated in boldface

other hand, UTP did not compete for the binding site indicating that UTP is no potential substrate.

In contrast to several other viral NTB-motif containing enzymes (Kadaré and Haenni, 1997), there was no stimulation by polyribonucleotides. After addition of unspecific poly(A)-RNA (Boehringer) to the reaction mixture, neither ATPase nor GTPase activity of GST-2C was increased (Fig. 4A). Even concentrations of 0.1 μg poly(A)-RNA/μl did not alter the amount of phosphate released (data not shown).

4. Discussion

Although the poliovirus 2C protein has been studied thoroughly there are only few data on 2C of other enteroviruses. However, 2C of picornaviruses is a highly conserved protein (Mirzayan and Wimmer, 1992), and sequence comparisons with other proteins showed similarities to the so-called NTB protein family (Walker et al., 1982; Dever et al., 1987; Gorbalenya et al., 1990). The amino acid alignment of polypeptide 2C of echovirus 9 and poliovirus reveals nearly identical NTB-motif sequences, suggesting NTPase activity for both poliovirus and echovirus 2C (Table 1). For poliovirus 2C, ATP/GTP binding and splitting activities have been demonstrated (Rodriguez and Carrasco, 1993; Mirzayan and Wimmer, 1994). In this communication, the ability of echovirus 2C to dephosphorylate ribonucleotides in vitro is demonstrated for the first time. Our data reveal that echovirus 2C and 2BC are able to recognize ATP as well as GTP as substrate, whereby the affinity to ATP is approximately three times higher than that to GTP.

The biological functions of NTPases are widely spread and include, for example, signal transduction, membrane trafficking, synthesis of macromolecules, and unwinding of double stranded RNA or DNA (Walker et al., 1982; Kadaré and Haenni, 1997). All of these activities have one common feature: they are strictly energy consuming.

Several lines of evidence suggest that 2C is involved in viral RNA replication and/or RNA packaging (Li and Baltimore, 1990; Wimmer et al., 1993). In the context of viral RNA replication, the exact function of 2C is still unclear. However, 2C appears to be a multifunctional protein involved — among others — in binding of membranes (Bienz et al., 1990; Cho et al., 1994; Echeverri and Dasgupta, 1995; Schlegel et al., 1996) as well as RNA (Rodriguez and Carrasco, 1993; Rodriguez and Carrasco, 1995; Banerjee et al., 1997). It is noteworthy that both functions are supposed to be independent of the NTPase activity of 2C, since deletion mutants without motifs A and B are still able to bind membranes as well as RNA (Rodriguez and Carrasco, 1993; Teterina et al., 1997). Hence, there must be at least one more, energy-dependent function of 2C. On the basis of sequence comparisons it is proposed that picornavirus 2C belongs to the helicase family (Gorbalenya et al., 1988; Gorbalenya et al., 1990), however, experimental verification was not yet possible. One feature of helicases with established activity is the stimulated NTPase activity in the presence of polyribonucleotides (Kadaré and Haenni, 1997). Our finding that echovirus 2C is, if at all, only weakly stimulated, is in accordance with the described, at most twofold stimulation of postulated helicases without assured activity, e.g. poliovirus 2C (Kadaré and Haenni, 1997). If the stimulatory effect of single-stranded nucleic acids on NTPase activity is a typical character of helicases, polio- as well as echovirus 2C would not belong to that enzyme family.

In this case, it could be hypothesized that NTPase 2C supplies energy for another step of the viral life cycle. Involvement of 2C in uncoating and/or RNA packaging was already proposed. Li and Baltimore (1990) created a poliovirus 2C mutant with an uncoating defect, and Vance et al. (1997) reported that molecular determinants of resistance to the encapsidation inhibitor 5-(3,4-dichlorophenyl) methylhydantoin map to poliovirus 2C. In this context, too, an energy-consuming role of 2C remains to be detected.

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