Echovirus-9 protein 2C binds single-stranded RNA unspecifically

Marcus Klein, Hans J. Eggers and Birgit Nelsen-Salz

Institut für Virologie der Universität zu Köln, Fürst-Pückler-Str. 56, 50935 Köln, Germany

Polypeptide 2C is essential for picornavirus replication. Although many data on multiple functions of this highly conserved protein are available, the mechanism of RNA binding is still obscure. In this work, protein 2C of echovirus-9 strain Barty was expressed as a histidine-tagged protein in E. coli followed by nondenaturing purification to homogeneity. After incubation of 2C protein with different kinds of RNA fragments, binding was shown in gel retardation assays. Competition experiments revealed that 2C targets linear RNA unspecifically; however, single-stranded linear DNA does not react with this protein. In contrast to poliovirus, protein 2C of echovirus-9 only recognizes RNA with a low content of secondary structures. This may be a first hint of a different binding specificity of 2C in echo- and polioviruses.

Echovirus-9 is a member of the picornavirus family. In contrast to the related polioviruses, only limited information is available on the function(s) of its nonstructural proteins. In particular, the role of 2C, one of the most conserved picornavirus proteins (Argos et al., 1984), is incompletely understood.

As part of the replication complex (Bienz et al., 1992), the 329-amino-acid protein 2C is involved in viral RNA replication. This was proven by the finding, among others, that relevant mutations for both resistance to or dependence on the 2C helicase (Gorbalenya et al., 1988; Kadáré & Haenni, 1997), but the experimental evidence for this activity is still missing.

One further function of 2C has been reported for poliovirus: the ability to bind RNA, first demonstrated by Rodriguez & Carrasco (1993). Experiments with truncated 2C revealed that two regions (aa 21–45 and 312–319) are involved in RNA binding (Rodriguez & Carrasco, 1995). Recently, specific binding of poliovirus 2C to a distinct part of the negative-stranded 5′-nontranslated region (ntr) was reported (Banerjee et al., 1997).

The aim of this study was to identify and characterize a putative RNA-binding function of 2C protein of echovirus-9 strain Barty. The protein was expressed in E. coli and purified as a histidine-tagged (His)-protein, according to earlier work (Klein et al., 2000). In order to control for induction of the protein, an SDS–10% polyacrylamide gel was run with an equal amount of induced and uninduced bacteria (Fig. 1a). Inclusive of the 6His-tag and a short linker sequence of the expression vector, a total molecular mass of 40.9 kDa was calculated for His-2C. One clear and sharp protein band of the expected size was visible, confirming proper expression of full-length His-2C (Fig. 1a, lane 2). Since we preferred to work with native and undenatured protein, His-2C was purified from the supernatant fraction of the lysed bacteria by Ni²⁺ affinity chromatography according to the manufacturer’s protocol (Qiagen). After step-wise washing and elution, His-2C-containing fractions were analysed by SDS–12% PAGE. Most of the His-protein was eluted at 60 mM imidazole, possibly due to the His-tag not being completely exposed at the N terminus of 2C (Fig. 1b, lane 3). To ensure the use of highly purified His-2C protein, only fractions eluted with 100, 150 and 200 mM imidazole were pooled (Fig. 1b, lanes 5–7), concentrated with spin columns (Amicon), and dialysed against 20 mM HEPES–KOH (pH 7.5), 100 mM KCl, 5 mM DTT and 25% glycerol.

For the RNA-binding assay, radioactively labelled RNA was synthesized as follows. After KpnI digestion of echovirus-9 strain Barty, full-length clone E9B-fl.20 (Zimmermann et al., 1996), the 353 bp fragment comprising nucleotides 2844–3196, was cloned into a KpnI-linearized pT7/T3-z-18-vector (Gibco-BRL). In order to obtain viral RNA fragments of positive or negative polarity, plasmids containing the insert in each orientation were SmaI-linearized and transcribed in vitro.

**Author for correspondence**: Marcus Klein.
Fax +49 221 4783902. e-mail marcus.klein@medizin.uni-koeln.de
Fig. 1. Nondenaturing purification of echovirus-9 protein 2C. (a) Expression of His-2C in E. coli BL21. Whole cells of uninduced (lane 1) and IPTG-induced (lane 2) bacteria were harvested and subjected to SDS–10% PAGE. (b) Step-wise elution of His-2C after binding on Ni$^{2+}$–agarose. Samples (2 µl) of each fraction were analysed by SDS–12% PAGE. The proteins were eluted with 20, 40, 60, 80, 100, 150 and 200 mM imidazole, respectively (lanes 1–7). M, protein marker (kDa). The position of His-2C is marked by arrowheads.

Fig. 2. Binding of echovirus-9 His-2C to viral RNA. Radiolabelled transcripts comprising the genome of echovirus-9 from position 2844 to 3196 were incubated with the indicated amounts of His-2C to form RNA–2C complexes. Thereafter, a 3–5% EMSA was run and the dried gels were subjected to autoradiography. (a) Binding of various amounts of His-2C to viral RNA transcripts of the indicated sense. (b) Competition of RNA binding of 100 ng His-2C by different types of unlabelled nucleic acids. Lane 1, no protein. Lane 2, His-2C without competitor. Lane 3, 100 ng of echovirus-9 full-length in vitro transcript of negative sense. Lane 4, 100 ng of echovirus-9 full-length in vitro transcript of positive sense. Lane 5, 10 µg of yeast tRNA. Lane 6, 1 µg of synthetic poly(A) RNA. Lane 7, 1 µg of denatured single-stranded salmon sperm DNA.

It was shown for the first time that echovirus-9 protein 2C binds to RNA (Fig. 2): 20 ng of 2C protein led to noticeable complex formation and 100 ng (approx. 200 nM) completely retained the inserted labelled RNA (Fig. 2a). As a negative control, the same amounts of another viral His-tagged protein (human papillomavirus type 8 E2) were used, and no binding was observed (data not shown). However, the RNA–His-2C complex did not appear as an individual band with reduced mobility; instead, the emerging complexes failed to enter the gel, suggesting a very high molar ratio of protein to RNA (Fig. 2). It has been reported that picornavirus 2C tends to oligo- or multimerize (Mirzayan & Wimmer, 1994; Tolskaya et al., 1994). We suggest that this effect – probably strongly pertaining to echovirus-9 – may be the reason for the abolished mobility of the RNA–2C complexes. Similar findings have been reported for the P30 protein of tobacco mosaic virus,

using $\alpha^32$PCTP according to standard protocols (Ausubel et al., 1998). The resulting RNAs were diluted in 20 mM HEPES–KOH (pH 7.5), 100 mM KCl and 3 mM MgCl$_2$.

The binding reaction was performed with 50000 c.p.m. labelled RNA and His-2C of high purity in 10 mM HEPES–KOH (pH 7.5), 50 mM potassium acetate, 3 mM magnesium acetate, 10 mM DTT, 2.5 mM ATP, 0.02% BSA, 0.5% Tween 20 and 5% glycerol. After incubation for 15 min at 37 °C, the reactions were subjected to a 3.5% electrophoretic mobility shift assay (EMSA) in 0.25 M Tris–borate buffer at 4 °C. The dried gels were autoradiographed or analysed by phosphorimager.

It was shown for the first time that echovirus-9 protein 2C binds to RNA (Fig. 2): 20 ng of 2C protein led to noticeable complex formation and 100 ng (approx. 200 nM) completely retained the inserted labelled RNA (Fig. 2a). As a negative control, the same amounts of another viral His-tagged protein (human papillomavirus type 8 E2) were used, and no binding was observed (data not shown). However, the RNA–His-2C complex did not appear as an individual band with reduced mobility; instead, the emerging complexes failed to enter the gel, suggesting a very high molar ratio of protein to RNA (Fig. 2). It has been reported that picornavirus 2C tends to oligo- or multimerize (Mirzayan & Wimmer, 1994; Tolskaya et al., 1994). We suggest that this effect – probably strongly pertaining to echovirus-9 – may be the reason for the abolished mobility of the RNA–2C complexes. Similar findings have been reported for the P30 protein of tobacco mosaic virus,
Unspecific RNA binding by echovirus 2C

which binds to linear nucleic acids only if a minimum number of nucleotides is exceeded (Citovsky et al., 1992).

Translations of either polarity were bound to an equal extent, suggesting an unspecific binding mechanism independent of sequence (Fig. 2a). This hypothesis is supported by competition/binding studies, which revealed that interaction of 2C and positive-stranded RNA is abolished in the presence of both positive- and negative-stranded viral full-length RNA as well as synthetic poly(A) RNA (Fig. 2b). However, 2C/RNA binding was not impeded by single-stranded DNA, even when used at 1000-fold excess (Fig. 2b), indicating at least the necessity of RNA for successful interaction.

Although the influence of RNA length was not examined explicitly, a substantial criterion for binding by 2C seems to be the secondary structure of the target RNA. Obviously, the echovirus 2C protein ignores strongly folded RNA. Using up to 1 µg of tRNA, successful competition of the 2C/linear RNA interaction was not detectable (Fig. 2b). This is in accordance with further binding experiments performed using as RNA target the first 119 nucleotides of the 5′-ntr of echovirus-9 in both positive- and negative-stranded orientation, which are supposed to build up complex secondary structures. In these experiments, almost no binding of His-2C to this kind of RNA was detectable (Fig. 3).

For poliovirus, a highly specific interaction of de/renatured His-2C with a specific double-stranded RNA structure within the negative-stranded 5′-ntr region has been described (Banerjee et al., 1997). In our hands, this binding specificity could not be observed in the case of natively purified echovirus-9 His-2C under various reaction conditions. However, analogous to echovirus, Rodriguez & Carrasco (1995) demonstrated, using northwestern techniques, interaction between unadenatured poliovirus 2C and the internal viral sequence (nt 2099–4600). They located a potential RNA-binding site—an arginine-rich region—between amino acids 312 and 319 of 2C (Rodriguez & Carrasco, 1995). These regions are often found in RNA-binding proteins supposed to be involved in sequence-independent interaction, and, in addition, even nonspecific nucleic acid binders, e.g. histones, exhibit similarities to Arg-rich regions (Lazinski et al., 1989).

For the first time, the interaction with RNA of the highly conserved echovirus 2C protein has been characterized at the molecular level. The finding that this obviously multifunctional protein binds preferably to single-stranded RNA fits well with current opinion concerning participation of 2C in RNA replication and/or RNA encapsidation.

We thank Andreas Voosen for excellent technical assistance. This project has been supported by the Deutsche Forschungsgemeinschaft (NE 586/2-2). B.N.-S. was maintained by a grant from the Lise-Meitner-Stiftung.

References


Received 28 April 2000; Accepted 21 June 2000