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Characterization of enterovirus 71 capsids using subunit protein-specific polyclonal antibodies

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Enterovirus 71 (EV71), a member of the *Enterovirus* genus of the *Picornaviridae* family, is one of the major causative agents of hand-foot-and-mouth disease (HFMD), which is prevalent in the Asia-Pacific region. In this article, a set of capsid subunit protein-specific antibodies was used to characterize the EV71 structural protein processing and to determine the composition and assembly of EV71 capsids. SDS-PAGE and Western blot analyses showed that the capsids of a purified EV71 preparation, which lacked viral infectivity, were composed of processed VP0, VP1 and VP3, all of which co-assembled into particles. Analyses of infectious EV71-containing cell lysate revealed the presence of VP2, in addition to VP0, VP1 and VP3, suggesting that the cleavage of VP0 into VP2 and VP4 is important for infectivity. Immunofluorescent staining with the three specific antibodies demonstrated that the capsid subunit proteins co-localized in the cytoplasm of cells infected with EV71. The results add new information on the processing, assembly and localization of EV71 capsid proteins, and demonstrate the usefulness of the capsid protein-specific antibodies for virological investigation and for development of vaccines and diagnostic reagents.

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1. Introduction

Enterovirus 71 (EV71) is the major causative agent of hand-foot-and-mouth disease (HFMD) (Lee et al., 2009; Wong et al., 2010), which is prevalent currently in the Asia-Pacific region (Wong et al., 2010; Xu et al., 2010), China in particular (Xu et al., 2012; Yang et al., 2011; Zhang et al., 2009, 2010). EV71 infection occurs mainly in children under the age of five. A portion of patients infected with EV71 develop severe neurological and cardiopulmonary complications, such as polio-like paralysis, brain stem encephalitis, meningitis, and pulmonary edema, which may ultimately lead to death (reviewed in Lee et al., 2009; McMinn, 2002; Wong et al., 2010).

EV71 is a member of the *Enterovirus* genus of the *Picornaviridae* family. Its genome is a single-stranded, positive sense RNA molecule of ∼7410 bases, and encodes a single large polyprotein which can be divided into structural P1 and nonstructural P2 and P3 regions (reviewed in McMinn, 2002). In many enteroviruses, such as poliovirus and foot-and-mouth disease virus, the structural P1 region is processed by viral proteinase to yield the subunit proteins VP0, VP1 and VP3, all of which co-assemble to form empty capsids (Fry et al., 2005; Hellen and Wimmer, 1992); further cleavage of VP0 into VP2 and VP4 by an autocatalytic mechanism involving the encapsidating RNA is required for generation of infectious mature virions (Basavappa et al., 1994; Curry et al., 1997; Hellen and Wimmer, 1992; Hindiyeh et al., 1999). However, whether this is the case for EV71 has not been investigated sufficiently, due partly to the lack of suitable immunological reagents to identify subunit protein species.

In this study, a set of capsid subunit protein-specific antibodies was used to characterize the EV71 structural protein processing and to determine the composition and assembly of EV71 capsids. In addition, the localization of capsid proteins within infected cells was investigated by immunofluorescent staining.

2. Materials and methods

2.1. Cells and viruses

RD and Vero cells were grown in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 100 unit/ml penicillin and 100 μg/ml streptomycin) at 37 °C with 5% CO_2. EV71 strain G081 (genotype C4) was propagated in RD cells. Virus titers was determined by the microtitration method using RD cells and were

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expressed as the 50% Tissue Culture Infectious Dose (TCID50) according to the Reed–Muench method (Reed, 1938). Purified EV71 was obtained from Hualan Inc. (Henan, China).

2.2. Capsid subunit protein-specific polyclonal antibodies

The anti-VP0 guinea pig polyclonal antibody was described previously (Feng et al., 2011). The anti-VP3 guinea pig polyclonal antibody was generated by immunization of guinea pigs with the recombinant VP3 protein of Coxsackievirus A16 (CVA16) (Liu et al., 2011b), this antibody was found to cross-react strongly with EV71 (data not shown) and hence was used in this study. The anti-VP1 polyclonal antibody was generated by immunization of rabbit with recombinant EV71 VP1 protein produced from *E. coli*. To construct the EV71 VP1 prokaryotic expressed vector, RNA was extracted from EV71-infected RD cells using Trizol (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed using oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to Manufacturer's instructions. VP1 fragment was amplified from the resultant cDNA with primers (forward 5′-GGTCCATGGGAGATAGGGTGGCAGATG-3′ and reverse 5′-GCGCTCGAGAAGAGTGGTGATCGCTGAG-3′), digested with *NcoI* and *XhoI*, and then inserted into prokaryotic expression vector pET26b from the same sites, giving rise to pET26-VP1. Prokaryotic expression of pET26-VP1 and purification of recombinant VP1 protein were carried out following protocols described previously (Liu et al., 2011b). One rabbit was immunized subcutaneously with 50 μg of VP1 protein in the presence of complete Freund's adjuvant (Sigma, St. Louis, MO, USA) to generate VP1-specific antibody, and boosted twice with the same amount of antigen plus incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) at 3 and 6 weeks post-priming, respectively. At two weeks after the last booster injection, the animal was killed and sera collected. This animal experiment was approved by the SJTU Ethics Committee for Animal Care and was carried out in the SJTU animal facility. Specific IgG titers were determined by endpoint titer ELISA as described previously (Liu et al., 2011b) except that the 96-well ELISA plates were coated with 100 ng/well of the recombinant EV71 VP1 protein.

2.3. SDS-PAGE and Western blotting

Protein samples were mixed with SDS-PAGE sample buffer, boiled and then separated on 12% polyacrylamide gels. Proteins were either visualized by Coomassie blue staining or transferred onto PVDF membranes for Western blot analysis. Membranes were probed with a capsid subunit protein-specific antiserum, followed by a corresponding horseradish peroxidase (HRP) conjugated secondary antibody. Positive signals on the membranes were developed by chemiluminescence using the BeyoECL Plus kit (Cat# P0018, Beyoime, Shanghai, China), recorded using a LAS-4000 Luminescent Image Analyzer (Fujifilm Life Science).

2.4. Sucrose gradient analysis

Protein samples (purified EV71 or cell lysates) were layered onto 10–50% sucrose gradients and subjected to ultracentrifugation in a Beckman SW60Ti rotor at 156,000 × g for 3 h at 4 °C. Ten fractions of 0.4 ml each were taken from top to bottom and subjected to ELISA and/or Western blot analyses.

2.5. Indirect ELISA

Each sucrose gradient fraction (10 μl/well) was added into 96-well microtiter plates and incubated at 37 °C for 2 h, followed by washing three times with PBST (PBS containing 0.05% Tween-20). Wells were blocked with 200 μl PBST plus 5% milk at 37 °C for 1 h, washed, then incubated with 50 μl/well of the guinea pig anti-VP0 antisera diluted (1:2000) in PBST plus 1% milk at 37 °C for 2 h. After washing, wells were incubated with 50 μl/well of goat anti-guinea pig conjugated to horseradish peroxidase diluted (1:5000) in PBST plus 1% milk at 37 °C for 1 h. For color development, wells were washed then 50 μl/well of TMB mixture was added and incubated for 5–10 min, followed by 50 μl/well of 1 N H_3PO_4 to stop the reaction. Absorbance was measured at 450 nm in a 96-well plate reader.

2.6. Electron microscopy

Purified EV71 preparations were stained negatively with 0.5% aqueous uranyl acetate, and transmission electron microscopy was carried out with a Philips CM-12S microscope.

2.7. Immunofluorescent staining

Vero cells infected with EV71 were fixed with 4% paraformaldehyde for 20 min, followed by treatment with 1% NP40 diluted in PBS for 10 min at room temperature. The fixed cells were incubated with 10% normal goat serum for 30 min, then with a rabbit or guinea pig primary antibody for 30 min, followed by a corresponding secondary antibody for 30 min. The secondary antibodies used included FITC-conjugated (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Alexa Fluor 488-conjugated (Cat# A11073, Invitrogen) anti-guinea pig IgG for emitting green fluorescence, and Texas red-conjugated (Santa Cruz Biotechnology) or Alexa Fluor 555-conjugated (Cat# A21429, Invitrogen) anti-rabbit IgG for emitting red fluorescence. At last, the cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min. All incubation steps were carried out at 37 °C and the cells were rinsed four times with PBS between steps. The stained cells were examined using a conventional epifluorescent microscope (Leica, Wetzlar, Germany) or a confocal microscope (Leica).

2.8. Confocal microscopy

Confocal microscopy was carried out using a Leica SP5 confocal laser scanning microscope. In general, the following parameters were used, pinhole aperture: 1 P AU; UV laser (405 nm) intensity: 12.5%; Ar laser (488 nm) intensity: 15%; He–Ne laser (543 nm) intensity: 36%; scan speed: 200 Hz. Images in 1024 × 1024 format were acquired in the DAPI, Alexa Fluor 488 and Alexa Fluor 555 channels, and processed using the Leica LAS AF version 2.6.0 software.

3. Results

3.1. Protein composition of EV71 capsids

To determine the protein composition of EV71 capsids, we first analyze a purified EV71 preparation by SDS-PAGE analysis. Three major Coomassie blue-stained bands with molecular weight of approximately 38-, 34-, and 26-kDa, respectively, were evident (Fig. 1A, lane 1). Detection with capsid subunit protein-specific polyclonal antibodies revealed that, the 38-kDa band reacted only with the anti-VP0 polyclonal antibody (Fig. 1A, lane 2), the 34-kDa band with the anti-VP1 (Fig. 1A, lane 3), and the 26-kDa band with the anti-VP3 (Fig. 1A, lane 4). These results indicated that the 38-, 34- and 26-kDa bands represent VP0, VP1 and VP3 subunit proteins, respectively. To analyze further the EV71 capsid composition, lysate from cells infected with EV71 was subjected to Western blotting. As expected, positive signals of ∼34 and ∼26 kDa were solely detected by the anti-VP1 and anti-VP3, respectively (Fig. 1B, lanes 2 and 3). However, two bands, one of ∼38 kDa and another of

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