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Efficient production of foot-and-mouth disease virus empty capsids in insect cells following down regulation of 3C protease activity

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A B S T R A C T

Foot-and-mouth disease virus (FMDV) is a significant economically and distributed globally pathogen of *Artiodactyla*. Current vaccines are chemically inactivated whole virus particles that require large-scale virus growth in strict bio-containment with the associated risks of accidental release or incomplete inactivation. Non-infectious empty capsids are structural mimics of authentic particles with no associated risk and constitute an alternate vaccine candidate. Capsids self-assemble from the processed virus structural proteins, VP0, VP3 and VP1, which are released from the structural protein precursor P1-2A by the action of the virus-encoded 3C protease. To date recombinant empty capsid assembly has been limited by poor expression levels, restricting the development of empty capsids as a viable vaccine. Here expression of the FMDV structural protein precursor P1-2A in insect cells is shown to be efficient but linkage of the cognate 3C protease to the C-terminus reduces expression significantly. Inactivation of the 3C enzyme in a P1-2A-3C cassette allows expression and intermediate levels of 3C activity resulted in efficient processing of the P1-2A precursor into the structural proteins which assembled into empty capsids. Expression was independent of the insect host cell background and leads to capsids that are recognised as authentic by a range of anti-FMDV bovine sera suggesting their feasibility as an alternate vaccine.

1. Introduction

Foot-and-mouth disease virus (FMDV) is the prototypic aphthovirus within the family *Picornaviridae* (reviewed by (Grubman and Baxt, 2004)). Economic losses from foot-and-mouth disease outbreaks are among the highest of all livestock diseases and widespread vaccination is the method of choice for disease control (Rodriguez and Grubman, 2009). The current vaccine is a killed whole virus vaccine whose limitations have been widely discussed; growth of the live virus prior to inactivation is not without risk, the appropriate serotype for each outbreak is required, some field strains grow poorly before adaptation and immunogenicity can be lost upon storage (Rodriguez and Grubman, 2009). To address these issues alternate vaccines have been sought, among them the use of empty capsids which are structural and immunogenic mimics of virus particles but lack the potential for causing disease outbreak (Rowlands et al., 1975; Rweyemamu et al., 1979).

As for all picornaviruses, the icosahedral FMDV capsid is assembled from mature proteins derived from a structural precursor, P1-2A, following cleavage *in trans* by the 3C protease (reviewed by (Belsham, 2005)). The 3C protease is one of many non-structural proteins synthesised in the infected cell but expression of P1-2A and 3C in the absence of any other FMDV encoded protein in recombinant systems is sufficient to afford authentic precursor cleavage (Lewis et al., 1991; Roosien et al., 1990). Accordingly there have been a number of reports of the assembly of recombinant FMDV empty capsids following the use of expression systems such as vaccinia virus (Abrams et al., 1995), adenovirus (Mason

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et al., 2003), *E. coli* (Cao et al., 2010; Lewis et al., 1991), transgenic plants (Pan et al., 2008) and baculovirus (Li et al., 2008; Oem et al., 2007; Roosien et al., 1990). In some cases, sufficient empty capsid material has been prepared to immunise cattle and protection against homologous challenge was demonstrated (Li et al., 2008, 2011) but in the main the configuration of the P1and 3C coding sequences used to achieve empty capsid expression and the efficiency of capsid assembly has been highly variable, particularly in insect cells. For example, using the successful expression of swine vesicular disease empty capsids as an exemplar (Ko et al., 2005), usage of a dual promoter vector in which the P1 and 3C coding sequences of an O serotype of FMDV were under the control of the baculovirus polyhedrin and p10 promoters respectively, resulted in incomplete precursor cleavage and predominantly pentameric assemblies rather than complete capsids (Oem et al., 2007). Similarly, a dual expression strategy of an Asia 1 serotype of FMDV led to incomplete cleavage of the P1-2A precursor (Cao et al., 2009). More recently, forsaking the use of 3C to generate the mature capsid proteins, VP0 and VP3-2A-VP1 from an O serotype of FMDV were co-expressed relying on self-cleavage at the 2A site (Donnelly et al., 2001) to generate the requisite structural proteins for assembly, which resulted in partial success (Cao et al., 2010). In a further example, a Bombyx (silk worm) baculovirus system encoding a P1-2A-3C sequence of an FMDV Asia 1 isolate was used as a single transcription unit driven by the polyhedrin promoter and the empty capsid material harvested from the haemolymph of the infected silk worms was immunogenic in cattle and led to levels of neutralising antibody associated with protection (Li et al., 2008, 2011). Despite these successes, variation associated with both the FMDV serotype and the host cell background used mean that a uniform genetic design capable of producing empty capsids for any serotype has yet to be reported. Recently, the yield of empty capsids from insect cells for another picornavirus, human enterovirus 71, was improved by use of a dual vector in which a less active promoter, the CMV early promoter, was used for transcription of the 3C coding unit whilst the strong polyhedrin promoter directed expression of the P1 structural precursor (Chung et al., 2010). However, yield improvement was restricted to Sf9 cells as infection of T.ni cells, an alternative insect cell line that generally gives higher expression levels (Davis et al., 1993), resulted in poor capsid expression plausibly as a result of low levels of promoter-specific transcription factors present in T.ni cells. Picornaviruses traditionally exhibit a strong "host cell shutoff" phenotype which is partly the result of 3C protease cleavage of host cell proteins in addition to its action on the virus structural precursor P1 (Li et al., 2001; Strong and Belsham, 2004). As a result, picornavirus replication cycles are typically rapid and exhibit extensive cytopathic effect (*e.g.* (Rodriguez Pulido et al., 2007)). It follows that if cleavage of host cell proteins by FMDV 3C protease was to occur in a recombinant empty capsid expression system it would curtail the expression period and limit the yield of capsids observed. Thus, variable levels of 3C expression could account for the variation in the levels of FMDV empty capsids reported to date and purposeful moderation of 3C activity might enhance the yield of capsid observed, allowing a more thorough exploration of their virtues as vaccine candidates. Here a new genetic design is described for the expression of empty FMDV capsids in insect cells following infection by recombinant baculoviruses expressing P1-2A-3C with a number of modifications to reduce 3C activity.

2. Materials and methods

2.1. Cell culture and virus growth

Sf9, T.ni and T.nao38 cells were cultured in BioWhittaker® Insect-Xpress (Lonza, Basel, Switzerland) supplemented with 2% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Cells were grown at 28 °C as monolayers or in suspension with agitation at 100 rpm. Baculoviruses were generally amplified in monolayer cultures but large scale infections for capsid isolation were done in suspension. Virus stocks were titreed using plaque assay on Sf9 monolayers.

2.2. Sequences and cloning

The sequence for FMDV A22 Iraq (AY593764.1) was that deposited in the database. DNA was synthesised *de novo* (Lifetechnologies, Carlsbad, USA). The transfer vector used for all expressions was based on pOPINE (Berrow et al., 2007), itself a derivative of pTriEx1.1 (EMD Biosciences, Billerica, USA) and a fragment encoding P1-2A-3C of FMDV serotype A22 was cloned downstream of the p10 promoter by use of In-Fusion Technology (Clontech, Mountain View, USA). Mutations within this cassette were introduced by the swapping of appropriate fragments *via* unique restriction sites introduced during gene synthesis. Recombination between pOPINE vectors and AcMNPV bacmid KO1629 in insect cells was as described (Zhao et al., 2003). Routine DNA procedures *in vitro* made use of standard protocols or, when kits were used, those recommended by the vendor. All vectors were confirmed by DNA sequencing prior to use for expression.

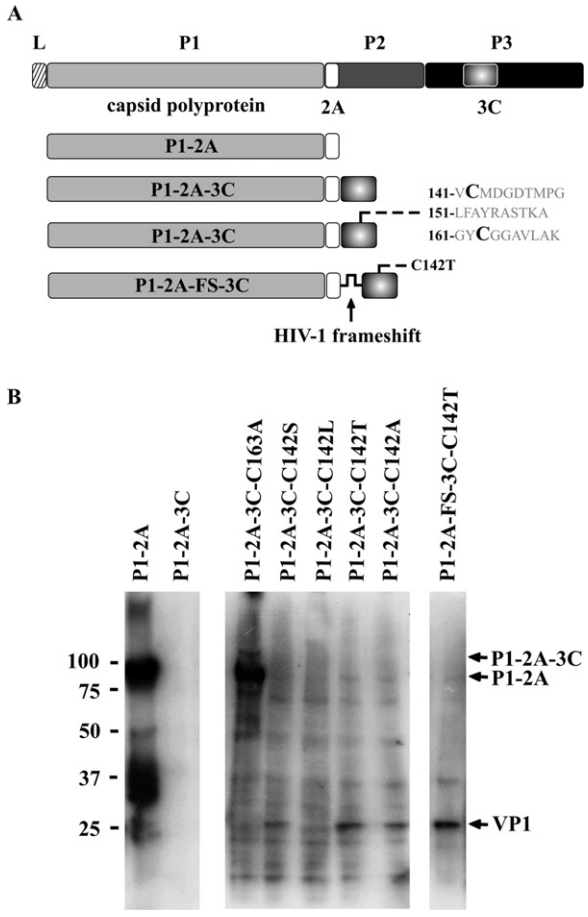

Fig. 1. Expression screening of various FMDV P1-2A ± 3C cassettes. (A) Cartoon representation of the genetic designs tested. The text near the bottom right represents the amino acid sequence of FMDV 3C between residues 141–170 with Cys 142 and Cys 163 highlighted. (B) Outcome of screening the various A22 Iraq constructs by immunoblotting using a polyvalent A serum. The expected migration positions of P1-2A-3C, P1-2A and VP1 are indicated although very little P1-2A-3C is visible. Numbers to the left are the migration positions of protein markers and are in kilodaltons.