PROKARYOTIC EXPRESSION OF PORCINE EPIDEMIC DIARRHOEA VIRUS ORF3

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1. ABSTRACT

Wild type (wt) and cell culture adapted (ca) strains of the coronavirus PEDV differ in their ability to cause diarrhea in neonate piglets: the wt strains are virulent; the ca strains are attenuated. Comparison of the available nucleotide sequences obtained from the different viral isolates revealed almost complete sequence identity with the exception of variations and truncations in open reading frame 3 (ORF3) observed exclusively in ca-PEDV isolates. In order to study the biological function(s) of the putative ORF3 product, the molecule was expressed as a heterodimeric fusion protein in *E.coli*. ORF3 was fused in frame to the alkaline phosphatase gene. Simultaneously, the construct was designed to form specific heterodimers by inclusion of the well known leucine zipper motiv of Jun and Fos. The heterodimerization partner contained the *E.coli* heat-labile enterotoxin subunit B (LTB) to allow specific binding to the eukaryotic cell receptor GM1. Our results indicate that heterodimeric fusion protein containing a truncated form of ORF3 was produced in high amounts, carried the expected ORF3 epitope, showed phosphatase activity, and was able to bind to the GM1 receptor. In contrast, a fusion protein containing the entire sequence of the ORF3 product was produced in minute amounts, indicating that it may have biological activity in prokaryotes, which led to the reduction of the amounts of proteins expressed.

2. INTRODUCTION

Comparison of the available nucleotide sequences obtained from wild type (wt) and either the corresponding or a different cell culture adapted (ca) strain of the coronavirus

PEDV revealed almost complete sequence identity with the exception of variations and truncations in the open reading frame 3 (ORF3) observed exclusively in ca-PEDV isolates (Duarte et al., 1994; Tobler and Ackermann, 1995). A Spanish wild type isolate of PEDV acquired mutations in ORF3 during adaptation to growth in cell culture (Carvajal, Utiger, and Ackermann, unpublished observations).

Attempts to express ORF3 in eukaryotic cells, either by transient expression or in recombinant viruses, e.g. Baculovirus or Vacciniavirus, were unsuccessful. In contrast, it was possible to translate ORF3 mRNA *in vitro* or to produce ORF3 in the cytosol of *E.coli* (Utiger et al., 1995).

To study the functions of the putative ORF3 product, we planned to express ORF3 as a heterodimeric fusion protein in *E.coli*. The N-terminal part of the fusion protein was designed to contain the pelB leader to guide the polypeptide to the periplasmic space of the bacteria in order to allow correct folding of the aminoacid (aa) chain. Fused to this component the entire ORF3 or a fragment thereof was arranged to follow. A third component of the fusion protein consisted of *fos* sequences, and alkaline phosphatase and was designed to represent the C-terminus of one part of the heterodimeric protein. The second part was composed to contain again the pelB leader, followed by the *E.coli* heat-labile enterotoxin subunit B (LTB) and the *jun* polypeptide. A well known leucine zipper motiv of Jun and Fos is known to cause specific dimerization of the two components (Crameri and Suter, 1993). The LTB module was included in order to allow binding of the fusion protein to the eukaryotic cell receptor GM1 (Holmgren, 1973).

Here we show that the PEDV ORF3 product may have biological activity in prokaryotes, which led to the reduction of the amounts of proteins expressed. Heterodimeric fusion proteins containing truncated forms of ORF3 were produced in high amounts, they carried the expected ORF3 epitope, showed phosphatase activity, and were able to bind to the GM1 receptor.

3. MATERIALS AND METHODS

3.1. Plasmid Constructions

To construct pAS1, the sequence encoding the *E.coli* heat-labile enterotoxin subunit B (LTB) was amplified by PCR from pLTB0 (Aitken et al., 1994), and integrated as a *SalI/Hind*III fragment into pJuFo (Crameri and Suter, 1993) containing the gene encoding the *E.coli* alkaline phosphatase (phoA). For the construction of pAS6, pAS1 was partially digested with *Nco*I and cut with*Sac*I before the complete ORF3 was inserted. The plasmid pAS12 was created by digestion of pAS6 with *Sac*I and religation, deleting the 3' last 574 nucleotides of ORF3. Gene expression in all constructs was under the control of *lac*Z promoters. The nucleotide sequence of the plasmid inserts was verified by sequencing.

3.2. Protein Expression and Extraction

For the induction of protein expression, bacteria were cultured at 37°C for 3h in LB containing 0.2% glucose, 100μg/ml ampicillin, and 1mM IPTG.

After the induction period, the cells were centrifuged and resuspended in 1/50 volume of 20% sucrose 50mM Tris pH 8.0. 1/1000 volume of 10mg/ml lysozyme in 50mM Tris pH 8.0 was added and the cells were kept on ice for 15min. 1/1000 volume of 0.5M EDTA pH 8.0 was added and incubation on ice continued for 10min. 1/500 volume of 10%

Brij58 in 50mM Tris pH8.0 and 1/1000 volume of 10mM PMSF were added and the cells were incubated on ice for an additional 30min. The cells were centrifuged and the supernatant containing the periplasmic extract was removed.

3.3. Nitrocellulose Test

Serial dilutions of the recombinant proteins in PhoA buffer (100mM Tris pH 9.5, 100mM NaCl, 50mM MgCl2) were sptted onto nitrocellulose sheets. PhoA activity was monitored by addition of substrate solution (0.45mg/ml of 4-nitroblue tetrazolium chloride and 0.175mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate, Boehringer, in PhoA buffer). Crude bacterial alkaline phosphatase (Sigma) bound in defined concentrations to the same membrane served as a standard for evaluation of the enzymatic activity.

3.4. Immunodetection of Proteins on Western Blots

Periplasmic isolates were solubilized in loading buffer, boiled and separated on standard 10% polyacrylamide gels containing SDS before being transferred to nitrocellulose by electroblotting. For the immunodetection of proteins, the Western blots were first blocked in washing buffer (50mM Tris pH 7.4, 140mM NaCl, 5mM EDTA, 0.05% Nonidet P40, 0.25% gelatine) containing 10% skimmed milk (s.m.), then incubated with either a rabbit anti-peptide serum (1:100) specific for an N-terminal epitope of the putative ORF3product (Utiger et al., 1995) or the corresponding preimmune serum. Then, the blots were washed, incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunological Laboratories), and washed again before being developed by addition of the substrate solution containing 0.01% 4-chloro-1-naphtol and 0.015% H_2O_2.

Subsequently, the same blots were probed for PhoA using a mAb (Anawa) specific against this protein and the glucose oxidase mouse IgG kit (ABC-GO) and the GO-subrate kit III (Vector Laboratories). Blots were incubated and developed by addition of the GO substrate containing INT tetrazoium salt in Tris pH 9.5 essentially according to the protocols of the manufacturer.

3.5. GM1-Enzyme-Linked-Assay (GM1-ELA)

ELISA plates were coated over night at 4°C with 100µl of 1µg/ml GM1 (Sigma) in PBS pH 7.4. The plates were blocked for 1h at 37°C with 100µl of 3% BSA in PBS. 100µl of undiluted periplasmic extracts were applied to the wells and incubated for 1h. The plates were washed three times before the substrate (1.5mg/ml of 4-nitrophenyl phosphate, Sigma, in 1M diethanolamine buffer pH 9.8 containing 0.5M MgCl2) was added and PhoA activity measured at 405nm after 15, 75 and 800 min.

4. RESULTS

4.1. Construction of Vectors for the Expression of ORF3 Fusion Proteins

We attempted to express ORF3 in prokaryotes in order to analyse the protein in vitro and to deliver the product into eukaryotic cells by the LTB fusion approach.

Complete and truncated ORF3 constructs were integrated into a cassette (pAS1) consisting of the E.coli LTB and alkaline phosphatase, linked together by the leucine zipper formation of jun and fos sequences, which were also part of the constructs. A plasmid termed pAS6 contained the entire ORF3 coding sequence within the cassette. For the construction of pAS12, a SacI fragment was removed from pAS6. Thus, the new plasmid encoded, apart from the LTB-jun fusion protein, a truncated second polypeptide consisting of the 33 aa nearest to the N-terminus of the putative ORF3 product fused to fos and the alkaline phosphatase. The nt sequence of the constructs was confirmed by sequencing (data not shown) before attempts were made to express the corresponding proteins in bacteria following induction with IPTG. Periplasmic protein extracts were harvested and serial dilutions thereof were spotted on nitrocellulose sheets. The relative amounts of recombinant proteins were monitored by measuring phosphatase activity on the nitrocellulose sheets. Interestingly, considerable phosphatase activity was observed with the periplasmic extracts of bacteria transformed with pAS1 or pAS12 but only minute activity was seen with pAS6 (data not shown).

4.2. Analysis of Prokaryotically Expressed ORF3 Fusion Proteins

In order to verify that the product obtained from pAS12 contained both alkaline phosphatase and the PEDV ORF3 tag, Western blot analyses were performed. The results are shown in Fig. 1. Alkaline phosphatase, visualized by monoclonal antibody and an anti-mouse glucose-oxidase conjugate (red color), was present in extracts of both pAS12 and the positive control. In contrast, the PEDV ORF3 tag was labelled exclusively in pAS12 extracts, using a rabbit anti-peptide serum (but not preimmune serum) specific for the N-terminus of the putative ORF3 product and an anti-rabbit peroxidase conjugate (purple color). Neither phosphatase nor the ORF3 tag was detected in extracts obtained from control bacteria.

The sheet containing lanes 1 through 3 was then re-incubated using preimmune rabbit serum (PI), whereas a rabbit anti-peptide serum (I) specific for the N-terminus of the putative ORF3 product was used for the sheet containing lanes 5 through 7. The reactions were visualized using anti-rabbit peroxidase conjugate and chloronaphtol.

The circles in the left panel refer to bands stained unspecifically by the preimmune rabbit serum. These bands allow inference on the abundancy of protein loaded in each lane. The arrow points to the only band in the figure which was stained both by the monoclonal antibody against phosphatase and the rabbit anti-ORF3 serum. The numbers on the right of the figure indicate the relative mobility of the proteins of the molecular weight marker (MW, lane 4).

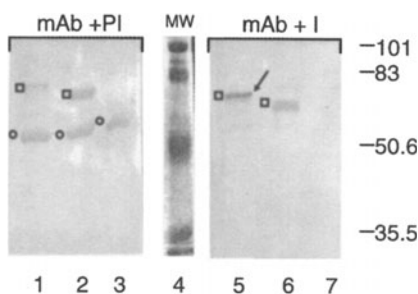
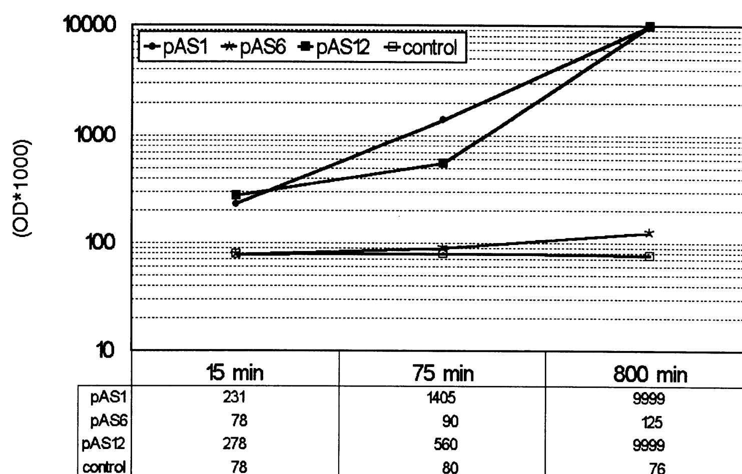


Figure 1. A double stained Western immunoblot is shown. Periplasmic extracts of *E.coli* transformed with pAS12 (lanes 1 and 5), pAS1 (lanes 2 and 6) or not transformed (lanes 3 and 7) were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose sheets were first stained for the detection of alkaline phosphatase by a monoclonal antibody (mAb) and the mouse ABC-GO system as described in Materials and Methods. Since colors cannot be discriminated in this Figure, the red-stained bands are indicated by squares.



	15 min	75 min	800 min
pAS1	231	1405	9999
pAS6	78	90	125
pAS12	278	560	9999
control	78	80	76

Figure 2. Enzyme-linked binding assay (ELA). Microtiter plates were coated with GM1 before being incubated with periplasmic extracts of *E.coli* transformed with either pAS1 (dots), pAS6 (asteriks), pAS12 (filled squares) or a control plasmid (open squares). After washing, binding of LTB-containing fusion proteins was visualized and quantified by the addition of phosphatase substrate. Absorption (OD405 nm) was measured in an ELISA reader at 15, 75, and 800 minutes after the addition of substrate. The values for each construct at each time point are indicated in the table below the figure.

4.3. Functional Analysis of the LTB-Binding Domain

In order to test the functional capacity of the LTB-domain to bind to the eukaryotic cell receptor GM1, extracts of bacteria transformed with pAS1, pAS6, pAS12, or control plasmids were incubated on GM1 coated microtiter plates and bound phosphatase activity was quantified at different time points after the addition of substrate (Fig. 2). Extracts of both, pAS1 and pAS12 showed almost linear increase of phosphatase activity over time, whereas no phosphatase activity was observed with the negative control. As expected, slight but reproducibly measurable phosphatase activity was detected in association with GM1 when the product was expressed from plasmid pAS6.

5. DISCUSSION

Several conventional eukaryotic expression systems, e.g. transient expression in transfected Vero cells, as well as the recombinant baculovirus- and vaccinia virus systems, were inadequate to provide sufficient quantities of the Porcine Epidemic Diarrhoea coronavirus putative ORF3 product to study its properties in detail (Tobler and Ackermann, unpublished observations). On the other hand, there was clear evidence from *in vitro* translation (Siréjols, Tobler, and Ackermann, unpublished) and cytosolic expression in *E.coli* (Utiger et al., 1995) that the ORF3 gene encoded a product which could be translated.

Therefore, our plan was to generate the ORF3 product in a prokaryotic system in order to deliver it to eukaryotic cells. To this aim, a heterodimeric fusion protein was con-

structed (Crameri and Suter, 1994). One part of the heterodimer consisted of the pelB-leader followed by either full length or truncated ORF3 sequences, and fused to fos and the alkaline phosphatase. The second part consisted of the pelB-leader, followed by the *E.coli* heat-labile enterotoxin subunit B (LTB) and jun at the carboxy terminus. The pelB-leader was used to guide the growing amino acid chains into the periplasmic space of *E.coli* in order to allow correct folding of the protein. The jun-fos leucine zipper was included to allow heterodimerization of the two fusion proteins. Phosphatase activity permitted a read-out for the correct reading frame and translation of the ORF3 fusion protein, and LTB was added as a means to bind the heterodimeric protein to the eukaryotic cell receptor GM1 for internalization (Aitken et al., 1994).

Our results clearly indicated that all of the components, pelB-leader, phosphatase, LTB, and the jun-fos leucine zipper were intact when expressed from pAS12, which encoded a truncated ORF3 product. Since pAS12 was a deletion mutant produced from pAS6, which encoded the entire ORF3, we conclude that the differences seen between the pAS12 and pAS6 expression products must be due to the biological activities of the ORF3 product. It appears as if ORF3 was able to reduce the net amounts of protein produced in *E.coli*. On the other hand, the work presented here confirmed the specificity of the rabbit anti-peptide serum for the N-terminus of the ORF3 protein (Utiger et al., 1995) as well as the possibility to use the N-terminal 33 amino acids of the ORF3 product as a tag in fusion proteins. The leucine zipper of jun-fos allowed heterodimerization of as large macromolecules as LTB and alkaline phosphatase without disturbing each others functions. These constructs will now be used to fine map the biological activities of the ORF3 product in more detail.

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REFERENCES

Aitken R., Brock J., Sinclair M.C., 1994, Receptor-binding subunits from cholera toxin and heat-labile enterotoxin as immunological carriers, in: *Bacterial protein toxins*, Zbl. Bakt. Suppl. **24**, (Freer et al., eds), Gustav Fischer, Stuttgart, pp 467–473.

Crameri R., Suter M., 1993, Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production, *Gene* **137**: 69–75.

Duarte M., Tobler K., Bridgen A., Rasschaert D., Ackermann M. and Laude H., 1994, Sequence analysis of the porcine epidemic diarrhea virus genome between the nucleocapsid and spike protein genes reveals a polymorphic ORF, *Virology* **198**: 466–476.

Holmgren J., Lonnroth I., Svenerholm, L., 1973, Tissue receptor for cholera exotoxin: postulated struture from studies with GM1 ganglioside and related glycolipids, *Infect. Immun.* **8**: 208–14.

Tobler K. and Ackermann M., 1995, PEDV leader and junction sites. In: Corona- and Related Viruses (P.J. Talbot and G.A. Levy, eds.), *Adv. Exp. Med. Biol.* **380**: 541–542.

Utiger A., Tobler K., Bridgen A., Suter M., Singh M., Ackermann M., 1995, Identification of proteins specified by porcine epidemic diarrhoea virus. In: Corona- and Related Viruses (P.J. Talbot and G.A. Levy, eds.), *Adv. Exp. Med. Biol.* **380**: 287–290.