Cleavage of rotavirus VP4 in vivo

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The infectivity of rotavirus particles is dependent on proteolytic cleavage of the outer capsid protein, VP4, at a specific site. This cleavage event yields two fragments, identified as VP5* and VP8*. It has been hypothesized that the particle is more stable, but non-infectious, when VP4 is in the uncleaved state. Uncleaved VP4 and the resultant increased stability might be advantageous for the virus to resist environmental degradation until it infects a susceptible host. When VP4 is cleaved in the lumen of the host's gastrointestinal tract, the virus particle would become less stable but more infectious. To test this hypothesis, a series of experiments was undertaken to analyse the cleavage state of VP4 on virus shed by an infected host into the environment. Immuno-bLOTS of intestinal wash solutions derived from infant and adult BALB/c mice infected with a virulent cell culture-adapted variant of the EDIM virus (EW) or wild-type murine rotavirus EDIM-Cambridge were analysed. Virtually all of the VP4 in these samples was in the cleaved form. Moreover, cell culture titration of trypsin-treated and untreated intestinal contents from pups infected with EW indicated that excreted virus is fully activated prior to trypsin addition. It was also observed that trypsin-activated virus has no disadvantage in initiating infection in naive animals over virions containing an intact VP4. These studies indicate that VP4 is cleaved upon release from the intestinal cell and that virus shed into the environment does not have an intact VP4.

Introduction

Rotaviruses are the leading cause of severe infantile gastroenteritis in the world, infecting up to 90% of children under the age of 3 (Kapikian & Chanock, 1990; Estes & Cohen, 1989). These viruses can cause severe dehydrating diarrhoea leading to significant morbidity and mortality.

Rotaviruses are triple-layered icosahedral particles with a dsRNA genome containing 11 gene segments (Bellamy & Both, 1990). The outer capsid of the virus is comprised of two coat proteins, the 37 kDa VP7 glycoprotein and the 84 kDa spike protein, VP4. Neutralizing antibodies to the virus have been found to recognize either VP7, the product of gene segment 9, or VP4, the product of gene segment 4. The binding epitopes of neutralizing antibodies on VP7 and VP4 have been characterized (Matsui et al., 1989). A role for VP4 in cell attachment and virus virulence has also been shown (Méndez et al., 1993; Ludert et al., 1996). VP4 has been implicated as an important determinant of rotavirus stability during purification (Chen & Ramig, 1992). It has been shown that trypsin cleavage of the VP4 spike protein is important for virus infectivity in vitro; the role of VP4 cleavage in vivo has yet to be elucidated (Kaljot et al., 1988; Konno et al., 1993).

VP4 is a protein comprised of 775 or 776 amino acids. It is proteolytically cleaved to yield two polypeptides, VP5* and VP8*. This cleavage activates the virus (i.e. enhances virus infectivity) and permits penetration of the virus into the cell (Clark et al., 1981; Espejo et al., 1981; Estes et al., 1981). Cell binding is not dependent upon VP4 cleavage (Keljo & Smith, 1988). Cleavage sites have been located on the C-terminal side of Arg441 and Arg447, creating VP8* from the N-terminal end and VP5* from the C-terminal portion of the molecule (López et al., 1985). The rationale for why the virus produces a full-length molecule which subsequently must be cleaved to render it infectious is not known.

It has been theorized by Nibert et al. (1991) that VP4 acts in a similar fashion to the μ1 and ε1 proteins of reovirus, another member of the family Reoviridae. In reoviruses, the coat protein μ1 is proteolytically cleaved upon entry into the host organism. When μ1 is intact, the virus particle is much more stable but less infectious than
when it is cleaved. These observations led to the hypothesis that when μ1 is in its native uncleaved state, the virus is better able to survive in the relatively harsh external environment. When cleavage of μ1 occurs upon entry into the host organism, the virus becomes more unstable. This cleavage, however, is advantageous as it ‘activates’ the virus, making it much more infectious, presumably by enhancing its ability to enter cells. Extending this hypothesis to rotavirus would seem reasonable since both viruses are members of the same family and both must survive in a relatively harsh external environment. In addition, Chen & Ramig (1992) found that intact VP4 confers stability to certain rotavirus strains during purification but not during storage in gentle conditions. On the other hand, newly released rotaviruses are shed into a veritable ‘sea’ of proteases (including trypsin) in the gastrointestinal lumen. It was therefore unclear whether progeny virions shed into the lumen of the gastrointestinal tract could escape the host uncleaved, despite the theoretical advantages of remaining in this state.

We sought to investigate the role of VP4 cleavage in vivo in more detail. We carried out studies to determine if rotavirus VP4 is cleaved or uncleaved upon exit from the infected host and, if VP4 is cleaved, to ascertain whether cleavage occurs before or after the virus is shed from the host. We also investigated if cleavage of VP4 prior to inoculation is disadvantageous in the initiation of infection in the mouse model system.

**Methods**

*Cells and viruses.* Three previously described strains of rotavirus were used in this study. The cell culture-adapted strains included the EDIM strain of murine rotavirus EW and the simian rhesus rotavirus (RRV). The EW strain, even though adapted to grow in cell culture, still retains infectivity and virulence in mice (Burns et al., 1995; Ward et al., 1990). All tissue culture-adapted viruses were propagated in MA-104 fetal monkey kidney cells (Greenberg et al., 1986). The virus was grown in stationary culture as previously described (Burns et al., 1995). The non-trypsin-activated RRV stock was produced by inoculating MA-104 cells at an m.o.i of 10 with trypsin-activated virus for 1 h. The inoculum was then removed, the monolayer washed extensively and fed with Medium 199 (M199) containing a cocktail of protease inhibitors (0.2 mM-AEBSF, 1 μg/ml aprotinin, 1 mM-benzamidine, 1 mM-EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin) and 0.5% fetal calf serum. At 12 h post-infection (p.i.) the medium was discarded, the monolayer washed twice with M199 and cells frozen with a minimum volume of medium.

The wild-type murine EDIM-Cambridge (EC) strain was obtained by infecting 4-6-day-old mouse pups with an intestinal homogenate derived from mice infected with the wild-type virus. The pups were washed twice with M199 and cells frozen with a minimum volume of medium.

**Immunoblotting. SDS-PAGE in conjunction with Western blotting** was used to detect uncleaved VP4 and the VP5* cleavage product. Aliquots of undiluted intestinal wash samples were treated with 0.4% SDS and 0.5% 2-mercaptoethanol, boiled for 5 min and electrophoresed on 12% polyacrylamide vertical slab gels at 125 V until the dye front reached the bottom of the gel. The proteins were transferred onto an Immobilon PVDF membrane (Millipore) pretreated in accordance with the manufacturer’s instructions. Transfer was accomplished overnight at 200 mA (4 °C). The Immobilon filter was then blocked for 2 h at room temperature with Blotto [5% (w/v) Carnation non-fat dried milk and 0.2% Tween 20 in PBS] (Johnson et al., 1984). Filters were probed with purified HS2 monoclonal antibody (MAb), which is reactive with both intact VP4 and the VP5* cleavage product of virtually all type A rotaviruses, diluted 1:1000 in Blotto for 1 h at room temperature. Membranes were washed (three times with Blotto for 10 min each), probed with anti-mouse IgG antibody conjugated to hors eradish peroxidase (HRP; Kirkegaard & Perry) diluted 1:3000 in Blotto and incubated for 1 h at room temperature. After another series of washes, the proteins were visualized using the ECL chemiluminescence system (Amersham) in accordance with the manufacturer’s instructions.

**Intestinal washes.** Ten-day-old rotavirus antibody-negative BALB/c suckling mice (Simonsen) were inoculated with 10¹⁴ f.f.u. of EW virus and 4-6-week-old adults with 10¹⁵ ‘shedding dose 50s’ of EC virus by oral gavage. Pups and adult mice were sacrificed 3 or 4 days p.i., respectively, and their entire small intestines were excised. The intestinal contents were obtained by gently rinsing the lumen of the intestine with 0.5 ml of M199 for the pups or 10 ml of PBS for the adults containing a protease inhibitor cocktail (0.2 mM-AEBSF, 1 μg/ml aprotinin, 1 mM-benzamidine, 10 μg/ml leupeptin and 10 μg/ml pepstatin) and collecting the effluent on ice. Samples were aliquoted and stored at −70 °C until analysed by immunoblotting or ELISA. The ELISA assays to detect the presence of rotavirus in the pups’ intestinal contents was performed as previously described (Burns et al., 1995).

**Focus titration.** The intestinal content of six mice inoculated with 10¹⁴ f.f.u. of EW virus were collected by flushing the intestine with 0.5 ml of M199 containing 200 μg/ml of gentamicin. Immediately after collection the sample was divided into two aliquots; one was treated with trypsin (10 μg/ml) and incubated for 30 min at 37 °C, while the other was treated with the cocktail of protease inhibitors and kept on ice until used.

To determine the titre of the two samples, a focus assay was done as previously described (Feng et al., 1994). Tenfold serial dilutions of the samples were added to MA-104 cells and incubated overnight at 37 °C. The next day the cells were fixed with ice-cold methanol for 20 min at room temperature. The fixed cells were then rinsed twice with PBS. After washing, fixed cells were incubated with an anti-rotavirus antibody solution (rabbit polyclonal antiserum to RRV in PBS containing 1% BSA) for 1 h at room temperature. The cells were then probed with a HRP-conjugated goat anti-rabbit IgG antibody (Kirkegaard & Perry), 1:2000 in PBS-1% BSA for 1 h at room temperature. The infected cells were stained with AEC-developing solution and counted.

**Infectivity titration in mice.** Eight litters of BALB/c pups (3-5 days old) were inoculated by gastric gavage with serial 10-fold dilutions of RRV. Litters were dosed with 10⁵, 10⁶, 10⁷ or 10⁸ p.f.u. of virus per pup. Litters were inoculated with either stock virus that was not trypsin-activated or virus from the same stock treated with trypsin at a concentration of 10 μg/ml for 30 min at 37 °C prior to serial dilution and inoculation. The mice were checked for diarrhoea for 3 days after inoculation by applying gentle pressure to the abdomen as previously described (Greenberg et al., 1986). Antibody-naive mice inoculated with RRV always develop diarrhoea by day 3 if diarrhoea is going to develop. The cleavage state of the VP4 in the inoculated virus dilutions was assessed by immunoblotting using MAb HS2 as probe.
Results

To determine whether VP4 shed into the gastrointestinal tract during acute infection was in a cleaved or uncleaved state, we used MAb HS2, which is directed to a non-conformational epitope on VP5*, to probe Western blots of virus protein derived from faecal material. We were unable to detect any uncleaved VP4 in the stool of acutely infected pups or adult mice (data not shown). However, these studies could not distinguish whether the observed cleavage of VP4 was due to intestinal proteases or bacterially mediated degradation occurring in the colon or post-excretion. We therefore directly examined the contents of the intestine of mouse pups acutely infected with EW and adult mice infected with EC. Luminal washes were obtained on day 3 or 4 p.i. to correspond to maximum shedding. Once again, we were unable to detect uncleaved VP4 in any of the tested specimens (Fig. 1). However, all specimens contained readily detectable levels of VP5*, indicating that most, if not all, VP4 had been cleaved prior to release from the mouse. Comparable results were obtained in pups and adult animals, although more VP5* was seen in the adult specimens. ELISA analysis of the intestinal washes confirmed the presence of rotavirus antigen in all the samples collected from the EW-inoculated pups, while no antigen was detected in the intestinal washes collected from mock-inoculated pups (data not shown). The VP5* fragment detected in the intestinal washes from the pups as well as the adult mice appeared to migrate slightly faster during gel electrophoresis than the VP5* fragment obtained from RRV treated with trypsin in vitro (Fig. 1).

We next sought to determine the sensitivity of our assay for detecting uncleaved VP4 in the presence of excess VP5*. Presumably if only limiting amounts of VP5* were present in the intestinal wash samples, we might not be able to identify residual VP4. MAb HS2 is equally efficient at detecting VP4 or VP5* (Figs 1 and 4). A representative sample of VP5*-positive intestinal wash was serially diluted and probed by Western blot (Fig. 2). VP5* was clearly detectable in wash samples diluted 1:100. Hence, if a small amount of uncleaved VP4 is present in this sample it is unlikely to be present in amounts greater than 1–2% of VP5* as measured by Western blot.

The Western blot data indicated that greater than 98% of all virus VP4 was present in a cleaved form in the stools of mice infected with murine rotavirus. However, small amounts (less than 2%) of uncleaved VP4 might not be detected by this assay. Therefore, we used a biological assay to detect more sensitively small amounts of uncleaved VP4 shed in faeces. We studied the ability of exogenous trypsin to activate (cleave VP4) potentially infectious rotavirus (i.e. uncleaved VP4) in the intestinal contents from infected mice. Intestinal contents from these mice were titrated on MA-104 cells with and without the proteolytic activation step before titration (Fig. 3). The titres of murine rotavirus EW in all six animals were very similar irrespective of exogenous
We sought to determine if this hypothesis was accurate. It could be assumed that the virus should contain predominantly intact VP4 upon exit from the host animal. In support of this hypothesis is the finding that intact VP4 confers stability (Chen & Ramig, 1992). If the hypothesis is correct, one would be found predominantly uncleaved in the relationship of VP4 to certain rotavirus strains during CsCl purification.

Recently, Nibert et al. (1991) suggested that cleavage of rotavirus VP4 might be similar to the cleavage of the μ1 protein of reovirus. They hypothesized that since the particle with uncleaved VP4 was more stable but less infectious than the particle with cleaved VP4, the virus would be found predominantly uncleaved in the relatively harsh external environment. In support of this hypothesis is the finding that intact VP4 confers stability to certain rotavirus strains during CsCl purification (Chen & Ramig, 1992). If the hypothesis is correct, one could assume that the virus should contain predominantly intact VP4 upon exit from the host animal. We sought to determine if this hypothesis was accurate.

We used Western blot analysis to study the condition of VP4 in the gut lumen of acutely infected mice. Shed virus was collected into buffer containing a number of protease inhibitors making it unlikely that cleavage would occur after collection. Nevertheless, we were unable to detect any full-length VP4 in the gut lumen by Western blot. We estimate that if some VP4 remained uncleaved, it made up less than 2% of the total VP4 in the specimen.

However, it remained theoretically possible that a very small percentage of virions with uncleaved VP4 accounted for most of the potential infectious rotavirus found in faeces. If this theory were true, the infectivity titre of shed virus should increase when treated with trypsin prior to cell culture titration. As shown in Fig. 3, our data do not support this hypothesis. In fact, virtually no increase in infectivity was observed when intestinal wash virus was pre-treated with trypsin prior to titration, supporting the Western blot observations that virus shed into the environment has already been trypsin-activated. We also carried out studies to determine whether infection with cleaved virus was disadvantageous as compared to infection with uncleaved virus. Theoretically, for example, uncleaved virus could be more resistant to destruction in the harsh environment of the stomach and hence be more infectious. However, our data suggest that infection with non-activated virus does not differ significantly from infection with preactivated virus, probably due to the likelihood that any uncleaved input virus is readily activated by trypsin when it reached the small intestine.

We observed some differences in VP5* migration in gel electrophoresis. We do not know why the VP5* fragments of EW and EC migrate more quickly than the comparable fragment of RRV. Presumably, this is caused by differences in primary amino acid sequence that have subtle effects on gel migration. Alternatively, it is possible that murine strains are cleaved at Arg269 or Arg274 rather than at Arg241 or Arg247, as is the case for SA-11 (López et al., 1985). Further studies will be required to explain this observation.

We noted a non-specific band in all the virus preparations studied by Western blot (Fig. 1), migrating just ahead of VP5*. This protein probably represents immunoglobulin heavy chains found in the intestinal wash since it reacted with anti-mouse immunoglobulin in the absence of primary antibody (data not shown).

From our findings, it seems unlikely that stability in the external environment explains the need to translate VP5* and VP8* as the single polypeptide VP4. The question as to why intact VP4 is produced in the first place remains unanswered, but several plausible hypotheses may account for this; these hypotheses could be tested in the future. One is that the intact VP4 molecule...
is needed for proper assembly of the outer capsid. It is possible that the mechanism to assemble efficiently infectious rotavirus requires a single VP4 peptide in order to form the spike on the outer capsid, due to either energetic or physical constraints. A corollary to this hypothesis is that intact VP4 and its stability is required for intracellular transport and maturation of the virus. If this hypothesis is true, then virus-like particles should not be formed or be formed less efficiently using recombinant VP5* and VP8* in place of VP4. A second possibility is that VP5* and/or VP8* are in some way toxic to the host cell when expressed as separate proteins. In this case, translation of VP4 as a single polypeptide would act as a safety measure, allowing the host cell to survive until progeny virus production is maximized. This possibility could also be studied in cell culture using recombinant clones expressing full-length VP4 versus those expressing VP5* and VP8*.

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