A new cysteine in rotavirus VP4 participates in the formation of an alternate disulfide bond

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Most animal rotaviruses bind to a cell surface molecule that contains sialic acid (SA). We have recently isolated variants from simian rotavirus RRV which show an SA-independent phenotype. The VP4 protein of these variants was shown to have three amino acid changes with respect to the wt protein, one of them being Tyr-267→Cys. In this work, we have investigated whether the new cysteine could interfere with the disulfide bond (Cys-318/Cys-380) present in the VP5* subunit of the wt protein. Cysteine residues 318 and 380 were mutagenized in gpr8 and RRV VP4 genes, and the wt and mutant genes were transcribed and translated in vitro. The protein products were analysed by electrophoresis under reducing and non-reducing conditions. This approach showed that, in the VP4 protein synthesized in vitro, Cys-267 is capable of forming an alternate disulfide bond with Cys-318. This alternate bond also seems to occur in the VP4 protein present in the variant gpr8 virus particles.

Rotaviruses are the single most important aetiological agents of severe dehydrating infantile gastroenteritis in developed and developing countries (De Zoyza & Feachem, 1985). The genome of these viruses is composed of 11 segments of dsRNA and is surrounded by three concentric layers of proteins (Estes & Cohen, 1989). The outermost layer is formed by two proteins, VP4 and VP7. VP7 forms the external surface of mature virions, while dimers of VP4 form spikes that extend from the virus surface. VP4 has essential functions in the virus life-cycle, including receptor binding and cell penetration (Estes & Cohen, 1989). The properties of this protein are therefore important determinants of host range, virulence and induction of protective immunity.

In vitro treatment of virions with trypsin results in the specific cleavage of VP4, which is 776 amino acids (aa) in length, into polypeptides VP8* (aa 1–231) and VP5* (aa 248–776), with the concomitant enhancement of virus infectivity (Arias et al., 1996; López et al., 1985).

VP4 has some other distinctive structural features. It contains a hydrophobic region, between aa 384 and 404, that shares sequence similarity with an internal fusogenic hydrophobic domain of the E1 glycoproteins of Sindbis and Semliki Forest viruses (Mackow et al., 1988), although the functionality of this region in rotaviruses has not been established. Also, VP4 contains the integrin ligand site Asp-Gly-Glu at aa 308–310, and peptides that mimic this sequence were shown to block the infectivity of rotaviruses (Coulson et al., 1997). In addition, the VP4 of many animal rotaviruses contains two intrachain disulfide bonds in the protein, one in VP8* between Cys-203 and Cys-216, and the second in VP5* between Cys-318 and Cys-380 (Patton et al., 1993).

Haemagglutination (HA) and the initial attachment of most animal rotaviruses to the target cell are mediated by VP4. These interactions have been shown to depend on the presence of sialic acid (SA) on the cell (Fukudome et al., 1989; Keljo & Smith, 1988; Yolken et al., 1987). In contrast, human rotaviruses do not have HA activity and do not require SA to infect the cells. Recently, we isolated variants from rotavirus RRV whose infectivity is no longer dependent on the presence of SA on the cell surface (Méndez et al., 1993). This SA-independent interaction is mediated by VP4, via a different site from the SA-binding domain located in VP8* (Fiore et al., 1991; Ruggieri & Greenberg, 1991; Isa et al., 1997; Méndez et al., 1993, 1996). Sequence analysis of the VP4 gene of the SA-independent variants showed three amino acid changes, with respect to the parental RRV gene, at positions 37 (Leu→Pro), 187 (Lys→Arg) and 267 (Tyr→Cys). Since one of the amino acid changes is a replacement of a tyrosine by a cysteine, we sought to determine if the new cysteine (at position 267) could be involved in the formation of an alternate disulfide bridge in the VP5* protein of the variant gpr8.

cDNA clones of the VP4 gene of RRV and its SA-independent variant gpr8 were obtained by RT–PCR and the PCR products were cloned into the BamHI site of the

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transcription vector pGEM-3Z (Promega). To generate plasmid constructs encoding the VP5* protein of RRV and gpr8, plasmids containing the full-length VP4 constructs were digested with SmaI and EcoRV, which cut the vector downstream of the T7 promoter and at nucleotide position 760 of gene 4, respectively, and the digestion products were religated. The VP5* protein encoded by these vectors started at Met-

To determine if Cys-267 is involved in the formation of an alternate disulfide bridge in VP5*, the plasmids encoding the VP5* domain of wt and variant VP4 proteins were transcribed and translated in vitro, as described by López et al. (1994), and the synthesized 35S-labelled proteins were analysed by SDS–PAGE under reducing and non-reducing conditions, essentially as reported by Patton et al. (1993). Briefly, before electrophoresis the samples were mixed with Laemmli sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue) with or without 100 mM dithio-erythritol (DTE), boiled for 3 min, and the proteins were separated by electrophoresis in SDS–12% polyacrylamide gels (15 × 18 cm) using the discontinuous buffer system of Laemmli. To resolve the reduced and oxidized forms of the proteins, the gels were electrophoresed for double the time taken for the bromophenol blue to run out of the gel. Under these conditions, the non-reduced products have an increased mobility, apparently due to a more compact conformation of the proteins when stabilized by disulfide bonds (Patton et al., 1993).

Electrophoretic analysis of in vitro translated gpr8 VP5* and RRV VP5* showed that these proteins migrate faster under non-reducing conditions than under reducing conditions (Fig. 1a), suggesting that they both have at least one intramolecular disulfide bridge. To determine whether Cys-267 in the RRV variant gpr8 was involved in formation of a disulfide bond, site-directed mutagenesis of the gpr8 and wt RRV VP5* constructs was carried out. Specific nucleotide changes in the VP5* gene were introduced by PCR such that the codons encoding either Cys-318 or Cys-380 were replaced by serine residues. Electrophoretic analysis of the in vitro translated mutant proteins showed that the RRV VP5* protein in which the Cys-380 was replaced by serine (RRV-VP5380) had the same migration in the presence or absence of DTE (Fig. 1b), indicating the absence of a disulfide bridge in the protein, as previously observed by Patton et al. (1993). In contrast, when either Cys-318 or Cys-380 was replaced in gpr8-VP5* by serine, the translated protein had a different electrophoretic mobility under reducing and non-reducing conditions, indicating the presence of a disulfide bridge in both mutant proteins (gpr8-VP5318 and gpr8-VP5380). This observation suggests that Cys-267 is able to substitute for either Cys-318 or Cys-380 in the formation of a disulfide bond in VP5*. In the absence of DTE, about half of the gpr8-VP5318 is in the reduced form (Fig. 1b), suggesting that the putative disulfide bond between Cys-267 and Cys-380 might not be very stable.

To determine if Cys-267 is also able to form a disulfide bond in the complete VP4 protein, a SmaI–MscI fragment from the VP5* constructs, containing the Cys → Ser mutations, was used to replace the equivalent region in the plasmids that contained the cDNA copy of the full-length gene 4 of wt RRV and gpr8 viruses; the in vitro translated proteins were then analysed by SDS–PAGE (Fig. 2). Under non-reducing conditions, the VP4 proteins of RRV and gpr8 contained two bands that migrated below the reduced form of the protein, which have been previously attributed to VP4 species having intrachain disulfide bridges present in VP5* and VP5* (Patton et al., 1993). The RRV VP4 protein in which Cys-380 was replaced by serine (RRV-VP4380) showed only one band under non-reducing conditions (in addition to one slower migrating band which seems to be the reduced form of the protein), which presumably corresponds to the intrachain disulfide bridge present in VP5*. In contrast, when gpr8-VP4380 was analysed under non-reducing conditions, two bands were detected, suggesting that this product has two disulfide bonds even in the absence of Cys-380. These results suggest that
Cys-267 is able to form a disulfide bond with Cys-318 in the VP5* portion of the gpr8 VP4 protein.

Unlike gpr8-VP4<sub>380</sub>, the mutant gpr8 VP4 protein in which Cys-318 was substituted by serine (gpr8-VP4<sub>381</sub>) showed only one band in the absence of the reducing agent, as was the case for RRV-VP4<sub>380</sub>; this band presumably corresponds to the protein species that contain the disulfide linkage between Cys-203 and Cys-216 in VP4<sup>*</sup>. It is interesting to note that the behaviour of the gpr8-VP4<sub>381</sub> protein is different from that observed when this mutation is present in the VP5<sup>*</sup> fragment, where Cys-267 is apparently able to form a disulfide bridge with Cys-380 (gpr8-VP5<sub>381</sub>; Fig. 1b). This difference might be due to the fact that in the complete VP4 protein the alternate disulfide bridge between Cys-267 and Cys-380 is not conformationally favoured, whereas in the VP5<sup>*</sup> portion this same bond might be less conformationally restricted, since the translation of the VP5<sup>*</sup> domain starts at Met-265.

To determine if the disulfide bonds found in the in vitro translated proteins were present in the virus, semi-purified preparations of RRV and variant gpr8 were obtained. Rotavirus RRV, kindly provided by H. B. Greenberg (Stanford University, Stanford, CA, USA) and variant gpr8 (Méndez et al., 1993) were propagated in MA104 cells as described previously (Espejo et al., 1980). At the time of harvesting, 50 mM iodoacetamide (Sigma) was added to the media in order to block the reactive sulfhydryl groups and, therefore, the formation of non-authentic disulfide bonds (Patton et al., 1993). Semi-purified viruses were obtained by extracting the infected cell lysate with freon, and centrifugation through a 35% sucrose cushion. Viral pellets were resuspended in TNC buffer (10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 10 mM CaCl<sub>2</sub>). The viral proteins were separated by SDS–PAGE in the presence or absence of reducing agent, and then transferred to nitrocellulose, and stained with MAb HS2, which reacts specifically with VP4 and with its cleavage product VP5* (Arias et al., 1996; Padilla-Noriega et al., 1993). The antigen–antibody reaction was then revealed with an anti-mouse antibody coupled to peroxidase, and detected using the ECL peroxidase kit (Amersham).

Under non-reducing conditions, the VP4 protein of both virus strains migrated as two bands (Fig. 3a; labelled S–S), which shifted to a single band in the presence of DTE (labelled as VP4 SH). In contrast, the VP5* protein showed important differences between RRV and gpr8; in the absence of DTE, the VP5<sup>*</sup> protein of RRV appeared to contain, in addition to the reduced form, a single faster migrating unreduced form. In the VP5<sup>*</sup> protein of the gpr8 variant, there was more than one band (Fig. 3a) that migrated faster than the reduced form in the absence of DTE. This difference in the migration of VP5<sup>*</sup> could be best visualized when the virus samples were treated with trypsin (10 µg/ml for 30 min at 37 °C) before electrophoresis (Fig. 3b). The heterogeneity of bands, observed almost as a smear under non-reducing conditions for gpr8 VP5<sup>*</sup> (but not for RRV VP5<sup>*</sup>), suggests the presence of several VP5<sup>*</sup> electrophoretic species in this virus.

These results support the idea that Cys-267 participates in the formation of a disulfide bond in the virus, and suggest that, in variant gpr8, the original Cys-318/Cys-380 and the alternate Cys-267/Cys-318 disulfide bonds might both be present.

The variant virus gpr8 can interact, through VP4, with an SA-independent cell surface receptor during attachment of the virus to the cell, surpassing the need for the initial interaction with SA shown by RRV (Méndez et al., 1993). Thus, it has been suggested that the VP4 protein of gpr8 has a conformation that is slightly different from that of RRV VP4, and it has been presumed that this change in conformation is the result of one or more of the three amino acid differences (including the Ser → Cys change at position 267) that exist between these two proteins (Méndez et al., 1996). In this context, the alternate disulfide bonds present in gpr8 VP5<sup>*</sup> (Cys-267/Cys-318 and Cys-318/Cys-380) might alter the conformation of this protein, probably favouring the interaction of the gpr8 virus with the SA-independent cell surface molecule.

The structural and functional integrity of many virus particles is maintained by disulfide bridges. The two major envelope proteins of lactate dehydrogenase-elevating virus (LDV), VP-2 and VP-3, are linked by disulfide bonds and these

![Fig. 2. Detection of disulfide bonds in in vitro translated VP4 proteins. The indicated VP4 proteins were synthesized in a cell-free system in the presence of [35S]Met, and the labelled proteins were resolved in a 12% polyacrylamide gel in the presence (+) or absence (−) of DTE, and detected by fluorography. The completely reduced form of VP4 (SH), as well as the non-reduced VP4 protein forms (S–S) containing heterogeneous disulfide bonds are indicated. All samples were run on the same gel except for RRV-VP4<sub>380</sub> which was run on a separate gel.](image-url)
are essential for LDV infectivity (Faaberg et al., 1995). In the case of human immunodeficiency virus, two highly conserved cysteine residues in the extracellular region of the TM protein have been shown to be essential for transport and processing of the gp160 precursor into the active gp120 and gp41 mature forms (Dedera et al., 1992; Tschachler et al., 1990). In the murine leukaemia viruses, there are three conserved cysteine residues within the extracellular domain of their TM protein; substitutions of these residues result in non-viable virus and mutagenic analysis revealed that the disulfide bonds are essential for transport and processing of the precursor TM protein throughout the endoplasmic reticulum (Thomas & Roth, 1995).

In rotavirus, the biological significance of the disulfide bonds in VP4 has not been established. It has been suggested that the disulfide bridge in VP5*, which is highly conserved among rotaviruses, might serve to bring the region around the trypsin cleavage sites, the integrin binding site and the putative fusogenic peptide into close proximity (Coulson et al., 1997; Patton et al., 1993). Here, we have shown that a mutant virus which no longer requires the presence of SA to infect the cell, contains mixed species of disulfide bonds in VP5*, which might alter the overall conformation of the protein and contribute, at least in part, to the phenotype of these mutants. In addition, the fact that Cys-267 can form a disulfide bond with Cys-318 provides further evidence for the structural features of VP4 mentioned above, suggesting that these two cysteine residues are close to each other. Although further studies are needed, the data presented in this work suggest that the disulfide bridge present in the VP5* domain of VP4 is important for initial rotavirus–cell interactions.

This work was partially supported by grants 75197-527106 from the Howard Hughes Medical Institute, G0012-N9607 from the National Council for Science and Technology, Mexico, IN207496 from DGAPA-UNAM, ERB3514PL950019 from the INCO programme of the European Community, and from The Miguel Aleman Foundation.

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Received 9 March 1998; Accepted 15 July 1998