

Point Mutations in the S Protein Connect the Sialic Acid Binding Activity with the Enteropathogenicity of Transmissible Gastroenteritis Coronavirus

CHRISTINE KREMPL,¹ BEATE SCHULTZE,¹ HUBERT LAUDE,² AND GEORG HERRLER^{1*}

Institut für Virologie, Philipps-Universität Marburg, 35037 Marburg, Germany,¹ and Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas, France²

Received 3 September 1996/Accepted 30 December 1996

Enteropathogenic transmissible gastroenteritis virus (TGEV), a porcine coronavirus, is able to agglutinate erythrocytes because of sialic acid binding activity. Competitive inhibitors that may mask the sialic acid binding activity can be inactivated by sialidase treatment of virions. Here, we show that TGEV virions with efficient hemagglutinating activity were also obtained when cells were treated with sialidase prior to infection. This method was used to analyze TGEV mutants for hemagglutinating activity. Recently, mutants with strongly reduced enteropathogenicity that have point mutations or a deletion of four amino acids within residues 145 to 155 of the S protein have been described. Here, we show that in addition to their reduced pathogenicity, these mutants also have lost hemagglutinating activity. These results connect sialic acid binding activity with the enteropathogenicity of TGEV.

Transmissible gastroenteritis virus (TGEV) is an enteropathogenic porcine coronavirus (13). Infections are most severe in newborn piglets, which in general develop a fatal diarrhea. TGEV is an enveloped virus with a positive-stranded RNA genome. Among the proteins that are inserted into the viral membrane, surface glycoprotein S is the main target of the immune response to TGEV and contains four major antigenic sites (2, 4). This protein mediates the binding of the virus to the cell surface by specific interaction with porcine aminopeptidase N, the cellular receptor for TGEV (6). The S protein is probably also responsible for the fusion between the viral and the cellular membranes. In addition, it has hemagglutinating (HA) activity (11, 12). Sialic acid has been shown to serve as ligand for the binding of TGEV to erythrocytes (18). The virus is able to recognize *N*-acetylneuraminic acid, but more efficient binding to receptors containing another type of sialic acid, *N*-glycolylneuraminic acid, was observed (18). The biological importance of sialic acid binding activity is not known. Interestingly, a related virus, porcine respiratory coronavirus (PRCV), lacks HA activity (18). PRCV replicates with high efficiency in the respiratory tract but with very low efficiency in the gut (3). The main differences found between TGEV and PRCV are several deletions which affect the S gene and one open reading frame coding for a nonstructural protein. As a consequence, the genes of PRCV strains encode S proteins with large deletions near the N termini (e.g., 224 amino acids lacking at position 5 in the mature protein of the European isolates of PRCV) (14, 16, 19). The deletion within the spike protein of PRCV is responsible for the loss of two antigenic sites, as indicated by the lack of reactivity with monoclonal antibodies (10, 15). The other antigenic sites are not affected by the deletion, and this explains the serological relatedness between PRCV and TGEV. The lack of HA activity of PRCV suggested that the sialic acid binding site is located in that portion of the S protein of TGEV that is deleted in the cor-

responding protein of PRCV. The presence of HA activity in enteropathogenic TGEV and its absence in the respiratory variant PRCV raised the possibility that sialic acid binding activity contributes to the enteropathogenicity of TGEV.

Recently mutants of TGEV that have lost enteropathogenicity have been described (1). The mutants had been selected for resistance to a neutralizing monoclonal antibody directed against antigenic site D (in the Paris nomenclature; site B in the Madrid nomenclature; see Fig. 2). As pointed out in the previous report (1), these site D mutants differed from the wild-type virus only by point mutations within that portion of the S protein that is missing in the PRCV glycoprotein (residues 145 to 155). This finding shows that this part of the surface protein is important for enteropathogenicity. As the HA activity is also located within or close to site D (18), it was of interest to analyze these mutants for HA activity.

The high-passage Purdue-115 strain (9) of TGEV was propagated in swine testicular (ST) and LLC-PK1 cells. The selection and characterization of the escape mutants have been described elsewhere (1, 4, 5). The mutants were grown in ST cells and harvested 1 day after infection. The supernatant was clarified by low-speed centrifugation (2,000 × *g*, 10 min) and, following the addition of 10% fetal calf serum, was stored at –80°C.

We have shown recently that the HA activity of TGEV can be observed only transiently during infection. By 48 h postinfection (p.i.), HA virus is detectable in the supernatant only if the sample is treated with sialidase (neuraminidase), an enzyme that releases terminal sialic acids, both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid (18). It appears that the enzyme treatment is required to inactivate competitive inhibitors on the viral surface. We found that the HA activity of TGEV can be induced not only by sialidase treatment of virions but also by sialidase treatment of cells prior to infection. For this purpose LLC-PK1 cells were incubated with 200 mU of sialidase from *Clostridium perfringens* (type X; Sigma) in 200 μl of phosphate-buffered saline (PBS). After 1 h, the enzyme was removed by three washings with PBS and the cells were infected with TGEV. At different times after infection, the supernatant was analyzed for HA activity. As shown in

* Corresponding author. Mailing address: Institut für Virologie, Philipps-Universität Marburg, Robert-Koch-Str. 17, 35037 Marburg, Germany. Phone: 6421/28-5360. Fax: 6421/28-5482. E-mail: herrler@mail.uni-marburg.de.

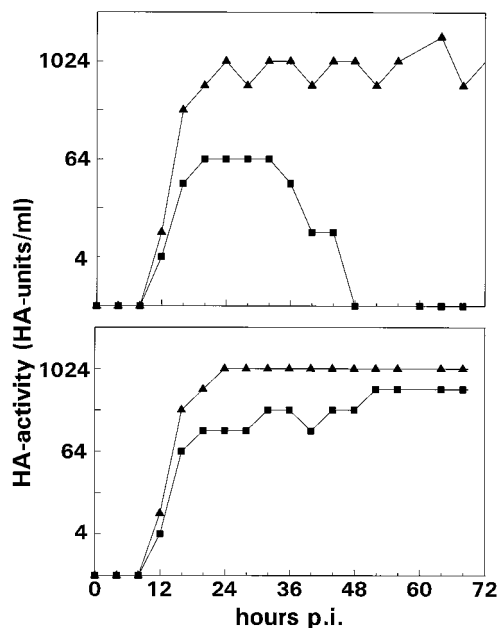
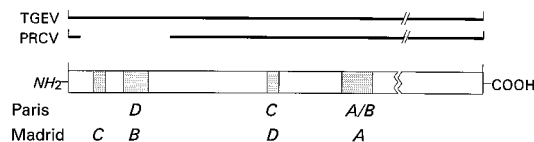


FIG. 1. Effect of sialidase treatment of LLC-PK1 cells on infection by TGEV. Untreated (upper panel) and sialidase-treated (lower panel) cells were infected with TGEV. At different times p.i., aliquots of the supernatant were analyzed for HA activity with chicken erythrocytes. Prior to the HA assay, the samples were incubated in the presence (triangles) or absence (squares) of sialidase from *Vibrio cholerae*.

Fig. 1, lower panel, virions released from the pretreated cells had HA activity that was detectable at 12 h p.i. and reached the maximum value around 60 h p.i. Sialidase treatment of these virions resulted in somewhat increased HA titers. By contrast, for virions released from untreated cells hemagglutination was detectable only at the earlier time points, with a maximum between 20 and 30 h after infection. After 48 h p.i. no HA activity was measurable (Fig. 1, upper panel). The HA activity of the control virus could be recovered by treatment of the virions in the medium with sialidase (upper panel). Essentially the same result as that shown in Fig. 1 was obtained when ST cells were treated with sialidase prior to infection by TGEV. These findings suggest that hemagglutination inhibitors are present on the cell surface prior to infection. Obviously, these inhibitors are not regenerated by the confluent cells during infection. Sialidase treatment of cells is more convenient than sialidase treatment of virions, because the enzyme can easily be washed away from the adherent cells, while a centrifugation step is required in the case of virions. Therefore, this method was used to analyze the site D mutants of TGEV described in the introductory section for HA activity.

As shown in Fig. 2, among the five site D mutants, only mutant m9 was able to agglutinate erythrocytes. The enteropathogenicity of this mutant was only somewhat reduced compared to that of the wild-type virus (1). On the other hand, no HA activity was detected with site D mutants m5, m6, m8 and m10, which had lost enteropathogenicity. The presence of virus in the mutant preparations is indicated by the infectivity titers. These results show that the defined mutations in the S protein within positions 145 to 155 affect both the enteropathogenicity and the HA activity of TGEV. As a control, we analyzed mutants that had been selected for resistance to antibodies directed against site A, B, or C (4). These mutants have not lost pathogenicity. As shown in Fig. 2 (lower four lines), each of



mutant	amino acid exchange	position	entero-pathogenicity	infectivity (pfu/ml)	HA-activity (HA-units/ml)
m5	C - F	155	-	3.3×10^8	< 2
m6	P - L	145	-	2.5×10^8	< 2
m8	C - R	147	-	8.9×10^8	< 2
m9	S - P	149	+	6.0×10^8	512
m10	4 aa del	146-149	-	5.0×10^8	< 2
wildtype	---	---	++	5.7×10^8	256
48.1 (A/B)	T - P	533	++	2.6×10^8	128
25b.21 (B)	D - A	547	++	3.8×10^8	128
20.9 (A)	D - N	570	++	2.0×10^8	128
3b.5 (C)	S - I	367	n.d.	1.8×10^8	128

FIG. 2. Analysis of site D mutants of TGEV for HA activity. At the top, the S genes of TGEV and PRCV are shown schematically to illustrate the deletion in the S gene of PRCV. Below this, a schematic drawing of the S protein of TGEV is shown, with the antigenic sites indicated as grey boxes. Both the Paris nomenclature (4) and the Madrid nomenclature (2) of the antigenic sites are given. In the lower part of the figure, the infectivity and the HA activity of TGEV mutants are shown. The m mutants were selected for resistance to monoclonal antibodies directed against site D (Paris nomenclature). The other mutants were selected for resistance to antibodies directed against the antigenic sites indicated in brackets. The enteropathogenicity is indicated according to the mortality rates (number of animals that died/number of animals tested) described by Bernard and Laude (1): 0/3 (-), 2/3 (+), and 3/3 (++) n.d., not determined. The infectivities of the wild-type and the mutant viruses were determined by a plaque assay. HA activity was determined according to published procedures (17, 18) with a 0.5% suspension of chicken erythrocytes.

these mutants (48.1, 25b.21, 20.9, and 3b.5) was able to agglutinate chicken erythrocytes. This result supports our conclusion that there is a connection between sialic acid binding activity and the enteropathogenicity of TGEV. The comparison of TGEV and PRCV had already suggested that the portion of the S protein that is missing in PRCV is involved in sialic acid binding activity (18). As 224 amino acids within the S protein of PRCV are lacking compared to the surface protein of TGEV, the location of the sialic acid binding domain could only roughly be determined. Our findings pinpoint amino acids 145 to 155 as being part of the sialic acid binding site. In addition, our results provide strong evidence for our view that sialic acid binding activity is involved in the enteropathogenicity of TGEV. This is consistent with a previous report that pathogenic strains other than the Purdue-115 strain also have HA activity (11, 12). We would like to point out that loss of pathogenicity is not necessarily associated with the loss of HA activity. Factors other than sialic acid binding activity contribute to pathogenicity. Loss or inactivation of any of these factors would result in an attenuated virus that retains HA activity.

How sialic acid binding activity may contribute to the enteropathogenicity of TGEV is presently not clear. The binding activity is dispensable for virus growth in cell culture. Both

TGEV and PRCV grow well in cell culture with aminopeptidase N as a receptor (6, 7). Also, the virus yield of the site D mutants in ST or LLC-PK1 cells was comparable to that of the wild-type virus. As aminopeptidase N is an abundant protein in the intestinal tract, it is not obvious why additional binding activity is required for infection of intestinal cells. Moreover, sialic acid binding activity is sensitive to competitive inhibitors. Other enveloped viruses that use sialic acid as a receptor determinant for binding to cells contain a receptor-destroying enzyme, a neuraminidase or an acetylsterase (8). This enzyme is able to inactivate inhibitors. These findings do not exclude a role for sialic acid binding activity in the attachment of TGEV to intestinal cells. However, an alternative explanation for the importance of sialic acid binding activity should also be considered. Coronaviruses like TGEV are exceptional among enterotropic viruses because they contain a lipid envelope. How TGEV can cope with the unfavorable conditions in the alimentary tract is not known. One possibility is that sialic acid binding activity may increase the stability of the virions by the binding of virus to sialoglycoconjugates. An increased stability would help virions to survive the passage through the gastrointestinal tract.

(Part of this work was done by C.K. in partial fulfillment of the requirements for the Dr. rer. physiol. degree at Philipps-Universität Marburg, Fb 20.)

The technical assistance of Anja Heiner and Lydia Besnardeau is gratefully acknowledged.

Financial support was provided by Deutsche Forschungsgemeinschaft (He1168/2-3).

REFERENCES

- Bernard, S., and H. Laude. 1995. Site-specific alteration of transmissible gastroenteritis virus spike protein results in markedly reduced pathogenicity. *J. Gen. Virol.* **76**:2235-2241.
- Correa, I., G. Jimenez, C. Sune, M. J. Bullido, and L. Enjuanes. 1988. Antigenic structure of E2-glycoprotein of transmissible gastroenteritis coronavirus. *Virus Res.* **10**:77-94.
- Cox, E., M. B. Pensaert, P. Callebaut, and K. van Deun. 1990. Intestinal replication of a porcine respiratory coronavirus closely related antigenically to the enteric transmissible gastroenteritis virus. *Vet. Microbiol.* **23**:237-243.
- Delmas, B., J. Gelfi, and H. Laude. 1986. Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer protein. *J. Gen. Virol.* **67**:1405-1418.
- Delmas, B., D. Rasschaert, M. Godet, J. Gelfi, and H. Laude. 1990. Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike glycoprotein S. *J. Gen. Virol.* **71**:1313-1323.
- Delmas, B., J. Gelfi, R. L'Haridon, L. K. Vogel, H. Sjostrom, O. Noren, and H. Laude. 1992. Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* **357**:417-420.
- Delmas, G., J. Gelfi, H. Sjostrom, O. Noren, and H. Laude. 1993. Further characterization of aminopeptidase N as a receptor for coronaviruses. *Adv. Exp. Med. Biol.* **342**:293-298.
- Herrler, G., J. Hausmann, and H.-D. Klenk. 1995. Sialic acid as a receptor determinant of ortho- and paramyxoviruses, p. 315-336. *In* A. Rosenberg (ed.), *Biology of the sialic acids*. Plenum Press, New York, N.Y.
- Laude, H., J. M. Chapsal, J. Gelfi, S. Labiau, and J. Grosclaude. 1986. Antigenic structure of transmissible gastroenteritis virus. I. Properties of monoclonal antibodies directed against virion proteins. *J. Gen. Virol.* **67**:119-130.
- Laude, H., J. Gelfi, D. Rasschaert, and B. Delmas. 1988. Caractérisation antigénique du coronavirus respiratoire porcine à l'aide d'anticorps monoclonaux dirigés contre de la gastro-entérite transmissible. *J. Rech. Porcine* **20**:89-94.
- Noda, M., F. Koide, M. Asagi, and Y. Inaba. 1988. Physicochemical properties of transmissible gastroenteritis virus hemagglutinin. *Arch. Virol.* **99**:163-172.
- Noda, M., H. Yamashita, F. Koide, K. Kadoi, T. Omori, M. Asagi, and Y. Inaba. 1987. Hemagglutination with transmissible gastroenteritis virus. *Arch. Virol.* **96**:109-115.
- Pensaert, M., P. Callebaut, and E. Cox. 1993. Enteric coronaviruses of animals, p. 627-696. *In* A. Z. Kapikian (ed.), *Viral infections of the gastrointestinal tract*. Marcel Dekker, New York, N.Y.
- Rasschaert, D., M. Duarte, and H. Laude. 1990. Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. *J. Gen. Virol.* **71**:2599-2607.
- Sánchez, C. M., G. Jiménez, M. D. Laviada, I. Correa, C. Suné, J. B. María, F. Gebauer, C. Smerdou, P. Callebaut, J. M. Escribano, and L. Enjuanes. 1990. Antigenic homology among coronaviruses related to transmissible gastroenteritis virus. *Virology* **174**:410-417.
- Sánchez, C. M., G. Jiménez, A. Méndez, J. Dopazo, and L. Enjuanes. 1992. Genetic evolution and tropism of transmissible gastroenteritis coronaviruses. *Virology* **190**:92-105.
- Schultze, B., H. J. Gross, R. Brossmer, H.-D. Klenk, and G. Herrler. 1990. Hemagglutinating encephalomyelitis virus attaches to N-acetyl-9-O-acetylneuraminic acid-containing receptors on erythrocytes: comparison with bovine coronavirus and influenza C virus. *Virus Res.* **16**:185-194.
- Schultze, B., C. Krempl, M. L. Ballesteros, L. Shaw, R. Schauer, L. Enjuanes, and G. Herrler. 1996. Transmissible gastroenteritis virus, but not the related porcine respiratory coronavirus, has a sialic acid (N-glycolylneuraminic acid) binding activity. *J. Virol.* **70**:5634-5637.
- Wesley, R. D., R. D. Woods, and A. K. Cheung. 1991. Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. *J. Virol.* **65**:3369-3373.