MOLECULAR BASES OF TROPISM IN THE PUR46 CLUSTER OF TRANSMISSIBLE GASTROENTERITIS CORONAVIRUSES

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ABSTRACT

Transmissible gastroenteritis coronavirus (TGEV) infects both, the enteric and the respiratory tract of swine. S protein, that is recognized by the cellular receptor, has been proposed that plays an essential role in controlling the dominant tropism. The genetic relationship of S gene from different enteric strains and non-enteropathogenic porcine respiratory coronaviruses (PRCVs) was determined. A correlation between tropism and the genetic structure of the S gene was established. PRCVs, derived from enteric isolates have a large deletion at the N-terminus of the S protein. Interestingly, two respiratory isolates, attenuated Purdue type virus (PTV-ATT) and Toyama (TOY56) have a full-length S gene. PTV-ATT has two specific amino acid differences with the S protein of the enteric viruses. One is located around position 219, within the deleted area, suggesting that alterations around this amino acid may result in the loss of enteric tropism.

To study the role of different genes in tropism, a cluster of viruses closely related to PUR46 strain was analyzed. All of them have been originated by accumulating point mutations from a common, virulent isolate which infected the enteric tract. During their evolution these viruses have lost, virulence first, and then, enteric tropism. Sequencing analysis proved that enteric tropism could be lost without changes in ORFs 3a, 3b, 4, 6, and 7, and in 3′-end untranslated regions (3′-UTR). To study the role of the S protein in tropism recombinants were obtained between an enteric and a respiratory virus of this cluster. Analysis of the recombinants supported the hypothesis on the role in tropism of S protein domain around position 219.

INTRODUCTION

TGEV, infects both, the enteric and the respiratory tract of swine. TGEV must attach to host cells through the S glycoprotein, since monoclonal antibodies (MAbs) specific for

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the S glycoprotein, but not MAbs specific for the N or M proteins inhibit the binding of the virus to ST cells[16]. Then it should be expected that S protein plays an important role in the control of dominant tropism. In fact, there are data from different laboratories establishing a correlation between the S protein gene structure and tropism. PRCVs have been originated, independently, in Europe[12, 3] and in North America[18], from enteric isolates[14]. PRCVs show a large deletion at the N-terminus of the S protein, including the antigenic sites C and B[14]. Interestingly, there are two respiratory isolates, PTV-ATT and TOY56, with a full-length S gene, without deletion. The S genes of these viruses have been sequenced and compared to S genes from the enteric isolates. In PTV-ATT, only two nucleotide differences, leading to two specific amino acid changes were found. One was located around amino acid 219, within the deleted area, while the other was outside[14]. Our laboratory has proposed that alterations around position 219 may be responsible for the loss of enteric tropism in these viruses.

However, it cannot be excluded that other genes could be involved in tropism. ORF3 has accumulated a large number of nucleotide changes among enteric isolates and PRCVs, including both, insertions and deletions[13, 2, 17, 9]. Most important, ORF3a is expressed in the enteric isolates but not in PRCVs, due to a nucleotide change in the ORF3a consensus region. In contrast, in the nearest genes 3b and 4, there are no specific mutations between enteric viruses and PRCVs. To analyze the role of different genes in tropism a cluster of viruses closely related to the PUR46 strain was studied.

MATERIALS AND METHODS

Cells and Viruses

All viruses were grown on swine testis (ST) cells[11]. The characteristics of TGEV strains: PUR46-SW11[6], provided to us by M. Pensaert and PUR46 [1, 15, 14], have been described. PTV-ATT, a Purdue type virus, was previously named NEB72. Due to the relationship between NEB72 and other TGEVs in the epidemiological tree developed[14] and to its sequence homology with the PUR46 isolate we have renamed this virus strain. Both, PTV-ATT and a temperature sensitive mutant, PTV-ATT*ts*, derived from it, were kindly provided by M. Welter and L. Welter. PTV*tsdmar* was derived from PTV-ATT*ts*. This virus is temperature sensitive and has modified the antigenic subsites Aa and Ab of the S protein.

Recombinants were obtained by coinfection with two parental strains: respiratory PTV*tsdmar* and enteric PUR46. The progeny was selected using MAbs and restrictive temperature.

RNA Analysis

RNA was sequenced by different procedures: directly from viral RNA[5] or deriving cDNA fragments by RT-PCR; cDNA fragments were sequenced using *fmol* system (Promega) or cloned into pBluescript to be sequenced using the *Sequenase* kit (USB).

To analyze a point mutation in nucleotide 655 of S gene, cDNA fragments containing the mutated position were RT-PCR derived and their susceptibility to *Bsm*AI restriction endonuclease enzyme studied.

Virus Tropism

Tropism was studied in conventional, non-colostrum-deprived, newborn mini-swine. Piglets were orally inoculated by stomach tube and slaughtered at 24, 48, and 72 hours

post-infection. Animal room was kept at 22°C. Lungs and small intestine were collected and homogenized in PBS. A sample was separated and virus titer determined by plaque-assay[7].

RESULTS

To study the role of different genes in tropism a cluster of closely related viruses was studied (Fig. 1). All of them have been originated, by accumulating point mutations, from a common and virulent, ancestor PUR46-SW11 that infects the enteric tract. All these isolates have, as a trade mark, a small deletion of six nucleotides in the S gene. These viruses have evolved, losing their virulence (PUR46) and their enteric tropism (PTV-ATT and PTV*tsdmar*).

ORFs 2, 3a, 3b, 4, 6, and 7 and the 3′-UTR were sequenced in PUR46, PTV-ATT and PTV*tsdmar*. Sequence comparison of the genomes of the enteric isolate PUR46 and its respiratory derivative PTV-ATT showed that no nucleotide was changed in 3′-UTR and in ORFs 7, 6, and 4. In ORFs 3a and 3b only two nucleotide differences were found. One was located in the non-coding region between the two genes, and the other in ORF3b. Viruses of this cluster do not express ORF3b because they have a point mutation in the consensus region. These data show that, in contrast to what has been previously suggested[17], enteric tropism may be lost without changes in ORF3a.

Between the S genes of PUR46 and PTV-ATT, only two nucleotides have mutated leading to two specific amino acid changes. One was nucleotide 655, located within the deleted area of the PRCVs. The other was nucleotide 2098. To determine if these positions were involved in tropism, ST cells were coinfected with the enteric PUR46 and the respiratory PTV*tsdmar*. Recombinants were obtained having either, both or only one nucleotide changed. The recombinant isolation frequency was estimated to be very low

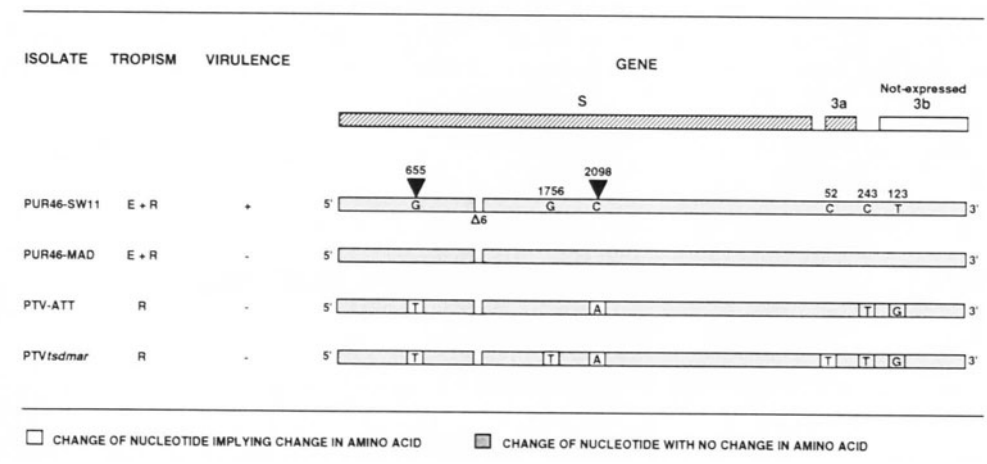

Figure 1. Summary of nucleotide changes in genes S, 3a, and 3b of PUR46-related viruses. Genes 2, 3a, and 3b were sequenced and compared in the cluster of PUR46-related viruses, including PUR46-SW11, PUR46-MAD, PTV-ATT, and PTV*tsdmar* isolates. Bars represent the studied genes. White and dotted squares indicate the position of nucleotide causing a change or no modification, respectively, in the amino acid sequence. Letters represent nucleotides. Numbers above these letters indicate the location of nucleotides referring to the ATG of each gene. Δ6 indicates a deletion of 6 nucleotides, in relation to the sequence of the MIL65 strain. Virus tropism is indicated with E+R when virus infects both, the enteric and the respiratory tract, and with R when virus only infects the respiratory tract. Virulence is designed by + if virus kills piglets or by - when not, in the assay conditions described in Materials and Methods.

Table 1. Phenotypic characterization of potential recombinants between enteric PUR46 and respiratory PTV*tsdmar*

Recombinant group	Neutralization index (MAbs)	Inactivation index (temperature)	Number of clones	%
PUR46*wt*	3.7±0.3	0.5±0.3	0	—
PTV*tsdmar*	0.5±0.3	3.0±0.2	3	9.7
Group 1	3.7±0.3	3.0±0.2	0	—
Group 2	0.5±0.3	0.5±0.3	15	48.4
Group 3	1.2>NI>0.5	2.4>II>0.5	13	41.9

($<1.0 \times 10^{-6}$) in this cluster of viruses. The progeny phenotype was determined (Table 1). Recombinants were classified in three groups according to their resistance to MAbs and restrictive temperature. Selection was performed against group 1 (sensitive to both, neutralization by MAbs and inactivation at restrictive temperature) and, in fact, no virus with this phenotype was isolated. Group 3 contained viruses having an intermediate phenotype between the two parental strains. Almost 50% of the progeny showed the expected recombinant phenotype (group 2), resistant to both, neutralization by MAbs and inactivation at restrictive temperature.

To genotypically characterize group 2 recombinants two nucleotide differences between the S genes of the two parental viruses were used. According to the position of the crossing-over, recombinants were split up into two groups (Fig. 2). Group 2A had recombined in the S gene, between the two nucleotide differences, taking position 655 from the enteric parental (PUR46), while nucleotide 2098 from the respiratory one (PTV*tsdmar*). The mutation responsible for the lack of antigenic subsites Aa and Ab had been previously mapped at nucleotide 1756 of ORF2. The crossing-over of this group of recombinants was located between nucleotides 655 and 1756. A high number of the analyzed recombinants, more than 40%, showed this structure. Group 2B had recombine 5′ upstream of the S gene, although the exact position of the crossing-over has not yet been mapped. Group 2B

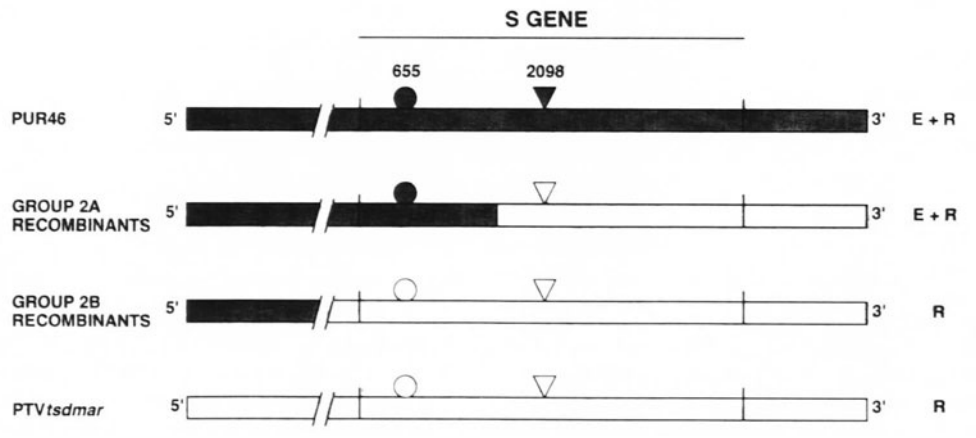

Figure 2. Genetic structure of the recombinants obtained by crossing the enteric PUR46 and the respiratory PTV*tsdmar*. Full and empty bars indicate sequences coming from PUR46 or PTV*tsdmar*, respectively. The nucleotide difference of position 655 is marked by a circle and the difference at position 2098 by a triangle. Two Kb of ORF1b 3′-end were sequenced.

recombinants has taken the entire protein from the respiratory parental virus. If the predicted role of the S protein domain around amino acid 219 in tropism is correct, group 2A recombinants should infect the enteric tract, while those of group 2B should be respiratory. The tropism of PUR46-cluster of viruses, as well as group 2A of recombinants was tested. All these viruses could efficiently replicate in lungs. PUR46-SW11 and PUR46 could be isolated in the enteric tract, while PTV-ATT and PTV*tsdmar* were not, as expected. Recombinants of group 2A were able to infect the small intestine. These results support the hypothesis that the area around position 219 of the S protein, taking in this group from the enteric parental strain, is involved in tropism.

DISCUSSION

PUR46-cluster of viruses does recombine. The estimated frequency of recombinants isolation ($<1.0 \times 10^{-6}$ per 10^3 nucleotides) is lower than that described for murine coronaviruses[10]. Due to this low frequency, selective pressure has to be used to eliminate parental viruses in order to isolate recombinants. The use of selective markers mapping in distal parts of the genome increases the distance to recombine. The markers that were used to select the recombinants (*dmar* and *ts* mutations) were located, at least, at 4 kb from each other. Nevertheless, 40% of the isolates that were analyzed have recombined within nucleotides 655 and 1756 of the S protein. This concentration of recombinants having the crossing-over in this region could be due either, to a high frequency of recombination in the area, or to a selective advantage of viruses having this genome.

The respiratory PTV-ATT was originated from the enteric PUR46. Sequencing analysis has shown that enteric tropism has been lost in PTV-ATT without changes neither in 3′-UTR nor in the proteins encoded by ORFs 3a, 3b, 4, 6, and 7, when compared to PUR46 strain. Although in this cluster of viruses enteric tropism could be lost without changes in these genes, it does not imply that mutations affecting ORFs 7, 6, 4, 3b, and 3a could not lead to a change of tropism. In fact, several laboratories have reported data suggesting a possible involvement of ORF3a in TGEV enteropathogenicity[9] and attenuation.

The tropism of group 2A recombinants strongly suggests that the area around nucleotide 655 of the S gene is involved in the enteric tropism of this cluster of viruses. To make a definitive statement group 2B recombinants has to be further characterized. S protein plays an essential role in the attachment of virions[16, 14] and in the fusion of viral and cellular membranes[9]. Aminopeptidase N (APN) is known to act as a major receptor for TGEV in cell culture[4]. MAbs specific for the antigenic sites A and D are the best inhibitors of virus binding to ST cells, suggesting that the domain recognized by the cellular receptor on ST cells must be located spatially close to these sites[16]. PRCVs, that have a large deletion at the 5′-end of the S gene including the area around nucleotide 655, may also use APN to entry into cells[9]. In fact, binding of APN to both TGEV and PRCVs is mediated by residues located between amino acids 500-800 of S protein[8]. All these data indicate that the viruses of this cluster having nucleotide 655 mutated which do not infect the enteric tract still recognize APN. Different mechanisms could be proposed to explain how the area around position 219 of the S protein is involved in tropism. One possibility is the presence of a second cellular receptor needed for *in vivo* virus entry into cells of the enteric tract. The receptor binding-site would map around position 219 in the S protein. Another possibility is, as proposed for MHV[19], the need of a spike protein-dependent cellular factor, other than a virus receptor, required for a productive virus cycle in the enteric tract. The cellular factor would interact with the spike protein in the area around amino acid 219. The analysis of group 2 recombinants will be very helpful to explain the role of S protein in tropism.

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