ELSEVIER

Gene 179 (1996) 211–218

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

The major subunit ClpG of *Escherichia coli* CS31A fibrillae as an expression vector for different combinations of two TGEV coronavirus epitopes

Marie-Claire Méchin, Maurice Der Vartanian *, Christine Martin

Laboratoire de Microbiologie, Institut National de la Recherche Agronomique, Centre de Recherches de Clermont-Ferrand-Theix, 63122 Saint-Genès-Champanelle, France

Received 16 October 1995; revised 25 March 1996; accepted 2 April 1996

Abstract

Previously, two B-cell epitopes from the entero-pathogenic transmissible gastroenteritis virus (TGEV), namely the C epitope (TGEV-C) amino acids (aa) 363–371 and the A epitope (TGEV-A) aa 522–531 of the spike S protein (TGEV-S), have been separately expressed on the CS31A fibrillae at the surface of *Escherichia coli* following insertion into a same region of ClpG. However, the resulting chimeras induced a marginal TGEV-neutralizing antibody (Ab) response in mice. Here, with the view to improving this response, we introduced TGEV-C alone or in different tandem association with TGEV-A (A::C or C::A) in twelve putatively exposed regions of ClpG. Among the 28 resulting engineered proteins only 15, carrying up to 51 extra aa, had not essentially disturbed the correct CS31A fibrillae formation process. Six partially permissive sites accepting only TGEV-C and three highly permissive sites tolerating A::C or C::A tandem peptide, were identified throughout ClpG. Intact bacteria or extracted CS31A hybrid fibrillae expressing TGEV epitopes at any of the permissive sites, were recognized by Ab directed against the foreign parent protein, providing a direct argument for exposure of the corresponding ClpG region at the cell surface and for antigenicity of the epitopes in the polymeric CS31A fibrillae context. The potential of CS31A fibrillae as carriers of the TGEV peptides indicates that there may be three positions (N terminus, aa 202–204 and 202–218) in ClpG which may turn out to be important fusion sites and therefore be relevant for the eventual design of TGEV vaccines. Unexpectedly, TGEV-A, whatever its position in ClpG, mediated the partial proteolytic degradation of the hybrid proteins, suggesting that it functions as a substrate for a cellular protease, and thereby that its suitability as a vaccine antigen candidate is doubtful.

Keywords: Recombinant DNA; Genetic fusion; Hybrid protein; Tandem insertion; Transmissible gastroenteritis virus; Peptide presentation; Surface exposure

1. Introduction

Epitope-based recombinant vaccine technologies offer the potential for oral or mucosal delivery, especially when the relevant epitope genetically fused to a carrier protein, is displayed as a heterologous peptide on the surface of a bacterial strain (Rabinovich et al., 1994). However, coupling foreign peptides to carriers can result in a poor immunogenicity of the chimeric antigens due to the local conformational restrictions imposed on epitopes by the embedding structure (Benito et al., 1995). Therefore, approaches to enhancing immunogenicity are critical. Since the conformation of a foreign sequence within a carrier varies widely depending on the flanking sequences (Tishminetzky et al., 1994), the insertion of an antigen into different exposed regions of a delivery protein, thus changing antigen conformation, could permit a strategy for finding appropriate environments for peptide presentation among the complex assortment of molecular contexts offered by the carrier. A second strategy to improving immunogenicity is the fusion of

* Corresponding author. Tel.: +33 4 73624243; Fax: +33 4 73624581; e-mail: dvartan@clermont.inra.fr

Abbreviations: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); BSA, bovine serum albumin; ClpG, major CS31A fibrillar subunit; *clpG*, gene encoding ClpG; kb, kilobase(s) or 1000 bp; LB, Luria-Bertani (medium); mAb monoclonal Ab; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; pAb, polyclonal Ab; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (0.14 M NaCl/2.7 mM KCl/1.47 mM KH_2PO_4/20 mM Na_2HPO_4, pH 7.4); SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline (0.15 M NaCl/10 mM Tris-HCl, pH 7.4); TGEV, transmissible gastroenteritis virus; TGEV-A, peptide/site/epitope A of TGEV-S; TGEV-C, peptide/site/epitope C of TGEV-S; TGEV-S, spike S glycoprotein of TGEV; wt, wild type; ::, novel junction (fusion or insertion); [ ], denotes plasmid-carrier state.

0378-1119/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
PII S0378-1119(96)00348-4

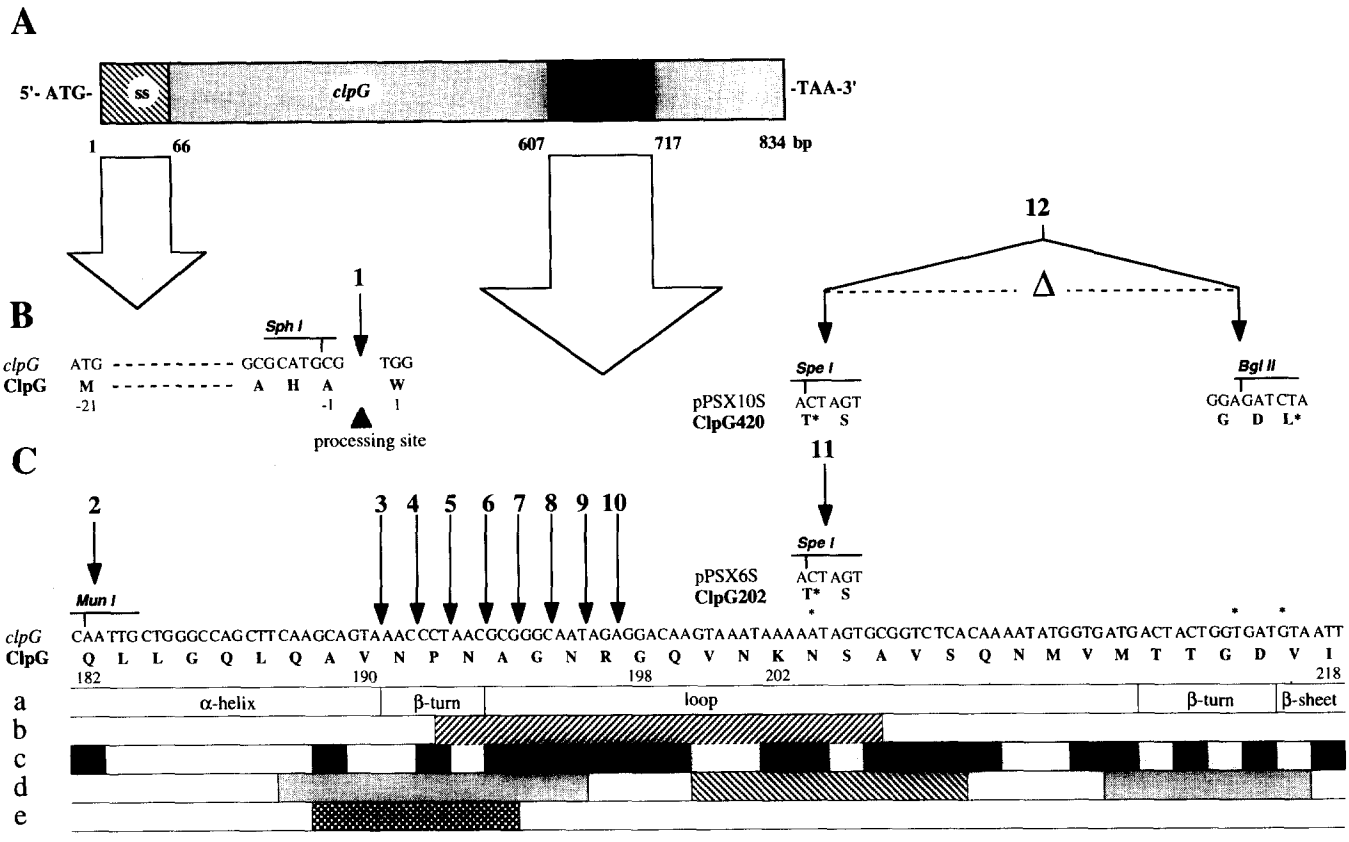

Fig. 1. Construction strategy of the ClpG hybrids. (A) Overview of the *clpG* gene. *ss*, signal sequence. (B and C) Distribution and accurate location of insertion sites in the nucleotide (nt) sequence parts of *clpG* corresponding to the aa −21 to 1 (B) and aa 182 to 218 (C) of the ClpG precursor. The one-letter code for aa designation is used. Numbered vertical arrows above the nt sequence indicate the number and position of the twelve selected insertion sites. Asterisks denote the nt modified by site-directed mutagenesis and the corresponding mutated aa residue. The resulting engineered restriction sites *Spe*I and *Bgl*II in plasmids pPSX6S and pPSX10S (Bousquet et al., 1994), coding for mutants ClpG202 and ClpG420, are mentioned. (a–e) in panel C represent (a) predicted secondary structured, (b) hydrophilic (hatched box), (c) variable (black boxes) or conserved (open boxes) (Girardeau et al., 1991; Méchin et al., 1995), (d) accessible (dotted and hatched boxes) and immunodominant (dotted boxes) domains of ClpG, and (e) the only natural, continuous, immunodominant, accessible-surface epitope of ClpG (chequered box) capable of binding with Ab directed against the native CS31A fibrillum (Méchin, unpublished). *Δ*, deletion:insertion into site 12 = substitution. Site-directed mutagenesis was either accomplished on single-stranded DNA according to a gapped-duplex DNA method (Stanssens et al., 1989), or performed on double-stranded DNA using an adapted protocol from the methods of Jung et al. (1992) and Deng and Nickoloff (1992). Mutations were verified by DNA sequencing (Sanger et al., 1977). The oligos used in this study were synthesized and, when necessary, PAGE-purified and 5′-phosphoryled (Eurogentec, Belgium); in the oligos shown below, names are indicated, numbers in parentheses refer to nt position in the coding strand of the *clpG* gene (see C), and bold characters represent mutations resulting of the *Spe*I site introduction: VN1, (559)CTTCAAGCAGTA**ACTAGT**AACCCTAACGCG(582); NP2, (562)CAAGCAGTAAAC**ACTAGT**CCTAACGCGGGC(585); PN3, (565)GCAGTAAACCCT**ACTAGT**AACGCGGGCAAT(588); NA4, (568)GTAAACCCTAAC**ACTAGT**GCGGGCAATAGA(591); AG5, (571)AACCCTAACGCG**ACTAGT**GGCAATAGAGGA(594); GN6, (574)CCTAACGCGGGC**ACTAGT**AATAGAGGACAA(597); NR7, (577)AACGCGGGCAAT**ACTAGT**AGAGGACAAGTA(600); RG8, (600)GCGGGCAATAGA**ACTAGT**GGACAAGTAAAT(603).

tandem peptides to immunogenic carrier proteins (Broekhuijsen et al., 1986; Martineau et al., 1992; Khan et al., 1994). A third strategy might be the insertion of epitopes in an accessible surface region previously observed as a natural immunodominant site on the native carrier molecule.

Here, we developed these different approaches simultaneously by using the major ClpG subunit of the *E. coli* CS31A fibrillae as the carrier protein (Bousquet et al., 1994; Der Vartanian et al., 1994), and two B-cell epitopes from TGEV consisting of the site C (aa 363–371) and site A (aa 522–531) of TGEV-S as the foreign antigenic determinants (Delmas et al., 1990; Gebauer et al., 1991). The continuous site C elicits neutralizing Ab and the site A is part of highly immunogenic conformational antigenic region (Delmas et al., 1990; Correa et al., 1990). TGEV-A or TGEV-C inserted in the position aa 202–218 of ClpG was previously shown to induce a low neutralizing Ab response in mice (Bousquet et al., 1994). The aim of this work was to explore recombinant ClpG::TGEV proteins for the display of different combinations of the two TGEV peptides in particular contexts that could result in improved epitope performance, and thus allow the design of vaccine antigens. For this purpose, we examine the permissivity (Charbit et al., 1991) of 12 sites of ClpG by

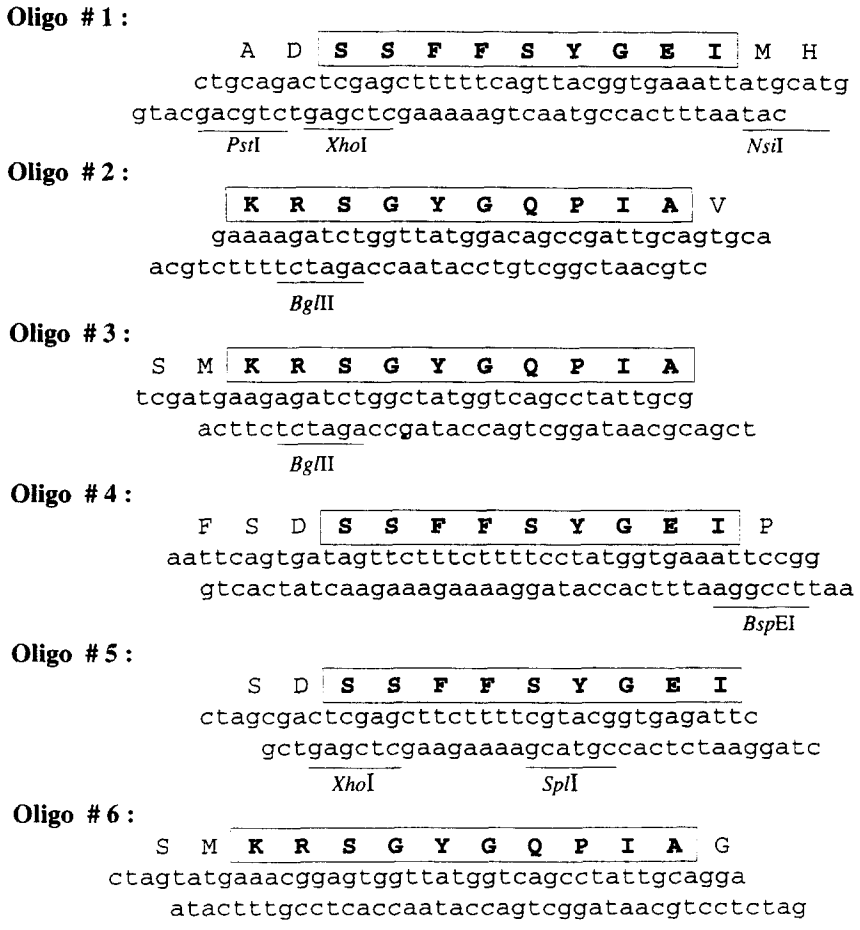

Fig. 2. Foreign sequences used in this study for the construction of the chimeric *clpG* genes. The six double-stranded synthetic oligos coding for TGEV-C (oligos #1, #4 and #5) and TGEV-A (oligos #2, #3 and #6) of TGEV-S of the porcine Purdue-115 strain of TGEV are shown. The bolded aa residues correspond to the residues 361–371 and 522–531 of TGEV-S, spanning the sites C and A, respectively. Restriction sites are underlined.

introducing TGEV-C alone or in tandem with TGEV-A in these sites and by investigating the influence of the resulting modifications on the expression of CS31A fibrillae. We discuss the nature of permissive sites and the positioning and tandem insertion effects of the epitopes.

2. Experimental and discussion

2.1. Construction of ClpG::TGEV hybrid proteins

Twelve sites within ClpG were selected for insertion of TGEV peptides (Fig. 1). One of them is located at the N terminus (Fig. 1B), and the others are distributed along the aa 182–218 region (Fig. 1C). This region contains a variable flexible loop structure (aa 190–217) carrying a hydrophilic domain and several accessible continuous immunodominant epitopes, one of which (aa 189–194) is exposed on the native ClpG subunit at the surface of the polymeric CS31A fibrillae (Fig. 1C). For these reasons, we hypothesized that the region aa 182–218 is naturally favorable to the presentation of the TGEV epitopes.

To construct insertion plasmid vectors as a preliminary step for viral epitopes insertion in the selected regions aa 190–198 and 202–218 of ClpG (Fig. 1C), the corresponding *clpG* sequences were submitted to oligodeoxyribonucleotide (oligo) site-directed mutagenesis to create unique restriction sites at different positions within these sequences. Thus, a *Spe*I site was independently engineered after each codon expressing every one of aa composing the region aa 190–198 (Fig. 1C), resulting in the addition of the dipeptide threonine-serine (TS) (Fig. 3). The aa 202–218 peptide-encoding sequence was 5′-ended by a *Spe*I site and 3′-ended by a *Bgl*II site after two rounds of mutagenesis which induced changes in ClpG (Fig. 1C, mutants ClpG202 and ClpG420); the first round allows insertions into the site aa 202–204 and the second the replacement of the region aa 203–217. The strategy of ClpG::TGEV hybrids construction was to use these engineered restriction sites, and two naturally occurring sites, *Sph*I (Fig. 1B) and *Mun*I (Fig. 1C), to introduce suitable TGEV peptides-encoding oligos

Site[a]	Plasmid[b]	ClpG mutant[c]	Mutation[d]	Insert size[e]	CS31A[f]
1	pGisa 226	ClpG1-C	-1 A **AD** ▨ **MHA** W 1	14	+
	pGCA 41155	ClpG1-CA	-1 A **AD** ▨ **MQ** ■ **VHA** W 1	26	+
	pGAC 524	ClpG1-AC	-1 A **ADSM** ■▨ **MHA** W 1	26	+
2	pCO6	ClpG182-C	182 Q **FSD** ▨ **PE** L 183	14	-
3	pVN1	ClpG190	190 V **TS** N 191	2	+
	pVN1C	ClpG190-C	190 V **TSD** ▨ **PS** N 191	14	-
	pVN1AC	ClpG190-AC	190 V **TSDSM** ■▨ **PS** N 191	26	-
	pVN1XC	ClpG190-XC	190 V **TSDS** □▨ **PS** N 191	26	-
4	pNP2	ClpG191	191 N **TS** P 192	2	-
	pNP2C	ClpG191-C	191 N **TSD** ▨ **PS** P 192	14	-
	pNP2XC	ClpG191-XC	191 N **TSDS** □▨ **PS** P 192	26	-
5	pPN3	ClpG192	192 P **TS** N 193	2	+
	pPN3C	ClpG192-C	192 P **TSD** ▨ **PS** N 193	14	+
	pPN3AC	ClpG192-AC	192 P **TSDSM** ■▨ **PS** N 193	26	-
	pPN3XC	ClpG192-XC	192 P **TSDS** □▨ **PS** N 193	26	-
6	pNA4	ClpG193	193 N **TS** A 194	2	+
	pNA4C	ClpG193-C	193 N **TSD** ▨ **PS** A 194	14	+
	pNA4AC	ClpG193-AC	193 N **TSDSM** ■▨ **PS** A 194	26	-
7	pAG5	ClpG194	194 A **TS** G 195	2	+
	pAG5C	ClpG194-C	194 A **TSD** ▨ **PS** G 195	14	+
	pAG5XC	ClpG194-XC	194 A **TSDS** □▨ **PS** G 195	26	-
8	pGN6	ClpG195	195 G **TS** N 196	2	+
	pGN6C	ClpG195-C	195 G **TSD** ▨ **PS** N 196	14	+
	pGN6AC	ClpG195-AC	195 G **TSDSM** ■▨ **PS** N 196	26	-
	pGN6XC	ClpG195-XC	195 G **TSDS** □▨ **PS** N 196	26	-
9	pNR7	ClpG196	196 N **TS** R 197	2	+
	pNR7C	ClpG196-C	196 N **TSD** ▨ **PS** R 197	14	+
	pNR7AC	ClpG196-AC	196 N **TSDSM** ■▨ **PS** R 197	26	-
10	pRG8	ClpG197	197 R **TS** G 198	2	+
	pRG8C	ClpG197-C	197 R **TSD** ▨ **PS** G 198	14	+
11	pGC326	ClpG202-C	202 K **TSD** ▨ **P** S 204	13	+
	pGAC326	ClpG202-AC	202 K **TSDSM** ■▨ **P** S 204	25	+
	pGXC326	ClpG202-XC	202 K **TSDS** □▨ **P** S 204	25	+
12	pGA102	ClpG203-A	202 K **TSM** ■ GD**L** I 218	13	+
	pGCA102	ClpG203-CA	202 K **TSD** ▨ **PSM** ■ GD**L** I 218	25	+
1 + 12	pDEV2CA	ClpG1/203-CA	-1 A **AD** ▨ **MQ** ■ **VHA** W 1 ⌐ // ⌐ 202 K **TSD** ▨ **PSM** ■ GD**L** I 218	51	+

Fig. 3. Engineering of viral epitopes in ClpG carrier. (a) Site refers to the number and the location of the twelve selected insertion sites as shown and defined in Fig. 1 and its legend. (b) Plasmids were constructed as follows: pGC326 was made by inserting the oligo #5 (Fig. 2) comprising unique *Xho*I site with *Spe*I-compatible ends into the *Spe*I site of pPSX6S (see Fig. 1 and its legend); pGAC326 and pGXC326 were constructed by inserting the oligo #3 (Fig. 2) with *Xho*I-compatible ends into the *Xho*I site of pGC326, resulting in the addition of the oligo #3 in the correct and in-frame reverse orientations, respectively; pGA102 (Bousquet et al., 1994) was made by replacing the 38-bp *Spe*I–*Bgl*II fragment from pPSX10S (see Fig. 1 and its legend) with oligo #6 (Fig. 2) having 5′-*Spe*I and *Bgl*II-3′ compatible ends; pGCA102 was constructed by inserting the oligo #5 containing unique *Xho*I site with *Spe*I-compatible ends into the *Spe*I site of pGA102; pGisa226 was engineered by cloning the oligo #1 (Fig. 2) comprising unique *Xho*I site with *Sph*I-flanked ends into the unique *Sph*I site of pDSPH524 (Der Vartanian et al., 1994); pGAC524 was obtained by inserting the oligo #3 with *Xho*I-compatible ends into the *Xho*I site of pGisa226; pGCA41155 was constructed in two steps from pDEV41155 (Der Vartanian et al., 1994): in the first step, the oligo #1 including unique *Nsi*I site with *Sph*I-flanked ends was cloned into the *Sph*I site of *clpG* (Fig. 1B) and in the second step, the *Nsi*I site in oligo #1 allowed the insertion of oligo #2 (Fig. 2) with *Nsi*I compatible ends downstream of oligo #1; pCO6 was made from pDEV41155 by inserting the oligo #4 (Fig. 2) with *Mun*I-compatible ends into the *Mun*I site of *clpG* (Fig. 1C); pVN1, pNP2, pPN3, pNA4, pAG5, pGN6, pNR7 and pRG8 were constructed from pDEV41155 as follows: a *Spe*I site within *clpG* was introduced after each codon expressing every one of aa residues covering the region 190–197 of ClpG (Fig. 1C) by in vitro mutagenesis using oligos VN1, NP2, PN3, NA4, AG5, GN6, NR7 and RG8 (Fig. 1 legend); pVN1C, pNP2C, pPN3C, pNA4C, pAG5C, pGN6C, pNR7C and pRG8C were obtained by inserting the oligo #5 containing *Xho*I site with *Spe*I compatible ends into the *Spe*I site of pVN1, pNP2, pPN3, pNA4, pAG5, pGN6, pNR7 and pRG8 respectively; pVN1AC, pPN3AC, pNA4AC, pGN6AC and pNR7AC were made by cloning the oligo #3 with *Xho*I-compatible ends into the *Xho*I site of pVN1C, pPN3C, pNA4C, pGN6C and pNR7C, respectively; pVN1XC, pNP2XC, pPN3XC, pAG5XC and pGN6XC were engineered from pVN1C, pNP2C, pPN3C, pAG5C and pGN6C, respectively, as indicated just above except that oligo #3 was inserted in-frame in the reverse orientation, as designated by the letter X; pDEV2CA was made by replacing a 0.3-kb *Mun*I–*Sac*I fragment from pGCA41155 with the 0.32-kb *Mun*I–*Sac*I fragment from pGCA102. (c) The presence ofTGEV-C and -A is specified by the letters C and A respectively; CA or AC,

(Fig. 2). All these constructions summarized in Fig. 3 are described in detail in the Fig. 3 legend. In total, 36 mutants were obtained. Twenty-eight of them contained at least one TGEV peptide, among which 10 had both TGEV-C and -A as an A::C or C::A fusion. Finally, modifications in ClpG resulted in an insert of 2, 13, 14, 25, 26 or 51 aa in length (Fig. 3).

2.2. Effect of the insertions on CS31A biogenesis

Each of the 36 mutant plasmids (Fig. 3) was transferred into *E. coli* DH5α bearing the *trans*-complementing plasmid pDSPH524. The cell-surface location of the corresponding hybrid ClpG subunits (Fig. 3) on CS31A fibrillae was determined on intact cells by in situ colony immunoblotting and from isolated mutated CS31A polymers by immunodot analysis using a CS31A-specific polyclonal Ab (pAb), the TGEV-C-specific 3b.5 mAb and the TGEV-A-specific 1AF10 mAb (not shown). Out of 28 recombinant CS31A fibrillae carrying at least one TGEV peptide only 13 failed to react whatever Ab. In contrast, the 15 remaining hybrids were capable of exposing the TGEV peptides at the cell surface on the correctly assembled CS31A fibrillae. Nine permissive were identified throughout ClpG. The positions aa −1/1, 202–204 and 202–218 appeared to be the most permissive targets since the largest insertions (25 or 26 aa), did not interfere with the CS31A fibrillae formation. Even the hybrid ClpG1/203-CA protein with an insert of 51 aa long, resulting from the simultaneous tandem addition of the two TGEV peptides in both positions aa −1/1 and 202–218, was incorporated in CS31A polymer. In positions aa 192 to 197 less residues (14 aa) was tolerated. Positions aa 182–183, 190–191 and 191–192 were nonpermissive since, except for ClpG190 with only two extra aa, no hybrid was detected. All nonpermissible insertions were located in or near a predicted α-helix structure, and targeted conserved aa residues (Fig. 1, aa 182–193). By contrast, all permissive sites, excluding the ClpG-N terminus, were included in a predicted loop (Fig. 1, aa 194–212) that is more likely to be flexible enough to accommodate large inserts, as indicated by the proposed ClpG topology (Méchin et al., 1995). While the two fully permissive sites (aa 202–204 and 202–218) were located between the top and the end of the loop, the six partially permissive sites (aa 192 to 197) targeted region immediately beginning this loop. These data suggest a relationship between the permissivity of ClpG and the local ClpG structures into which the epitope was inserted. On the basis of these results, we conclude that ClpG as a carrier is very flexible since insertions varying in length and nature can be made in different sites without affecting CS31A formation.

2.3. Characterization of the expressed ClpG::TGEV hybrids

Only hybrid ClpG subunits displaying at least one TGEV peptide on CS31A were characterized by Western immunoblot analysis (Fig. 4). Proteins ClpG192-C, ClpG193-C, ClpG194-C, ClpG195-C, ClpG196-C, Clp G197-C, ClpG202-XC, ClpG1-CA, ClpG1-C, GlpG1-AC and ClpG202-C migrated as a single band which corresponds to the expected full-length hybrid since revealed by anti-ClpG pAb, 3b.5 mAb and 1AF10 mAb (Fig. 4a, b, c). Mutants ClpG203-A, ClpG203-CA and ClpG202-AC showed two protein products, all reacting with anti-ClpG (Fig. 4a); their upper band detected by the three Ab represents the whole fusion molecule which was more abundant in ClpG203-CA. In ClpG203A and ClpG202-AC, the major lower band was recognized by only anti-ClpG while in ClpG203-CA the minor lower band was additionally by 3b.5 mAb (Fig. 4b). In ClpG1/203-CA the two uppermost bands were lighted whatever Ab (Fig. 4a, b, c) and the two lowermost bands only with anti-ClpG and 3b.5 Ab (Fig. 4a, b). These observations indicated that most of the hybrids carrying TGEV-A was subjected to an incomplete proteolysis which, however, did not prevent the CS31A formation (Fig. 3 and Section 2.2)). In a general way, ClpG hybrids containing TGEV-A reacted faintly with 1AF10 mAb (Fig. 4c).

---

peptides C and A in tandem; X, cryptic peptide encoded by oligo #3 (Fig. 2) inserted in-phase but in the reverse orientation. (d) aa changes resulting from the engineering of the TGEV epitopes-encoding oligos (Fig. 2) in ClpG: the numbers refer to the indicated first and last aa residues of the wild-type (wt) ClpG sequence; residues from the original ClpG protein are in small characters and additional residues are in large bold type. Hatched boxes, TGEV-C; black boxes, TGEV-A; open boxes, cryptic peptide X: TQQADHSQISS. (e) Total number of added aa with respect to wt ClpG. (f) CS31A fibrillae biogenesis: + and −, synthesis and no synthesis, respectively. *Methods*: The production of hybrid CS31A polymers was detected by in situ colony immunoblotting and immunodot analysis. For colony blots analysis, single colonies were streaked on a solid agar LB plate containing appropriate antibiotics. After overnight incubation at 37°C, a nitrocellulose filter (pore diameter, 0.45 μm; Schleicher and Schuell) was carefully applied on agar surface. Blots were blocked and then washed with 1% BSA-0.01% Tween 20 in PBS until the bulk of bacteria was removed. The filters were further incubated with appropriate primary Ab in 1% BSA in PBS. Bound primary Ab were detected by incubation of the filters with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary Ab, and developed with H_2O_2-α-chloronaphthol. For the immunodot analysis of the extracted mutated CS31A fibrillae, bacteria growing overnight on LB agar medium with the appropriate antibiotics were carefully scraped and suspended in PBS. This suspension was then vigorously agitated for 1 min with a top mix shaker, and placed at 60°C for 20 min (thermo-elution of the CS31A polymer). After centrifugation at 12 000 × *g* for 10 min, the resulting supernatant was used for experiments. CS31A fibrillae-specific rabbit antiserum (anti-CS31A) was obtained as described by Girardeau et al. (1988). mAb 3b.5 (Delmas et al., 1990) and mAb 1AF10 (Gebauer et al., 1991) raised, respectively, against the C and A sites of TGEV-S on native coronavirus, were used.

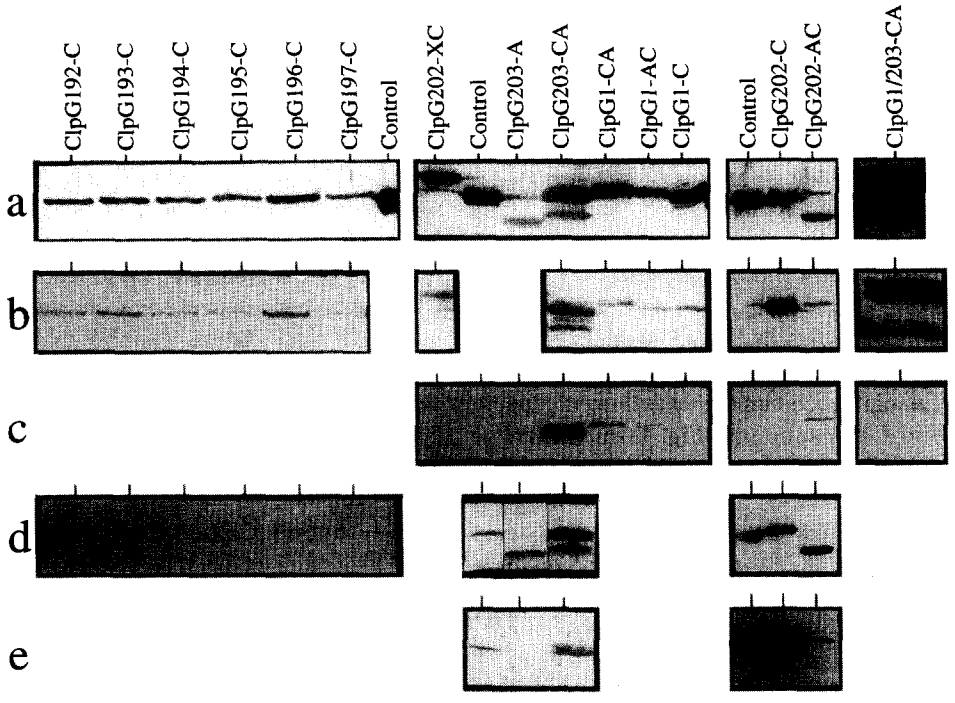

Fig. 4. Characterization of the ClpG::TGEV fusion proteins by Western immunoblotting. Samples of the thermo-eluted chimeric CS31A fibrillae (see Fig. 3 legend) were mixed with an equal volume of 2 × Laemmli buffer, boiled for 5 min, separated by 15% SDS-PAGE and semi-dry electro-transferred onto nitrocellulose sheets (Towbin et al., 1979). Control, wt CS31A fibrillae produced by *E. coli* DH5α [pDEV41155, pDSPH524] (Der Vartanian et al., 1994). Western blots were then treated as described in the Fig. 3 legend by using as primary Ab either (a) anti-ClpG pAb, or (b) 3b.5 mAb, or (c) 1AF10 mAb, or (d) anti-G15Q pAb, or (e) anti-T15P pAb. ClpG subunit-specific rabbit antiserum (anti-ClpG pAb) was obtained as described by Girardeau et al. (1988). The G15Q peptide (GQLQAVNPNAGNRGQ) and the T15P peptide (TFTNPVVSTTQWSAP) correspond to the aa residues 185–199 and 235–249 of ClpG, respectively. The synthetic G15Q and T15P peptides were obtained from Neosystem (Strasbourg, France) and their purity was >75% as determined by high-performance liquid chromatography. To prepare anti-G15Q and anti-T15P antisera, peptides G15Q and T15P were coupled to BSA using glutaraldehyde (Sigma); rabbits were primed by intradermic injection of 250 μg of peptide emulsified with incomplete Freund's adjuvant, then boosted 20 and 40 days later, and finally bled 15 days after the last immunization.

Although we have not sequenced the N- and C-terminal parts of cleaved products from ClpG::TGEV hybrids, several lines of evidence suggest that a proteolytic event occurs within the TGEV-A oligopeptide: (1) no truncated products from any of the hybrids containing one copy of TGEV-A was detected with 1AF10 mAb; (2) no proteolysis happened with ClpG alone or in association with TGEV-C; (3) ClpG202-XC chimera that differs from ClpG202-AC in only a TGEV-A sequence in reverse orientation, was not cleaved; (4) the immunoblot patterns of ClpG1/203-CA, carrying two copies of TGEV-A, are consistent with the presence of a cleavage site within each copy since four fragments were visualized. This implies that TGEV-A fused to the N terminus of ClpG was cleaved in a fashion similar to TGEV-A in the C-terminal part of ClpG. In this case, proteolytic cleavage would result in a N-terminal fragment too small (<20 aa) to be detected, that explaining why no truncated product was visualized from ClpG1-CA or ClpG1-AC (Fig. 4). To probe more precisely the N- and C-terminal parts of the truncated proteins generated by ClpG203-A, ClpG203-CA and ClpG202-AC we used two additional specific pAb, anti-G15Q and anti-T15P, that recognize the peptides G15Q (aa 185–199) and T15P (aa 235–249) in ClpG, respectively; insertions in G15Q sequence abolished the binding of anti-G15Q (Fig. 4d, mutants ClpG192-C to ClpG197-C). In these constructs, G15Q is placed 10 aa upstream from the ClpG::TGEV epitope fusion junction and T15P 20 to 30 aa downstream. Western immunoblots indicated that the intact ClpG203-CA and ClpG202-AC molecules reacted with both anti-G15Q and anti-T15P; in ClpG203-A the intact molecule is undetectable because too low in amount (Fig. 4d, e). By contrast, their degraded forms were probed by anti-G15Q but not by anti-T15P (Fig. 4d). Together with the facts that the cleaved form of ClpG203-CA was detected with 3b.5 mAb (Fig. 4b) but not with 1AF10 mAb (Fig. 4c), and that the truncated product of ClpG202-AC did not react with any of the two mAb (Fig. 4b, c), these data suggest that protease cleavage occurs within TGEV-A. Furthermore, the uncleaved form of ClpG203-CA was substantially more abundant than its truncated form (Fig. 4a). In contrast, ClpG203-A and ClpG202-AC showed a prominence in amount of the cleaved products, indicating that C::A fusion, rather than A::C fusion, significantly reduced cleavage process, probably due to the protective placement of the hydrophobic

TGEV-C motif at the N-terminal end of TGEV-A. Therefore, we speculate that the site of cleavage must be N-terminally located with respect to the TGEV-A sequence. Supporting this hypothesis, the two first aa residues of TGEV-A consist of lysine (K) and arginine (R) which are often involved in the proteolytic maturation of viral envelope glycoproteins (Moulard et al., 1995).

3. Conclusions

(1) Three nonpermissive sites (aa 182–183, 190–191 and 191–192), six partially permissive sites (aa 192 to 197) and three fully permissive sites (aa −1/1, 202–204 and 202–218) were identified throughout ClpG. The latter sites appeared as the most versatile targets since the largest insertions consisting of 26 áa residues did not interfere with CS31A biogenesis. Even an insert of 51 aa long resulting of the addition of the TGEV-C::TGEV-A tandem peptide in both positions aa −1/1 and 202–218 was normally incorporated in CS31A polymer. The binding of the TGEV-specific mAb with hybrid fibrillae indicates that the TGEV antigenic determinants expressed at any of the permissive sites are exposed on both ClpG and CS31A proteins at the *E. coli* cell-surface. We show here that the potential of ClpG as a carrier for foreign peptides may be influenced by the general predicted properties of the local sequences.

(2) Unlike TGEV-C, TGEV-A expressed at any permissive site of ClpG rendered the hybrid proteins partially susceptible to proteolytic degradation, indicating that it was influential in, but not critical for, fibrillae integrity, presumably because providing a protease cleavage site.

(3) CS31A fibrillum appears as a potent cell-surface presenting vector for TGEV epitopes. Such hybrid fibrillae might be valuable tools in the development as components of a subunit vaccine. Whether these hybrid fibrillae induce specific Ab is currently under investigation. The ClpG exposure vector system described here provides a number of insertion sites and therefore a variety of flanking aa sequences for the expression of TGEV epitopes. Consequently, a further study of the immunogenicity of the TGEV epitopes in different contexts of ClpG will improve our understanding of the relationship between flanking sequences and the immunogenicity of the epitopes.

Acknowledgement

The authors thank Y. Bertin, M. Chavarot, B. Gaillard-Martinie, B. Girard and B. Jaffeux for technical assistance, S. Dutilloy for secretarial assistance, Dr. L. Enjuanes and Dr. H. Laude for providing mAb 1AF10 and mAb 3b.5, respectively. This work was supported by grants AGRE-0008-C from European Economic Community (ECLAIR program).

References

Benito, A., Mateu, M.G. and Villaverde, A. (1995) Improved mimicry of a foot-and-mouth disease virus antigenic site by a viral peptide displayed on β-galactosidase surface. Bio/Technology 13, 801–804.

Bousquet, F., Martin, C., Girardeau, J.P., Méchin, M.C., Der Vartanian, M., Laude, H. and Contrepois, M. (1994) CS31A capsule-like antigen as an exposure vector for heterologous antigenic determinants. Infect. Immun. 62, 2553–2561.

Broekhuijsen, M.P., Blom, T., Ven Rijn, T., Pouwels, P.H., Klassen, E.A., Fasbender, M.J. and Enger-Valk, B.E. (1986) Synthesis of fusion proteins with multiple copies of an antiigenic determinant of foot-and-mouth disease virus. Gene 49, 189–197.

Charbit, A., Ronco, J., Michel, V., Werts, C. and Hofnung, M. (1991) Permissive sites and topology of an outer membrane protein with a reporter epitope. J. Bacteriol. 173, 262–275.

Correa, I., Gebauer, F., Bullido, M.J., Suné, C., Baay, M.F.D., Zwaagstra, K.A., Posthumus, W.P.A., Lenstra, J.A. and Enjuanes, L. (1990) Localization of antigenic sites of the E2 glycoprotein of transmissible gastroenteritis coronavirus. J. Gen. Virol. 71, 271–279.

Delmas, B., Rasschaaert, D., Godet, M., Gelfi, J. and Laude, H. (1990) Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike protein. J. Gen. Virol. 71, 1313–1323.

Deng, W.P. and Nickoloff, J.A. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. 200, 81–88.

Der Vartanian, M., Méchin, M.C., Jaffeux, B., Bertin, Y., Félix, I. and Gaillard-Martinie, B. (1994) Permissible peptide insertions surrounding the signal peptide-mature protein junction of the ClpG prepilin: CS31A fimbriae of *Escherichia coli* as carriers of foreign sequences. Gene 148, 23–32.

Gebauer, F., Posthumus, W.A.P., Correa, I., Suné, C., Sanchez, C.M., Smerdon, C., Lenstra, J.A., Meloen, R. and Enjuanes, L. (1991) Residues involved in the formation of the antigenic sites of the S protein of transmissible gastroenteritis coronavirus. Virology 183, 225–238.

Girardeau, J.P., Bertin, Y., Martin, C., Der Vartanian, M. and Boeuf, C. (1991) Sequence analysis of the clpG gene, which codes for surface antigen CS31A subunit: evidence of an evolutionary relationship between CS31A, K88 and F41 subunit genes. J. Bacteriol. 173, 7676–7683.

Girardeau, J.P., Der Vartanian, M., Ollier, J.L. and Contrepois, M. (1988) CS31A, a new K88-related fimbrial antigen on bovine enterotoxigenic and septicemic *Escherichia coli* strains. Infect. Immun. 56, 2180–2188.

Jung, R., Scott, M.P., Oliveira, L.O. and Nielsen, N.C. (1992) A simple and efficient method for the oligodeoxyribonucleotide-directed mutagenesis of double-stranded plasmid DNA. Gene 121, 17–24.

Khan, C.M.A., Villarreal-Ramos, B., Pierce, R.J., Demarco de Hormaeche, R., McNeill, H., Ali, T., Chatfield, S., Capron, A., Dougan, G. and Hormaeche, C.E. (1994) Construction, expression and immunogenicity of multiple tandem copies of the *Schistosoma mansoni* peptide 115–131 of the P28 glutathione *S*-transferase expressed as C-terminal fusions to tetanus toxin fragment C in a live Aro-attenuated vaccine strain of *Salmonella*. J. Immunol. 153, 5634–5642.

Martineau, P., Guillet, J.G., Leclerc, C. and Hofnung, M. (1992) Expression of heterologous peptides at two permissive sites of the

MalE protein: antigenicity and immunogenicity of foreign B-cell and T-cell epitopes. Gene 113, 35–46.

Méchin, M.C., Bertin, Y. and Girardeau, J.P. (1995) Hydrophobic cluster analysis and secondary structure predictions revealed that major and minor structural subunits of K88-related adhesins of *Escherichia coli* share a common overall fold and differ structurally from other fimbrial subunits. FEBS Lett. 364, 319–324.

Moulard, M. and Bahraoui, E. (1995) Role of proteolytic processing of viral envelope glycoproteins. Médecine/sciences 11, 73–80.

Rabinovich, N.R., McInnes, P., Klein, D.L. and Hall, B.F. (1994) Vaccine technologies: view to the future. Science 265, 1401–1404.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Stanssens, P., Opsomer, C., McKeown, Y.M., Kramer, W., Zabeau, H. and Fritz, H.J. (1989) Efficiency oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternative selectable markers. Nucleic Acids Res. 17, 4441–4454.

Tishminetzky, S.G., Scodeller, R.A., Evangelisti, P., Chen, Y., Schiappacassi, M., Porro, F., Bizik, F., Zacchi, T., Lunazzi, G., Miertus, S. and Baralle, F.E. (1994) Immunoreactivity of chimeric proteins carrying the HIV-1 epitope IGPGRAF: correlation between predicted conformation and antigenicity. FEBS Lett. 353, 1–4.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.