Successful mimicry of a complex viral antigen by multiple peptide insertions in a carrier protein

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Abstract The antigenic properties of a viral peptide from the surface of foot-and-mouth disease virus particles have been successfully mimicked by multiple insertion in solvent-exposed regions of *Escherichia coli* β-galactosidase. By increasing the number of viral peptides per enzyme monomer, the average IC_{50} of hybrid proteins in a competitive enzyme-linked immunosorbent assay) have decreased to values close to that presented by natural virions. Moreover, the antigenic diversity of these new recombinant enzymes when measured with different anti-virus antibodies has also been largely reduced, indicating a better presentation of the epitopes located in the viral peptide. Although bivalent antibody binding could have been favoured by multiple presentation, conformational modifications of the viral peptide, due to the presence of other insertions or a cooperative antibody binding cannot be excluded. In addition, a multidimensional antigenic analysis have grouped together the multiple-inserted proteins with the native virus, suggesting that increasing the number of insertions could be a good strategy to reproduce the antigenic properties of an immunoreactive peptide in a natural multimeric disposition.

Key words: Epitope tagging; Recombinant protein; β-Galactosidase; Antibody; Foot-and-mouth disease virus

1. Introduction

Small peptides reproducing continuous B-cell epitopes have been widely used as antigens due to their simplicity and innocuousness [1–4]. Although many of these peptides are recognised by monoclonal antibodies (MAb) elicited against the native protein, in general this binding is somewhat less efficient than where the peptide is placed in its natural location [5]. This decrease in antibody binding can be attributed to both the absence of large surface areas involved in antibody/antigen interaction [6–7] and the conformation adopted by the peptide in solution or absorbed to a solid phase [3], most often different to that presented in the protein. Several approaches with different results have been taken to mimic the structure of the peptide as in the intact protein, like cyclisation by a disulphide bridge or through lactam formation using linkers of different length [8–9].

A major immunodominant region of foot-and-mouth disease virus (FMDV) serotype C_1, also called site A, is located in a hypervariable region of capsid protein VP1, which corresponds to a segment of the G-H loop [10–11]. This viral stretch, spanning residues 138 to 150, is composed of multiple, continuous B-cell epitopes ([12] and references therein) and it is involved in virus-cell attachment through an Arg-Gly-Asp motif [13]. The VP1 G-H loop is an exposed, highly mobile, disordered protrusion on the surface of virus particle, as determined by X-ray crystallography of native virions [14–15]. This particular behaviour allows this segment to display multiple presentations to which several specific antibodies could be directed [16]. Linear synthetic peptides containing the site A sequence have effectively reproduced the reactivity and specificity of FMDV with neutralising monoclonal antibodies [17–18]. Nevertheless, this binding is usually lower than in the native virus, especially when used as free peptide in competitive enzyme-linked immunosorbent assay (ELISA), owing to its largely unstructured conformation. The cyclisation has not improved the antigenicity of the peptide either [8]. To better reproduce the characteristics of site A and its surrounding area, an interesting approach would be the insertion of the viral sequence in a carrier protein, which would favour the native peptide mobility and flexibility.

The *Escherichia coli* β-galactosidase protein is a well-known molecular marker [19], because of its ability to hydrolyse lactose analogues, like *ortho*-nitrophenyl-β-D-galactopyranoside, chlorophenol red galactopyranoside or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, rendering coloured compounds that can be easily visualised in situ or spectrophotometrically quantified [20]. This enzyme has also been used as a carrier protein to stabilise heterologous proteins produced in bacteria [21–22] and, more recently, several permissive sites have been determined in exposed solvent regions of the protein [23–24]. Moreover, due to its tetrameric structure, four copies of the heterologous domain are presented per molecule of hybrid enzyme [25]. In previous work, we have analysed the antigenicity of a 23 amino acid peptide from FMDV VP1 protein (residues 134–156), namely GH23, inserted at different sites on the surface of β-galactosidase [26]. This study pointed out the influence of the particular acceptor site in the reactivity with antibodies, showing the importance of the molecular context where the insertion has been made. However, the antigenic reactivity of the inserted peptide was only similar to native FMDV for some specific antibodies and only in particular insertion sites. To improve the antigenicity of recombinant, peptide-based antigens we have constructed new hybrid proteins with more than one insertion per monomer. In these chimeras, the antigenic structure is much more similar to that of the virus than that exhibited by single insertion proteins. In addition, a greater antigenicity is observed in all the analysed cases that cannot be attributed to a mere molar

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increase in the number of antibody binding sites, but to a better reactivity between recombinant epitopes and monoclonal antibodies.

2. Materials and methods

2.1. Vectors and plasmid construction

Plasmids pJLACZ, pM278VP1, pJX795A and pJX8CA are pJLA602 derivatives that encode the *E. coli* β-galactosidase enzyme. All of them, except the pseudo-wild type protein LACZ, encode a segment of VP1 protein, named GH23 (residues 134–156), from FMDV (isolate C-Sta. Pau, Spain/70, clone C-S8c1, serotype C [27]) inserted into different solvent-exposed loops of the enzyme [23–24].

In order to obtain new chimeras with more than one insertion of the viral peptide per monomer (hybrid proteins) we used the *Sac*I and *Bsi*WI restriction sites present in the *lacZ* gene. Specifically, pHD72CA was obtained by ligation of the 5.2 and 2.7 kb restriction fragments from pJX8CA and pJX795A, respectively, digested at *Nco*I and *Bsi*WI restriction sites. For pHD78CA construction, the *Nco*I/*Sac*I fragment from pM278VP1 was inserted into pJX8CA after removal of the 2 kb *Nco*I/*Sac*I segment from this plasmid. For pHD7278A, pM278VP1 and pJX795A were both digested at *Sac*I and *Eco*RI sites and the respective 6.8 and 1.1 kb fragments ligated. Finally, to construct a hybrid protein with three copies of the viral peptide, pHT7278CA, plasmids pM278VP1 and pHD72CA were cut with *Nco*I and *Sac*I and the respective 1.9 and 6 kb fragments ligated. All of the engineered *lacZ* genes are under the control of the CI857^{ts}-repressed p_R p_L lambda promoters.

All of the constructs were transformed into *E. coli* K12 strain MC1061, *araD139*, *galE15*, *galK16*, Δ(*araA-leu*)*1697*, Δ(*codB-lacI*)*3*, *hsdR2*, *mcrA*, *mcrB*, *relA1*, *rps150*(*strR*), *spoT1* [28], and the proteins were produced in BL26 strain, a Δ*lacZ* derivative of BL21, *dcm*, *gal*, *hsdS*, *lon*, *ompT* [29].

2.2. Production and purification of recombinant proteins

Bacterial cultures were grown in 200 ml of LB medium [30], plus 100 μg/ml ampicillin and 50 μg/ml streptomycin (when needed) in 1-l shaker flasks, at 32°C and 250 rpm. To induce the transcription of recombinant genes, cell cultures were shifted to 42°C when the OD reached between 0.3 and 0.4 U, and incubated for further 4 h at 250 rpm. Cells were then harvested by centrifugation and sonicated as previously described [31]. Purification of recombinant proteins was done by affinity chromatography with *p*-aminophenyl-β-D-thiogalactoside (TPEG)–Sepharose columns [32] and the purified protein dialysed against Z buffer [20] without β-mercaptoethanol. The purified protein concentration was determined spectrophotometrically at 280 nm, with $\varepsilon = 1.5 \times 10^5$ M^{-1} cm^{-1} [24] or by polyacrylamide gel electrophoresis followed by immunoblotting [26].

2.3. Enzymatic assay and determination of specific activity

β-Galactosidase activity was determined according to Miller's method [20] in a 1-ml reaction volume. One enzymatic unit is defined as the amount of protein that produces 1 μmol *o*-nitrophenol per minute at 28°C. The specific activity was calculated from cell culture samples with known enzymatic activity. The recombinant protein concentration was obtained by densitometric analysis of Western blot sheets, using appropriate standards. Determinations were performed up to three times for each protein.

2.4. Analysis of protein stability

All the analyses were done at 23 μg/ml of protein, in Z buffer [20]. To determine the resistance to heat denaturation, the proteins were incubated at different temperatures from 28 to 52°C. Samples were collected after 30 min, cooled at 4°C and further incubated at 28°C for Miller's assay [20]. Alternatively, the recombinant enzymes were also incubated at 37°C in the presence of 100 μg/ml trypsin in Z buffer. Aliquots were taken at different times and proteolysis was immediately stopped by adding 10 μM soybean trypsin inhibitor. Enzyme activity was determined as described above. Half-lives of the active proteins under these conditions are given as t_{50}. To analyse the resistance to urea denaturation the proteins were incubated for 2 h at 28°C in Z buffer, in the presence of different concentrations of urea, from 0 to 8 M. After a 10-fold dilution, the activity of each sample was rapidly determined as described above. The urea concentration that inactivated the 50% of protein initially present in the sample is given as C_{50}. All the determinations were done at least in triplicate.

2.5. Competitive ELISA

Competitive ELISA was performed as previously described [26]. Briefly, 20 pmol GH23-KLH or 5 pmol purified LACZ protein were incubated overnight in microtitre plates at 4°C as coating antigen. After saturating for 1 h with 3% (p/v) bovine serum albumin in phosphate-buffered saline (PBS), 100 μl of the monoclonal antibodies with purified recombinant proteins were added. These solutions containing a non-saturating amount of monoclonal antibody were previously incubated for 1.5 h at room temperature with different concentrations of the proteins (competitive antigen). Plates were then incubated for 1 h at room temperature, washed with PBS and further incubated with 100 μl of peroxidase-conjugated goat anti-mouse IgG (Bio-Rad), followed by extensive washing. The amount of bound antibody was detected with 0.01% (p/v) H_2O_2, 0.8 mM 3-dimethylaminobenzoic acid and 40 mM 3-methyl-2-benzotriazolinone in PBS buffer. The reaction was stopped with 50 μl of 2 N H_2SO_4. All the monoclonal antibodies used in this work were elicited against either wild-type β-galactosidase (Roche) or against native FMDV, directed against one of the multiple continuous epitopes within site A [33].

The IC_{50} value for each protein, defined as the amount of competitor antigen required to inhibit 50% of the binding of a particular monoclonal antibody to the coating antigen, was calculated as described elsewhere [34].

2.6. Clustering analysis

For cluster analysis, the IC_{50} value (in nM) obtained for each protein and mAb were used to compute an Euclidean distance matrix between pairs of proteins. To identify putative clusters, a multidimensional scaling was performed, as a first approach, with STATISTICA 4.5 for Windows. Then, several amalgamation methods were used for hierarchical clustering (single and complete linkage, centroid, median, weighted and unweighted pair-group average, Ward's method), in order to test consistency of the obtained clusters.

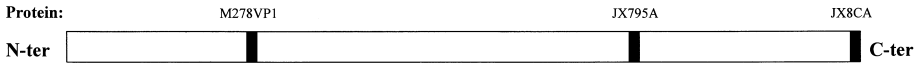
3. Results

3.1. Design and production of chimeric proteins

In previous work, we engineered several β-galactosidases with a segment of FMDV VP1 protein fused or inserted at different permissive sites on the enzyme surface [23–24]. To further explore the tolerance of β-galactosidase to accommodate foreign peptides we have constructed new hybrid enzymes with more than one copy of the viral segment per

Table 1
Stability and enzymatic properties of recombinant proteins

Proteins	Specific activity (U/μg)	Urea stability (M)	Thermal stability (°C)	Proteolytic stability (min)
LACZ	2765 ± 347	2.62 ± 0.005	50.8 ± 0.3	109.4 ± 10.2
M278VP1	170 ± 43	0.68 ± 0.008	45.3 ± 0.3	5.6 ± 0.1
JX795A	675 ± 102	1.99 ± 0.014	49.5 ± 0.3	22.1 ± 1.6
JX8CA	2840 ± 218	2.54 ± 0.002	50.7 ± 0.3	90.4 ± 5.4
HD78CA	99 ± 15	2.07 ± 0.014	44.1 ± 0.3	6.1 ± 0.7
HD72CA	385 ± 64	1.03 ± 0.021	46.8 ± 0.4	28.7 ± 3.7
HD7872A	8 ± 3	1.47 ± 0.052	47.9 ± 0.6	33.5 ± 6.3
HT7278CA	3 ± 4	0.54 ± 0.084	40.5 ± 2.3	1.6 ± 0.1



GS*TTYTASARGDLAHLTTT*HARHLPGS

Fig. 1. Top: Lineal schematic representation of the β-galactosidase sequence with the insertion sites indicated by black boxes. Bottom: Amino acid sequence of the GH23 peptide from FMDV clone C-S8c1. In italics, the amino acids encoded by the *Bam*HI restriction site and underlined the major immunodominant region site A [12].

monomer. For this purpose, we selected proteins with a low antigenicity of the epitopes present in GH23 (Table 2), in an attempt to improve it by increasing the number of insertions. By using proteins M278VP1, JX795A and JX8CA we have designed all the possible combinations of two and three insertions. The new enzymes HD78CA, HD72CA and HD7872A carry two copies at amino acid positions 278 and C-terminus, 795 and C-terminus, and 278 and 795, respectively, whereas HT7278CA presents three copies of GH23 at positions 278, 795 and C-terminus (Fig. 1).

During production of these new recombinant proteins, no inhibition of cell growth was detected and a very similar yield was reached (not shown), indicating low toxicity of these enzymes and comparable turn over for all the proteins. An important feature of these chimeras is the maintenance of their enzymatic activity even in the triple hybrid (Table 1), which allows its efficient purification with TPEG–Sepharose columns (not shown).

The study of the specific activity revealed important differences between the new enzymes and the respective parental proteins with a single insertion (Table 1). This trait is especially dramatic in HD7872A and HT7278CA with an activity of nearly 100-fold lower than M278VP1 or JX795A, and 1000 times lower than the C-terminal GH23 fusioned protein JX8CA or the pseudo wild-type enzyme LACZ. Nevertheless, these proteins can still be easily quantified and their production monitored by the Miller's colourimetric assay [20].

3.2. Structural stability of hybrid β-galactosidases

The stability of all new proteins was determined by their resistance to denaturation against temperature, urea and proteolysis in vitro (Table 1). In all the performed experiments, an important decrease in the stability of these chimeras has been observed, due to the presence of more than one insertion per monomer. As expected, there is a direct effect between the number of insertions and denaturation, HT7278CA being the most sensitive construct in all the parameters analysed. However, it is worthy to note the higher resistance to denaturation by temperature and proteolysis of HD7872A as compared to HD78CA or HD72CA, despite the presence of two foreign insertions in this construction and the important decrease of about 300-fold in the specific activity of this enzyme compared with LACZ (Table 1).

On the other hand, whereas M278VP1 is the most sensitive enzyme among proteins with a single insertion, the double hybrid chimeras with an insertion at this position (HD78CA and HD7872A) are equal or more resistant to denaturation than M278VP1, especially in the presence of urea or trypsin. This unexpected result indicates a stabilisation of M278VP1 by the presence of an additional insertion.

3.3. Reactivity of chimeric proteins with site A-specific MAbs

The antigenicity of engineered β-galactosidases was analysed in competitive ELISA by using different monoclonal antibodies directed against FMDV site A. All the examined enzymes were well recognised by MAbs, with slight differences between antibodies (Table 2). Only in the case of MAb SD6, the binding was always lower than in the other MAbs, with values of IC_{50} up to 10 times higher, depending on the antibody or the enzyme analysed.

The average IC_{50} has been calculated to estimate the overall antigenicity of each protein in particular, but considering together all the MAbs used in this work (Table 2). This approach could be done because this set of MAbs is representative of a larger panel of neutralising MAbs which recognise antigenic features of site A similar to those recognised by antibodies of swine, a natural host for FMDV [26]. Thus, despite JX8CA being a good antigen with an average IC_{50} similar to that of the peptide conjugated to KLH, the recombinant enzymes M278VP1 and JX795A are less reactive to MAbs than GH23-KLH (Table 2). However, hybrid proteins with multiple copies of the tested antigen show not only

Table 2
Antigenic features of modified β-galactosidases

Proteins	Monoclonal antibodies (nM[a])					Average IC_{50} (nM[a])	Variation coefficient (%)
	3E5	6D11	7FC12	7JD1	SD6		
GH23-KLH	9.70[b]	11.77	92.12	12.65	62.10	37.7	100
M278VP1	69.00	66.00	298.00	267.00	218.00	183.6	60
JX795A	9.15	12.79	75.21	17.14	954.17	213.7	194
JX8CA	10.83	13.70	2.01	0.15	175.63	40.5	187
HD78CA	1.63	1.38	0.67	1.27	6.05	2.2	99
HD72CA	2.73	2.09	0.64	0.76	21.82	5.6	162
HD7872A	4.06	2.77	2.01	1.89	7.78	3.7	66
HT7278CA	3.17	6.33	1.83	1.96	4.41	3.5	53
FMDV[c]	2.70	10.50	3.50	2.50	15.00	6.8	82

[a]Concentration of competitive antigen.
[b]IC_{50} of synthetic peptide and recombinant proteins in competitive ELISA.
[c]FMDV stands for VP1 protein in FMDV particles, clone C-S8c1.

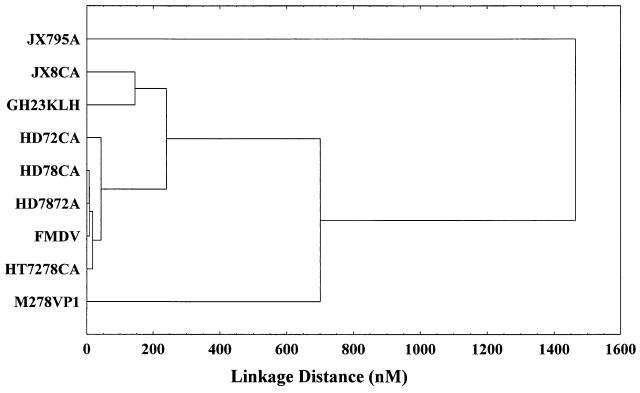


Fig. 2. Hierarchical clustering tree of the different recombinant proteins obtained by Ward's amalgamation method using Euclidean distances.

an important decrease in the average IC_{50} values in relation to their parental proteins, suggesting a clear improvement in the recognition by antibodies, but also a better binding to antibodies than GH23-KLH and similar to FMDV particles. Furthermore, the variation coefficient value is lower in most of the analysed hybrid enzymes than in parental ones (Table 2), indicating a narrower deviation in the antibody recognition of the epitopes of site A than when presented in single-inserted proteins.

To better analyse the effect of multiple peptide presentation we have also examined the antigenic profile of all the hybrid enzymes in a multidimensional antigenic space generated by the analysed epitopes (Fig. 2). By considering clusters below 100 nM, FMDV virions and hybrid proteins with several copies of the antigen are grouped together. On the other hand, the viral peptide coupled to KLH appears in another cluster with protein JX8CA, while enzymes M278VP1 and JX795A with a single internal insertion of the peptide split from the rest of the antigens by 700 and 1500 nM, respectively.

3.4. Effect of peptide insertions and MAb binding on β-galactosidase immunoreactivity

To determine the effect of the viral segment insertion on the immunoreactivity of other enzyme regions, we explored if the structural changes promoted by the inserted peptide could modulate the binding of monoclonal antibodies against the carrier protein. A competitive ELISA using a monoclonal antibody against β-galactosidase and protein LACZ as competitive antigen shows a significant improvement in the β-galactosidase epitope recognition of JX8CA and M278VP1, when compared with enzyme LACZ (Table 3). Only in protein JX795A does the presence of the viral peptide impair the antibody recognition of β-galactosidase, perhaps by steric hindrance.

To further investigate if the binding of an antibody can modulate the coupling of a secondary antibody in a different site, we examined in competitive ELISA the effect of the presence of a site A-specific MAb (SD6) on the binding of a MAb directed against β-galactosidase. Even though the results render no significant data, there is a different tendency depending on the protein studied (Table 3). Thus, while in mutant JX795A and in the pseudo-wild type protein LACZ the presence of SD6 has no effect in the recognition of β-galactosidase by its specific MAb, in the case of M278VP1 or JX8CA, the pre-incubation with SD6 improves the binding of the MAb anti β-galactosidase to the carrier protein.

4. Discussion

The β-galactosidase enzyme has been used as a carrier protein for many purposes [32,35–36]. Among them, one of the most recent applications is the engineering of β-galactosidase to be used as an epitope tagging for the detection of antibodies [23–24]. To generate good antibody sensors and in general recombinant ligands it is important to keep the reactivity of the probe as similar as possible as in its natural location. Up to now, only single insertions had been made in β-galactosidase looking for particular sites that would reproduce the epitope environment as in the original protein. Unfortunately, the antigenicity achieved only reached levels similar to those presented by FMDV for some specific monoclonal antibody and depending on the insertion site [26]. An alternative but still unexplored strategy to improve the antigenicity of an inserted epitope in this carrier protein would be the increase of copies of the foreign peptide per molecule of carrier protein.

β-Galactosidase protein can tolerate up to three copies of a 27-mer viral peptide at amino acid positions 278, 795 and C-terminal, without loosing its activity (Table 1). However, the results obtained with hybrid enzymes denote that the structural changes promoted by the simultaneous presence of more than one peptide per enzyme monomer have an unpredictable outcome in the stability and activity of the new recombinant proteins, as noticed by comparing proteins HD78CA and HD7872A with their parental enzymes M278VP1, JX795A and JX8CA. An interesting observation is the increase in protein stability upon peptide insertion or fusion at C-terminus besides the one at position 278 (Table 1). This region of β-galactosidase, amino acids from 272 to 288, is involved in the monomer–monomer contacts through a protruding loop that extends across the activating interface to the neighbouring monomer [37]. Insertions in this area

Table 3
Statistical analysis of antibody binding by ANOVA tests

Protein	Without MAb SD6	P^a	With MAb SD6	P^b
LACZ	2.41[c] ± 0.22	–	2.47[c] ± 0.18	0.624
M278VP1	2.01 ± 0.05	**0.0089**	1.89 ± 0.15	0.372
JX8CA	1.78 ± 0.04	**0.0006**	1.57 ± 0.07	0.090
JX795A	2.82 ± 0.13	**0.0087**	2.85 ± 0.16	0.756

In bold face values with significance level below 0.01.
[a]Differences between enzymes with the inserted viral peptide and the pseudo-wild type LACZ.
[b]Differences between proteins pre-incubated with monoclonal antibody SD6 and without SD6.
[c]IC_{50} of native β-galactosidase and recombinant proteins in competitive ELISA.

cause an important decrease in the enzymatic specific activity and also less resistance to denaturation [24]. Nevertheless, the presence of a fusion at the C-terminus or a new insertion at position 795, stabilise the recombinant enzyme, reaching values of relevant parameters close to those exhibited by the chimera with either one single insertion at position 795 or a fusion at the C-terminus. In the tetramer of β-galactosidase, the position 795 from a monomer is very close to residue 278 from the monomer beside [37]. Owing to this proximity, the conformational changes promoted by the presence of a foreign peptide at position 278 could be modified by a new insertion at 795, improving the stability of the new protein when compared with the enzyme with a single insertion. Yet, this recovery has not been observed in the specific activity (Table 1), probably because residue 794 of β-galactosidase is involved in the catalytic function of the enzyme [38], and insertions close to this position lead to a dramatic decrease in the catalytic constant of the protein [24].

All the constructs used in this work contain the loop G-H of VP1 protein from FMDV, spanning residues from 134 to 156 [14]. This peptide, named GH23, contains the major antigenic site of FMDV (site A), made up of several overlapping B-cell epitopes [12,33]. The insertion of this segment on the surface of β-galactosidase has improved its antigenicity in relation to the peptide alone in solution or coupled to KLH [26,39]. Moreover, by increasing the number of copies of GH23 per monomer, the average IC_{50} has significantly decreased (Table 1), suggesting a better reactivity between antibodies and epitopes. Despite having more determinants per protein, the binding enhancement is not additive. Thus, this improvement cannot only be attributed to an increase of the binding sites per antibody, but to a greater affinity.

The chance of a bivalent binding is clearly favoured in hybrid proteins with two or three copies of the viral peptide per monomer. However, it also has to be considered that the conformational modifications promoted by the presence of several insertions in a same protein could either facilitate the accessibility of the antibody to the epitope or lead to a structure of the determinant which is best recognised by antibodies than in proteins with a single insertion. In addition, the stability and especially the activity of hybrid proteins have been impaired when increasing the number of insertions, suggesting important structural changes. The antigenicity analysis of a β-galactosidase epitope showed significant differences between proteins with the inserted viral peptide and the pseudo-wild type protein LACZ (Table 3), indicating that the insertional sites used in this work can alter the protein structure enough to improve or impair the antibody binding in a different place.

The binding of an antibody can induce both local conformational changes in a protein or in the whole molecule, which can even affect its function [40–41]. Such a mechanism has been broadly analysed in β-galactosidase where a series of inactive mutants can be restored in their activity upon interaction with monoclonal antibodies [42]. More recently, it has been proven that low active recombinant β-galactosidases with a 27-mer foreign peptide inserted near the active site, can be modulated by antibodies directed against the peptide inserted [43]. It has been previously described that in proteins with multiple determinants, the antibody binding may be cooperative between antibody pairs that recognise different epitopes [44], probably by conformational changes promoted by the attachment of the first antibody. The same mechanism has also been noticed in multivalent proteins using different monoclonal antibodies [45]. In the case of single-insertion β-galactosidase proteins we have not observed significant differences in the recognition of a MAb directed against the carrier protein between enzymes that have been previously incubated or not with a MAb against the viral peptide (Table 3). Nevertheless, the binding of an antibody to the foreign peptide can modulate the enzyme activity at least in proteins JX795A and M278VP1 [43,46]). This suggests that antibodies are able to promote conformational changes but these modifications are not sufficient to improve the recognition of another epitope in single-inserted proteins.

In a previous study, we examined the antigenic profile of site A inserted at different positions of two carrier proteins and compared it with this peptide coupled to KLH or with native FMDV [34]. In this former investigation, proteins M278VP1, JX795A and JX8CA were in distinct groups, indicating a different antigenic behaviour, and JX8CA only appeared in the same cluster as FMDV. Now, by increasing the number of antibody binding sites, the variability in the recognition of the epitopes by all the MAbs decreases in the hybrid proteins, down to values similar to that presented by FMDV particles. This result suggests a better presentation of the antigenic region located in the inserted segment and consequently, an antigenic convergence of the whole recombinant antigen with FMDV (Table 2 and Fig. 2).

The G-H loop from FMDV is a highly mobile, disordered protrusion that could display multiple presentations through hinge movements [14–15]. The fact that a G-H loop containing recombinant protein is only well recognised by some specific antibodies could be due to a restriction of the flexibility of this peptide by selective insertion in β-galactosidase [26,39]. However, hybrid proteins with several insertions are more unstructured than enzymes with single insertions, as observed in their stability and specific activity (Table 1). This situation could allow the viral peptide to be more exposed or even more flexible as in the capsid of virus, thus mimicking the antigenicity of FMDV site A.

The usual procedure of obtaining peptide-displaying recombinant antigens is to look for permissive sites in the carrier protein that would reproduce not only the determinant structure but also its environment. Despite the fact of being a good strategy, the limitation of permissive sites in a protein and the difficulties in finding them makes this approach tedious and uncertain. In addition, for β-galactosidase, the high values obtained in the variation coefficient with single-inserted recombinant enzymes (Table 2) indicate that we have only partially reproduced the characteristics of the epitopes. An alternative method of improving the antigenicity of the viral epitopes, as demonstrated in this work, would be to increase the peptide copy number per enzyme, once we know some tolerant acceptor sites. In this way, we have obtained chimeric β-galactosidases that successfully have mimicked the antigenicity site A capsid protein VP1 in the FMDV.

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