Discriminating Foot-and-Mouth Disease Virus-Infected and Vaccinated Animals by Use of β-Galactosidase Allosteric Biosensors

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Recombinant β-galactosidases accommodating one or two different peptides from the foot-and-mouth disease virus (FMDV) nonstructural protein 3B per enzyme monomer showed a drastic enzymatic activity reduction, which mainly affected proteins with double insertions. Recombinant β-galactosidases were enzymatically reactivated by 3B-specific murine monoclonal and rabbit polyclonal antibodies. Interestingly, these recombinant β-galactosidases, particularly those including one copy of each of the two 3B sequences, were efficiently reactivated by sera from infected pigs. We found reaction conditions that allowed differentiation between sera of FMDV-infected pigs, cattle, and sheep and those of naïve and conventionally vaccinated animals. These FMDV infection-specific biosensors can provide an effective and versatile alternative for the serological distinction of FMDV-infected animals.

Foot-and-mouth disease (FMD) is one of the most important and highly transmissible diseases of livestock that causes severe production losses, and it is a major constraint to international trade of live animals and their products (42, 50). FMD affects extensive areas worldwide and is included in the list of diseases notifiable to the World Organization for Animal Health (http://www.oie.int/eng/en_index.htm). The etiological agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51). FMDV is the prototypic agent FMD virus (FMDV) infects artiodactyla, mostly cattle, swine, sheep, and goats (38, 51).
zynamically to antibodies directed to foreign peptides displayed on the enzyme surface (53), is highly promising (23). We previously showed that multiple insertions of a major FMDV B-cell epitope from the VP1 capsid protein near the active site of recombinant β-galactosidases dramatically increased the enzyme responsiveness to specific antipeptide antibodies, including sera from infected animals (4, 17). In this study, we report that recombinant β-galactosidases accommodating one or two different peptides from the FMDV NS protein 3B per enzyme monomer can be reactivated by anti-3B monoclonal antibodies (MAbs). Interestingly, these recombinant β-galactosidases, particularly those including one copy of each of the two 3B sequences, could also be efficiently reactivated by sera from infected animals. We found reaction conditions that permitted differentiation between sera from infected animals and those from naïve and conventionally vaccinated pigs. These infection-specific FMDV biosensors can provide an effective and versatile alternative for the serological distinction of FMDV-infected animals.

**MATERIALS AND METHODS**

**Plasmids, bacterial strains, and antibodies.** Plasmid pX795A (6) encodes a segment of VP1 protein (residues 134 to 156) from an FMDV isolate of serotype C; C-St8C1 (46), inserted at amino acid residue 795 of β-galactosidase from a BamHI restriction site. This plasmid was derived from pJLacZ (5), which encodes a pseudo-wild-type Escherichia coli β-galactosidase enzyme. In pX795A, recombinant gene expression was under the control of the rB75ra-repressed lambda pR and pL, strong promoters placed in tandem (43). MAbs 1A10 and 1F8 (27) were kindly provided by E. Brocchi (Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna, Brescia, Italy). Both MAbs were mapped by peptide scanning and were shown to react with the FMDV 3B peptides inserted into recombinant β-galactosidases (5). MAbs 4B12 (against FMDV 3D) and SD6 (against FMDV VP1) were used as non-3B-specific control antibodies (16). The naïve rabbit polyvalent antibody P1 (M. González-Magaldi and R. Armas-Portela, unpublished results) was also used as a control. The animal sera analyzed in this study are detailed in Table 1.

**Construction of pX795A derivatives displaying FMDV 3B peptides.** DNA fragments corresponding to nucleotides 5827 to 5895 or 5827 to 5931 of the FMDV RNA were amplified from pC8.2 (37) by using BioTaq DNA polymerase (Bioline) and the oligonucleotide primers shown in Table 2 (which included a BamHI site), according the methods described in reference 52. The fragments amplified encoded 23 of 24 aa of FMDV protein 3B-2 alone (insert 23) or the complete 3B-2 protein followed by the first 11 aa of protein 3B-3 (insert 35) (Fig. 1A). Each of these amplicons was first cloned into the pGEM-T Easy vector (Promega), and the resulting plasmids were digested with BamHI and ligated into pX795A, from which the fragment BamHI-BamHI containing the VP1 protein insert had been excised previously. The plasmids recovered were named pJB23 and pJB35. To obtain recombinant β-galactosidases harboring two FMDV inserts, pJB23 and pJB35 were digested with ClaI endonuclease, which cuts at nucleotide 819 of the lacZ gene in pLacZ, corresponding to residue 278 of the β-galactosidase monomer (24). Each digested plasmid was ligated with amplified DNA fragments containing the FMDV 3B sequences of inserts 23 or insert 35, including the ClaI restriction site sequence, which were obtained by PCR amplification from pC8.2, using the primers Cla S and 35 Cla (Table 2), cloned, and digested with ClaI, using the strategy described above. The inserts harbored by the resultant plasmids, which were named pJB23_C35 and pJB35_C35, are summarized in Fig. 1B. Recombinant plasmids were selected by restriction enzyme analysis, and the adequate orientation of the inserts determined by specific PCR amplifications using primers P1L-f and TDIa (Bam) or EXT-S and TDIa (Cla) (Table 2). The expected nucleotide sequences of the plasmids selected were confirmed by nucleotide sequencing using primer pairs P1L-f and P1L-r and EXT-S and EXT-A (Table 2).

**Production, identification, and purification of recombinant proteins.** Transformed E. coli cells from strain BL26, a lacZ derivative of BL21 deon gal hsdS leu omprT (49), were grown in 200 ml of LB medium plus 100 μg/ml ampicillin. To induce the transcription of the recombinant genes, the temperature was shifted to 42°C when the optical density at 550 nm reached about 0.3 units, and cultures were incubated for a further 4 h. Bacterial cells were then harvested by centrifugation, and protein expression was confirmed by Western blotting of cell
extracts by using a polyclonal anti-β-galactosidase (24) or MAb 1F8 and peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Dako) as reported previously (27). The engineered enzymes were purified by affinity chromatography with p-aminophenyl-β-D-galactopyranoside (17) and lyophilized, and protein concentrations were determined spectrophotometrically at an absorbance of 280 nm (19). Purified enzymes were stored at −80°C.

Enzymatic assays. β-Galactosidase enzymatic activity was analyzed according to Miller’s method (35) as described previously (3) using chlorophenol red

### TABLE 2. Oligonucleotides used for PCR amplification

| Oligonucleotide | Sequence (5′-3′) | Genetic material (first nucleotide) |
|-----------------|------------------|-------------------------------------|
| Cla S           | TATAATCGATGGACCTTACGCCGGTGCA | FMDV (5827) |
| 35 Cla          | TATAATCGATGCAGGCTTCTTCACCGGGTCCTT | FMDV (5931) |
| Bam S           | TATAAGATCCCGAAGCTTTCAGCGCCGTCGA | FMDV (5827) |
| 23 Bam          | TATAAGATCCCGAAGCTTTCAGCGCCGTCGA | FMDV (5895) |
| 35 Bam          | TATAAGATCCCGAAGCTTTCAGCGCCGTCGA | FMDV (5931) |
| EXT-S           | TATAATTTTTATGGCACTTGGATGACCCGCGAGG | LacZ gene (756) |
| EXT-A           | TATAATTTTTATGGCACTTGGATGACCCGCGAGG | LacZ gene (868) |
| PJL-f           | AACAACCTGCTGACGC | LacZ gene (2303) |
| PJL-r           | AACAACCTGCTGACGC | LacZ gene (2450) |
| TDIA            | ITGCTTTACCTTCAGCGGC | FMDV (5879) |

*a Forward primers are indicated by S or f. Those without either are reverse primers.

*b Restriction sites introduced (ClaI for Cla primers and BamHI for Bam primers) for cloning into pJX795A vector are shown in boldface. The FMDV and LacZ gene nucleotides in each primer are underlined; FMDV sequences are in italics.

*c Genomic positions of the first FMDV nucleotide (52) and of the first LacZ gene nucleotide (29) included in the primers are given in parentheses.

FIG. 1. Construction and expression of recombinant β-galactosidases. (A) FMDV 3B protein sequences. Amino acid sequences of the two FMDV peptides, of 23 and 35 aa, respectively, inserted into β-galactosidase are indicated. (B) Schematic representation of the recombinant β-galactosidase proteins in which the FMDV insertion sites are depicted in gray (23 aa) and black (35 aa) boxes. BamHI and ClaI insertion sites are indicated. (C) Expression of the recombinant proteins. Thermally induced cultures of transfected E. coli BL26 cells were lysed. The released proteins were resolved using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining. Purified β-galactosidase (β-gal) and extracts from E. coli BL26 transformed with pLacZ or not transformed were included as controls. (D) The produced proteins were purified by affinity chromatography with 1-aminophenyl-β-D-galactopyranoside and analyzed as described for panel C. (E) Expression of 3B protein was confirmed by Western blotting with the 3B-specific MAb 1F8. Migration of molecular size markers (in kilodaltons) is shown to the left of panels D and E.
β-galactosidase (CPRG) as the substrate. Kinetic parameters of recombinant proteins were calculated through standard procedures previously described and were used to calculate the \(K_a\) and \(K_m\) values (11).

The enzymatic activity modulation assay for detection of anti-FMDV antibodies was performed as described previously (3). Briefly, different amounts of recombinant protein were incubated at 28°C with 1% (wt/vol) bovine serum albumin, in the presence or absence of different dilutions of anti-FMDV antibodies or animal sera (Table 1). After the incubation period, different concentrations of the substrate CPRG were added and the reaction product was determined by measuring the absorbance at 540 nm at different times, using a multiscan spectrophotometer (Tecan Spectrafluor). All the assays were done at least in triplicate. Percentages of reactivation were calculated relative to values determined in the absence of antibodies. Data were represented using the SigmaPlot scientific graphing software, version 8.0.

Detection of antibodies against 3ABC protein by ELISA. Serum samples were examined for the presence of antibodies against NS FMDV protein 3ABC, indicative of virus replication, by using an in-house ELISA (7) and the commercial kit Cedilis FMDV-NS (9).

RESULTS

Single and double insertions of FMDV 3B protein sequences render stable recombinant β-galactosidases with decreased enzymatic activity. To evaluate the feasibility of a biosensor-mediated detection of antibodies specific for FMDV infection, plasmid pJX795A (17) was engineered to accommodate insertions of two peptides of 23 and 35 aa in length. The sequences of the 3B gene region spanned by each peptide are shown in Fig. 1A. These peptides had been previously reported to allow ELISA detection of infection-specific anti-FMDV antibodies from all seven serotypes of the virus (44). Four plasmids were constructed, and each one harbored one or two FMDV 3B peptides in restriction sites (BamHI and/or ClaI) close to the active site of the β-galactosidase monomer (18) (Fig. 1A and B). The integrity of the recombinant DNAs was confirmed by restriction enzyme analyses and by nucleotide sequencing of the corresponding plasmids.

As shown in Fig. 1C, the amount of β-galactosidase expressed from the recombinant plasmids was similar to that observed in E. coli cells transformed with pJLacZ. Also, comparable amounts of β-galactosidase were recovered upon purification by affinity chromatography with N-(p-aminophenyl)-β-D-galactopyranoside (Fig. 1D). The presence of FMDV inserts in the different constructions was confirmed by Western blotting with an anti-3B MAb (Fig. 1E).

As previously observed with other foreign inserts, peptide insertion in the selected sites dramatically reduced the enzymatic activity of the accommodating enzyme by differently affecting its enzymatic constants (Table 3). A general, strong reduction in the \(K_a/K_m\) ratio was observed. Despite the standard errors found in the constants determined for recombinant β-galactosidases, a trend toward a greater decrease in \(K_a/K_m\) ratio was found for proteins with double insertions. This reduction was a positive predictor of their allosteric performance, as the extent of antibody-mediated enzymatic reactivation has been shown to be highly dependent on the reduction of such values (25).

Antibodies against FMDV 3B protein can reactivate the enzymatic activity of recombinant β-galactosidases. We first assessed the capacity of the recombinant β-galactosidases to be reactivated by MAbs that react against the 3B peptides inserted in the recombinant proteins (see Materials and Methods), using reaction conditions employed in previous studies (20). The addition of 3B-specific MAbs 1F8 and 1A10 to the enzymatic reaction resulted in increased reactivation values for the constructions accommodating two copies of FMDV 3B, reaching B23C35 protein reactivation percentages up to 300% (Fig. 2A and B). The reactivations observed with the corresponding control, isotype-matched MAbs never exceeded 140% (see Fig. S1 in the supplemental material). Interestingly, higher levels of reactivation were observed when rabbit poly-

### TABLE 3. Enzymatic constants of engineered β-galactosidases

| Protein | Sp act (U/µg) | \(K_a\) (s\(^{-1}\)) | \(K_m\) (mM) | \(K_a/K_m\) (mM\(^{-1}\) s\(^{-1}\)) |
|---------|---------------|-----------------|-------------|-----------------|
| LacZ*  | 2.765 ± 347   | 18.300 ± 779 | 0.19 ± 0.033 | 97.340 ± 1.757 |
| B23    | 138 ± 33.84   | 0.65 ± 0.59    | 1.45 ± 1.36 | 0.45 ± 0.73     |
| B35    | 283.95 ± 41.98| 0.33 ± 0.25    | 0.85 ± 1.10 | 0.39 ± 0.37     |
| B23C35 | 19.94 ± 6.15  | 0.05 ± 0.05    | 1.05 ± 1.23 | 0.04 ± 0.07     |
| B35C35 | 49.01 ± 5.64  | 0.01 ± 0.02    | 1.02 ± 1.38 | 0.01 ± 0.02     |

* Data from the parental protein LacZ, devoid of any peptide insertion obtained from use as the substrate (24), is shown for comparison.
clonal hyperimmune serum 346, produced against a recombinant protein spanning the three copies of the 3B protein (R1; Table 1), was added to the reaction (Fig. 2C). In this case, the higher-level reactivations were also induced by construct B23C35, and these were observed at high dilutions of sera (e.g., 1/1,280). The reactivations observed with a naïve rabbit polyclonal antibody never exceeded 200% (see Fig. S1 in the supplemental material). Thus, recombinant β-galactosidases accommodating FMDV 3B peptides can be efficiently reactivated by specific antiviral antibodies.

**Optimization of the biosensing reaction with swine sera.**

The increase of the enzymatic activity of recombinant β-galactosidases by MAbs and rabbit antibodies led us to explore their reactivation by FMDV-specific pig sera. We initially determined the kinetics of enzymatic reactivation (0.5 pmol of protein and 1 mg/ml of substrate) by using serum I1 from an FMDV-infected pig. By 20 min of the reaction, a plateau of reactivation was observed for the four recombinant β-galactosidases analyzed (Fig. 3A). Percentages of reactivation with control serum N5 (Table 1) were lower than and never exceeded 300% (Fig. 3B). A similar analysis conducted to determine the effect of serum dilution in the reaction revealed that a dilution of 1/32, producing a reactivation close to saturation values, allowed high reactivation levels of the four recombinant proteins analyzed, as shown in Fig. 3C.

As observed for rabbit polyclonal serum 346, reactivation percentages induced by pig sera were higher than those induced by MAbs under the same experimental conditions (Fig. 2).

Next, a serial study of the effect of a range of substrate and enzyme concentrations on the biosensing reaction was performed, using the reaction time and the serum dilutions selected above. This study included sera from infected and naïve, noninfected pigs. As shown in the topographical representation of the allosteric responses detected (Fig. 4), sera from infected animals induced higher sensing signals than sera from noninfected animals under the range of tested conditions. Proteins accommodating the two FMDV insertions, B23C35 and B35C35, showed higher-level-reactivation factors. For B23, B35, B23C35, and B35C35 proteins, conditions that allowed reactivation in the presence of sera from infected animals that was consistently greater than that observed with control sera from naïve animals were selected (0.5 pmol protein, 1.5 mg/ml CPRG, and a 20-min reaction time). Similar conditions were found with 30-min, 40-min, and 50-min reaction times (data not shown).

**Recombinant β-galactosidases can differentiate FMDV-infected sera from those of naïve and vaccinated animals.** To assess the diagnostic potential of the recombinant β-galactosidases, we determined the reactivation level induced by a panel of swine sera in the enzymatic activity of the four proteins, using the reaction conditions selected above. Sera from infected animals promoted higher-level enzymatic signals than those triggered by sera from noninfected or vaccinated animals (Fig. 5). Greater differences were found for protein B23C35, for which the reactivation factors induced by sera from control noninfected and vaccinated pigs were, in all cases, lower than 40%. The potential of sera from different species to reactivate recombinant β-galactosidases was also analyzed. Interestingly, a similar reactivation pattern was observed with these sera. As shown in Fig. 5B, a serum from infected cattle induced high-level reactivation of proteins B23C35 and B35C35 relative to that induced by the control, noninfected serum. When sheep sera were analyzed, higher-level reactivations of proteins B35, B23C35, and B35C35 were also induced by serum from the infected animals (Fig. 5C).

All the infected sera tested were positive in a parallel analysis using an ELISA design to detect antibodies to 3ABC protein, confirming the specificity of the reactivations observed (Table 1). These results indicate that recombinant β-galacto-
sidases can act as sensors for FMDV-specific sera from different species.

**DISCUSSION**

Allosteric biosensors allow detection of antibodies against different viruses by accommodating peptide sequences from surface viral proteins, acting as antibody receptors, into permissive sites of allosterically responsive recombinant β-galactosidases. Among the advantages of such biosensors as diagnostic tools is the homogeneous nature of the assay, the short time required for the enzymatic reaction and antibody detection, and the potential for handling large number of samples and for automatic processing, as recently shown for human immunodeficiency virus (20, 22).

Reliable and efficient detection of antibodies specific for FMDV is a crucial bottleneck for vaccine administration. This is due to the risk of the presence of infectious virus in vaccinees, which is behind the restrictions on exports of meat products and derivatives to FMDV-free, nonvaccinating countries (30). In this report, we describe a novel approach to generating efficient β-galactosidase biosensors in which, for the first time to our knowledge, two different peptide sequences from a viral NS protein were accommodated at two independent sites in the enzyme. As expected from a double insertion, B23C35 and B35C35 enzymes showed $K_{cat}/K_m$ ratios that were much lower than those observed for recombinant β-galactosidases accommodating a single insertion. This lower enzymatic activity correlated with higher-level reactivations in the presence of murine MAbs to 3B, which was more prominent when a rabbit hyperimmune serum was added to the enzymatic reaction. Indeed, B23C35 was the recombinant β-galactosidase that exhibited the highest reactivation values among those for the constructions studied, suggesting that apart from generating the improved reactivation expected from low-$K_{cat}/K_m$ enzymes, the multivalent strategy first tested here consisting of the display of more than one B-cell epitope (acting as receptors for antibodies) in a single enzyme could render unusually high-sensitivity sensors. This is probably due to the cooperative enzymatic activation promoted by different antibody populations.

The capacity of recombinant β-galactosidases for specific reactivation extended also to sera from natural FMDV host
species. Indeed, sera from infected pigs efficiently reactivated recombinant enzymes at reaction times (20 min) and dilutions (1/32) suitable for the development of a diagnostic test (Fig. 3). An ample screening of other reaction parameters (enzyme and substrate concentration) revealed conditions that allowed discrimination between sera from FMDV-infected and noninfected pigs (Fig. 4). When one such condition was used for the screening of pig sera, greater differences between the reactivation levels induced by sera from FMDV-infected and noninfected or vaccinated pigs were found with recombinant \( \beta \)-galactosidases accommodating two copies of 3B. Interestingly, the reactivations observed with recombinant B23C35 discriminated between these two groups of pig sera (Fig. 5A). Such discrimination was also possible when sera from cattle and sheep were included in the assay (Fig. 5B). The significant correlation found with the ELISA detection of 3ABC-specific antibodies, using a validated routine assay for FMD diagnosis (9), in the sera tested (Fig. 5 and Table 1) confirmed the specificity of the analytical signal produced by B23C35. Although the analysis of a higher number of sera would be obviously required to define the best-tailed recombinant \( \beta \)-galactosidases and optimal reaction conditions for diagnostic purposes, our results provide the proof of principle for the optimization of a biosensing assay for diagnosis of FMDV infection. This assay is simple, homogeneous, and short in time to perform, and it could be used for different species without the need for species-specific secondary antibodies required by current ELISA tests. Furthermore, its simplicity and the fact that allosterically sensing \( \beta \)-galactosidases perform very efficiently once immobilized on solid phases (21) permit the envisaging of sensor prototypes that could be designed for use under field conditions by nonskilled personnel. Our results confirm also the high-throughput, multiscreening potential of allosteric biosensors that, because of their simplicity, could be fully automated and miniaturized to be adapted to macroscopic detection or to nanoscale devices.

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