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Screening HIV-1 antigenic peptides as receptors for antibodies and CD4 in allosteric nanosensors

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We have analyzed the suitability of six antigenic peptides from several HIV-1 structural proteins (namely gp41, gp120, p17, and p24), as anti-HIV-1 antibody receptors in an allosteric enzymatic biosensor. These peptides were inserted in a solvent-exposed surface of *Escherichia coli* (*E. coli*) beta-galactosidase by means of conventional recombinant DNA technology. The resulting enzymes were tested to allosterically respond to sera from HIV-1-infected individuals. Only stretches from gp41 and gp120 envelope proteins were able to transduce the molecular contact signal in the presence of immunoreactive sera. Intriguingly, the enzyme displaying the CD4 binding site segment KQFINMWQEVGKAMYAPP was activated by soluble CD4, suggesting that it produces conformational modifications on the allosteric enzyme as those occurring during antibody-promoted induced fit. This fact is discussed in the context of the design of smart protein drugs and markers targeted to $CD4^+$ cells. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: biosensor; antibody; virus receptor; CD4; cell binding

INTRODUCTION

Protein–protein interactions can be transmitted across folded polypeptide chains through conformational modifications, for short distance straightforward transduction of molecular signals. Such natural principle, also observed in allosteric enzymes, is exploited in man-made biosensors based on enzymes or fluorescent proteins that allosterically respond to a specific effector [Ferraz *et al*., 2006c]. In this regard, several functional polypeptides including beta-galactosidase, alkaline phosphatase, beta-lactamase, and green fluorescent protein have been engineered to display, in specific solvent-exposed areas of the protein, foreign protein stretches that interact specifically with ligands of analytical interest, especially antibodies [Villaverde, 2003]. The docking between the effector antibody and the receptor peptide at the sensor surface promotes conformational modifications that modulate the enzymatic activity, usually by enhancing it up to an analytical level. The amount of product derived from the enzymatic reaction depends, quantitatively, on the effector's concentration. Antibodies, because of the perturbations produced on the epitope's conformation (induced fit) seem to be particularly good analytes for allosteric biosensing [Villaverde, 2003]. For that reason, these enzymatic biosensors appear as especially appropriate for the diagnosis of infectious diseases.

We have previously developed a series of genetically related beta-galactosidases using antigenic peptides from foot-and-mouth disease virus (FMDV) [Benito *et al*., 1996] and human immunodeficiency virus (HIV)-1 [Ferrer-Miralles *et al*., 2001] as receptors for specific antibodies in sera samples, which respond allosterically in a dose-dependent manner. In particular, the beta-galactosidase variant NF795gpC displays the immunodominant epitope P1 from the envelope glycoprotein gp41 of HIV-1, between residues 795–796 [Ferrer-Miralles *et al*., 2001]. By protein engineering [Cazorla *et al*., 2002], adjusting the reaction conditions [Ferraz *et al*., 2004] and selecting a convenient beta-galactosidase substrate [Ferraz *et al*., 2006b], the analytical signal (that is, the amount of substrate processed per time unit) has been enhanced up to more than 10 fold than that observed in absence of effector (the background signal). This has permitted the use of this enzyme for high-throughput functional screening of human sera [Ferraz *et al*., 2006a] and to explore the functional modification of the humoral response promoted by antiretroviral therapy [Ferraz *et al*., 2008]. For further diagnostic applications and molecular screening of sera samples, it would be highly convenient to identify other immunogenic HIV-1 peptides suitable to be used as receptors in beta-galactosidase sensors, to expand the spectrum of sensed antibody subpopulations. We

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have here inserted selected stretches from gp120, p17, and p24 in the convenient accommodation site of the bacterial enzyme, and measured the allosteric activation of the sensor in front of sera samples in comparison with the NF795gpC performance. Two new suitable peptidic receptors from gp120 have been identified. Intriguingly, the gp120 peptide KQFINMWQEVGKA-MYAPP, acting in the virus envelope as the CD4 binding site, responds to soluble CD4 glycoprotein in a dose dependent way. The biolotechnological implications of this finding are discussed in the context of potential development of $CD4^{+}$-targeted drugs.

MATERIALS AND METHODS

Protein construction, production, and purification

Several highly antigenic peptides from HIV-1 structural proteins were selected to be displayed on the *Escherichia coli* (*E. coli*) beta-galactosidase protein (between amino acids 795 and 796) as allosteric receptors. The parental expression vector pJX795 [Feliu and Villaverde, 1998] contains a *Bam*HI restriction site siding the codon 795 of *lacZ* gene in which pairs of complementary, synthetic oligonucleotides with overhanging *Bam*HI-linking stretches were inserted by conventional cloning strategies [Ferrer-Miralles *et al*., 2001]. This plasmid permits the tempera-ture-controled induction of recombinant gene expression regulated by *p*R-*p*L lytic lambda promoters and the thermo-sensitive CI^{857} repressor. The sequences of displayed peptides and features of the source HIV-1 proteins are summarized in Table 1. Chimerical enzymes were produced in *E. coli* BL26 [Sambrook *et al*., 1989] in Luria-Bertani (LB) medium [Sambrook *et al*., 1989] by a temperature shift to 42°C. Four hours after induction of gene expression, cells were low speed centrifuged, rapidly frozen at −80°C and further de-frozen, and sonicated by 25 min jacketed in ice. Details of the cell disruption profile can be found elsewhere [Feliu *et al*., 1998b]. Proteins were purified from cell extracts in a single step by affinity chromatography with Sepharose-TPEG (phenylethyl-beta-D-galactopyranoside) columns [Ullmann, 1984]. Finally, the obtained protein was dialyzed against Z buffer [Miller, 1972] and quantified by Bradford analysis [Sambrook *et al*., 1989].

Biosensing assay

The biosensing reaction was performed in 96-well ELISA Costar microplates by mixing 10 μl of protein solution with 20 μl of serum. Sera were always used at a dilution 1:4. After incubation for 1 h at 28°C, 40 μl of the beta-galactosidase substrate CPRG (chlorophenol red *β*-D-galactopyranoside) at a defined concen-tration (Table 2) was added, and absorbance at 540 nm was determined 30 min later in a Labsystems IEMS plate reader. The sensing signal was obtained as the percent ratio between the product obtained in the presence and in the absence of sera [Ferraz *et al*., 2004].

The biosensing assay was specifically adapted to the new proteins as done previously for NF795gpC with a sera pool from 36 HIV-1-infected individuals [Ferraz *et al*., 2006b]. For that, several concentrations of both protein and substrate were assayed following a central composite rotatable experimental design (CCRD, Essential Experimental Design tool from Microsoft Excel 2003), and results analyzed by the Essential Regression

Table 1. Features of antigenic HIV-1 peptides selected for display on *E. coli* beta-galactosidase

Biosensor name	Sequence	Peptide length (aa)	Protein	Region	Reference
NF795gpC	^{579}GIKQLQARILAVERYLKDQQLLGIWGCSGKLICTT^{613}	35	gp41	P1/P2	[Ferrer-Miralles *et al*., 2001]
JX795-MN	^{299}INCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQA^{333}	35	gp120	V3 (MN strain)	[Broliden *et al*., 1992]
JX795-IIIB	^{271}INCTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQ^{305}	35	gp120	V3 (IIIB strain)	[Broliden *et al*., 1992]
JX795-B138	^{421}KQFINMWQEVGKAMYAPP^{438}	18	gp120	CD4BS	[Morrow *et al*., 1992]
JX795-p17	^{2}GARASVLSGGELDRWEKIRLRPGGKKKYKLKHIVW^{36}	35	p17	nd	[Fiorentini *et al*., 2004]
JX795-p24	^{196}AAMQMLKETINEEAAEWDRVHPVHAGPIAPGQMRE^{230}	35	p24	nd	[Janvier *et al*., 1991]

nd: not defined.

Table 2. Concentrations of both protein and substrate (CPRG) used in the assays, for each specific sensor

Biosensor	Protein (nM)	CPRG (mg/ml)
NF795gpC	4.16	1
JX795-MN	2.5	2.5
JX795-IIIB	4.16	1
JX795-B138	8.33	3.5
JX795-p17	8.33	0.5
JX795-p24	8.33	1

software from Microsoft Excel 2003 (Microsoft Corporation, Redmont, WA) and Minitab 14 software (MINITAB. Release 14.12.0. (2004) Minitab Inc). Those concentrations giving the maximal enzymatic activity for any specific enzyme in presence of an immune sera pool (Table 2) were selected for the analysis of individual sera samples.

Western blot

Briefly, 1 μg of each enzyme was used for SDS-PAGE after heating at 98°C for 20 min in Laemli buffer [Sambrook *et al*., 1989] plus 8M urea. After running and transfer, bands were identified with a sera pool [Ferraz *et al*., 2006b] from 36 HIV-1 infected patients (1/300), and visualized with goat anti-human IgG antibody peroxidase conjugated (1/2000) from Pierce. Beta-galactosidase immunoreactivity was determined similarly as described [Ferrer-Miralles *et al*., 2001].

Sera samples and clinical parameters

HIV-1 immune sera samples and clinical parameters data (viral load, $CD4^+$ and $CD8^+$ T cell count) were kindly provided by Miguel Ángel Martinez from the Hospital Universitari Germans Trias i Pujol, Badalona, Spain.

Statistical analysis

Regression analysis between activation factors and clinical parameters were calculated with Sigma Plot v.10. *p*-values under 0.05 were considered as statistically significant.

CD4 sensing

Pure soluble CD4 glycoprotein from Affinity Bioreagents was used to analyze the activation of protein JX795-B138. Briefly, appropriate dilutions of JX795-B138 and CD4 glycoprotein in Z buffer were mixed in a final volume of 120 μl and incubated at 28°C for 1 h. Then, 40 μl of CPRG was added and its hydrolysis product determined after 30 min.

RESULTS AND DISCUSSION

Antibody-mediated sensor activation

Several antigenic HIV-1 peptides were selected from bibliographical sources to be tested as receptors in allosteric sensors (Table 1). All of them were inserted between amino acids 795 and 796 of the bacterial beta-galactosidase by means of recombinant DNA technology, and the resulting enzymes were produced in *E. coli* and purified by affinity chromatography as described elsewhere [Ferraz *et al*., 2006b]. After production, all the proteins were well recognized by anti-beta-galactosidase antibodies in Western blots, showing sufficient proteolytic stability (data not shown). Further functional testing was carried out by using protein NF795gpC [Ferrer-Miralles *et al*., 2001] as reference. Before sera analysis, beta-galactosidase reaction conditions optimal for the sensing reaction were determined for each protein by using an HIV-1 immune sera pool (Table 2). Under these conditions, purified proteins were exposed to different sera from HIV-1-infected individuals to evaluate the extend of the enzymatic activation. As observed (Figure 1A), only NF795gpC, JX795-MN and JX795-IIIB were notably activated by the tested sera. The signal produced by JX795-IIIB hardly doubled the background value, being lower than that rendered by NF795gpC and JX795-MN (up to 1200 and 800% the background, respectively) but sufficiently high for analytical purposes and within the range offered by other allosteric enzymatic sensors [Ferrer-Miralles *et al*., 2000]. The other three proteins were not activated by any of the tested sera. When exploring the sensing signals induced by particular sera samples we noted that different enzymes were differently activated by a given serum (a few examples are depicted in Figure 1B), indicating that the populations of antibodies directed to particular B-cell epitopes were different in different patients. Altogether, these data showed that the two novel peptides were highly promising

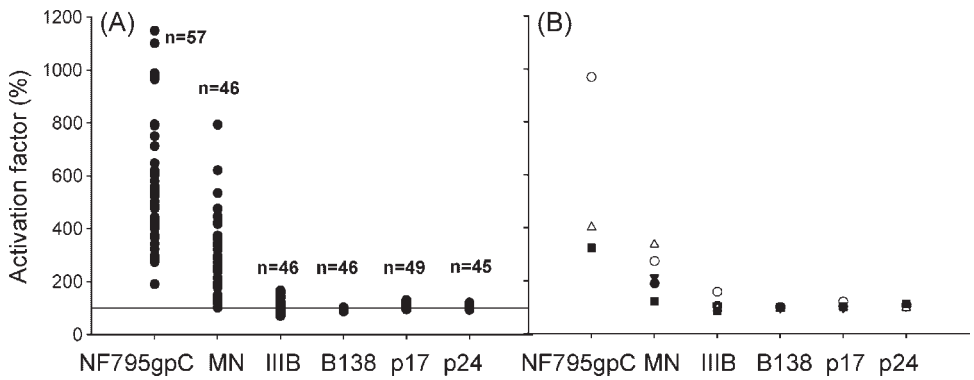

Figure 1. Beta-galactosidase activation promoted by HIV-1 immune human sera analyzed on different enzyme variants. The horizontal bar indicates the background value (in absence of sera) as reference. Protein names have been shortened for a better presentation. (A) The total number of sera and individual sensor activation values are indicated. (B) Activation values of only five are represented (indicated by different symbols) for comparison of activation levels of the sensor set.

allosteric receptors whose use in multiple sensing arrays would widen the spectrum of anti-HIV-1 antibodies detectable by enzymatic reactions. In this regard, the recent finding that allosteric beta-galactosidase sensors show high performance upon solid-phase immobilization [Ferraz *et al*., 2007] offers the possibility to develop miniaturized devices in which several sensing elements reactive to complementary analytes (in this case, different populations of antibodies) could offer matricidal data profiling the immune response. The null responses of JX795-p17 and JX795-p24 are probably due to low titres of antibodies directed against p17 and p24 in most of the tested sera, since such proteins were also hardly recognized in western blot (data not shown). In fact, anti-p17 and -p24 antibodies rise immediately after infection but their concentration in blood might decline a few weeks later [Chargelegue *et al*., 1995]. On the other hand, the low activation of JX795-B138 could be due to a receptor peptide shorter than the length required for efficient interaction with antibodies [Ferrer-Miralles *et al*., 2001], since while this stretch being a prevalent antigenic determinant of the virus, the carrying protein was not recognized by immune sera pool (data not shown). Interestingly, this peptide accommodated in beta-galactosidase shows the lowest average hydrophilicity index (−0.3) among the tested peptide set (up to 0.4, not shown) according to the Hopp & Woods scale [Hopp and Woods, 1981; Hopp and Woods, 1983], a fact that could severely impair its role as antibody ligand.

CD4-mediated sensor activation

As a routine analysis, we determined if the individual allosteric signal induced by particular sera in all the engineered enzymes would correlate with clinical parameters of the patients who were the source of sera. Such correlation was not expected since the only known effectors of responsive beta-galactosidases are antibodies targeted to antigenic receptor peptides [Ferraz *et al*., 2006a]. However, the activation of protein JX795-B138 clearly correlated with the level of $CD4^+$ cells in blood, a fact never observed in previous studies in which the sensing response of NF795gpC was deeply characterized. Such correlation was consistently observed when analyzing the sera from patients receiving an antiretroviral treatment and also naïve patients (Table 3). Since the viral peptide exposed on JX795-B138 contains the CD4 binding site, which enables the virus to attach the target cells, we suspected that CD4 itself could be acting as an effector for sensor activation, in a similar way than antibodies do. Being fully plausible, this possibility was not supported by previous data. In fact, cell surface integrins were unable to activate a similar beta-galactosidase sensor displaying a viral, RGD-based integrin binding site, that was instead efficiently activated by anti-RGD antibodies [Feliu *et al*., 1998c; Alcala *et al*., 2001]. However, although up to a level lower than that usually observed for antibodies (130%), soluble CD4 enhanced the enzymatic activity of JX795-B138 in a dose-dependent way (Figure 2), being this the

Table 3. Correlation parameters between activation factor of each protein and viral load (VL), $CD4^+$ T cell (CD4), and $CD8^+$ T cell (CD8) count in sera samples samples

	VL		CD4		CD8	
HIV-infected individuals (*n*)	*p*	r^2	*p*	r^2	*p*	r^2
NF795gpC (57)	0.1936	0.0309	0.3051	0.0190	0.5800	0.0057
JX795-MN (46)	0.8996	0.0003	0.5139	0.0099	0.7165	0.0031
JX795-IIIB (46)	0.2305	0.0332	0.5212	0.0096	0.1451	0.0498
JX795-B138 (46)	0.4772	0.0121	**0.0291**	0.1082	0.5333	0.0095
JX795-p17 (49)	0.6144	0.0056	0.1209	0.0526	0.2155	0.0346
JX795-p24 (45)	0.1543	0.0476	0.8374	0.0010	0.7632	0.0018
Non-treated individuals (*n*)[a]						
NF795gpC (18)	0.6420	0.0138	0.5058	0.0281	0.0574	0.2202
JX795-MN (12)	0.4393	0.0609	0.6032	0.0279	0.8633	0.0034
JX795-IIIB (12)	0.2859	0.1127	0.5366	0.0393	0.2090	0.1690
JX795-B138 (11)	0.9263	0.0013	**0.0461**	0.3727	0.1614	0.2293
JX795-p17 (12)	0.9957	0.0000	0.9288	0.0008	0.7584	0.0110
JX795-p24 (11)	0.8284	0.0055	0.4739	0.0584	0.3970	0.0909
Treated individuals (*n*)[a]						
NF795gpC (20)	0.4008	0.0395	0.3821	0.0426	0.4764	0.0285
JX795-MN (17)	0.1307	0.1555	0.4869	0.0351	0.2511	0.0928
JX795-IIIB (17)	0.0878	0.1938	0.4000	0.0510	0.7851	0.0054
JX795-B138 (18)	0.2001	0.1143	**0.0162**	0.3479	0.9686	0.0001
JX795-p17 (20)	0.3799	0.0484	0.4434	0.0371	0.2478	0.0825
JX795-p24 (17)	0.2307	0.1008	0.9557	0.0002	0.9877	0.0000

Significant *p*-values ($p < 0.05$) are indicated in bold.

[a]Subpopulations of HIV-infected individuals, for which the absolute lack of antiretroviral treatment or its regular administration was documented, were in addition separately analyzed from the whole group.

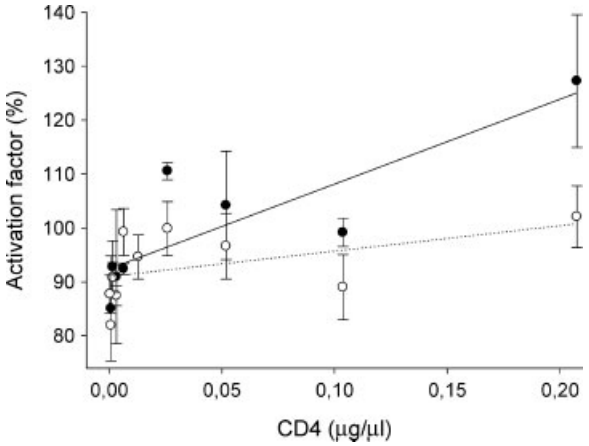

Figure 2. Dose-dependent beta-galactosidase activation promoted by soluble CD4 on JX795-B138 (continuous line, filled dots; $r^2 = 0.713$, $p = 0.008$). As a control, data obtained with NF795gpC (discontinuous line, empty dots; $r^2 = 0.230$, $p = 0.159$) are also plotted. Shown values are the mean from three independent experiments.

first report of a protein other than an antibody being able to activate beta-galactosidase allosteric sensors. Besides, the ability of CD4 to allosterically activate JX795-B138 demonstrated that the mere interaction between CD4 and the KQFINMWQEVGKAMYAPP peptide was able to promote conformational modifications in the active site of the enzyme in absence of additional ligands and the gp120 framework. Since denatured gp120 does not bind CD4 [Robey *et al*., 1996], the allosteric activation observed here promoted by the interaction between JX795-B138 and CD4 suggests that the viral peptide adopts, as accommodated in the bacterial enzyme, a native or native-like conformation, as it has been suggested by molecular dynamics for other viral peptides acting as efficient receptors in allosteric sensors [Feliu *et al*., 1998a; Ferrer-Miralles *et al*., 2001].

In the context of viral infection, the structural reorganization of gp41 that exposes its N-terminal fusion peptide for cell penetration is promoted by conformational modifications induced in gp120 by its binding to viral receptor and also co-receptor molecules [Eckert and Kim, 2001; Follis *et al*., 2002]. In fact, the CD4 binding site is a main neutralizing epitope of the virus that, as others, seems to be sensitive to conformational perturbations promoted by the antibody-induced fit [de Rosny *et al*., 2004]. Also, the introduction of single amino acid substitutions inactivate distant B-cell epitopes not only within any of gp120 or gp41 proteins but also between them [Thali *et al*., 1994; Watkins *et al*., 1996; Park *et al*., 1998, 2000; Zhang *et al*., 2002], stressing the particular molecular flexibility of gp41-gp120 trimeric complexes. In fact, such a particular molecular flexibility of gp120 and gp41 is assumed to permit signal transduction between both proteins during viral attachment [Myszka *et al*., 2000; Yingliang *et al*., 2007]. Very recently, the conformation of the gp120 CD4 binding site has been modeled in both CD4 bound and unligated states [Berkower *et al*., 2008]. CD4 binding promotes important reorganizations in surrounding gp120 loops that are responsible of the conformational masking of the neutralizing epitope in the CD4 binding site.

Such a particular flexibility of gp120 during receptor binding could explain that the interaction between CD4 and the HIV-1 CD4 binding site can be transmitted to a heterologous carrier protein were the viral peptide is accommodated, and produce a remarkable macroscopic event as observed here. This fact can be of relevant interest for the design of smart protein drugs for gene and cell therapy, that need to respond to specific stimuli such as specific receptor binding of endosomal acidification for transmembrane crossing [Ferrer-Miralles *et al*., 2008]. Since $CD4^+$cells are potential therapeutic targets in HIV-1 infection, the availability of a ligand peptide that could activate a coupled enzyme upon CD4 binding (or more generally, modulate the conformation or activity of a partner protein), is of pharmacological interest and its therapeutic applicability should be further investigated.

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REFERENCES

Alcala P, Feliu JX, Aris A, Villaverde A. 2001. Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins. *Biochem. Biophys. Res. Commun.* **285**: 201–206.

Benito A, Feliu JX, Villaverde A. 1996. Beta-galactosidase enzymatic activity as a molecular probe to detect specific antibodies. *J. Biol. Chem.* **271**: 21251–21256.

Berkower I, Patel C, Ni Y, Virnik K, Xiang Z, Spadaccini A. 2008. Targeted deletion in the beta20-beta21 loop of HIV envelope glycoprotein gp120 exposes the CD4 binding site for antibody binding. *Virology* **377**: 330–338.

Broliden PA, von Gegerfelt A, Clapham P, Rosen J, Fenyo EM, Wahren B, Broliden K. 1992. Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. *Proc. Natl Acad. Sci. USA* **89**: 461–465.

Cazorla D, Feliu JX, Ferrer-Miralles N, Villaverde A. 2002. Tailoring molecular sensing for peptide displaying engineered enzymes. *Biotechnol. Lett.* **24**: 467–477.

Chargelegue D, Stanley CM, O'Toole CM, Colvin BT, Steward MW. 1995. The affinity of IgG antibodies to gag p24 and p17 in HIV-1-infected patients correlates with disease progression. *Clin. Exp. Immunol.* **99**: 175–181.

de Rosny E, Vassell R, Jiang S, Kunert R, Weiss CD. 2004. Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes. *J. Virol.* **78**: 2627–2631.

Eckert DM, Kim PS. 2001. Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* **70**: 777–810.

Feliu JX, Benito A, Oliva B, Aviles FX, Villaverde A. 1998a. Conformational flexibility in a highly mobile protein loop of foot-and-mouth disease virus: distinct structural requirements for integrin and antibody binding. *J. Mol. Biol.* **283**: 331–338.

Feliu JX, Cubarsi R, Villaverde A. 1998b. Optimized release of recombinant proteins by ultrasonication of *E. coli* cells. *Biotechnol. Bioeng.* **58**: 536–540.

Feliu JX, Ramirez E, Villaverde A. 1998c. Distinct mechanisms of antibody-mediated enzymatic reactivation in beta-galactosidase molecular sensors. *FEBS Lett.* **438**: 267–271.
Feliu JX, Villaverde A. 1998. Engineering of solvent-exposed loops in *Escherichia coli* beta-galactosidase. *FEBS Lett.* **434**: 23–27.
Ferraz RM, Aris A, González G, López-Santín J, Villaverde A, Alvaro G. 2007. Allosteric molecular sensing of anti-HIV antibodiesmby an immobilized, engineered beta-galactosidase. *Enzyme Microb. Technol.* **41**: 492–497.
Ferraz RM, Aris A, Martinez MA, Villaverde A. 2006a. High-throughput, functional screening of the anti-HIV-1 humoral response by an enzymatic nanosensor. *Mol. Immunol.* **43**: 2119–2123.
Ferraz RM, Aris A, Villaverde A. 2004. Profiling the allosteric response of an engineered beta-galactosidase to its effector, anti-HIV antibody. *Biochem. Biophys. Res. Commun.* **314**: 854–860.
Ferraz RM, Aris A, Villaverde A. 2006b. Enhanced molecular recognition signal in allosteric biosensing by proper substrate selection. *Biotechnol. Bioeng.* **94**: 193–199.
Ferraz RM, Martinez MA, Cubarsi R, Villaverde A. 2008. Antiretroviral therapy-induced functional modification of IgG4 and IgM responses in HIV-1-infected individuals screened by an allosteric biosensor. *J. Biomol. Screen* **13**: 817–821.
Ferraz RM, Vera A, Aris A, Villaverde A. 2006c. Insertional protein engineering for analytical molecular sensing. *Microb. Cell Fact.* **5**: 15.
Ferrer-Miralles N, Feliu JX, Villaverde A. 2000. Molecular mechanisms for antibody-mediated modulation of peptide-displaying enzyme sensors. *Biochem. Biophys. Res. Commun.* **275**: 360–364.
Ferrer-Miralles N, Feliu JX, Vandevuer S, Muller A, Cabrera-Crespo J, Ortmans I, Hoffmann F, Cazorla D, Rinas U, Prévost M, Villaverde A. 2001. Engineering regulable *Escherichia coli* beta-galactosidases as biosensors for anti-HIV antibody detection in human sera. *J. Biol. Chem.* **276**: 40087–40095.
Ferrer-Miralles N, Vazquez E, Villaverde A. 2008. Membrane-active peptides for non-viral gene therapy: making the safest easier. *Trends Biotechnol.* **26**: 267–275.
Fiorentini S, Marini E, Bozzo L, Trainini L, Saadoune L, Avolio M, Pontillo A, Bonfanti C, Sarmientos P, Caruso A. 2004. Preclinical studies on immunogenicity of the HIV-1 p17-based synthetic peptide AT20-KLH. *Biopolymers* **76**: 334–343.
Follis KE, Larson SJ, Lu M, Nunberg JH. 2002. Genetic evidence that interhelical packing interactions in the gp41 core are critical for transition of the human immunodeficiency virus type 1 envelope glycoprotein to the fusion-active state. *J. Virol.* **76**: 7356–7362.
Hopp TP, Woods KR. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl Acad. Sci. USA* **78**: 3824–3828.
Hopp TP, Woods KR. 1983. A computer program for predicting protein antigenic determinants. *Mol. Immunol.* **20**: 483–489.
Janvier B, Baillou A, Archinard P, Mounier M, Mandrand B, Goudeau A, Barin F. 1991. Immune response to a major epitope of p24 during infection with human immunodeficiency virus type 1 and implications for diagnosis and prognosis. *J. Clin. Microbiol.* **29**: 488–492.
Miller JH. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
Morrow WJ, Williams WM, Whalley AS, Ryskamp T, Newman R, Kang CY, Chamat S, Köhler H, Kieber-Emmons T. 1992. Synthetic peptides from a conserved region of gp120 induce broadly reactive anti-HIV responses. *Immunology* **75**: 557–564.
Myszka DG, Sweet RW, Hensley P, Brigham-Burke M, Kwong PD, Hendrickson WA, Wyatt R, Sodroski J, Doyle ML. 2000. Energetics of the HIV gp120-CD4 binding reaction. *Proc. Natl Acad. Sci. USA* **97**: 9026–9031.
Park EJ, Vujcic LK, Anand R, Theodore TS, Quinnan GV Jr. 1998. Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. *J. Virol.* **72**: 7099–7107.
Park EJ, Gorny MK, Zolla-Pazner S, Quinnan GV Jr. 2000. A global neutralization resistance phenotype of human immunodeficiency virus type 1 is determined by distinct mechanisms mediating enhanced infectivity and conformational change of the envelope complex. *J. Virol.* **74**: 4183–4191.
Robey FA, Harris-Kelson T, Robert-Guroff M, Batinic D, Ivanov B, Lewis MS, Roller PP. 1996. A synthetic conformational epitope from the C4 domain of HIV Gp120 that binds CD4. *J. Biol. Chem.* **271**: 17990–17995.
Sambrook J, Fritsch E, Maniatis T. 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
Thali M, Charles M, Furman C, Cavacini L, Posner M, Robinson J, Sodroski J. 1994. Resistance to neutralization by broadly reactive antibodies to the human immunodeficiency virus type 1 gp120 glycoprotein conferred by a gp41 amino acid change. *J. Virol.* **68**: 674–680.
Ullmann A. 1984. One-step purification of hybrid proteins which have beta-galactosidase activity. *Gene* **29**: 27–31.
Villaverde A. 2003. Allosteric enzymes as biosensors for molecular diagnosis. *FEBS Lett.* **554**: 169–172.
Watkins BA, Buge S, Aldrich K, Davis AE, Robinson J, Reitz MS Jr, Robert-Guroff M. 1996. Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. *J. Virol.* **70**: 8431–8437.
Yingliang W, Hong Y, Zhijian C, Wenxin L. 2007. Conformation of trimeric envelope glycoproteins: the CD4-dependent membrane fusion mechanism of HIV-1. *J. Biomol. Struct. Dyn.* **25**: 1–9.
Zhang PF, Bouma P, Park EJ, Margolick JB, Robinson JE, Zolla-Pazner S, Flora MN, Quinnan GV Jr. 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. *J. Virol.* **76**: 644–655.