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Efficient Accommodation of Recombinant, Foot-and-Mouth Disease Virus RGD Peptides to Cell-Surface Integrins

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The engineering of either complete virus cell-binding proteins or derived ligand peptides generates promising nonviral vectors for cell targeting and gene therapy. In this work, we have explored the molecular interaction between a recombinant, integrin-binding foot-and-mouth disease virus RGD peptide displayed on the surface of a carrier protein and its receptors on the cell surface. By increasing the number of viral segments, cell binding to recombinant proteins was significantly improved. This fact resulted in a dramatic growth stimulation of virus-sensitive BHK_{21} cells but not virus-resistant HeLa cells in protein-coated wells. Surprisingly, growth stimulation was not observed in vitronectin-coated plates, suggesting that integrins other than $\alpha_v\beta_3$ could be involved in binding of the recombinant peptide, maybe as coreceptors. On the other hand, both free and cell-linked integrins did not modify the enzymatic activity of RGD-based enzymatic sensors that contrarily, were activated by the induced fit of anti-RGD antibodies. Those findings are discussed in the context of a proper mimicry of the unusually complex architecture of this cell-binding site as engineered in multifunctional proteins. © 2001 Academic Press

Key Words: **RGD; recombinant protein; cell receptor; integrin; FMDV.**

Modified viruses are useful DNA-delivering instruments for gene therapy purposes (1, 2). However, undesirable reactions to viral administration have been eventually observed (3, 4). This fact, together with the growing concern about the dissemination of modified organisms, firmly prompt the development of non-infectious vehicles that could represent realistic alternatives to viral-mediated gene transfer. In this regard, multifunctional proteins engineered to succeed in the mimicking of viral functions such as DNA condensation, cell attachment and internalisation, represent intriguing challenges that have resulted in the generation of promising prototypes (5–7).

In previous works, we have inserted a recombinant peptide from foot-and-mouth disease virus (FMDV) containing the RGD-based, integrin $\alpha_v\beta_3$-targeted cell binding motif, in different solvent-exposed surfaces of *E. coli* β-galactosidase (8, 9). This protein segment, that on the virus surface shows an extreme architectural complexity (10–12), promotes in these unnatural molecular frameworks cell attachment and protein internalisation (12, 13), being thus a suitable cell-binding tag in nonviral recombinant vehicles for targeted gene delivery (14). On the other hand, the same viral peptide contains several overlapping B-cell epitopes, corresponding to a major immunodominant site of FMDV (15) Recently, we have shown that by increasing the number of inserted peptides in the bacterial enzyme, the immunoreactivity of the resulting proteins is dramatically improved (16). Since this viral peptide is also the basis of prototype vehicles for cell-targeted gene delivery based on multifunctional proteins (14, 17), we wanted to examine the influence of multivalent RGD presentation on cell binding properties of hybrid β-galactosidases to cell surface integrins acting as main FMDV receptors. The obtained results prove a dramatic influence of multiple peptide presentation enhancing specific cell binding and strongly suggest an efficient molecular accommodation of the recombinant viral peptide to integrin $\alpha_v\beta_3$ or/and to alternative or cooperative cell receptors.

MATERIALS AND METHODS

Proteins and antibodies. Protein M278VP1 is a hybrid *E. coli* β-galactosidase protein in which a foot-and-mouth disease virus

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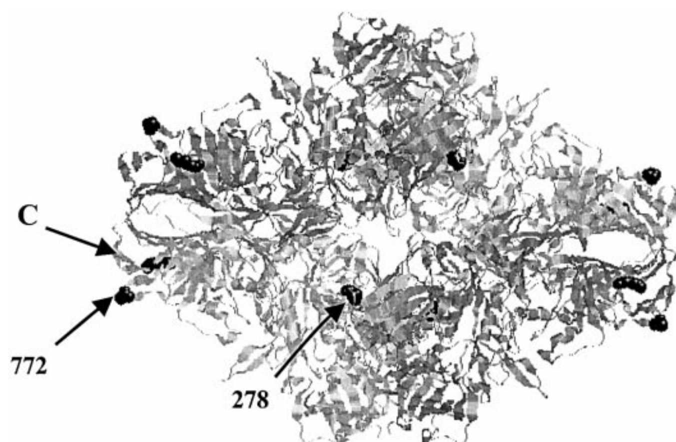

FIG. 1. A RasMol[R] representation of *E. coli* β-galactosidase indicating on each monomer the insertion (residues 278 and 772) and fusion (C-terminus) sites for RGD-containing peptides in the hybrid protein HT7278CA. In a single monomer, these positions are indicated by arrows.

(FMDV) protein segment has been accommodated in a solvent-exposed region, in the vicinity of the active site (17). This peptide, that reproduces the G-H loop of the VP1 capsid protein (clone C-S8c1) (10), includes the complete, antigenic site A that also embraces the RGD-based cell attachment motif (18) responsible for cell infection. Upon binding of anti-peptide antibodies, the enzymatic activity of M278VP1 increases in a titre-dependent fashion (19). Protein HT7278CA, contains three copies per monomer of the same viral peptide sequence, that have been accommodated in the same insertion site than in M278VP1 plus in the position 795 and in the carboxy terminus of the enzyme (20). The activity of this protein, that in the tetrameric form exposes 12 copies of the viral peptide (Fig. 1), is also modulated by antibody binding (Feliu *et al.,* submitted). Proteins HD72CA, HD78CA, and HD7872A contain two copies per monomer of the peptide in the different combinations of these three insertion sites (21). All of these proteins have been produced in temperature-dependent bacterial expression systems and purified as described (22).

Neutralising monoclonal antibodies SD6 and 3E5 have been elicited against FMDV and both recognise RGD-containing B-cell epitopes within the site A (15, 23, 24).

Cell binding assay. Cell binding to β-galactosidase recombinant proteins was performed as described previously (25) with slight modifications. Briefly, 10 pmols of protein in PBS were bound to ELISA microtitre plates overnight at 4°C. Wells were then washed in PBS and blocked with 3% BSA in PBS for 1 h at 37°C. Trypsinized HeLa and BHK cells were added at about 5×10^5 cells/ml, further incubated for 90 min at 37°C and gently washed in PBS, after which the remaining cells were fixed with methanol. Cells were then stained with crystal violet, washed in PBS and lysed with 1% SDS. The absorbance was determined at 620 nm in a microtiter reader and blanks were obtained by measuring cell binding to BSA. The specificity of cell binding was confirmed by exposition of protein HT7278CA to anti-FMDV 3E5 monoclonal antibody before incubation with cells, as previously described (12). All the assays were done at least in triplicate.

Analysis of cell growth kinetics. Cell cultures flasks of 25 cm^2 were blocked with 35 pmols of protein HT7872CA and vitronectin in PBS, for 2.5 h at 37°C. After washing with PBS, trypsinized BHK and HeLa cells were added at about 8.5×10^4 cells/ml and further incubated at 37°C. At different times cells were then collected by trypsinization and counted in a hemocytometer slide. Determinations were performed up to three times.

Modulation assay in liquid phase. The modulation assay was done in microtiter ELISA plates by slight modification of a previously described procedure (19). Two picomols of M278VP1 in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, pH 7.0) plus 1% BSA and 1 mM $MnCl_2$, were incubated at 28°C with different concentrations of the anti FMDV mAb 3E5 (23) or either the integrins $\alpha_V\beta_3$ or $\alpha_5\beta_1$ (Chemicon, Inc.) in a range between 0.11 and 4×10^{-5} μM. After 1 h, 40 μl of 2 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG) substrate were added. When the reaction mixture turned yellow, the enzymatic reaction was stopped by adding 50 μl of 1 M Na_2CO_3 and the absorbency read at 405 nm (A_{405}) in an iEMS Reader MF (Labsystems). Values are expressed as the A_{405} at a given ligand concentration relative to that found in its absence. All the experiments were done at least in duplicate. In some assays, $MnCl_2$ was omitted as indicated in the figure legends.

In situ modulation assay. BHK_{21} cells, the currently used target for *in vitro* FMDV propagation (26), were cultured at different concentrations in 96-well tissue culture grade plates (Corning), in DMEM supplemented with 20% fetal calf serum. From to 1.5×10^3 to 1.5×10^4 cells were plated per well and incubated for 3 days, reaching different degrees of confluence ranging from about 30 to 90%. Control wells were also processed without cells. Wells were then washed twice in Z buffer and then blocked with 1% BSA in Z buffer for one hour at 37°C. After washing three times with Z buffer plus 1 mM $MnCl_2$, two picomols of M278VP1 in BSA and $MnCl_2$-containing Z buffer were added and incubated at 4°C. At this temperature, integrin binding but not ligand internalisation takes place (13, 27). After 2 h of incubation, 40 μl of 2 mg/ml ONPG were added and the reaction performed at 4°C as described before. Controls for 3E5-mediated reactivation under these incubation and reaction conditions were also included. All the experiments were done at least in triplicate.

Binding assay in solid phase. Binding of M278VP1 to either antibody or integrin ligands was done in solid phase by sandwich ELISA (28). Alternatively, binding was performed using a modification of a previously described procedure (29). Wells in ELISA microtiter plates were coated overnight at 4°C with integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ and mAbs SD6 and 3E5, by adding 100 μl of different dilutions of ligands in coating buffer (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Ligand concentration ranged between 0.055 and 2.05×10^{-5} μM. After washing with coating buffer, wells were blocked with 200 μl of binding buffer (50 mM Tris–HCl [pH 7.4], 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) with 5% BSA at room temperature for 3 h and washed three times with binding buffer plus 1 mg/ml BSA. Further incubation was done with 5 pmols of M278VP1 in binding buffer plus 1 mg/ml BSA at 30°C for 3 h. After washing three times with BSA-containing binding buffer, bound ligands were detected by rabbit sera against β-galactosidase (obtained in our laboratory). After washing, goat anti-rabbit linked to peroxidase (from Pierce) was added and incubated for 1 h at 37°C. After further washing, peroxidase reaction was done by adding a standard solution of H_2O_2, 3-dimetilaminobenzoic acid and 3-methyl-2-benzothiazolinone as substrates (Sigma). The reaction was stopped with 2 M H_2SO_4 and the resulting blue colour was read at 620 nm (A_{620}). Background was determined in wells using BSA as bound ligand, and it was subtracted to the obtained values. The assays were done at least in duplicate.

RESULTS

Differential cell binding to RGD-displaying, engineered β-galactosidases. The cell binding properties of engineered β-galactosidases presenting an RGD-containing FMDV peptide were investigated. While the presence of two copies per monomer of the viral peptide improved cell attachment only moderately (compare

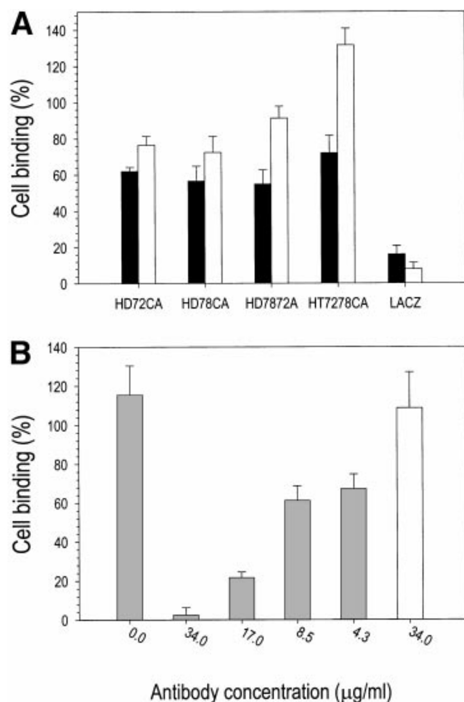

FIG. 2. (A) Binding of HeLa cells (black bars) or BHK_{21} cells (white bars) to β-galactosidase recombinant proteins. LACZ protein, the parental, pseudo-wild type β-galactosidase protein that lacks the amino seven residues, has been also included as a control. Values are given as the increase of the number of cells bound relative to that obtained with vitronectin. (B) Binding of BHK_{21} cells to β-galactosidase recombinant protein HT7278CA (grey bars), previously incubated with different concentrations of the anti-FMDV mAb 3E5. Values are given as the percentage of cell binding relative to that obtained with vitronectin. As a control of 3E5 specificity, vitronectin (white bar) was also included as cell ligand in the competition assay.

binding to HD proteins in Fig. 2A and in Ref. 16), protein HT7278CA (with three copies per monomer) showed a significant enhancement of cell binding above the values shown by vitronectin ($P < 0.01$), the natural ligand for integrin $\alpha_v\beta_3$ (Fig. 2A). Interestingly, binding improvement was only observed on BHK_{21} cells, the common *in vitro* system for FMDV multiplication. HeLa cells, to which FMDV attaches but does not replicate (26), were retained by the HD protein set and the HT construct with a similar efficiency, also comparable to that observed in β-galactosidase proteins with single peptide insertions (12). Cell binding to HT7278CA was completely inhibited by a monoclonal antibody directed against the viral segment (Fig. 2B), confirming the specificity of the cell-protein interaction.

The properties of the multivalent cell ligand HT7278CA were further explored by assaying its ability to promote growth of cultured cells in protein-coated plates. Significant cell growth stimulation was observed in BHK_{21} ($P < 0.001$) but not in HeLa cells ($P > 0.05$, Fig. 3), a result that is in the context of the differential binding data presented in Fig. 2. Interestingly, vitronectin, the natural ligand for integrin $\alpha_v\beta_3$, showed a null stimulating effect on BHK_{21} proliferation.

Responsiveness of an RGD-based enzymatic sensor to integrin binding. The same FMDV RGD peptide used in this work had been previously employed as a biosensor component for antibody detection (19), since this amino acid segment also contains the antigenic, immunodominant site A of FMDV (8). As inserted in specific positions of *E. coli* β-galactosidase, the induced fit mediated by the antibody affects the enzymatic constants of the enzyme, resulting in detectable changes in the activity (30–32) and offering an interesting tool to monitor specific molecular interactions. Therefore, to better explore the molecular basis of the cell binding abilities exhibited by the recombinant peptide, we analysed the responsiveness of such sensor to integrin binding by using the regulatable enzyme M278VP1 (19). In this protein, carrying one copy per monomer of the FMDV peptide, the insertion site is one of the used in HT7278CA and it has been fully characterised regarding the mechanics of the enzymatic modulation (19, 30, 31), being then useful to evaluate the impact of integrin-peptide interaction on the peptide conformation. Figure 4 shows that while monoclonal antibody 3E5 reactivated protein M278VP1, integrin $\alpha_V\beta_3$ did not have any impact on the enzymatic activity of the sensor. This lack of responsiveness occurred also in presence of divalent cations, which are required for efficient integrin binding (Fig. 4B). Integrin $\alpha_5\beta_1$, recently recognised as a receptor for FMDV (33) and to which recombinant FMDV RGD peptides also bind (12, 34), is also unable to induce enzymatic changes in M278VP1. Binding of these purified integrins to recombinant enzymes was confirmed by solid phase assays

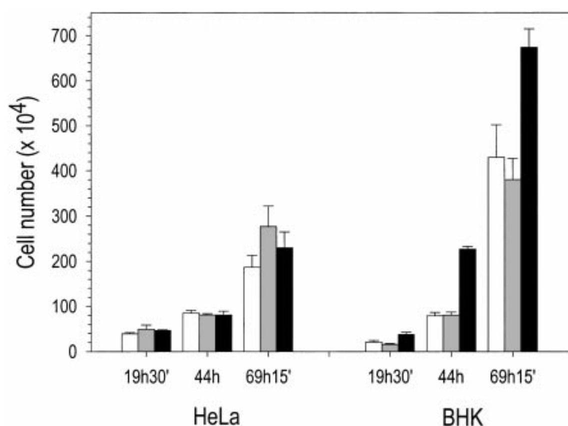

FIG. 3. Cell growth of HeLa or BHK_{21} cells in flasks previously coated with 35 pmols of vitronectin (grey bars) or HT7872CA protein (black bars). As a control, flasks treated with only PBS have been included (white bars).

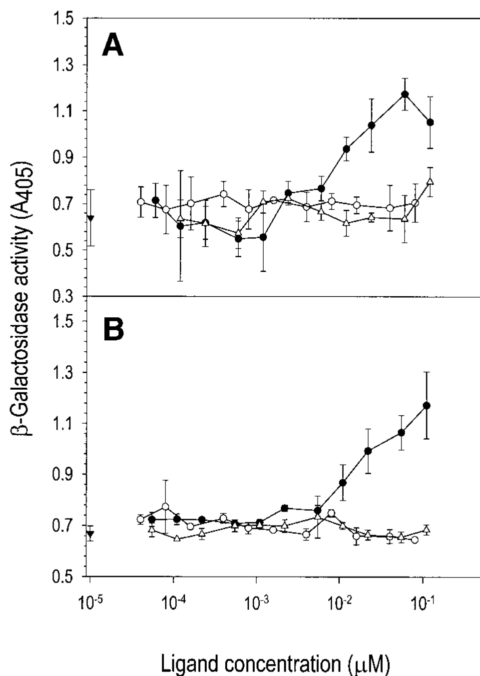

FIG. 4. Variations in the enzymatic activity of a M278VP1 solution in presence of mAb 3E5 (●) and integrins $\alpha_V\beta_3$ (△) and $\alpha_5\beta_1$ (○), in absence (A) or in presence (B) of Mn^{2+}, which has been proven to be critical for integrin binding (29, 34). The activity of M278VP1 alone is also indicated (▼).

(not shown), as previously done with intact FMDV particles (29).

To better reproduce the natural context for ligand interaction, integrin mediated sensor regulation was also explored with whole cells at 4°C, a temperature in which integrin-binding ligands bind but do not internalise (13, 27). The performance of this experiment was convenient since it could not be discarded that soluble integrins would promote a different binding pattern than when presented at the cell surface. However, as in the case of pure integrins, enzymatic reactivation was again not observed up the maximum cell concentration achieved in cell culture (Fig. 5).

DISCUSSION

Cell targeting for drug and DNA delivery is a matter of increasing interest, especially in the context of the complete human genome sequencing and the expected identification and molecular characterisation of genetic diseases. Multifunctional proteins represent a safer alternative to viral vectors as targeting and delivery vehicles (3, 35) and allow extended engineering possibilities for continuous improvement and adaptation to specific situations. In this context, the use of recombinant, cell binding peptides of viral origin could represent an interesting strategy to avoid undesirable infection risks during gene transfer, keeping the high selectivity of the viral adsorption. We have previously shown that a cell-binding, RGD-containing peptide motif from FMDV, as inserted in *E. coli* β-galactosidase, binds and internalise cultured cells (13). Note that this bacterial enzyme, although initially convenient as marker for internalisation assays (13), offers important advantages as a carrier because of its tolerance to insertional mutagenesis (9), easy, single-step purification from crude cell extracts (22), enzymatic responsiveness to molecular interactions (19), and DNA-protecting activities in appropriate protein-DNA complexes (14). In this line, efficient gene expression has been achieved through the use of this FMDV targeting peptide combined with β-galactosidase and DNA condensing protein segments in a multifunctional protein (7, 14), at levels higher than those achieved by synthetic, similar lengthen RGD peptides of nonviral origin (36). For these reasons, we were prompted to explore in more detail the cell binding mechanics of this recombinant peptide, and to scrutinise the impact of multiple peptide presentation on the efficiency of cell attachment. This interest partially arose from the dramatic improvement in the immunoreactivity of chimeric β-galactosidases resulting from a higher copy number of this viral segment (20).

Data presented in Fig. 2 prove a significant stimulation of cell binding in HT7278CA when compared with single insertion mutants, that also results in a more rapid cell proliferation in protein-coated plates (Fig. 3). The non-additive profile exhibited by the protein series (Fig. 2) strongly suggests that the spatial orientation of the integrin ligands as displayed in HT7278CA (Fig. 1), could be relevant for the cell binding. This is supported by the fact that the three peptide insertion sites in this protein are a combination of those involved in individual members of the HD series. In addition, HT7278CA seems to bind tighter than vitronectin to relevant re-

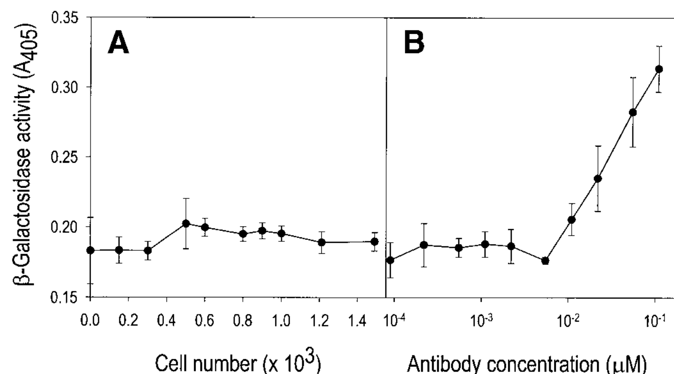

FIG. 5. (A) Variations in the enzymatic activity of M278VP1 exposed to cultured BHK_{21} cells, measured at 4°C. Figures indicate cell inoculum sizes. After 3 days of culture, cell density ranged from 30 to 90%. (B) Reactivation of M278VP1 mediated by 3E5, under the same exposure and reaction conditions than in A.

ceptors on cell surface (Figs. 2 and 3). Although we can not suggest a definitive explanation, important differences in the stability of RGD-integrin complexes have been proven depending on the structure of the RGD-containing molecule or the framework of the RGD peptide motif (37). In this context, the specific spatial presentation of RGD peptides in HT7278CA would favour integrin binding.

On the other hand, the interaction between both soluble and cell-associated integrins and protein M278VP1 does not result into detectable changes in the enzyme activity of this protein (Figs. 4 and 5). Interaction with anti-peptide antibodies, however, induces an enzymatic reactivation (Fig. 5) trough important, antibody-induced alterations in enzymatic constants (30–32), that reveal conformational modifications of the enzyme active site. These data suggests a confortable molecular accommodation of the recombinant RGD residues to integrins in which conformational changes induced in the enzyme, if any, are functionally undetectable. This could be explained by a high molecular flexibility in the RGD triplet as modelled on the enzyme surface (16). Apart from that, only antibodies (19, 38, 39) and enzyme inhibitors (40) have been so far observed as targets for regulatable enzymes.

The intriguing discrimination between HeLa and BHK_{21} cells regarding HT7278CA attachment (Figs. 2 and 3) remains unexplained. The vitronectin receptor $\alpha_V\beta_3$ has been proved to be the main target for FMDV attachment (26, 41, 42). However, other integrins like $\alpha_5\beta_1$ (33) or $\alpha_V\beta_6$ (43) have been also shown as alternative virus receptors. Although a detailed catalogue of integrins expressed in HeLa cells is not available, different direct and indirect observations indicate that integrins within those listed above are expressed in HeLa (44, 45). Moreover, both FMDV (26) and FMDV-β-galactosidase hybrid proteins (12) bind to this cell line. Therefore, the lack of susceptibility to viral infection (26), the failure of HT protein in enhancing cell binding and proliferation and the null effect of vitronectin (Figs. 2 and 3) could suggest that coreceptor elements involved in RGD-mediated cell attachment (or alternative receptors) would be missing in HeLa cells.

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