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Molecular Organization of Protein–DNA Complexes for Cell-Targeted DNA Delivery

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Multifunctional proteins are interesting candidates for nonviral gene transfer to and expression in their target cells. Since at difference of viral vectors, the performance of these vehicles depends on their functional optimisation, a better comprehension of the molecular organisation within protein–DNA complexes would be of great help in reaching their full delivery potential. In this work, we have characterised an RGD-tagged, cell-targeted multifunctional β-galactosidase carrying a poly-lysine-based DNA-binding domain. In solution, the engineered enzyme spontaneously forms proteinaceous particles of between 20 and 40 nm in diameter that might contain around 10 molecules of enzymatically active protein. Plasmid DNA is efficiently condensed into these particles without modification of the shape, morphology or enzymatic activity, indicative of a comfortable molecular accommodation to the DNA-binding domains. Although the RGD peptide remains equally solvent-exposed and immunoreactive at different DNA–protein ratios, an optimal expression level of cell-delivered genes and integrin-binding specificity are both achieved at 0.02 μg of DNA per μg of protein, indicative of influences of the packaged nucleic acid on the interaction between filled vehicles and the receptors of target cells. © 2000 Academic Press

Key Words: **recombinant protein; protein folding; β-galactosidase; RGD; integrin; gene therapy.**

The identification of relevant genes and their functions emerging from the completed human genome sequence will offer extended possibilities for therapeutic approaches to inheritable diseases. The stable introduction of functional genes into target cells requires appropriate vehicles for DNA delivery to the cell nucleus. In the last decade, engineered adenoviruses and retroviruses have been largely explored as such transfer vehicles with an important degree of success (1, 2). However, a set of adverse reactions observed in some clinical trials (3, 4), in addition to possible risks associated with the release of manipulated infectious agents, strongly encourage the development of safer and more efficient non-viral vehicles for gene transfer (3, 5). In this context, cationic lipids (2) and synthetic peptides (6), independently or in combination, have been also explored as coating elements to deliver expressible DNA sequences.

Alternatively, a few multifunctional proteins for cell targeted DNA delivery, that do not require chemical modifications after production, have been constructed by combining bioactive proteins or protein domains from different origins (7–9). These joined regions supply DNA-condensing, cell binding, internalisation and eventually endosome-disrupting and nuclear targeting activities. Although this family of constructs is still in an early stage of development, its intrinsic flexibility and the possibility of further improvement by protein engineering offer wider perspectives for the generation of promising vehicle prototypes that could mimic features of the viral infection process (5). Contrary to viral particles and nucleoprotein, in which the nucleic acid-protein interactions are becoming intensively investigated, the molecular organisation of recombinant, DNA-binding vehicles remain largely unexplored. This represents an obstacle for the structural optimisation of protein–DNA delivery complexes and for a rational design of better prototypes. In this work, we have investigated relevant features of an engineered *Escherichia coli* β-galactosidase containing both DNA- and integrin-dependent, cell-binding peptides, as a carrier for transferable plasmid DNA. Interestingly, functional tetramers of the engineered enzyme assemble spontaneously, to form virus-sized particles that display a fully accessible cell binding domain, and into which the DNA accommodates without disturbing protein conformation and organization.

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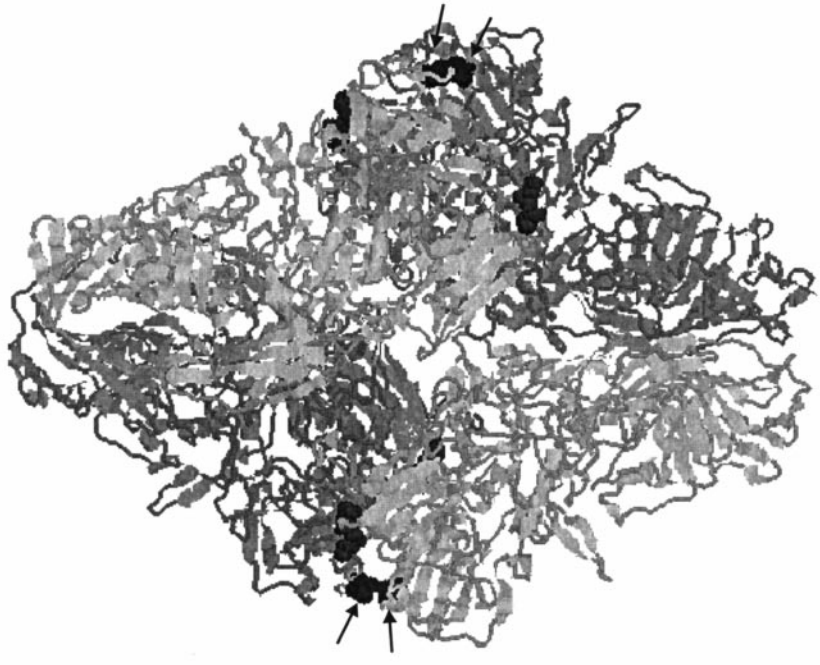

FIG. 1. A RasMol[R] representation of *E. coli* β-galactosidase, indicating on every monomer the insertion sites for both the RGD-containing peptide (light grey circles for residues 249 and 250) and the position of the poly-lysine tail (black circles, arrowed).

MATERIALS AND METHODS

Plasmids, proteins, and plasmid construction. Plasmids pJLACZ (10), pJX249A (11) and pTrC249AL (9) encode the engineered and enzymatically active *E. coli* β-galactosidases LACZ, JX249A and 249AL, respectively. The protein JX249A is derived from the pseudo-wild-type LACZ and displays a 27 mer peptide containing an integrin-targeted, RGD-based motif between residues 249 and 250. The inserted segment, reproducing a region of the FMDV VP1 capsid protein (12), binds mammalian cells through $\alpha_v\beta_3$ (and to a lesser extent $\alpha_5\beta_1$) cell surface integrins (13) and directs the internalisation of the hybrid enzyme in a functional form (14). The addition of a deca-lysine tail to the amino terminus of JX249A, resulting in the construct 249AL, enables β-galactosidase to promote efficient DNA delivery and expression in cultured cells (9). A 249AL derivative (K_{10}LACZ protein), was constructed by removing the DNA region that encodes the FMDV peptide from pTrC249AL. This was done by digesting at the *Aoc*I and *Cla*I sites flanking the viral DNA segment and replacing it by the analogous *Aoc*I-*Cla*I fragment from pJLACZ, that only encodes the corresponding β-galactosidase residues. The resulting plasmid construct, pTK_{10}LACZ, encodes a recombinant β-galactosidase that only carries the N-terminal lysine tail. In Fig. 1, a Rasmol representation of 249AL shows the multidomain nature of the assembled tetramer and the spatial orientation of both sets of cell- and DNA-binding domains.

Protein production, purification, and complex formation. Production of recombinant proteins was induced by temperature shift from 28 to 42°C for MC1061/pJLACZ and MC1061/JX249A cultures, or by addition of 1 mM IPTG to MC1061/pTrC249AL and MC1061/pTK_{10}LACZ cultures, while growing exponentially in LB medium (15) in the presence of 100 μg/ml ampicillin. Proteins were purified in a single step by affinity chromatography with a nonhydrolysable β-galactosidase substrate (16). Purification procedures as well as conditions for protein–DNA complex formation have been previously described in detail (9). Briefly, protein and DNA solutions were mixed and incubated in Hepes buffered saline (HBS) at room temperature for 1 h. The chosen ratios were 0.08, 0.04, 0.03, 0.02 μg DNA per μg protein, corresponding to 1, 2, 3, 5 retardation units, respectively (one retardation unit is defined as the amount of lysine tail-containing protein that fully retards the mobility of 1 μg of plasmid DNA in 0.8% agarose gel) (9).

Cell-binding analysis and ELISA. The cell-binding assay was described in detail elsewhere (13, 14). Briefly, 15 pmols of either pure or DNA-associated protein in PBS were incubated in ELISA COSTAR EIA/RIA plates overnight at 4°C. Wells were then washed in phosphate buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA). Trypsinized HeLa cells from 80% confluent cultures were added to about 5×10^4 cells/well and incubated for 2 h at 37°C. For the competition binding assay, cells were mixed gently by rotation for 1 h at 4°C with 100 μM of synthetic peptide GPenGRGDSPCA from Gibco BRL before being added to the wells. This peptide is a potent inhibitor of cell binding to vitronectin (17). After washing with PBS, cells were fixed with absolute methanol and stained with 0.1% (w/v) crystal violet. The excess of dye was removed by washing with PBS and the stained cells were lysed by addition of 1% (w/v) SDS. The absorbance at 570 nm was measured in a microtiter reader.

To evaluate the accessibility of cell-binding motifs to 3E5 mAb, directed against the RGD containing epitope (18), an indirect ELISA was performed following standard procedures. In this ELISA, protein was incubated overnight at 5 pmols/well to ensure that the assay was carried out under nonsaturating conditions. After blocking for 1 h with 3% (w/v) BSA in PBS, 100 μl of 5.75 nM 3E5 mAb were added. 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 bound antibody was detected with 0.01% (w/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 2N H_2SO_4 and measured at 570 nm in a microtiter reader.

TABLE 1

Stability and Enzymatic Activity of Engineered β-Galactosidases

Protein	Thermal stability (t_{50}[a])	(%)	Specific activity (U/ml)	(%)
LACZ	135.3 ± 17.8	100	2761.6 ± 361.8	100
JX249A	10.5 ± 0.8	6.8	2196.4 ± 222.1	79.5
249AL	9.0 ± 1.5	5.9	1671.03 ± 121.9	60.5
K_{10}LACZ	825.7 ± 137.9	538.6	2451.1 ± 392.4	88.7

[a] t_{50} is the half life of the active proteins under 50°C.

Characterisation of recombinant proteins. The specific activity of 249AL, LACZ, JX249A and K_{10}LACZ was determined by Western blot analysis of cell extracts and independent enzymatic determination (19). Temperature stability of these proteins was determined as described (11). The enzymatic activity of different recombinant proteins was evaluated in comparison to those incubated previously with DNA in HBS for 1 h at room temperature. Two picomoles of protein, either free or incubated with different amounts of plasmid DNA, were transferred to a 96-well plate in 80 μl of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.04 M β-mercaptoethanol, pH 7.0). Then, 40 μl of 4 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG) substrate were added. When the yellow colour was evident (usually after 1 min), the enzyme was inactivated with 50 μl of 1 M Na_2CO_3. The intensity of the reaction was determined at 414 nm in a microtiter reader. Four independent analyses were performed on each sample.

Electron microscopy. DNA–protein complexes were prepared for electron microscopy by direct adsorption on protein-free carbon supporting films. Two nanometer basic carbon layers were prepared by using a MED 020 modular high vacuum coating system BAL-TEC INC (Balzers, Principality of Liechtenstein), transferred onto 400-mesh copper grids and drained in air. The films were contacted with 0.002% (w/v) Alcian blue dye for 5 min., further washed in distilled water for 5 min. and drained by filter paper. To prepare the samples, 0.2 μg of plasmid DNA were incubated with different amounts of 249AL and LACZ proteins in a final volume of 27 μl HBS for 1 h at room temperature. The negative controls corresponded to DNA-free proteins and HBS. After 1/100 dilution in ddH_2O, samples were placed as 10 μl drops for 5 min. on the carbon films. After washing in distilled water, the grids were dehydrated in ethanol, dried in air and finally rotary shadowed with platinum-carbon at an angle of 85°. Micrographs were taken with a Hitachi H-7000 transmission electron microscope (Hitachi LTD. Tokyo, Japan) at a magnification of 20,000× and 100 kV.

Measurement of transferred gene expression. Detailed procedures for detection of transferred DNA expression have been described elsewhere (9). The vector pGL3 (Promega, Madison, WI), carrying the American firefly (*Phonitus pyralis*) luciferase gene was incubated with different amounts of 249AL. Caco-2 (human colonic epithelial) cells were growth in 24-well plates to 50% confluence. Then, cells were washed and further incubated in OptiMEM medium (Gibco-BRL). The media was removed and the DNA complexes containing 1 μg of pGL3 were added in a total volume of 200 μl/well. After 6 h of incubation, the media was replaced by Glutamax-containing DMEM, supplemented with 20% foetal calf serum and penicillin-streptomycin (100 units/ml each). Cells were washed after 48 h and further harvested in lysis buffer (Promega). Luciferase activity was measured by standard protocols (20).

RESULTS

β-Galactosidase enzymatic activity in DNA–249AL complexes. Protein 249AL, containing both an RGD cell binding peptide and a DNA-condensing poly-lysine tail, is able to interact with plasmid DNA and to mediate its efficient uptake and expression in cultured cells (9). The presence of the foreign peptides conferring the multifunctional character to 249AL has different effects on protein stability and activity. While the amino-terminal fusion tends to stabilise the protein, thermal stability is significantly impaired by RGD peptide insertion at position 249 (Table 1). Note that the parental LACZ protein is a pseudo-wild-type β-galactosidase lacking the eight amino terminal residues (10), and also that the amino terminus is involved in building the activating interface (21). Similar stabilising effects of other non homologous amino terminal peptides have been previously observed in our laboratory (Corchero *et al.*, unpublished results). However, the specific activity of the modified β-galactosidases is much less affected by the heterologous peptides and it is so in an additive fashion (Table 1), suggesting that the tetrameric enzyme structure is retained in the majority of 249AL molecules. Interestingly, the formation of DNA–protein complexes does not have any effect on the enzymatic activity even at high DNA–protein ratios (Fig. 2), indicating that monomer conformation, monomer-monomer contacts and substrate diffusion and processing are not disturbed by the presence of condensed DNA. The stability of DNA–protein complexes in the Z buffer used for activity assays was assessed by gel retardation analysis (9) (not shown).

DNA–protein complex formation and morphology. We have previously reported spontaneous *in vitro* aggregation of 249AL (9), that may account for the slight reduction in specific activity of the engineered

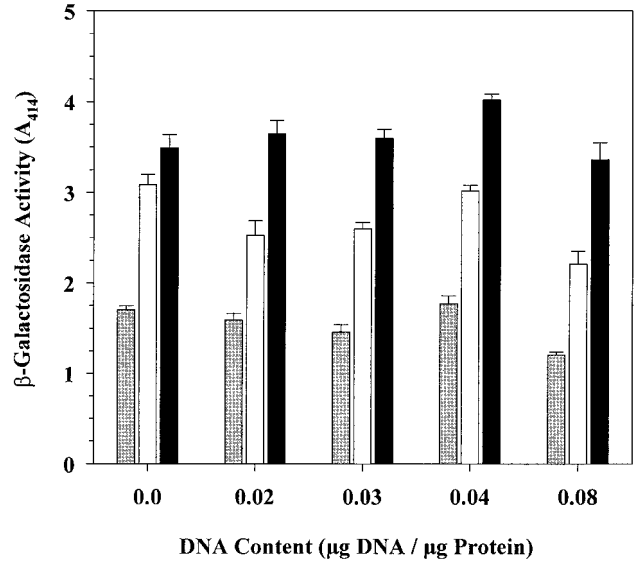

FIG. 2. β-Galactosidase enzymatic activity of proteins 249AL (grey bars), JX249A (white bars) and LACZ (black bars), in presence of different amounts of plasmid DNA under conditions promoting complex formation with 249AL.

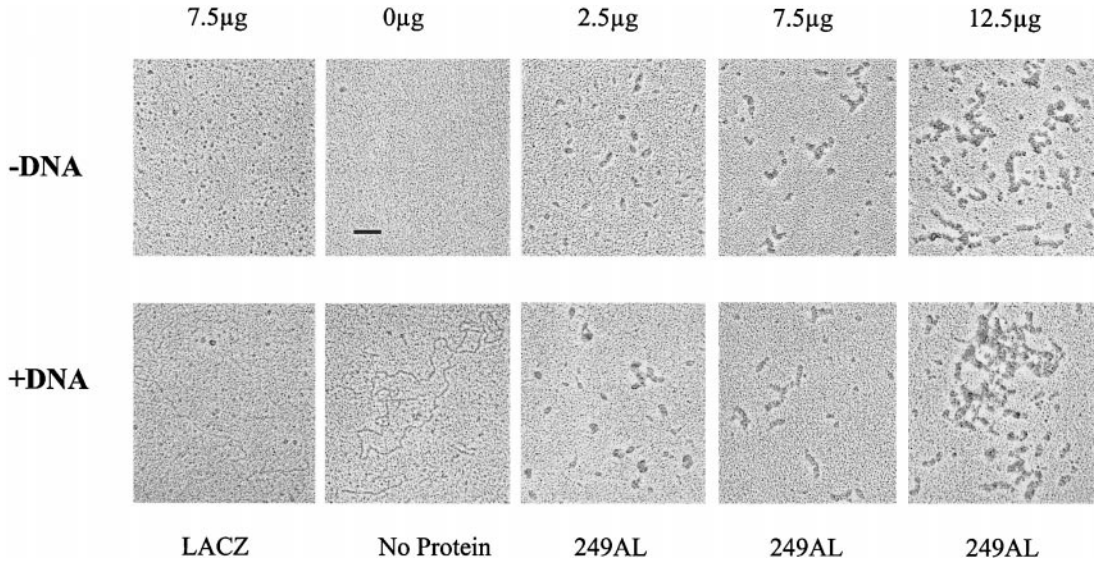

FIG. 3. Electron micrographs of 249AL and LACZ solutions without DNA (top) and incubated with different molar ratios of plasmid DNA (bottom). Protein concentration is indicated by the figures at the top. Plasmid DNA concentration was always 7.4 μg/ml. The resulting DNA/protein ratios were 0.08, 0.03, and 0.02 respectively. The bar indicates 100 nm.

β-galactosidase (Table 1). The presence of regular-sized proteinaceous nanoparticles rather than amorphous aggregates has been observed by transmission electron microscopy of 249AL samples. These particles associate in pseudo-filaments up to 200 nm in length, following a protein concentration-dependent pattern (Fig. 3, top). The diameter of individual particles ranges between 20 and 40 nm, and no filaments were observed at low protein concentrations. These filaments are not seen in the LACZ solutions at 7.5 μg either with or without DNA. Since practically all of the protein molecules are involved in these protein complexes, the slight reduction of the enzymatic activity observed in protein 249AL (Table 1) is unlikely to be due to inactivation of a small protein fraction by aggregation. It therefore appears to be caused by direct modulation of the catalytic activity by the peptide insertion itself, individual molecules thus remaining active as members of the macromolecular assemblies. In fact, insertational mutagenesis of β-galactosidase, even involving solvent-exposed loops as insertion sites, often results in a reduced enzyme performance (11, 12).

The addition of DNA to a solution of protein 249AL, followed by incubation under conditions in which DNA–protein complexes are formed, results in efficient DNA condensation. Note that free DNA molecules are not seen in any of the 249AL samples but they are apparent when the DNA is mixed with the non condensing LACZ protein (Fig. 3, bottom). On the other hand, size and organisation of 249AL particles are barely affected by the presence of DNA. However, a moderate enhancement of inter-particle aggregation is observed, specially at a high protein concentration. Again, these results strongly suggest that the DNA–protein interaction in these recombinant vehicles is accounted for by a non-disturbing accommodation of DNA molecules into the protein particles.

DNA-mediated modulation of cell binding. Protein 249AL, like other related, RGD-containing protein constructs (11, 13), binds mammalian cells efficiently through the vitronectin receptor (Fig. 4A). 249AL-DNA complexes also direct efficient delivery and expression of plasmid DNA in cultured cells (9). However, the possible influence of nucleic acid in the cell binding process remained unexplored. Surprisingly, the accommodation of DNA into 249AL particles significantly decreases their cell-binding potential in a dose-dependent fashion, up to around 0.08 μg per μg of protein (Fig. 4B). A similar impairing effect is also observed in K_{10}LACZ (Fig. 4B), an RGD-lacking 249AL derivative exhibiting only background integrin-specific binding (see Fig. 4A). This would indicate nonspecific inhibitory effects by the packaging of DNA on the binding properties of the whole vehicle. However, from the experiments presented in Fig. 5A, in which the vitronectin-receptor specificity is evaluated at different DNA–protein ratios, a DNA-promoted decrease of integrin-binding specificity can be inferred from the declining difference between complex binding in absence and in presence of a competing RGD peptide (17). At 0.04 μg of DNA per μg of protein, a molar excess of this peptide has no detectable influence on cell attachment of these vehicles. This suggests that at this DNA–protein ratio, cell attachment of the vehicle does not occur through binding to integrins $\alpha_V\beta_3$ and $\alpha_V\beta_1$. In-

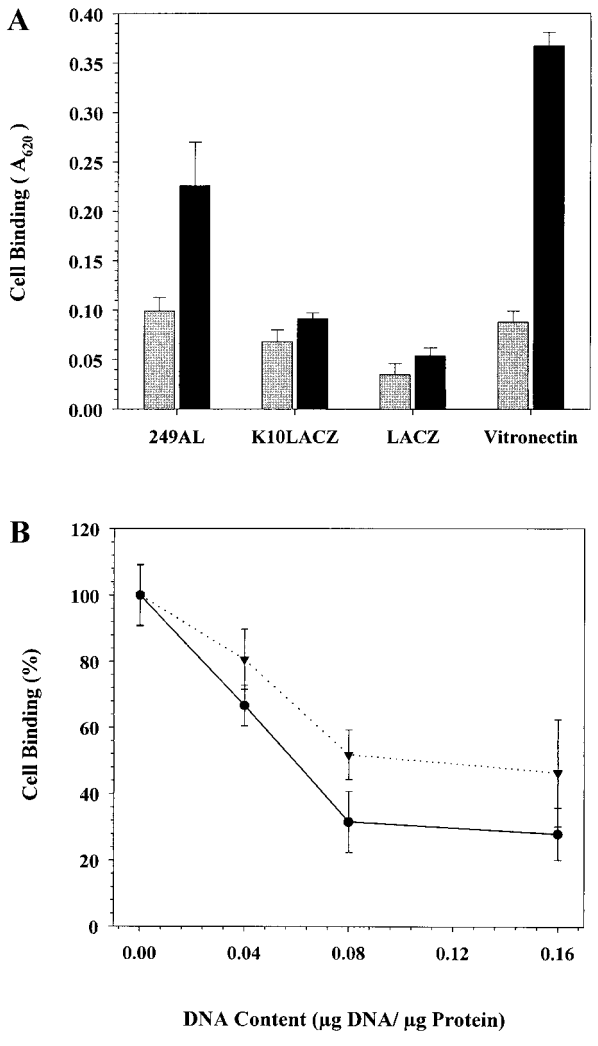

FIG. 4. (A) Cell binding of different proteins in presence of either the competing GPenGRGDSPCA peptide (grey bars) or the noncompeting GRGESP peptide (black bars). (B) Cell binding of proteins 249AL (circles) and K_{10}LACZ (triangles) in presence of plasmid DNA.

terestingly, note that the optimal efficiency of delivered gene expression takes place between 0.02 and 0.03 μg of DNA per μg of protein, a range in which the cell-binding specificity to integrins is maximum.

To evaluate if the observed modulation of cell attachment properties by DNA could be caused by a DNA-promoted masking of the cell targeting RGD motif, the solvent-exposure and reactivity of this viral peptide in complexes was explored by ELISA with the anti-viral, RGD-directed 3E5 monoclonal antibody. As shown in Fig. 6, the immunoreactivity of this peptide remains invariable at different DNA–249AL protein ratios. In addition, the presence of free DNA does not interfere in the reactivity of protein JX249A, that lacking the DNA-binding domain, displays the viral, cell-binding peptide inserted at the same position than in 249AL.

DISCUSSION

In an attempt to develop safer gene transfer vehicles as alternatives to viral vectors, a set of recombinant proteins have been designed to confer cell-targeting properties to transferable DNA. In some of them, poly-lysine peptides must be either chemically conjugated to the protein (22, 23) or added to DNA–protein complexes (8, 24), in order to stabilise the vehicles and to promote efficient DNA delivery. However, single-polypeptide, multifunctional proteins that do not require modification after production are more robust vectors, at the same time offering convenient flexibility for further optimisation. Histones (25), DNA topoisomerase I (26) and GAL4 protein (7, 8) are among the explored DNA-binding domains in recombinant constructs. However, although poly-lysine peptide seg-

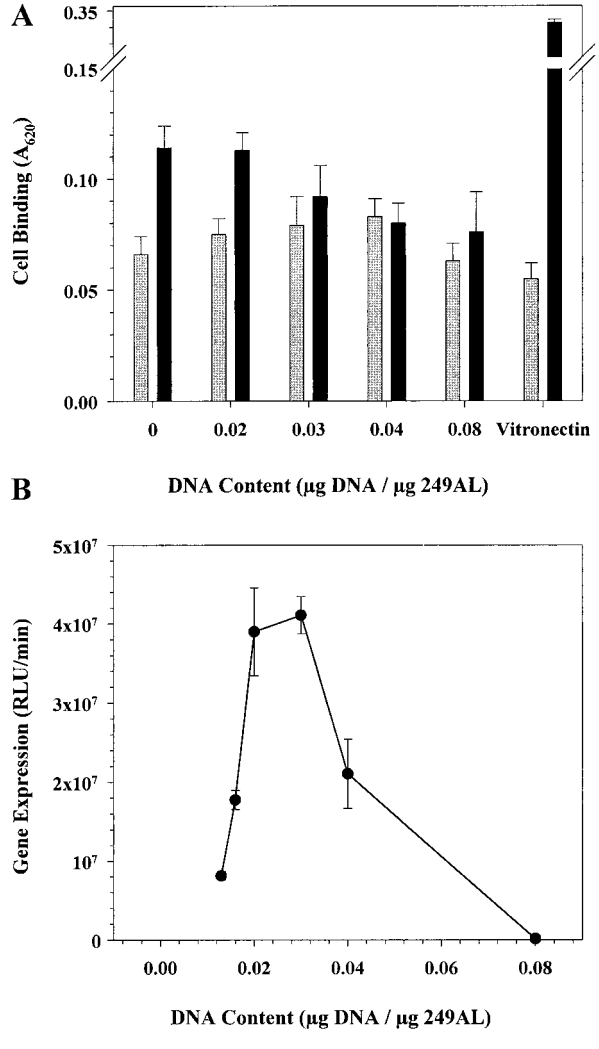

FIG. 5. (A) Cell binding of DNA–249AL complexes in presence (grey bars) and in absence (black bars) of the competing GPenGRGDSPCA peptide. (B) Luciferase gene expression after transfection with complexes formed at different DNA–249AL ratios.

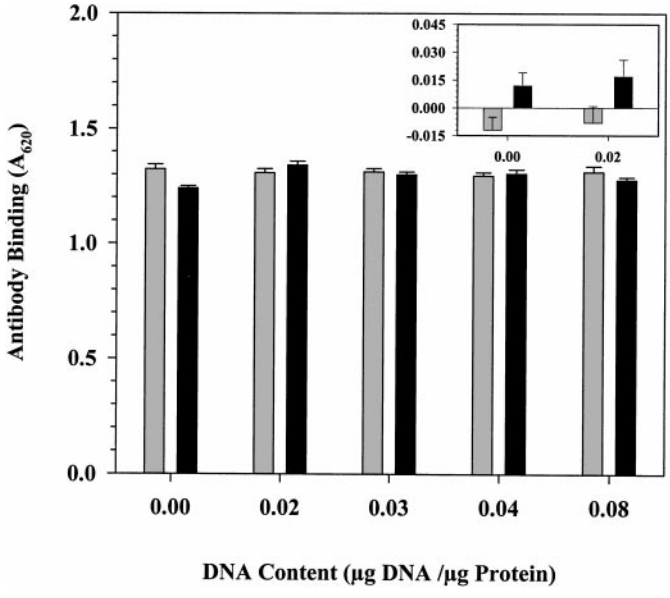

FIG. 6. Immunoreactivity in ELISA of proteins 249AL (grey bars) and JX249A (black bars), in the presence of different amounts of plasmid DNA. In the inset, the analysis of proteins K_{10}LACZ (grey bars) and LACZ (black bars) is also shown.

ments are commonly used as efficient DNA-condensing motifs in short synthetic peptides of defined length (2, 6), this ligand had not been successfully explored as a part of a multifunctional protein.

Protein 249AL, is an engineered *E. coli* β-galactosidase that displays two foreign molecular moieties, namely a viral, RGD-containing cell-binding region inserted in a solvent-exposed loop within the domain II of the enzyme (11), and a poly(10)-lysine peptide fused at its amino terminus. Since these peptides do not interfere with enzyme tetramerization (Table 1), they are presented as four copies each for enzyme molecule in all the spatial orientations (Fig. 1). Protein 249AL in solution forms regular sized, ellipsoid aggregates of between 20 and 40 nm (Fig. 3). According to the molecular dimensions of the assembled enzyme (21) they could be formed by about 12 spatially ordered, active molecules. Since the parental JX249A protein does not exhibit any aggregation tendency (not shown), the poly-lysine tail seems to be a main contributor of particle formation. Despite reports that poly-lysine peptides form regular sized particles when combined with DNA (24), this is, as far as we know, the first observation of a similar effect on covalently-linked domains in a multifunctional recombinant protein.

Interestingly, when plasmid DNA is added to a 249AL protein solution, enzyme activity (Fig. 2), particle shape and morphology (Fig. 3) and antigenicity of the integrin-binding domain (Fig. 6) remain unmodified. This is true for all the DNA–protein ratios with proven biological significance explored in this work. Note that the stability and enzymatic activity of the protein vehicle is maintained while DNA molecules are efficiently condensed (Fig. 3), in spite of the proximity of the DNA-binding domain and the active site (21) (Fig. 1). These observations suggest that the assembled protein incorporates plasmid molecules in cationic regions without significant alterations in their multidomain organisation. The comfortable accommodation of DNA into 249AL nanoparticles seems to be a different molecular event than DNA coating by histone-based delivery vectors and the progressive DNA condensation promoted by short peptides in a dose-dependent fashion (compare Fig. 3 from this report with Fig. 3 from reference (25) and from reference (20), respectively). The high molecular mass of *E. coli* β-galactosidase and the larger-scale intermolecular association of 249AL could offer appropriate cavities for electrostatic DNA accommodation up to around 0.03 μg of DNA per μg of protein. At higher DNA–protein ratios, the recombinant poly-lysine peptides seem to be insufficient to compensate the negative charges within the particles, and cell binding and consequently the level of gene delivery and expression starts to decline (Figs. 4 and 5).

The results presented here suggest mechanisms of molecular interaction between protein 249AL and transferable DNA, which are distinguishable from those observed in other recombinant and synthetic non-viral vehicles. The virus-like DNA-harbouring and cell binding properties exhibited by 249AL particles support the presented multifunctional approach in the development of non-viral alternatives for therapeutic gene delivery.

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