TREBALL IV

NON-VIRAL GENE DELIVERY TO THE CNS BASED ON A NOVEL INTEGRIN TARGETING MULTIFUNCTIONAL PROTEIN

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Enviat a Human Gene Therapy

Els experiments presentats en aquest treball han estat realitzats principalment en el Laboratori d'Histologia, del Departament de Biologia Cel.lular, Fisiologia i Immunologia a la Universitat Autònoma de Barcelona. Tot i que no conformen els resultats principals d'aquesta tesi doctoral, han estat inclosos dins d'aquest apartat perquè en aquest estudi s'avalua el potencial *in vivo* de la construcció 249AL en aplicacions al SNC. Es va demostrar que després de la injecció intracortical de complexes formats pel plasmidi codificant per GFP i la proteïna quimèrica 249AL, hi havia expressió significativa de GFP en cervells intactes o que prèviament havien estat lesionats excitotòxicament. Després de la lesió, s'observava una expressió generalitzada en tota l'àrea afectada i la presència de la proteïna GFP en nuclis talàmics llunyans, la qual cosa suggeria que el vector podia ser transportat retrògradament mitjançant el motiu RGD, tal i com s'ha descrit per alguns virus. La proteïna 249AL és capaç de transfectar neurones, astrocits, microglia i endoteli sense mostrar, com a mínim durant els primers 6 dies post-transfecció, l'existència de processos inflamatoris ni estimulació del sistema immunològic, demostrant que és un prototip de vector alternatiu als vectors vírics en el SNC.

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Running title: 249AL *in vivo* CNS transgene delivery

Abstract

Successful introduction of therapeutic genes into the central nervous system (CNS) needs the further development of efficient transfer vehicles, avoiding viral vector-dependent adverse reactions but maintaining a high transfection efficiency. The multifunctional protein 249AL was recently generated for *in vitro* gene delivery. We explore here the performance of this vector for the *in vivo* gene delivery to the postnatal rat CNS. After intracortical injection of DNA-containg 249AL vector, significative transgene expression was observed both in excitotoxically injured and non-injured brain. After injury, a widespread expression occurred in the entire lesioned area, and retrograde transport of the vector towards distant thalamic nuclei and transgene expression were observed. Neurons, astrocytes, microglia, and

endothelial cells expressed the transgene. No recruitment of leukocytes, demyelination, interleukin-1β expression, or increase in astrocyte/microglial activation was observed after 6 days of vector injection. In conclusion, the 249AL vehicle exhibits promising properties for gene therapy intervention to the CNS, including the targeting to different but limited cell populations.

Overview summary

The introduction of therapeutic genes for the treatment of CNS pathologies requires the development of flexible vehicles devoid of inflammatory and immune reactions triggered by many viral vectors. In this regard, a multifunctional protein vector termed 249AL was recently generated based on *E. coli* β-galactosidase. Taking advantage of the intrinsic nuclear localization motifs located in this protein, two additional functional domains were introduced, namely an Arg-Gly-Asp (RGD) integrin-binding and cell internalization peptide, and a polylysine tail with DNA condensing properties. The resulting construct was then efficiently delivering expressible DNA to cultured cells. We report here the competent *in vivo* 249AL-mediated transgene delivery to the intact and injured postnatal rat CNS, in the absence of an inflammatory reaction or immune response at 6 days post injection. Following an excitotoxic insult, transgene expression is clearly enhanced, occurs more widespreadly, and it is observed in neurons, astrocytes, microglia and endothelium. In summary, the 249AL vector displays promising properties for future gene therapy intervention to the CNS specially under tissue damage conditions, offering also a flexible, modular design adaptable to other specific therapeutical requirements.

Introduction

In the context of the sequencing and expected solving of the complete human genome, gene therapy approaches will probably acquire an extremely high importance in the improvement of human health. The transitory or stable introduction of therapeutic genes in order to be expressed into target cells requires appropriate vehicles for DNA delivery and nuclear transfer. In the last years, adenoviruses, parvoviruses, herpesviruses and retroviruses have been engineered to generate DNA transfer vehicles, exploiting relevant viral properties such as cell attachment, internalization, nuclear transport and stable DNA expression. These efforts resulted in prototypes which showed an important degree of success under experimental conditions (Brooks et al., 2002; Costantini et al., 2000; Mountain, 2000; Sapolsky and Steinberg, 1999). However, a set of viral-dependent adverse reactions have been eventually observed in clinical trials (Jane et al., 1998; NIH Report 2002), accompanied by an increasing concern about the possible risks associated to the release of manipulated infectious agents and their scale up production difficulties. For these reasons, development of safer, stable and eventually more efficient non-viral vehicles for gene transfer is very convenient (Jane et al., 1998; Navarro et al.,

1998). Cationic lipids (Li and Ma, 2001; Mountain, 2000) and synthetic peptides (Sparrow et al., 1998) have been explored as coating devices for expressible DNA, and multifunctional proteins for cell targeted DNA delivery have been constructed by combining bioactive protein domains from different origins (Arís et al., 2000; Fominaya and Wels, 1996; Paul et al., 1997). These independent elements can supply DNA-condensing, cell binding, cell internalization, endosome-disrupting and nuclear targeting activities to the resulting vehicles without most of the inconveniences of potentially infective material (Navarro et al., 1998). The intrinsic flexibility of these vectors offers wider perspectives for the generation of promising vehicle prototypes for specific therapeutical needs.

For the central nervous system (CNS) the value of these constructs is of special relevance, as DNA delivery by different viral species has been achieved (Brooks et al., 2002; Costantini et al., 2000; Dewey et al., 1999; Sapolsky and Steinberg, 1999), but accompanied by unacceptable toxicity (Nilaver et al., 1995), persistent inflammation (Dewey et al., 1999), immune activation (Byrnes et al., 1996; Wood et al., 1996), and demyelination (Byrnes et al., 1996; Nilaver et al., 1995). In addition, viral delivery has been shown to potentially break tolerance to endogenous proteins (Zinkernagel et al., 1990). Likewise, the peripheral re-administration of viral vectors to animals previously injected intracerebrally with the same vectors induces a delayed-type hypersensitivity reaction in the neural parenchyma (Byrnes et al., 1996).

Alternative gene delivery strategies for the CNS, such as intracerebral injection of polyethylenimine/DNA (Bousiff et al., 1995) or lipid/DNA complexes (Hecker et al., 2001; Shi and W.M., 2000) have been explored, although they still need further improvement (Li and Ma, 2001). In this regard, we have previously reported the construction of 249AL, a non-viral vector based on an engineered β-galactosidase protein (Arís et al., 2000). Taking advantage of β-galactosidase nuclear localization motifs (McInnis et al., 1995), two additional functional domains were introduced into this bacterial protein. An Arg-Gly-Asp (RGD) integrin-binding and cell internalization peptide and a polylysine tail with DNA condensing properties, rendered this multifunctional protein an efficient vector for gene delivery into cultured cells (Arís et al., 2000).

In this study, we have characterized 249AL as a vector for *in vivo* gene delivery to the intact and damaged postnatal rat CNS. Gene therapy attempts of damaged brains would be of special relevance for the therapeutic approches of perinatal hypoxic-ischemic insult (or related pathologies), involving severe neurological consequences as spastic paresis, choreo-atheosis, ataxia, disorders of sensomotor coordination, or impairment of intellectual ability (Berger and Garnier, 1999), and with a significant incidence in human populations (Ohrt et al, 1995). In this regard, excitotoxicity by NMDA receptor activation has been largely used as a model for hypoxic-ischemic injury to the postnatal brain (Ikonomidou et al, 1989; Olney 1990).

In the context of our approach, we have been specially focused on the evaluation of the transfection efficiency mediated by 249AL in neurons and glia, the extent of transgene expression, but also in the exploration of putative induction of inflammatory-immune response.

Materials and methods

PROTEIN, DNA AND FORMATION OF PROTEIN-DNA COMPLEXES

Protein 249AL is an engineered form of *Escherichia coli* β-galactosidase that displays a 27-mer peptide containing an integrin-targeted RGD-based motif. This segment, inserted between residues 249 and 250 of the bacterial enzyme, reproduces the cell-attachment region of the VP1 capsid protein of foot-and-mouth disease virus (Arís and Villaverde, 2000). The additional presence of a decca-lysine tail joined to the amino terminus of the construct and an still unidentified enzyme segment with nuclear targeting properties (McInnis et al., 1995) enables 249AL to promote DNA delivery. The 249AL protein was produced in bacteria and purified from crude cell extracts as described previously (Arís et al., 2000). A red-shift variant of jellyfish Aequorea Victoria green fluorescent protein (GFP) gene, transported by plasmid pEGFP-C1 (Clontech) under the control of the cytomegalovirus promoter and the SV40 enhancer element, was used as reporter gene to monitor the efficiency of DNA delivery. In all cases, protein and DNA complexes were formed by incubation in 0.9 % NaCl at room temperature for 1 hour, at 0.04 μg DNA per μg of 249AL protein. Details of complex formation are provided elsewhere (Arís and Villaverde, 2000).

EXPERIMENTAL ANIMALS

Experimental animal work was conducted according to Spanish regulations in agreement with European Union directives. A total of 43 nine-day old Long-Evans black-hooded rat pups (15-20 gr., both sexes; Janvier, France) were used, either non-lesioned or excitotoxically pre-lesioned. Experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering in every step.

INJECTION OF 249AL INTO NON-LESIONED CORTEX

Both the 249AL protein/pEGFP-C1 DNA complexes or naked pEGFP-C1 DNA were injected intracerebrally in two independent experiments and animals were sacrificed 24 hours later. Intracerebral injections were performed under anesthesia, into the right sensorimotor còrtex at the level of the coronal suture (2 mm lateral of bregma and 0.5 mm depth), by using a stereotaxic frame adapted to new-borns (Kopf Instruments). The skull was opened with a surgical blade and injections of either 1 μl of pEGFP-C1 DNA (0.032 μg/μl in NaCl 0.9 %) or 249AL protein/pEGFP-C1 complexes (0.032 μg/μl pEGFP-C1 in NaCl 0.9 %) were made using an automatic injector at 0.33 μl/min. The needle was left in place for 3 additional minutes to allow

diffusion. To evaluate possible toxicity associated to 249AL , only purified 249AL protein (0.8 µg/µl) was injected intracerebrally as described above, and animals sacrificed 6 days later. Control animals, injected only with saline solution, were also included in the experiment. Inflammation was assessed by immunohistochemical analysis of sections for the distribution of glial markers such as the astrocyte glial fibrillary acidic protein (GFAP) and the microglial/macrophage tomato lectin, the pro-inflammatory cytokine interleukin 1β (IL-1β) expression, the presence of infiltrating CD8+ or CD4+ T lymphocytes, and myelin marker myelin basic protein (MBP).

INJECTION OF 249AL INTO PRE-LESIONED CORTEX

Excitotoxic lesions were done as previously described (Acarin et al., 1999), by injecting 37 nmol of N-methyl-D-aspartate (NMDA) (Sigma) diluted in 0.15 µl of saline solution (0.9 % NaCl) or control saline solution at 0.05 µl/min, at the same coordinates as described for the 249AL injections. After suture, pups were placed in a thermal pad for 2 hours at 36 ºC to maintain normothermia. Six hours after, excitotoxically lesioned animals were injected with 249AL/pEGFP-C1 or pEGFP-C1, and sacrificed at 24 hours for immunohistochemical or western blot analysis. One additional group was injected with 249AL/pEGFP-C1 and sacrificed at 7 days post-lesion for immunohistochemical analysis.

INJECTION OF FLUORO-GOLD

In order to comparatively assess retrograde transport from sensorimotor còrtex toward connected thalamic nuclei, the retrograde tracer Fluoro-Gold was injected (0.15 µl, 2 %) in non-lesioned rat pups by an identical procedure as for injection of 249AL vector. Rats were sacrificed 5 days afterwards.

FIXATION AND HISTOLOGY

Animals were anaesthetized by inhalation and perfused intracardially for 7 minutes with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed immediately, immersed in the same fixative for 4 hours, cryoprotected in a 30 % sucrose and quickly frozen with dry CO2. Alternate cryostat sections were mounted on gelatin-coated slides (15 µm thick) or stored free-floating in antifreeze buffer (30 µm thick).

IMMUNOHISTOCHEMISTRY

Sections were processed for endogenous peroxidase inactivation and blocked for 1 hour in Tris-buffered saline (TBS, pH 7.4), 10 % fetal calf serum. Sections were incubated overnight at 4 ºC in the same blocking solution with primary antibodies against either GFP (1:1500 Abcam ab290 rabbit polyclonal, or 1:100 Clontech JL-8 mouse monoclonal), GFAP (1:1800 Dakopatts Z-0334 rabbit polyclonal), IL-1β (1:200

Santa Cruz sc-1252 goat polyclonal), CD8 (hybridoma supernatant) (Malissen et al., 1982), CD4 (hybridoma supernatant) (Carrera et al., 1987), or MBP (1:300 Dakopatts A623 rabbit polyclonal). After several washes, they were incubated for 1 hour with biotinylated anti-rabbit (1:200, Amersham RPN-1004) or biotinylated anti-mouse (1:200, Amersham RPN-1001). Specific staining was evidenced by incubation with avidin-peroxidase (1:400 Dakopatts P0364) for 1 hour and subsequent 3,3´-diaminobenzidine (DAB)-hydrogen peroxide developing procedure in all cases except for GFP immunohistochemistry. In that case, the biotin-streptavidin LSAB2 System (Dakopatts K0675) was used, followed by developing solution of 0.1 M acetate buffer pH 6 containing 0.027 g/l glucose oxidase, 0.4 g/l ammonium chloride, 24 g/l ammonium nickel sulfate, 2 g/l glucose, and 0.2 g/l DAB. Microglia was evidenced by histochemistry for tomato lectin Lycopersicon esculentum, performed by incubation for 1 hour at room temperature in TBS with the biotinylated lectin (6 µg/ml, Sigma L-9389) followed by avidin-peroxidase incubation and DAB-hydrogen peroxide developing procedure as described previously (Acarin et al., 1994).

For double-staining procedures, sections were first processed as described for GFAP immunolabeling or tomato lectin histochemistry, and developed with DAB-hydrogen peroxide. These sections were then incubated as described with primary antibody to GFP (Abcam ab290), amplified by avidin-biotin complex and developed by incubation in buffer phosphate 10 mM pH 7.4, 390 µM filtered benzidine, 840 µM sodium nitroprusside dehydrate and 0.008 % hydrogen peroxide. Control sections were processed in parallel for detection of GFAP or tomato lectin followed by GFP detection, but omitting the GFP primary antibody.

WESTERN BLOTTING

Western blots for GFP were performed in order to comparatively assess transgene expression between 249AL/pEGFP-C1 (n=5) and pEGFP-C1 (n=4) injected lesioned animals. Animals were decapitated, the brains quickly extracted and the hemispheres separated, chopped and frozen in liquid nitrogen. Samples were resuspended in SDS 1 %, aprotinin (1 µg/ml), PMSF (1 mM), pepstatin A (1 µM), leupeptin (100 µM), and disrupted with a Braun Labsonic U sonicator (20 KHz, 50 W). Total protein concentration was measured by the bicinchoninic acid method and equal quantities of protein samples were used for 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrotransfered protein samples to polyvinylidene fluoride (PVDF) membranes were incubated with TBS 5 % non-fat milk, 0.05 % Tween 20 overnight at 4 ^{o}C and then exposed to primary antibody to GFP (1:1000 Clontech JL-8) for 2 hours at room temperature in the same solution. Specific staining was visualized by incubations in secondary biotinylated anti-mouse (1:800, Amersham RPN-1001), avidin-peroxidase (1:3000 Dakopatts P0364) and finally in chemiluminiscent substrate SuperSignal West Pico (Pierce) combined with exposure on Hyperfilm ECL (Amersham).

DENSITOMETRICAL MEASUREMENTS

GFP immunohistochemically processed sections were digitized with a Nikon DXM1200 digital camera attached to a E600 Nikon microscope and further densitometrical measurements were performed by using the NIH Image 1.52 software as previously described (Acarin et al., 1997). Briefly, images from two areas (0,6 mm2 each) from the còrtex injection site and one from contralateral còrtex were digitized from three parallel sections of each animal (249AL/pEGFP-C1 n=6; pEGFP-C1 n=5). Data are expressed as mean densitometrical ratio of each section (ipsilateral/contralateral). Western blot protein signals were estimated by measuring band areas with one-dimension Quantity One (BioRad) software after high resolution scanning. All results are reported as mean values ± SEM. Differences between two groups were evaluated for equal variance (F test) followed by unpaired Student´s t test, and probabilities < 0,05 were considered statistically significant.

Results

249AL DELIVERED THE TRANSGENE IN DAMAGED AND INTACT BRAIN

The potential of 249AL-vector as a vehicle for gene delivery to postnatal CNS was evaluated after stereotaxic intracortical injection in intact and excitotoxically-damaged animals. Injection of 249AL vector complexed with pEGFP-C1 DNA (249AL/pEGFP-C1) into intact brains, showed a local expression of the GFP reporter gene in the ipsilateral neocòrtex along the needle track and the subjacent corpus callosum at one day post-injection. This expression was observed by immunohistochemistry using two different GFP antibodies (Fig. 1C, D) and confirmed by epifluorescence for GFP (not shown). The GFP protein was still present 7 days post-injection in neurons of the injected còrtex (Fig. 1E, F), and in sparse neurons of the contralateral còrtex. In contrast, animals injected with naked pEGFP-C1 DNA showed only a mild immunoreactivity for GFP at the injection site (Fig. 1B). Densitometrical analysis of GFP immunohistochemistry processed sections from intact animals, showed a significantly ($p<0.05$) higher cortical transgene expression levels in 249AL/pEGFP-C1 injected animals in comparison to those injected with naked pEGFP-C1 DNA (Fig. 1G).

The vector 249AL also rendered high expression levels of GFP transgene expression in NMDA-injured brains. NMDA administration is a well known model of excitotoxic damage that triggers rapid neuronal death and tissue injury, which expands rostro-caudally and includes part of the cortex, corpus callosum, dorsal striatum and septum (Fig. 2A, Acarin et al., 1999). When 249AL/pEGFP-C1 was injected into damaged brains, there was a widespread GFP transgene expression, covering the entire lesioned area at 24 hours post-injection (Fig. 2B) and remaining for at least 7 days. Interestingly, after this period, these animals also showed GFP

green fluorescence (Fig. 2G) as well as GFP immunoreactivity (Fig. 2E) in several thalamic nuclei anatomically connected with the injected còrtex (Faull and Mehler, 1985; Sherwood and Timiras, 1970). This GFP expression profile correlated with the staining pattern of thalamo-cortical projecting nuclei, labeled after intracortical injection of the Fluoro-Gold retrograde tracer into the còrtex of intact animals (Fig. 2F).

In addition to immunohistochemical techniques, the 249AL transgene delivery efficiency in damaged brains was also assessed by comparative western blot and further densitometrical analysis of 27 KD bands which co-migrated with recombinant GFP. A significantly ($p<0,05$) 132 ± 17 % higher GFP expression was observed in 249AL/pEGFP-C1 injected animals in relation to pEGFP-C1 DNA injected ones (Fig. 3).

Both in damaged and intact brains, GFP was efficiently expressed in neurons, scattered endothelial cells and glia. Double labeling with GFAP astroglial marker or tomato lectin microglial marker showed enhanced GFP expression in these cells after the lesion (Fig. 4).

Finally, appropriate controls were performed to avoid unspecific immunostaining for GFP, and none of them showed immunoreactivity. These included: (i) sections from intact saline injected brains (Fig. 1A); (ii) sections from damaged saline injected brains (Fig. 2C); (iii) sections without incubation with both primary antibodies; (iv) sections processed with primary antibody preincubated with recombinant EGFP peptide (0.1 mg/ml, not shown).

ABSENCE OF HISTOPATHOLOGICAL ALTERATIONS AFTER 249AL INTRACEREBRAL INJECTION

In order to evaluate possible immune/inflammatory responses elicited by 249AL, we injected intracerebrally the 249AL vector alone, devoid of DNA, to exclude putative GFP protein-derived effects. Six days after the vector injection, sections stained with toluidine blue showed a well-organized brain parenchyma lacking inflammatory infiltrates or degenerating neurons (Fig. 5A, B). The immunohistochemical staining for GFAP showed local astroglial hypertrophy at the needle track and subjacent corpus callosum (Fig 5C), which was indistinguishable from that observed in saline injected controls. Similarly, sections processed for tomato lectin for the demonstration of microglia/macrophages did not show any significant reactivity (Fig. 5D). Only a slight increase in macrophage number was observed into the needle track, whereas normal ramified resting microglia were observed in the neural parenchyma (Fig. 5E). No infiltration of CD8 or CD4 positive T lymphocytes was observed besides from occasional positive cells in the parenchyma (Fig. 5F). Regarding the expression of the pro-inflammatory cytokine IL-1β, 249AL injected animals presented similar results to saline injected controls,

showing only some immunopositive endothelial cells (not shown). Finally, MBP immunohistochemistry showed only a slight demyelination in the corpus callosum at the injection site in both saline and 249AL injected animals, thus probably associated to the injection procedure itself (Fig. 5G).

Discussion

In this study, we propose a multifunctional vector prototype (249AL) as an alternative to viral vectors for the *in vivo* CNS gene delivery, maintaining the beneficial properties of viral attachment and internalization processes and avoiding most of the inconveniences associated to potentially infective material.

249AL EFFICIENTLY DELIVERED THE GFP TRANSGENE

Upon intracerebral injection into intact or damaged postnatal rat brain, the 249AL vector efficiently delivered the GFP transgene. The 249AL vector used in the present work displays an RGD motif, which interacts with αVβ3, αVβ5 and at least some other αV integrins (Wickham et al., 1993). In this sense, the observed high transfection efficiency is probably due to an enhanced cell internalization of the DNA, mediated by interactions between the RGD motif and the plasma membrane αV integrins which are expressed in the brain (Akiyama et al., 1991; Ellison et al., 1998; Masumura et al., 2001; Pinkstaff et al., 1999). In fact, cell internalization via receptor dependent endocytosis of many viruses, including most adenovirus serotypes and foot-and-mouth disease virus, is specifically mediated by interactions of a viral RGD peptide with cellular αV integrins (Wickham et al., 1993). In addition, 249AL polylysine-DNA condensation properties have been shown to protect DNA from nuclease activity and increase cell internalization (Fominaya and Wels, 1996), thus contributing further to the enhanced transfection efficiency. Interestingly, an additional increase in transgene expression was observed when DNA complexed to the 249AL vector was injected into damaged brain. An explanation for this observation could be the lesion-triggered upregulation of αVβ3 integrin expression, which has been reported in hypertrophic astrocytes, microglia and microvessels after ischemia (Ellison et al., 1998; Masumura et al., 2001; Okada et al., 1996), Alzheimer (Akiyama et al., 1991) or experimental autoimmune encephalomyelitis (Previtali et al., 1997). The increased glial transgene expression after the lesion reported here further supports this idea. Noteworthy, the 249AL vector was capable of transfecting cells of the entire excitotoxically lesioned area, from the most rostral portions to caudal ones and including different brain regions. This then fulfills one of the most important requirements for successful CNS gene therapy, i.e. whole-lesion widespread gene delivery and expression (Sapolsky and Steinberg, 1999). Therefore, 249AL could be an appropriate vector to preferentially induce transgene expression in injured areas, sparing most of the normal parenchyma.

249AL DOES NOT INDUCE AN IMMUNE/INFLAMMATORY RESPONSE

One of the biggest challenges facing vectors is the host immune response . Whereas at the cellular level, recruitment of cytotoxic T lymphocytes eliminates vector-targeted cells and triggers tissue injury (Byrnes et al., 1996; Yang et al., 1994), at the humoral level, antibodies against the vector preclude the repeated administration (Kajiwara et al., 2000). In this regard, viruses have been shown to potentially break tolerance to endogenous, previously non-immunogenic proteins, acting as potent adjuvants in the generation of immune responses (Zinkernagel et al., 1990). Particularly in the brain, unacceptable persistence of inflammation has been observed upon injection of different viral vectors (Dewey et al., 1999), as well as immune activation (Wood et al., 1996) and demyelination (Byrnes et al., 1996; Nilaver et al., 1995). Accordingly, the intracerebral injection of adenoviral vectors induces a rapid increase of IL-1β (Cartmell et al., 1999), followed by activation of microglia/macrophages and astroglia (Wood et al., 1996), an enhanced expression of major histocompatibility complex class I (Wood et al., 1996) and a rise in anti-adenovirus antibodies in serum at the first 48 hours (Kajiwara et al., 2000). Moreover, accumulation of activated CD8+ and CD4+ lymphocytes occurs in the neural parenchyma by the first week (Byrnes et al., 1996). In contrast, our results show that neither of these histopathological changes occurred 6 days after 249AL injection, suggesting that this vector does not trigger any inflammatory reaction or immune activation in the host in a time frame where viral vectors do.

249AL TRANSPORT AND TRANSGENE EXPRESSION IN DISTAL BRAIN AREAS

Several viruses including adenoviruses (Akli et al., 1993) are internalized by axonal terminals and retrogradely transported to the neuronal soma, hence making possible the selective transfection of a population of distant neurons by a non-invasive approach. It is believed that terminal internalization of viruses into neurons is mediated by interactions of αV integrins with RGD motifs of viral capsid proteins, as it has been described for other cell types (Wickham et al., 1993). Interestingly, in our study, transgene expression was observed in sparse neuronal soma in the contralateral còrtex and in several thalamic nuclei, which are known to have afferent/efferent cortical projections (Faull and Mehler, 1985). Thus, it is conceivable that cortically injected 249AL vector would be internalized by somatosensory thalamo-cortical axonal terminals, and by retrograde transport, transfect distant neuronal soma. In this context, the presence of the Fluoro-Gold retrograde tracer in contralateral cortical neurons and in the MDL and Po thalamic nuclei further supports the idea of efficient 249AL retrograde transport. A similar spatial pattern of transgene expression was reported after adenoviral intracortical injection into the ischemic brain (Abe et al., 1997). These results suggest that the 249AL vector retains the retrograde transfecting property of several viruses, and hence it could be used for transgene delivery by a non-invasive administration route to target specific distal neuronal populations in the CNS.

Conclusions and future directions

Non-viral gene delivery using 249AL vector offers promising insights of future therapies for acute CNS injuries, preserving most of the relevant viral properties but avoiding the inflammatory, immune-activating, infectious, and scale-up production derived difficulties of viral vectors. The intrinsic flexibility of this modular vector provides wider perspectives for the generation of specific prototypes for different therapeutical needs.

In this regard, gene therapy mediated neuroprotection following acute neurological insults such as hypoxia-ischaemia, hypoglycaemia, seizures or traumatic brain injury, has been achieved in different animal models (Costantini et al., 2000; Sapolsky and Steinberg, 1999) including postnatal rats (Hagan et al, 1996). These strategies involve the transgenic overexpression from neuron-survival promoters, including genes of calcium binding proteins like calbindin D28K, heat shock proteins as Hsp70, glucose transporters such as Glut1, hyperpolarizing ion channels like potassium channels, anti-inflammatory proteins such as IL-1 receptor antagonist, neurotrophic factors as glial-derived neurotrophic factor (GDNF), or antiapoptotic genes like Bcl2 (Reviewed in Sapolsky, 2003). It should be noted that several of the neuroprotective effects of these molecules are based on a transient post-injury overexpression, and not on a constitutively enhanced expression. As an example, it has been shown that constitutive overexpression of calbindin disrupts neuronal plasticity (Chard et al, 1995). In this context, the rapid and transient expression of the transgene after an acute injury, as observed with the 249AL vector in the postnatal brain, is likely to be an efficient clinical approach.

Finally, it should be noted that upregulation of αV integrins in cerebral microvessels after ischemia (Okada et al., 1996) or in tumor microvasculature (Pasqualini et al., 1997) opens the possibility of an intravenous route for vector administration targeting these specific areas of interest. Future studies must be undertaken to establish therapeutically relevant transgene expression levels achievable with the 249AL vector under different experimental paradigms.

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Legends to figures

FIGURE 1. 249AL transgene delivery. GFP immunoreactivity (Abcam antibody) at different survival times after the intracerebral injection of saline solution (A), pEGFP-C1 DNA (B) or 249AL/pEGFP-C1 (C-F). Saline-injected brains do not show any immunoreactivity (A). One day after injection with pEGFP-C1 DNA, only a mild immunoreactivity was observed in the vicinity of the injection site (B). Brains injected with 249AL/pEGFP-C1 show GFP immunoreactivity in the parenchyma along the needle track and subjacent corpus callosum at 1 day post-injection (C, D) and in sparse neurons in the ipsilateral còrtex (E, F) at 7 days post-injection. D and F are magnifications from squares in C and E respectively. Densitometrical analysis for GFP immunoreactivity 1 day after injection show a significant (*p<0,05) increase in sections from animals injected with 249AL/pEGFP-C1 in relation to naked pEGFP-C1 DNA injected animals (G). Scale bars: A, B, C, E=300 μm, D=50 μm, F=20 μm.

FIGURE 2. Widespread transgene delivery after an excitotoxic lesion. GFP immunoreactivity in coronal sections at the level of striatum (B-C) and thalamus (D-G) of excitotoxically lesioned animals post-injected with 249AL/pEGFP-C1, at 1 day (A-C) and 7 days post-injection (D-G). At 1 day, the neurodegenerative area, clearly visualized with Nissl stain (A), is entirely matching with GFP immunoreactivity (B). No immunopositive signal was observed in any of the NMDA lesioned animals injected only with saline solution (C). In the retrogradely connected thalamic nuclei, as identified in Nissl stained sections (D), diffuse GFP immunoreactivity is found in the mediodorsal lateral nucleus (MDL), posterior nuclear group (Po), reticular nucleus (Rt) and lateral nuclear group (LP-LD) (E). Non-lesioned animals injected intracortically with the retrograde tracer Fluoro-Gold (F) show fluorescence in MDL, Po and ventral posterolateral nucleus (VPL). Similar findings are observed by GFP epifluorescence in thalamus (G), although GFP expression is also seen in Rt nucleus located on the other side of the external medullary lamina (eml). Scale bars: A-C=1mm, D-G=400μm.

FIGURE 3. 249AL-mediated transgene expression in NMDA lesioned brain. Twenty-four hours after injection, protein samples from lesioned brain hemispheres of 249AL/pEGFP-C1 or naked pEGFP-C1 DNA injected animals were extracted and submitted to SDS-PAGE and western blot for GFP (A). Only one immunopositive band per lane was observed, which comigrated with recombinant EGFP (rEGFP). Densitometrical analysis of the immunopositive bands revealed a significant (*p<0,05) increase in transgene expression in 249AL/pEGFP-C1 injected animals in comparison with naked pEGFP-C1 injected animals (B).

FIGURE 4. 249AL-mediated transgene expression in different cell types. GFP immunoreactivity showed transgene expression in neurons (A) as well as in glial cells (B-C) at 1 day post-injection. Double immunohistochemistry indicated that

both astrocytes (B) and microglia (C) express the transgene as shown by colocalisation of GFAP (brown in B) and GFP (green in B) or tomato lectin (TL, brown in C) and GFP (green in C) respectively. Scale bars: A=10 μm, B, C=20 μm.

FIGURE 5. Absence of histopathological alterations after 249AL injection. Nissl staining (A, B), several markers of inflammation and immune activation (C-F) and myelin staining (G) in 249AL-injected brains after 6 days survival. Nissl staining does not show degenerating neurons, perivascular leukocyte infiltrates (blood vessel: bv) or general alterations in parenchymal structure apart from the needle track canal (*) (A, B). GFAP immunostaining show no differences in astrocytosis (C). Microglial marker tomato lectin shows normal ramified resting microglia (D, E) and only some scattered macrophages at the needle track (arrow in E). No further differences in immunoreactivity of CD8 lymphocyte marker (F) or myelin protein MBP (G) were observed in comparison to saline-injected animals. Scale bars: A, C, D, F=500 μm, B=30 μm, E=50 μm, G=2 mm.

Figure 1. Peluffo et al.

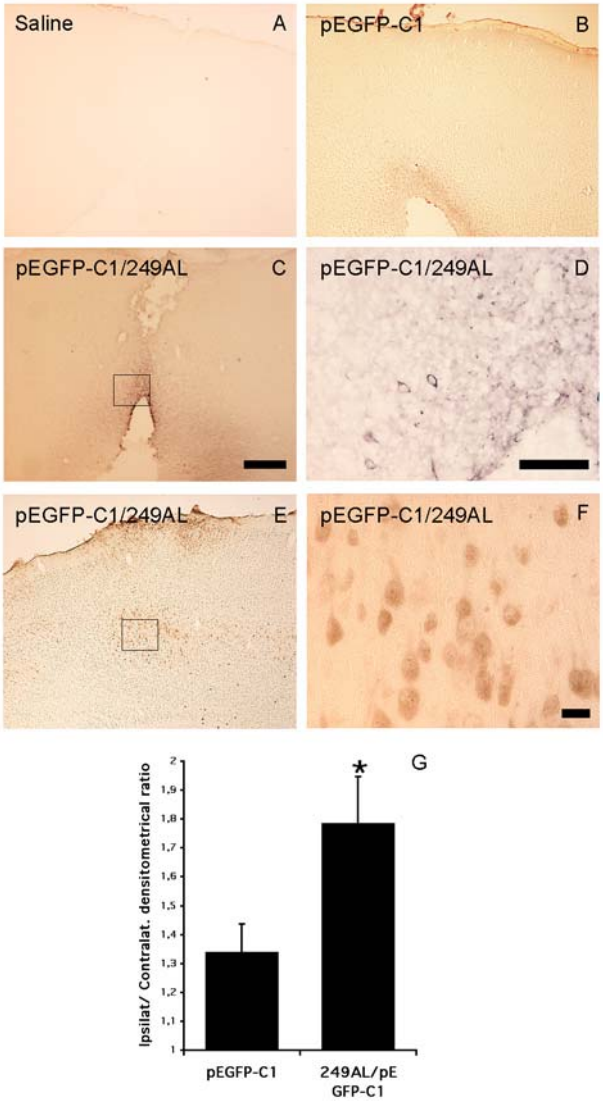


Figure 5. Peluffo et al.

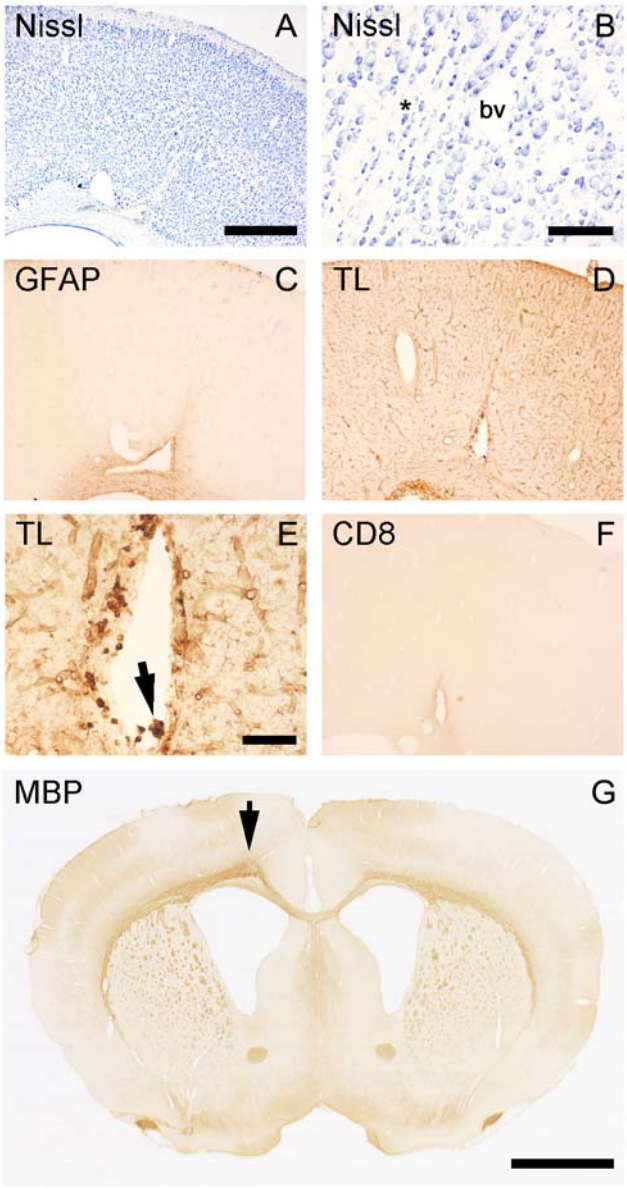
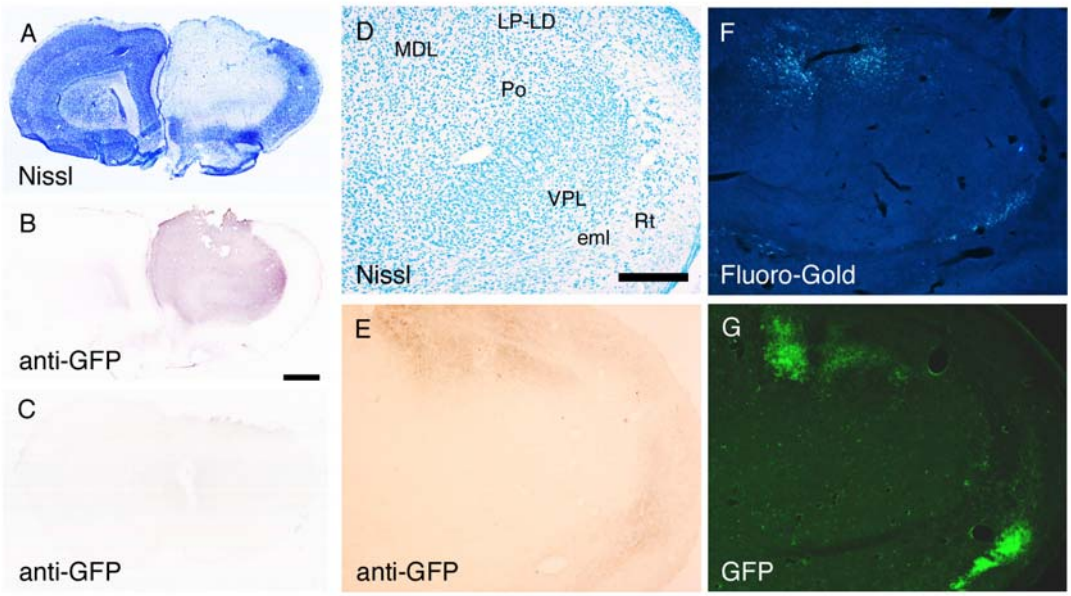


Figure 2. Peluffo et al.



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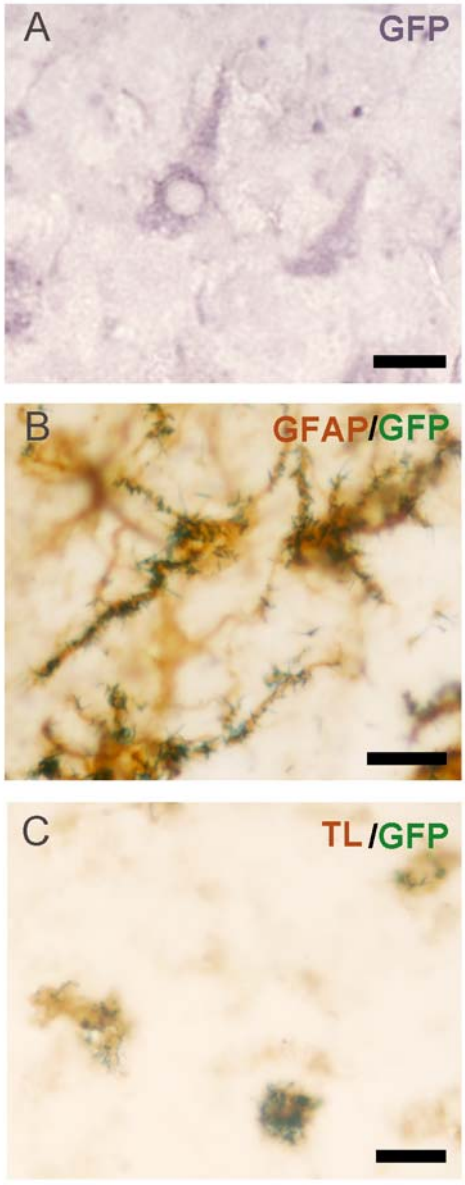
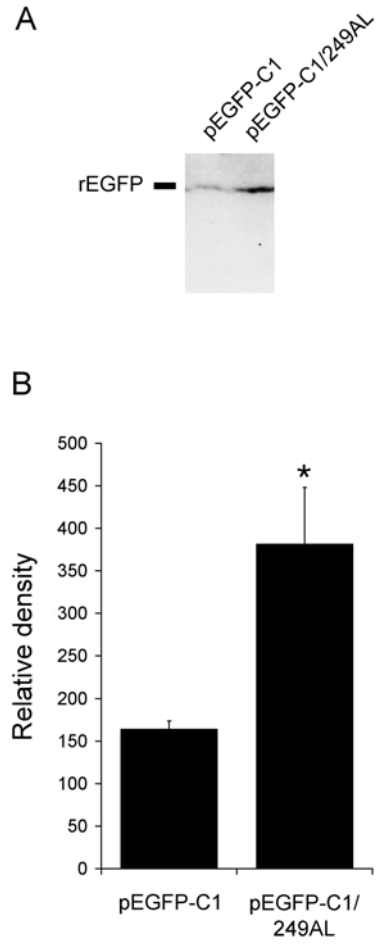


Figure 3. Peluffo et al.



TREBALL V

ENGINEERING NUCLEAR LOCATION SIGNALS IN MODULAR PROTEIN VEHICLES FOR GENE THERAPY

Anna Arís and Antonio Villaverde

Enviat a FEBS Letters

En aquest estudi hem analitzat la millora del transport nuclear dirigida pel vector 249AL. S'ha insertat el segment comprés entre els aminoàcids 126-135 del antigen T del virus SV40, en dos llocs acceptors alternatius d'aquest enzim, ja que aquest ha estat descrit com un senyal de localització nuclear eficient quan es presentat per proteïnes recombinants. Els resultats d'aquest treball mostren que la presència del SLN de SV40 incrementa l'eficiència de transfecció de 249AL fins a trenta vegades però que els nivells d'expressió gènica es troben altament influenciats pel lloc d'inserció i per l'impacte estructural que produeix l'acomodació del pèptid heteròleg en la proteïna presentadora.

ENGINEERING NUCLEAR LOCATION SIGNALS IN MODULAR PROTEIN VEHICLES FOR GENE THERAPY

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Keywords: αvβ3; β-galactosidase; molecular interactions; nuclear location signal; gene therapy; SV40 T antigen

Abstract

Amino acids from 126 to 135 of the SV40 virus T antigen act as efficient nuclear location signal during infection but also when fused to recombinant proteins. This peptide has been inserted into two alternative acceptor sites of a modified *Escherichia coli* β-galactosidase which also displays a DNA-binding domain, a cell-binding motif for integrin αvβ3 targeting and cell internalisation and a cryptic nuclear targeting signal naturally present in the bacterial enzyme. In cultured cells, the presence of the SV40 peptide enhances the expression of a delivered DNA up to thirty-fold. However, the DNA expression levels are largely depending on the chosen insertion site for the SV40 segment concomitant to the structural impact of peptide accommodation on the protein vehicle. The structural stability of the hybrid protein, apparently critical for efficient gene transfer, is discussed in the context of modular protein engineering to develop non-viral vectors for gene therapy.

1. Introduction

Conventional gene therapy requires safe and efficient tools for directed transfer of expressible DNA into target cells [1]. In addition, the silencing of selected genes by interfering RNA (iRNA) offers a new healing potential for cancer, genetic diseases and viral infections [2-3]. In this context, there is an increasing need for appropriate

vehicles to deliver DNA (encoding therapeutic molecules, either proteins or RNA), small iRNA molecules or their hairpin precursors. Viral-based vectors are the first choice for DNA targeting by exploiting relevant properties of the viral cycle, such as receptor-mediated cell recognition and internalisation, endosomal escape, nuclear transport and DNA integration. Obviously, prior to their therapeutic use, viral genomes must be conveniently engineered to eliminate any chance of virulence. Being undoubtedly efficient for these purposes, an important number of clinical trials are being carried out with a significant degree of success [4]. However, the appropriateness of therapeutic viruses is controversial, not only because of the dissemination of modified but potentially replicable, randomly integrable and/or recombinable genomes but also on the basis of non-desirable side effects observed upon massive inoculation of viral particles [5-8]. Moreover, the use of viral vectors must overcome the eventual immunization against related viruses in previous natural infections or against resident viruses.

In this context, multifunctional proteins, upon appropriate combination of different functional cassettes, could offer interesting alternatives for DNA delivering tools through the mimicry of relevant viral functions [9]. In addition, the versatility offered by protein engineering would allow the easy adaptation of such vehicles to new deliverers like small RNA molecules, or to new targets by the inclusion of appropriate cell-binding domains. On the other hand, the industrial production of recombinant proteins is feasible upon moderate efforts while the large-scale production of modified viruses has been observed as an additional, but also main obstacle for their use as therapeutics [10].

We have previously constructed an integrin αVβ3-targeted β-galactosidase enzyme that condenses and delivers DNA, and allows significant levels of encoded gene expression both in cell culture and mammalian tissues [11]. Upon internalisation, the nuclear delivery was mediated by a cryptic nuclear location signal (NLS) present in the bacterial enzyme [12-13], but that is only moderately efficient when compared with fully functional viral signals [12]. In this work, we have introduced an additional NLS from the SV40 T antigen in two alternative acceptor sites of the enzyme, offering differential structural frameworks for its presentation. While in both cases the viral NLS rises above the bacterial motif and enhances gene expression levels, the extent of this improvement is dramatically affected by the chosen accommodation site, which is thus revealed as a critical factor for the efficiency of the whole delivery system.

2. MATERIAL AND METHODS

2.1 PROTEINS AND PLASMID CONSTRUCTION

Plasmids pJLACZ [14], pJX249A [15], pTrC249AL [11] and pTK10LACZ [16] encode the engineered *E. coli* β-galactosidases LACZ, JX249A, 249AL and K10LACZ

respectively. Despite carrying different foreign peptides inserted in alternative accommodation sites (Figure 1), all of them are enzymatically active. Protein JX249A derives from the pseudo-wild type LACZ and displays a 27-mer FMDV peptide containing an integrin-targeted RGD-based motif between residues 249 and 250 [17]. The joining of a deca-lysine tail to the amino terminus of JX249A resulted in the β-galactosidase variant 249AL that binds, efficiently condenses and delivers plasmid DNA to target cells [11]. The K10LACZ control protein is a closely related β-galactosidase that only carries the N-terminal lysine tail. This protein was constructed by removing the cell binding peptide from 249AL [16].

NLS278 and NLSCt proteins (encoded by pTNLS278 and pTNLSCt respectively) are 249AL derivatives constructed here by the incorporation of the SV40 NLS (PKKKRKVEDP) [12], either inserted between residues 279 and 280 or fused to the carboxy terminus of the enzyme respectively (Figure 1). For NLS278 construction, two partially complementary oligonucleotides (5'-CGCGCCGAAGAAAAAACGC-AAAGTGGAAGATCCGCTAGC-3' and 5'-CGGCTAGCGGATCTTCCACTTTGC-GTTTTTTCTTCGGCG-3') were designed to encode the NLS. Once hybridized, the overhanging ends permitted the ligation with ClaI-digested pTrC249AL. The presence of an internal NheI site and a stop codon in the inverted oligonucleotide segment allowed the identification of those clones receiving the segment in the correct orientation. For NLSCt construction, plasmid pTrC249AL was linearized by PstI site and partially digested with BamHI. A 7320 bp fragment was selected for ligation with the hybridized 5'-GATCCCGAAGAAAAAACGCAAAGTGGAA-GATCCGAACTAGTGCA-3' and 5'-CTAGTTCGGATCTTCCACTTTGCGTTTTTT-CTTCGG-3' oligonucleotides that encode the SV40 NLS and a carboxy terminus stop codon.

2.2 PROTEIN PRODUCTION, PURIFICATION AND PROTEIN-DNA COMPLEX FORMATION

The production of modified β-galactosidases was induced by temperature shift from 28º to 42º C for MC1061/pJLACZ and MC1061/pJX249A cell cultures or by the addition of 1 mM IPTG for MC1061/pTrC249AL, MC1061/pTK10LACZ, MC1061/pTNLS278 and MC1061/pTNLSCt cell cultures. The recombinant gene expression was triggered within the early exponential phase in LB medium [18] in the presence of 100 µg/ml ampicillin. Proteins were purified in a single step affinity chromatography with the non-hydrolysable β-galactosidase substrate TPEG as described previously [11]. Protein-DNA complexes were prepared in microcentrifuge tubes by the incubation of plasmid DNA at 0.01 µg/µl in Hepes buffered saline (HBS) or OptiMEM (Gibco-BRL) when for the *in vitro* transfections, with different concentrations of the engineered proteins for 1 hour at room temperature. The chosen ratios were 0.02, 0.03, 0.04, and 0.08 µg DNA per µg of protein corresponding to 1, 2, 3, 5 retardation units respectively (see below for definition).

2.3 DETERMINATION OF SPECIFIC ACTIVITY, PROTEIN SOLUBILITY AND STABILITY

The specific activity of LACZ and all the derivatives was determined by Western blot with appropriate standards of commercial β-galactosidase and independent enzymatic analysis as described [19]. Intrinsic tryptophan fluorescence was determined in an AMINCO SLM 8000 spectofluorometer on recombinant proteins alone or previously incubated with plasmid DNA at different ratios. Excitation wavelength was set at 283 nm and the emission spectra were recorded between 300 and 400 nm using the maximum fluorescence value to compare conformational variations in different samples. The fraction of soluble complexes was estimated by Bradford dye-binding procedure [20] after centrifugation at 15500 g for 15 min.

For electron microscopy, complexes were prepared at 0.02, 0.03, 0.04, 0.08 DNA-protein rations [16]. Micrographs were taken with a Hitachi H-7000 transmission electron microscope (Hitachi LTD.Tokyo, Japan) at a magnification of 50000x and 100 kV.

2.4 ANALYSIS OF DNA AND CELL BINDING

The cell-binding assay has been described in detail elsewhere [17, 21]. Briefly, 15 pmols of pure protein in phosphate buffered saline (PBS) were incubated in ELISA COSTAR EIA/RIA plates overnight at 4ºC. Wells were then washed in PBS and blocked with 3 % (w/v) bovine serum albumin (BSA). Trypsinized BHK cells from 80 % confluent cultures were added to the wells to about 5x104 cells/well and incubated for 1.5 h at 37ºC. After washing with PBS, cells were fixed with 100 % 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 the addition of 1 % (w/v) SDS. The absorbance at 620 nm was measured in a microtiter reader. For retardation assay, 25 µl of protein-DNA complexes in HBS were analyzed on 0.8 % agarose gels to determine the minimal amount of protein required to fully impede DNA mobility (one retardation unit).

2.5 MEASUREMENT OF TRANSGENE EXPRESSION

The vector pGL3 (Promega, Madison, WI), carrying the American firefly (Phonitus pyralis) luciferase gene was incubated with different amounts of chimeric proteins. CaCo-2 (human colonic epithelial) cells were grown in 24-wells plates to 50 % confluence. Then, cells were washed and further incubated with OptiMEM medium (Gibco-BRL). The media was removed and the DNA complexes containing 1 µg pGL3 were added to 200 µl OptiMEM per well. After 6 h of incubation, the media was replaced by Glutamax-containing DMEM, supplemented with 20% foetal calf serum and penicillin-streptomycin (at 100 units/ml each). After 48 h, cells were washed and further harvested in lysis buffer (Promega). Luciferase activity was measured through standard protocols [22]. Further details can be found elsewhere [11].

3. RESULTS

3.1 DESIGN AND PRODUCTION OF ENGINEERED B-GALACTOSIDASES

Protein 249AL is a hybrid, *E. coli* β-galactosidase displaying both an integrin-targeted, cell binding motif and a DNA condensing domain on each enzyme monomer (Figure 1). This construct is able to direct cell internalisation and expression of a transported DNA [11], with the participation of a cryptic NLS located in the bacterial enzyme [13]. To explore if an additional NLS could enhance the efficiency of DNA transfer and expression we have inserted a well characterized, SV40 NLS as an additional module of the chimeric protein. Two acceptor sites, previously recognized as exposed to the enzyme surface [23], were selected for these constructions, namely the activating interface, involved in monomer-monomer contacts and in the active site construction, and the carboxy terminus, overhanging at the end of the long interface [24]. Our aim was to evaluate the possible influence of the structural framework for the accommodation of NLS on the global performance of the DNA delivery vehicle. The resulting proteins, NLS278 and NLSCt respectively were produced in *E. coli* without symptoms of toxicity in cultures exhibiting β-galactosidase enzymatic activity and they were easily purified by single-step, TPEG affinity chromatography (not shown).

3.2 DNA CONDENSATION AND CELL ADHESION IN SV40 NLS-CONTAINING CHIMERIC B-GALACTOSIDASES

Since NLS278 and NLSCt were both active and produced at reasonable yields, we inferred that the addition of a third foreign peptide (the SV40 NLS stretch) was not disturbing the formation of tetramers and the global stability and activity of the bacterial enzyme. However, we wanted to explore if the SV40 segment could be affecting the more delicate DNA-condensing and cell binding properties determined by the already present additional motifs, that are both essential for the DNA delivery. In agarose gels, both NLS278 and NLSCt retarded plasmid DNA as efficiently as the parental control protein 249AL (Figure 2A). Although NLS278 might be slightly more efficient in DNA-binding than the other two proteins (Figure 2B), the three constructs completely impeded DNA migration between 11 and 12 μg protein/μg DNA (Figure 2B). On the other hand, the cell binding through target integrins was also not affected in any of these extensively engineered β-galactosidases (Figure 2C).

Protein 249AL alone without DNA, spontaneously forms aggregates that are slightly heterogeneous in size and morphology, to which the DNA easily accommodates [16]. We explored if the different location of the viral NLS peptide could influence the formation of the resulting protein-DNA complexes. Their occurrence was studied under transmission electron microscopy at 0.02, 0.03 and 0.04 DNA-protein ratios without observing significant differences beyond the

internal morphological diversity. As an example, those formed at 0.04 DNA-protein ratio are shown in Figure 3.

3.3 Enhanced but differential gene expression promoted by SV40-NLS

Since the preliminary characterization described above revealed the conservation of relevant functions in the protein vehicles NLS278 and NLSCt, they were then tested for delivery of expressible DNA in cultured cells. While the SV40 NLS in NLS278 promoted gene expression levels similar to those obtained with 249AL, the placement of the viral peptide at the carboxy terminus of the enzyme (in NLSCt), dramatically enhanced the performance of the vehicle. In that case, luciferase gene expression reached levels between 2 and 30-fold higher than when mediated by 249AL (Figure 4). The improvement of gene expression was particularly important at 3 RU (0.03 μg DNA/μg protein), a DNA-protein ratio within the range proved useful for DNA transfer [11, 16]. Therefore, it was evident that the presence of the SV40 NLS could overcome the nuclear delivery potential offered by the bacterial enzyme, but also that this capability is largely dependent on the specific accommodation site.

3.4 Structural stability of DNA delivering vehicles

Since the different expression levels observed in NLS278 and NLSCt (Figure 4) were not due to deviations in the binding profile of these proteins (Figure 2) or in the general morphology of the resulting complexes (Figure 3), we explored other protein features that could be relevant to the vehicle performance upon internalization and responsible for their differential efficiency. We reasoned that although both proteins were enzymatically active, the different accommodation sites could affect at different extents protein structure and/or stability, especially upon DNA binding. Therefore, to explore this possibility, we followed several approaches considering both the protein alone and also the protein as part of the complexes.

The inserted peptide, when accommodated in the activating interface, produced a dramatic impact on the specific activity, which dropped from 730 U/μg (in the parental JX249A) to 21 U/μg (in NLS278). The carboxy terminal fusion had, however, no detectable impact on enzyme activity (Table 1). Thus, the effect of the foreign stretch seemed to be not restricted to a mere local perturbation in the active site that could specifically affect the substrate uptake or processing, but it rather altered the global stability of the protein. In this context, the accommodation of the DNA into NLS278 protein complexes had a major effect on the conformation of the vehicle, as revealed by the dispersion of the tryptpophan spectrum at different DNA-protein ratios (Table 1). In this context, while the solubility of NLSCt is comparable to that of the parental 249AL, the extent of soluble NLS278-DNA complexes was significantly affected mainly at 0.08 DNA-protein ratios (Figure 5).

An important background of gene expression had been observed at this DNA-protein ratio (not shown), proving that these insoluble complexes are still able to deliver expressible DNA. However, this differential solubility also indicates that the structural properties of the protein could have been importantly affected by the peptide insertion at the activating interface, then the conformational stability of this construct resulting more sensitive to DNA binding. The also considerable insolubility of (K)10LAZ, which interestingly, is irrespective of the molar DNA-protein ratio of the complexes, could be attributed to a greater solvent-exposure of the polylisine tails in absence of other foreign peptides. In fact, it is known that long basic amino acid stretches importantly significantly impair protein solubility [26].

4. DISCUSSION

In animal DNA viruses that replicate in the cellular nucleus, specific signals are required to drive relevant proteins to this compartment. In SV40, a minimum of eight contiguous amino acids are responsible for the nuclear transport of T antigen [12]. This and other NLS-containing peptides have been explored as elements to deliver expressible genes to target cells, mainly through chemical conjugation with the encoding plasmid DNA [26]. In addition, NLS peptides are able to promote the nuclear transport of large non-mammalian proteins such as, among others, *E. coli* β-galactosidase, as end-terminal fusions [12] or as inner insertions located in permissive sites of the enzyme [27]. This ability represents an interesting possibility for the construction of multifunctional proteins as vehicles for gene therapy. However, the possible influence of the particular NLS(s) accommodation site on their performance as nuclear drivers has yet to be done. The position of the NLS in the chimeric protein might be of relevance for its activity, since the specific molecular framework has been proven to be critical for other biological properties of inserted peptides, such as the reactivity with peptide-targeted antibodies [28]. We have here explored the performance of the SV40 T antigen NLS, one of the most employed peptides to artificially promote nuclear transport [12], in two structurally different acceptor sites of *E. coli* β-galactosidase, namely an inner tolerant site between amino acids 279 and 280, within the activating loop of the enzyme protruding from one monomer to the other [24], and the overhanging carboxi terminus. Peptides of different lengths accommodated in either both sites are solvent exposed for complex protein-protein molecular interactions [29, 30].

Insertions were made on protein 249AL (Figure 1), that containing both a cell-binding and a DNA-binding motifs [11], is able to internalize and deliver condensed DNA to the cell nucleus, via cell surface integrin receptors [21]. The nuclear transport for gene expression mediated by 249AL was achieved by a background nuclear targeting activity observed in the bacterial enzyme [12, 13, 17]. The results obtained here show a biological impact of SV40 NLS peptide in promoting gene expression of delivered DNA, that is clearly different in the two tested accommodation sites (Figure 4). In protein NLS278, the SV40 peptide is inefficient,

since its presence does not enhance the expression levels over those achieved by the cryptic β-galactosidase NLS. However, protein NLSCt promotes gene expression levels up to 30-fold higher than those reached by 249AL (Figure 4). This improvement of the vector performance occurs without any noticeable modification in the DNA- and cell-binding properties (Figure 2), and in DNA-protein complex organization (Figure 3) and solubility (Figure 5). This indicates that SV40 NLS is functional when present in the bacterial enzyme and that its activity prevails over the background but biologically relevant nuclear targeting properties of the bacterial enzyme itself.

The failure of the SV40 segment in improving gene expression as mediated by NLS278, indicates the importance of the acceptor site for the whole performance of the DNA vehicle. This is not merely depending on peptide accommodation as an inner protein insertion or as an end-terminal fusion, since a NLS peptide inserted between amino acids 375 and 376 of β-galactosidase has been proven to be fully active [27]. On the other hand, the differences observed between NLS278 and NLSCt should be attributed to a lower solvent exposure of SV40 NLS in NLS278 that could eventually impair the molecular contacts required for nuclear penetration. In fact, the binding of a hybrid β-galactosidase to membrane-associated cell integrins is about two-fold more efficient when the foreign ligand peptide is inserted between residues 279-280 respective to the equivalent carboxy terminal fusion [23], and the immunoreactivity of antigenic peptides in these two sites is comparable [29]. However, it is not possible to discard that the conformational presentation of the SV40 NSL in the activating interface would be inappropriate for the proper interaction with importins and/or other nuclear translocators [31].

Interestingly, the insertion of SV40 NLS in NLS278 had an important impact on the enzyme specific activity (Table 1). In addition, binding of NLS278 to DNA, although not resulting in morphologically aberrant protein-DNA complexes (Figure 4), significantly impairs solubility (Figure 5) and promotes important conformational modifications in the whole protein, as revealed by thryptophan absorbance (Table 1). While the impaired solubility itself seems to be not a direct cause of low reporter gene expression levels (Figure 4, 5 and results not shown), these results indicate a loss of structural stability in NLS278 that might affect delivery steps other than cell binding and internalization or DNA condensing. Although poor is still known about the mechanics of protein nuclear transport, the multiple interactions with the nuclear pore complexes [32] would require a minimal degree of native structure in the target proteins, and differently from in other organelles, nuclear transport occurs on fully folded proteins [33]. Furthermore, the final dissociation of the DNA-protein complexes, believed to occur in the nucleus trough competition of the cellular DNA for the protein vehicle [34], could be impaired by a poorly structured or insoluble protein. In this context, different peptide insertions previously performed in the β-galactosidase activating interface have often resulted in more

unstructured proteins [15], that even exhibited more susceptibility to cell proteolysis [29].

Results presented in this work prove that the addition of a viral NLS can dramatically enhance the resulting gene expression levels mediated by a chimeric, multifunctional β-galactosidase (up to 30 times), but also that this upgrading is largely dependent on the particular accommodation site and probably on the resulting structural stability of the whole construct. Interestingly, the proficient protein NLSCt carries three foreign modules in each monomer of the bacterial enzyme, being all of them and the enzyme itself, highly functional. This represents a further evidence of the usefulness of *E. coli* β-galactosidase as an extraordinarily flexible carrier for the multifunctional approach to new generation, gene delivery vehicles.

Acknowledgments

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Table 1. Biochemical parameters of engineered β-galactosidases

Protein	Specific activity (U/ug)	λ_{max} (nm)[b]
LACZ	920 ± 1	336-337
JX249A	730 ± 0.79	nd (336)
249AL	557 ± 0.60	336-337
K_{10}LACZ	817 ± 0.88	336-337
NLSCt	765 ± 0.83	337-338
NLS278	21 ± 0.02	334-339

[b] Range of tryptophan absorbance in protein alone and in DNA protein complexes from 0.02 to 0.08 DNA/protein ratios.

nd JX249A does not possess any DNA binding domain. Thryptophan absorbance is shown here for the protein alone.

FIGURE 1. Schematic representation of the engineered β-galactosidase proteins, indicating the approximated location of the foreign stretches as follows: in white boxes, the FMDV cell-binding segment; in grey, the DNA-binding domain; in black, the SV40 NLS. At the bottom, the specific amino acid sequences of each of these peptides are indicated. Residues typed in small-case are those encoded by restrictions sites used in the cloning process, and those in boldface are those belonging to *E. coli* β-galactosidase. Numbering of enzyme residues is according to Kalnins and coworkers [35]. Underlined residues in the FMDV peptide are those shown critical for integrin binding [36].

FIGURE 2. A). Retardation of DNA in agarose gels, mediated by 249AL, NLS278 and NLSCt. Figures at the top indicate the protein-DNA ratio used in each lane. The amount of DNA loaded was 0.25 μg in all the cases. B) Amount of migrating DNA versus protein amount in each of the conditions indicated in panel A. 249AL data are shown by squares, NLS278 by triangles and NLSCt by circles. DU are densitometric units. C) Cell binding mediated by the complete set of engineered enzymes. Vitronectin, that binds integrin αvβ3, is shown here as a control.

FIGURE 4. Luciferase gene expression in CaCo-2 cells 48 hours post transfection, as mediated by different protein vehicles and with naked plasmid DNA alone. The DNA/protein ratios (w/w) were 0.04 (black bars), 0.03 (light grey bars) and 0.02 (dark grey bars).

FIGURE 3. Electron microscopy micrographs of DNA protein complexes taken at 0.04 DNA/protein (w/w) ratio.

FIGURE 5. Fraction of soluble DNA-protein complexes. From darker to lighter grey: 0.08, 0.04, 0.03 and 0.02 DNA/protein ratio.

Figure 1. Arís and Villaverde

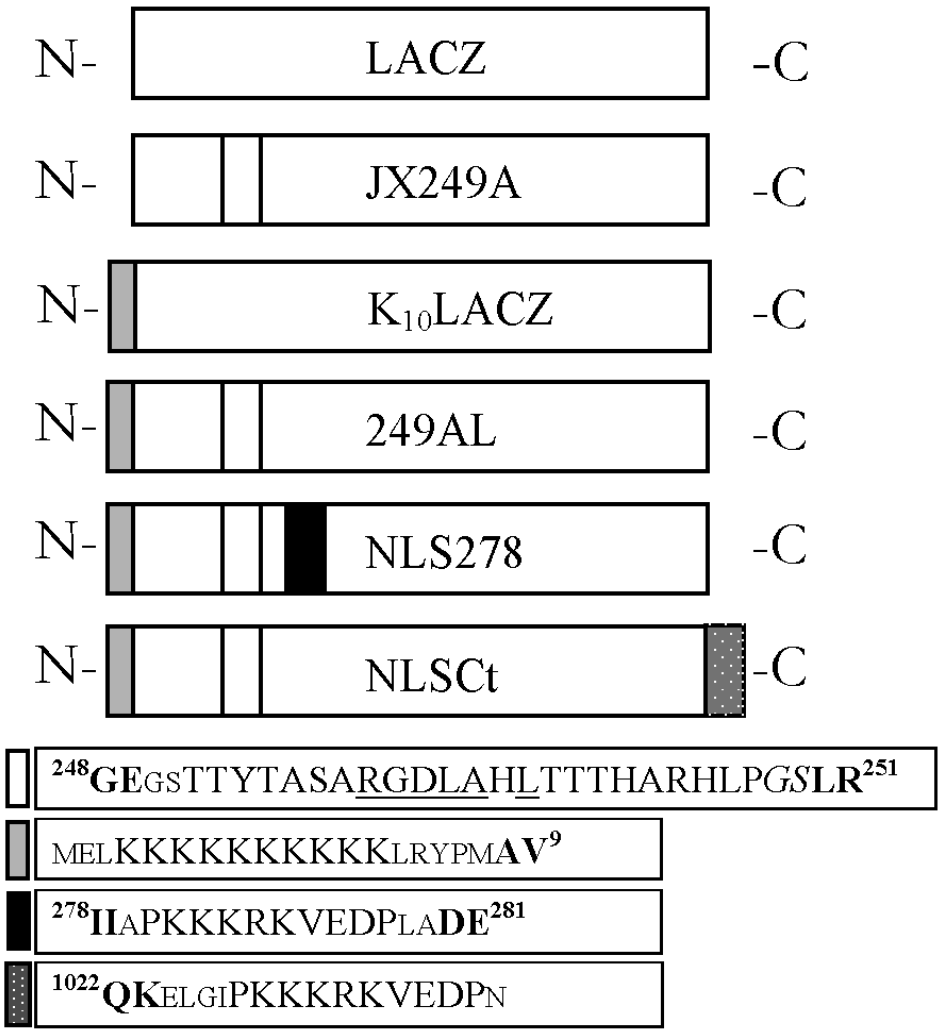


Figure 2. Arís and Villaverde

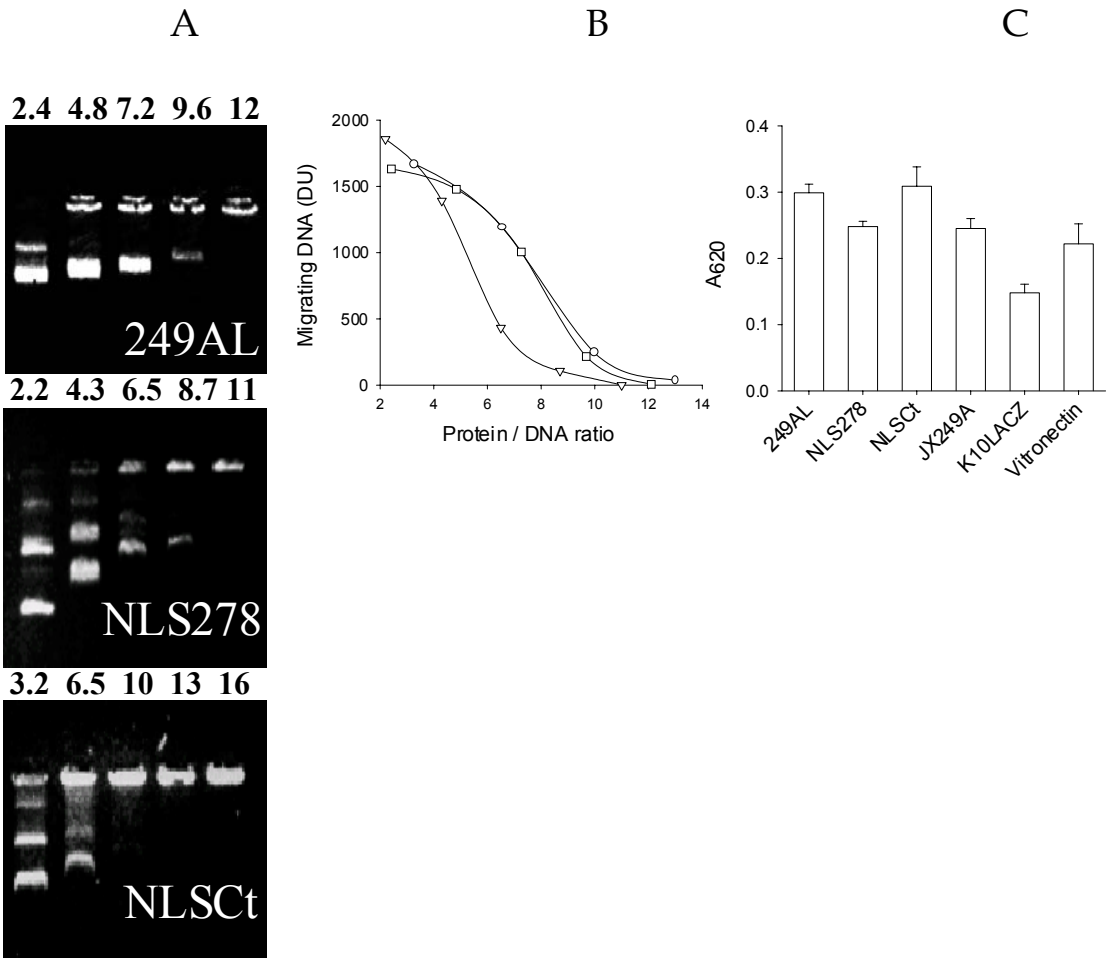


Figure 3. Arís and Villaverde

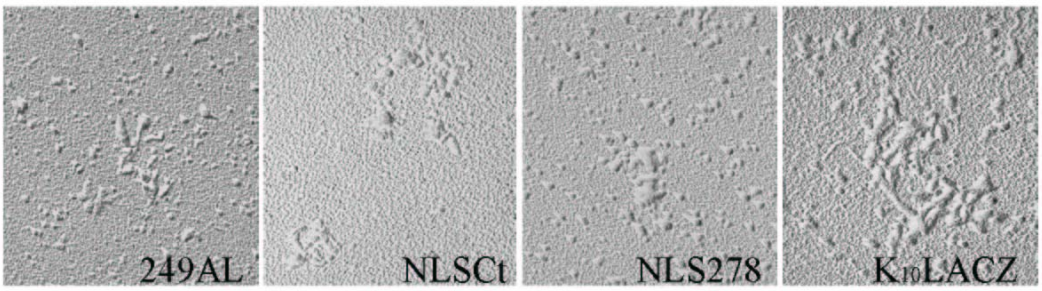


Figure 4. Arís and Villaverde

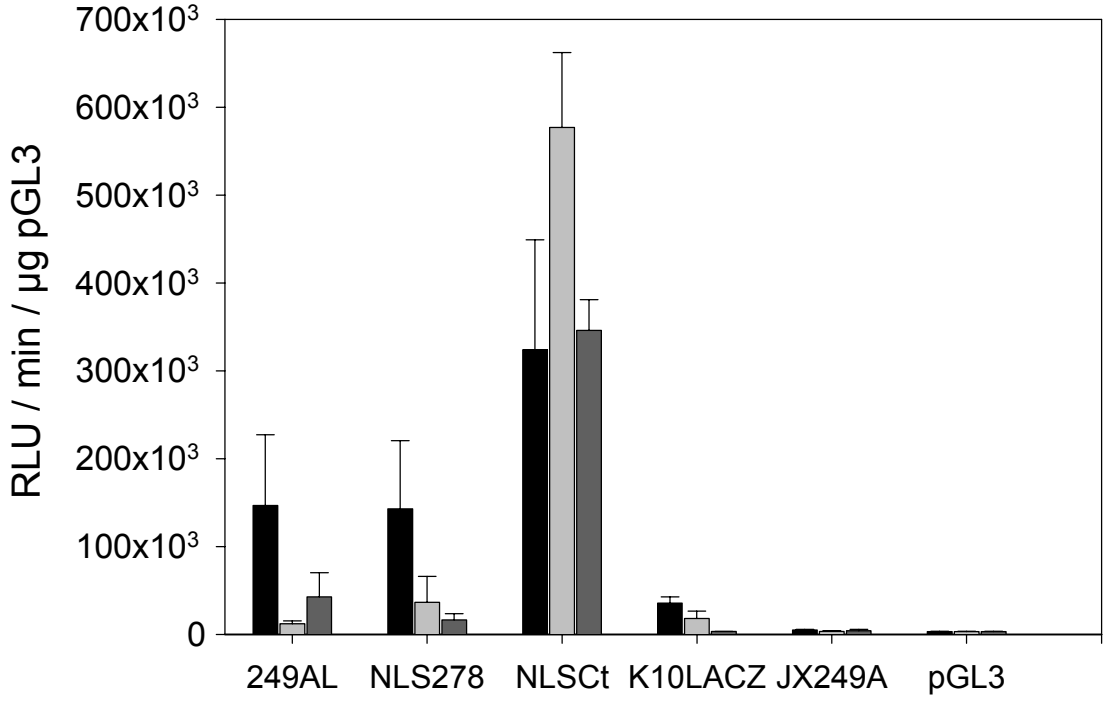
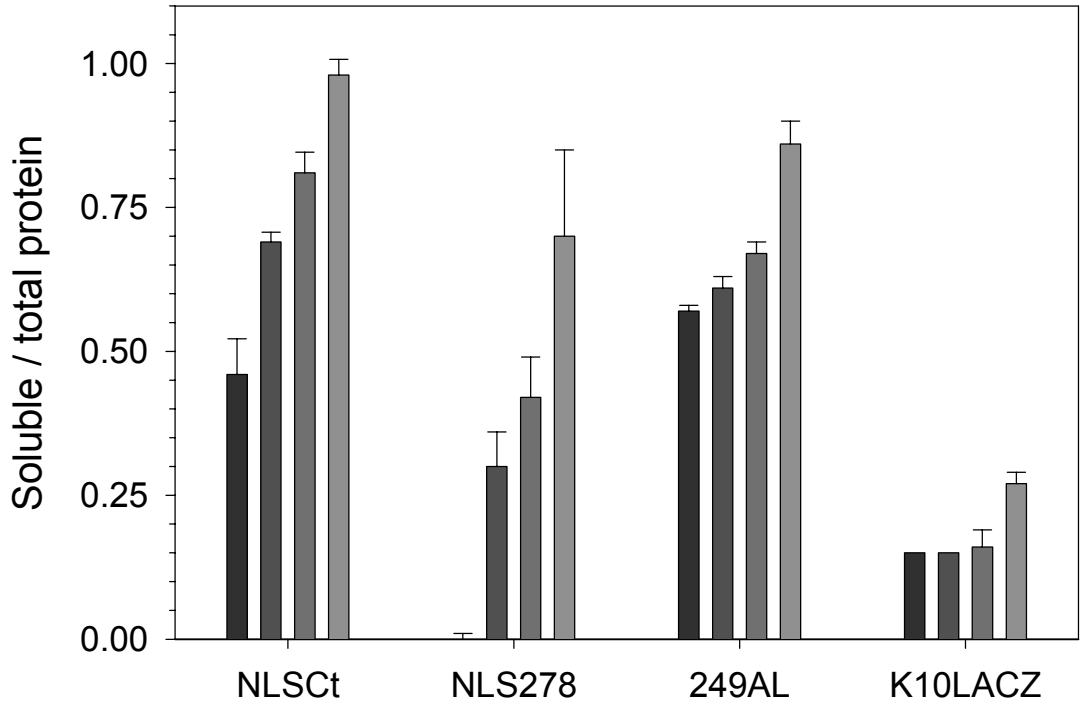


Figure 5. Arís and Villaverde



DESENVOLUPAMENT DE PROTEÏNES QUIMÈRIQUES MULTIFUNCIONALS COM A VECTORS DE TRANSFERÈNCIA GÈNICA MEDIADA PER RECEPTOR

IV.DISCUSSIÓ

En els últims anys, l'ús de sistemes no vírics en teràpia gènica ha anat prenent importància, ja que, tot i que s'ha demostrat que els vectors vírics són eines molt eficients en la transferència de DNA, presenten limitacions associades, com ara la difícil obtenció de títols elevats de partícules víriques, la inducció de respostes immunològiques i riscs de seguretat biològica com la mutagènesi insercional i la possible reversió a vectors recombinants competents en replicació. A més a més, la mida del DNA transportat és limitada i sovint presenten poca especificitat cel·lular o bé una especificitat inadequada. L'enginyeria de les partícules víriques ha permès millorar i adequar aquests vehicles a determinats procediments de teràpia gènica, però generalment accentua els problemes de producció d'aquests vectors a gran escala (vegeu la introducció).

És, però, molt important assegurar dins de les estratègies no víriques, processos d'endocitosi mediada per receptor, no tan sols per dirigir el procés d'internalització cel·lular eficientment, sinó per poder mantenir l'especificitat per a un tipus cel·lular diana.

Les proteïnes quimèriques recombinants són candidats molt interessants en la transferència gènica no vírica. La generació d'aquests vehicles ha estat poc explorada dins la teràpia gènica, i es basa en la combinació de diverses proteïnes o dominis proteics bioactius en una sola cadena polipeptídica, garantint l'obtenció d'una proteïna transportadora de DNA mitjançant un procés de producció ràpid, senzill i barat. Aquestes regions heteròlogues combinades, aporten la capacitat d'unir molècules de DNA, el reconeixement i la internalització cel·lular i possibles funcions potenciadores de la transferència cel·lular com ara la lisi d'endosomes i el transport nuclear. Els senzills processos de producció i purificació a gran escala i la seva naturalesa modular, fan que siguin vehicles fàcilment optimitzables i adequables a noves aplicacions de teràpia gènica.

IV.A.1.ENZIMS β-GALACTOSIDASA PRESENTADORS DE PÈPTIDS VÍRICS: POSSIBLES VECTORS DE TRANSFERÈNCIA GÈNICA

En el nostre grup s'havia demostrat prèviament que el pèptid GH23, que comprèn el lligand d'unió cel·lular del VFA, podia ser presentat en enzims β-galactosidasa recombinants, de manera que aquests adquirien la capacitat d'unir-se a cèl·lules de mamífer a través de receptors de vitronectina i, amb menys eficiència, a través de receptors de fibronectina (203) (vegeu antecedents del treball de recerca). Aquests estudis, i el fet de disposar d'un model ben caracteritzat de proteïna portadora, van promoure l'inici d'aquest treball dirigit al desenvolupament de vectors de transferència gènica a cèl·lules de mamífer, basats en enzims β-galactosidasa quimèrics i multifuncionals.

En el treball I, presentat en els resultats, es demostra que la presentació del pèptid GH23 en la β-galactosidasa no només dirigeix la unió de l'enzim a cèl·lules de mamífer, sinó que també promou la seva internalització cel·lular. També s'observa que la proteïna M275VP1, que presenta el pèptid entre els residus 275 i 276, dins l'*activating interfase* de l'enzim, mostra un patró d'unió cel·lular més eficient que la proteïna AB1, presentadora del pèptid en la posició N terminal (figura 2B, treball I). Els resultats corroboren que el lloc d'inserció del fragment víric en la proteïna portadora és crític, malgrat que s'hagi descrit prèviament que els dos llocs d'inserció estan exposats al solvent (199) i toleren insercions o fusions peptídiques (248;249). Sembla que l'exposició del segment GH23 en l'*activating interfase* reprodueix les propietats d'unió del VFA molt millor que en l'extrem N terminal, la qual cosa confirma la importància de mantenir la conformació del motiu RGD en un llaç tancat (250).

L'estudi, mitjançant microscòpia confocal de fluorescència, de la internalització cel·lular de la proteïna AB1275VP1, construcció híbrida de les proteïnes M275VP1 i AB1, mostrava que, una hora després d'afegir proteïna soluble a cultius cel·lulars, la major part de l'enzim recombinant es localitzava a dins de les cèl·lules seguint un patró vesicular. Aquestes observacions indicaven que gran part de la proteïna es troba internalitzada en els endosomes mentres que tan sols una petita part resta associada a la membrana (figura 3, treball I). Fent un seguiment de l'activitat enzimàtica en cèl·lules incubades amb la proteïna a 37ºC, temperatura a la qual pot haver-hi internalització mediada per integrines, i a 4ºC, temperatura a la qual tan sols es produeix la unió però no la internalització cel·lular (251), es demostrava que les proteïnes recombinants mantenen la seva activitat enzimàtica després de ser internalitzades (figura 5, treball I) i que en l'espai intracel·lular la vida mitjana de l'enzim funcional és una mica més llarga d'una hora (figures 4, 5 i 6, treball I). Els resultats discutits en aquest treball confirmen que la presentació de pèptids vírics que contenen motius d'unió cel·lular com ara l'RGD, poden ser eines eficients per dirigir quimeres que conserven la seva activitat biològica quan estan unides a la superfície cel·lular i també un cop han estat internalitzades. Aquesta aproximació pot ser rellevant en casos de marcatge de superfície cel·lular, lliurament de toxines i teràpia proteica. La incorporació de dominis adequats d'unió a DNA ofereix la possibilitat d'utilitzar aquests constructes com a vectors de transferència gènica mediada per integrines cel·lulars.

Les integrines són receptors heterodímers d'adhesió cel·lular que estan involucrats en unions cèl·lula-cèl·lula i en interaccions entre la cèl·lula i els components de la matriu extracel·lular (252). Interaccionen amb una gran varietat de lligands entre els que trobem glicoproteïnes de la matriu extracel·lular, com ara la fibronectina i la vitronectina i, alhora, els seus dominis intracel·lulars interaccionen amb el citoesquelet (253). Les integrines són els lligands naturals per a l'entrada mediada per receptor de molts microorganismes patògens, com ara el virus de la febre aftosa (254), els adenovirus (251) i els bacteris com *Yersinia pseudotuberculosis* (255) i

Bordetella pertussis (256). Estudis del cicle d'infecció del VFA indiquen que la integrina $\alpha_v\beta_3$ és el seu principal receptor durant una infecció natural (257), i, de fet, aquesta integrina també és el principal receptor de les proteïnes β-galactosidasa portadores del pèptid víric GH23 que han estat construïdes en aquest treball de recerca (figura 3A, article II).

La integrina $\alpha_v\beta_3$ és el receptor principal de la vitronectina, i tot i que és força ubiqua en teixits de mamífers, s'ha demostrat que té un paper molt important en l'angiogènesi (258), de manera que lligands antagonistes d'aquest receptor tenen un efecte antitumoral i provoquen l'apoptosi de les cèl·lules dels vasos sanguinis angiogènics (259;260). A més a més, s'ha demostrat que els bacteriòfags presentadors de pèptids RGD de reconeixement específic de $\alpha_v\beta_3$, s'acumulen en tumors després de la seva injecció intravenosa en animals (261;262). Aquests fets suggereixen el potencial antitumoral de vectors de transferència gènica basats en el reconeixement de la integrina $\alpha_v\beta_3$.

IV.A.2.L'ENZIM 249AL, UN NOU PROTOTIP DE VECTORS QUIMÈRICS MULTIFUNCIONALS EN TERÀPIA GÈNICA

La construcció de proteïnes quimèriques recombinants per a la transferència dirigida de DNA, és una aproximació dins la teràpia gènica que fins ara ha estat poc estudiada. La naturalesa d'aquests vectors ofereix un gran potencial, però la combinació dels diferents polipèptids heteròlegs no deixa de ser un procés d'assaig i d'error en el qual no tan sols s'ha de comprovar que es mantenen les funcions dels diferents dominis sinó que també s'ha d'analitzar els possibles efectes deleteris sobre els processos de producció i purificació del vector. Els objectius d'aquest treball de recerca es centren en la construcció d'una proteïna eficaç en la transferència gènica i també en la producció d'un prototip que permeti aprofundir en el coneixement de les bases moleculars que dirigeixen la combinació de diversos dominis funcionals, en el context de vectors no vírics. Per tal de minimitzar els possibles problemes d'estabilitat d'aquest prototip, vam escollir una β-galactosidasa presentadora del pèptid GH23 entre els residus 249 i 250: la proteïna JX249A. Aquest lloc d'inserció ha estat descrit com un dels llocs permissius de l'enzim β-galactosidasa en el que s'altera menys l'estabilitat conformacional i l'activitat de la proteïna, i que alhora, manté les propietats funcionals del pèptid insertat (200). La preservació de l'estabilitat enzimàtica de la β-galactosidasa ens permet explotar al màxim els avantatges que ofereix com a proteïna portadora de dominis heteròlegs. Entre aquests aventatges podem destacar que és una proteïna no tòxica per a *Escherichia coli* com a organisme productor; que és soluble i resistent a la proteòlisi; que el gen *lacZ* que la codifica presenta un crivellatge senzill (colònies blaves i blanques); que pot ser fàcilment quantificada i controlada, durant el seu procés de producció, mitjançant assatjos enzimàtics; que pot ser purificada de forma ràpida i senzilla amb cromatografia d'afinitat amb substrats no hidrolitzables de la β-

galactosidasa (TPEG); que presenta una estructura tridimensional resolta i, per acabar, que és un enzim portador d'un senyal de localització nuclear (SLN) críptic en cèl·lules eucariotes que podria millorar el rendiment dels processos de transfecció (180;263).

Tot i que la proteïna JX249A es produïa sota els promotors $p_R p_L$ a concentracions suficients, la unió posterior de dominis funcionals podria accentuar la toxicitat i proteòlisi en l'organisme productor. Així doncs per evitar processos de toxicitat en la producció, es va construir el vector de transferència a partir de la proteïna JX249A en plasmidis pTrC99A, basats en promotors induibles per IPTG, en els quals no s'havia vist estimulació del sistema SOS lligada a la sobreexpressió de proteïnes recombinants (vegeu els antecedents del grup i l'annex).

El polipèptid que codifica per JX249A va ser enginyeritzat perquè incorporés una seqüència de deu lisines a la seva posició N terminal. S'havia vist que fusions a l'extrem N terminal de la proteïna pseudosalvatge LACZ milloraven la seva estabilitat (José Luís Corchero, resultats no publicats), possiblement a causa de la falta, en aquest gen, dels vuit primers aminoàcids de la β-galactosidasa. Posteriorment aquests resultats van ser corroborats amb els nostres estudis (taula 1, article III). Les cues de lisines han estat molt utilitzades com a agents d'unió a DNA en conjugats moleculars i s'ha comprovat que poden unir i transferir molècules d'RNA tan eficientment com les de DNA (264). Així doncs, els vectors portadors d'aquests dominis poden ser utilitzats en la tecnologia de l'*RNA interference* (RNAi), que es basa en la inhibició de l'expressió de determinats gens mitjançant la utilització de petites molècules d'RNA anomenades *small interfering RNA* (siRNA) (265). Aquests siRNA són RNA de doble cadena d'una longitud aproximada de 21-23 pb que es troben associats a un nombre elevat de proteïnes, algunes de les quals encara no han estat identificades. Aquests complexos reconeixen el seu mRNA diana (de cadena senzilla) i, després d'un procés d'hibridació, dirigeixen la degradació dels mRNA que es complementen perfectament amb qualsevol de les dues cadenes dels siRNA. Aquest mecanisme permet a la cèl·lula controlar l'expressió de determinats gens i és una aproximació molt prometedora en determinades aplicacions terapèutiques, com ara les antivíriques.

Abans de la construcció d'aquesta nova proteïna quimèrica recombinant, no havíem trobat en la bibliografia cap descripció de vectors de transferència gènica codificats en una sola cadena polipeptídica, que utilitzessin cues de lisines per unir i condensar àcids nucleics. Tot i així, recentment s'han dissenyat altres proteïnes quimèriques que també utilitzen polilisines com a domini d'unió a DNA (171). En alguns vectors recombinants s'han utilitzat dominis basats en proteïnes estructurals nuclears, però en la majoria dels casos el domini d'unió a DNA més emprat ha estat el de l'activador transcripcional GAL4 de *Saccharomyces cerevisae*. Aquest domini presenta desavantatges en comparació amb la polilisina (PLL), ja que només és capaç d'unir eficientment molècules de DNA a través del reconeixement d'una

seqüència específica que ha de ser incorporada en el plasmidi codificant pel transgen. En canvi, el procés d'unió a DNA mediat per la polilisina es basa en unions electrostàtiques entre les càrregues negatives de l'àcid nucleic i les càrregues positives de les lisines i per tant no requereix la introducció de seqüències de reconeixement específic. A més a més, el domini de GAL4 no és capaç de condensar el DNA i necessita polilisines o altres agents de condensació per formar complexos compactes en els quals l'àcid nucleic quedi protegit de les nucleases.

La proteïna que va resultar de la incorporació de la cua de lisines a la JX249A va ser anomenada 249AL, i aquesta es produïa sense mostrar problemes de toxicitat envers la cèl·lula productora. Mitjançant la seqüenciació proteica de la regió N terminal es va demostrar que la cua de lisines es mantenia estable i que la seva incorporació no semblava que hagués produït alteracions importants en l'estructura de la proteïna parental, ja que mostrava valors d'activitat específica, estabilitat a urea i a temperatura i espectres d'emissió del triptòfan semblants als de la proteïna JX249A (taula 1, treball II i treball III). A més a més, la incorporació de la polilisina no altera la unió específica a cèl·lules de mamífer a través de la integrina $\alpha_v\beta_3$ (figura 3A, article II) i aporta capacitat d'unir DNA, tal i com es veu en el seguiment de la seva mobilitat electroforètica en un gel d'agarosa (figura 2, article II). A partir de gels de retard, establíem el valor d'una unitat de retard que correspon a la mínima quantitat de proteïna capaç de retardar completament la mobilitat electroforètica d'un microgram de DNA, en un gel d'agarosa 0,8%. Per la proteïna 249AL, una unitat de retard correspon a uns 12 μg i equival a complexos formats a una relació de 0,08 μg DNA/μg proteïna.

Cal indicar que, segurament, la cua de lisines indueix l'agregació d'una petita part de la proteïna 249AL, ja que s'observava certa terbolesa en els vials de proteïna purificada. Aquest fet concorda amb que la quantitat mínima de 249AL requerida per a la unió cel·lular era lleugerament més elevada que en JX249A, cosa que es podria explicar per una fracció de 249AL menys eficient en la unió cel·lular (figura 3B, article II). Els complexos entre la proteïna i el DNA es formaven en tampó HBS, ja que prèviament s'havia descrit que la incubació de conjugats moleculars amb DNA en tampons a pH fisiològic i sense o amb un baix contingut de fosfats, com el HBS, permetia la formació de complexos de mides regulars de forma controlada i repetitiva (118;131). Tal i com s'havia fet per altres β-galactosidases recombinants, es va fer el seguiment de l'activitat de l'enzim 249AL en cèl·lules preincubades amb la proteïna a 37ºC, i ,com a control negatiu, es va realitzar el mateix experiment a 4ºC, temperatura a la qual hi ha unió a les integrines cel·lulars però no es produeix el procés d'endocitosi. Es va detectar activitat intracel·lular de l'enzim 249AL a 37ºC després de 30 i 120 minuts d'incubació entre les cèl·lules en cultiu i la proteïna recombinant, de manera que es comprovava que l'enzim era estable i enzimàticament actiu durant el seu transport cel·lular (figura 3C, treball II).

L'anàlisi de l'eficiència de transfecció de la proteïna 249AL es va fer utilitzant com a marcador el gen que codifica la proteïna luciferasa (*Firefly luciferase*). S'ha demostrat que la luciferasa és una proteïna útil per realitzar cinètiques d'expressió gènica en cèl·lules transfectades, ja que és força làbil i la seva activitat reflexa l'activitat transcripcional en el temps analitzat (266). En tots els experiments de transfecció, incloíem un control realitzat amb lípids catiònics (LipofectAMINE®). Aquests dirigeixen la transferència de DNA de forma inespecífica però presenten rendiments molt elevats en transfeccions *in vitro.* Això ens permetia tenir un control positiu intern per comprovar el bon funcionament dels diferents estocs del DNA plasmídic que codificava pel gen marcador, i alhora podíem comparar dos procediments de transfecció no vírics amb bases moleculars diferents.

La proteïna 249AL no tan sols és capaç d'entrar a les cèl·lules de mamífer sinó també de promoure la transferència del DNA plasmídic al nucli. Tot i que el treball I ens fa pensar que l'entrada mediada pel motiu RGD dirigeix les proteïnes als endosomes, la proteïna 249AL podria arribar al nucli com un fons inespecífic d'escapament de material endosòmic o bé perquè presentés un domini críptic d'escapament, tot i que fins ara aquest no s'ha descrit.

L'expressió gènica aconseguida amb 249AL és més elevada que la mediada per pèptids RGD sintètitcs units a polilisines ((K)16RGD), els quals han estat descrits com a conjugats moleculars eficients en la transferència no vírica de DNA mediada per receptor (131). Dependent de les relacions vector/DNA utilitzades s'aconseguien expressions entre tres i deu vegades més elevades amb la proteïna 249AL respecte els aconseguits amb el pèptid (K)16RGD (figures 4A i 4B, treball II). És necessari optimitzar la proporció utilitzada entre el vector i el DNA perquè sigui més favorable al procés de transfecció i, en el cas de la proteïna 249AL, es mostra propera a 0,03 μg DNA/μg proteïna, valor que equival a tres unitats de retard (figura 4B, treball II). Està clar, doncs, que el lliurament de DNA per la proteïna quimèrica recombinant és més eficient que el que dirigeixen els pèptids (K)16RGD, tot i que aquests presenten una polilisina de 16 residus que, en principi, podria condensar més eficientment l'àcid nucleic. Aquest fet podria estar determinat per una major protecció del DNA a causa de la massa molecular de la pròpia proteïna que no pas en el cas de pèptids petits i, d'altra banda, per la presència del SLN críptic descrit en l'enzim β-galactosidasa (180;263).

Els màxims nivells d'expressió gènica, dirigits per la proteïna 249AL, s'aconseguien després de 90 minuts de contacte entre els complexos i les cèl·lules CaCo-2 en cultiu, i es mantenien constants fins a temps d'incubació de 16 h (figura 5A, treball II). En canvi, la cinètica de transferència de la LipofectAMINE ® (liposomes formats pel lípid policatiònic DOSPA amb el co-lípid neutre DOPE en una relació de 3:1 en aigua) era més lenta i s'aconseguia el màxim d'expressió després de 5 hores d'incubació amb les cèl·lules, abans d'assolir un *plateau* (figura 5A, treball II). El fet que per aconseguir els màxims nivells d'expressió amb LipofectAMINE® es precisi

d'un temps d'incubació entre els complexos i les cèl·lules més llarg que en el cas de la proteïna 249AL vol dir que els liposomes catiònics tenen un procés d'entrega més llarg que proteïnes quimèriques recombinants del tipus de la 249AL, segurament perquè les integrines promouen la internalització cel·lular d'una forma més ràpida i eficient.

Els valors d'expressió gènica aconseguits amb LipofectAMINE® eren més elevats que no pas amb 249AL, possiblement a causa de l'escapament dels liposomes catiònics dels endosomes ja que en la seva composició presenten DOPE, un lípid fusogènic que promou la lisi endosòmica. Amb un mateix temps d'incubació entre les cèl·lules i els lípids catiònics o amb la proteïna quimèrica recombinant, el màxim d'expressió pels lípids s'assolia més tard, segurament perquè hi ha més quantitat de DNA que arriba al nucli i requereix més temps per ser expressat. Tot i així, el fet que siguin dos mecanismes de transferència de DNA amb una base molecular totalment diferent, pot fer que hi hagin altres punts del procés de transferència gènica responsables de les variacions finals en l'expressió gènica, com ara una arribada més ràpida de la proteïna 249AL al nucli per la presència del SLN de l'enzim β-galactosidasa o bé, perquè tota la quantitat de proteïna que aconsegueix escapar dels endosomes ho fa en els primers moments de l'entrada cel·lular com un fons inespecífic del procés endocític. En canvi, tot i que part de la LipofectAMINE®pugui arribar al citosol dins d'aquest fons d'escapament dels endosomes, una part dels complexos internalitzats és capaç de superar barreres endosòmiques per l'activitat fusogènica del DOPE i potser aquest procés requereix més temps. S'ha postulat que després de l'endocitosi d'agregats d'àcids nucleics amb líposomes catiònics, els lípids catiònics indueixen un moviment de lípids aniònics des de la cara citoplasmàtica de la membrana endosòmica a la cara interna de l'endosoma (moviment *flip-flop*) i que resulta en la desestabilització del complex i l'alliberament dels àcids nucleics (86;267). A causa de que les cèl·lules utilitzades *in vitro* es troben en divisió, el DNA nu alliberat pels lípids catiònics podria arribar al nucli després de la descomposició de la membrana nuclear durant el procés de divisió cel·lular, a diferència del procés mediat per la proteïna recombinant en el que arribaria formant part dels complexos. Així doncs el DNA s'alliberaria dels complexos de la 249AL de forma més progressiva que en el cas de la LipofectAMINE® i en combinació amb la menor quantitat de DNA internalitzada, podria explicar que els valors d'expressió mediats per 249AL siguin més constants, al llarg de les 80 hores post-transfecció analitzades, que en el cas del liposomes catiònics, els quals presenten un pic d'expressió seguit d'una disminució important en els nivells de luciferasa (figura 5B, article II).

IV.A.3.OPTIMITZACIÓ DEL PROCÉS DE TRANSFECCIÓ MEDIAT PER 249AL

La proteïna 249AL és capaç de dirigir l'expressió de gens marcadors en cultius cel·lulars. Amb l'objectiu d'optimitzar aquest procés vam creure convenient estudiar

l'organització molecular d'aquests complexos en diferents relacions i la seva influència en el procés de transferència gènica.

Fins ara, no havíem explorat quina era la influència de les molècules de DNA presents en els complexos proteics respecte la unió i la internalització cel·lular. La incorporació de DNA en els complexos disminueix el potencial d'unió cel·lular, en un patró dependent de dosi, fins a valors de 0,08 μg DNA/μg proteïna (figura 4B, treball III). S'obervava el mateix efecte en els complexos formats amb la proteïna K10LACZ (una β-galactosidasa recombinant portadora de la polilisina però que no presenta el pèptid GH23), indicant que el DNA inhibeix les unions inespecífiques mediades per les interaccions electrostàtiques de la polilisina i els fosfolípids de membrana. A més a més, un ELISA indirecte realitzat amb l'anticòs monoclonal 3E5 anti-RGD va demostrar que la immunoreactivitat d'aquest pèptid es manté invariable a diferents relacions de DNA/proteïna, i per tant podem concloure que la presència de la cua de lisines i del DNA no emmascara els pèptids RGD en la conformació final de la proteïna recombinant dins dels complexos (figura6, treball III).

Molts lipoplexos i poliplexos es preparen a una relació lípid o polímer/DNA lleugerament positiva que propiciï les unions a membranes cel·lulars carregades negativament i faciliti la seva internalització. Cal, però, trobar un òptim en aquesta raó, ja que un excés de càrregues positives pot estimular la unió amb molts components del sèrum quan es realitzi una administració intravenosa del vector i estimular el sistema del complement (110). A banda, cal tenir en compte que un excés de càrregues positives pot apantallar l'acció de lligands específics presents en el vector i que, tot i que polilisines llargues condensen més eficientment el DNA, també poden accentuar la insolubilitat i inestabilitat dels complexos. De fet, s'ha descrit que polilisines d'uns vuit aminoàcids són adequades per mantenir processos d'endocitosi mediada per receptor en un context de conjugats moleculars basats en pèptids sintètics (119;120).

Nosaltres vam utilitzar una cua de 10 lisines tot i que desconeixíem la seva funcionalitat en el context d'una proteïna quimèrica d'elevat pes molecular i en el que l'estabilitat de la proteïna podria afectar a l'exposició d'aquest domini d'interacció amb àcids nucleics, tal i com després han suggerit resultats obtinguts en el treball V. En aquests resultats s'observa que complexos de la proteïna K10LACZ-DNA són més insolubles que els complexos formats amb proteïnes quimèriques portadores del pèptid GH23 (figura 5, treball V). Aquesta proteïna sembla ser més estable que les altres construccions, tal i com indiquen els valors de la seva activitat específica i de la seva termoestabilitat (taula 1,treball III), de manera que la PLL podria ser més activa i condensar més eficientment el DNA, accentuant la insolubilitat dels complexos.

El nombre de lisines utilitzat en aquest clonatge ens permet trobar una relació òptima perquè hi hagi condensació de DNA, es mantinguin les unions cel·lulars específiques i els complexos no presentin problemes d'insolubilitat. Realitzant un assaig d'unió cel·lular de competició dels complexos DNA-249AL amb pèptids RGD sintètics i comparant-lo amb la unió total, s'obtenia una mesura del component específic involucrat en el procés d'unió (figura 5A, treball III). Es va veure que, a 0,04 μg DNA/μg proteïna, un excés molar del pèptid competitiu ja no produïa cap efecte en la unió cel·lular d'aquests vehicles, cosa que suggeria que en aquestes relacions de DNA/proteïna la unió no és produeix a través de la integrina $\alpha_v\beta_3$, sinó que està mediada pel component inespecífic. Cal destacar que l'eficiència òptima de transferència de DNA en 249AL es troba en el rang de 0,02 i 0,03 μg DNA/μg proteïna, en el que l'especificitat d'unió es màxima (figura 5B, treball III).

Mitjançant microscòpia electrònica de transmissió (MET), vam veure que la proteïna 249AL en solució forma espontàniament agregats elipsoides de mida regular, amb un diàmetre entre 20 i 40 nm (figura 3, treball III). D'acord amb les dimensions moleculars de l'enzim en la seva forma tetramèrica, sembla que podrien estar formats per dotze molècules actives ordenades espaialment. Aquestes partícules s'associen en pseudofilaments de fins a 200 nm de longitud, seguint un patró dependent de la concentració de proteïna. Ja que la proteïna JX249A no presenta cap tendència a l'agregació (resultats no mostrats), sembla que la cua de polilisines és el principal inductor de la formació d'aquestes partícules. Les molècules de DNA són condensades eficientment per la proteïna, ja que no se n'observa cap en les preparacions de microscòpia de transmissió fetes per a la construcció 249AL, però sí que n'hi ha quan estan incubades amb la proteïna LACZ. Sembla que les molècules de DNA s'acomoden en les cavitats d'aquests agregats i interaccionen amb les regions catiòniques sense modificar la conformació i l'organització molecular de la proteïna recombinant (figura 3, treball III); tan sols s'observa un lleu increment de la interacció interpartícules, especialment a concentracions elevades de proteïna. La formació de complexos DNA-proteïna, fins i tot a relacions elevades, no té cap efecte sobre l'activitat enzimàtica (figura 2, treball III), cosa que indica que la conformació del monòmer, els contactes monòmer-monòmer i la difusió i el processament del substrat no es veuen influenciats per la condensació del DNA, tot i la proximitat del domini d'unió a DNA i el lloc actiu (199) (figura 1, treball III).

Sembla que aquest acomodament confortable del DNA és un procés diferent del recobriment del DNA pels vectors basats en histones (199;268), en els que s'observen estructures *rod-like* o bé de la condensació progressiva, dependent de concentració, de vectors basats en petits pèptids conjugats a PLL (131;199). S'ha descrit que complexos formats amb PLL adquireixen morfologies toroïdals, i tenen una mida aproximada de 80-100 nm (118). Aquest volum reduit pot ser beneficiós dependent de la via d'entrada a la cèl·lula, ja que permet que complexos com els de transferrina-PLL puguin ser incorporats per vesícules recobertes de clatrina en les que hi ha una limitació de grandària entre els 100 i 200 nm (118;131;269). En altres

casos però, com en el cas de pèptids RGD conjugats a PLL, les estructures toroïdals no corresponen a la forma més activa que poden adoptar els complexos. La relació òptima entre el pèptid (K)16RGD i les molècules de DNA dóna lloc a complexos més condensats i amb tendència a agregar entre ells. Aquests són més semblants a les morfologies que nosaltres observem a la concentració òptima de la proteïna 249AL (0,03 μg DNA/μg proteïna). Segurament en aquest punt, el DNA es troba més protegit de les nucleases i encara es capaç de dissociar-se del vector un cop arriba al nucli. Tal i com ha estat descrit pels pèptids K16RGD (131), a relacions molt baixes de DNA/proteïna en les que el plasmidi està més condensat, el procés de dissociació dels complexos a dins la cèl·lula podria ser menys eficient, correlacionant-se amb una disminució de l'expressió gènica (figura 5, article III). Tot i que la internalització mediada per integrines permet l'entrada de partícules de la mida de microorganismes (256;270) i per tant no hi ha una limitació en la grandària dels complexos, la mida dels agregats formats per la proteïna 249AL no seria un punt limitant per ser incorporats per altres vies endocítiques, en cas que substituíssim el lligand de reconeixement cel·lular per dirigir-lo a noves aplicacions.

Sembla que l'elevada massa molecular de l'enzim β-galactosidasa i també la interacció intermolecular que presenta la proteïna 249AL ofereix cavitats adequades per a l'acomodació eficient del DNA i mantenen un potencial adequat de transfecció cel·lular a unes relacions de 0,02-0,03 μg DNA/μg proteïna. Quantitats més elevades de DNA en els complexos sembla que tampoc no són adequades, possiblement perquè les polilisines són insuficients per apantallar les càrregues negatives del DNA i hi ha una disminució considerable de la unió cel·lular i dels nivells d'expressió gènica (figures 4B i 5, treball III).

IV.A.4.ESTUDI *IN VIVO* DEL VECTOR 249AL:APLICACIÓ AL SNC

Experiments *in vivo* en el sistema nerviós central (SNC) demostren que la proteïna 249AL és un prototip de vector alternatiu a l'ús de vectors vírics en la teràpia gènica del SNC. Es va realitzar la injecció intracortical, en cervells no lesionats i en cervells lesionats, de 249AL acomplexada amb plasmidis portadors del gen de la GFP. Es va creure que la GFP era el gen marcador més adequat perquè permetia observar les seccions directament al microscopi i corroborar els resultats de les proves immunohistoquímiques, i, a més a més, perquè la seva elevada estabilitat i la seva resistència eren adequades per a la manipulació que s'havia de fer de les mostres en alguns casos, com ara en l'obtenció de llisats per western blot. L'eficiència de transfecció observada era probablement deguda a la internalització cel·lular dels complexos proteïna-DNA, mediada per interaccions entre el motiu RGD i les integrines α_v que s'expressen en el cervell (271-274). La injecció en cervells no lesionats donava lloc a una expressió gènica localitzada en el neocòrtex ipsilateral i restringida a la zona de la injecció i al *corpus callosum* subjacent, a un dia post-injecció (figures 1C i D, treball IV). Als 7 dies postinjecció, es trobava proteïna GFP

en neurones del còrtex injectat (figures 1E i F, treball IV) i en escasses neurones del còrtex contralateral. Hi havia, però, un increment de l'expressió transgènica quan s'injectaven complexos de DNA-249AL en cervells lesionats excitotòxicament i això podria ser degut a l'augment en l'expressió de gens de les integrines $\alpha_v\beta_3$ estimulat per la lesió, fet que s'ha descrit en astròcits hipertròfics, microglia i microvesícules després d'isquèmia (272;273;275), en Alzheimer (271) o bé en encefalomielitis autoimmune experimental (276). La proteïna 249AL era capaç de transfectar cèl·lules de tota l'àrea lesionada al cap de 24 hores (figures 2A i 2B, treball IV) sense transfectar la gran part del parènquima normal. Aquests resultats indiquen que la proteïna 249AL és un vector adequat per transferir DNA principalment a zones que han estat lesionades. Tot i que el cervell és un teixit immunoprevilegiat, la injecció de diferents vectors vírics s'ha vist lligada a processos inflamatoris inacceptables (277), activació immunològica (278) i desmielinització (279;280). La injecció intracerebral de vectors adenovírics indueix el increment en IL-1β (281), seguit d'una activació de la microglia/macròfags i de l'astroglia (278), un augment de l'expressió del complex major d'histocompatibilitat de classe I (278) i un increment dels anticossos antiadenovirus en sèrum durant les primeres 48 hores (282). A més a més, hi ha una acumulació en el parènquima neural de limfòcits CD8+ i CD4+ activats a la primera setmana (283). Sis dies després de la injecció de 249AL, no s'observa cap d'aquests canvis histopatològics, cosa que suggereix que no hi ha resposta inflamatòria ni cap activació del sistema immunològic (figura 5, treball IV).

Alguns virus són internalitzats per terminals axonals i són transportats retrògradament al soma neuronal mitjançant interaccions de motius RGD amb integrines α_v (251). En els resultats d'aquest treball (figures 2D-G, treball IV), es veu expressió transgènica en alguns somes neuronals del còrtex contralateral i en alguns nuclis talàmics, els quals és sabut que tenen projeccions corticals aferents i eferents (284). Aquests resultats suggereixen que la presència del pèptid víric GH23, en la proteïna 249AL, podria permetre que aquest vector recombinant mantingués la capacitat de transport retrògrad d'alguns virus, la qual cosa explicaria la transfecció d'aquests somes neuronals llunyans. Aquesta capacitat podria ser utilitzada per la transferència gènica selectiva d'una població distant de neurones mitjançant rutes d'administració no invasives.

S'ha comprovat, doncs, que aquest vector té potencial de transfectar neurones, astròcits, microglia i endoteli mitjançant la utilització de motius basats en RGD, i que en cap cas es troba associat a processos de toxicitat. Així doncs, aquest estudi demostra l'ús potencial d'aquest vector en el SNC i que gràcies a la seva base modular, podria utilitzar-se en aplicacions dirigides a tipus cel·lulars específics tan sols canviant el domini d'unió a cèl·lules per altres més adequats i utilitzant promotors específics.

IV.A.5.MILLORA DE 249AL

Un cop optimitzats els procediments d'ús *in vitro* de la proteïna 249AL i veient que aquesta oferia un potencial considerable en aplicacions *in vivo*, vam centrar el treball en la millora dels dominis funcionals presents en el vector.

A causa de que *in vivo* la majoria de tipus cel·lulars no es troben en divisió activa, el transport nuclear esdevé el punt crític d'un procés de transferència gènica. Un dels punts que volíem explorar era la millora del transport nuclear portat a terme per la proteïna 249AL, ja que el SLN present en aquest enzim bacterià és moderadament eficient comparat per exemple amb senyals de localització nuclear vírics (180;263). Així doncs, vam estudiar si la incorporació del SLN de l'antigen T de SV40, un dels SLN més ben descrits i testats fins ara, podia incrementar l'eficiència d'aquest procés. Kalderon i colaboradors van descriure que el segment comprés entre els aminoàcids 126-132 del antigen T de SV40 constituia la mínima seqüència necessària perquè actués com a senyal de localització nuclear (180). Tot i així, la possibilitat que aminoàcids com la Prolina 135 tinguessin un paper cooperatiu no estava del tot descartada i per aquesta raó vam utilitzar el segment ^{126}PKKKRKVEDP^{135}.

La posició del SLN en la proteïna quimèrica podria tenir una rellevància especial per a la seva activitat, ja que s'ha demostrat que l'entorn molecular de les insercions és crític per a altres activitats biològiques de pèptids inserits, com ara la unió cel·lular (treball I) i la reactivitat enfront d'anticossos (201). A més a més, hi ha treballs previs en els que la incorporació d'aquest mateix SLN no ha donat bons resultats. Per exemple, el SLN del Ag-T del SV40 ha estat acoblat a PLL per procediments convencionals de *cross-linking* i després d'acomplexar-se amb plasmidis DNA era incapaç de reconéixer les importines α/β. El rendiment millorava considerablement quan aquesta era fusionada prèviament amb un pèptid (185). Dins del context de les proteïnes quimèriques recombinants es va comprovar que la fusió d'aquest SLN a vectors de condensació de DNA basats en histones no produia cap increment de l'eficiència de transport nuclear (153).

En el treball V describim la inserció del segment 126-135 del antígen-T de SV40 en dos llocs que es troben exposats al solvent en l'enzim β-galactosidasa d'*Escherichia coli* (200) però que estructuralment són molt diferents: un lloc d'inserció intern, entre els aminoàcids 279 i 280, en l'*activating interfase* de l'enzim (199), i en l'extrem carboxi terminal sobresurtint de la *long interfase* (199). Les dues proteïnes resultants, NLS278 i NLSCT respectivament, presentaven rendiments de producció i purificació per cromatografia tan bons com els de la proteïna parental 249AL i no mostraven símptomes de toxicitat en cultius d'*E.coli*. La incorporació del SLN no alterava la capacitat d'unió a les integrines cel·lulars ni el patró d'unió i condensació de DNA, ja que, en els tres casos, 11-12 μg de proteïna eren capaços de retardar completament la mobilitat de les molècules plasmídiques en un gel d'agarosa (figura2, treball V). Tampoc es van observar, mitjançant MET, patrons

d'organització molecular marcadament diferents dels que havien estat descrits per la proteïna 249AL (figura 3, treball III i V). El càlcul de les activitats específiques (taula 1, treball V) indica que la incorporació del SLN en l'*activating interfase* produeix un impacte important en l'activitat enzimàtica de la β-galactosidasa (taula1, treball V). Sembla que aquest efecte no estaria restringit tan sols a una pertorbació del lloc actiu i a la capacitat d'incorporar i processar el substrat sinó que també alteraria l'estabilitat global de la proteïna ja que l'acomodació de les molècules de DNA en els complexos de la proteïna NLS278 té un major efecte en la conformació de la proteïna, tal com indica la dispersió de l'espectre del triptòfan a diferents relacions de DNA-proteïna (taula 1, treball V). Tanmateix, aquesta idea és corroborada per la tendència a la insolubilitat dels complexos NLS278-DNA, principalment a raons de 0,08 µg DNA/µg proteïna, en comparació amb les altres proteïnes 249AL i NLSCt que mantenen patrons de solubilitat similars (figura 5, treball V).

La proteïna NLSCt, que presentava el SLN a l'extrem C terminal, dirigia nivells d'expressió gènica de dues a trenta vegades més elevats que en el cas de la proteïna 249AL, dependent de la relació de DNA/proteïna utilitzada en la formació dels complexos (figura 4, treball V). L'increment de l'expressió gènica era més accentuat a una raó de 0,03 µg DNA/µg proteïna, relació adequada per mantenir una transferència gènica mediada per receptor eficient (figura 5, treball III). Aquesta millora de la funcionalitat del vector és independent de qualsevol modificació en la capacitat d'unió i condensació del DNA i de les propietats d'unió cel·lulars, ja que tant ambdues construccions com la parental eren capaces d'unir el DNA i les cèl·lules amb la mateixa eficiència (figura 2, treball V). Això indica que el SLN de SV40 és funcional un cop presentat en la proteïna recombinant i que preval per sobre del fons de localització nuclear presentat per l'enzim β-galactosidasa. A més a més, a causa de que els senyals de localització nuclear són rics en residus carregats positivament com les lisines, es podria establir una competició per la unió al DNA que després tingui conseqüències en el reconeixement de les importines, però en aquest cas tant la cua de lisines com el SLN mantenen intacte la seva funcionalitat. En canvi, la proteïna NLS278 no mediava nivells d'expressió gènica superiors als aconseguits amb el SLN críptic de la β-galactosidasa, cosa que indica que el lloc d'inserció d'un domini funcional és crític per obtenir un bon funcionament del vector. Tot i que prèviament s'havia demostrat que pèptids d'unió cel·lular presentats en aquest punt eren més eficients que en l'extrem C terminal (285), en aquest cas el SLN de SV40 podria quedar poc exposat per poder interaccionar amb les importines β1/α i travessar el NPC. Aquest fet no depèn exclusivament de si la presentació del SLN és interna o bé és una fusió terminal, ja que s'ha descrit la presentació de SLN entre els aminoàcids 375 i 376 de la β-galactosidasa, que ha demostrat ser completament actiu (286). No descartem però, que la pèrdua de l'estabilitat estructural de la NLS278 pugui afectar alguns punts de la transferència gènica, a part dels processos d'unió a DNA i a cèl·lules, com ara el travessament

mateix del NPC, la dissociació final de les molècules de DNA de la proteïna o la major susceptibilitat a la proteòlisi, com han demostrat altres β-galactosidases amb insercions en l'*activating interfase* en contextes de cèl·lules bacterianes (287). El fet que el SLN incrementi l'eficiència de transport nuclear ens indica que el procés de dissociació es produeix a l'interior del nucli, segurament per intercanvi amb el DNA genòmic (85).

Els resultats obtinguts fins ara ratifiquen el potencial de la proteïna 249AL i dels seus derivats com a vectors de transferència gènica. Fins ara, els nivells d'expressió aconseguits són baixos comparat amb els que s'obtenen amb vectors vírics, però són suficients per mediar efectes terapèutics en contextes en els que elevats nivells d'expressió *per se* no són importants, com el desencadenament d'una resposta immunològica en teràpia anticancerígena o bé l'expressió de factors de creixement o de CFTR en l'epiteli pulmonar. Futurs estudis en la millora del vector, anirien dirigits a la incorporació d'activitats d'escapament d'endosomes dels complexos DNA-proteïna. La utilització d'agents lisosomotròpics que inhibeixen l'acció lisosomal com la cloroquina són inadequats per aplicacions *in vivo* i tenen un efecte limitat a causa de les seves propietats citotòxiques. La incubació dels complexos amb una certa dosi de lípids fusogènics com DOPE permetria incorporar activitats d'escapament d'endosomes amb facilitat, però amb el risc que s'incrementin les interaccions indesitjades amb proteïnes del sèrum en administracions intravenoses (90),(288) de manera que provocaria la desestabilització del complex i la conseqüent pèrdua de l'activitat fusogènica. Així doncs, podria ser més adequat la incubació amb polímers de PEI que també presenten mecanismes de desestabilització endosòmica (99;289) gràcies a la seva capacitat de tamponació, que disminueix el nivell d'acidificació en els endosomes i permet l'alliberament del DNA al citoplasma. La interacció posterior de la proteïna amb polímers o lípids podria modificar les propietats funcionals dels dominis heteròlegs de manera que caldria optimitzar el procés o bé plantejar-se la incorporació d'un pèptid fusogènic, com la seqüència amino-terminal de la subunitat HA2 de la hemaglutinina del virus de la grip (97).

En resum, aquest treball demostra que la proteïna 249AL i els seus derivats, com la proteïna NLSCt, són vehícles atractius per portar a terme processos de transferència dirigida de DNA. La seva naturalesa modular i els seus processos de producció i purificació fàcils, ràpids i totalment adapatables a procediments a gran escala els hi confereixen un potencial prou elevat perquè siguin una bona alternativa al ús dels vectors vírics en teràpia gènica. És necessari, però, aprofundir en l'estudi d'aquesta nova generació de vectors per optimitzar el seu rendiment, i, en aquest sentit, hem confirmat que l'enzim β-galactosidasa és extremadament útil i flexible com a proteïna portadora, per explorar la combinació de diferents dominis funcionals en la generació de nous vectors recombinants per a la transferència gènica.

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V.CONCLUSIONS

1. El pèptid víric GH23 presentat en proteïnes β-galactosidases quimèriques confereix propietats de reconeixement i internalització cel·lular específiques, alhora que es manté l'activitat dels enzims presentadors, tant adsorbits en superfície com en l'espai intracel·lular.

2. La proteïna AB1275VP1 és internalitzada per cèl·lules en cultiu a través de motius RGD i es localitza principalment en vesícules endosòmiques.

3. L'exposició del segment GH23 entre els residus 275 i 276 de l'*activating interfase* de la β-galactosidasa reprodueix molt millor les propietats d'unió del VFA que en l'extrem N terminal, fet que confirma la importància de la presentació del motiu RGD en un llaç tancat.

4. Les proteïnes 249AL, NLSCt, NLS278 i la proteïna control K10LACZ són solubles, es produeixen eficientment sense mostrar símptomes de toxicitat per *Escherichia coli* com a organisme productor i són fàcilment purificables per cromatografia d'afinitat amb substrat no hidrolitzable de β-galactosidasa.

5. La cua de lisines insertada en l'extrem N terminal de la β-galactosidasa es manté estable i permet que l'enzim 249AL uneixi i condensi DNA plasmídic eficientment. La seva incorporació no produeix alteracions importants en l'estructura de la proteïna parental JX249A i conserva el reconeixement específic de la integrina $\alpha_v\beta_3$, principal receptor de la vitronectina.

6. La inserció de la polilisina a l'extrem N terminal de l'enzim pseudo-salvatge LACZ millora la seva estabilitat tèrmica (t50), probablement perquè compensa la manca dels primers 8 aminoàcids de la β-galactosidasa, mentre que la inserció del pèptid GH23 entre els residus 249 i 250, la disminueix considerablement.

7. La proteïna 249AL dirigeix la transferència de DNA plasmídic a cèl·lules de mamífer assolint-se nivells d'expressió gènica que poden arribar a ser deu vegades superiors als aconseguits amb pèptids sintètics basats en motius RGD. Aquest fet podria estar determinat per una major protecció de l'àcid nucleic, per la gran massa molecular de la proteïna i per la presència del SLN críptic en l'enzim β-galactosidasa.

8. L'òptim d'expressió gènica mediada per 249AL s'assoleix entre 0,02 i 0,03 μg DNA/μg proteïna, rang en el que la unió específica a les integrines cel·lulars és màxima.

9. La cinètica d'internalització dels complexos de DNA amb la proteïna 249AL és més curta que amb liposomes catiònics (LipofectAMINE®), probablement perquè les integrines promouen la internalització cel·lular de forma ràpida i eficient.

10. La màxima expressió gènica aconseguida amb el vector 249AL s'assoleix en un temps més curt i es manté de forma més constant que en el cas de la LipofectAMINE® , tot i que aquesta dirigeix nivells d'expressió més elevats. La presència en els liposomes del lípid fusogènic DOPE, el SLN descrit per la β-galactosidasa i el mecanisme de dissociació dels dos tipus de complexos podrien explicar les diferències observades en el procés de transferència gènica.

11. La incorporació de DNA en complexos de 249AL i de K10LACZ disminueix el potencial d'unió cel·lular en un patró dependent de dosi fins a valors de 0,08 μg DNA/μg proteïna, la qual cosa indica que l'àcid nucleic inhibeix les unions inespecífiques mediades per les interaccions electrostàtiques de la polilisina amb els fosfolípids de la membrana cel·lular.

12. La incorporació del SLN del Ag-T de SV40 en la posició C terminal i entre els residus 279 i 280, en l'*activating interfase*, no modifica el patró d'unió cel·lular ni la morfologia dels agregats observada per MET. Les proteïnes NLSCt i NLS278 uneixen i condensen DNA amb eficiències similars a la proteïna parental 249AL, establint-se el valor corresponent a una unitat de retard com a 11-12 μg proteïna/μg DNA. Els complexos amb K10LACZ mostren patrons d'insolubilitat més accentuats de manera que suggereixen que la polilisina podria quedar més exposada i condensar més eficientment el DNA.

13. La proteïna 249AL en solució forma espontàniament agregats elipsoides de tamany regular amb un diàmetre entre 20 i 40 nm, que s'associen en pseudofilaments de fins a 200 nm de longitud, en un patró dependent de concentració de proteïna.

14. Les molècules de DNA interaccionen amb les cues de lisines acomodant-se en les cavitats dels agregats proteics sense modificar la seva morfologia ni la immunoreactivitat del pèptid RGD i sense produir cap efecte en l'activitat enzimàtica, tot i la proximitat del domini d'unió a DNA i el centre actiu de l'enzim.

15. La proteïna 249AL és un prototip de vector alternatiu al ús de vectors vírics en el SNC, especialment en aplicacions terapèutiques a zones lesionades en les que es produeix un increment de l'expressió dels gens de les integrines $\alpha_v\beta_3$. És capaç de dirigir la transfecció de neurones, astrocits, microglia i endoteli sense induir processos inflamatoris ni estimular el sistema immunològic, com a mínim, en els 6 primers dies postinjecció, tal i com s'ha observat en alguns sistemes de transferència vírics.

16. El pèptid GH23 presentat en la proteïna recombinant podria ser el responsable que el vector 249AL retingui les capacitats víriques de transport retrògrad i adquireixi el potencial de transfectar una població distant de neurones mitjançant rutes d'administració no invasives.

17. La incorporació del SLN del AgT de SV40 en l'*activating interfase* de la β-galactosidasa, produeix un impacte important en l'activitat enzimàtica i l'acomodació de les molècules de DNA té un major efecte en la conformació de la proteïna NLS278, com indica la dispersió de l'espectre de triptòfan i la insolubilitat més accentuada dels seus complexos.

18. La proteïna NLSCt dirigeix nivells d'expressió gènica fins a trenta vegades més elevats que els aconseguits amb la 249AL en la relació de 0,03 μg DNA/μg proteïna, fet que indica que el SLN del Ag-T és funcional en l'extrem C terminal de la proteïna 249AL i prevaleix sobre el fons de localització nuclear presentat per l'enzim β-galactosidasa. Aquest fet ens confirma que el procés de dissociació dels complexos entre el DNA i les proteïnes quimèriques es realitza en el nucli.

19. El lloc d'inserció del SLN del AgT de SV40 és crític per obtenir una bona funcionalitat ja que en la proteïna NLS278 no produeix cap increment de la transferència gènica. Tot i la possibilitat que, en aquest punt d'inserció, el senyal de localització no adopti la conformació adequada per interaccionar amb les importines β1/α, no podem descartar que la pèrdua de l'estabilitat estructural de la proteïna NLS278 pugui afectar alguns punts de la transferència gènica, que no siguin la interacció amb el DNA ni la unió cel·lular.

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VI.ANNEX

The Expression of Recombinant Genes from Bacteriophage Lambda Strong Promoters Triggers the SOS Response in *Escherichia coli*

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Abstract: The production of several non-related heterologous proteins in recombinant *Escherichia coli* cells promotes a significant transcription of *recA* and *sfiA* SOS DNA repair genes. The activation of the SOS system occurs when the expression of plasmid-encoded genes is directed by the strong lambda lytic promoters, but not by IPTG-controlled promoters either at 37 or at 42°C, and it is linked to an extensive degradation of the proteins after their synthesis. The triggering signal for the SOS response could be an important arrest of cell DNA replication observed within the first hour after the induction of recombinant gene expression. The stimulation of this DNA repair system can partially account for the toxicity exhibited by recombinant proteins on actively producing *E. coli* cells. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **60**: 551–559, 1998.

Keywords: *Escherichia coli;* SOS; DNA repair; recombinant proteins; promoter; proteolysis

INTRODUCTION

The bacterium *Escherichia coli* is the most widely used biological system for the expression of recombinant genes and the analysis of their products. However, the overproduction of many heterologous proteins causes an important metabolic burden, and the products themselves often exhibit toxicity over the producing cells (Bentley et al., 1990; Dong et al., 1995; Glick, 1995; Marston, 1986). These events result in poor growth and product yield, death of recombinant cells, and an increase of plasmid-free cells during prolonged bacterial culture. Despite the fact that the genetics and physiology of *E. coli* are well characterized, the nature of the cell damage caused by heterologous proteins is far from being understood. In some cases, the toxicity can be attributed to the natural enzymatic activities of the recombinant proteins (Bedouelle et al., 1990; Laity et al., 1993; Pham and Coleman, 1985). For other products, the presence of particular amino acid sequences can be responsible for their toxicity (Viaplana and Villaverde, 1996; Yike et al., 1996), and their removal renders non-toxic products and increases the yield (Sheu and Lo, 1995; Sisk et al., 1992; Vidal et al., 1991). Toxicity on harboring cells has been also associated with the proteolytic degradation of the encoded products (Viaplana et al., 1997), although the responsible mechanisms remain unexplored. Altogether, these data suggest that different features of recombinant proteins can disturb the cell physiology at different levels, and that there is not a general mechanism responsible for toxicity on producing cells. Along this line, diverse cellular activities have usually been reported upon overproduction of recombinant proteins, such as the heat shock (Allen et al., 1992; Goff and Goldberg, 1985; Rinas, 1996; Wild et al., 1993) and stringent responses (Anderson et al., 1996).

One of the more intriguing cellular stress responses in bacteria is the activation of the SOS DNA repair system, which is induced by DNA damage or by the arrest of intact DNA replication (Walker, 1984). The key event in the SOS response is the activation of the RecA coprotease, that promotes the self-digestion of LexA and lambda CI repressors (Kim and Little, 1993). More than 20 genes, whose transcription is controlled by the repressor LexA, are then transiently expressed. The SOS proteins cooperate in repairing the DNA ,and also in preventing cell division until DNA replication is restored. In a previous work, we have shown that a recombinant RNA polymerase from foot-and-mouth disease virus (FMDV) triggers a strong SOS response in carrier cells by promoting a dramatic and immediate arrest of the cell DNA synthesis, that could be attributed to the enzymatic activities displayed by this protein (Benito et al., 1995a). In the present study, and by monitoring the expression of the more responsive *sfiA* (*sulA*) and *recA* SOS genes, we have shown that the production of other non-related recombinant proteins is also able to stimulate a significant SOS response. This fact is not dependent on any enzymatic

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activity of the encoded protein, and it has only been observed when the recombinant gene expression is directed by the strong lambda lytic promoters. Although the exact nature of the inducing events remains unclear, the observed SOS response could be connected to the proteolytic processing of recombinant proteins, which are produced at very high rates.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *E. coli* strain MC1061 *hsdR2, mcrB, araD139,* Δ(*ara-ABC-leu*)*7679,* Δ*lacX74, galU, galK, rpsL, thi, strA* (Studier and Moffat, 1986) was commonly used during the construction of recombinant vectors as well as for their maintenance. To monitor the extent of SOS induction under different situations, we transformed the desired plasmids into the *E. coli* strains GC4581, F^- *supE44 thr1 leu6 proA2 argE3 thi1 lacY1 galK2 ara14 xyl15 mtl1 tsx33 rpo122 rpo122 rpsL* [λd (*sfiA::lacZ*) CI *ind1*] $RecA^+$ Ap^s (Huisman and D'Ari, 1983), and IC2015, *top*$^+$ *uvrA6 thr leu thi pro argE3 his-4 sup-37 strA* (λGE272 *recA′*::*lacZ*$^+$) $RecA^+$ Ap^s (Urios et al., 1990; Weisemann et al., 1984). Plasmids carrying lambda promoters are derivatives of pJLA602 (Schauder et al., 1987), a multicopy expression vector that harbors an ampicillin resistance gene and the CI857 repressor gene under the control of its own promoter. pCI857 is a helper construct formerly used in combination with p_L-based expression vectors to provide the CI857 repressor to strains devoid of the encoding gene (Remaut et al., 1983). This vector also confers kanamycin resistance, allowing a simultaneous maintenance with ampicillin-resistant vectors.

Culture and Induction Conditions

Unless otherwise indicated, Luria-Bertani (LB) medium (Sambrook et al., 1989) with 20 μg/mL streptomycin and 100 μg/mL ampicillin was used for all the experiments. When required, kanamycin was added to 50 μg/mL. Overnight cultures were used to inoculate fresh medium at a dilution 1:50, in 100 mL-shaker flasks. The usual working volume was 20 mL. The new cultures were incubated at 250 rpm, and unless otherwise indicated, at either 37°C or 28°C for IPTG- and temperature-inducible systems, respectively. When the optical density at 550 nm (OD_{550}) reached between 0.2 and 0.3 units, the recombinant gene expression was induced, either by a temperature shift to 42°C or by the addition of different amounts of IPTG. In positive-control cultures, the genotoxic drug mitomycin C (MC) was also added at this moment to a final concentration of 1.6 μg/mL.

Analysis of Recombinant Proteins and β-Galactosidase Activity

Recombinant proteins were detected by Western blot of crude cell extracts. Samples of 1 mL were taken from the cultures, centrifuged, and the cells resuspended in 100 μL denaturing buffer (Laemmli, 1970) and boiled for 5 min. Then, appropriate volumes were loaded onto SDS-polyacrylamide gels to analyze the same biomass. After the electrophoresis and blotting, immunoreactive bands were visualized with appropriate rabbit sera, and their amount determined by densitometric analysis of membranes as previously described (Benito et al., 1995b). β-Galactosidase activity was assayed by standard procedures (Miller, 1972) in culture samples permeabilized with chloroform.

Measurement of Cell DNA Synthesis

Cultures carrying the desired plasmids were growing in minimal CAM9 medium (Maniatis et al., 1982) plus 20 μg/mL streptomycin, 100 μg/mL ampicillin and 2 g/L glucose at 28°C until the early exponential phase. Then, cultures were submitted to temperature shift or treated with 1.6 μg/mL MC. DNA synthesis was measured at different times before and after induction of recombinant gene expression, by 2-min pulse-labeling of 0.5 mL samples with [^{3}H]-thymidine (1 μCi; specific activity 85 Ci/m*M*) as described (Benito et al., 1995a).

RESULTS

Induction of *sfiA* Transcription by Expression of Recombinant Genes

In a previous work (Benito et al., 1995a), we have used the tightly repressed *cea* promoter to detect SOS expression stimulated by a recombinant FMDV RNA polymerase (the VIA antigen). The production of other recombinant proteins only rendered basal levels of the LexA-controlled gene expression in the same system (Benito et al., 1995a). However, in further analysis using the more responsive *sfiA* SOS promoter (Peterson and Mount, 1987; Schnarr et al., 1991), we also observed significant levels of *sfiA* transcription in cells expressing other non-related proteins (not shown). To explore this fact in more detail, we transformed the strain GC4581, carrying a *sfiA::lacZ* gene fusion, with a set of different plasmid constructs encoding heterologous proteins and also with parental plasmids and unrelated cloning vectors. The presence in the cell of either cloning and expression vectors such as pBR322, pET22b(+), pTrc99A, pJLA602 and pUC18, did not promote detectable transcription of *sfiA* gene, even when the culture was submitted to conditions allowing derepression of the promoters transported by some of these plasmids (Table I). As expected, the addition of the genotoxic drug MC induces a high transcription of *sfiA* in all the cell clones. In cells harboring foreign genes downstream IPTG-inducible promoters, SOS induction was not detected under both inducing and non-inducing conditions (Table II). Surprisingly, when cells carried CI857-based expression vectors, SOS induction was evident upon gene expression. The expression of all the tested genes

Table I. *sfiA* gene expression in *E. coli* GC4581 cells carrying either cloning or expression vectors.

Vector	Promoter	Inducing agent[a]	Induction conditions	MC[b]	Growth temperature	*c1857* gene[c]	U/mlOD[d]	References
none	none	none	–	–	28°C	–	64	–
			–	–	42°C	–	65	
pBR322	none	none	–	–	28°C	–	73	Bolivar et al., 1977
			–	–	42°C	–	22	
			–	+	42°C	–	3025	
pET22b(+)	p_{T7}	IPTG	–	–	37°C	–	64	Viaplana and Villaverde, 1996
			0.5 m*M*	–	37°C	–	64	
			1 m*M*	–	37°C	–	57	
			–	+	37°C	–	3454	
pTrc99A	p_{Trc}	IPTG	–	–	37°C	–	74	Amann et al., 1988
			0.5 m*M*	–	37°C	–	77	
			1 m*M*	–	37°C	–	83	
			–	+	37°C	–	3297	
pUC18	p_{Lac}	IPTG	–	–	37°C	–	81	Messing, 1983
			0.5 m*M*	–	37°C	–	80	
			1 m*M*	–	37°C	–	84	
			–	+	37°C	–	2995	
pJLA602	$p_L\, p_R$	temperature	–	–	28°C	+	62	Schauder et al., 1987
			–	–	42°C	+	56	
			–	+	28°C	+	2007	
			–	+	42°C	+	5740	

[a]Note that despite inducing agents added, no foreign genes were present downstream the promoters.
[b]MC was added simoultaneously to either IPTG and/or temperature shift.
[c]Indicates the presence (+) or absence (−) of the *cI857* gene in the recombinant cells.
[d]β-galactosidase activity was measured 2.5 h after the treatment.

controlled by the CI857 repressor resulted in significant levels of *sfiA* gene transcription in a temperature-dependent fashion. The only exception was the RHDV *VP60* gene, carried by the vector pJRV26, whose expression was not linked to any detectable *sfiA* gene transcription (Table II). Cells carrying the parental pJLA602 only showed basal levels of β-galactosidase activity (Table I). Similar results were obtained with minimal medium (not shown).

Simultaneous Expression of *sfiA* and *recA* SOS genes in Recombinant *E. coli* Cells

The *sfiA* gene product prevents septum formation until the DNA is repaired by the coordinated activity of other SOS proteins, although other LexA-controlled proteins could also be involved in preventing division of cells carrying damaged DNA (Hill et al., 1997). The activation of *sfiA* transcription is, like in other SOS genes, exclusively achieved by the LexA-RecA pathway (Peterson and Mount, 1987). However, to eliminate the possibility that the observed *sfiA* gene transcription in recombinant cells could be due to an unknown SOS-independent mechanism, we have monitored the effects of the CI857-controlled overproduction of two recombinant proteins, namely FMDV VP1 and P22 TSP, on the expression of *sfiA* and *recA* SOS genes under the same inducing conditions. In Figure 1 it is shown that in addition to the *sfiA* expression, the transcription of *recA* gene is also triggered by the overproduction of both recombinant proteins, indicating that the results shown in Table I are not restricted to *sfiA* gene, but are representative of a genuine SOS response in recombinant cells. On the other hand, data presented in Table II and Figure 2 indicate that a truncated VP1 protein of 147 amino acids in length is also able to promote a detectable SOS response, although less intense than that triggered by the full-length VP1.

System Dependence of SOS Activation in Recombinant Cells

Results presented in Table I suggest that the observed SOS response is linked to the CI857-based lambda expression system, because no β-galactosidase activity over the background was detected in cultures that produce foreign proteins directed by p_{Trc} or p_{Lac} IPTG-inducible promoters. It is known that both CI and LexA repressors exhibit limited autoprotease activities, both repressors share their target sequences—also cross-proteolysis between LexA and CI can occur in vitro (Kim and Little, 1993). Although experimental data suggest that the CI857 mutant repressor is not hydrolyzed during the SOS response (Petranovic et al., 1979), it could not be excluded that CI857 could have some residual trans-protease activity over LexA, that could be enhanced when the repressor is thermally inactivated. However, the introduction of a constitutively expressed *cI857* gene (in plasmid pCI857) into cells producing IPTG-controlled proteins, does not promote any detectable SOS response (Table III).

Another possible explanation for the observed system dependence could be that the stimulation of the SOS response is dependent on a high growth temperature, and therefore

Table II. *sfiA* gene expression in *E. coli* GC4581 cells carrying recombinant expression vectors under non-inducing and inducing conditions.

Construction	Vector[a]	Encoded protein	Promoter	Inducing agent	Induction conditions	MC	Growth temperature	*c1857* gene	U/mLOD
pTrc99A9	pTrc99A	TSP[b]	p_{Trc}	IPTG	−	−	28°C	−	79
					−	−	37°C	−	58
					−	−	42°C	−	23
					0.5 m*M*	−	37°C	−	56
					1 m*M*	−	37°C	−	56
					0.5 m*M*	−	42°C	−	14
					1 m*M*	−	42°C	−	19
					−	+	28°C	−	1894
					−	+	37°C	−	2822
					−	+	42°C	−	3547
					1 m*M*	+	37°C	−	2948
					1 m*M*	+	42°C	−	685
pTRV1	pBR322	VP60	p_{Trc}	IPTG	–	–	37°C	−	74
					0.5 m*M*	−	37°C	−	66
					1 m*M*	−	37°C	−	83
					−	+	37°C	−	2775
pTTSPA	pTrc99A	TSPA	p_{Trc}	IPTG	–	–	37°C	−	79
					0.5 m*M*	−	37°C	−	61
					1 m*M*	−	37°C	−	70
					−	+	37°C	−	2490
pUTSP	pUC18	TSP	p_{Lac}	IPTG	−	−	37°C	−	77
					0.5 m*M*	−	37°C	−	85
					1 m*M*	−	37°C	−	78
					−	+	37°C	−	3023
p3D12	pJLA602	VIAA	$p_L\,p_R$	temperature	−	−	28°C	+	84
					−	−	42°C	+	1684
					−	+	42°C	+	6234
p3DC1a	pJLA602	VIAA(−)	$p_L\,p_R$	temperature	−	−	28°C	+	67
					−	−	42°C	+	2456
					−	+	42°C	+	5621
pM652VP1	pJLA602	M652VP1	$p_L\,p_R$	temperature	−	−	28°C	+	54
					−	−	40°C	+	302
					−	−	42°C	+	898
					−	+	28°C	+	1560
pM751VP1	pJLA602	M751VP1	$p_L\,p_R$	temperature	−	−	28°C	+	57
					−	−	40°C	+	240
					−	−	42°C	+	757
					−	+	28°C	+	2139
pJRV26	pJLA602	VP60	$p_L\,p_R$	temperature	−	−	28°C	+	155
					−	−	40°C	+	97
					−	−	42°C	+	64
					−	+	42°C	+	3464
pJVP1	pJLA602	VP1	$p_L\,p_R$	temperature	−	−	28°C	+	33
					−	−	40°C	+	216
					−	−	42°C	+	1676
					−	+	28°C	+	1462
					−	+	42°C	+	5740
pJVP1(−)	pJLA602	VP1(−)	$p_L\,p_R$	temperature	−	−	28°C	+	136
					−	−	42°C	+	903
					−	+	42°C	+	5184
pJXC1	pJLA602	TSP	$p_L\,p_R$	temperature	−	−	28°C	+	32
					−	−	40°C	+	46
					−	+	42°C	+	3803
					−	−	42°C	+	928

[a]Indicates the parental plasmid.

[b]Abreviations are: TSP, P22 tail-spike protein; VP60, RHDV capsid protein; TSPA, TSP C-terminal fusion protein; VIAA, FMDV RNA polymerase; VIAA(−), truncated VIAA; M652VP1, mutant, inactive *E. coli* β-galactosidase; M751VP1, mutant, inactive *E. coli* β-galactosidase; VP1, FMDV capsid protein; VP1(−), truncated VP1.

More details of the employed plasmids are given elsewhere: pTRC99A and pUTSP (Carbonell and Villaverde, 1998); pTRV1 and pJRV26 (Viaplana and Villaverde, in preparation); pTTSPA and pJXC1 (Carbonell and Villaverde, 1996); p2D12 and p3DC1a (Benito et al., 1995a); pM652VP1 and pM751VP1 (Benito et al., 1995b); pJVP1 and pJVP1(−) (Corchero and Villaverde, 1998).

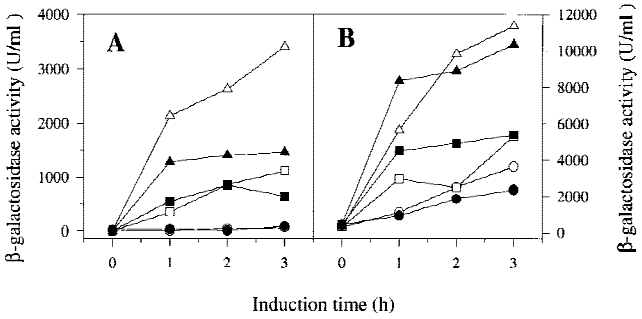

Figure 1. Expression of *lacZ* gene directed by *sfiA* (**A**) and *recA* (**B**) promoters, in cultures carrying either pJXC1 (white symbols) or pJVP1 (black symbols) plasmids. All the cultures were incubated at 28°C until they reached an OD_{550} of 0.3. At this moment (time 0), the cultures were either left at 28°C (circles), incubated at 42°C (squares) or incubated at 42°C simultaneously with the addition of 1.6 μg/ml MC (triangles). Note that basal levels of gene expresion are higher for the *recA::lacZ* fusion.

stimulated by a mechanism that would remain inactive at 37°C. To test this possibility, we investigated whether the SOS response could become apparent when the IPTG induction was accompanied by a temperature shift. In Figure 3 it is shown that the *sfiA* transcription during the IPTG-mediated production of TSP protein cannot be stimulated by a synchronic temperature shift to 42°C. The lack of SOS induction is clear even in the presence of the CI857 protein (Table III). However, the expression of the same gene from lambda promoters does indeed result in important *sfiA* transcription levels (Fig. 4).

Protein Degradation in Thermoinducible Expression Systems

Results presented above show that recombinant proteins are able to stimulate a cell DNA repair response depending on the expression system that directs their synthesis. On the other hand, data from Table I and Figure 2 clearly demonstrate that high temperature, if involved itself in this activation, is not a sufficient condition. A possible explanation for the different effects of the expression systems could be the stronger transcription directed from lambda lytic promoters, when compared with other promoters commonly used for recombinant gene expression in *E. coli* (Brunner and Bujard, 1987; Deuschle et al., 1986). This high transcription could yield amounts of protein above a threshold critical to either trigger or detect a SOS response. However, the Western blot analysis shown in Figure 5, reveals that the model TSP is hardly detected in cell extracts when its synthesis is directed by temperature, but clearly visible in IPTG-induced cultures. Therefore, we can conclude that high amounts of recombinant protein (at least in its intact form) are not required to generate the SOS-inducing signal. This does not exclude the fact that the synthesis rate could inded be a participating factor.

An extensive degradation of TSP has been previously observed when produced from pJXC1 (Carbonell and Villaverde, 1996), and this prompted us to use alternative constructs such as pTRC99A9 for its production and further purification. This proteolytic attack seems to be responsible for the very low levels of TSP detected in cell extracts. Similar observations have been done in our laboratory regarding other heat-inducible proteins encoded in pJLA602-derived vectors (not shown). In fact, among those studied here, the only protein that accumulates in the cells is the RHDV VP60, when produced from pRV26. The proteolytic stability of this protein can be attributed to its strong tendency to aggregate as inclusion bodies (Viaplana and Villaverde, unpublished results). Interestingly, this protein is unique in that overproduction from a pJLA602 derivative does not stimulate detectable levels of *sfiA* transcription (Table II).

The SOS-Inducing Signal

Because results shown above strongly suggest that CI857 has not RecA-independent activities affecting LexA stability, the SOS response is expected to occur according to the normal steps, namely RecA activation followed by the subsequent LexA inactivation (Little and Mount, 1982). Therefore, irrespective of the events that could be involved in the induction of this response in overproducing recombinant cells, a RecA-activating signal must be generated to allow transcription of LexA-repressed genes. In the SOS induction caused by DNA damage, nucleoside triphosphates, plus single-stranded DNA regions resulting from the arrest of DNA replication, stimulate the RecA coprotease activities (Phiziky and Roberts, 1981). In Figure 6, the incorporation of labeled DNA into recombinant cells before and after heat-induction is shown. The temperature shift produces a

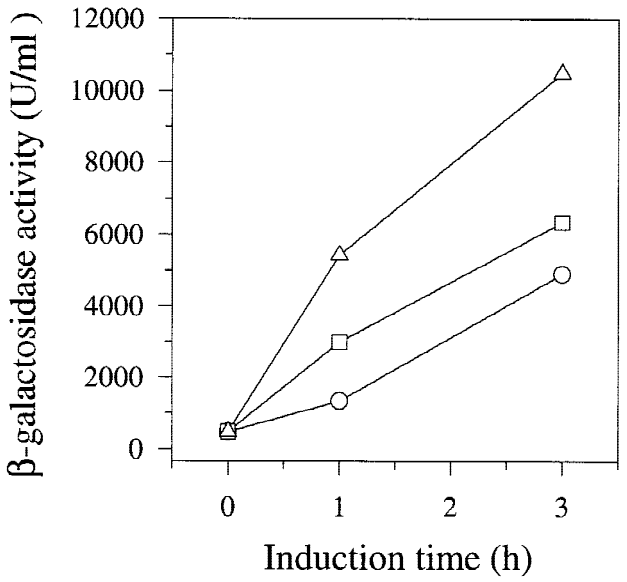

Figure 2. β-Galactosidase activity in IC2015/pJVP1(−) cultures at 28°C (circles), 42°C (squares), and 42°C plus 1.6 μg/mL MC (triangles). Time 0 indicates the onset of induction.

Table III. Effect of *cI857* gene on *sfiA* gene expression in *E. coli* GC4581 cells carrying recombinant expression vectors under non-inducing and inducing conditions.

Construction	Vector	Encoded protein	Promoter	Inducing agent	Induction conditions	MC	Growth temperature	U/mLOD
pcI857	pcI857	none	none	none	−	−	28°C	78
					−	−	42°C	10
					1 m*M*	−	42°C	43
					1 m*M*	+	42°C	2408
pTrc99A + pcI857	pTrc99A + pcI857	none	p_{Trc}	IPTG	−	−	28°C	99
					−	−	42°C	59
					1 m*M*	−	42°C	68
					1 m*M*	+	42°C	1910
pTrc99A9 + pcI857	pTrc99A	TSP	p_{Trc}	IPTG	−	−	28°C	65
					−	−	42°C	76
					1 m*M*	−	42°C	53
					1 m*M*	+	42°C	1206

rapid, but transient increase of the DNA synthesis rate, which is followed by a dramatic arrest in cells producing TSP, but not in those devoid of the recombinant plasmid. In control cells in which MC has been added simultaneously to the temperature shift, the effects of the genotoxic drug are immediately detected after its addition, and this drug prevents the transient enhancement of DNA synthesis. Interestingly, the influence of the recombinant protein on DNA synthesis is not dramatic until about 1 h after the induction, although already observed at 30 min. This suggests that the protein itself has no direct activities on DNA replication, and indicates that some indirect influences are able to reduce about 80% of the DNA synthesis rate, consequently activating the SOS DNA repair response.

DISCUSSION

The synthesis of heat shock and other stress proteins has been often observed as a response to the overproduction of foreign polypeptides in bacterial cells (Arora and Pedersen, 1995; Parsell and Sauer, 1989; Rinas, 1996). On the other hand, the induction of SOS genes has also been reported in *E. coli* cultures expressing viral genes, whose products are known or supposed to interfere with the cell DNA metabolism (Benito et al., 1995a; Colasanti and Denhardt, 1985). The experimental data presented in this study reveal that the heat-induced expression of recombinant genes from the strong lambda p_L-p_R promoters activates the SOS system in the harboring cells (Figs. 1 and 4), irrespective of the nature of the encoding gene, but coincident with the proteolytic instability of the resulting proteins (Table II, Fig. 5). This effect, which has not been observed in other expression systems, is not exclusively dependent on features of the temperature-inducible system itself, such as the thermal shift or the presence of the lambda promoters or their CI857 repressor (Fig. 3, Table III), and it requires the synthesis of the encoded protein. Non-sense transcripts (of about 600 nt in length) from the parental vector pJLA602 result in only background levels of SOS induction (Table I). On the other hand, the proteolysis of the recombinant proteins, if not the triggering event itself, seems to be at least a potent positive modulator of the SOS response. In overproducing cells, RecA protein could be activated by a progressive arrest of DNA replication after heat induction that is already detected at 30 min, but becomes dramatic at about 1 h after the temperature shift (Fig. 6). The SOS response in recombinant bacteria could explain previous observations, such as the increase of the RecA-dependent mutation rate during recombinant gene expression (Lukacsovich and Venetianer, 1991) and the cell filamentation frequently observed in actively producing cultures. Although undetected influences of the specific plasmids and strains used in this study on the generation of the SOS response cannot be completely excluded, the presented data prompt us to suggest that the SOS activation could be a general effect caused by recombinant proteins on recombinant cells. On the other hand, because

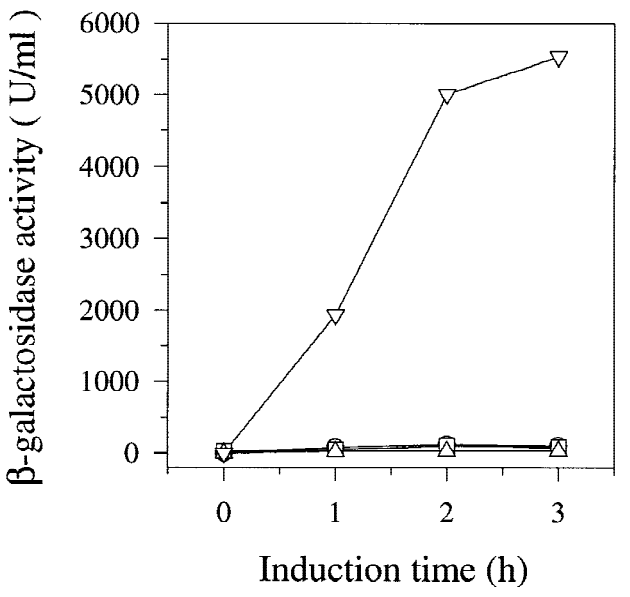

Figure 3. β-Galactosidase activity in GC4581/pTrc99A9 at 37°C (circles), at 37°C plus 1 m*M* IPTG (squares), at 42 °C plus 1 m*M* IPTG (triangles) and at 42 °C plus 1 m*M* IPTG and 1.6 μg/mL MC (inverted triangles).

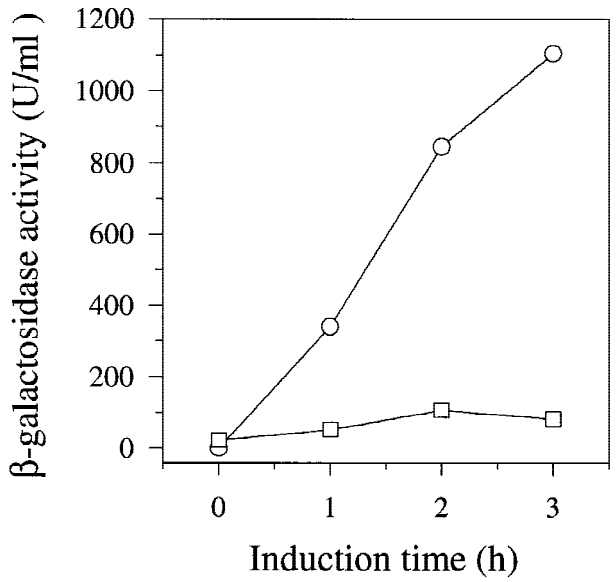

Figure 4. β-Galactosidase activity in GC4581 carrying either pJXC1 (circles) or pTrc99A9 (squares) after shifting to 42°C or adding 1 m*M* IPTG, respectively. Time 0 indicates the onset of induction.

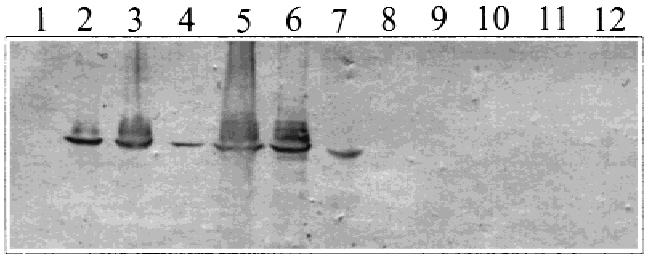

Figure 5. Western blot analysis of crude cell extracts of GC4581 carrying either pTrc99A9 (lanes *1* to *8*) or pJXC1 (lanes *9* to *12*). Cell extracts were obtained 1 h after the onset of induction. In lanes *1* to *4*, cultures were kept at 37°C without IPTG (*1*), plus 0.5 m*M* IPTG (*2*), plus 1 m*M* IPTG (*3*) or plus both 1 m*M* IPTG and 1.6 μg/mL MC (*4*). In lanes *5* to *8*, cultures were shifted to 42°C, plus 0.5 m*M* IPTG (*5*), plus 1 m*M* IPTG (*6*), plus 1 m*M* IPTG and 1.6 μg/mL MC (*7*) and without any drug (*8*). For pJXC1-carrying cells, the culture was kept at 28°C (*9*), at 40°C (*10*) at 42 °C (*11*), and at 42°C plus 1.6 μg/mL MC (*12*). Anti-P22 serum was obtained as described (Carbonell and Villaverde, 1996).

some of the tested proteins are enzymatically inactive (the insertional β-galactosidase mutants M652VP1 and M751VP1, and the FMDV VP1 capsid protein), no specific enzymatic activities seem to be involved.

The main question arising from the obtained data, is how the arrest of cell DNA replication can be linked to unspecific recombinant protein production, and why the consequences of this effect are only detectable when the recombinant gene expression is directed by the lambda expression system. It is known that the lambda lytic promoters are among the strongest ones used for recombinant gene expression in *E. coli*. For instance, the strength of p_L promoter is 10-fold higher than p_{lacUV5}, 5.6 than p_{lac}, 2 than p_{tacI} and 1.7 than T7 p_{A1} and p_{A2} promoters (Deuschle et al., 1986). Similarly, a comparison between the yield of the same protein controlled by either a p_{lac}- or the same p_L-p_R-based system used here revealed 10-fold more protein in the temperature-inducible system (Seeger et al., 1995). Such a high rate of protein synthesis could result in the extensive proteolysis of gene products such as TSP (Fig. 5), whose folding intermediates are highly thermally unstable (King et al., 1996, and references therein), but also of other proteins unable to fold correctly in bacterial cells (Parsell and Sauer, 1989). A critical amount of unfolded nascent polypeptides is probably required to stimulate this proteolytic attack, that could not be reached with the IPTG-inducible promoters used in this work. Note that in the heat-inducible system a shift to 40°C, that only allows about 50% the CI857-controlled transcription rate observed at 42°C (Villaverde et al., 1993), renders lower values of *sfiA* expression, which in some cases are proximal to background levels (Table II).

More detailed studies would be required to establish the precise basis of the SOS response in recombinant cells, and several non-exclusive mechanisms could be involved in the generation of the inducing signal. However, according to the experimental data, we would favor the hypothesis that a high protein synthesis rate allowed by strong promoters can induce or stimulate a dramatic proteolysis of the resulting misfolded proteins, provided they are produced above a critical threshold rate. This degradation is probably enhanced by the temperature shift used for induction (Chesshyre and Hipkiss, 1989; Kosinsky and Bailey, 1991). It is known that an intense proteolysis, either promoted by a high content in cell proteases or by an overproduction of misfolded proteins, has deleterious effects on the cells, and it causes a remarkable growth inhibition (Goff and Goldberg, 1987; Viaplana et al., 1997). Apart from La protein, which is one of the main proteases hydrolyzing misfolded proteins (Goldberg et al., 1981), other ATP-dependent pro-

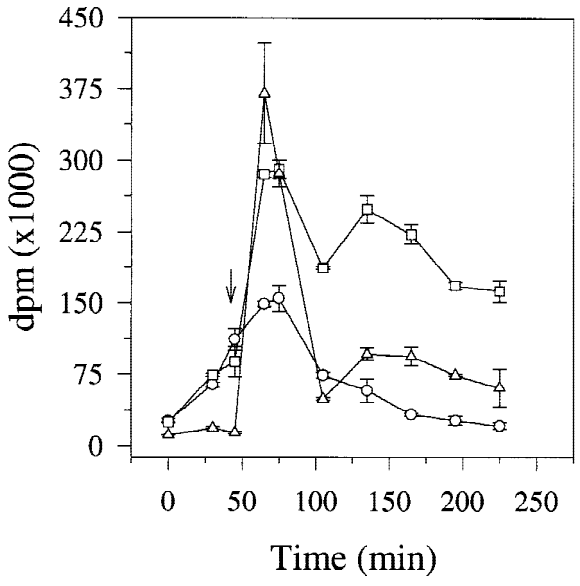

Figure 6. DNA synthesis in GC4581/pJXC1 at 42°C (triangles), GC4581 at 42 °C (squares), and GC4581 at 42 °C plus 1.6 μg/mL MC (circles). The arrow indicates the time of induction.

teases are actively involved in proteolytic events (Kosinski et al., 1992). ATP-Consuming activities elicited by CI857-controlled gene overexpression could promote a decrease in the ATP levels that could affect strongly ATP-dependent cell functions such as DNA replication. In agreement with this assumption, the shortening by about 35% of the VP1 protein in VP1(−) also reduces the intensity of the SOS response (Table II and Fig. 2). Because the truncated protein was simply generated by a frameshift mutation introduced in the cloned gene, similar molar rates of protein synthesis would be expected for VP1 and VP1(−), but less energy must be then required for a complete degradation of the truncated version. Moreover, the intracellular ADP/ATP ratio is presumed to increase in cells overproducing the protease La (Fischer and Glockshuber, 1993).

Estimations of the intracellular amount of proteins GroEL and GroES indicate that in the natural cell context, 95% of the cellular proteins do not require these chaperonins for their folding because the amounts of both GroEL/ES can only account for the assisted folding of the remaining 5% (Lorimer, 1996). However, the intracellular concentration of chaperonins seems to be near the limiting values because in underproducing $RpoH^-$ mutant strains, the aggregation of cell proteins increases (Gragerov et al., 1992). In a stressful situation such as the overproduction of foreign peptides, the molar amount of chaperonins, even enhanced by the temperature shift, could be too low to promote folding of the foreign products above a given synthesis rate, allowing their degradation. Given this, the recombinant protein yield is very low or even undetectable (Roman et al., 1995), but it can be recovered, in some cases, by an additional supply of chaperonins (Georgiou and Valax, 1996, and references therein). Moreover, it also cannot be discounted that in overproducing cells, the concentration of free chaperonins could be insufficient for the folding of cell proteins with a rapid turnover, that could be involved in functions such as DNA replication. Note that DnaK and other chaperonins are required for DNA replication (Zylic, 1993).

The activation of the SOS response, with their associated activities such as inhibition of cell division, alleviation of respiration and error-prone DNA replication (Walker, 1984), can account for an important part of either the metabolic burden and the toxicity associated to the production of recombinant proteins in *E. coli.*

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DESENVOLUPAMENT
DE PROTEÏNES QUIMÈRIQUES MULTIFUNCIONALS
COM A VECTORS DE TRANSFERÈNCIA GÈNICA
MEDIADA PER RECEPTOR

VII.BIBLIOGRAFIA

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