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A purified truncated form of yeast Gal4 expressed in *Escherichia coli* and used to functionalize poly(lactic acid) nanoparticle surface is transcriptionally active *in cellulo*

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ABSTRACT

Gal4/UAS system is a powerful tool for the analysis of numerous biological processes. Gal4 is a large yeast transcription factor that activates genes including UAS sequences in their promoter. Here, we have synthesized a minimal form of Gal4 DNA sequence coding for the binding and dimerization regions, but also part of the transcriptional activation domain. This truncated Gal4 protein was expressed as inclusion bodies in *Escherichia coli*. A structured and active form of this recombinant protein was purified and used to cover poly(lactic acid) (PLA) nanoparticles. *In cellulo*, these Gal4-vehicles were able to activate the expression of a Green Fluorescent Protein (GFP) gene under the control of UAS sequences, demonstrating that the decorated Gal4 variant can be delivery into cells where it still retains its transcription factor capacities. Thus, we have produced in *E. coli* and purified a short active form of Gal4 that retains its functions at the surface of PLA-nanoparticles in cellular assay. These decorated Gal4-nanoparticles will be useful to decipher their tissue distribution and their potential after ingestion or injection in UAS-GFP recombinant animal models.

Introduction

In the nanomedicine field, nanoparticle vectors have gained more and more interest over the last decade, either as drug delivery systems [1,2] or vaccine vehicles [3]. Among the factors that hampered their development [4], we can cite the presence of toxic components in some nanoparticles (inorganic compounds, for example), a reliable reproducibility for the production of stable nanosystems, the lack of long-term survey studies to examine their potential deleterious side effects, and the real cell/tissue specificity of these vehicles [5,6]. There is also a lack of information about their biodistribution in the whole body after their administration. The biodegradable particles that are based on a PLA or poly (lactic acid-co-glycolic acid) (PLGA[1] copolymer) backbone are among the most promising nanocarriers [3,7]. They are made of non-toxic polymers that have been approved by the Food and Drug Administration (FDA) [8]. The transport of PLA/PLGA nanoparticles through intestinal and pulmonary mucosa has been studied with the use of fluorescent particles and powerful imaging techniques [9–11]. However, quenching effects, fluorescent fading, and the small size (120 – 250 nm) of PLA/PLGA nanoparticles have precluded a precise analysis of their fate upon parenteral or mucosal administration, and still limit their precise location in whole body [12].

To overcome such physical and structural limitations, we propose to use the Gal4/UAS system as a biological tool to monitor the fate of PLA particles after cell uptake [13]. Indeed, as any reporter gene under the control of an UAS promoter could be switched on upon binding of a dimeric GAL4 protein, we hypothesize that nanoparticles loaded with GAL4 protein could be tracked in an indirect manner after cell uptake. After endocytosis, GAL4 protein will be released from PLA-nanoparticles, transported to the nucleus, and switched on UAS-GFP reporter gene.

To assess the proof of concept of such tools, we have designed, constructed, and expressed in *Escherichia coli* a GAL4 minimal protein in order to optimize loading of PLA nanoparticles with a shorter polypeptide. The truncated protein possesses the essential

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[1] *Abbreviations used:* PLA, poly(lactic acid); PLGA, poly (lactic acid-co-glycolic acid); GFP, Green Fluorescent Protein; S2, Schneider's D. melanogaster line 2; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; CD, circular dichroism.

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functions of the Gal4 transcription factor (UAS binding and dimerization regions, the nuclear localization sequence, part of the transcriptional activation domain). After purification and refolding, this recombinant Gal4 protein was successfully loaded on the surface of PLA-nanoparticles. Finally, we provided evidence that GAL4 decorated particles were efficiently ingested by mammalian cells, and that desorbed Gal4 protein retained its full transcription factor capacities as demonstrated by activation of a UAS-GFP reporter gene.

Materials and methods

Bacterial strains and plasmids

The *E. coli* JM109 was used for standard cloning. *E. coli* BL21(DE3), BL21(DE3)pLysS and Rosetta-gami(DE3) strains were used as hosts for overexpression. The truncated Gal4-coding sequence was synthesized by Proteogenix (Schiltigheim, France). The coding sequence of Gal4min (residues 1–100 and 841–881, according to the GenBank reference **CAA91596.1**) was flanked by attB1 and attB2 gateway sequences. The *Gal4min* gene was delivered in the pUC57 cloning vector. Gateway vectors (donor plasmid pDONR207 and drosophila expression plasmid pAWR) were purchased from Life Technologies (Saint Aubin, France). pAc5.1B-EGFP was a gift from Elisa Izaurralde (Addgene plasmid # 21181), and is a drosophila GFP constitutive expression vector. pAC-GAL4 was a gift from Liqun Luo (Addgene plasmid # 24344) [14], and is a plasmid expressing Gal4 in insect cells. pJFRC28-10XUAS-IVS-GFP-p10 was a gift from Gerald Rubin (Addgene plasmid # 36431) [15]. This insect expression vector contains a Gal4-responsive promoter. pT7/7-6His and pETG-10a are *E. coli* overproducing plasmids designed to generate recombinant proteins with C-terminal [16] or N-terminal (Life Technologies) 6 His-tag, respectively.

Schneider's Drosophila melanogaster line 2 cells

Schneider's *D. melanogaster* line 2 (S2) cells were obtained from the CelluloNet of the UMS3444/US8/SFR BioSciences Gerland Lyon-Sud. S2 cells were cultured in Schneider's drosophila medium (Revised) (Life Technologies) plus 10% fetal bovine serum (Life Technologies), and grown at 28 °C without CO_2 as a loose and semi-adherent monolayer in tissue culture T-25 flasks. Twenty-four hours prior transfection, S2 cells were seeded in 6-well plates at a density of 10^6 cells/mL. S2 cells were then transiently transfected with constructs (1 μg of each plasmid per well) with the Effectene™ transfection kit (Qiagen; Courtaboeuf, France). Twenty-four hours after transfection, approximately 2000 uncoated or Gal4min formulated PLA-nanoparticles were added to the cell medium. GFP observations were performed with a Zeiss Axioplan epifluorescence microscope equipped with a Coolsnap fx digital camera (Roper scientific).

Construction of a Gal4min expression vectors

A BP recombination reaction in the presence of the Gateway® BP Clonase® II enzyme was performed between the linearized attB-pUC57-Gal4min clone and the attP-pDONR207 donor vector to generate an entry clone. This newly constructed attL-containing entry clone was used in an LR recombination reaction with the attR pAWR or attR pETG-10a destination plasmids and the Gateway® LR Clonase® II enzyme to get the pAWR-Gal4min or pETG-10a-H-Gal4min expression clones, respectively. All these Gateway reactions were done according to the manufacturer's recommendations (Life Technologies). To generate pT7/7 derivative constructs expressing a Gal4min protein fused or not at its C-terminus to a 6His-tag, we first amplified by PCR the Gal4min coding sequence using the forward primer (5′-ATGAATTCTTAAACTGCTGAGCAGCATTGAAC-3′) and the reverse primer (5′-TATCTGCAGAAGTTCTTTTTTCGGGTTCG-3′; His-tagged isoform) or the reverse primer (5′-TATCTGCAG**TTA**TTCTTTTTTCG-GGTTCGG-3′; untagged Gal4min protein). The *Eco*RI and *Pst*I restriction endonuclease sequences were underlined in these oligonucleotides, and the stop codon UAA is indicated in bold characters. *Eco* RI-*Pst* I digested PCR fragments were gel purified using the Wizard® SV gel and PCR clean-up system (Promega; Charbonnières-les-Bains, France), ligated with gel purified *Eco* RI/*Ps t*I DNA fragment of pT7/7-6His, and then transformed into JM109 bacterial strain. The cloned sequences were checked by direct sequencing (Sigma–Aldrich; Saint-Quentin de Fallavier, France).

Gal4min protein expression

E. coli (BL21DE3, BL21(DE3)pLysP or Rosetta-gami(DE3)) clones containing pETg-10a or pT7/7 derivatives were grown at 37 °C in LB broth (Luria low salt; Sigma–Aldrich) supplemented with 50 μg/mL ampicillin and 60 μM $ZnCl_2$. Protein induction were realized when the absorbance at 600 nm of the cell culture reached approximately 0.6 with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM or 0.4 mM for pETG-10a or pT7/7 expression vectors, respectively). After IPTG induction, bacterial cultures were grown overnight at 18 °C or 2–3 h at 37 °C before harvesting by centrifugation. To obtain a total crude extract, pellet from 2 mL cell culture was resuspended in 100 μL of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer (60 mM Tris–Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled during 5 min, and centrifuged at 4 °C for 10 min. A 10 μL aliquot of supernatant was analyzed by SDS–PAGE. To obtain insoluble and soluble protein fractions, cell pellet from 2 mL induced-culture was resuspended in 500 μL of 10 mM Tris–Cl, 1 mM EDTA pH 8. The bacterial solution was chilled on ice and broken up by sonication. The cell lysate was harvested by centrifugation at 10,000*g* for 10 min at 4 °C, and supernatant and pellet corresponding to soluble and insoluble proteins were analyzed by SDS–PAGE.

His-tagged Gal4min purification

Cells from an IPTG induced culture (400 mL) were harvested by centrifugation at 10,000*g* for 20 min at 4 °C. Pellet was resuspended in 40 mL of lysis buffer (50 mM NaCl, 60 μM $ZnCl_2$, 1% Triton-X100, 20 mM β-mercaptoethanol, 50 mM Tris–Cl pH8), and then lysed by sonication. The cell lysate was centrifuged at 13,000*g* for 30 min at 4 °C, and the pellet was resuspended in 40 mL of lysis buffer. After one more cycle of sonication–centrifugation, the pellet containing mostly the inclusion body proteins was resuspended in 40 mL of denaturing buffer (10 mM Imidazole, 50 mM NaCl, 60 μM $ZnCl_2$, 8 M urea, 20 mM β-mercaptoethanol, 50 mM Tris–Cl pH8), kept at 4 °C for 30 min, and centrifuged at 4 °C for 30 min at 13,000*g*. Supernatant fraction was applied to a nickel-affinity chromatography column preequilibrated with denaturing buffer. After extensive washing of the Ni–NTA agarose (Qiagen) column, the His-tagged proteins were recovered with elution buffer (200 mM Imidazole, 300 mM NaCl, 60 μM $ZnCl_2$, 8 M urea, 20 mM β-mercaptoethanol, 20 mM Tris–Cl pH 7.5). Aliquots of each eluted protein fraction were analyzed by SDS–PAGE.

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