

ARTICLE

Different protein composition and functional properties of adeno-associated virus-6 vector manufactured from the culture medium and cell lysates

Jerome Denard¹, Christine Jenny¹, Véronique Blouin², Philippe Moullier^{2,3} and Fedor Svinartchouk¹

Vectors based on recombinant adeno-associated viruses (rAAV) attract a growing interest for human gene therapy. Recently, it was shown that many rAAV serotypes produced by transient transfection of human embryonic kidney 293 cell line (HEK293) are efficiently released into culture medium and functionally equivalent to those purified from cell lysates. Here, we report that HEK293 cells produce and secrete Galectin 3-binding protein (huG3BP), a protein that efficiently binds rAAV6 *in vivo*. Importantly, intracellular G3BP and secreted G3BP have different properties: while the secreted protein had the same electrophoretic mobility as serum huG3BP and interacted with rAAV6, intracellular protein migrated faster and did not bind rAAV6. Consequently, rAAV6 purified from culture medium (secreted, rAAV6-S) was physically associated with huG3BP while rAAV6 harvested from cell lysates (cellular, rAAV6-C) was huG3BP-free. After systemic injections, rAAV6-S bound to huG3BP was 3 times less efficient compared to rAAV6-C and induced an immune response against huG3BP protein. Our findings show that protein content of rAAVs purified from culture medium or from cell lysates can be different and these differences may impact vector efficacy and/or immune response.

Molecular Therapy — Methods & Clinical Development (2014) **1**, 14031; doi:10.1038/mtm.2014.31; published online 30 July 2014

INTRODUCTION

Vectors based on recombinant adeno-associated virus (rAAV) are becoming tools of choice for many applications in human gene therapy. rAAV can be produced either in baculovirus system or by transient transfection of human embryonic kidney 293 cell line (HEK293). Recently, it was reported that many rAAV serotypes are efficiently released into a culture medium after transient transfection of HEK293 and vector particles isolated from the culture medium are functionally equivalent to those purified from cell lysates.¹ Moreover, for some serotypes (rAAV6, rAAV7, rAAV8), secreted particles presented the main fraction of the produced vectors. The possibility to harvest vectors from culture medium rather than cell lysates could simplify purification of rAAV.¹

Previously, we showed that human G3BP protein interacts with some rAAV serotypes and especially impacts on rAAV6 efficiency *in vivo*.^{2,3} G3BP coding mRNA was found in many human cell lines and all tested tissues.⁴ Here, we report that the cell line HEK293, routinely used for rAAV production, produces and secretes huG3BP. Importantly, intracellular G3BP and secreted G3BP have different properties: while secreted protein has the same electrophoretic mobility as serum huG3BP and interacts with rAAV6, intracellular protein migrates faster and does not bind rAAV6.

Consequently, we found that rAAV6 purified from culture medium (secreted, rAAV6-S) is physically associated with huG3BP

while rAAV6 harvested from cell lysates (cellular, rAAV6-C) is not. Association with huG3BP protein seriously impacts the *in vivo* properties of rAAV6 by decreasing its transduction efficiency after systemic injections and also results in the induction of an immune response against huG3BP protein.

Our findings show that protein content of rAAVs purified from culture medium or from cell lysates can be different and these differences may impact vector efficacy and/or immune response.

RESULTS

HEK-293 cells produce huG3BP

Expression of secreted and intracellular huG3BP protein by HEK293 cells was quantified by an ELISA assay 3 days after cell seeding. The main portion of the protein (90% of 1,500 ng) produced by 40×10^6 cells was found in secreted form in the culture medium. Less G3BP protein was found in the cells, where it was concentrated in the cytoplasmic fraction and a trace quantity of G3BP (less than 2% of cytoplasmic fraction) was found in the nucleus fraction. Importantly, secreted (huG3BP-S) and intracellular (huG3BP-C) huG3BP had different electrophoretic mobilities when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis (Figure 1). Electrophoretic mobility of the secreted protein was the same as for huG3BP obtained from human serum, while several bands between 45 and 70 KD reacted with

¹Biomarkers Department, Genethon, 1 bis rue de l'Internationale, Evry, France; ²Université de Nantes–Inserm UMR649, Nantes, France; ³Molecular Genetics and Microbiology Department, University of Florida, Gainesville, Florida, USA. Correspondence: F Svinartchouk (svinart@genethon.fr)

Received 20 March 2014; accepted 5 June 2014

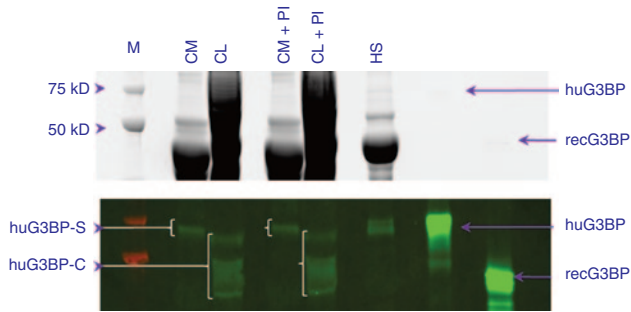


Figure 1 Secreted and cellular huG3BP have different electrophoretic motilities. Secreted and cellular huG3BP were analyzed by western blot. Culture medium (CM) and cell lysates (CL) of HEK293 cells were prepared as described in the Materials and Methods. CM+PI and CL+PI are culture medium and cell lysates prepared in the presence of protease inhibitors. HS, human serum; M, molecular weight markers; huG3BP, purified huG3BP; recG3BP, human recombinant G3BP produced in *Escherichia coli*. Upper panel: Coomassie staining; lower panel: corresponding western blot analysis of hu-G3BP.

anti-huG3BP antibody when the cell lysate was analyzed. Inhibition of proteases with “complete protease inhibitor cocktail” (Roche, Basel, Switzerland) during cell lysis did not change the western pattern (Figure 1). Because the molecular weight of all forms of cellular huG3BP were between mature glycosylated protein and recombinant nonglycosylated huG3BP, we suspect that these intermediate bands correspond to partially glycosylated forms of huG3BP.

rAAV6 produced from cell culture medium but not from cell lysates is associated with HuG3BP

Because some rAAV serotypes interact with huG3BP protein,² production of huG3BP protein by HEK293 cells raises the possibility of vector association with this protein. In order to check this hypothesis, we analyzed different preparations of rAAV6 vector, which has the highest affinity to huG3BP (an association constant of $\approx 10E9 M^{-1}$).² rAAV6 was purified by density gradient canonical procedures either from culture medium (secreted, rAAV6-S) or from cell lysate (cellular, rAAV6-C) and the presence of huG3BP was analyzed by western blot. Interestingly and despite similar purity patterns on SDS-PAGE, G3BP was detected only in the rAAV6-S but not in the rAAV6-C (Figure 2).

Using human recombinant G3BP as a reference standard, the quantities of secreted (huG3BP-S) and intracellular (huG3BP-C) proteins produced by HEK293 cells in one T75 flask were estimated as $10E13$ and $2 \times 10E12$ molecules respectively. Taking into account that vector yield from a T75 flask does not exceed $1 \times 10E12$ particles (our unpublished data) and,⁵ it was surprising that no G3BP protein was detected in rAAV6-C harvested from cell lysates. To explain the absence of G3BP in rAAV6-C, we suggested that huG3BP-C and huG3BP-S bind rAAV6 differently. The results of a coimmunoprecipitation assay (Figure 3) fully validated this hypothesis: while huG3BP-S, similar to huG3BP from serum, was able to bind rAAV6, no binding was detected for huG3BP-C (Figure 3).

Presence of G3BP in the rAAV6-S fractions indicates that this complex is not dissociated even in high concentrations of CsCl (from 3 to 4 mol/l) during the two runs of cesium chloride ultracentrifugation. To check the stability of the huG3BP/rAAV6 complex under purification conditions, immobilized rAAV6 vector was incubated with HEK293 conditioned culture medium or cell lysate and then washed in the presence of growing concentrations of CsCl (Figure 4). Importantly, the complex of rAAV6 with huG3BP from culture medium was stable at all concentrations tested. No complex was

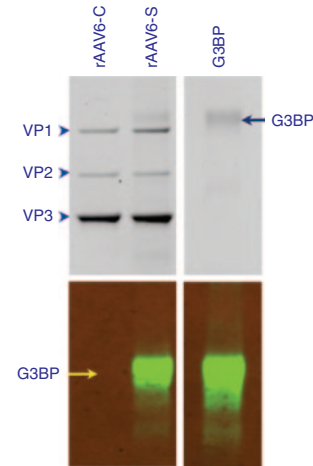


Figure 2 Only rAAV6-S purified from culture medium contains huG3BP protein. $1 \times 10E11$ pp of rAAV6 produced in HEK293 cells and purified either from culture medium (rAAV6-S) or from cell lysates (rAAV6-C) by CsCl centrifugation were analyzed by SDS-PAGE and western blot analysis. G3BP: 100 ng of purified huG3BP protein. Upper panel: Coomassie staining; lower panel: corresponding western blot analysis of hu-G3BP using anti-huG3BP antibodies. The ratio (number of rAAV6 physical particles)/(number of huG3BP molecules) in rAAV6-S fraction is equal to 1/5.

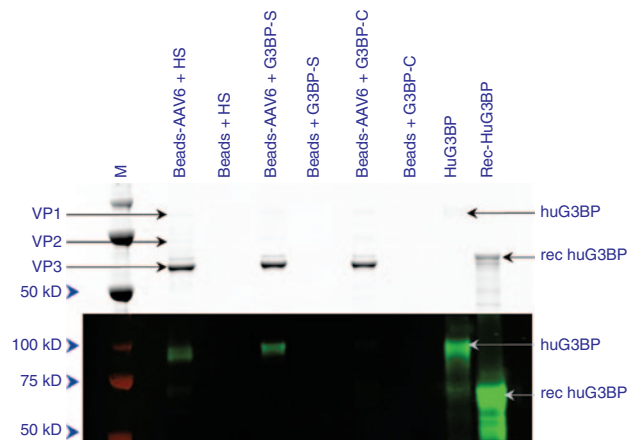


Figure 3 Interaction of secreted and intracellular G3BP with rAAV6. Interactions of secreted (G3BP-S) and intracellular (G3BP-C) G3BP with rAAV6 were assessed by coimmunoprecipitation assay with the vector immobilized on AVB-Sepharose beads. M, molecular weight markers; Beads-AAV6 or beads alone, immobilized rAAV6 or beads alone incubated respectively with human serum (HS), G3BP-S or G3BP-C. The same quantity of G3BP (70 ng) was used in each coimmunoprecipitation assay. Reference standard huG3BP: 100 ng of glycosylated human protein. Rec-huG3BP: 100 ng of nonglycosylated human protein. Upper panel: Coomassie staining; lower panel: corresponding western blot analysis of hu-G3BP using anti-huG3BP antibodies. Positions of G3BP and viral capsid proteins are indicated by arrows.

formed with huG3BP from cell lysate (Figure 4b). Stability of the huG3BP/rAAV6 complex in the presence of up to 4.1 mol/l of CsCl (Figure 4a) strongly suggests that huG3BP copurifies as a complex with rAAV6 particles as opposed to colocalization at the same CsCl gradient density.

We also tempted to dissociate rAAV6/huG3BP complexes by affinity purification of rAAV6 from rAAV6-S fraction.⁶ To this purpose, rAAV6-S were incubated with AVB Sepharose affinity beads and contaminating proteins were washed either with phosphate-buffered saline (PBS) or PBS+1% Triton X-100. While more than 90% of rAAV6 were

bound to the beads under these conditions, the ratio AAV6/huG3BP in the affinity-bound AAV6 and nonpurified rAAV6-S was the same (Supplementary Figure S1). Because the routinely used conditions of

rAAV purification do not separate rAAV6 from huG3BP, we suggest producing G3BP free rAAV6 from the cellular fraction of HEK293 cell.

Most of rAAV6 particles in rAAV6-S fraction are bound to HuG3BP. By using known quantities of huG3BP as standard, we identified the ratio (number of rAAV6 physical particles)/(number of huG3BP molecules) in rAAV6-S fraction as 1/5 (Figure 2). This ratio is very close to the previously determined ratio AAV6/huG3BP in the complex (one rAAV-6 particle bound up to six huG3BP monomers),² suggesting that all or nearly all rAAV6 particles in the secreted fraction are bound to huG3BP. To further approve this statement, we used the propensity of rAAV6/huG3BP aggregates to precipitate after low-speed centrifugation.² According to this experimental procedure, 60 μ l of rAAV6-S or rAAV6-C were centrifuged either at 2,000 *g* for 10 minutes or at 10,000 *g* for 30 minutes and the supernatant (50 μ l) was carefully discarded. Ten microliter aliquots from the supernatant and precipitate were analyzed by SDS-PAGE. Vectors were equally recovered in both supernatant and precipitate after rAAV6-C fraction centrifugation. After centrifugation of rAAV6-S fraction at 2,000 *g*, 70% of rAAV6 was found in the pellet fraction and at higher speed (10,000 *g*) the percentage of rAAV6 in the precipitate increased up to 90% thus indicating that at least 90% of rAAV6 in rAAV6-S fraction are bound to huG3BP (Figure 5). More efficient precipitation of rAAV6/huG3BP complexes at higher speed can be due to different sizes of the complexes, where bigger complexes precipitate first while the smaller need higher speed/more time to be precipitated.

To answer the question if the remaining in the supernatant rAAV6 is free or bound to huG3BP, rAAV6 in the supernatant was captured with AVB beads and the quantities of free and immobilized on the AVB beads rAAV6 and huG3BP were estimated by the respective western blot analyses. Importantly, recovering of the remaining in the supernatant rAAV6 by AVB beads led to the complete clearance of huG3BP protein from the supernatant (Figure 5). The ratio of (number of rAAV6 physical particles)/(number of huG3BP molecules) in the captured by AVB beads rAAV6/huG3BP complex was very close to 1/5, thus confirming that the major part of rAAV6-S is presented by rAAV6/huG3BP complexes.

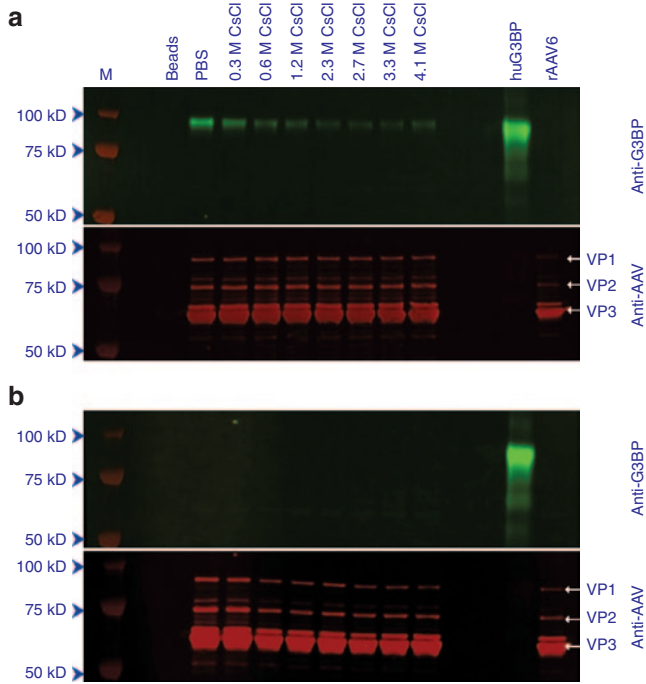


Figure 4 HuG3BP-S/rAAV6 complex resists to high CsCl concentrations. rAAV6, immobilized on NHS-activated Sepharose, was incubated with HEK293 conditioned culture medium (a) or cell lysate (b) and then washed in the presence of phosphate-buffered saline (PBS) or increasing concentrations of CsCl (from 0.3 to 4.1 mol/l). Anti-G3BP, western blot analysis of huG3BP protein bound to rAAV6; Anti-AAV6, western blot analysis of VP proteins; M, molecular weights markers; Beads: beads alone incubated with conditioned culture medium or cell lysate and washed in PBS; HuG3BP: 100 ng of glycosylated human protein; rAAV6: 5×10^{10} of rAAV6 particles. Positions of viral capsid proteins are indicated by arrows.

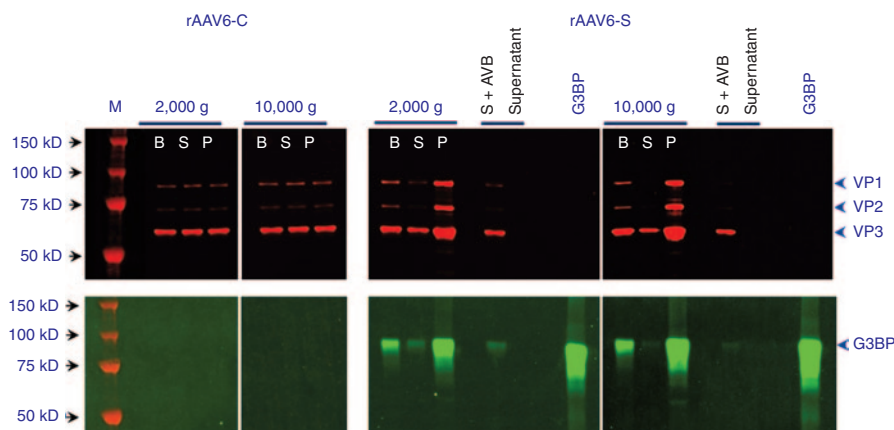


Figure 5 Most of the rAAV6 particles in rAAV6-S fraction are bound to HuG3BP. rAAV6-C (left panel) or rAAV6-S (right panel) were centrifuged either at 2,000 *g* for 10 minutes or at 10,000 *g* for 30 minutes and the supernatant was carefully discarded. The quantities of rAAV6 and G3BP protein before centrifugation (B), in the supernatant (S) and precipitate (P) were estimated by the respective western blot analyses. Vectors were equally recovered in both supernatant and precipitate after rAAV6-C fraction centrifugation. After centrifugation of rAAV6-S fraction at 2,000 *g* 70% of rAAV6 was found in the pellet fraction and at 10,000 *g*, the percentage of rAAV6 in the precipitate increased up to 90%. The remaining in the supernatant rAAV6 was captured with AVB beads. Importantly, recovering of the remaining in the supernatant rAAV6 by AVB beads (S+AVB) led to the complete clearance of huG3BP protein from the supernatant (Supernatant). The percentage of the precipitated rAAV6 was calculated by using a ratio of VP1 intensities in rAAV6 before centrifugation and in the supernatant. Upper panels: western blot analysis using anti-VP antibodies; lower panels: corresponding western blot analysis of hu-G3BP. Positions of G3BP and viral capsid proteins are indicated by arrows.

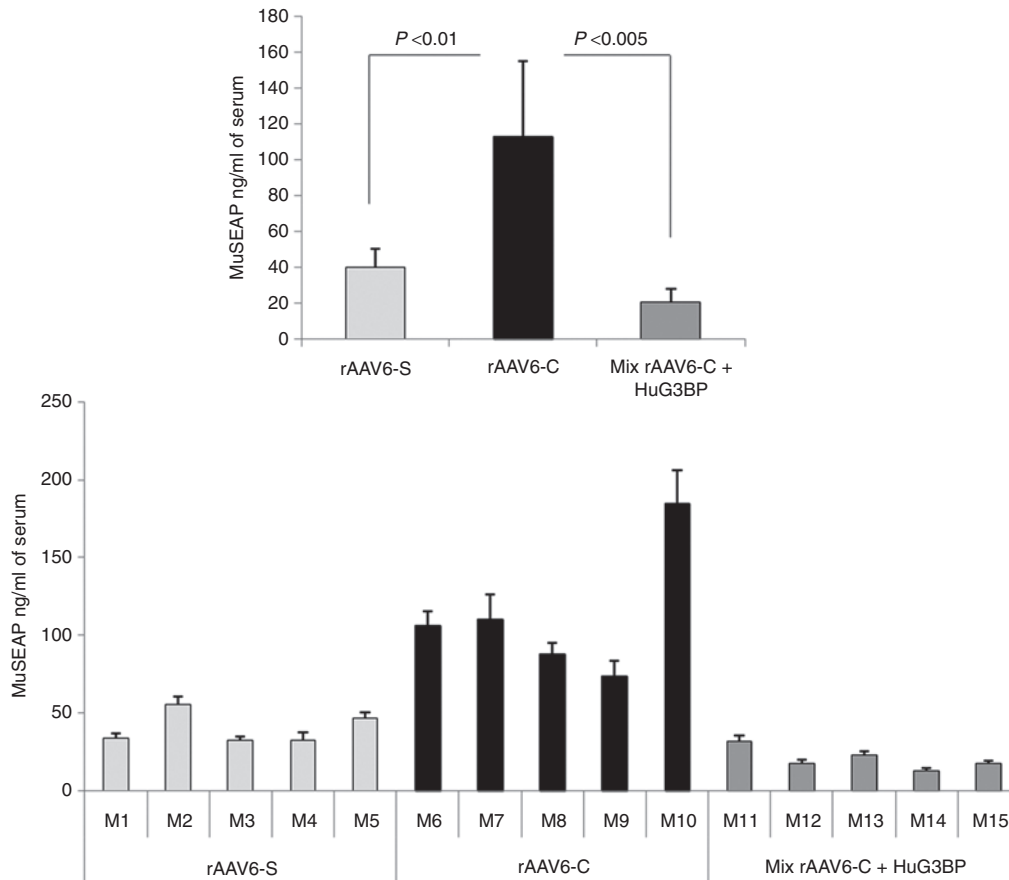


Figure 6 Presence of huG3BP in rAAV6-S attenuated transduction efficacy. Serum levels of MuSEAP were measured 2 weeks after intravenous injections of MuSEAP encoding rAAV6-S (M1 to M5), rAAV6-C alone (M6 to M10), or rAAV6-C preincubated with purified huG3BP at 20 $\mu\text{g}/\text{ml}$ (M10 to M15). Upper panel: transduction is expressed as mean of MuSEAP serum levels (ng/ml) \pm SD; lower panel: the same experiment, expression of MuSEAP in each injected mouse \pm SD of technical triplicates is shown.

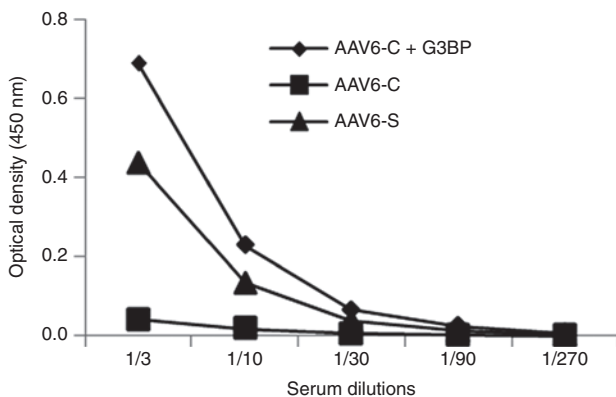


Figure 7 Systemic injections of rAAV6-S induce immune response against huG3BP. 1×10^{11} vg of either rAAV6-S or rAAV6-C or rAAV6-C preincubated with serum huG3BP coding for the MuSEAP under the CMV promoter were injected in the tail vein. The level of anti-huG3BP IgG was determined by enzyme-linked immunosorbent assay 2 weeks after injection.

huG3BP impacts rAAV6 efficiency *in vivo*

Previously we demonstrated that serum huG3BP initiates formation of rAAV6 aggregates leading to vector precipitation impairing vector efficiency.² Similar to the serum huG3BP, huG3BP present in rAAV6-S also initiates formation of rAAV6/huG3BP

aggregates *in vitro* (Figure 5). To test if the presence of huG3BP in rAAV6-S can influence the vector efficacy *in vivo*, huG3BP and rAAV6 seronegative mice (C57BL/6) received intravenous injections of: (i) the murine secreted embryonic alkaline phosphatase encoding rAAV6-S, (ii) rAAV6-C, or (iii) AAV6-C preincubated with purified huG3BP at the physiological concentration (20 $\mu\text{g}/\text{ml}$). rAAV6-S was less efficient than rAAV6-C, as illustrated by the threefold lower level of MuSEAP in serum 2 weeks after injection (40 ± 10 ng/ml for rAAV6-S versus 113 ± 42 ng/ml for rAAV6-C) (Figure 6). Importantly, addition of serum huG3BP to rAAV6-C decreased its activity to the level of rAAV6-S (21 ± 7 ng/ml), thus confirming the negative impact of huG3BP on rAAV6-S efficacy *in vivo*.

Systemic injections of rAAV6-S induce a humoral response against huG3BP

Human and mouse G3BP proteins have 68% of homology, and it is expected that injection of human protein can induce a humoral response in mice. Indeed, the quantity of huG3BP protein associated with rAAV6-S was able to induce an immune response in mice, while no anti huG3BP antibody formation could be detected when the animals received the rAAV6-C vector. Consistently, addition of serum huG3BP to rAAV6-C confirmed the immunogenicity of huG3BP in the mouse model (Figure 7).

DISCUSSION

Growing interest in AAV-based vectors requires easy and versatile vector production protocols. Conventionally, AAV vector production was based on purification from cell lysates, but recent studies demonstrated that most of the AAV vectors are released at high yields in culture medium.^{1,7} Comparison of *in vivo* properties of some serotypes (rAAV1, rAAV8, and rAAV9) purified either from cell lysates or from culture medium demonstrated that they were functionally equal when tested in the mouse model.¹ Assuming that protein composition of culture medium is simpler compared to cell lysates, purification procedures of secreted particles may be easier with high recovery yields. Nevertheless secreted proteins can specifically interact with rAAV vectors,^{2,3} potentially endangering rAAV vectors harvested from cell medium with specific contaminants absent in cell lysates. In the present study, we confirm this possibility by demonstrating that rAAV6 vector purified from cell culture medium but not from cellular lysate of HEK293 cells contains huG3BP protein.

G3BP is ubiquitously expressed, highly glycosylated glycoprotein with seven *N*-glycosylation sites occupied with complex oligosaccharide chains containing sialic acid,⁸ but not heparan sulfate.⁹ From a structural point of view, G3BP is composed of several domains consisting of a single scavenger receptor cysteine-rich domain at the *N* terminus; two central domains related to the dimerization domains BTP/POZ and IVR of the *Drosophila* kelch protein; and a *C*-terminal domain.^{8–11} Many of scavenger receptor cysteine-rich-containing proteins are expressed by cells involved in immunity (B cells, T cells, and macrophages) and are implicated in host defense and immune regulation.¹² G3BP interacts with galectin-3 and other extracellular proteins such as collagens IV, V, and VI, fibronectin, and nidogen. The protein forms ring-shaped oligomers with five to seven 14-nm long segments made of two 92 kD G3BP monomers.^{8,11} These ring structures form complexes with rAAV6 particles in which many rAAV6 are bridged with G3BP rings between them and impact the vector efficiency and biodistribution.²

Importantly, while huG3BP was present in the cytoplasm of HEK293 cells, this protein had higher electrophoretic mobility compared to the secreted G3BP and was unable to bind rAAV6 in the immunoprecipitation assay. We suggest that the cellular forms of huG3BP are not completely glycosylated and thus cannot form functional ring structures and therefore cannot bind rAAV6.

High affinity of huG3BP binding to rAAV6 (association constant of $\approx 10E9 M^{-1}$) and resistance of the complex to up to 4.1 mol/l of CsCl (the highest concentration used to purify rAAVs by cesium chloride ultracentrifugation), indicate that the codetection of huG3BP protein in rAAV6-S vector stocks is due to huG3BP/rAAV6 complex formation during vector cell release. Most importantly, the presence of huG3BP in rAAV6-S impacts on *in vivo* properties of the vector by diminishing its transduction efficiency as compared to rAAV6-C and also resulting in an anti-huG3BP humoral response in the mouse model. Even if such immune response may not occur in humans, it can undermine interpretation of data obtained from animal models.

Our results demonstrate that only the secreted, and not intracellular, form of huG3BP can react with the rAAV6 vector. As a consequence, rAAV6-S was purified as a rAAV6/huG3BP complex while rAAV6-C was not found associated with G3BP. Because *in vivo* properties of these two forms are different, special attention has to be paid when choosing between “culture medium” and “cell lysis” method of rAAV production.

MATERIALS AND METHODS

rAAV production

Adeno-associated virus vector-6 (rAAV6) was generated in a Corning CellSTACK—5 Chamber system by cotransfection of HEK293 cells with two plasmids (one coding for the vector genome and another for Rep, Cap and adenovirus helper genes) as previously described.^{13,14} Twenty-four hours after transfection, Dulbecco's modified Eagle's medium cell culture medium with 10% fetal bovine serum was replaced with the same medium without serum and 48 hours later culture medium and cells were harvested separately for vector purification. Vector particles from culture medium were precipitated by PolyEthylene Glycol (PEG-400). Cells were lysed by three rounds of freezing/thawing and vector particles were precipitated by ammonium sulfate. Both precipitates were treated by benzonase and purified by two cycles of cesium chloride centrifugation. For the first cycle, pellets were dissolved in 1.37 g/ml CsCl solution. Twenty milliliters of virus sample in 1.37 g/ml CsCl were overloaded on 0.5 ml of 1.5 g/ml CsCl in 12.5 ml Ultra-clear tubes. The samples were centrifuged for 36 to 48 hours at 288,000 $\times g$, 15 °C (41,000 rpm in a Beckman SW-41 rotor) and 10 drop fractions were collected from the bottom of the gradient. Repartition of viral particles through the gradient was followed up by Dot/Blot analysis with biotinylated DNA-probe specific for the transgene. Fractions containing rAAV were pooled, the volume was adjusted to 12 ml with 1.37 g/ml CsCl solution for the second round of CsCl purification under the same conditions. Pooled fractions containing rAAV after the second gradient were dialyzed in MWCO 10,000 Slide-A-Lyzer dialysis cassettes against three 500 ml changes of sterile 1 \times PBS for at least 3 hours each at 4 °C and stored at –80 °C.

The number of viral genomes (vg) was obtained by quantitative polymerase chain reaction of extracted vector DNA and the number of vector physical particles (pp) by an ELISA-based method (PRAAV1; Progen, Heidelberg, Germany) The ratio viral genome to physical particles in thus prepared vectors varied from 1/3 to 1/10.

Animals

Healthy, 4-week-old C57BL/6 mice were used in the study. All the procedures involving animals were performed according to the guidelines of our Institute's Animal Ethical Committee. For systemic delivery, 1 $\times 10E11$ vg of rAAV6 coding for the murine secreted embryonic alkaline phosphatase MuSEAP¹⁵ under the CMV promoter were injected into the lateral tail vein. Vectors and huG3BP at 20 $\mu g/ml$ (physiological concentration in human) were incubated for 1 hour at ambient temperature before injections. For intravenous injections, the final volume was adjusted to 120 μl with PBS. Mice were sacrificed 2 weeks after injection and serum levels of MuSEAP were evaluated by chemoluminescence reporter assay (TROPiX, Bedford, MA).

Production of huG3BP by HEK-293 cells

To estimate production of huG3BP by HEK-293, the cells were cultured in T-225 flasks for 3 days in Dulbecco's modified Eagle's medium culture medium with 10% fetal bovine serum. After culture medium harvest, cells ($40 \times 10E6$) were washed two times with PBS and detached from plastic by treatment with 12.5 mmol/l solution of ethylenediaminetetraacetic acid in PBS. Cells were collected by centrifugation and lysed in a buffer containing 150 mmol/l NaCl, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 50 mmol/l Hepes, pH 7.6 by four rounds of freezing/thawing. The lysed cells were clarified by centrifugation at 10,000 g for 10 minutes at 4 °C and the supernatant fraction (“intracellular”) was used in further experiments. Concentrations of secreted and intracellular huG3BP were determined by an ELISA assay (LGALS3BP (human) ELISA kit (Abnova)) according to the manufacturer instructions.

As reference standards, we used serum human G3BP (huG3BP) purified as described before² and human recombinant nonglycosylated G3BP (recG3BP, 65 kD MW) from GenWayBio (San Diego, CA).

Subcellular fractionation

Cells cultivated in T-flasks were washed five times with PBS and collected by scraping. After centrifugation, the cell pellet was lysed in a buffer containing 0.32 mol/l sucrose, 3 mmol/l CaCl₂, 2 mmol/l magnesium acetate, 0.1 mmol/l ethylenediaminetetraacetic acid 1% Triton X-100, 1 mmol/l DTT, 1 mmol/l sodium vanadate, protease inhibitor cocktail (Roche), and 20 mmol/l TrisHCl pH 7.5. Cytoplasmic fraction was recovered after centrifugation at 2,000 g for 10 minutes. Nuclear fraction was obtained by protein extraction from the precipitate with the same buffer supplemented with 0.42 mol/l NaCl and

centrifugation at 17,000 *g* for 30 minutes. The purity of the fractions was confirmed by western blot analysis with anti-hsp and anti-lamin A/C antibodies.

Coimmunoprecipitation assay

Coimmunoprecipitation of proteins from the culture medium or lysed cells with rAAV6 was performed with the vector immobilized either on immunoaffinity beads (AVB-Sepharose beads) (GE Healthcare Life Sciences, Piscataway, NJ) or on N-hydroxysuccinimide (NHS)-activated Sepharose (GE Healthcare Life Sciences). For one western blot assay, immobilized rAAV6 vectors (8 μ l of AVB Sepharose/ 1×10^{11} vector particles or 5 μ l NHS-activated Sepharose/ 1×10^{11} vector particles) were incubated for 1 hour with a volume of culture medium or cell lysate containing ~ 70 ng of huG3BP. Beads were collected by centrifugation, washed four times with PBS containing 0.25% Triton X-100 and the immunoprecipitates were analyzed by SDS-PAGE electrophoresis and western blot. To test the stability of the huG3BP/rAAV6 complex in the high CsCl concentrations, the first wash was performed in 500 μ l of CsCl solution (concentrations of CsCl varied from 0.3 to 4.1 mol/l) followed by the washing in the presence of PBS as described before.

Low-speed centrifugation

Putative aggregate formation of rAAV6 was assessed by low-speed centrifugation as described before with some modifications.² rAAV6 (2×10^{11} physical particles) was incubated in 60 μ l of PBS for 1 hour, centrifuged at 2,000 *g* for 10 minutes or at 10,000 *g* for 30 minutes and then the supernatant (50 μ l) was carefully discarded. Ten microlitre aliquots from the supernatant and precipitate were analyzed by SDS-PAGE and repartition of rAAV and huG3BP in the two compartments was estimated by the respective western blots analysis.

Western blot analysis

Proteins were separated by SDS-PAGE (4–12% gradient gel) and transferred onto a PVDF-Plus membrane (Millipore, Darmstadt, Germany). The following primary antibodies were used: goat polyclonal antibody to huG3BP (1:1,000; R&D Systems, Minneapolis, MN) and B1 mouse monoclonal anti-adenovirus-associated virus capsid proteins (1:300; Progen, Heidelberg, Germany) followed by IRDye-800CW or IRDye-600CW conjugated secondary antibodies (1:10,000) according to the manufacturer's instructions (LI-COR Biosciences, Lincoln, NE). Infrared fluorescence of the secondary antibodies was read on an Odyssey Imaging System.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Genethon *in vivo* evaluation core for the technical support and Anatolii Kokoza for proofreading of the manuscript. This work was supported by the Association Française contre les Myopathies.

REFERENCES

- Vandenbergh, LH, Xiao, R, Lock, M, Lin, J, Korn, M and Wilson, JM (2010). Efficient serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing. *Hum Gene Ther* **21**: 1251–1257.
- Denard, J, Beley, C, Kotin, R, Lai-Kuen, R, Blot, S, Leh, H *et al.* (2012). Human galectin 3 binding protein interacts with recombinant adeno-associated virus type 6. *J Virol* **86**: 6620–6631.
- Denard, J, Marolleau, B, Jenny, C, Rao, TN, Fehling, HJ, Voit, T *et al.* (2013). C-reactive protein (CRP) is essential for efficient systemic transduction of recombinant adeno-associated virus vector 1 (rAAV-1) and rAAV-6 in mice. *J Virol* **87**: 10784–10791.
- Iacobelli, S, Bucci, I, D'Egidio, M, Giuliani, C, Natoli, C, Tinari, N *et al.* (1993). Purification and characterization of a 90 kDa protein released from human tumors and tumor cell lines. *FEBS Lett* **319**: 59–65.
- Yuan, Z, Qiao, C, Hu, P, Li, J and Xiao, X (2011). A versatile adeno-associated virus vector producer cell line method for scalable vector production of different serotypes. *Hum Gene Ther* **22**: 613–624.
- Smith, RH, Levy, JR and Kotin, RM (2009). A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. *Mol Ther* **17**: 1888–1896.
- Okada, T, Nonaka-Sarukawa, M, Uchibori, R, Kinoshita, K, Hayashita-Kinoh, H, Nitahara-Kasahara, Y *et al.* (2009). Scalable purification of adeno-associated virus serotype 1 (AAV1) and AAV8 vectors, using dual ion-exchange adsorptive membranes. *Hum Gene Ther* **20**: 1013–1021.
- Hellstern, S, Sasaki, T, Fauser, C, Lustig, A, Timpl, R and Engel, J (2002). Functional studies on recombinant domains of Mac-2-binding protein. *J Biol Chem* **277**: 15690–15696.
- Sasaki, T, Brakebusch, C, Engel, J and Timpl, R (1998). Mac-2 binding protein is a cell-adhesive protein of the extracellular matrix which self-assembles into ring-like structures and binds beta1 integrins, collagens and fibronectin. *EMBO J* **17**: 1606–1613.
- Hohenester, E, Sasaki, T and Timpl, R (1999). Crystal structure of a scavenger receptor cysteine-rich domain sheds light on an ancient superfamily. *Nat Struct Biol* **6**: 228–232.
- Müller, SA, Sasaki, T, Bork, P, Wolpensinger, B, Schulthess, T, Timpl, R *et al.* (1999). Domain organization of Mac-2 binding protein and its oligomerization to linear and ring-like structures. *J Mol Biol* **291**: 801–813.
- Resnick, D, Pearson, A and Krieger, M (1994). The SRCR superfamily: a family reminiscent of the Ig superfamily. *Trends Biochem Sci* **19**: 5–8.
- Wang, L, Blouin, V, Brument, N, Bello-Roufai, M and Francois, A (2011). Production and purification of recombinant adeno-associated vectors. *Methods Mol Biol* **807**: 361–404.
- Vandenbergh, LH, Breous, E, Nam, HJ, Gao, G, Xiao, R, Sandhu, A *et al.* (2009). Naturally occurring singleton residues in AAV capsid impact vector performance and illustrate structural constraints. *Gene Ther* **16**: 1416–1428.
- Wang, M, Orsini, C, Casanova, D, Millán, JL, Mahfoudi, A and Thuillier, V (2001). MUSEAP, a novel reporter gene for the study of long-term gene expression in immunocompetent mice. *Gene* **279**: 99–108.



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>

Supplementary Information accompanies this paper on the *Molecular Therapy—Methods & Clinical Development* website (<http://www.nature.com/mtm>)