On the possible involvement of bovine serum albumin precursor in lipofection pathway

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Protein factors involved in lipofection pathways remain elusive. Using avidin-biotin affinity chromatography and mass finger printing analysis technique, herein we report the identification of a 70 kDa size protein (bovine serum albumin precursor, BSAP) which binds strongly with lipoplexes and may play role in lipofection pathway. Using multiple cultured animal cells and three structurally different cationic transfection lipids, we show that the efficiencies of liposomal transfection vectors get significantly enhanced (by ~2.5- to 5.0-fold) in cells pre-transfected with lipoplexes of reporter plasmid construct encoding BSAP. Findings in the cellular uptake experiments in A549 cells cultured in DMEM supplemented with 10% (w/w) BODIPY-labelled BSAP are consistent with the supposition that BSAP enters cell cytoplasm from the cell culture medium (DMEM supplemented with 10% FBS) used in lipofection. Cellular uptake studies by confocal microscopy using BODIPY-labelled BSAP and FITC-labelled plasmid DNA revealed co-localization of plasmid DNA and BSAP within the cell cytoplasm and nucleus. In summary, the present findings hint at the possible involvement of BSAP in lipofection pathway.

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1. Introduction

Viruses, owing to their natural abilities to infect cells, have been widely used in gene therapy as vectors for delivering genetic materials into our body cells (Seymour and Thrasher 2012). However, viral vectors are capable of inducing adverse immunogenic response (Hollon 2000). They can either inhibit tumour suppressor genes or activate oncogenes through ectopic chromosomal integration of viral DNA within the host chromosomes (Schröder *et al.* 2002; Woods *et al.* 2003; Li *et al.* 2002; Hacein-Bey-Abina *et al.* 2003). Thus, efforts are now being directed towards developing alternative non-viral transfection vectors, mainly cationic liposomes and cationic polymers. Cationic liposomes, due to their biocompatibility, less immunogenicity as well as easy handling techniques, are finding increasing applications as non-viral transfection vectors in gene therapy (Karmali and Chaudhuri 2007; Bhattacharya and Bajaj 2009).

Since the pioneering development of the cationic transfection lipid (*N*-(1-(2,3-dioleyloxy)propyl))-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) (Felgner *et al.* 1987), design of more efficient liposomal vectors have been based mostly on empirical structure-activity studies (Karmali and Chaudhuri 2007; Bhattacharya and Bajaj 2009; Rajesh *et al.* 2007) and biophysical investigations (Koltover *et al.* 1998; Koynova *et al.* 2006, 2009). Despite significant

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progresses, poor gene transfer efficiency remains a limiting factor impeding the clinical success of liposomal transfection vectors. The lipofection pathway, at the most, remains incompletely understood. Currently believed mechanistic steps involved in liposomal gene delivery (lipofection) include: (a) formation of liposome-DNA complex (lipoplex); (b) initial binding of the lipoplex to the cell surface; (c) endocytotic internalization of the lipoplex; (d) trafficking in the endosome/lysosome compartment and escape of DNA/lipoplex from the endosome/lysosome compartment to the cytosol; and (e) transport of the endosomally released DNA to the nucleus followed by its transgene expression (Zabner *et al.* 1995; Xu and Szoka 1996; Zelphati and Szoka 1996; Zuhorn *et al.* 2007; Szoka and Nguyen 2012). However, not much is known about protein factors involved in lipofection pathway. Rational design of efficient liposomal transfection vectors will be less of an arduous task with detailed molecular understanding of such factors, if any. To this end, in the present investigation we incubated lipoplexes of biotinylated plasmid DNA with cytoplasmic extract of cells. Thereafter, using avidin-biotin affinity chromatography and mass finger printing analysis technique, herein we report on the identification of a 70 kDa size protein (bovine serum albumin precursor, BSAP) which binds strongly with lipoplexes and therefore may play role in lipofection pathway. Using multiple cultured animal cells and three structurally different cationic transfection lipids, we show that the efficiencies of liposomal transfection vectors can significantly be enhanced (by ~2.5- to 5.0-fold) in cells pre-transfected with lipoplexes of reporter plasmid construct encoding BSAP. Cellular uptake studies by confocal microscopy using a fluorescently labelled BSAP revealed co-localization of plasmid DNA and BSAP within the cell cytoplasm and nucleus. In summary, the present findings hint at the possible involvement of BSAP in lipofection pathway.

2. Materials and methods

2.1 *Isolation of cytoplasmic proteins from CHO cells*

Total cytoplasmic proteins from CHO cells were isolated using conventional cell lysis buffer **A** (20 mM Hepes, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), antipain (1 μg/mL) and leupeptin (1 μg/mL)) and cytoplasmic extraction clarification buffer **B** (20 mM Hepes, pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 40% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 μg/mL) and leupeptin (1 μg/mL)).

CHO cells were cultured in T-75 tissue culture flasks. When the cells reached 70–80% confluency, they were collected using rubber polishman and gentle tapping. The cell pellets were washed twice with cold PBS (2 × 200 μL) and centrifuged. The supernatant were discarded and the pellets were suspended in 200 μL (two pellet volumes) of buffer A and left on ice for 10–15 min with occasional tapping. The nuclei were pelleted by centrifuging at 2000 rpm for 5 min at 4°C. The cytoplasmic supernatant fractions were collected in another eppendorf tube. To the cytoplasmic fraction, 1/3 volume of buffer B was added for further clarification. The mixture was kept on ice at 4°C for 30 min (to equilibrate the cytoplasmic proteins with the buffer contents), centrifuged at 13000 rpm for 15 min and the supernatant was used as cytoplasmic proteins extract.

2.2 *Isolation of BSAP by avidin-biotin affinity chromatography*

Liposome of lipid **1** was complexed with 1 μg of biotinylated β-gal DNA at lipid: DNA charge ratio 4:1 and was incubated for 15–30 min at 37°C. The total cytoplasmic proteins of the cells obtained from a T-75 flask were added to the lipoplexes and incubated for 1 h at 37°C. The protein-lipoplex mixture was loaded on an immobilized neutravidin (Promega, USA) column having 2 mL gel volume and was allowed to pass through the column. When the entire sample entered the gel, 1 mL of PBS was added on the top of the column and was incubated at 4°C for 1 h. The unbound fraction was eluted first with 12 mL (six column volumes) of PBS and the bound lipoplex-binding proteins were eluted with 12 mL (six column volumes) of 2 mM biotin solution. The column was regenerated by washing two times with 4 mL of regeneration buffer containing 2 mM glycine solution. The column was prepared for storage at 4°C by washing with 5 mL PBS containing a preservative such as 0.01 % sodium azide. The biotin eluted proteins were concentrated (by centrifuging over a 10 Kd amicon filter) to 500 μL which ensured removal of all the free biotin. After avidin-biotin affinity chromatography, the biotin eluted proteins may contain lipoplex-binding proteins as well as biotin-binding proteins. To remove the biotin-binding proteins, if any, the concentrated solution was placed on a biotin-coated plate (Promega, USA) at 4°C for 15 min. The supernatants containing only lipoplex-binding proteins were collected. Control affinity experiments were carried out by loading the following solutions onto the neutravidin column: (i) only total cytoplasmic proteins, (ii) the cytoplasmic proteins incubated with only liposomes and (iii) the cytoplasmic proteins incubated with only biotinylated DNA.

2.3 *Evaluating transfection efficiencies of the lipoplexes of lipids* **1–3** *in cells pre-transfected with lipoplexes of BSAP, β-galactosidase or GFP encoded plasmids*

Cells were seeded at a density of 10,000 cells per well in a 96-well plate 12 h prior to transfection and were transfected

with lipoplexes of 0.30 μg of *p*DNAs (encoding both BSAP and GFP, only β-galactosidase or only GFP) and liposomes of lipids **1–3** as described previously (Sen and Chaudhuri 2005; Singh *et al.* 2002, see supplementary material, Syntheses). After 48 h, the resulting pre-transfected cells were washed twice with PBS (100 μL), incubated with the (second) lipoplexes of the reporter p-CMV-luc plasmid for additional 48 h. Cells were then washed with PBS (100 μL) and lysed in 50 μL 1X lysis buffer (promega). The luciferase activity per well was estimated by adding 8 μL of luciferase substrate solution (Promega, Madison, WI, USA) to the 5 μL lysate in a white (non-transparent) 96-well plate in a Microplate Luminometer (FL_x800, Bio-Tek Instruments, USA). The transfection values reported are the average values from two replicate experiments performed in the same plate on the same day. Each transfection experiment was performed three times on three different days.

2.4 *Cellular uptake study by confocal microscopy*

Cells were seeded on a cover slip placed into a six well plate 12 h prior to transfection and were transfected with the ternary complex made of liposome of lipid **1**, BODIPY-labelled BSAP and FITC-labelled p-CMV-β-gal. After 4 h of transfection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% TritonX-100 in PBS for 5 min. The cover slip was then mounted on a slide using mounting medium (VectaShield) containing DAPI in 1 μg/mL concentration and was imaged under confocal microscope (Olympus, FV1000).

2.5 *Cellular uptake study with lyso-tracker*

A549 cells were seeded on a cover slip placed into a six well plate 12 h prior to transfection. The seeded cells were then transfected with the ternary complex of lipid **1**, p-CMV-β-gal and BODIPY-labelled BSAP in serum free medium. After 2 h, media was removed and the cells were treated with 50 nM green lyso-tracker (Molecular Probes, Invitrogen) solution in serum free medium. After 1 h, media was removed; the cells were washed twice with PBS, fixed with 4% para-formaldehyde in PBS for 20 min and permeabilized with 0.1% TritonX-100 in PBS for 5 min. The cover slip was finally mounted on a slide using mounting medium (VectaShield) containing DAPI in 1 μg/mL concentration and imaged under confocal microscope (Olympus, FV1000).

2.6 *Statistical analysis*

Statistical analyses were performed by a two-tailed Student's *t*-test and $P < 0.05$ was considered as significant.

3. Results

3.1 *Biotin-avidin chromatography reveals BSAP as a lipoplex-binding protein in lipofection pathway*

With a view to identifying important protein factor(s) involved in lipofection pathway using avidin-biotin affinity interactions, we first biotinylated the plasmid DNA (pCMV-SPORT-βgal) with Bio-Prime DNA labeling kit (Invitrogen, USA). The total cytoplasmic proteins isolated from the CHO cells were then incubated with lipoplexes of the biotin-labelled β-gal plasmid DNA and the cationic liposomes of a previously reported (Sen and Chaudhuri 2005) efficient guanidinylated cationic transfection lipid **1** (figure 1A). The mixture was then allowed to pass through a ready-to-use immobilized neutravidin column (Promega, USA). After eluting the unbound flow-through cytoplasmic protein fractions, the bound proteins were eluted with six column volumes of 2 mM free biotin. The pure biotin-binding proteins were separated from the lipoplex-binding proteins by incubating the biotin-eluted fraction with biotin-coated plates. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) of the lipoplex-binding protein mixtures showed essentially one major band of molecular weight ~70 kDa (lane 3, figure 1B). As expected, the unbound fraction eluted by PBS wash showed numerous cytoplasmic protein bands (lane 6, figure 1B). In the control experiment the cytoplasmic proteins were incubated with only biotinylated β-gal pDNA, and then loaded on to the neutravidin column. The biotin-eluted fractions obtained after the affinity purification did not show any proteins bands (lane 2, figure 1B) thereby ruling out the possibility that the isolated ~70 kDa size protein (lane 3, figure 1B) is just a pDNA-binding cytoplasmic protein. Toward confirming that the affinity purified protein band (lane 3, figure 1B) did not result from some non-specific binding interactions of a cytoplasmic protein with the neutravidin column matrix, we repeated the affinity chromatography by loading only cytoplasmic proteins on the neutravidin column. No band was detected in the biotin-eluted fraction in such control experiment (lane 1, figure 1B). Interestingly, a faint protein band (at ~70 kDa) was observed (lane 4, figure 1B) when the affinity chromatography was repeated after incubating cytoplasmic protein mixtures with only liposomes of lipid **1**. This finding supports the notion that the affinity purified 70 kDa protein (lane 3, figure 1B) is likely to possess some binding affinity for cationic liposomes. Importantly, the 70 kDa protein isolated from the biotin-eluted fraction (lane 3, figure 1B) closely matched in size with the major component of the bovine serum albumin (lane 5, figure 1B) (Sigma, Fraction V). The Coomassie stained gel band in 1D SDS-PAGE (lane 3, figure 1B) was air dried, trypsin digested and the resulting peptide fragments extracted as described

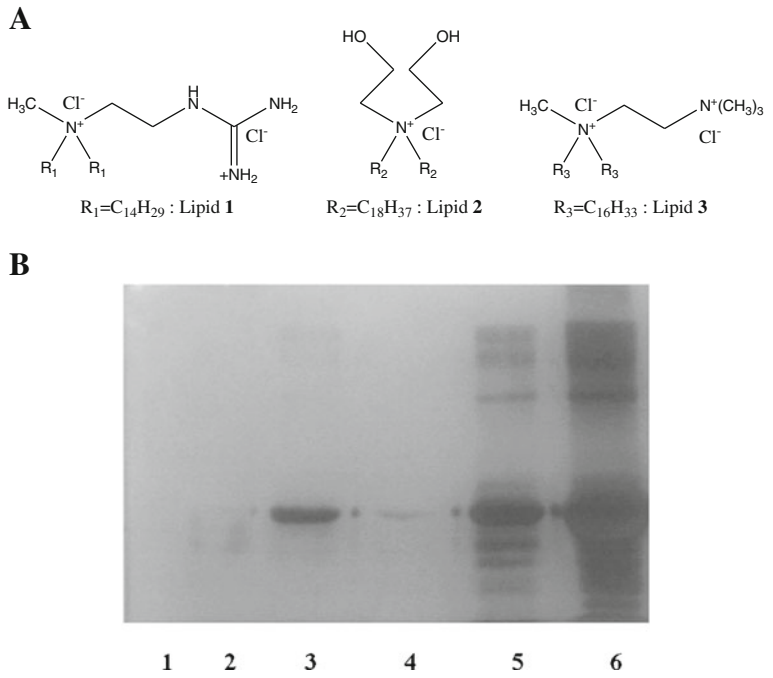

Figure 1. (**A**) Structures of the lipids **1–3**; (**B**) SDS-PAGE for fractions in avidin-biotin chromatography. Lane 1, only cytoplasmic proteins; lane 2, cytoplasmic proteins incubated with biotinylated DNA; lane 3, cytoplasmic proteins incubated with lipoplexes containing biotinylated DNA; lane 4, cytoplasmic proteins incubated with liposome only; lane 5, pure BSA protein; and lane 6, pass through fraction.

previously (Bringans *et al.* 2008). Peptide fragments were analyzed at Proteomics International, Australia by MALDI-TOF-TOF mass spectrometer using a 4800 Proteomics analyzer (Applied Biosystems) and Mascot sequence matching software (Matrix Science). The Mass Finger Printing Analysis with Mascot search revealed maximum hit for the affinity purified protein band with bovine serum albumin precursor (BSAP) of MW 70858 with Primary accession No. P02769.

3.2 *Reporter gene with encoded BSAP induces significant transfection enhancement*

Toward gaining insight into whether or not BSAP plays transfection enhancing role in lipofection, we envisaged, lipofection efficiencies in cells pre-transfected with BSAP encoded plasmid DNA should be significantly higher than those in untreated cells or in cells pre-transfected with control plasmid encoding non-BSAP proteins such as GFP, β-galactosidase, etc. With a view to address this issue, three cultured animal cells (CHO, COS-1 and A549) were first pre-transfected with lipoplexes of a reporter plasmid DNA encoding both green fluorescence protein (GFP) and BSAP under the same CMV promoter (pCMV-BSAP-GFP plasmid, RAS Life Sciences, Hyderabad). Control transfection experiments were carried out using cells pre-transfected with lipoplexes of two different reporter genes namely, p-CMV-GFP and pCMV-SPORT-β-gal (i.e. reporter genes with no encoded BSAP). Liposomes of three different cationic transfection lipids **1–3** (figure 1A) were used in these *in vitro* transfection experiments. Importantly, the transfection efficiencies of the all the lipoplexes were found to be significantly (by ~2.5–5.0 folds) enhanced in all the three cells pre-transfected with lipoplexes of pCMV-BSAP-GFP. Contrastingly, transfection efficiencies were not significantly enhanced when the cells were pre-transfected with the control lipoplexes of pCMV-GFP and pCMV-SPORT-β-gal (figure 2A–C). These findings are consistent with some transfection enhancing role of BSAP in lipofection pathway.

3.3 *BSAP co-localizes with plasmid DNA inside cell cytoplasm and nucleus*

Toward gaining insights into sub-cellular localization of BSAP, we prepared a fluorescently labelled BSAP. To this end, we first over expressed and purified BSAP using a second pET28a-BSAP plasmid (RAS Life Science, Hyderabad) in a widely used bacterial gene expression host BL21(DE3) which is superior to other commonly used strains of *E. coli* (Studier *et al.* 1990). The expressed BSAP protein was purified from Ni-NTA matrix following the Novagen purification manual and its purity was checked by FPLC (Bio-Rad). The purified BSAP protein was

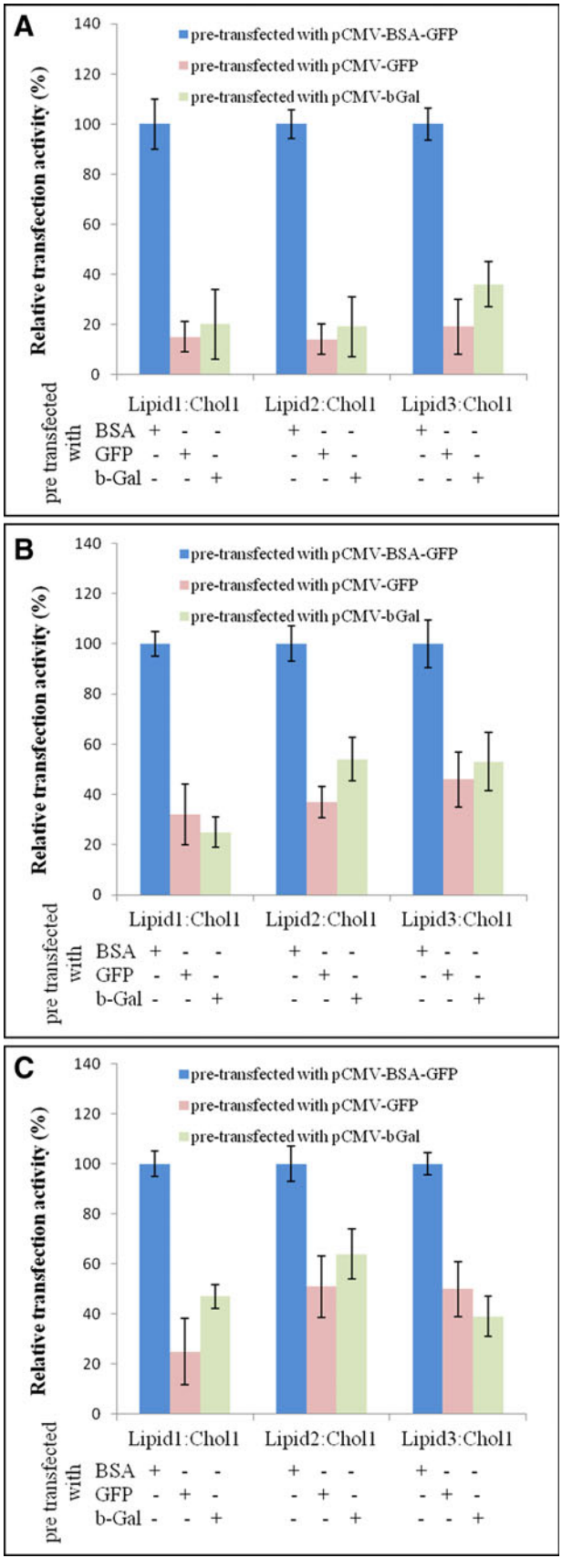

Figure 2. The transfection efficiencies of cationic amphiphiles are significantly enhanced in cells pre-transfected with BSAP-encoded plasmid DNA. A549 (**A**), CHO (**B**) and COS-1 (**C**) cells were pre-transfected with lipoplexes of different cationic lipids (lipids **1**–**3**) and three different reporter plasmids pCMV-BSAP-GFP, pCMV-GFP and pCMV-β-gal. 48 h after treatment, the cells were again transfected with lipoplexes of same cationic lipids **1**–**3** and pCMV-Luc. 48 h post pCMV-Luc lipoplex treatment, the luciferase expression were measured. Relative transfection efficacies in cells pre-transfected with lipoplexes of pCMV-GFP or pCMV-β-gal are shown are relative to the average luciferase expression efficiencies in cells pre-transfected with lipoplexes of pCMV-BSAP-GFP (shown as 100).

fluorescently labelled (red) with BODIPY (630/650) following the Instruction Manual of Molecular Probes, Invitrogen. Purities of both BSAP and BODIPY-labelled BSAP were confirmed by FPLC (supplementary figure 1). Next, A549, CHO and RAW 264.4 (mouse macrophage cells) were transfected with the ternary complexes of liposomes of lipid **1**, BODIPY-labled BSAP (red) and FITC-labelled p-CMV-β-gal (green). Superimposed confocal laser scanning microscopic (CLSM) images of the transfected cells revealed co-localization of green and red labels both in the cytoplasm and nucleus in all the three cells (figure 3). Fluorescent pDNA nuclear localization was confirmed through confocal z-scans consisting of 4 optical sections (1 μm step size) (supplementary figure 2A–B, Confocal micrographs for A549 and CHO cells are shown as representative examples) superimposed on their corresponding phase contrast images from the top to the bottom of the nuclei. Such co-localization of BODIPY-labelled BSAP and FITC-labelled plasmid DNA both in cytoplasm and cell nucleus (figure 3) does not confirm but hints on the possibility of BSAP helping nuclear translocation of reporter genes in lipofection pathway.

3.4 *Cell culture medium is a likely source of the affinity purified BSAP protein*

With a view to explore the possible source of the affinity purified BSAP protein, A549 cells were incubated in DMEM supplemented with 10% (w/w) BODIPY-labelled BSAP for 2 h, 4 h and 12 h. Cells were washed three times with PBS to remove excess BODIPY-labelled BSAP and then viewed under an inverted phase contrast fluorescence microscope. Presence of BODIPY-labelled BSAP inside the cell cytoplasm (figure 4) is consistent with the supposition that the BSAP enters cell cytoplasm from the cell culture medium (DMEM supplemented with 10% FBS) used in lipofection. Taking such possible culture medium origin and the significantly enhanced transfection properties of the lipoplexes of BSAP encoded reporter genes (figure 2) into account, we further envisaged that the transfection efficiencies of the presently described lipoplexes should, in

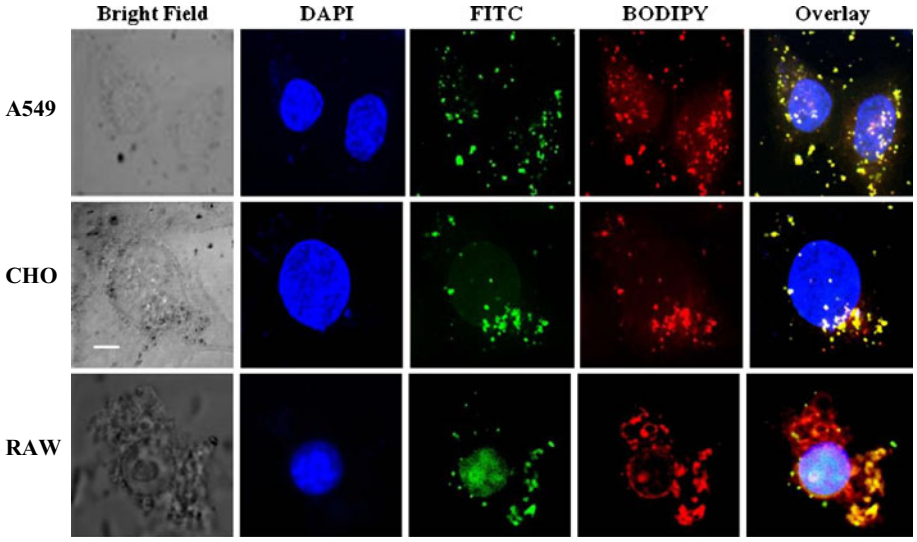

Figure 3. Co-localization of plasmid DNA and BSAP in both cell cytoplasm and nucleus. Confocal micrographs of the cells (A549, CHO, RAW) transfected with the ternary complex of lipid **1**, FITC-labelled p-CMV-β-gal and BODIPY-labelled BSAP protein. DAPI-405 (blue) channel, FITC-488 (green) channel, BODIPY (PI)-543 (red) channel. Confocal z-scan consisting of 4 optical sections (1 μm step size) superimposed on their corresponding phase contrast images from the top to the bottom of the nuclei to verify fluorescent pDNA nuclear localization. Bar (in CHO cell): 2 μm.

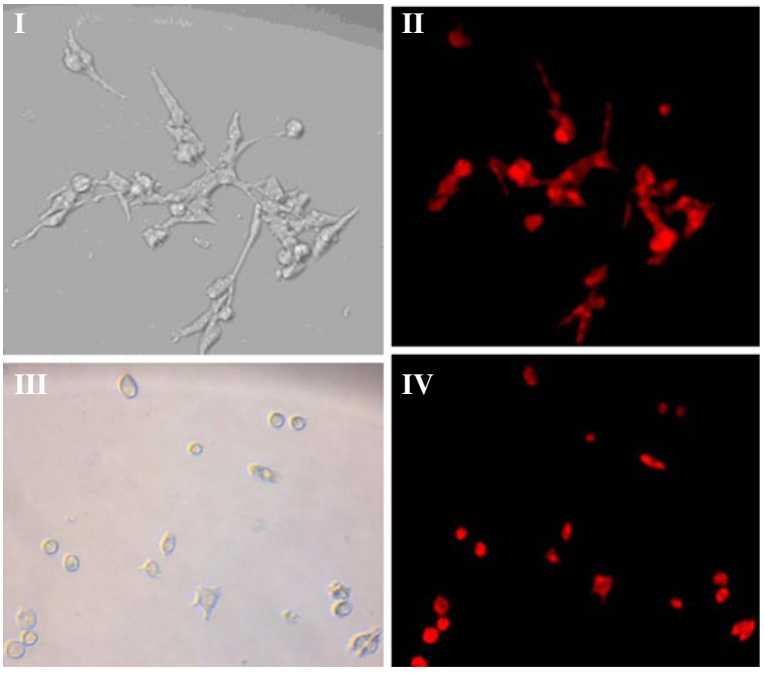

Figure 4. BSAP enters cell cytoplasm presumably from culture medium. Inverted fluorescence microghaphs of B16F10 and RAW cells cultured with medium containing DMEM and 10% BODIPY-labelled BSAP. **I** and **III** are the bright field micrographs and **II** and **IV** fluorescent micrographs.

principle, be severely compromised if the transfection experiments are carried out in cells slowly adapted to serum-substituted culture media.

3.5 *Lipoplex-associated BSAP localizes in lysosomal compartments in cell cytoplasm*

After obtaining insights into the culture medium origin of BSAP, we examined whether or not lipoplex associated BSAP ends up within lysosomal compartments of cells. To this end, we carried out a cellular uptake experiment using a ternary complex of liposomes of lipid **1**, BODIPY-labelled BSAP (red) and plasmid DNA in A549 cells lysosomes of which were pre-labelled with commercially available lyso-tracker (green). While most of the BODIPY-labelled BSAP was found to be present in the cell cytoplasm, only few were found to be within lysomal compartments (figure 5).

4. Discussion

Till date, most of the reported studies are centered on understanding cytoplasmic factors involved in transfection of cells

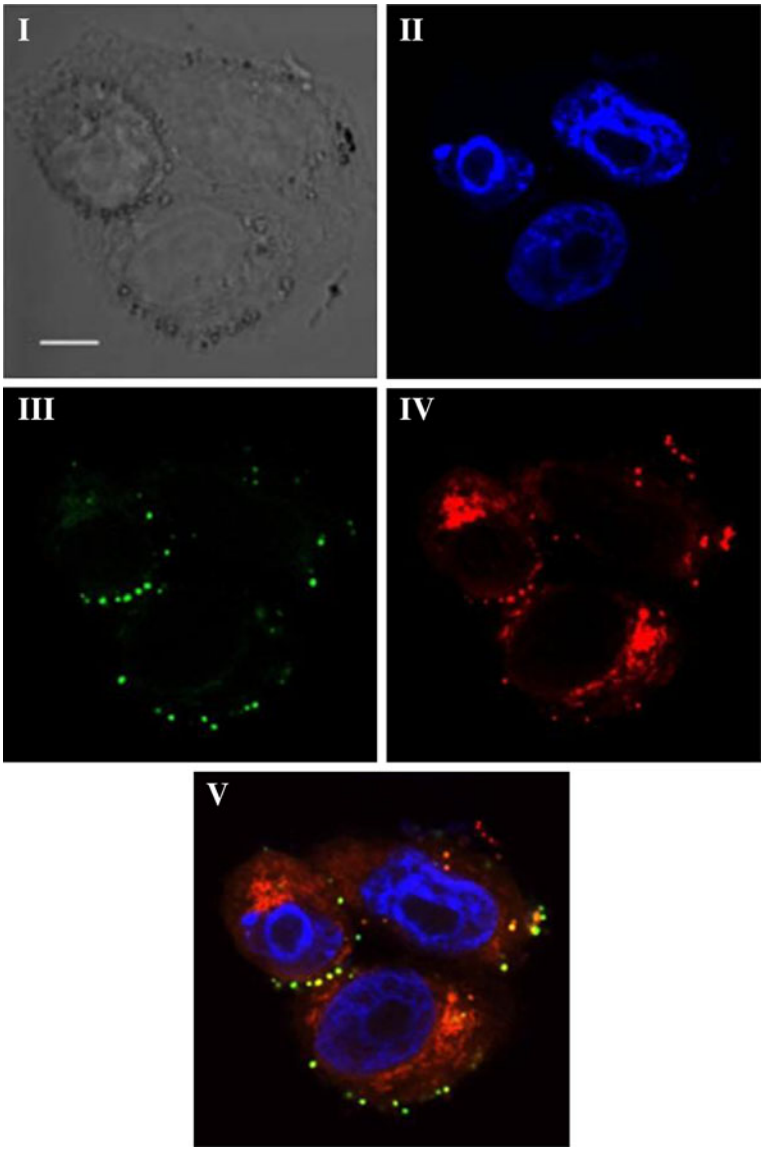

Figure 5. Lipoplex-associated BSAP gets localized to lysosomal compartments. Confocal microghaphs of cellular uptake in A549 cells with the ternary complex of liposome of lipid **1**, pCMV-β-gal, BODIPY-labelled BSAP protein and green lyso-tracker. DAPI-405 (blue) channel, FITC-488 (green) channel, BODIPY (PI)-543 (red) channel. I = Bright field, II = DAPI, III = green lyso-tracker, IV = BODIPY-labelled BSAP, V = overlay of confocal micrographs II, III and IV. Bar: 10 μm.

with naked plasmid DNA. For instance, using pCMV-β-DTS immobilized on Sepharose 4B-linked PNA, Munkonge *et al.* (2009) identified a number of pDNA nuclear shuttle proteins from cytoplasmic extract of HeLa cells including NM23-H2, a ubiquitous-Myc transcription-activating nucleoside diphosphate kinase and the core histone H2B. Using plasmid affinity chromatography, mass spectrometry and live-cell pulldowns of transfected plasmid constructs in smooth muscle cells, Miller *et al.* showed that importin β1 enhances nuclear uptake of plasmid DNA (Miller *et al.* 2009; Lam and Dean 2010). However, studies aimed at identifying unique protein factors with possible role in lipofection pathway have hardly been undertaken. Toward identifying transfection modulating protein factors in liposomal gene delivery, in the present investigation we have carried out an avidin-biotin affinity chromatography of cell cytoplasmic extract over a neutravidin column after incubating it with electrostatic complexes (lipoplexes) of cationic liposomes and biotinylated reporter plasmid DNA. A single protein band of ~70 kDa size was identified in the biotin eluted fraction. Mass fingerprinting analysis with Mascot search revealed maximum hit for the affinity purified protein band with bovine serum albumin precursor (BSAP) of MW 70858 (Primary accession No. P02769). It is worth mentioning at this point of discussion that the molecular mass of our affinity purified BSAP protein (70858 Da) is somewhat higher than that of serum albumin with protein accession no. P02769 reported in the website (*http://www.uniprot.org/uniprot/P02769*). The number P02769 is the primary accession number used for the entry name ALU_BOVIN. Application of 2D-PAGE gel characterization technique has revealed existence of several serum albumin proteins (with same primary accession number of P02769) with varying molecular weights around 70 kDa (Kim *et al.* 2010). The molecular mass of 70858 for BSAP reported in the present study exactly matches that of a serum albumin protein reported in the supplementary information (*http://www.rsc.org/suppdata/nr/c3/c3nr32551b/c3nr32551b.pdf*) of a very recently published work (Mahmoudi *et al.* 2013). BSAP cannot be just a biotin-binding protein since no significant bands were detected in the biotin-eluted fractions when affinity chromatography was repeated with cell cytoplasmic mixture incubated with only biotinylated plasmid DNA. We incubated the biotinylated lipoplexes with cytoplasmic protein mixture only after pelleting down the nuclei. This could be why we did not detect any nuclear histone proteins in the bound fraction. However, it may not be impossible that the essentially single protein band observed in one-dimensional SDS-page of the affinity purified fraction contains more than one co-eluted proteins of similar molecular weight. Careful isoelectric focusing study (2D SDS-page) needs to be carried out in future toward gaining more insights into such possibility. Essentially one protein being identified in the present avidin-biotin affinity approach presumably originates from high lipoplex binding affinities of serum proteins (serum proteins are well known for their high affinity for fatty molecules e.g. fatty acids) coupled with the high cytoplasmic concentrations of serum proteins. With a view to probe transfection modulating role of the affinity purified BSAP, if any, we next compared the gene transfer efficiencies of cationic lipids **1–3** in three different cells (CHO, COS-1 and A549) pre-transfected with lipoplexes of reporter plasmids encoding BSAP. As controls, the gene transfer efficiencies of the same three cationic lipids were also measured when cells were pre-transfected with lipoplexes of reporter plasmids not encoding BSAP but encoding other control proteins namely, β-galactosidase enzyme and green fluorescence protein. The findings that all the three cationic lipids **1–3** showed significantly enhanced (by~2.5- to 5.0-folds) reporter luciferase gene expressions when cells were pre-transfected with lipoplexes of BSAP-encoded plasmid DNA strongly hint at transfection enhancing role of BSAP in lipofection pathway. Absence of any significant enhancements in transfection effiencies of lipids **1–3** in cells pre-transfected with lipoplexes of plasmid DNA encoding control non-BSAP proteins (β-galactosidase or GFP) further confirmed such proposition. Such transfection enhancing role of the currently described affinity purified BSAP is consistent with previous report in which it was shown that coating of cationic liposomes with human serum albumin and subsequent complexation with reporter luciferase plasmid greatly enhances luciferase expression in epithelial and lymphocytic cells when compared with transfection of plain lipoplexes (Simões *et al.* 2000).

With a view to gain some mechanistic insights into the origin of enhanced transfection efficiencies in cells pre-transfected with BSAP-encoded plasmid DNA, we performed a confocal microscopy study using FITC-labelled plasmid DNA and BODIPY-labelled BSAP. Co-localization of BSAP and plasmid DNA in both cell cytoplasm and nucleus indirectly hints at some possible carrier role of BSAP in translocating plasmid DNA from cell cytoplasm to nucleus. However, caution needs to be exercised not to over-interpret such findings in confocal microscopy studies since mere co-localization of FITC-labelled plasmid DNA and the BODIPY-labelled BSAP in cell nucleus can never be an evidence for BSAP playing a carrier role in nuclear translocation of plasmid DNA in liposomal gene delivery. It may well be that it is just a coincidence. It may also be possible that nuclear localization of BSAP is mediated by some other cytoplasmic adaptor protein factor(s) containing nuclear localization signal (NLS). Clearly, further mechanistic experiments need to be conducted in future toward understanding the origin of enhanced transfection efficiencies of cationic amphiphiles in cells pre-transfected with BSAP-encoded plasmid DNA.

It is believed that endocytotic cellular uptake of lipoplexes leads to close physical proximity of cationic lipids and anionic lipids of endosomal membrane components, which in turn induces formation of ion-pair between them. Such ion-pairing leads to transition from lamellar to non-lamellar inverted hexagonal phase with the concomitant release of plasmid DNA from endosome to cell cytoplasm (Zabner *et al.* 1995; Xu and Szoka 1996; Zelphati and Szoka 1996; Zuhorn *et al.* 2007; Szoka and Nguyen 2012). The findings in our cellular uptake experiments using ternary complex of liposome of lipid **1**, plasmid DNA and BODIPY-labelled BSAP (red) revealed majority of the BODIPY-labelled BSAP not to be co-localized with lysosomal compartments pre-labelled with green lysotracker. This finding combined with the significantly enhanced transfection efficiencies of lipids **1–3** in cells pre-transfected with BSAP-encoded plasmid DNA raises the interesting possibility of BSAP in complexation with lipoplexes promoting lamellar to non-lamellar structural transition of lipoplexes in lipofection pathway. Clearly, further cell biology experiments need to be conducted in future toward confirming such lipoplex structure perturbing capabilities of BSAP. Not much is known about the protein factors playing role in lipofection pathway. The presently identified BSAP might have played important role in many previously conducted empirical *in vitro* transfection optimization studies where the transfection efficiencies of cationic liposomes were found to be significantly enhanced with increasing amounts of added serum (DeLima and Simões 2004; Mukthavaram *et al.* 2009; Srujan *et al.* 2011; Mukherjee *et al.* 2008).

In summary, exploiting avidin-biotin affinity chromatography, we have shown that bovine serum albumin precursor (BSAP) possesses strong affinity for lipoplexes. Our findings that the transfection efficiencies of three different cationic amphiphiles in three different cultured animal cells get remarkably enhanced (by ~2.5- to 5.0-fold) when cells are pre-transfected with plasmid DNA encoding BSAP strongly support transfection enhancing role of BSAP in liposomal gene delivery. Findings in the cellular uptake experiments in A549 cells cultured in DMEM supplemented with 10% (w/w) BODIPY-labelled BSAP are consistent with the supposition that BSAP enters cell cytoplasm from the cell culture medium (DMEM supplemented with 10% FBS) used in lipofection. Cellular uptake studies by confocal microscopy using BODIPY-labelled BSAP and FITC-labelled plasmid DNA revealed co-localization of plasmid DNA and BSAP within the cell cytoplasm and nucleus. Such findings hint at the possibility of involvement of BSAP in nuclear translocation of plasmid DNA in lipofection. In summary, the present findings hint at the possible involvement of BSAP in lipofection pathway.

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