

In-vitro Interaction of α B-Crystallin on Serum Amyloid A and Serum Amyloid A Fibrils with Hepta 1-6 Cells

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ABSTRACT

The interactions of SAA and SAA protofibrils with protecting role of α B-Crystallin with hepta 1-6 cells of the mouse are dealt with in detail to study the binding of SAA protofibrils in various conditions. Specifically, interaction of serum amyloid A fibrils with a cell surface binding site/receptor might alter the local environment to cause cellular dysfunction and to be more favorable for amyloid formation and prevention with α B-Crystallin. This is important in relation to the activity of membrane proteins, because losing the activity of such systems will ultimately lead to malfunction or death of the cell. The interactions of Serum Amyloid A (SAA) and Serum Amyloid A protofibrils with hepta 1-6 cells of the mouse are dealt with in detail to study the binding of SAA protofibrils in various conditions. The induced fluorescence, induced circular dichroism, FACScan and MTT assay results have shown the SAA and SAA protofibrils binding and cell toxicity with the hepta 1-6 cells with different concentrations of α B-Crystallin 0.15-15 nM. Specifically, cells were incubated with 1.25-6.25 μ M SAA-FITC and SAA protofibrils-FITC assayed. The 50% viable hepta 1-6 cells at 4–6 μ M with an LD_{50} of 3.5 μ M. The interaction of serum amyloid A fibrils with a cell surface binding site/receptor might alter the local environment to cause cellular dysfunction and to be more favorable for amyloid formation. In the present study, concluding that the SAA fibrils and SAA protein binding and cell cytotoxicity was reduced in the presence of α B-Crystallin.

Key words: Serum Amyloid A, Crystallin, hepta 1-6 Cells, FITC, Fibril, Protofibril.

Date of Submission: 05 August 2015



Date of Accepted: 15 August 2015

I. INTRODUCTION

Small heat-shock proteins (sHSPs) are one of four families of heat-shock proteins (HSPs) expressed in response to heat shock and other forms of stress (Parsell and Lindquist 1993). They are a diverse family of proteins that appear to be ubiquitous in nature, being found as surface antigens in eukaryotic parasites, as inclusion body-binding protein in E. Coli, and as structural proteins in the vertebrate lens (Caspers et al 1995). Despite their low molecular mass (12-40 kDa), sHSPs are isolated as large oligomeric complexes of 2-40 subunits depending on the physiological state of the cell. Distant members of the family have relatively low sequence similarity except for a highly conserved stretch of 100 amino acids, often called the α -Crystallin domain (Caspers et al 1995). Although their cellular function under physiological conditions is still largely unknown, the increased expression of sHSP is associated with cell survival under heat stress (Arrigo and Landry 1994). The role of these proteins in thermotolerance appears to be a consequence of their ability to function as molecular chaperones and to modulate actin filament dynamics (Lee et al 1995). sHSPs from many species inhibit the unfolding-induced aggregation of proteins in an ATP-independent manner and form a stable complex with their protein substrate. Thus, during periods of stress, sHSPs act as energy-independent traps preventing the irreversible aggregation of proteins. Recently, it has been shown that upon establishment of refolding conditions, proteins bound to sHSPs are efficiently refolded in cooperation with other chaperones (Lee et al 1997)

The evaluation of the role of the α -Crystallin domain and consequent insight into the molecular mechanisms involved in these diverse functions are hampered by the lack of structural information on HSP 27 and sHSPs in general. The major difficulty relates to the considerable flexibility of their quaternary structure (Groenen et al 1994). This flexibility, believed to be critical for their function, results in conformational heterogeneity that to date has prevented the crystallization of these proteins. Therefore, there is limited information concerning the tertiary structure of the subunits and their arrangement in the oligomer. It has been proposed that mammalian sHSPs have a tertiary structure similar to that proposed for lens α -Crystallin subunits (De Jong et al 1993). The model, proposed by Wistow, consists of two domains, each of two structurally similar

motifs with a dynamic C-terminal arm (Wistow 1993). On the secondary structure level, HSP 27 appears to be composed mainly of β -sheets as revealed by far-UV CD (Merck et al 1993). Electron microscopy studies indicate that mouse HSP25 forms ring-like particles, Behlke et al. (1991) proposed that these particles are composed of 32 monomers in hexagonal packing.

The research subunit is investigating the in-vivo functions of the lens α -Crystallin proteins (α A and α B). These proteins, abundant in vertebrate lenses, were originally thought to be solely structural proteins, but in recent years have been shown to possess a myriad of activity in-vitro including molecular chaperone activity, autokinase activity, DNA binding activity and binding to and regulating the Polymerization State of several cytoskeletal proteins. Mice lacking the α -Crystallins have been generated and are being analyzed. Mice lacking α B-Crystallin appear to be completely normal. Mice lacking α A however develop cataracts beginning early in life and progressing in severity with age. In the absence of α A, the α B forms large insoluble masses, 1 to 3 μ m in diameter, in the cytoplasm in lens fiber cells. Lenses of mice lacking α B-Crystallin appear to be completely normal. However, these mice exhibit dystrophy of select skeletal muscles, particularly those of the head (especially tongue) and those surrounding the spine. Mice lacking both α -Crystallins exhibit a severe cataract very early in life and also develop the muscular dystrophy observed in α B-Crystallin knockout mice (Xu et al 2000).

The role of α B-Crystallin in the development of these diseases is largely unknown but may be related to its functional role as a small heat shock protein (Caspers et al 1995). Recently, α -Crystallin was reported to act as a chaperone molecule capable of protecting other proteins from denaturation (Horwitz, 1992; Horwitz 1993). Whether this chaperone function is active in-vivo either in the lens, or, in other tissues, is unknown. To understand the effect of α B-Crystallin on A β fibril formation, we need to determine whether they interact with each other. In the present study, in-vitro studies on the role of the interaction of SAA/SAA fibrils with chaperones were investigated. The results indicated that there was interaction between SAA and α B-Crystallin when they were incubated together with hepta 1-6 Cells. A possible mechanism for this interaction and its implicated significance in-vitro are discussed.

II. MATERIALS AND METHODS

Isolation of α B-Crystallin

Bovine lenses (8-10 lenses) were decapsulated and homogenized (Luthra and Balasubramanian 1993) in 0.1 M Tris buffer, pH 7.4 containing 0.5 M NaCl, 1 mM EDTA, and 0.1% NaN₃. The insoluble protein fraction and membrane debris was removed by centrifugation at 30,000g for 30 min. The supernatant was chromatographed on a Sephacryl-S200 (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (0.8 \times 90 cm) to separate the alphaB-Crystallin using Gradifrac™ FPLC system (Pharmacia Biotech, Uppsala, Sweden). Each of the alphaB-Crystallin fractions was dialyzed repeatedly against water using FILTRON concentrating 10000 D. Molecular weight cut off membrane (Gelman Sciences, India) and lyophilized and stored at -20°C. Protein was determined by Lowry et al (Lowry et al 1951). Homogeneous 12% SDS-polyacrylamide gels were run on the mini gel electrophoresis system (Laemmli, 1970).

Isolation of SAA

SAA was isolated from the plasma of casein injected (Botto et al 1997) mice as reported previously by Lindhorst et al (Lindhorst et al 1997). It was then characterized using 17% SDS-polyacrylamide gels. Isolated SAA was purified by Waters reverse phase-high performance liquid chromatography (Kaplan et al 1999) (RP-HPLC) analytical column 4.6 \times 250 mm Spherisorb ODS2 LC₁₈ (Waters, Milford, Massachusetts, USA) and size exclusion chromatography (SEC) columns using a series of 7.8 \times 300 mm Ultrahydrogel 250™ and Ultrahydrogel 500™ the molecular weight was determined (Waters, Milford, Massachusetts, USA).

Fluorolabelling of SAA and SAA fibrils

Purified SAA and SAA protofibrils (400 μ g/400 μ l 0.001% NH₄OH, pH 9.0) were labeled with fluorescein isothiocyanate (FITC) (Fluka, USA) by dialysis through a 1000 D cutoff membrane. FITC (0.4 mg) was dissolved in 40 μ l DMSO and added to 40 ml of PBS adjusted to pH 9.0 with NH₄OH. After 12 hrs at 4°C the SAA-FITC and SAA protofibrils-FITC were dialyzed against milli-Q water, sterile filtered (0.45 μ m) and held at 4°C (Linke et al 1991). 1.25-6.25 μ M of SAA-FITC and SAA protofibrils-FITC were assayed with 0.15-15 nM of α B-Crystallin.

Single Photon Counting Life-time Measurements

1.25-6.25 μ M of SAA-FITC and SAA protofibrils-FITC was assayed with 0.15-15 nM of α B-Crystallin for lifetime measurements (FL, at λ_{\max} 530 \pm 15 nm) (Spectra physics, IBH Consultants, Scotland, UK).

Induced Circular Dichroic Spectroscopic Studies

1.25-6.25 μ M of SAA-FITC and SAA protofibrils-FITC were assayed with 0.15-15 nM of α B-Crystallin for induced CD measurements (Jasco J-715 Spectropolarimeter, Tokyo, Japan).

FACScan Analysis: hepta 1-6 Cells.

Mice hepta 1-6 cells were incubated with 1.25-6.25 μ M of SAA-FITC and SAA protofibrils-FITC assayed with 0.15-15 nM of α B-Crystallin for 24 hrs at 4°C, washed in PBS, fixed immediately in 1% paraformaldehyde in PBS, and assayed for cellular fluorescence (FL, at λ_{\max} 530 \pm 15 nm), forward scatter (FSC), and side scatter (SSC). Binding was analyzed on a FACScan flow cytometer using FACScan analysis software WINMDI (Becton Dickinson, San Jose, CA, USA).

MTT assay

1.25-6.25 μ M of SAA-FITC and SAA protofibrils-FITC were assayed with 0.15-15 nM of α B-Crystallin (Rousset 2001) for incubation with hepta 1-6 cells for 24 hrs. hepta 1-6 cells were fed with 200 μ L fresh medium at the ends of the growth period and added 50 μ L of MTT to all wells in columns 1 to 6. Wrapped plates in aluminum foil and incubated for 4 hrs in a humidified atmosphere at 37°C. This is a minimum incubation time and plates can be left for up to 8 hrs. Removed the medium and MTT from the wells and dissolved the remaining MTT-formazan crystals, adding 200 μ L of DMSO to all wells in columns 1 to 6. Added glycine buffer (25 μ L per well) to all wells containing the DMSO. Recorded absorbance at 570 nm immediately, since the product was unstable. The wells in column 1, which contained medium, MTT, but no cells, were used to blank the plate reader.

III. RESULTS

The fluorescence spectral changes observed on α B-Crystallin binding to FITC-SAA was shown in spectrum Fig. 1a. During addition of α B-Crystallin, the fluorescence at 516 nm decreased gradually indicating that FITC-fluorescence was quenched by the disulfide bonds in α B-Crystallin. The fluorescence intensity was increased during addition of α B-Crystallin to SAA protofibril-FITC. This increase in FITC intensity may be associated with the conversion of protofibrils by α B-Crystallin in lower concentration (at 5 nM). However, further addition of α B-Crystallin does not change the fluorescence intensity. The effect of α B-Crystallin concentration on lifetime of FITC-SAA protofibril is indicated in Fig. 1b.

The average fluorescence lifetime decreased from 3.0 to 1.2 ns indicating that the fluorophore is in highly polar environment than the previous state. In high α B-Crystallin concentration (30 nM), there was quenching of FITC in SAA by disulfide groups around the environment. The amplitude for long lifetime (τ_1) was decreased significantly from 50% to 40% during addition of α B-Crystallin. However, short-lived species was constant during further α B-Crystallin incubation (Fig. 2a and b).

From the induced CD studied, changes in FITC environment of SAA were measured at different concentrations of α B-Crystallin. The CD band at 450 and 505 nm were changed during addition of α B-Crystallin. The measured ellipticity values were plotted against various concentrations of α B-Crystallin which, is given in Fig. 3a. The ellipticity attained saturation at 2 nM α B-Crystallin. Further addition of α B-Crystallin did not change the ellipticity at 505 nm. FITC labeled SAA protofibril also showed similar effect on addition of α B-Crystallin. The CD band at 450nm gradually decreased as a function of α B-Crystallin (Fig. 3b). The negative band at 505 nm increased even at very low concentration of α B-Crystallin. Further addition (7.5 nM) did not change the ellipticity significantly.

Flow cytometry revealed that the SAA-FITC positive binding readily occurred in the presence of α B-Crystallin in hepta 1-6 brain cells and is shown in histograms. Both mean fluorescence intensity, as well as percentage of positive cell increased as a function of α B-Crystallin. However, the binding of SAA-FITC was almost completely prevented by simultaneous addition of α B-Crystallin. The histogram (Fig. 4a) showed the decrease in the percentage of positive binding than in the FITC-SAA control experiment during addition of α B-Crystallin. The mean fluorescence intensity was decreased to 45% of control values 55% in hepta 1-6 cells, respectively (Fig. 4a). The cell volume and granulater changes were also studied by using forward and side scatter plot. In hepta 1-6 cells studied the forward scatter values were not varied significantly indicating that the volume of cells remain constant during α B-Crystallin addition. However, Side scatter values were increased significantly indicating that the α B-Crystallin treated cells were highly granular (Fig. 4b, c and d).

We next studied the cellular binding of SAA protofibril-FITC in the presence of various concentration of α B-Crystallin. The SAA protofibril-FITC binding to hepta 1-6 cells decreased markedly during the addition of α B-Crystallin. The mean fluorescence intensity decreased from 70% to 30% as shown in FL plot. From the histogram, the shifting of cell population from fluorescence positive region towards control cells (Fig. 5a) is seen. Further the cell cytotoxicity studies were carried out by MTT assay.

IV. DISCUSSION

The principal role of SAA during the acute-phase reaction appears to be the association with HDL-particles and subsequent changes of apolipoprotein composition and metabolic properties of its physiological carrier. Because SAA may displace apolipoprotein A-I, the major apolipoprotein of HDL, it is hypothesized that SAA could alter the protective function of HDL during 'reverse cholesterol transport (Artl 2000). SAA binding acute phase reaction was studied with macrophages (representative peripheral cells) revealed that the binding affinity for SAA is enhanced, Earlier findings suggest that extracellular matrix proteins appear to serve as a temporary anchorage sites for SAA and amyloid A (Preciado-Patt et al 1996).

The FITC fluorescence quenching ability of protein varies in proportion to disulfide groups. In monomeric SAA, addition of α B-Crystallin causes quenching of fluorescence. This clearly indicates that FITC environment in SAA is near to the disulfide groups of α B-Crystallin. However, the mechanism of binding of α B-Crystallin to SAA is still unknown. The SAA protofibril-FITC also interacts with α B-Crystallin resulting in enhanced fluorescence intensity. This is possibly due to exposure of FITC region of SAA to a more polar environment. In the case of the α B-Crystallin binding to protofibril, the FITC region is far away from the disulfide groups of α B-Crystallin. Decay of FITC labeled SAA showed multiple fluorescent lifetime species and addition of α B-Crystallin significantly decreased the average fluorescence lifetime of FITC. The decrease in lifetime of FITC is possibly due to the association of α B-Crystallin to the SAA. The interaction of disulfide groups in α B-Crystallin on FITC groups of SAA quenched the fluorescence of probe, which is accompanied by a decrease in fluorescence lifetime.

From the induced CD studies, it is clear that the folding of SAA occurs up to 2 nM concentration of α B-Crystallin. Further addition of α B-Crystallin did not change the FITC region of SAA. However, in the protofibrils, FITC-environment changes were observed only upto 1 nM of α B-Crystallin. Further addition of α B-Crystallin did not altered the conformation of FITC molecular environment indicating the completion of the folding process at low concentration of α B-Crystallin in protofibrillar than in native form of SAA.

Using flow cytometric studies, the competing nature of α B-Crystallin with monomeric and protofibrillar SAA binding was studied in hepta 1-6 cells. α B-Crystallin did not compete with monomeric SAA binding to hepta 1-6 cells. This is possibly due to the occurrence of a large number of high affinity SAA binding sites on hepta 1-6 cells. However, hepta 1-6 cells the SAA binding decreased in the presence of α B-Crystallin. This effect is either due to the presence of low affinity SAA binding site or competing effect of α B-Crystallin with SAA in the binding process. α B-Crystallin may prevent the SAA mediated toxic mechanism in hepta 1-6. Studies shown in hepta 1-6 cells increases in both muscle protein degradation and levels of mRNAs encoding components of the ubiquitin-proteasome pathway have been found in association with other catabolic conditions, including sepsis, cancer, burns, starvation, and denervation. Consequently, insights into the mechanisms leading to increased mRNAs for components of this pathway could have widespread interest (Bailey et al 1996). The metabolic acidosis causes infants and children to grow poorly, and showed that induction of metabolic acidosis in normal adults by administration of ammonium chloride accelerated catabolism of both protein and branched-chain amino acids (BCAAs). Experimental metabolic acidosis in rats reduces growth and stimulates whole body proteolysis and BCAA catabolism (Isozaki et al 1971).

Protofibril binding to hepta 1-6 cells significantly decreased in the presence of α B-Crystallin. This effect may be either due to α B-Crystallin complexation of SAA protofibrils. The α B-Crystallin incubation with SAA significantly increased the granularity in cell lines. Endocytosed proteins and most membrane components are degraded by another proteolytic system, acidic proteases in lysosomes, but this organelle also appears to be important in degrading certain cytosolic proteins, especially in catabolic states (Mitch et al 1994).

V. CONCLUSION

In conclusion, the interaction and binding of SAA protein and protofibrils with hepta 1-6 cells decreases in the presence of α B-Crystallin and the cytotoxicity of the SAA and SAA protofibrils reduced.

ACKNOWLEDGEMENTS

We thank to Council of Scientific Industrial Research-Senior research fellowship, New Delhi. India. Dr. T. Ramasami, Director, Central Leather Research Institute, Chennai. India for his kind permission to publish this work. We are also thankful to Dr. A. Meenakshi, Cancer Institute, Chennai for her valuable help in fluorescence microscopy facility. We also thank Prof. Ramurthy, University of Madras, and Chennai for his kind permission to use fluorescence spectroscopy and single photon counting facility.

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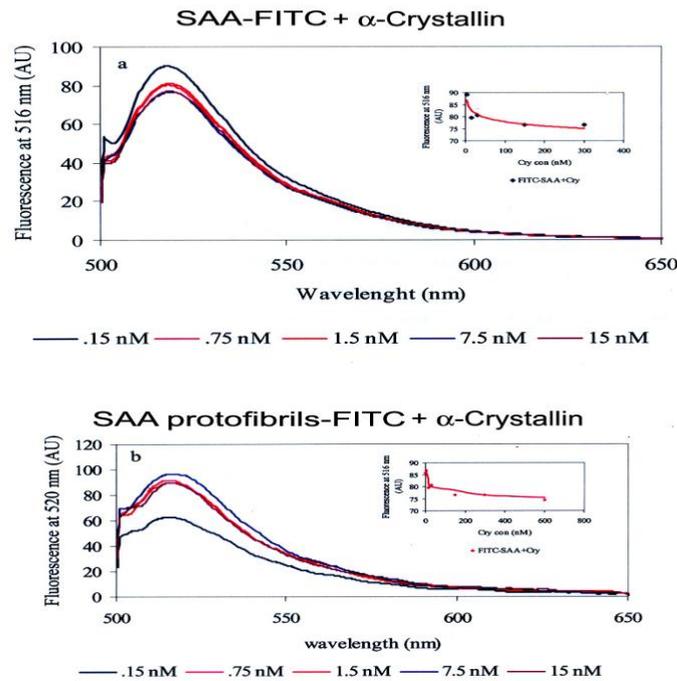


Fig. 1. Fluorescence spectra of SAA-FITC (a) SAA-FITC (2.5 mM) with increasing concentration of (0.15-15 nM) α B-Crystallin and insert plot shows concentration Vs Fluorescence, (b) SAA-FITC protofibrils (2.5 mM) with increasing concentration (0.15-15 nM) α B-Crystallin and insert plot shows concentration Vs Fluorescence.

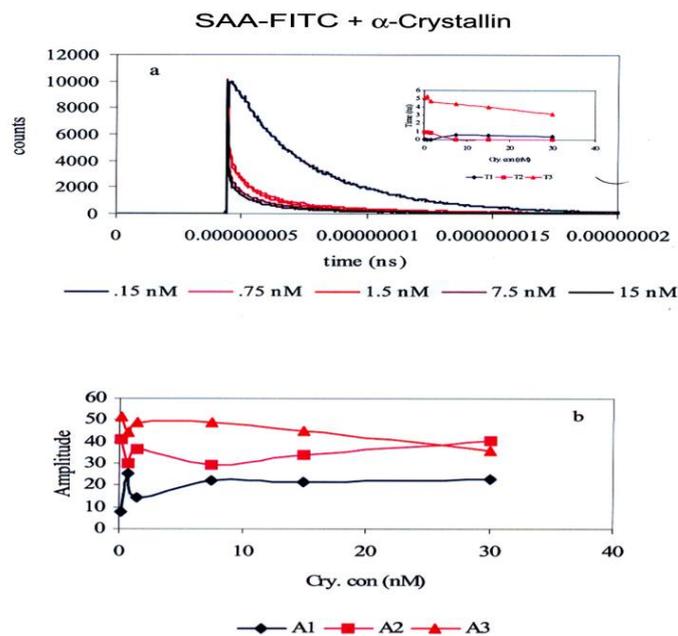


Fig. 2. Life time measurement fluorescence decay of SAA-FITC (a) SAA-FITC (2.5 mM) with increasing concentration of (0.15-15 nM) α B-Crystallin and insert plot shows concentration Vs time, (b) plot shows concentration Vs amplitude.

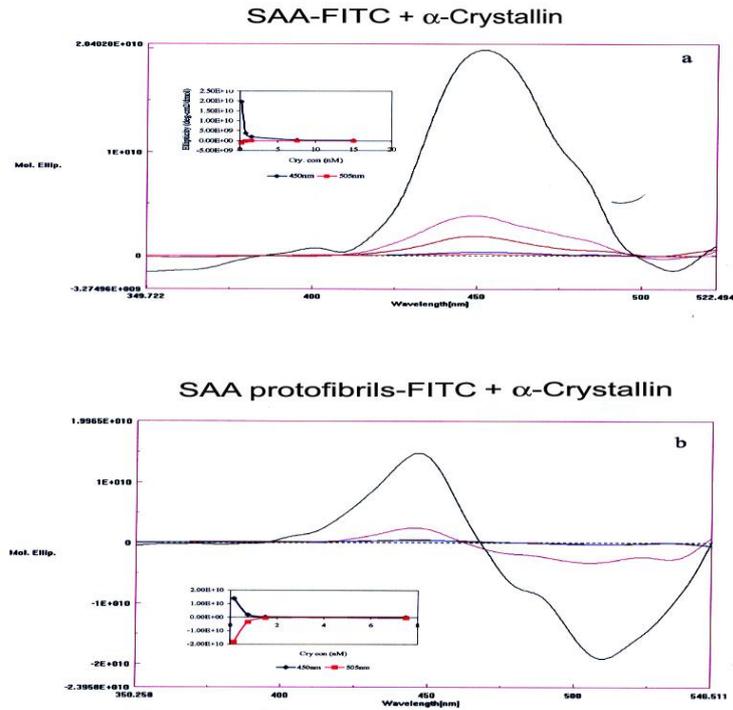


Fig. 3. CD spectra of SAA-FITC (a) SAA-FITC (2.5 mM) with increasing concentration of (0.15-15 nM) α B-Crystallin and insert plot shows concentration Vs ellipticity at 450 and 505 nm, (b) SAA-FITC protofibrils (2.5 mM) with increasing concentration (0.15-15 nM) α B-Crystallin and insert plot shows concentration Vs ellipticity at 450 and 505 nm.

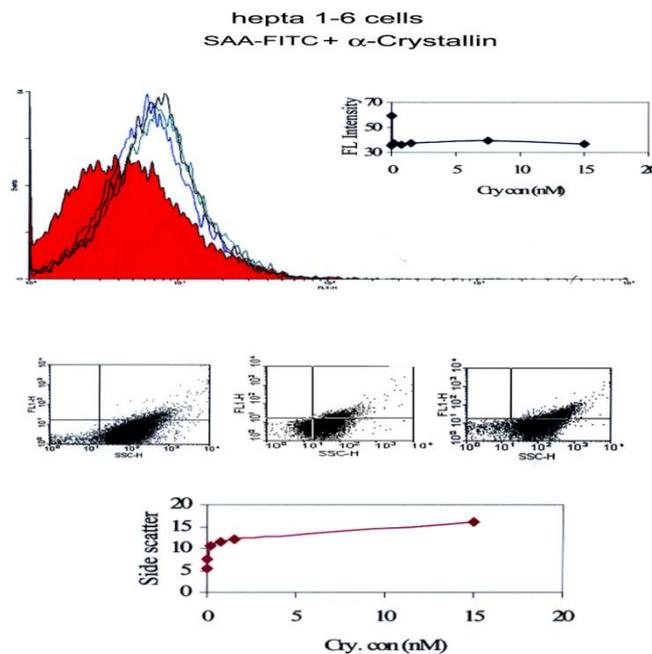


Fig. 4. Effect of α B-Crystallin on SAA-FITC binding to mouse hepta 1-6 cells (a) histogram shows filled curve control cells, black curve (6.25 mM) SAA-FITC, green curve shows addition of (0.75 nM) α B-Crystallin and insert plot shows increasing concentration of α B-Crystallin (0.15-15 nM) Vs mean fluorescence (b) side scatter dot plot of hepta 1-6 cells and incubated with SAA-FITC (2.5 mM) (c) SAA-FITC with (0.15-15 nM) α B-Crystallin, (d) plot shows α B-Crystallin concentration Vs side scatter (upper right).

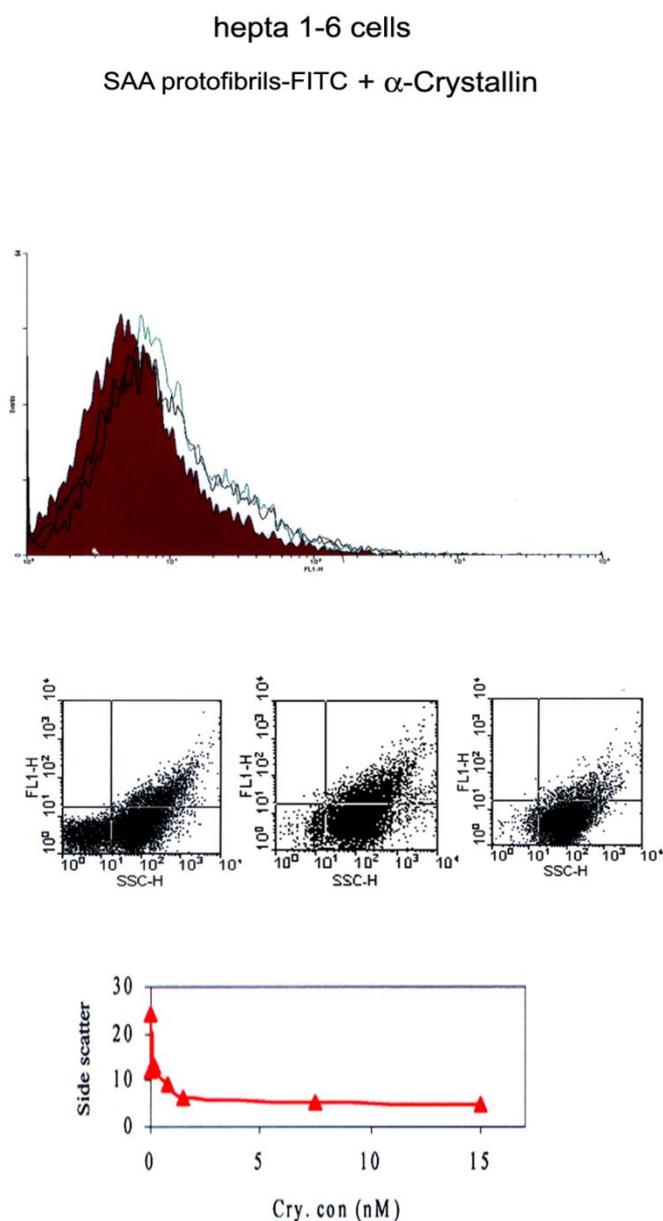


Fig. 5. Effect of α B-Crystallin on SAA protofibrils-FITC binding to mouse hepta 1-6 cells (a) histogram shows filled curve control cells, black curve (6.25 mM) SAA protofibrils-FITC, green curve shows addition of (0.75 nM) α B-Crystallin and insert plot shows increasing concentration of α B-Crystallin (0.15-15 nM) Vs mean fluorescence (b) side scatter dot plot of hepta 1-6 cells and incubated with SAA protofibrils-FITC (2.5 mM) (c) SAA protofibrils-FITC with (0.15-15 nM) α B-Crystallin, (d) plot shows α B-Crystallin concentration Vs side scatter (upper right).