Investigation of truncated variations for structural and functional analysis of Hyaluronan binding protein 1 (HABP1)
5.1 INTRODUCTION

Biochemical studies on proteins over the years have unearthed a baffling range of functions for proteins in the cell. The advancements in the field of structural biology have revealed detailed atomic intricacies about proteins and successfully established the functional aspects with respect to their structure. From its establishment, the Protein Data Bank (PDB) has substantially increased with the release of new proteins. Scientists have always pondered over the correlation between the structure-function relationships of proteins. Dealing with a small protein with a single function is easy, but when it comes to evaluate a multifunctional oligomeric protein, the task becomes rather complicated. One question that comes to one’s mind at this point is “Why do proteins oligomerize?” Though numerous explanations can be given for this notion, Schultz and Schirmer have summarized some of them (Schultz and Schirmer, 1979). The first postulate states that the functionality of a protein can be enhanced through interaction of sub-units, as in many cases, the active site of an oligomeric protein is located at the interface between sub-units. The second one describes the specificity and efficiency in substrate channeling for oligomeric proteins. The third and an important factor is the reduction of osmotic pressure in the cell. The oligomerization of proteins buries portions of the protein surface, thus reducing the number of ions needed to neutralize the exposed charges and reduce the amount of ordered water required to hydrate the protein surface (Goodsell and Olson, 1993). Having said all these, one is forced to ask one-self once again, “But why oligomerization, when they can have a long polypeptide?” This brings us to a discussion on the advantages of having an oligomeric protein rather than one long polypeptide. Klotz et al. have postulated that it is better to build a big structure with smaller molecules rather than one big molecule, as the chances of error in a big molecule would be more (Klotz et al., 1975). Constant association and dissociation of the oligomers can eliminate a faulty sub-unit, but a
deleterious error in a big molecule could be lethal for the cell. Another advantage of having an oligomer is that it enhances the functionality of the protein. An oligomer can function per se at one place and at the same time can dissociate and diffuse to another place to function as a monomer (Honzatko et al., 1985). Thus, oligomerization ascertains the evolutionary hierarchy in a protein and establishes functional diversity.

The oligomeric nature of HABP1 has been established through experiments with the crystal structure substantiating it. The cDNA sequence revealed the presence of a single cysteine in each protomer in the polypeptide chain of HABP1 (Deb and Datta, 1996). The crystal structure showed it to be a doughnut shaped homo-trimer, with a unique non-crystallographic three-fold axis of symmetry, a structure hitherto unknown for other proteins (Jiang et al., 1999). The monomers have seven consecutive β-strands, forming a highly twisted antiparallel β-sheet, with β1 nearly perpendicular to β7. The β-strands are flanked by one N-terminal (α-A) and two C-terminal (α-B and α-C) α-helices, where the N-terminal helix (α-A) forms an antiparallel coiled-coil with the C-terminal helix (α-C) of the adjacent sub-unit. This coiled-coil interaction is responsible for the homo-trimerization of HABP1 (Figure 1.5). The crystal structure also exhibits a solvent-exposed HA-binding motif in the trimeric assemblage. Recent studies have reported the formation of a cysteine-mediated dimer of trimers for HABP1 (Jha et al., 2002). This oligomerization has functional implications, as the compact structure is shown to have highest affinity for HA. The protein also has an asymmetric charge distribution, with positive and negative charges being distributed at two different faces of the molecule. This asymmetric distribution causes a highly flexible conformational state of HABP1, which is essential for its structural integrity and functional diversity (Jha et al., In press). The binding of HABP1 to HA occurs only under oligomeric conditions, i.e. the monomer does not bind HA. However, interactions of HABP1 with C1q and viral proteins have been shown to
take place under monomeric conditions as well (Ghebrehiwet et al., 1994; Luo et al., 1994; Yu et al., 1995; Tange et al., 1996; Wang et al., 1997; Matthews and Russell, 1998; Kittlesen et al., 2000). Thus it is speculated that the oligomeric protein HABP1 might possess the ability to function *per se* at one place and can dissociate and diffuse to another place in the cell to function as a monomer.

The present study focuses on the role of terminal α-helices in maintaining the oligomeric assembly, secondary structure of the resultant protein and its ligand affinity, by examining the interaction of HA with truncated variants of HABP1. Following *in vitro* analysis, the truncated variants, along with mature HABP1 have been transiently transfected in COS-1 cells and the morphology of the transfected cells have been carried out. Concurrently, based on the morphology, studies were carried out to analyze the cellular localization of structural proteins like f-actin in the transfected cells. Based on these studies, the truncated variants have been used as molecular tools to investigate the structural and functional analysis of HABP1.

5.2 MATERIAL AND METHODS

5.2.1 Material

Unless otherwise mentioned, all chemicals were procured from Sigma Chemicals Co., (St. Louis, MO, USA). Chemicals for PCR were acquired from Perkin Elmer, Norwalk, CT, USA, while DIG-labelled UTP and DIG Easy Hyb hybridization buffer were acquired from Roche Biochemicals, Basel, Switzerland. DNA molecular weight markers, mammalian cell culture reagents, antibiotics and bacterial culture media are products of Life Technologies, UK. Hybond-NTM nylon membrane, Hybond-CTM nitrocellulose membrane, agarose and Coomassie brilliant blue were acquired from Amersham, Buckinghamshire, UK. All the restriction enzymes used in the study were procured from New England Biolabs, Beverly, MA, USA. Individual ELISA strips and plastic culture-
wares for mammalian cell culture were purchased from Corning Costar Corp., Corning, NY, USA. The polyclonal anti-HABP1 antibodies used in the study were raised against rat tissue HABP1.

5.2.2 Bacterial strains and growth conditions

_E. coli_ strain DH5α was the recipient for all the plasmids used in sub-cloning. The BL21(DE3) strain was used for the bacterial expression of HABP1 and the truncated variants. Luria-Bertani (LB) medium (Life Technologies) with 30 μg/ml of kanamycin or 50 μg/ml of ampicillin was used for growing the _E. coli_ cells harbouring the plasmids (Sambrook et al. 1989).

5.2.3 Preparation and transformation of competent cells

The preparation of competent cells and transformation were performed by the method of Hanahan (1985). In short, a primary culture 5 ml of LB media was inoculated with DH5α or BL21(DE3) strains of _E. coli_ and incubated overnight at 225 rpm at 37°C. A secondary inoculum of 40 ml of LB was set up with 400 μl of overnight culture and allowed to grow at 37°C for 2-2.5 h till it reached an OD₆₀₀ of 0.38-0.42. The culture was chilled on ice for 15 min with vigorous shaking and then centrifuged at 6000 rpm at 4°C for 5 min. The pellet was resuspended in 20 ml of 0.1 M CaCl₂ (freshly prepared) by tapping and kept in ice for 15 min with stirring. The cells were centrifuged at 6000 rpm at 4°C for 5 min. The pellet thus obtained was resuspended in 2 ml of ice-cold 0.1 M CaCl₂ and 15% glycerol and stored at −70°C in aliquots of 100 μl each for further use.

When required, the competent cells (stored at −70°C) were first thawed on ice for 30 min and then proceeded for transformation. For transformation, 20-100 ng of plasmid DNA was added to 100 μl of competent cells and incubated at 4°C for 45 min. The cells were given a heat shock for 90 sec at 42°C and immediately chilled on ice for 1-2 min. Cells were then grown in 1 ml of LB for 1 h, without antibiotics. Finally,
they were plated on LB plates containing proper antibiotics and grown for 16 h at 37°C to analyze the transformed cells.

5.2.4 Mini scale DNA preparation by modified alkaline lysis method

Rapid mini scale DNA purification from E. coli was done by the method described by Ahn et al. (2000). E. coli cells were grown in 2 ml of LB with appropriate antibiotics and harvested by centrifugation at 11000 rpm for 1 min. Cells were then resuspended in 100 μl resuspension buffer (50 mM Tris-Cl, pH 8.0; 10 mM EDTA and 20 μg RNase A) and 100 μl of lysis buffer (200 mM NaOH; 1% SDS) was added to it and mixed thoroughly. The lysed suspension was neutralised with 120 μl neutralising buffer (3 M potassium acetate, pH 5.5). It was mixed properly and incubated at room temperature for 3 min. The bacterial debris were removed by centrifugation at 11000 rpm for 1 min. The supernatant was then added to 200 μl of isopropanol and incubated for 1 min at room temperature to precipitate the plasmid DNA. The DNA pellet was collected by centrifugation at 11000 rpm for 30 sec and washed with 500 μl of 70% (v/v) ethanol. Finally, the pellet was air dried and resuspended in 100 μl of sterile water or TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0).

5.2.5 Midi scale DNA preparation by alkaline lysis method

Medium scale DNA purification from E. coli was done after minor modifications of methods described by Birnboim and Doly (1979). E. coli cells grown overnight in 50 ml LB medium containing appropriate antibiotics were collected by centrifugation (5000 rpm at 4°C for 15 min) and resuspended in 1 ml of GTE Buffer (50 mM Glucose, 25 mM Tris-Cl, pH 8.0 and 10 mM EDTA) containing 2 mg/ml lysozyme, and kept on ice for 10 min. To the resuspended cells, 2 ml of denaturing solution (1% SDS and 0.2 N NaOH) were added and mixed gently by inversion to lyse the cells and then incubated on ice for 5-10 min. To the lysed cells, 1.5 ml of neutralizing solution (3 M potassium acetate, pH 5.5) was added
and incubated on ice for 10 min, till a flocculent white precipitate was formed. The lysate was then centrifuged at 12000 rpm for 20 min to selectively remove the flocculent chromosomal DNA-high molecular weight RNA-potassium/SDS/protein membrane complex. The pellet thus obtained was discarded and 0.6 volume of isopropanol was added to the supernatant, mixed well and incubated at room temperature for 10 min. The nucleic acids were recovered by centrifugation of the solution at 6000 rpm for 15 min at room temperature. The pellet thus obtained was rinsed with 70% ethanol and dissolved in 1 ml of TE or nuclease-free water. The RNA was digested by treating the dissolved nucleic acids with 10 µl of RNase A (10mg/ml) at 37°C for 45 min. The RNA digested preparation was further purified by extracting twice with an equal volume of phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] and once with equal volume of chloroform/isoamyl alcohol [24:1 (v/v)]. The plasmid DNA was precipitated by the addition of two volumes of pre-chilled ethanol and one-third volume of 7.5 M ammonium acetate (final concentration 2.5 M) with an incubation of 30 min at -70°C. The precipitate was collected by centrifugation at 12000 rpm for 30 min at 4°C. The pellet was resuspended in 100 µl of TE or nuclease free water.

5.2.6 Midi scale DNA preparation by QIAGEN column

Ultrapure, supercoiled-rich plasmid DNA for transfection into the mammalian cells was prepared using QIAGEN Plasmid Midi Kit by following the manufacturer's instructions. In short, 10 ml of LB medium with appropriate antibiotics was inoculated with a single bacterial colony and incubated for ~8 hours at 37°C with vigorous shaking (~300 rpm). This culture was diluted 1/500 into 100 ml of LB medium with appropriate antibiotics and further grown for 12-16 h with vigorous shaking (~300 rpm). Bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C and resuspended by pipetting in 4 ml of Buffer P1 (50 mM Tris, pH 8.0; 10 mM EDTA and 20 µg RNase A). To the resuspended cells, 4 ml of Buffer P2 (200 mM NaOH; 1% SDS) was added
and mixed gently followed by incubation at room temperature for 5 min, for lysing the cells. Neutralization of the lysed cells was done by the addition of 4 ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5) and incubation on ice for 15 min. The sample was then centrifuged at 12000 rpm for 30 min and the supernatant containing plasmid DNA was collected promptly. If the supernatant was not absolutely clear, it was centrifuged again or filtered through a pre-wetted folded sterile filter to remove the debris. In the mean time, a QIAGEN-tip 100 was equilibrated with 4 ml of Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100). The supernatant containing the plasmid DNA was carefully applied to the column and allowed to enter the resin by gravity flow. The column was then washed with 2 x 10 ml of Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) to remove the contaminants. DNA was eluted with 5 ml of buffer QF (1.25 NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol) and precipitated by adding 3.5 ml (0.7 volumes) room-temperature isopropanol. The DNA was centrifuged immediately at 12000 rpm for 30 min at 4°C and the supernatant was carefully decanted. The DNA pellet thus obtained was washed with 2 ml of 70% ethanol and centrifuged at 12000 rpm for 10 min. The supernatant was carefully decanted and the pellet was air-dried and redissolved in suitable volume of 10 mM Tris-Cl, pH 8.5.

5.2.7 Generation of N-and C-terminal truncated clones of HABPI

The HABPI cDNA was cloned under the NdeI and BamHI restriction sites of pET-30b and was designated as pET.AS.HABPI. DNA segments for the truncated variants were generated by PCR (Mullis et al., 1986) with pET.AS.HABPI as the template. The forward primers used in the study had an NdeI site, while the reverse primer for the N-terminal deleted fragment had a BamHI site and that for the C-terminal deleted fragment had an EcoRV site. The primers selected for the study are tabulated below and a schematic representation for the generation of the truncated clones is presented in figure 5.1. AS1 and AS20 primers were
used to generate the C-terminal truncated fragment, while AS12 and AS2 primers were used for the N-terminal truncated fragment.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS1</td>
<td>5'-GGAGATATACATATGCACACCGAC-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>AS2</td>
<td>5'-GCAGCCGGATCCGTAAAACTC-3'</td>
<td>Reverse</td>
</tr>
<tr>
<td>AS12</td>
<td>5'-CCCTCCCTCATATGTCTGGGA-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>AS20</td>
<td>5'-CCCCGATATCTACTCCTGGTGCTCCAGGGC-3'</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

PCR amplification for the generation of the truncated variants was performed using AmpliTaq™ DNA polymerase (Perkin Elmer) according to the manufacturer's protocol with the following modifications. For a 50 µl reaction, 40 ng of template DNA, 0.15 µM of each primer and 0.25 mM of each dNTP were used. The samples were incubated in a thermal cycler (MJ Research) at 94°C for 5 min and then kept for 30 cycles of amplification. Each cycle included 45-sec denaturation (94°C), 60-sec annealing (55°C) and 90-sec chain elongation (72°C). After 30 cycles, the reaction was extended for an additional 5 min at 72°C. Following analysis on an agarose gel, the N-terminal truncated fragment (900 bp) and the C-terminal truncated fragment (600 bp) were purified using Wizard™ SV PCR-purification kit (Promega, Madison, USA). The N-terminal truncated fragment was digested with Ndel and BamHI and cloned at the Ndel–BamHI restriction sites of pET-30b and designated as pET.AS.ΔN.HABP1, while the C-terminal truncated fragment was digested with Ndel and EcoRV and cloned at the Ndel–EcoRV restriction sites of pET-30b and designated as pET.AS.ΔC.HABP1.

**5.2.8 Confirmation of the clones**

The cloning junction and presence of the truncated variants were confirmed by restriction digestion and Southern hybridization (Southern, 1975). The probe used for Southern hybridization was DIG-labelled cDNA, as described in section 2.2.4. The authenticity of the clones was
established by automated sequencing on a Perkin Elmer automated sequencer (Perkin Elmer, USA).

**Figure 5.1** Schematic representations of HABP1 variants created in this study. The figure shows the scheme for generating the truncated HABP1 variants. The position of primers on the plasmid template is shown along with the fragments generated through PCR. The fragments were ligated to pET-30b to generate the truncated clones. Mature HABP1 and the truncated variants, showing the HA-binding motif, are shown alongside. The amino acids 74-104 of the mature protein have been deleted to generate ΔN.HABP1, while amino acids 268-282 have been deleted to generate ΔC.HABP1. In ΔC.HABP1, a stop codon has been introduced after the glutamic acid (Glu-267). In the recombinant mature protein, leucine (Leu-74) has been replaced with methionine (Met-74).
5.2.9 Expression and purification of the recombinant proteins

HABP1 and its truncated variants were overexpressed in *E. coli* strain BL21(DE3) and purified by ion-exchange chromatography. A single *E. coli* colony was inoculated in 10 ml of LB containing 30 µg/ml of kanamycin and grown at 37°C for 9-12 h. About 1% of the cells were inoculated into 100 ml of LB containing 30 µg/ml of kanamycin and grown at 37°C, till it reached an OD$_{600}$ of ~ 0.6. Protein expression was induced with 1 mM isopropyl 1-thio-β-galactopyranoside (IPTG). Cells were grown at 37°C for 3 h after induction and collected by centrifugation. The bacterial pellet was suspended in 10 ml of buffer A (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 5% glycerol, pH 7.5) and disrupted by sonication, until it was optically transparent. The cell extract was centrifuged at 10,000 rpm for 30 min at 4°C to remove debris. The supernatant was subjected to 30-90% ammonium sulfate fractionation at 4°C, keeping the pH between 7.5-7.8. The ammonium sulfate precipitate was resuspended in buffer A and subjected to SDS-PAGE analysis for optimal percentage of ammonium sulfate required for the precipitation of the desired protein. The fractions thus obtained were pooled and dialyzed against 1 l of buffer A for 16 h. The dialyzed sample was centrifuged at 10,000 rpm to remove any particulate matter and then loaded onto Mono-Q™ column HR16 (Pharmacia, Uppsala, Sweden), pre-equilibrated with buffer A. After washing the column with buffer A, the protein was eluted with 0-1 M sodium chloride gradient, using buffer B (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 5% glycerol, 1 M NaCl, pH 7.5). The peak fractions obtained were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. Fractions containing the purified protein of interest were pooled, desalted and concentrated.

5.2.10 Immunoblot Analysis

The purified proteins were separated on a 12.5% SDS-PAGE under reducing conditions, as described in section 3.2.9 and immuno detected as described in section 3.2.10.
5.2.11 Size exclusion chromatography

To determine the native molecular mass of HABP1, ΔN.HABP1 and ΔC.HABP1 in solution, gel filtration experiments were performed on a Superose6™ analytical grade gel filtration column interfaced with Pharmacia FPLC system. The column was calibrated with the standard proteins; aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa) (Pharmacia, Uppsala, Sweden). The buffer used in this study was 10 mM Phosphate Buffered Saline with 150 mM NaCl to keep the ionic strength constant. The elution speed was kept constant throughout the experiment at 0.3 ml/min. The molecular weight and size of the unknown protein was calculated by using the methods of Seigel and Monty (1966).

5.2.12 Fluorescence Emission spectra

Intrinsic fluorescence emission spectra of HABP1 and its truncated variant were recorded on Cary Eclipse™ spectroflourimeter (Varian Inc., Australia), keeping the excitation and emission slit width at 2.5 and 5.0 nm respectively. Typically, the samples were excited at 282 nm and the emission spectra were recorded from 290-500 nm. The background correction for Raman scattering was done using buffer alone on the spectrofluorimeter. The protein concentration was kept such that the absorbance at 282 nm was always ≤ 0.1 to avoid any inner filter effect. Each solution was passed through 0.22 μm membrane filter prior to recording the spectra and the temperature was kept at 25°C throughout the experiment.

5.2.13 Circular Dichroism Spectroscopy

The dichroic spectra were recorded on Jasco 740 spectropolarimeter attached with water circulated thermostatic cell holder, in a cell of 1 cm path length. The concentration of the proteins were kept between 5-10 μM in 10mM phosphate buffer containing
150mM NaCl and molar elipticity was calculated for each sample. Typically, 30-40 scans per sample were given at a scan rate of 50 nm/min. The temperature was kept constant at 10°C throughout the experiment.

5.2.14 Binding of HABP1 and its variants to immobilized HA

Binding of HABP1 and its variants to immobilized HA was measured in 96-well microtiter plates. The wells were coated overnight at 4°C with 2 mg/ml HA in coating buffer (15 mM sodium carbonate and 34 mM sodium bicarbonate, pH 9.3) and blocked for 2 h in 0.25% BSA and 0.05% Tween-20 in PBS. After the plates were washed three times with PBS, they were incubated with the purified proteins for 2 h at room temperature. After washing off the unbound proteins, the plates were incubated with polyclonal anti-HABP1 antibodies for 1 h at room temperature. Following 5 x 5 washes with PBS, the plates were incubated with AP-conjugated goat anti-rabbit IgG for 1 h at room temperature. After the unbound secondary antibody was washed off from the plates, the reactions were developed using AP-substrate solution (Sigma 104 phosphate) till the desired colour developed. The absorbance of each well was recorded at 405 nm using a Bio-Rad microtitre plate reader (Bono et al., 2001). For control experiment, immobilized HA was first overlaid with BSA and subsequently treated with the antibodies used in the study.

5.2.15 Cloning of mature HABP1 and its truncated variants into a mammalian expression vector

The mature HABP1 cDNA and its truncated counterparts were cloned in the mammalian expression vector pBI-EGFP at the PvuII site. The fragments were amplified through PCR from the clones generated in pET-30b. The cloning strategy for the HABP1 fragment is presented in figure 5.2 and a similar approach was taken in cloning the truncated variants.
Figure 5.2 Schematic diagram of the HABP1 gene cloned in the mammalian expression vector pBI-EGFP. The HABP1 cDNA, encoding the mature protein, was PCR amplified with Vent® Polymerase using the Mamm 1 and Mamm 2 primers. The mammalian vector pBI-EGFP contains a bi-directional promoter P_{bi-1}, which has the multiple cloning site on one side and EGFP gene on the other side. It was digested with PvuII to generate a blunt end linear fragment. The PCR amplified HABP1 cDNA fragment and the linearized vector were ligated using T4 DNA-ligase. A similar scheme was followed for the generation of the truncated clones.
5.2.16 Transient transfection in COS-1 cells

Transient transfection in COS-1 cells was done with liposomal based Lipofectamine 2000™ according to the following protocol. Exponentially growing cells were split into 35 mm dishes containing cover-slips, to a confluency of around 50-60% the day before transfection and grown for 10-12 h in complete medium. Next day, 1 ml of DMEM was added to each 35 mm dish and the cells were equilibrated in it for 30 min prior to transfection. For each transfection, 500 ng of plasmid DNA (QIAGEN column purified) was added to 100 μl of DMEM in a microcentrifuge tube and incubated at room temperature for 5 min. Simultaneously, 6 μl of Lipofectamine 2000™ was added to 100 μl of DMEM in another microcentrifuge tube and incubated at room temperature for 10 min. The contents of the two tubes were mixed properly by gentle pipetting and further incubated for 30 min. Following this incubation, the 200 μl DNA-Lipofectamine complex was added to each 35 mm dish and incubated in a CO₂ incubator for 7 h. After this transfection period, DMEM was replaced with complete medium and the cells were grown for additional 21-25 h. These transiently transfected cells were further processed according to experimental requirements.

5.2.17 Preparation of lysate from the transiently transfected cells

The transfected COS-1 cells were washed once with PBS and then mechanically detached from the surface using a cell-scraper. The cells were collected in a microcentrifuge tube and centrifuged at 2000 rpm for 5 min to pellet the cells. The supernatant was removed and the pellet was re-suspended in 100 μl of 1X Laemmli buffer containing β-mercaptoethanol and boiled directly for 10 min. The boiling was followed by centrifugation of the lysate at 12000 rpm for 5 min and the supernatant containing the lysate proteins was collected and stored at 4°C for further use.
5.2.18 Detection of autophagic vacuoles in the transiently transfected cells with monodansylcadaverine (MDC)

The transfected cells grown for 30 h were washed with PBS to remove the culture medium. Following washes, the cells were incubated with 0.05 mM MDC in PBS at 37°C for 10 min (Biederbick et al., 1995). After incubation, the cells were washed four times with PBS and immediately analyzed by fluorescence microscopy. The images were obtained with the AxioCam™ CCD camera.

5.2.19 Detection of f-actin in transiently transfected cells

The transfected cells were permeabilized as described in section 4.2.4. Following permeabilization, the cells were washed once with PBS and incubated with rhodamine-conjugated phalloidin for 15 min at room temperature. After incubation, the cells were rinsed with PBS over a period of 15 min, following which they were incubated with Hoechst for 30 min. The cells were washed with PBS, mounted in glycerol-PBS and visualized under a fluorescent microscope.

5.2.20 Immunofluorescence studies in transiently transfected cells

Indirect immunofluorescence staining was performed on fixed, permeabilized transiently transfected COS-1 cells. Immunodetection of HABP1 and p53 were carried out in these cells using Cy3 conjugated secondary antibodies, as described in section 4.2.4. The resultant images were analyzed by fluorescence microscopy.

5.3 RESULTS

5.3.1 Generation and validation of the HABP1 truncated variants

Figure 5.1 shows a schematic representation for the generation of truncated variants of HABP1. The truncated variants generated through PCR exhibited molecular weights of 900 and 600 bp for the N-terminal and C-terminal deleted fragments respectively, as compared to 1030 bp for HABP1 (Figure 5.3). The fragments were subsequently cloned in
pET.30b to generate the truncated variants, as described in “Material and Methods”. The cloning junction and presence of all the truncated variants were confirmed by restriction digestion followed by Southern hybridization. The restriction digestion profile generated fragments of 900 bp, 600 bp and 1030 bp for ΔN.HABP1, ΔC.HABP1 and HABP1 respectively, which hybridized with DIG-labelled HABP1 cDNA probe, thus confirming their proper cloning (Figure 5.4 A, B). The clones were subsequently sequenced to establish their authenticity (data not shown).

Figure 5.3 Generation of truncated variants of HABP1. pET.AS.HABP1 was used as template for PCR, using the primer combinations mentioned, while pET-30b in combination with S1-S2 primers was used as negative control. The amplified products were analysed on a 1.2% agarose gel alongside 1 kb DNA molecular weight marker. The combination of S1-S2 generated a fragment of 1030 bp, while S12-S2 and S1-S20 generated fragments of 900 and 600 bp respectively.
5.3.2 Purification of the truncated variants of HABP1

Mature HABP1 and the truncated variants were successfully overexpressed in E.coli. The mature protein (HABP1), the N-terminal truncated variant (ΔN.HABP1) and the C-terminal truncated variant (ΔC.HABP1) were purified using an ion-exchange column on the FPLC. The proteins were purified to >95% homogeneity, as determined by Coomassie Blue staining of SDS-PAGE, where the sub-unit molecular weight of HABP1, ΔN.HABP1 and ΔC.HABP1 correspond to 34, 27 and 32 kDa respectively (Figure 5.5A). The antigenic specificity of the proteins...
was examined by immunodetection using anti-HABP1 antibodies (Figure 5.5B), which clearly demonstrated specific detection of mature HABP1 and its two truncated variants.

Figure 5.5 Homogeneous purification and immunodetection of HABP1 and its truncated variants. (A) HABP1, ΔN.HABP1 and ΔC.HABP1 were purified and resolved on a 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue to analyze their sub-unit molecular weight. (B) The mature protein and its truncated variants were immunodetected using anti-HABP1 antibodies after resolving them on a 12.5% SDS-PAGE. The truncated proteins also showed antigenic similarity to anti-HABP1 antibodies.

5.3.3 Truncation at the N- and C-terminal of HABP1 alters the structure of the resultant proteins

HABP1 is a homo-trimer, comprised of three chains A, B and C. Each chain has almost similar conformation, consisting of three α-helices and seven anti-parallel β-sheets. The first helix lies at the N-terminal and the other two lie at the C-terminal. It was tested whether the terminal deletions affected the folding and the stability of the
resultant variants. The circular dichroism (CD) spectra were measured for HABP1 and its truncated variants in 10 mM phosphate buffer containing 150 mM NaCl (pH 7.2) at 10°C. The spectra showed a negative peak between 210-225 nm for HABP1 and a small negative peak at 222 nm for ΔN.HABP1 (Figure 5.6). The negative peak however shifts to around 238 nm for ΔC.HABP1. Thus, deletion of the terminal helices generates variants, with an altered structure.

![CD Spectra](image)

**Figure 5.6 Circular Dichroism (CD) spectra of HABP1 and the truncated variants.**

The CD spectra is expressed as mean molar ellipticity (θ) for HABP1, ΔN.HABP1 and ΔC.HABP1 at ambient temperature. The results indicate a smaller negative peak for ΔN.HABP1 at 222 nm, as compared to HABP1 and a red shift for ΔC.HABP1.

5.3.4 Truncated variants of HABP1 lose their oligomerization property

Oligomeric state of HABP1 and the truncated variants were examined by size exclusion chromatography on Superose 6 column in 10 mM PBS. HABP1 exists primarily as a trimer (68 kDa) in a dynamic
equilibrium with disulfide mediated dimer of trimer (136 kDa) under non-reducing gel filtration chromatography. Under similar experimental conditions, the variants exhibited a monomeric and dimeric form corresponding to 20 kDa and 40 kDa for ΔN.HABP1 and 22 kDa and 44 kDa for ΔC.HABP1 (Figure 5.7B). However, in the presence of 0.02% β-mercaptoethanol, ΔN.HABP1 and ΔC.HABP1 exhibited only the monomeric species, while HABP1 exhibited trimeric species under similar conditions (Figure 5.7C). These results indicate that the variants generated through truncation at either N- or C-terminal are incapable of forming the trimer by coiled-coil interaction of the α-helices. It also shows that due to oxidative conditions, the variants tend to form a disulfide linkage, giving rise to the dimer apart from the monomer under near physiological conditions, while HABP1 primarily exists as trimer and hexamer under such conditions. The monomeric and dimeric species of the truncated variants are in a dynamic equilibrium. Thus, the truncated variants clearly lose their property to oligomerize.

5.3.5 Deletion of the terminal helices, alters the intrinsic fluorescence emission spectra of HABP1 variants

Intrinsic tryptophan fluorescence was used to monitor conformational change in the truncated variants. The intrinsic fluorescence of ΔN.HABP1 and ΔC.HABP1 shows different fluorescent spectra, though the intensity maxima of the two remain the same. However, the tryptophan fluorescence emission intensity decreases significantly in case of ΔN.HABP1 as compared to the full length HABP1 (Figure 5.8), indicating a major change around the tryptophan environment in the polypeptide chain. The fluorescence emission intensity changes to a lesser extent for ΔC.HABP1, as compared to the mature protein. The intrinsic tryptophan fluorescence emission intensity for HABP1 and its truncated variants is in the order of ΔN.HABP1< ΔC.HABP1< HABP1 (Figure 5.8).
Figure 5.7 Loss of oligomerisation of HABP1 upon deletion of N- and C-terminals.

(A) The elution profile of the column calibration markers plotted against the Log of molecular weight. The markers are labelled 1-5, in the order of decreasing molecular weight and the same labeling is followed in the experimental figures. The gel filtration analysis of HABP1, ΔN.HABP1 and ΔC.HABP1 were carried out in 10 mM phosphate buffer containing 150 mM NaCl (pH 7.2) on Superose 6 column, at a flow rate of 0.3 ml/min. The experiments were performed under non-reducing conditions (B) and reducing conditions, in the presence of 0.2% β-mercaptoethanol (C).
Figure 5.8 Loss of oligomerization leads to a change in the global conformation of the truncated variants of HABP1. Fluorescence emission spectra of HABP1, ΔN.HABP1 and ΔC.HABP1 were carried out in phosphate buffer (pH 7.2), as described in “Material and Methods”. The tryptophan fluorescence emission intensity changes slightly for ΔC.HABP1, but decreases significantly in case of ΔN.HABP1 as compared to mature HABP1.

5.3.6 The HABP1 variants retain their ability to bind HA

HABP1 possesses a solvent exposed HA-binding motif in its trimeric assembly. The variants however, do not possess this trimeric assembly, as evident from the size exclusion chromatography data (Figure 5.6). The HA-binding ability of each variant was examined using immobilized-HA, as described in “Material and Methods”. Results indicate that both ΔN.HABP1 and ΔC.HABP1 bind effectively to HABP1 (Figure 5.9) and the binding efficiency of the variants is comparable to that of HABP1. However, when the proteins were analysed on SDS-PAGE and probed with biotinylated HA, they did not show any binding to HA (data not shown). This suggests that the terminal deletions make the HA-
binding motif to be accessible to the solvent, which is reflected in the HA-binding ability of the variants. Thus, though the terminal deletions change the overall conformation of the variants, they retain their ability to bind HA under non-reducing conditions.

![Graph showing HA-binding activity of HABP1 and its truncated variants](image)

**Figure 5.9 HA-binding activity of HABP1 and its truncated variants.** Immobilized HA was used to compare the HA-binding activity of ΔN.HABP1 (---) and ΔC.HABP1 (-----) were compared with that of HABP1 (-----). The background reading for the control (BSA) has been subtracted from the experimental sample. The data presented represent averages of duplicate determinations in at least three identical experiments, carried out under similar conditions.

### 5.3.7 Cloning of HABP1 and its variants into mammalian expression vector

The generation of clones of HABP1 and its truncated variants in a mammalian expression vector pBI-EGFP are shown schematically in figure 5.2. Following ligation, the clones were digested with NdeI restriction endonuclease for the confirmation of cloning in sense-orientation. The cloning in sense orientation would generate fragments
corresponding to 1900 bp, 1800 bp and 1500 bp for HABP1, ΔN.HABP1 and ΔC.HABP1 respectively. The digested fragments upon analysis on a 0.7% agarose gel confirmed cloning in a sense orientation and their subsequent hybridization with the DIG-labelled HABP1 cDNA probe, established their authenticity (Figure 5.10). The clones were sequenced to establish their authenticity and named pBI-EGFP.HABP1, pBI-EGFP.ΔN.HABP1 and pBI-EGFP.ΔC.HABP1 respectively.

Figure 5.10 Confirmation of cloning of HABP1 and its truncated variants in sense orientation in mammalian vector. (A) The mature HABP1 and truncated fragments, cloned in pBI-EGFP, were digested with Ndel and resolved on a 0.7% agarose gel. The fragment from HABP1 was 1900 bp in length, while those from ΔN.HABP1 and ΔC.HABP1 were 1800 and 1500 bp respectively. This confirmed the cloning in a sense orientation, while Southern Hybridization established their authenticity (B).

In order to examine the morphological and cellular changes imparted by HABP1 and its truncated variants, they were cloned in the mammalian expression vector pBI-EGFP and transfected in COS-1 cells. The vector pBI-EGFP is a bi-directional vector, which simultaneously translates EGFP and the cDNA cloned in its multiple cloning site. This
aided us in our endeavour to study the cellular morphology, as EGFP would distribute uniformly in the cell and divulge any change in the cellular morphology.

5.3.8 Expression of mature HABP1 and the truncated variants in COS-1 cells induces morphological changes

The mammalian clones pBI-EGFP.HABP1, pBI-EGFP.ΔN.HABP1 and pBI-EGFP.ΔC.HABP1 generated in pBI-EGFP were transfected in COS-1 cells to generate transiently transfected clones for analysis. The vector pBI-EGFP was also transfected in COS-1 cells to serve as a control. On an average, the transfection efficiency was around 20% for COS-1 cells. Though no visible change in the shape and size of the cells were noticed, the transiently transfected cells showed an altered morphology, with numerous caveolar like circular structures resembling vacuoles (Figure 5.11). These structures have been henceforth referred to as vacuoles. The transfected cells were stained with Hoechst for visualizing the nucleus, while the presence of EGFP facilitated cell-morphology analysis. The presence of some vacuoles in cell lines in not an unknown feature, however in this study, it was observed that there was significant increase in the number and size of visible vacuoles upon transfection. As a result, a statistical analysis of around 20 transfected plates for each sample was carried out to estimate whether the vacuole formations were artifacts or a morphological manifestation of cellular changes due to transfection of COS-1 cells. The data revealed a four-fold increase in the percentage of transfected cells harbouring vacuoles, for HABP1 or its truncated variants, as compared to the vector control (Figure 5.13). However, it has to be noted here that vacuole formation was not an inherent property of the transfected cells, as evident from the percentage of cells manifesting them. In all the transfected, both vacuole harbouring and non-harbouring cells were present and an example of cellular morphology, used for scoring of vacuoles is presented in figure 5.12.
Figure 5.11 Altered morphology of the transiently transfected COS-1 cells. The COS-1 cells transfected with the vector alone (A) showed less vacuole formation than cells transfected with HABP1 (B), ΔN.HABP1 (C) or ΔC.HABP1 (D). The first column shows the morphology as depicted by the distribution of EGFP, while the second column reveals nuclear staining, visualized through Hoechst staining. The superimposed image corresponding to EGFP and Hoechst merged together is shown in third column. The bar represents 10 μm.
Morphology of transiently transfected Cos-1 cells. The transiently transfected COS-1 cells had both vacuole bearing and non-bearing cells. Some transfected cells not bearing vacuole are shown in panel A, while those, which harbour vacuoles, are shown in panel B. These are typical example of vacuolated and non-vacuolated cells used for scoring. The bar represents 10 μm.

Statistical analysis of vacuole formation in transfected cells. The transfected COS-1 cells illustrated both vacuole bearing and non-bearing cells. A statistical analysis for the percentage of vacuole bearing cells in the transfected population reveals a four-fold increase in the clones bearing HABP1 or its truncated variants, as compared to the vector control. The data is an average of 500 cells for each transfection.

5.3.9 Immunodetection of HABP1 in the lysate of transfected cells

Following the morphological analysis of transfected cells, an immunodetection of HABP1 in their lysate was carried out, using anti-
HABP1 antibodies. The lysate was prepared as described in section 5.2.17 and the relative level of HABP1 in cells transfected with pBI-EGFP.HABP1, pBI-EGFP.ΔN.HABP1 and pBI-EGFP.ΔC.HABP1 were compared to the one transfected with the vector pBI-EGFP alone (Figure 5.14A). The antibody recognizes only one band, corresponding to 34 kDa on the SDS-PAGE, which did not show any significant change. The antibodies however did not detect the truncated variants. The low transfection efficiency (as compared to the total cell population) could explain this observation, as the amount of truncated protein produced would constitute a very small portion of the total lysate. Immunodetection of actin in each lysate sample determined equal loading of the samples (Figure 5.14B).

![Figure 5.14 Immunodetection of HABP1 in the transfected cells. A. Cell lysates from the transfected cells were immunodetected using anti-HABP1 antibodies after resolving and transferring them from a 12.5% SDS-PAGE. Purified rHABP1 served as the positive control. B. The same lysate were probed with anti-actin antibodies to ascertain equal sample loading, with rHABP1 serving as a negative control.](image)
5.3.10 Subcellular localization of HABP1 in the transfected cells

The COS-1 cells upon transfection were processed for immunofluorescence with anti-HABP1 antibodies and the images thus obtained were analyzed by fluorescence microscopy. The total cell lysate prepared does not reflect the status/level of HABP1 being expressed in individual transfected cells as well as any change in the sub-cellular localization profile of the protein. This forced us to analyze the level of HABP1 in individual cells. At the individual cell-level, the data revealed a higher level of HABP1 in the transfected cells, as compared to the vector control, without any alteration in the sub-cellular localization profile (Figure 5.15). The increase in the level of HABP1 for a single cell was not exorbitant, which explained an unchanged profile for the total lysate. To ascertain the increase in the amount of HABP1 in the transfected cells, a statistical analysis was carried out to compare the intensities of EGFP or HABP1 (detected with Cy3-conjugated secondary antibody) with respect to the cell size, as described in section 4.3.5. A ratio between EGFP intensity (calculated as arbitrary units) and the cell size was plotted against the transfected samples (Figure 5.16). A similar pattern was followed for Cy3 intensity, for analyzing the increase in the amount of HABP1 (Figure 5.16). The increase in the intensity of EGFP and Cy3 in the transfected cells upon comparison with the untransfected cells has been represented as a column graph. The change in the intensity of the transfected cells (in percentage) is graphically represented for the vector pBI-EGFP, HABP1, ΔN.HABP1 and ΔC.HABP1. The ratio was calculated by scanning 150-200 transfected cells for each sample.
Figure 5.15 Immunodetection of HABP1 in transiently transfected COS-1 cells.

COS-1 cells transfected with the vector alone (A) did not show an alteration in the level of HABP1, while cells transfected with HABP1 (B), ΔN.HABP1 (C) or ΔC.HABP1 (D) showed a partial increase. The first column shows the distribution of EGFP, the second column reveals immunodetection of HABP1 using Cy-labelled secondary antibody, while the third column shows the nucleus, visualized through Hoechst staining. The superimposed images corresponding to EGFP, Cy3 and Hoechst merged together are shown in the fourth column. The bar represents 10 μm.
Figure 5.16 Change in the intensity of EGFP and Cy3 in transfected cells. The increase in the intensity of EGFP and Cy3 in the transfected cells upon comparison with the untransfected cells is represented as a column graph. A ratio of EGFP or Cy3 intensity and cell size was calculated in the transfected as well as the untransfected cells. The change in the intensity of the transfected cells (in percentage) is graphically represented for vector, HABP1, AN.HABP1 and AC.HABP1. The ratio was calculated by scanning 150-200 transfected cells for each sample.

5.3.11 Detection of vacuoles in the transfected cells using monodansylcadaverine (MDC)

The formation of cytoplasmic vacuoles upon transfection prompted us to study if they were autophagic in nature. As monodansylcadaverine specifically detects autophagic vacuoles in cells, the transfected cells were stained with MDC. Results reveal that these vacuoles are both autophagic and non-autophagic in nature (Figure 5.17). The data presented here shows some typical cells used for scoring MDC staining. While some of the vacuoles generated could stain perfectly well with MDC, the others did not. Thus it can be inferred from this data that the nature of the vacuoles is heterogeneous. Stress conditions often lead to the formation of autophagic vacuoles and that might be the case in some transfected cells. The vacuoles, which did not stain with MDC, might be generated through a different process and a detailed analysis of the
process has to be undertaken to ascertain the exact cause for their formation.

Figure 5.17 Detection of autophagic vacuoles through MDC in the transfected cells. MDC detects autophagic vacuoles in mammalian cells, as staining the transfected cells with MDC revealed some of the vacuoles being autophagic in nature, while the others are not. The nature of autophagic vacuoles in untransfected cells reveals staining at the perinuclear region (A). However, in the transfected cells some vacuoles are not autophagic, as they cannot be stained by MDC (B), while some are autophagic in nature (C). Column one depicts the distribution of EGFP, the second one shows detection of vacuoles with MDC and the third one shows the superimposed image corresponding to EGFP and MDC. The bar represents 10 μm.
5.3.12 Analysis of Programmed Cell Death in the transfected cells

Autophagy is often classified as Type II programmed cell death. In an attempt to check if the transfected cells were undergoing programmed cell death, the transfected cells were processed for immunofluorescence with anti-p53 antibodies to ascertain if the individual transfected cells were proceeding towards apoptosis. The images thus obtained were analyzed by fluorescence microscopy. The data revealed a similar amount of p53 in the transfected cells, as compared to the vector control, without any altered sub-cellular localization profile in the transfected cells (Figure 5.18). Taken together, these data explain that the cells analyzed after 28-32 hours after transfection did not depict a noticeable shift towards apoptosis for these cells. However, a few of the transfected cells did show apoptosis, as revealed in figure 5.11.

5.3.12 An insight into f-actin orientation in the transfected cells

Transfection and transformation are often related to a change in the distribution of structural proteins in the cell. In an attempt to study the effect of transfection of HABP1 or its truncated variants into COS-1 cells, f-actin was chosen as a representative molecule. The distribution and effective intensity of f-actin was measured in the transfected cells by using rhodamine-labelled phalloidin. Phalloidin specifically interacts with f-actin and gives a profile of the filaments in the cell. The analysis revealed that there was a decrease in the amount of f-actin in cells transfected with pBI-EGFP.HABP1 and pBI-EGFP.ΔC.HABP1, as compared to the untransfected cells or vector alone, while cells transfected with pBI-EGFP.ΔN.HABP1 showed an increase in f-actin intensity, but the filamentous nature of f-actin was perturbed and it was localized as a clump at perinuclear regions (Figure 5.19).
**Figure 5.18 Distribution of p53 in the transiently transfected COS-1 cells.** p53 shows a diffused pattern throughout the interphase nucleus. The profile of p53 was analyzed in the transfected cells to determine any change in its sub-cellular localization or intensity upon transfection. The immunofluorescence data showing COS-1 cells transfected with the vector alone (A), HABP1 (B), ΔN.HABP1 (C) or ΔC.HABP1 (D) did not show any change with respect to the sub-cellular localization or change in the intensity. The first column depicts the distribution of EGFP, the second one shows immunodetection of HABP1 using Cy3-conjugated secondary antibody, while the third one shows nuclear staining, visualized through Hoechst. The superimposed image corresponding to EGFP, Cy3 and Hoechst merged together is shown in the fourth column. The bar represents 10 μm.
Figure 5.19 Distribution of f-actin in the transiently transfected COS-1 cells. The distribution of f-actin in COS-1 cells transfected with vector alone did not undergo any change (A) as compared to the untransfected cells. However, a significant decrease in the level of f-actin was observed for HABP1 (B) and ΔC.HABP1 (D). The amount of f-actin shows an increase in cells transfected with ΔN.HABP1 (C), with the protein being localized as clumps at the perinuclear region. The first column depicts the distribution of EGFP, the second one shows detection of f-actin using rhodamine-labelled phalloidin, while the third one shows nuclear staining, visualized through Hoechst. The superimposed image corresponding to EGFP, rhodamine and Hoechst merged together is shown in the fourth column. The bar represents 10 μm.
5.4 DISCUSSION

The crystal structure has already established the trimeric nature of HABP1 (Jiang et al., 1999). The α-helix located at the N-terminal (α-A) forms a coiled-coil structure with the α-helix located at the C-terminal (α-C) of the adjacent sub-unit, a critical interaction for the maintenance of the trimeric assembly (Jiang et al., 1999). Within these structural arrangements, the HA-binding motif in each trimeric assembly is solvent exposed. Recent study from our laboratory has also shown that HABP1 exists as a trimer and dimer of trimers (hexamer) under physiological conditions, with the hexamer possessing a higher affinity for HA (Jha et al., 2002). Keeping these results in mind, the present study focuses on the structural variations of HABP1, by eliminating the terminal α-helices and analyzes its functional implications by probing the mutants for their HA-binding ability. The functional implications of HABP1 and its truncated variants were studied by examining the morphological changes imparted upon their expression in living cells.

The deletion of the N-terminal α-helix generated ΔN.HABP1, while that of the C-terminal α-helix generated ΔC.HABP1. The variants of HABP1, ΔN.HABP1 and ΔC.HABP1 have a sequence-based molecular weight of 20.1 and 22 kDa respectively, as evident from the size exclusion chromatography data, but exhibit molecular weights of 27 and 32 kDa on an SDS-PAGE. This anomaly in migration is due to the ratio of polar to aromatic amino acid residues, a phenomenon also noted previously for HABP1 (Deb and Datta, 1996).

This study depicts the inability of the truncated variants in forming a trimer, thus emphasizing the role of the terminal helices in maintaining the trimeric assembly. However, they are capable of forming a cysteine-mediated dimer. The deletion of the two terminal helices also alters the structure of the protein, as the Circular Dichroism spectra recorded for ΔN.HABP1 shows a decreased negative peak around 222 nm and a red
shifted peak in case of ΔC.HABP1. The decrease in the negative peak for ΔN.HABP1 is suggestive of a change in the helical content, while a red shift in case of ΔC.HABP1 indicates a change in the environment around the tryptophan residues. The C-terminal deletion may affect the overall fold of HABP1 in an extensive manner due to its dual role in maintaining the oligomeric assembly and supporting the monomer fold by a hydrophobic contact between α-B and the twisted anti-parallel β-sheets. The fluorescence emission data reflects a slight decrease in the intensity of ΔC.HABP1, while a drastic decrease is observed for ΔN.HABP1. The deletion of the N-terminal α-helix brings one of the tryprophan residues (W109), in close vicinity of the N-terminal of the truncated protein and the orientation of this tryptophan may render it incompetent in generating intrinsic fluorescence.

The HA-binding ability of each variant was examined using immobilized-HA as a probe and interestingly, both ΔN.HABP1 and ΔC.HABP1 bind effectively to HA, with their binding efficiency being comparable to that of HABP1. This suggests that though the variants have an altered structure, their HA-binding motif remains accessible to the solvent, a proposition being reflected in their HA-binding ability. As expected, the monomers do not have the ability to bind HA, but the variants forming cysteine-mediated dimers have the ability to bind HA. The results suggest that the HA-binding motif in the HABP1 variants may be unaltered and the ability of the variants to form dimers may juxtapose each monomer in a manner that allows the HA-binding motif to be accessible for binding. Thus, though the terminal deletions change the overall conformation of the variants, they retain their ability to bind HA under non-reducing conditions. These observations demand a detailed study on the HA-binding motif of HABP1.

Following in vitro analysis, the truncated variants, along with mature HABP1 were transiently transfected in COS-1 cells and analyzed for morphological changes in the transfected cells. The mammalian
expression vector chosen for this study was pBI-EGFP, which has a bidirectional promoter capable of simultaneously translating EGFP and the cDNA cloned in its multiple cloning site. This aided us in our study of the cellular morphology, as EGFP distributed uniformly in the cell and divulged changes in the cellular morphology in living cells. Though no significant change in the shape and size of the cells were evident, the transiently transfected cells showed an altered morphology, with numerous cytoplasmic vacuole-like structures. These vacuoles were randomly dispersed in the cytoplasm. This data is in concert with our earlier reports, where the formation of similar cytoplasmic vacuoles has also been shown in a cell-line stably expressing HABP1 (F-HABP07) (Meenakshi et al., 2003). HABP1 is a constitutive multifunctional protein and the expression of HABP1 or its truncated variants in the transfected cells might interfere with the functioning of the endogenous HABP1, leading to stress conditions, resulting in the formation of vacuoles.

Total lysate was analyzed through western blotting to determine changes in the level of HABP1 and actin following their transient expression. No substantial change in the level of either HABP1 or actin was recorded. This observation can be attributed to the fact that the cell transfection efficiency was around 20% in COS-1 cells and since the proteins cannot be induced in these cells, their contribution in the total lysate would be below detectable level. Therefore, to determine if there was any change in the level of HABP1 or its variants, at the individual cell level, immunodetection was carried out using anti-HABP1 antibodies and Cy3 conjugated secondary antibodies. The results indicate that on an average, the intensity of Cy3 increased by about 25% in the transfected cells, as compared to the untransfected cells. There is no alteration in the sub-cellular localization profile of HABP1 or its variants and it resemble that of indigenous HASP1.

Earlier studies from our laboratory have already established that the F-HABP07 cell-line, stably expressing HABP1 undergoes apoptosis
after 60 hours post-transfection (Meenakshi et al., 2003) and vacuolation has often been related to cell death (Hagen et al., 1997). Autophagic vacuoles have been reported to occur in physiological and diseased states, leading to programmed cell death (PCD) (Bursch et al., 2000). Thus, the next task undertaken was to analyze a protein, which can be used as a marker at individual cell level, to determine if the cells were undergoing PCD. The role of p53 has often been associated with PCD and recent studies have shown an altered sub-cellular localization profile for p53 in such cells (Mihara et al., 2003). Therefore, an immuno-detection of p53 was carried out in these cells to ascertain if they had undergone p53-associated PCD. Results indicated no sub-cellular change for p53 in the transfected cells as compared to the control. Thus, from this data, it can be concluded that between 28-32 hours following transfection, the cells did not undergo p53-associated PCD. Cytoskeletal proteins are often associated with PCD and the disruption of the microfilament network or depolymerization of f-actin in cell death has already been shown (Guénal et al., 1997; Korichneva and Hämmerling, 1999). In accordance with this, an investigation on the fate of f-actin in the transfected cells was carried out. Results indicated a decrease in the amount of f-actin in cells transfected with HABP1 and ΔC.HABP1. This decrease can be postulated to be due to depolymerization of f-actin. However, in cells transfected with ΔN.HABP1, there was a stress-induced aggregation of f-actin near the nuclear periphery, a structure resembling "Hirano bodies", which are cytoplasmic inclusions produced due to injury or stress (Hirano, 1994). Hirano bodies are often results of aggregated proteins and cells possessing them have normal total actin levels, but an increased amount of f-actin as compared with wild-type cells (Maselli, 2002). The N-terminal deleted variant has a greater variation in the helical content, as compared to HABP1 and may not be folded properly. This improper folding can result in the aggregation of the protein, leading to the formation of localized f-actin. Hirano bodies may act as a sink for f-actin,
sequestering actin and reducing the concentration of free actin available for cellular processes (Maselli, 2002). Vacuole formation has been attributed to stress conditions in transfected cells. Taken together, these data explain the sequestration of actin and their altered localization at the nuclear periphery.

Cell death is a natural phenomenon associated with differentiation, but in developmental biology early cell death was recognized as a "programmed" event, thus earning the name "Programmed Cell Death" (PCD) (Glucksmann, 1951; Lockshin and Williams, 1965). Subsequent studies later classified PCD into three types. Type I, which is identical to apoptosis; Type II, which is characterized by the formation of autophagic vacuoles ("autophagic cell death") and Type III, which occurs through disintegration of cells into fragments without involvement of the lysosomal system (Clarke, 1990; Schwartz et al., 1993). Autophagy and apoptosis were initially considered as mutually exclusive events, but recent studies showed that they should not be considered as mutually exclusive phenomena. Rather, they appear to reflect a high degree of flexibility in a cell's response to changes in environmental conditions, both physiological and pathological (Bursch et al., 2000).

In an attempt to characterize the vacuoles generated formed upon transient transfection, monodansylcadaverine (MDC) staining was carried out, as it specifically stains autophagic vacuoles in a cell (Biederbick et al., 1995) and this dye has been successfully used in determining the size of autophagic vacuoles (Munafo and Colombo, 2001). The staining revealed these vacuoles to be heterogeneous, being both autophagic and non-autophagic in nature. The MDC staining profile for vacuoles originating through transfection of HABP1 or its truncated variants were similar. The occurrences of small autophagic vacuoles are not unusual in cell-lines, but the vacuoles observed in HABP1/variants transfected cells were substantially increased in number and size. Autophagy has been considered as an adaptive response to nutrient
deprivation, stress conditions, tissue specific biogenesis and a housekeeping mechanism involved in cytoplasmic homeostasis, as it controls the turnover of peroxisomes, mitochondria and the size of the endoplasmic reticulum (Ogier-Denis and Codogno, 2003). Earlier studies on autophagic vacuoles have established their acidic nature (Dunn, 1990; Stromhaug and Seglen, 1993). HABP1 and its truncated variants manifest pl of around 4.2, consequently their acidic nature makes them probable candidates for enrotement into vacuoles. Stress conditions often lead to the formation of autophagic vacuoles (Munafó and Colombo, 2001, Schwartz et al., 1993) and transient expression of HABP1 or its variants in COS-1 cells might evoke a similar phenomenon, forcing the cells to take up autophagy as an adaptive response. Autophagy is also involved in maintaining a balance between protein synthesis and degradation (Hollenbeck, 1993). Expression of an exogenous protein having multiple functions may alter the cellular homeostasis, compelling the cell to take up autophagy for eliminating the exogenous protein.

Recent reports have shed light on the importance of autophagy in different pathological states (Dorn et al., 2002). Numerous viruses have developed strategies to prevent their autophagic degradation (Talloczy et al., 2002). The interaction of HABP1 with numerous viral proteins has already been recorded. The hijacking of HABP1 by viral proteins might affect the formation of autophagic vacuoles in these cases. On one hand, autophagy protects cancer cells from nutrient deficiency and therapeutics, while on the other, it is involved in the elimination of cancer cells by triggering Type II PCD, establishing its antagonistic role in tumour development (Bursch et al., 2000). It has also been observed that cell cycle arrest often manifests autophagy and drugs like 3-methyladenine and okadaic acid inhibit autophagy and suppress cell cycle arrest (Arai et al., 2001). The role of HABP1 in tumour formation, cellular differentiation (Gupta and Datta, 1991) and its dynamics during mitosis (section 4.4) has been reported. Taken together, the results
suggest a correlation between autophagy and the exogenous expression of HABP1. The formation of vacuoles might be indicative of PCD (Type I or II) and the cells manifesting them may undergo such a process soon thereafter.

The vacuoles, which did not stain with MDC, may have originated through a different process. A more detailed analysis of the process involved in the formation of such vacuoles needs to be undertaken to ascertain the exact cause for their formation. The HABP1 variants, though structurally dissimilar from parent molecule, bind HA with comparable efficiency. Taken together, the data postulates that the morphological events manifested through the formation of vacuoles might be related to HA-binding ability of the proteins.

Consequently, from these studies on mutant variants of HABP1, it can be concluded that:

1. **Deletion of N- and C-terminal helices of HABP1 prevents trimerization of the resultant variants, but they can form cysteine-mediated dimers. However, that does not affect their HA affinity, since the dimers of the truncated variants can bind HA with equal efficiency.**

2. **The cloning of HABP1 and its truncated variants in the mammalian expression vector pBI-EGFP enabled simultaneous monitoring of cellular morphology and structural changes acquired upon transient transfection.**

3. **Transient expression of HABP1 and its truncated variants in COS-1 cells generated cytoplasmic vacuoles, a phenomenon similar to a cell-line stably expressing HABP1 (F-HABP07). Since there is no change in the morphology and the HA-binding ability of native HABP1 and the variants, the phenomenon can be considered inter-related.**
4. Though vacuoles are formed in cells transiently expressing HABP1/truncated variants, they did not reveal an alteration in p53 distribution, negating the involvement of a p53 dependent apoptosis in the process. Cytoskeletal organization is disrupted by vacuole formation, as the expression of HABP1/truncated variants influence actin polymerization, thus altering the f-actin microfilament network.

5. Characterization reveals the vacuoles to be both autophagic and non-autophagic, establishing their heterogeneous nature. However, the morphological and structural changes incurred by the cells could be attributed to autophagic nature of the vacuoles.

These results highlight the structure-function relationship of HABP1 and establish the importance of this protein in cellular functioning. A change in the cellular level of HABP1 can drastically alter the morphology of the cell, forcing it into programmed cell death. Thus, from the data discussed in this work, coupled to earlier observations from our laboratory, it can be concluded that HABP1 is an essential cellular protein involved in the maintenance of cellular homeostasis.