CHAPTER-I

PURIFICATION AND CHARACTERIZATION OF THE 68 kDa HA-BINDING PROTEIN AND ITS IMMUNOLOGICAL RELATION TO THE 34 kDa HYALURONIC ACID BINDING PROTEIN FROM RAT SPLEEN
4.1 Introduction

Hyaluronic acid, (HA) is a molecule of paradoxes and contrasts. On one hand, it is a homopolymer composed of simple disaccharide units, endlessly repeated, present in some tissues (such as the cockscomb and vitreous humour) in large amounts, and doing very simple mechanical jobs as a space filler, or as a lubricant (in synovial joints). This is not the stuff of biological high drama. On the other hand, HA also takes part in cell surface phenomena of great specificity, at very low dilution (Laurent & Fraser, 1986). It is an important molecule of great evolutionary antiquity since it is made by streptococci as well as by connective tissue cells of all animals. Some of the tricks it performs in the contemporary biosphere must therefore be relatively new tricks, learnt during its evolutionary history. The simplicity of HA masks some important subtleties.

Hyaluronic acid has been shown to influence several types of cell behaviour, including cell differentiation (Kujawa et al., 1986), motility (Turley, 1994), proliferation (Brecht et al., 1986) and aggregation (Underhill, 1982), but its effects depend greatly on at least three factors. These are the size and concentration of the hyaluronic acid and the type of cell in question (Goldberg and Toole, 1987). One way in which hyaluronic acid is expected to affect cell behaviour is via interaction with its cell surface receptors. Studies on HA-cell interaction require the knowledge of the HA-binding proteins. Therefore, in order to establish the effect of HA on the various cellular activities, the study of the HA-binding proteins became inevitable. In this direction, work on one such HA-binding protein (Mr 34 kDa) has been going in our laboratory. In order to study the role of the 34 kDa HA-binding protein in cell functions, the rat spleen was chosen as a source, since spleen is an important organ involved in monitoring the blood, filtering unwanted elements in the blood by phagocytosis. It participates in the immune response to all the blood born antigens, and is also involved in all system inflammation, hematopoietic disorders and other metabolic disturbances.

The regulatory role of HA in inflammatory reaction has received considerable attention, since hyaluronic acid levels are increased during inflammation and some clinical reports have shown that HA has an inflammatory activity, suggesting a possible role of HA-binding proteins in pathological conditions. The principal objective of the present study was to purify, characterize and study the expression, localization and processing of the HA-binding protein in transformed cells.
4.2 Results

4.2.1 Purification of HA-binding protein from rat spleen tissue by established method

In order to purify the 34 kDa HA-binding protein from the rat spleen tissue, we made use of the established purification method described by Gupta et al (1991a). All the steps were carried out at 4°C, without using any protease inhibitors. For the purification of the 34 kDa spleen HA-binding protein, spleens were dissected out from the normal rats immediately after sacrificing. The tissue was washed in cold 0.01M phosphate buffered saline (PBS), pH 7.2 and homogenized in two volumes (v/w) of 0.2M glycine-HCl, pH 2.2 and centrifuged at 48,000xg for 30 min. The supernatant was neutralized, centrifuged, and dialysed against PBS and then fractionated by 60% saturated ammonium sulfate for 30 min. The precipitate obtained was removed by centrifugation at 10,000xg for 30 min. The clear supernatant fraction obtained was dialysed against PBS, loaded onto a HA-Sepharose-4B affinity column (pre-equilibrated with PBS) and incubated for 45 min. After washing with 20-bed volumes of PBS and then with 20 bed volumes of 0.01M phosphate buffer, pH 7.2 containing 0.5M NaCl, the column was eluted with 0.2M glycine-HCl, pH 2.2 at a flow-rate of 20 ml/h in fractions of 2ml each. The protein containing fractions were determined by measuring their absorbance at 280 nm. Only one peak was observed and the peak fractions were pooled and concentrated. Fig.4.1A shows the SDS-PAGE profile of protein samples collected from different purification steps, and Fig.4.1B shows the SDS-PAGE profile of concentrated HA-binding protein from the pooled (eluted from the HA affinity column) peak fractions. The SDS-PAGE data shows the presence of single prominent band at the 34 kDa position, thereby confirming the purity of the protein and the efficacy of the purification method. This purified protein was then used to develop polyclonal antibodies.

4.2.2 Evidence for the presence of higher Mr HA-binding proteins in rat spleen cell lysates

The polyclonal antibodies obtained after immunizing the rabbit with purified 34 kDa spleen HA-binding protein was tested for its cross-reactivity towards the spleen tissue lysate and purified HA-binding protein. The immunoblot analysis, clearly shows that the anti-34 kDa spleen HA-binding protein antisera recognises a protein of high Mr, a 68 kDa protein besides the 34 kDa HA-binding protein, (Fig.4.2) suggesting that either these two proteins have similar
Fig. 4.1 Purification profile of different steps of the HA-binding protein by the established method

(A) The protein pattern of each fractionation step were analysed by gel electrophoresis on 12.5% SDS-PAGE and visualized by Coomassie Brilliant Blue (CBB) staining. Equal aliquots of 10 μg of protein were applied to the gel. Lane 1, molecular weight markers; Lane 2, PBS extract; Lane 3, glycine-HCl extract; Lane 4, supernatant of neutralized extract; Lane 5, supernatant of ammonium sulfate fractions; Lane 6, pellet of ammonium sulfate fraction and Lane 7, purified HA-binding protein.

(B) SDS-PAGE profile of the 34 kDa HA-binding protein purified from rat spleen. Lane 1, purified spleen HA-binding protein and Lane 2, molecular weight markers. Resolution was carried out on 12.5% SDS-PAGE and protein bands were visualized by CBB staining.

Fig. 4.2 Evidence for immunologically related high molecular weight protein in rat spleen cells by using anti-34 kDa HA-binding protein anti sera by immunoblot. Lane 1, 4 μg purified HA-binding protein and Lane 2, 40 μg of spleen cell extract. Proteins were separated on 12.5% SDS-PAGE and the gel was electroblotted onto a nitrocellulose membrane, and probed with anti-HA-binding protein antisera.
epitopes or the signal is due to the non-specific interactions inherent to the polyclonal nature of antisera.

4.2.3 Co-purification of the 68 kDa and the 34 kDa HA-binding protein by modified method

The fact that the anti-34 kDa HA-binding protein antibodies shows cross-reactivity with 68 kDa protein suggests an existing relationship between the 34 kDa and the higher Mr 68 kDa protein. Therefore, an attempt to purify the 68 kDa protein from the HA-sepharose column was the next obvious step. This procedure involved a shorter purification protocol and also included protease inhibitors. To purify the 68 kDa protein along with the 34 kDa HA-binding protein, the spleens were dissected out from a normal rat, cleaned well, minced in cold 0.01M PBS at 4°C and passed through a steel mesh. The cell suspension was centrifuged at 3000xg at 4°C for 5 min. The cell pellet so obtained was suspended in lysis buffer containing phosphate buffered saline pH 7.4, 0.25M sucrose, 1mM PMSF, 5 mM EDTA and 0.1% Triton X-100. The cell lysate was then centrifuged at 48,000xg, the cell pellet discarded and the cell supernatant was dialysed against PBS and loaded onto the HA-affinity column and incubated for 45 min. After washing with 20 bed volumes of PBS and then with 20 bed volumes 0.01M phosphate buffer pH 7.2 containing 0.5M NaCl, the column was eluted with 0.2M glycine-HCl, pH 2.2 at a flow-rate of 20 ml/h in fractions of 2ml each. The protein containing fractions were determined by measuring their absorbance at 280 nm as shown in Fig.4.3A and the peak fractions were separated on a 12.5% SDS-PAGE (insert in the Fig.4.3B). The SDS-PAGE data shows that both the 68 kDa and the 34 kDa HA-binding proteins are present in all the fractions and the 68 kDa protein appears as a doublet. The peak fractions were pooled, concentrated and separated on SDS-PAGE. Fig.4.3C confirms the purification of the 68 kDa protein by this method along with the 34 kDa HA-binding protein, revealing the HA-binding properties of the 68 kDa protein.

4.2.4 Immunological cross-reactivity of the antibodies specific for the 34 kDa HA-binding protein

The proteins purified by the modified method were subjected to immunoblot analysis. In order to rule out the nonspecific interactions inherent to the polyclonal nature of the antiserum, the antibodies were affinity purified against the 34 kDa protein by using the blot
Fig. 4.3 Co-purification of the 68 kDa HA-binding protein with the 34 kDa HA-binding protein
(A) Co-purification profile of 68 kDa HA-binding protein from spleen cell lysate. The spleen cell extract was applied to the HA affinity column and washed with 0.01 M PBS, pH 7.2, followed by a wash with 0.01 M PBS containing 0.5 M NaCl, pH 7.2. The protein was eluted using 0.2 M glycine-HCl, pH 2.2 in fraction of 2 ml each with a flow rate of 18 ml/h. OD of all fractions was measured at 280 nm. The peak protein fractions were separated on a 12.5% SDS-PAGE shown in the inset.
(B) SDS-PAGE profile of the 34 kDa and 68 kDa HA-binding proteins purified from rat spleen; Lane 1, spleen cell lysates; Lane 2, molecular weight markers and Lane 3, purified spleen HA-binding protein. Electrophoresis was carried in 12.5% SDS-PAGE and the protein bands were visualized by CBB staining.

Fig. 4.4 Western blotting of purified spleen HA-binding protein using affinity purified antibodies. HA-binding protein was separated on 12.5% SDS-PAGE and transferred onto nitrocellulose and then probed with anti-34 kDa HA-binding protein antibodies.
procedure and again checked for its cross-reactivity. Similar to the result of the immunoblot analysis by the whole antiserum, the specific affinity purified anti 34 kDa-HA-binding protein antibodies also recognised (Fig.4.4) a 68 kDa doublet protein and additional 55 kDa protein along with the 34 kDa protein.

4.2.5 Native molecular weight and isoelectric point determination

The apparent Mr of the 34 kDa and the 68 kDa HA-binding proteins in the native state as revealed by a linear gradient of 4-20% polyacrylamide slab gel electrophoresis is 68 kDa and 136 kDa, respectively (Fig.4.5A). This was further confirmed by Western blotting (Fig.4.5B). The SDS-PAGE analysis of the purified HA-binding protein on a 12.5% gel showed two protein bands at 34 kDa and 68 kDa, thus indicating that the 68 kDa HA-binding protein is a homodimer of 34 kDa subunit and the 136 kDa is a homodimer of 68 kDa subunit.

The two dimensional gel electrophoresis was performed with spleen HA-binding proteins and then transferred onto the nitrocellulose membrane and probed with affinity purified anti-34 kDa antibodies. Prominent band corresponding to Mr 68 kDa and 34 kDa were observed (Fig.4.5C). Based on the molecular weight and isoelectric range estimates which were run simultaneously, the pls of the 68 kDa and 34 kDa proteins were estimated as 4.0 and 4.2, respectively.

4.2.6 Glycoprotein nature

The HA-binding proteins purified by HA column were separated by SDS-PAGE and transferred onto the nitrocellulose membrane and probed with radiiodinated Con-A. Con-A binding was seen with both the 68 kDa and the 34 kDa HA-binding proteins (Fig.4.6), suggesting the glycoprotein nature of both the proteins.

4.2.7 Protease sensitivity of spleen HA-binding proteins

Spleen lysates were prepared in different combinations of protease inhibitors [PMSF and Aprotinin for serine proteases, EDTA for metalloproteases, TLCK for trypsin, TPCK for chymotrypsin, leupeptin for serine and thiolproteases and pepstatin for acid proteases (Fig.4.7)]. These cell lysates were resolved under reducing conditions on SDS-PAGE and transferred onto the nitrocellulose membrane and probed with the affinity purified antibodies. Prominent bands at 68 kDa, 55 kDa and 34 kDa positions were seen when more number of
Fig. 4.5 PAGE and 2-D analysis

(A) Native polyacrylamide gel electrophoresis of rat spleen HA-binding protein. The purified HA-binding protein (6 µg) was electrophoresed at pH 8.9 on a 7.5% PAGE gel in the absence of 63 SDS at a constant voltage of 80 V as described under material and methods. The protein bands were visualized by CBB staining lane 1, BSA and lane 2, purified HA-binding protein.

(B) The purified HA-binding protein (6 µg) was electrophoresed at pH 8.9 in a 7.5% polyacrylamide slab gel in the absence of SDS at constant 80 V and gel was transferred onto nitrocellulose and probed with anti-34 kDa HA-binding protein antibodies. Lane 1, Brain HA-binding protein; Lane 2, Heart HA-binding protein and Lane 3, spleen HA-binding protein.

(C) Two dimensional electrophoresis analysis of purified HA-binding protein. 5 µg of HA-affinity column eluted protein was subjected to isoelectric focusing over a pH range of 3.0-10.0. The focussed proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose and probed with anti-34 kDa HA-binding protein antibodies.

Fig. 4.6 Binding of Con-A to HA-binding protein. 10 µg of purified HA-binding protein was separated on 12.5% SDS-PAGE and then transferred onto nitrocellulose membrane, blocked with 2% BSA in PBST for 2 h, incubated with 125I-Con-A, washed and autoradiographed.
Fig. 4.7 Evidence for protease sensitivity of HA-binding proteins from rat spleen. Rat spleen was homogenized with various combinations of protease inhibitors. Lane 1, more number of protease inhibitors (PMSF, EDTA, Benzamidine, TLCK, TPCK, Leupeptin, antipain, pepstatin and aprotinin); Lane 2, less number of protease inhibitors PMSF, EDTA, Leupeptin and Aprotinin; Lane 3, no protease inhibitors. Samples containing 20μg of protein were separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies.

Fig. 4.8 Immunodetection of the 34 kDa HA-binding protein in different cell lines and tissues. Equal amounts of proteins isolated from different cells and tissues were resolved on SDS-PAGE, transferred onto a nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies.

(A) Different transformed cell lines
(B) Rat tissue extracts
protease inhibitors (PMSF, EDTA, TLCK, TPCK, leupeptin, pepstatin, aprotinin) were used (Lane 1). Lane 2, shows a prominent band at 34 kDa and less prominent bands at the 68 kDa, 55 kDa positions, when lesser number of protease inhibitors (PMSF, EDTA, TLCK, TPLK) were used. When no inhibitors were used, only the 34 kDa protein could be detected with no bands at the higher molecular weight positions (Lane 3).

4.2.8 Expression of the 34 kDa HA-binding protein in various transformed cells and rat tissues

To study the expression of the 34 kDa HA-binding protein in various transformed cells and rat tissues, the stored transformed cell lysates prepared without protease inhibitors were separated on SDS-PAGE and transferred onto the nitrocellulose membrane and the HA-binding protein were detected using anti-34 kDa HA-binding protein antibodies. Fig.4.8A and Fig.4.8B show the presence of only the 34 kDa HA-binding protein in transformed cell lysate and tissue lysate of spleen, kidney and heart. This data indicates the ubiquitous expression of the 34 kDa HA-binding protein and confirms that the 34 kDa HA-binding protein is stable as it is observed even in the absence of protease inhibitors.

4.2.9 Co-expression of the 68 kDa and the 34 kDa HA-binding proteins in various tissues lysates and transformed cell lysates

After studying the expression of the 34 kDa HA-binding protein, conditions were designed to study the expression of the 68 kDa protein, since the 68 kDa protein is very unstable and protease sensitive. In order to inhibit the protease activity, the cell and tissues samples were prepared in the lysis buffer containing a cocktail of protease inhibitors. Equal amount of tissue lysates and cell lysate proteins were separated on SDS-PAGE and transferred onto the nitrocellulose membrane and probed with anti-HA-binding protein antibodies. Fig.4.9A, Fig.4.9B and Fig.4.9C show the expression of the 68 kDa, 55 kDa and 34 kDa HA-binding proteins in various tissues. Fig.4.9D shows the presence of the 68 kDa and 34 kDa HA-binding proteins in EL4, Raji and MCF-7 transformed cells, indicating the ubiquitous occurrence of the 68 kDa HA-binding protein along with the 34 kDa protein. For further studies, EL4 (Mice transformed T-cells), Raji (Human transformed B-cells) and MCF-7 (Breast carcinoma cells) cell lines were used.
Fig. 4.9 Immunodetection of the 34 kDa and 68 kDa HA-binding protein in various rat tissues and cell lines. Equal amounts of proteins (25 μg) from various rat tissues and cell lines were separated on 12.5% SDS-PAGE, and transferred onto nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies.

(A) Rat digestive tissues
(B) General rat tissues
(C) Lymphocytic tissues
(D) Transformed cell lines
4.2.10 Immunological relation of the 34 kDa HA-binding protein with other HA-binding proteins

In order to determine the relationship of the 34 kDa HA-binding protein with other known HA-binding proteins (e.g. CD44, RHAMM and Cdc 37), the purified 68 kDa and 34 kDa HA-binding proteins were subjected to immunoblot analysis and probed with anti-CD44, anti-P-32 (a Splicing Factor-2 co-purified protein), IVd4 monoclonal antibodies (of Cdc 37), and anti-RHAMM polyclonal antibodies. No bands could be detected in lanes containing purified HA-binding proteins, probed with CD-44 and Cdc-37 antibodies, indicating that these protein are immunologically distinct (Fig.4.10, Panel A and B). When the blot was probed with polyclonal antibodies (R.3.8) raised against a peptide sequence in RHAMM, crossreactivity between the 34 kDa and the 68 kDa position was seen indicating antigenic similarity (Fig.4.10, Panel C). On probing the blot with P-32 (a Splicing Factor-2 co-purified protein having complete sequence homology with the 34 kDa HA-binding protein) monoclonal antibodies; the 68 kDa, 55 kDa and 22 kDa HA-binding proteins along with the 34 kDa HA-binding protein could be detected in the cell lysate (Fig.4.10, Panel D).

4.2.11 Localization of the 34 kDa HA-binding protein in MCF-7 cells

To study the localization of the 34 kDa HA-binding protein, MCF-7 cells were incubated with the IgG of the pre-immune rabbit (as control) and with anti 34 kDa HA-binding protein antibodies. The antibodies bound to the cells were detected by incubation with FITC-conjugated goat anti-rabbit antibodies or HRPO labeled goat anti-rabbit antibodies. Approximately, 95% of the cell population got stained with anti- 34 kDa antibodies. As seen in Fig.4.11 (Panel 1A and 1B), pre-immune sera was used as a control and no signal could be seen. Fig.4.11 (Panel 2A and 2B) show the non permeabilized cells and the staining was observed on the cell surface. Fig.4.11 (Panel 3A and 3B) show the permeabilised cells where the staining was observed in the nuclei, indicating the nuclear localization of the 34 kDa or the 68 kDa HA-binding protein.

4.2.12 Subcellular analysis of HA-binding proteins in EL4 cells

To determine whether the 34 kDa and the 68 kDa proteins were located in the subcellular fractions, namely cytosol, nucleus and plasma membrane, the subcellular fractions were separated on SDS-PAGE and transferred onto the nitrocellulose membrane and probed
Fig. 4.10 Immunological relationship of the 34 kDa HA-binding protein with other known HA-binding proteins. The purified HA-binding protein and cell lysate proteins were separated on 12.5% SDS-PAGE and transferred onto nitrocellulose membrane and probed with the following:

(A) Mouse anti-CD44 monoclonal antibodies
(B) Rabbit anti-RHAMM polyclonal antibodies
(C) Mouse anti-IVd4 monoclonal antibodies
(D) Mouse anti-P-32 monoclonal antibodies
Fig. 4.11 Cell localization of the HA-binding protein

(A) Localization of HA-binding protein by immunofluorescence. Localization of HA-binding protein on the MCF-7 cell line by indirect immunofluorescence staining using anti-34 kDa HA-binding protein antibodies and FITC labelled secondary antibodies. MCF-7 cells were subcultured on glass coverslips for 24 h and permeabilized in methanol at -20°C for 4 min, fixed using 4% paraformaldehyde in PBS and exposed to antibodies against HA-binding protein and then the resultant immunocomplexes were visualized by FITC-labelled goat anti-rabbit IgG as described under material and methods. Pre-immune rabbit IgG staining showed negligible signal (Panel 1A), while the anti-HA-binding protein antibodies stained the cell surface in non-permeabilized cell (Panel 2A) and in permeabilized cells we could see the peri nuclear and nuclear region were stained (Panel 3A).
Fig. 4.11 Cell localization of the HA-binding protein

(B) Localization of HA-binding protein in MCF-7 cells by immunohistochemical staining. Monolayer cultures of subconfluent MCF-7 cells were permeabilized in methanol at -20°C for 4 min and fixed using 4% Paraformaldehyde. Further, the cells were first stained with rabbit anti-HA-binding protein antibodies and subsequently, with the complex of HRPO labelled goat-anti rabbit- IgG as described in materials and methods. The HA-binding protein was visualized by 3-3' diaminobenzidine. Pre-immune rabbit IgG staining showed negligible signal (Panel 1B), while the anti-HA binding protein antibodies stained on the cell surface in non-permeabilized cells (Panel 2B), and in permeabilized cells peri- nuclear and nuclear region were stained (Panel 3B).
with anti-HA-binding protein antibodies. Fig.4.12 shows that the anti-HA-binding protein antibodies recognize two proteins of Mr 34 kDa and 68 kDa in the plasma membranic, cytosolic and nuclear fractions.

4.2.13 Triton X-100 solubilization of HA-binding protein in EL4 cells

The EL4 cells were solubilized in different concentration of Triton X-100 and the insoluble fraction was separated on SDS-PAGE and transferred onto the nitrocellulose membrane and probed with anti-HA-binding protein antibodies. Fig. 4.13 shows that as the concentration of Triton X-100 is increased the amount of HA-binding protein present in the insoluble fraction reduces suggesting that it gets increasingly solubilized although, never completely (in the concentrations used). The anti-HA-binding protein antibodies recognized an additional 42 kDa band alongwith the expected 68 kDa and 34 kDa bands in the Triton X 100 insoluble fraction. This suggests that these proteins might be associated with either cytoskeletal proteins or with the other insoluble membranes in the cell.

4.2.14 Biosynthesis and processing of the 34 kDa HA-binding protein in EL4 transformed cells

In order to confirm the presence of the 68 kDa HA-binding protein and to study the processing of the 34 kDa HA-binding protein, we used EL4 cells and the standard pulse-chase technique. The EL4 cells were pulse-labeled with $[^{35}\text{S}]$ methionine (30, 60 and 90 min) and then the cells were lysed and immunoprecipitated with anti-34 kDa HA-binding protein antibodies Fig.4.14A shows the proteins detected after 30, 60 and 90 min. After 30 min of pulsing, a prominent 34 kDa protein and a less prominent 68 kDa and 95 kDa protein could be detected. However, after 60 and 90 min, the intensity of the 34 kDa, the 68 kDa and the 95 kDa bands increased and an additional 22 kDa band could also be seen.

For the chasing experiment, the EL4 cells were first pulsed for 20 min and then chased for various time intervals (0, 15, 30 and 60 min) in growth medium containing cold methionine. The cells were then lysed and immunoprecipitated using anti-34 kDa HA-binding protein antibodies. Fig.4.14B shows pulsing for 20 min and then chasing. Initially, at 15 min chasing, we could observe a 44 kDa and a 55 kDa proteins, then gradually the 44 kDa proteins began to disappear first, followed by the disappearance of the 55 kDa protein and the appearance of the 34 kDa protein, suggesting that the 34 kDa protein is synthesised from high
Fig. 4.12 Subcellular analysis to identify localization of the 34 kDa and the 68 kDa HA-binding proteins. The proteins from plasma membrane, cytosol and nucleus isolated from EL4 cells (lymphoma cell line) were separated on 12.5% SDS-PAGE under reducing conditions, transferred onto nitrocellulose membrane and probed with anti-HA-binding protein antibodies.

Fig. 4.13 Evidence for the presence of the 34 kDa and the 68 kDa HA-binding protein in Triton X-100 insoluble cell pellet. EL4 cells isolated and the pellets obtained with different concentrations of Triton X-100 were solublized in presence of SDS, separated on 12.5% SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies.
Fig 4.14 Biosynthesis of the 34 kDa and the 68 kDa HA-binding protein in lymphoma cell lines by [³⁵S] labelling studies

(A) Pulse labelling and immunoprecipitation of the 34 kDa and the 68 kDa HA-binding protein from T-lymphoma cells. Confluent cultures of EL4 cells were incubated in L-methionine free DMEM for 2 h followed by pulse labelling of cells with [³⁵S] methionine (100 μCi/ml) for 30 min (lane 1), 60 min (lane 2) and 120 min (lane 3). Cells were solubilized in RIPA buffer followed by anti-HA-binding protein antibodies mediated immunoprecipitation. The immunoprecipitates were analyzed by 10% SDS-PAGE followed by fluorography, as described under materials and methods.

(B) Biosynthetic processing of the 34 kDa HA-binding protein. Mouse EL4 lymphoma cells were first pulse-labelled with [³⁵S] methionine for 20 min. Cells were then incubated in unlabelled methionine-containing medium for various chase timings. Subsequently, cells were solubilized in RIPA buffer followed by anti-HA-binding protein mediated immunoprecipitation and analyzed by 10% SDS-PAGE followed by fluorography as described under material and methods. Lane 1, 15 min; Lane 2, 30 min; Lane 3, 60 min and Lane 4, 120 min.
molecular weight 44 kDa and 55 kDa proteins, which on processing give rise to the 34 kDa protein.

4.2.15 Biosynthesis of the 34 kDa HA-binding protein in MCF-7 cell lines

In order to confirm the processing of the 34 kDa HA-binding protein, MCF-7 cells were used for the standard pulse-chase technique. The MCF-7 cells were pulse-labeled with \[^{35}\text{S}]\text{methionine}\) for 3 and 4 h. The cells were then lysed and immunoprecipitated using anti-34 kDa HA-binding protein antibodies. Fig.4.15A shows prominent 44 kDa and 55 kDa proteins along with the 34 kDa and 68 kDa proteins after 3 and 4h pulsing. For the chasing experiment the MCF-7 cells were first pulsed for 2 h and then chased for 3 and 4 h in growth medium containing cold methionine. The cells were then lysed and immunoprecipitated using anti-34 kDa HA-binding protein antibodies Fig.4.15B shows the chasing. Initially, at 3 h chasing, the 44 kDa and the 55 kDa proteins were observed and then after 4 h the disappearance of 55 kDa protein followed by appearance of the 34 kDa protein was seen, indicating that the 34 kDa protein is presumably synthesized from higher Mr 44 kDa and 55 kDa proteins by processing, similar to our observation in EL-4 cells.

4.3 Discussion

This chapter documents for the first time, the presence of a novel 68 kDa HA-binding protein in rat spleen. This protein is found to be immunologically identical to the 34 kDa HA-binding protein reported earlier by Gupta et al (1991a). A modified procedure has been established to co-purify this protein with the 34 kDa HA-binding protein. The previous protocol, did not involve the usage of any protease inhibitors, and moreover, acidic conditions were used for extraction of the protein. Also, the procedure was time consuming and therefore, the probability of the experimental conditions being responsible for the degradation of these proteins was high. Taking this as a clue, the purification procedure was modified into a short one, which was carried out at neutral pH and in the presence of a cocktail of protease inhibitors. All the proteins detected by immunoblot could be purified by this method. Previously, a chondroitin sulphate proteoglycan from the human brain was purified in the presence of protease inhibitors using DEAE Sepharose column and the N-terminal amino acid sequence was found to be identical with the previously reported N-terminal sequence of versican, Hyaluronectin(Delpech and Halavent,1981,1982) and GHAP(Perides,1989,1992).
Fig. 4.15 Pulse labelling and immunoprecipitation of the 34 kDa HA-binding protein from MCF-7, breast tumor cell lines.

(A) Confluent cultures of MCF-7 cells were incubated in L-methionine free DMEM for 2 h followed by pulsing for 3 h and 4 h.

(B) Confluent culture of MCF-7 cells were first pulse labelled for 1 h. Cells were then incubated in unlabelled methionine for various chase timings (3h, Lane 1; 4h, Lane 2). Subsequently, the cells were solubilized in RIPA buffer followed by anti-HA-binding protein antibodies mediated immunoprecipitation and analysed on 12.5% SDS-PAGE followed by fluorography as described under materials and methods.
Zimmermann and Ruoslahti, (1989) have shown that GHBP and Hyaluronectin are derived from versican by protein degradation during protein purification, and the peptide sequence of GHBP and Hyaluronectin from brain are identical to the sequences in the amino terminal hyaluronic acid binding region of versican. Recently, Ponting et al (1995) purified Hyaluronectin from placental villi having Mr 47,52,57 and 67 kDa, and the differences in molecular weight was attributed to longer exposure to low pH, indicating hydrolysis of the protein on prolonged exposure to acid conditions. As per reports from these laboratories, the probability exists that 34 kDa HA-binding protein may be derived from the degradation of 68 kDa protein. However, the data already known, rules out this possibility.

Recently from our laboratory, Deb and Datta (1996) reported the isolation of a partial cDNA clone from an Agt11 cDNA expression library of human skin fibroblast. Immunoscreening with anti-HA-binding protein polyclonal antibodies was done and immunopositive clone was picked up and sequenced. Interestingly, the partial cDNA sequence of this clone and the internal polypeptides of the purified protein showed complete homology with the cDNA sequence of P-32, a protein co-purifying with human pre-mRNA splicing factor, SF2 (Krainer et al., 1991). Honore et al (1993) cloned and expressed the cDNA encoding P-32, which extends beyond 5' end of cDNA previously reported by Krainer et al (1991). Interestingly, cDNA fragment includes an ATG start codon which was absent from the previously reported cDNA. Honore et al (1993) has shown that the P-32 full length cDNA encodes protein of 282 amino acids, but when the clone was expressed in vaccinia virus, it synthesised a protein of 209 amino acid. N-terminal sequencing of the protein synthesized by cells infected with vaccinia virus construct gave the sequence of 73 amino acids away from start codon; thus, suggesting that the 34 kDa HA-binding protein/P-32 protein is synthesized as a pro-protein of 282 amino acids and it is post-translationally processed to remove the initial 73 amino acids to form a mature protein of 209 amino acids. It is also important to mention that the N-terminal amino acid sequence obtained from purified protein is completely identical with the predicted N-terminal amino acid sequence from cDNA encoding the 34 kDa HA-binding protein.

Characterization of the 68 kDa protein so purified, involved the determination of its native Mr, its pI, the glycoprotein nature and its sensitivity to proteases. The PAGE data
showed the apparent native Mr of the HA-binding proteins as 136 kDa and 68 kDa. In denatured state, the 136 kDa protein appeared as the 68 kDa and 68 kDa protein as the 34 kDa band, indicating that the 136 kDa HA-binding protein is a homodimer of 68 kDa subunits and the 68 kDa protein is a homodimer of 34 kDa subunits.

The 2-D analysis of the HA-binding proteins showed the acidic nature of these proteins and the Con-A binding studies showed the glycoprotein nature of the tissue purified 34 kDa and 68 kDa HA-binding proteins.

In the studies conducted by using different combination of inhibitors, it was seen that the higher molecular weight 68 kDa protein is extremely sensitive to proteolysis in comparison to the 34 kDa HA-binding protein. Sometimes, an additional protein bands were also observed between the 68 kDa and the 34 kDa proteins in protein purification gels and immunoblots. These proteins were found to be very unstable and appeared at Mr of 55 kDa and 44 kDa. It is speculated that either these proteins arise due to the degradation of the 68 kDa protein or they themselves are the precursors of the 34 kDa HA-binding protein.

The expression of these proteins in different rat tissues has also been demonstrated. A series of transformed cell lines were tested using immunoblot analysis and these proteins were found to be present in fibroblasts, macrophages, lymphocytes, epithelial cells, and plasma cells, suggesting that like CD44, the 34 kDa HA-binding protein, is also constitutively expressed. This is however, unlike other HA-binding proteins such as TSG-6, RHAMM and ICAM-1 that are tightly regulated in expression (Green and Tarone et al., 1988).

The localization studies of the HA-binding protein showed its presence on cell surface of non permeabilized Breast carcinoma cells. In permeabilized cells, the nuclear localization could also be seen. In an earlier study, it was also reported that the cDNA sequence of the 34 kDa protein contains the NLS motif in the deduced amino acid sequence of the mature HA-binding protein, indicating its role in nuclear import (Deb and Datta, 1996). Further, the subcellular analysis of EL4 transformed cells showed the existence of both the 34 kDa and the 68 kDa HA-binding proteins in nuclear, cytosolic and plasma membranic fractions. Immunoblot analysis of serum too, showed the existence of these proteins as soluble proteins, probably either due to plasma membrane shedding or the secretion of these proteins from the various leukocytes, involved in the inflammatory reactions.
Results on the Triton X-100 insoluble fractions showed the presence of the 34 kDa and the 68 kDa HA-binding protein along with the 42 kDa protein revealing the association of this protein with the cytoskeleton proteins, and in turn suggesting its role in intracellular traffic events like exocytosis and endocytosis.

In the studies using [35S] metabolic labelling in EL4 cells, a prominent 34 kDa and faint 68 kDa and 94 kDa bands were detected after pulsing for 1 h. Upon further labelling for 2 h and 3 h, prominent 68 kDa, 95 kDa and 22 kDa bands along with the 34 kDa protein band were seen. This data indicates that after the synthesis initially, the 34 kDa protein undergoes some modifications to give rise to the high molecular weight proteins. However, in MCF-7 cells, after 3 h and 4 h of labelling, the 44 kDa, 55 kDa and 34 kDa proteins were seen along with very faint bands at the 68 kDa position, indicating that the synthesis in EL-4 cells differs from that in MCF-7.

The pulse-chase experiment data clearly indicates that the processing of the 44 kDa and the 55 kDa proteins gives rise to the 34 kDa protein. However, during the chase experiment, proteins of 68 kDa and 95 kDa could not be detected, probably owing to the short pulse-chase time. The data, at present, does not clearly show the direct relation of the 34 kDa HA-binding protein with during the synthesis of the 68 kDa HA-binding protein.

The different identity of these proteins was established by the studies on antibody cross-reactivity of the 34 kDa and 68 kDa protein with CD44 and IVd4 monoclonal antibodies, which showed by that they are immunologically different proteins. The sequence data also showed that these proteins have very weak homology with CD44 and Cdc 37. The antibody cross-reactivity with RHAMM antibodies showed cross-reactivity with these proteins but as per the sequence analysis, it was seen that they are different.

The identification of 68 kDa HA-binding protein was done by immunoblot analysis using affinity purified polyclonal antibody raised against the 34 kDa HA-binding protein. Thus, as a continuation, we used the monoclonal antibody raised against P-32, a protein co-purified with splicing factor 2 (SF2) having sequence homology with the 34 kDa HA-binding protein. As apparent from our data, there is a cross-reactivity of the anti-P-32 antibodies with the 34 kDa and the 68 kDa HA-binding protein removing the doubt of non specific immunogenecity of polyclonal antibody against 34 kDa HA-binding protein. Besides being
completely identical with P-32, 34 kDa HA-binding protein was also found to be identical with two other proteins. The gClq-R (100% homology), the receptor of the complement protein C1q and a murine protein YL2 (92% homology), a protein which interacts with basic domain of Rev protein of HIV type 1. Ghebrehiwet (1994) recently discovered a gClq-R protein and purified this from Raji cells and cloned from a B-lymphocyte cDNA library. It was found to encode a pre-protein of 282 amino acids and N-terminal of the protein isolated from Raji cells started at the residue 74 of the predicted pre-protein sequence. The Northern blot analysis by using gClq-R cDNA showed that it contains only one transcript of 1.6 kb. This also indicates that the proteins which are detected in our purification, immunoblot and immunoprecipitation are synthesized by the same mRNA but molecular weight differences may arise due to post translational modifications. After a careful examination of the immunoblot presented by Ghebrehiwet et al (1994) in their paper, a visible band at 68 kDa along with the 34 kDa protein is detected, although, they have not mentioned about the 68 kDa protein in their paper. Another report made by Euggleton et al (1996) also supports our observation since they recognized a high Mr protein of 80-90 kDa by the antibodies raised against gClq-R.

In this chapter, the co-purification and partial characterization of the high Mr 68 kDa HA-binding protein from the rat spleen and its similarity with some of the properties of the 34 kDa HA-binding protein have been demonstrated. The results also showed that the 68 kDa protein is very unstable and it can be detected only in the presence of protease inhibitors. It needs to be mentioned here that the 68 kDa band can also be detected in the absence of protease inhibitors provided the tissue on the cells from which it is extracted is fresh (see chapter 2, section 5.2.3). The expression of the 68 kDa and the 34 kDa HA-binding proteins in various tissues and transformed cells and subcellular localization suggests some site specific functional role of this protein. [35S] labelling studies have shown that the 34 kDa HA-binding protein arises from the 55 kDa and the 44 kDa proteins. Although, the preliminary data presented here suggest that 68 kDa protein is related to 34 kDa HA-binding protein, some important questions still remain unanswered. In order to address these, our next attempt was to purify the 68 kDa protein.