CHAPTER-II

PRESENCE OF HIGH MOLECULAR WEIGHT HYALURONIC ACID BINDING PROTEINS IMMUNOLOGICALLY RELATED TO THE 34 kDa HYALURONIC ACID BINDING PROTEIN
5.1 Introduction

A major challenge in modern biology is to understand the structure-function relationship of mature proteins. All proteins translated from mRNA undergo one or more modifications in their covalent structure via post translational processing events. Collectively, these alterations are required for functions including activation, regulation, translocation and turnover.

Major post-translational modifications which occur in proteins, include phosphorylation (addition of phosphate group), glycosylation (addition of carbohydrate), prenylation (addition of C15 or C20 isoprenoids), acylation (addition of C16 to C18 fatty acids), sulphation (addition of sulphate) and protein-protein crosslinking by γ carboxyglutamic transferase. Hyaluronic acid (HA), a linear polysaccharide present in the extracellular matrix of all connective tissues, influences cell migration, proliferation and differentiation and plays a role in morphogenesis, angiogenesis, wound healing and immune response (Laurent and Fraser, 1992). Several specific HA-binding proteins, both extracellular and cell surface, collectively known as hyaladherins, have been identified and their functions are known to be regulated by the above mentioned post-translational modification.

The most well characterized HA-binding protein CD44, although derived from a single gene, can give rise different isoforms by alternative splicing (Shtivelman and Bishop, 1991). All of the isoforms of CD44 are highly glycosylated, containing both N- and O-linked carbohydrate side chains and variation in of glycosylation gives rise to multiple molecular mass of CD44 (Lokeshwer and Bourguigon, 1991).

CD38, another HA-binding protein of 45 kDa type II transmembrane glycoprotein is also reported to be modified post-translationally into a high Mr form of 190 kDa. CD38 has the enzymic activity for the synthesis of ADP-ribose from NAD+ and the hydrolysis of cADPR to ADP ribose, whereas the high molecular immunologically identified protein of 190 kDa has three times more cyclase activity, compared to that of CD38. Finally, it has been successfully demonstrated that CD38 as the substrate of transglutaminase confirming that P-190 is a post-translationally modified cross linked product of CD38 (Umar et al., 1996).

Another example of stable form of protein-protein interaction is the complex formation of serum protein, inter-α-inhibitor with another HA-binding protein, known as Tumor necrosis

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factor stimulated gene 6 (TSG-6). TSG-6, secretory, 35 kDa glycoprotein, inducible by TNF and IL-1, forms a complex readily with a serum protein and the complex is stable during SDS-PAGE under reducing and in presence of 8M urea. The protein from the complex has been identified, as inter-α-inhibitor and is shown to be cross linked with chondroitin sulphate. The possible functional implications of complex formation between TSG-6 and inter-α-inhibitor are still elusive. Though TSG-6 has been detected in human serum, it is most prominent at sites of local inflammation (Wisniewski et al., 1993). Therefore, TSG-6/interα-inhibitor interaction probably occurs at the inflammatory sites such as the synovial tissues in rheumatoid arthritis.

In our laboratory, we are working on a novel 34 kDa hyaluronic acid binding protein. This protein was shown to be involved in macrophage histiocytoma activation (Gupta and Datta, 1991b) and also its role in sperm maturation, motility and fertilization process has been described (Ranganathan et al., 1996). Recently, the predicted amino-acid sequence derived from cDNA sequence revealed its 100% homology with P-32, a protein co-purified with splicing factor 2 (SF2) as well as with gC1q-R protein and 92% homology with the YL2 protein, thus suggesting the multifunctional activity of this protein (Deb and Datta, 1996). In the previous chapter, we have identified and purified high Mr 68 kDa HA-binding protein along with the 34 kDa HA-binding protein. This seems to be an ideal model to study the interrelationship between two proteins. Thus, as an initial step, purification of the 68 kDa protein becomes inevitable. In this chapter, therefore, we made an attempt to homogenously purify and characterize the 68 kDa HA-binding protein.

5.2 Results

5.2.1 Purification of the 68 kDa HA-binding protein from rat spleen

To purify the 68 kDa HA-binding protein, the spleen was 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 3000 xg at 4°C for 5 min. The cell pellet so obtained was suspended in lysis buffer containing PBS (pH 7.2), with 0.25M sucrose, 1mM PMSF, 5 mM EDTA, leupeptin, pepstatin, aprotinin and 0.1% Triton X-100. The cell lysate was then, centrifuged at 48,000xg, the cell pellet was 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 of 0.5M NaCl buffered at pH 7.2 by 0.01M phosphate buffer, 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.5.1A and peak fractions separated on 12.5% SDS-PAGE was shown as insert in the Fig.5.1B.

5.2.2 Characterization of the 68kDa HA-binding protein

5.2.2.1 Native molecular weight determination

The apparent molecular mass of higher Mr HA-binding protein in native state was 136 kDa as determined by 4-20% polyacrylamide gradient slab gel electrophoresis under native conditions (Fig. 5.2).

5.2.2.2 Subunit molecular weight determination

Subunit molecular mass was determined by SDS-PAGE analysis of the purified HA-binding protein on the 12.5% slab gel. Presence of a protein band at 68 kDa, indicating that the higher Mr HA-binding protein is actually a homodimer of 68 kDa subunits. Furthermore, the same band appears both in the presence and absence of β-mercaptoethanol, which ascertains that the 68kDa HA-binding protein does not contain any interchain disulfide bonds (Fig. 5.3 A).

5.2.2.3 Immunological reactivity of the 68 kDa HA-binding protein with anti-34 kDa HA-binding protein antibodies

To examine immunological crossreactivity, the purified 68 kDa HA-binding protein was immunodetected with the affinity purified antibodies raised against the 34 kDa HA-binding protein. As shown in the immunoblot analysis (Fig.5.3B), it is evident that antibodies against the 34 kDa HA-binding protein, recognize the 68 kDa protein.

5.2.2.4 HA-binding ability of the 68 kDa protein

In order to examine the HA-binding property of the 68 kDa protein, the purified HA-binding protein was resolved on SDS-PAGE and transferred onto the nitrocellulose membrane and probed with biotinylated HA. Fig.5.3C shows the binding of biotinylated HA to the 68 kDa protein, thus confirming the HA-binding activity of the 68 kDa protein.

5.2.2.5 Glycoprotein nature

The 68 kDa HA-binding protein purified by HA affinity column was separated by
Fig. 5.1. 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 0.01M PBS pH 7.2, followed by wash with 0.01 M PBS containing 0.5M 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/1 h. OD of all fractions was measured at 280 nm (A). The peak protein fractions were separated on 12.5% SDS-PAGE and is shown in the inset (B).

Fig. 5.2. Native molecular mass of HA-binding protein purified from rat spleen. 2μg of HA affinity purified HA-binding protein (lane 1) and high Mr native PAGE marker (lane 2) were electrophoresed on 9-20% gradient native polyacrylamide gel and visualized by CBB staining.
Fig. 5.3. Characterization of the 68 kDa HA-binding protein

(A) Subunit molecular weight of HA-binding protein. Lane 1, 4μg protein in the presence of β-mercaptoethanol; Lane 2, in the absence of β-mercaptoethanol. Electrophoresis was carried out in 12.5% gel in presence of SDS and protein bands were visualized by CBB staining.

(B) Detection of the purified 68 kDa HA-binding protein by immunoblot analysis. 4μg of purified HA-binding protein was separated on 12.5% SDS-PAGE and transferred onto nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies as described in materials and methods.

(C) Detection of HA-affinity purified 68 kDa protein by HA-biotin. The rat spleen purified 68 kDa HA-binding protein was subjected to 12.5% SDS-PAGE, transferred onto the nitrocellulose membrane and probed with biotinylated-HA as described in material and methods.

(D) [125I]-WGA binding assay. The rat spleen purified 68 kDa HA-binding protein was subjected to 12.5% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane, probed with [125I]-WGA and bands are visualized by autoradiography.

(E) Cell surface expression of 68 kDa HA-binding protein. The EL4 cells were cultured and the cells (1x10⁷) were labelled with Na[125I] using lactoperoxidase/glucose oxidase. The cells were solubilized in RIPA buffer and subjected to immunoprecipitation by using anti-34 kDa HA-binding protein antibodies. The immunoprecipitates were analyzed by 12.5% SDS-PAGE under reducing conditions followed by autoradiography, as described under materials and methods.
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SDS-PAGE and transferred onto the nitrocellulose membrane and probed with radioiodinated WGA. Fig.5.3D showed a positive signal, suggesting the glycoprotein nature of the 68 kDa HA-binding protein.

5.2.2.6 Cell surface expression

In order to study the cell surface expression of the 34 kDa HA-binding protein, EL4 cells were labelled with Na\(^{[125I}\) using lactoperoxidase and glucose oxidase method. The labelled cells were isolated and lysed in presence of protease inhibitors and the supernatant was immunoprecipitated using anti-34kDa HA-binding protein antibodies. Fig.5.3E shows a band at 68 kDa upon autoradiography, indicating the cell surface presence of this protein.

5.2.3 Evidence for instability of the 68 kDa HA-binding protein in EL4 cells

The freshly cultured EL4 cells and the 2 week stored EL4 cells pellets were lysed in lysis buffer. Equal amounts of cell lysate proteins were separated on 12.5% SDS-PAGE and transferred onto the nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies. Fig.5.4 shows that in freshly prepared cell lysate, only the 68 kDa protein is observed whereas in stored cell lysate the 34 kDa HA-binding protein could also be detected along with the 68 kDa protein though, the level of the 68 kDa protein was reduced.

5.2.4 Effect of N-glycosidase treatment on the 68 kDa HA-binding protein

The purified 68 kDa HA-binding protein was treated with N-glycosidase as described in materials and methods. Both, the N-glycosidase treated and untreated samples were separated on SDS-PAGE and transferred onto the nitrocellulose membrane and probed with anti-34 kDa HA-binding protein. Fig.5.5 shows that N-glycosidase treatment leads to the generation of the 60 kDa protein thus, indicating the presence of N-linked oligosaccharides attached to the protein core.

5.2.5 Effect of denaturants on the 68 kDa protein

To check whether or not the 68 kDa protein is a dimer of the 34 kDa protein, the purified 68 kDa protein was treated with denaturants to remove noncovalently linked molecules and then separated on 12.5% SDS-PAGE, transferred onto the nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies. Fig. 5.6 shows that the 68 kDa protein is resistant to all these denaturants indicating, the absence of any noncovalently linked interactions.
Fig. 5.4. Evidence for the instability of the 68 kDa HA-binding protein. The freshly prepared and stored EL4 cells were lysed and were subjected to SDS-PAGE and transferred onto nitrocellulose and probed with anti-34 kDa HA-binding protein antibodies.

Fig. 5.5. Evidence for the presence of N-glycosidase linked carbohydrates in the 68 kDa HA-binding protein. Purified 68 kDa HA-binding protein was subjected to N-glycosidase digestion as described in the instructions of the Boehringer Mannheim manual. The protein samples were separated on 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane and probed with anti-34 kDa HA-binding protein antibodies, Lane 1, N-glycosidase treated; Lane 2, untreated.
Fig. 5.6. Effect of denaturants on the 68 kDa HA-binding protein. In order to check whether the 68 kDa protein is a dimer of the 34 kDa HA-binding protein, the spleen purified HA-binding protein was treated with 2% SDS, 5% mercaptoethanol, 8M urea and 8 M Guanidinium hydrochloride, separated on SDS-PAGE and transferred onto nitrocellulose and probed with anti-HA-binding protein antibodies.

Fig. 5.7. Peptide digestion of the 68 kDa HA-binding protein. The purified 68 kDa HA-binding protein was treated with V8 protease (Panel A) and CNBr (Panel B) and was subjected to 12.5% SDS-PAGE.
5.2.6 Digestion of the 68 kDa HA-binding protein

The purified 68 kDa HA-binding protein was treated with V8 protease and CNBr as described in materials and methods and then run on a SDS-PAGE. The treated and the untreated samples were stained with CBB. Fig.5.7A shows that the V8 protease digestion gives rise to protein bands at 44 kDa and 40 kDa and Fig.5.7B shows that the CNBr digestion leads to generation of 42 kDa and 34 kDa proteins, suggesting that the 34 kDa protein might be arising by proteolytic cleavage of the 68 kDa HA-binding protein.

5.2.7 Comparison of N-terminal amino acid sequence of the 34 kDa and the 68 kDa HA-binding proteins

The immunological crossreactivity of the 34 kDa and the 68 kDa HA-binding protein raises the possibility of these two proteins having identical features. Thus, as a short gun experiment, we transferred these two proteins onto the PVDF membrane and the bands corresponding to the 34 kDa and 68 kDa positions were excised and subjected to N-terminal amino acid sequence analysis. The results shown in Table 5.1 indicate that these two proteins have identical N-terminals (the first 15 amino acids).

5.3 Discussion

In this chapter, the purification and characterization of the 68 kDa HA-binding protein from rat spleen has been documented. In the previous chapter the co-purification and expression of the 68 kDa protein in various tissues has been described. The protease sensitivity of the 68 kDa HA-binding protein was also demonstrated. Taking this as a clue, larger number of protease inhibitors were used in the lysis buffer in order to prevent the degradation of the 68 kDa protein during purification. Using the freshly prepared spleen lysate (prepared in presence of a cocktail of many protease inhibitors), the 68 kDa protein was isolated by using HA-affinity column. The purified protein was subjected to PAGE and SDS-PAGE. The PAGE data shows a molecular weight of 136 kDa and SDS-PAGE analysis shows a 68 kDa band confirming our previous observation (see Chapter-I, section 4.2.5). When the samples were processed under reducing and non-reducing conditions they showed no shift in band position, indicating the absence of inter chain disulphide bonds.

To study the relation between the 34 kDa and the 68 kDa proteins, the purified 68 kDa protein was subjected to immunoblot analysis and probed with affinity purified anti-34 kDa
Table 5.1  
**Comparison of the N-terminal sequence of the 34 kDa HA-binding protein and the 68 kDa HA-binding protein**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>N-terminal sequences</th>
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<tbody>
<tr>
<td>Rat kidney HABP</td>
<td>L H T D G D K A F V E F L T D E</td>
</tr>
<tr>
<td>Predicted HABP (human)</td>
<td>L H T D G D K A F V D F L S D E</td>
</tr>
<tr>
<td>34 kDa HABP (EL4)</td>
<td>M H T D G D K A F V D F L S D</td>
</tr>
<tr>
<td>68 kDa HABP (EL4)</td>
<td>G H T E G D K A F V E F L T D E</td>
</tr>
<tr>
<td>Predicted YL2 (mouse)</td>
<td>L H T E G D K D F V E F L T D E</td>
</tr>
</tbody>
</table>

The EL4 purified 68 kDa and 34 kDa HA-binding protein were separated onto SDS-PAGE and transferred onto the PVDF membrane and the bands corresponding to the 34 kDa and the 68 kDa positions were excised and subjected to automated Edman’s degradation with a gas phase sequencer. Table 5.1 shows the N-terminal sequence of the EL4 purified 34 kDa and 68 kDa HA-binding protein, along with the already reported 34 kDa HA-binding protein sequences for different sources.
HA-binding protein antibodies. The results show that the anti-34 kDa HA-binding protein antibodies recognise the 68 kDa protein, indicating that the 68 kDa protein is immunologically related to the 34 kDa protein. In order to further show its relation with the 34 kDa HA-binding protein, HA-binding studies were performed by subjecting the purified protein to transblot assay using biotinylated HA. The results show that HA-biotin recognizes the 68 kDa HA-binding protein, thus strengthening its similarity with the 34 kDa protein. The dependence of the expression of the 68 kDa protein on the storage conditions of the cells, was also checked. The freshly isolated cells and stored cells were lysed and subjected to immunoblot analysis. The result showed that the freshly prepared samples contained the 68 kDa protein only whereas the stored cells showed the presence of both the 34 kDa and 68 kDa protein. Simultaneously, the reduction in the levels of the 68 kDa protein in stored cells as compared to its level in the freshly prepared cell lysates was also seen.

Additionally, the presence of β-D-N-acetylglucosamine linked carbohydrate structures by using 125I-WGA is also shown. The N-glycosidase treatment of the 68 kDa protein resulted in reduction in Mr, indicating the presence of N-linked sugars. Subsequently, by labelling with Na125I the cell surface occurrence of this protein was also established.

N-terminal sequence analysis of the first 15 amino acid of both the 34 kDa and the 68 kDa proteins was shown to contain identical N-terminal sequences, indicating a relation between these proteins.

In order to rule out the possibility of the 68 kDa protein being a dimer of the 34 kDa protein, the purified 68 kDa was subjected to harsh treatment with denaturants, to remove the associated and non-covalently linked molecules. The results indicate that the 68 kDa protein is resistant to 2% SDS, 5% mercaptoethanol, 8M Urea and 8M guanidinium hydrochloride treatments, indicating its stability and the absence of any non-covalently linked structures.

The protease treatment of the 68 kDa protein with V8 protease gave rise to a protease fragment of around 44 kDa. Upon CNBr digestion, a 44 kDa and a 34 kDa protein band was seen.

The presence of identical epitopes and the identical N-terminal amino acid residues in both the 68 kDa and the 34 kDa HA-binding proteins, leaves the option to consider as to whether the dimerization of the 34 kDa protein generates the 68 kDa protein or the proteolytic
cleavage of 68 kDa protein forms the 34 kDa protein or the 34kDa protein is covalently linked with other proteins through its N-terminal sequence to form the 68kDa protein. The last possibility may arise due to the homotypic cross-linking of two 34 kDa proteins through the lysine and glutamic acid residues by transglutaminase enzyme, to form the 68kDa protein. This kind of cross-linking has been reported in CD38 (Umar et al., 1996), a known HA-binding protein and in other extra-cellular matrix proteins like fibronectin and fibrin (fibrinogen formation during blood coagulation, Pisano et al., 1968).

Another HA-binding protein TSG-6, is a secreted 35 kDa glycoprotein which forms a complex with a protein present in the serum (Lee et al., 1992). This complex was found to be stable during SDS-PAGE under reducing conditions and in the presence of 8M urea. The protein that binds TSG-6 was identified as inter-α-inhibitor (IαI) and is cross-linked through chondroitin sulfate (Wisniewski et al., 1994). Recently, it was shown that the inter-α-inhibitor is covalently linked with hyaluronan through the light chain. Other serum proteins like C1q, IgM and IgG also show covalent cross-linking with HA, in some inflammatory conditions (Prehm, 1995).

Thus, the relationship existing between the 34 kDa and the 68 kDa proteins still remain ambiguous. The present data on protease digestion and CNBr cleavage experiment is, unable to clarify the inter-relationship existing between these two proteins. Although, the N-terminal sequences of these two proteins are identical and they are immunologically similar, the internal peptide sequence of the 68 kDa protein is the only way to know the exact relation existing between these two proteins; which could be either the monomer-dimer relationship (since there exist some reports on dimeric proteins that are resistant to SDS treatment; Berkiey and Koshland, 1991) or it could be that they are covalently linked with homotyping transglutaminase activity (Umar, 1996), as described earlier.