CHAPTER-III

ROLE OF HYALURONIC ACID BINDING PROTEINS IN HA-INDUCED CELLULAR SIGNALLING
6.1 Introduction

The dynamic nature of the ECM, particularly during stages of tissue development and inflammation, is just beginning to be appreciated, and a great deal of recent research has focussed on the effects of ECM on effector cell functions (Jones et al., 1993; Wrenshall et al., 1994). One general concept which has emerged from these studies is that fragments or degradation products of ECM components may acquire activity not possessed by their larger, precursor molecules (Clark et al., 1988). This process allows for the recruitment of biologically active ECM fragments from the intact ECM when needed, for example during periods of tissue differentiation or inflammation. A major component of the ECM that undergoes dynamic regulation during inflammation is the glycosaminoglycan (GAG), hyaluronan (HA). HA is a nonsulfated, linear GAG consisting of repeating units of \((\beta,1-4)-D\text{-glucuronic acid-}(\beta,1-3)-N\text{-acetyl-D-glucosamine}\) (Laurent and Fraser, 1992). In its native state, such as in normal synovial fluid; HA exists as a high molecular weight polymer, usually in excess of \(10^6\) Da (Laurent and Fraser, 1992). However, under inflammatory conditions HA has been shown to be more polydisperse, with a preponderance of lower molecular weight forms (McNeil et al., 1985). The accumulation of lower molecular weight forms of HA has been postulated to occur by a variety of mechanisms including depolymerization by reactive oxygen species, enzymatic cleavage, and de novo synthesis of lower molecular weight species (Laurent and Fraser, 1992). Several studies have suggested that high and lower molecular weight HA may exhibit different biological effects on cells and in tissues (Forrester and Balazas, 1980).

HA has been suggested to play an important role in a number of biological processes including wound healing (Oksala et al., 1995), embryonic development (Underhill et al., 1993), and tumor growth (Bartolazzi et al., 1994). Further evidence has suggested that certain HA functions are mediated by interactions with the HA receptor (Bartolazzi et al., 1994). Recent work has provided evidence that, in addition to serving as a structural scaffold, HA may function as a cellular signalling molecule under certain circumstances (Bourguignon et al., 1993).

Recent studies have shown that the binding of low molecular weight fragments of hyaluronan (HA) to alveolar macrophages via CD44, elicits the expression of a number of pro-inflammatory chemokines. HA fragments are capable of activating NF-\(\kappa\beta\) (Noble et al.,...
1996) and induce the expression of insulin-like growth factor-I by murine macrophages (Noble et al., 1993). These studies bring together two disparate and previously unconnected observations regarding the expression of biological activity of HA fragments. The first observation is that HA fragments are present at abnormally high levels in the joints of patients with rheumatoid arthritis (RA) and in other inflammatory conditions (Bjermer et al., 1989). These HA fragments are thought to arise primarily as a result of activated leukocyte driven extracellular matrix degradation at sites of inflammation and their presence has been proposed as a biological marker of diseases. The second observation is that HA fragments but not HA polymers have angiogenic activity, a process which has been proposed to play a key role in the maintenance and progression of RA and other chronic inflammatory diseases (West et al., 1985). The findings by Nobel, (1996) and his colleagues suggest that the high levels of HA fragments found in inflammed tissues bind to leukocytes and other CD44 expressing cells, and trigger a cascade of signaling events which are involved in maintaining and/or amplifying the inflammatory response. It is of interest to note that the biological activities elicited by the HA fragments are distinct from those elicited by the HA polymers. While the HA polymer, a component of the extracellular matrix and a substrate for CD44 mediate cell adhesion, HA fragments are signaling molecules which alert the immune system that significant tissue damage has occurred at a site of inflammation.

Cell adhesion molecules mediate structural linkages between cytoskeletal elements and the extracellular matrix. They also initiate signal transduction processes that regulate, for example, cytoskeletal organization, differentiation and programmed cell death. In some cases, connections have been made to downstream effectors, for example, cyclins in the regulation of proliferation and transcription factors for gene expression. It has become apparent recently, that the focal adhesion, a highly organized supramolecular structure in which some integrins reside, functions as a signal transduction centre. It contains over 20 signal transduction components and may well be involved in a variety of integrin-initiated and growth-factor-induced signal transduction pathways. Members of both the cadherin and Ig family interact physically with some growth factor receptors (Horwitz.,1996).

From our laboratory, a 34 kDa HA-binding protein has been purified and its specific affinity towards HA has been demonstrated, earlier (Gupta et al., 1991a). In addition, its role in tumor formation has also been speculated (Gupta and Datta, 1991b). The aim of this study
was to identify the role of the 34 kDa HA-binding protein in HA induced signal transduction by examining the cellular phosphorylation of the HA-binding protein and the IP₃ formation under HA stimulation. In addition, we have also demonstrated the regulation of cellular phosphorylation of the 68 kDa and the 34 kDa HA-binding protein in various transformed cells.

6.2 Results

6.2.1 Evidence for the presence of the 34 kDa and the 68 kDa HA-binding proteins in lymphocytes by ligand binding assay and immunoblotting

In order to study the role of the HA-binding proteins in rat lymphocytes, we studied the binding of ¹²⁵I-HA to the lymphocytes in vivo. We observed that binding of ¹²⁵I-HA to lymphocytes is inhibited by anti-34 kDa HA-binding protein antibodies indicating the presence of the 34 kDa HA-binding protein on rat lymphocytes. The binding of ¹²⁵I-HA was also inhibited by HA and heparin, but not by chondroitin sulphate (Table 6.1), suggesting a competition for HA-binding sites by heparin.

In order to further confirm the possible binding sites for hyaluronic acid in lymphocytes, we incubated the blot containing lymphocyte proteins with ¹²⁵I-HA and detected the proteins of Mr 68, 34 and 22 kDa (Fig. 6.1). In parallel, the 34 kDa lymphocyte HA-binding protein detected by ¹²⁵I-HA-binding could also be identified by immunoblotting analysis using anti-34 kDa HA-binding protein antibodies. However, this may not be the complete profile of HA-binding proteins in lymphocytes, since the cysteine dependent loop structure containing the HA-binding site may get abolished under reducing conditions (Sherman et al.,1994).

6.2.2 Specific affinity of the 34 kDa HA-binding protein for HA polymer, HA oligomer and HA hexamer

In order to determine if the 34 kDa HA-binding protein binds specifically to HA of varied Mr, varying concentrations of the purified 34 kDa HA-binding protein were slot blotted on Hybond nitrocellulose membrane and probed with biotinylated HA. Fig. 6.2 shows that the binding of the 34 kDa HA-binding protein to biotinylated HA is competed with excess of (50 fold) unlabelled HA of different sizes. This result suggests the specific binding of the 34 kDa HA-binding protein to HA oligomer, HA hexamer and also the HA polymer.
Table 6.1: Effect of anti-34 kDa HA-binding protein antibodies and glycosaminoglycans on $^{125}$I-HA-binding in rat lymphocytes

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% of HA-binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>100</td>
</tr>
<tr>
<td>Anti-34 kDa HA-binding protein</td>
<td>74</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>56</td>
</tr>
<tr>
<td>Heparin</td>
<td>68</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>93</td>
</tr>
</tbody>
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Cells were pre-treated with anti-34 kDa antibodies (1:20), and 50 μg/ml of HA, 50 μg/ml of heparin and 50 μg/ml chondroitin sulfate. The values represent an average of triplicates of two separate experiments.
Evidence for the presence of the 34 kDa HA-binding protein in lymphocytes. Lymphocyte cell lysate was prepared in lysis buffer. Approximately, 20 μg of total protein was subjected to 12.5% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with [125I]-HA, washed with PBST, dried and autoradiographed (lane 2). Another blot containing lymphocyte cell lysate was probed with anti-HA-binding protein antibodies and immunodetected (lane 1) as described in the materials and methods.

Specific affinity of the 34 kDa HA-binding protein towards HA polymer, oligomer and hexamer. Purified HA-binding protein (1 to 10 μg) (slot 1, 10μg; slot 2, 8μg; slot 3, 6μg; slot 4, 4μg; slot 5, 2μg) was lot blotted on nitrocellulose (NC) and probed with biotinylated-HA (1:4000 dilution) after previously blocking the NC with milk protein for 2 h. The other four replicate blots containing the same amount of the protein are probed with same dilution of biotinylated HA in presence of 50 fold excess unlabelled HA. Blot (A) Sigma HA, Blot (B) Polymer HA, Blot (C) Oligomer HA and Blot (D) Hexamer HA. The amount of biotinylated-HA bound to the proteins was detected using avidin-peroxidase (1:5000 dilution) and ECL detection system.
6.2.3 **HA-induced early signalling events**

HA induced early signalling events in lymphocytes were studied by cell aggregation, tyrosine phosphorylation and cytoskeletal protein phosphorylation. From our result (Fig. 6.3), it was seen that 50 μg/ml of HA causes cell aggregation in lymphocytes which is inhibited on treatment with anti-34 kDa HA-binding protein antibodies. These observations confirm the involvement of the 34 kDa HA-binding protein in HA induced cell aggregation.

In continuation, we studied HA-induced total cellular phosphorylation, by metabolically labelling the lymphocytes with $[^{32}\text{P}]$ orthophosphate. It was found that HA at the concentration of 100 μg/ml induced the highest level of cellular protein phosphorylation (Fig. 6.4A). Using this concentration, HA induced tyrosine phosphorylation and cytoskeletal protein phosphorylation were also studied. Enhanced tyrosine phosphorylation of protein bands at 60, 47, 45 and 38 kDa were observed in comparison to the unstimulated cells (Fig. 6.4B). The enhanced cytoskeletal protein phosphorylation as compared to uninduced cells was also observed by HA stimulation in lymphocytes (Fig. 6.4C).

6.2.4 **HA-induced Inositol triphosphate formation in lymphocytes**

The total content of inositol 1,4,5-triphosphate (IP$_3$) in HA and Con-A activated lymphocytes was measured with or without pretreatment of the cells with anti-34 kDa HA-binding protein antibodies. As shown in Fig. 6.5, HA (50 μg/ml) and Con-A (5 μg/ml) stimulate the maximum IP$_3$ production at 60 sec (12 pmole/1X10$^5$ cells for HA) and at 30 sec (21 pmole/1X10$^5$ cells, for Con A) of incubation. After reaching the highest level, IP$_3$ production gradually comes down to lower levels at 90 sec. When cells were pre-incubated with protein A purified anti-HA-binding protein (IgG) antibodies and stimulated with same amount of HA and Con-A in the same time dependent manner, IP$_3$ formation lowered down to 9 pmole/1X10$^5$ cells for HA and 13.25 pmole/1X10$^5$ cells for Con-A at the time of highest IP$_3$ formation 60 sec of incubation for HA and 30 sec for Con-A).

6.2.5 **Mitogen induced enhanced phosphorylation of the 34 kDa HA-binding protein**

In order to study the specific role of the 34 kDa HA-binding protein in HA-mediated signal transduction in lymphocytes, we first metabolically labelled lymphocytes with $[^{32}\text{P}]$ orthophosphate and then, stimulated the cells with HA or Con-A. Cells were washed, lysed
Fig. 6.3. Cell aggregation of freshly isolated rat lymphocytes.
(A) normal cells (B) Cells incubated with 50 μg/ml of HA (C) blocking of aggregation by preincubation with the anti-34 kDa HA-binding protein antibodies before HA addition (1:20 dilution of the 34 kDa affinity purified antibodies from rabbit immune sera).
Effect of HA stimulation on the (A) total cellular protein phosphorylation (B) tyrosine phosphorylation and (C) cytoskeletal phosphorylation in lymphocytes. Freshly prepared lymphocytes after labelling with $[^{32}\text{P}]$ orthophosphate for 4 h, were stimulated with varying concentrations of HA for 15 min. Total cellular proteins were extracted by TCA precipitation and measured for radioactivity in a liquid scintillation counter. Fresh lymphocytes were stimulated with 100 $\mu$g/ml of HA and subjected to western blotting using anti-phosphotyrosine antibodies for studying tyrosine phosphorylation under HA-stimulation. Cytoskeletal protein phosphorylation was observed under HA (100 $\mu$g/ml) stimulation after lysing the fresh lymphocytes in RIPA buffer, subsequent to $[^{32}\text{P}]$ labelling for 4 h. Triton X-100 insoluble fraction chiefly contained the cytoskeletal proteins. These proteins were solubilized in 2% SDS, separated on 12.5% SDS-PAGE, dried and autoradiographed.
Fig. 6.5. Effect of HA and Con-A on the production of inositol 1,4,5-triphosphate (IP$_3$) in lymphocytes. Freshly prepared lymphocytes (1X10$^6$ cells) were stimulated with HA (50 μg/ml) and Con-A (5 μg/ml) in fresh RPMI 1640 medium for different time periods at 37°C. The insoluble precipitates using perchloric acid were removed by centrifugation at 2,000xg for 10 min. IP$_3$, was measured in the final supernatant by a IP$_3$ assay system (Amersham). For the antibody treatment, the cells were pre-incubated with saturable amount of anti-HA-binding protein antibodies, washed with RPMI-1640 and subjected to stimulation with HA or Con-A. The IP$_3$ content was determined using an authentic standard curve. Each bar represents the mean of duplicate assays.

Fig. 6.6 (A) Enhanced phosphorylation of the 34 kDa HA-binding protein in HA and Con-A stimulated lymphocytes. Freshly isolated lymphocytes 1X10$^6$ were metabolically labelled with $[^{35}P]$ orthophosphate for 4 h and stimulated with mitogens for 15 min. Then, the cells were washed and lysed with lysis buffer and immunoprecipitated with anti-34 kDa HA-binding protein antibodies, separated on 12.5% SDS-PAGE, dried and autoradiographed.

(B) Phosphoaminoacid analysis of the 34 kDa HA-binding protein. The band from dried gel corresponding to 34 kDa position was excised and acid digested as described by Neufeld et al., (1989). The mixture was separated on ascending paper chromatography along with standard phosphoamino acids and the spots were visualized both by spraying 2% ninhydrin and by autoradiography.
in RIPA buffer which did not contain the protease inhibitors and immunoprecipitated with antibodies raised against the 34 kDa HA-binding protein. The HA-binding protein was observed to have undergone enhanced phosphorylation in Con-A and HA stimulated cells (Fig. 6.6A). The immunoprecipitated 34 kDa HA-binding protein band was cut from the gel and processed for phosphoaminoacid analysis which revealed the phosphorylation of the 34 kDa protein band at the threonine residues (Fig. 6.6B).

6.2.6 Cell surface and secretory nature of phosphorylated HA-binding protein

We have already shown the cell surface localization of this protein. To examine whether the cell surface 34 kDa HA-binding protein was phosphorylated, the cells were metabolically labeled with $^{32}$P orthophosphate and stimulated (as described in figure legends). Without lysing, the cells were incubated with anti-HA-binding protein antibodies and excess antibodies were washed out. The cell surface bound antibody in the lysed cell extract (without protease inhibitors) was immunoprecipitated with protein A Sepharose-4B. From Fig. 6.7A, it is evident that phosphorylation of cell surface 34 kDa HA-binding protein is also stimulated significantly in the presence of PMA, calyculin-A and Ca$^{++}$ ionophore. However, no 68kDa and 44kDa proteins could be detected, probably due to the fact that protease inhibitors were not used in the lysis buffer.

The phosphorylation of a secretory form of this 34 kDa HA-binding protein was studied by immunoprecipitating the serum free medium of the $^{32}$P orthophosphate labeled EL4 cells. As shown in Fig. 6.7B, $^{32}$P labeled HA-binding protein can be detected in the medium up to 2 h showing that the secreted form is phosphorylated (Gupta and Datta, 1991b).

6.2.7 Regulation of cellular phosphorylation of the 34 kDa and the 68kDa HA-binding protein by protein kinase modulators in various transformed cells

After studying the phosphorylation of the 34 kDa HA-binding protein, our next aim was to study the phosphorylation of the 68 kDa HA-binding protein. In order to study this and the existing relationship between the phosphorylation of the 34 kDa and the 68 kDa proteins, EL4, Raji, HeLa and MCF-7 cells were labelled with $^{32}$P orthophosphate and then stimulated with protein kinase activators. The cells were then lysed in RIPA buffer containing a cocktail of protease inhibitors (this was done, since the 68 kDa protein is very unstable and protease sensitive) and the HA-binding proteins were immunoprecipitated using anti-34 kDa
Cell surface and secretory 34 kDa HA-binding proteins as phosphorylated proteins. Approximately, 1 x 10^6 cells of EL4 were labeled with [32P] orthophosphate and stimulated for 15 min with PMA (250 ng/ml). Lane 2; Calyculin-A (10ng/ml), Lane 3; Ca^2+ ionophore (250 ng/ml), Lane 4 and control, Lane 1. Intact cells were incubated with anti-34 kDa HA-binding protein antibodies, the excess antibodies were washed out and the lysed cells were immunoprecipitated as described in materials and methods.

For secretory phosphorylated HA-binding protein, cells were radiolabeled in the presence of serum and after labeling, the cells were washed and incubated in serum free medium for different time intervals. Lane 1, 1 h; Lane 2, 2 h and Lane 3, 3 h. Culture medium was immunoprecipitated as described in materials and methods.
HA-binding protein antibodies. Fig. 6.8A shows the enhanced phosphorylation bands at 68 kDa and 34 kDa in PMA and calyculin-A stimulated EL4 cells (transformed T cells). An additional band corresponding to 44 kDa also showed enhanced phosphorylation. This data indicates the higher level of 68 kDa phosphorylation as compared to the phosphorylation of the 34 kDa protein. However, in the absence of protease inhibitors only the 34 kDa protein was observed, thereby further confirming the protease sensitive nature of the 68 kDa and the 44 kDa proteins.

In Raji cells, (human transformed B cells), the phosphorylation bands at 68 kDa, 44 kDa and 34 kDa were detected. Fig. 6.8B shows the constitutive phosphorylation of these protein in Raji cells, but upon Calyculin-A and Staurosporine stimulation, the enhanced phosphorylation of the 34 kDa and the 68 kDa proteins could be seen. However, under PMA and calcium ionophore stimulation, reduction in the levels of phosphorylation of these proteins was observed. Here, too, the increased level of the 68 kDa protein was seen as compared to the 34 kDa protein.

In HeLa cells, the phosphorylation bands at 95 kDa, 68 kDa and 44 kDa positions were seen (Fig. 6.8C). In Calyculin-A stimulated cells, enhanced phosphorylations levels of all these proteins were seen. Fig. 6.8D shows enhanced phosphorylation bands at 68 kDa, 44 kDa and 34 kDa protein under HA stimulation in MCF-7 cells. These results indicate that these proteins undergo phosphorylation and may eventually play an important role in cellular signalling.

6.2.8 Evidence for the existence of HA-binding protein receptors in the plasma membrane of transformed cells

To characterize the mode of association of extracellular matrix 34 kDa HA-binding protein with cell surface, we studied the binding of the HA-binding protein to cell surface of intact Raji cells by the saturation and competition method using \[^{125}\text{I}]\) labelled HA-binding protein. Fig. 6.9A shows scatchard plot analysis of the binding of \[^{125}\text{I}]\) HA-binding protein to intact cells indicating the presence of only a single class of binding sites for HA-binding protein on the surface of EL4 cells with the apparent dissociation constant Kd of $1.78 \times 10^{-11}$ M. The specificity of \[^{125}\text{I}]\) HA-binding protein was confirmed by the addition of increasing amount of unlabelled HA-binding protein. In order to identify the specific binding protein for HA-binding protein, the plasma membrane protein from Raji, J774 and EL4 cells were
Fig. 6.8 Regulation of cellular phosphorylation of the 68 kDa and the 34 kDa HA-binding proteins in transformed cells. Approximately, $1 \times 10^6$ cells (EL4, Raji, HeLa and MCF-7) were cultured in 24 well culture plates. Cells were labelled in DMEM containing with $1\mu$Ci[32P]orthophosphate for 4 h and then stimulated with PMA, Calyculin-A, Ca2⁺ ionophore, staurosporine and HA for 15 min each. Cells were washed with PBS and lysed in ice cold RIPA buffer containing protease inhibitors. The cell lysates were centrifuged at 15,000xg for 30 min and the supernatants were immunoprecipitated using anti-HA-binding protein antibodies. Proteins were resolved by SDS-PAGE and the gel was dried and autoradiographed.

(A) EL4 (Transformed T-cells), (B) Raji (Transformed B-cells)
(C) HeLa (Cervical carcinoma cells), (D) MCF-7 (Breast carcinoma cells)
Fig. 6.9. Existence of HA-binding protein - binding protein on plasma membrane.

(A) Scatchard plot analysis of the binding of [125I]-HA-binding protein to EL4 cells. The binding data analyzed by plotting the ratio of the bound concentration of radiolabelled HA-binding protein to the free concentration as a function of the bound concentration of radiolabelled HA-binding protein. The scatchard plot analysis was used to determine the Kd of the HA-binding protein binding to EL4 cells.

(B) Binding of [125I]-labelled 34 kDa HA-binding protein to plasma membrane protein of EL4, Raji, J774 cells. The plasma membranes were collected on a sucrose gradient and separated by 10% SDS-PAGE. After the transfer onto a nitrocellulose membranes, the membranes were incubated with [125I]-labelled 34 kDa HA-binding protein and autoradiographed.
prepared by sucrose density gradient. The proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane by electroblotting. The plasma membrane HA-binding protein-binding protein was identified by overnight incubation of blot with \(^{125}\text{I}\) HA-binding protein. After extensive washing with a non-ionic detergent, two prominent bands of 37 kDa and 40 kDa were detected after autoradiography (Fig. 6.9B).

6.3 Discussion

The extracellular matrix molecules after binding to their receptors, are known to transmit the signals to the cytosol by inducing clustering of receptors and reorganization of the cytoskeletal network, leading to enhanced cellular protein phosphorylation/dephosphorylation and finally, resulting in intracellular cascade of signal transduction events (Gilmond and Aumailley, 1992). Hyaluronic acid (HA), a major glycosaminoglycan in the ECM, is shown to be associated with cell aggregation in lymphocyte and macrophages (Green et al., 1988), T-cell activation (Haynes et al., 1989), B cell maturation (Miyake et al., 1990), cell proliferation (West and Kumar, 1989) and lymphocyte homing (Toole, 1990). Although HA is considered to be a physiologically relevant ligand/adhesion molecule in many cell types, very little is known concerning HA-mediated early signal transducing events, leading to the onset of various cell functions.

Depending on the size (ie, high Mr vs low Mr) and the concentration, HA is known to induce different cellular activities in various cell types (West and Kumar, 1989). In this study, we have used only high Mr HA to examine signal transducing events that occur subsequent to the binding of HA to its specific receptors.

Recently, we developed a novel method to radioiodinate hyaluronic acid. Using this probe, we studied binding of high Mr \(^{125}\text{I}\) HA to lymphocytes. When the cells were pretreated with anti-34 kDa HA-binding protein antibodies, we observed inhibition in HA-binding indicating the presence of 34 kDa HA-binding protein on rat lymphocytes. Further, we confirmed its presence by ligand blotting and immunoblot analysis. Our results suggest that the 34 kDa HA-binding protein is one of the receptors of high Mr HA on the surface of rat lymphocytes (Lesley et al., 1990). Previously CD44, has also been shown to recognize high Mr HA on mouse T lymphocytes. As a continuation, we further confirmed by competition assay, that 34 kDa HA-binding protein binds with high Mr HA and also with HA fragments.
This is especially interesting, as HA fragments are known to accumulate in pathophysiological conditions and results in initiation of signalling cascades (Noble et al., 1996).

Thus, to examine the role of 34 kDa HA-binding protein in cellular signalling, our next step was to see whether this protein is involved in HA induced cell aggregation. Our results show that high Mr HA induced cell aggregation. However, when the cells were pretreated with anti-34 kDa HA-binding protein antibodies, we observed inhibition in HA induced cell aggregation implicating the role of the 34 kDa HA-binding protein in cell migration, cell adhesion and proliferation. The molecular mechanisms underlying HA regulation of cell functions are still unknown, though the interactions between ECM elements and cell surface receptors are known to be linked to signal transduction events. Similarly, it was believed that HA, on interacting with its receptors, triggers a signal transduction cascade that is responsible for orchestrating key functions during lymphocyte migration, cell adhesion and proliferation events.

One of the early rapid biochemical changes that takes place after triggering T cell receptors, is an increase in tyrosine phosphorylation and reorganization of cytoskeletal network. Several recent reports also suggest that PI-specific PLC γ undergoes tyrosine phosphorylation leading to hydrolysis of membrane phosphoinositides into \( \text{PIP}_2 \). \( \text{PIP}_2 \) hydrolysis generates two products, inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) which acts in concert, as secondary messengers to increase intracellular calcium concentration and activate protein kinase C, thereby stimulating subsequent events leading to cell activation (Sefton and Campbell, 1991).

In the present study, we have evaluated the HA stimulated total cellular protein phosphorylation, tyrosine phosphorylation and cytoskeletal protein phosphorylation. Our results show that HA induces tyrosine protein phosphorylation of 60, 55, 47, 45 and 38 kDa proteins. Similar kind of observation was also reported in rat spermatozoa by Ranganathan et al., (1996) and Hall et al., (1994) in fibroblast cells under HA stimulated conditions. However, the proteins which we observed, were different from those observed in spermatozoa and fibroblast excepting the 60 kDa and 38 kDa proteins. In addition, the observation on enhanced cytoskeletal protein phosphorylation under HA stimulation suggests that it might be linked to the tyrosine phosphorylation and secondary messenger formation.

The total content of IP$_3$ in HA stimulated lymphocytes was measured and an increase
in IP₃ production under HA as compared to control was observed. When cells were pre treated
with anti-HA-binding protein antibodies, a reduction in IP₃ formation was seen indicating the
involvement of the 34 kDa HA-binding protein. Recently, Deb and Datta, (1996) reported the
HA induced enhanced PLC γ phosphorylation in J774 macrophages.

In order to elucidate the specific role of the 34 kDa and 68 kDa HA-binding protein
in HA-mediated signal transduction events, the HA induced HA-binding protein
phosphorylation status in various normal and transformed cells was investigated. Interestingly,
enhanced 34 kDa and 68 kDa HA-binding protein phosphorylation in EL4, Raji, MCF-7 and
HeLa cells was observed. When the cells are lysed in lysis buffer in presence of protease
inhibitors, strong phosphorylation bands at the 68 kDa and the 44 kDa positions were seen,
whereas in the absence of protease inhibitors only the 34 kDa HA-binding protein band was
observed. Further, when the phosphorylated 34 kDa band was processed for phosphoaminoacid
analysis, it revealed threonine residue phosphorylation, which also correlates with the presence
of substrate phosphorylation site for serine/threonine kinases (CK II, PKC, cAMP and MAP
kinases) in the cDNA sequence of 34 kDa HA-binding protein (Deb and Datta, 1996).

In T-cells, interestingly, the presence of phosphorylated HA-binding protein on the cell
surface was seen, by treating the [³²P] loaded transformed T lymphocytes with anti-HA-
binding protein antibodies, before lysis. After washing away the excess antibodies, the cells
were washed, lysed and the immunocomplex was immunoprecipitated without additional anti-
34 kDa HA-binding protein antibodies, confirming the phosphorylation of the 34 kDa
HA-binding protein. Similar observations have been reported for CD44, in resident and elicited
macrophages by Camp et al, (1991). Our speculation from the above finding is that the
phosphorylated form of HA-binding protein that has higher affinity to HA is being transported
to the cell surface. This notion is supported by the intracellular phosphorylation and
extracellular translocation or secretion of interleukin-1α (Benscher et al., 1988).

It was observed that the 34 kDa HA-binding protein is secreted in the medium as a
phosphorylated form, suggesting a mechanism for the deposition of this protein in the GAGs
rich environment of ECM (Sfeir, 1995). As already reported, vitronectin, a heparin binding
ECM protein is also released from platelets following phosphorylation by a cAMP- dependent
protein kinase (Tsukita et al., 1994).
Finally, the regulation of phosphorylation of the 34 kDa and the 68 kDa HA-binding protein by HA, PMA, Calyculin-A, Calcium ionophore and Staurosporine was demonstrated in various transformed cells. Our results reveal enhanced phosphorylation of the 34 kDa, the 44 kDa and the 68 kDa HA-binding proteins under PMA stimulation but this phosphorylation was found less when compared to other stimulations, indicating downregulation of HA-binding protein expression. It is important to highlight some observations made by other groups, which are in agreement with the present data. The sequence analysis of the 34 kDa HA-binding protein confirms its identity with the receptor of globular head of Clq, known as gClq-R (Ghebrehiwet, 1994). Recently, Euggleton et al (1995) reported that PMA treatment of neutrophils result in downregulation of the gClq-R receptors from the cell surface and appearance of significant amount of soluble gClq-R in media supernatant, suggesting the shedding or secretion.

There is another very interesting observation on the stimulation of phosphorylation by staurosporine. Staurosporine is a indole carbazole chromophore, shown to inhibit protein kinases. Staurosporine is a very effective inhibitor of several serine/threonine and tyrosine specific protein kinases, including cAMP dependent protein kinases, phosphorylase kinase, ribosomal protein S6 kinase, pp60\textsuperscript{v-src} and the receptor tyrosine kinases. Staurosporine binds to a variety of protein kinases with an affinity greater than that of ATP. It is thought to bind to the ATP binding sites of the protein kinases. Though staurosprine is considered to be a kinase inhibitor, our present data suggests the enhanced phosphorylation of 34 kDa HA-binding protein in presence of staurosporine, apparently contradicting our earlier observation in J774 cells (Deb and Datta, 1996). There are several reports where indole carbazoles paradoxically appears to mimic the effect of phorbol esters, suggesting that these compounds may have actions other than inhibitors on protein kinases in intact cells (Hidaka and Kobayashi, 1993).

The involvement of phosphatases in the regulation of the 34 kDa HA-binding protein activation was studied using Calyculin-A, an inhibitor of the serine/threonine phosphatases PP1 and PP2A. From our results, an enhanced phosphorylation of the HA-binding protein under Calyculin-A stimulation was seen, suggesting the involvement of phosphatases in the phosphorylation of the 34 kDa HA-binding protein.
In Raji cells, we observed constitutively phosphorylated form of HA-binding proteins and stimulation with Calyculin-A, staurosporine and HA stimulation.

We observed a highly significant *in vivo* phosphorylation of the 34 kDa HA-binding protein in activated normal lymphocytes as well as transformed cells. Also, the observation that HA enhances HA-binding protein phosphorylation, indicates not only a specific binding of HA to the HA-binding protein, but also the regulation of HA-binding protein phosphorylation by HA.

It is therefore, clear from our data that the 34 kDa HA-binding protein is one of the potential HA-receptors in mediating HA-induced signalling. Few other HA-binding proteins are already reported to initiate the cellular signalling (Bourguignon et al., 1993; Hall et al., 1993, 1995).

The involvement of CD44, the transmembrane HA-receptor in cell aggregation, as confirmed by inhibition of cell aggregation by anti-CD44 monoclonal antibodies has been reported (Lesley et al., 1990). CD44 is reported to be involved in cellular signalling which is mediated by physical association with p56^ck^ (Taber et al., 1996) and also with cytoskeletal proteins like ankyrin (Bourguignon et al., 1993), actin, ezrin, radixin and moesin (Tsukita et al., 1994).

Recently, it has been shown that HA can induce the early signal transduction causing alteration of tyrosine kinase activity, inositol break down and calcium mobilization. CD44, an integral transmembrane glycoprotein is known to bind to HA and transduce a cascade of biochemical events. These events include an influx of Ca^{2+} and activation of G-protein (G) coupled phospholipase C, which hydrolyses phosphoinositides (PIP_{2}) into diacylglycerol (DAG) and inositol triphosphate (IP_{3}). DAG is known to activate protein kinase C (PKC). IP_{3} binds to IP_{3}-R causing internal Ca^{2+} release. Ankyrin may play a pivotal role in linking surface CD44 adhesion molecules and intracellular Ca^{2+} storage organelles to the cytoskeleton. This linkage may be very important for signal transduction and regulation of Ca^{2+} activities needed for the activation of Ca^{2+} dependent enzymes or protein kinases during the onset of physiological responses (Bourguignon, 1996).

Recently, RHAMM has also been reported to participate in HA mediated signalling in association with FAK and Src kinases during HA induced cell motility (Hall et al., 1994).
Autophosphorylation of pp$^{125}\text{FAK}$ results in stable binding of its SH2 domain of Src family kinases. It is also shown that PLC $\gamma$ is a potential target of PP$^{125}\text{FAK/}pp\text{Src}$ complex which after being activated, produce IP$_3$ by PIP$_2$ breakdown (Turley, 1989). The synthesis of HA, the RHAMM ligand, has been correlated to many cellular functions including cell proliferation and cell division. In particular, HA synthesis and synthase activity increase during mitosis when cells round and are loosely adherent, possibly because HA is required for detachment of cells from the supporting matrix (Brect et al., 1986). Synergistic interactions between growth factors and integrin-mediated signal transduction involving sustained tyrosine phosphorylation regulate cell proliferation and cell adhesion during G to S transition and cyclin A expression has been shown to be a target for adhesion dependent signals (Guadango et al., 1993).

Recently, a chicken homologue of Cdc37, known to influence the activity of p34$^{\text{cdc2}}$ kinase in cell cycle progression is also shown to bind hyaluronic acid, heparin and chondroitin sulfate, suggesting the role of GAG binding protein in cell division control (Grammatikakis et al., 1995). The physical interaction between cdc37 and cdk 4 suggest that cdc 37 may regulate the mammalian cell cycle through a direct effect on cdk 4.

CD38 molecules, a homologue of ADP-ribosyl cyclase protein having ecto NADase activity leads to synthesis of cyclic ribose an endogenous secondary messenger regulating Ca$^{2+}$ mobilization. CD 38 mediated signalling for activation and proliferation of T-cells and NK cells has also been reported (Lund et al., 1995).

Besides the extracellular localization, GAGs have been shown to be present in the cytoplasm and also in the nucleus. It also seen that heparin and related polysaccharides inhibit the action of Fos and Jun on transcription events involved in cell cycle progression (Busch et al., 1992; Noble et al., 1996), have recently demonstrated that HA induces the expression of IL-1$\beta$ and TNF $\beta$, both of which are regulated by NF-KB. Recently, they also showed that HA fragment but not native molcules, activate the transcriptional regulatory complex NF-KB/1-KB$\alpha$ in mouse macrophages. Recent evidences have suggested that heparin sulfate can activate NF-KB DNA binding in mouse macrophages. Rooney et al, (1995), showed that HA oligosaccharides rapidly initiate the transient expression of several immediate early response genes (ERG), including C-fos, C-jun and jun-B by endothelial cells. Native high Mr
HA did not induce ERG expression. The product of C-jun, jun-B and C-fos and related genes are DNA binding proteins, which associate to form a homodimeric (jun-jun) or heterodimeric (jun-fos) complex which binds to the same DNA binding site as activator protein 1 (AP1), a transcriptional factor closely involved in the initiation of cell proliferation (Curran and Franz, 1988). In addition, these gene products are transcriptional factors for metalloproteinase gene expression (Latchman, 1991). If C-fos, C-jun or jun B were expressed by endothelial cells, they could play an important role in initiation of extracellular matrix degradation prior to cell migration as well as initiating cell proliferation.

We also demonstrated the presence of two prominent bands of 37 kDa and 40 kDa on the plasma membrane of EL4, Raji and J774 cells. The plasma membrane protein can serve as a docking protein connecting cell surface HA-binding protein to cytoskeleton and transmitting the signal from extracellular matrix to cytosol, since the sequence analysis of the 34 kDa HA-binding protein as described by Deb and Datta (1996) does not encode a transmembrane membrane. We propose that the 34 kDa HA-binding protein may resemble the high-affinity elastin/laminin receptors and associate with the cell surface via an integral docking protein. RHAMM is also predicted to bind to a transmembrane docking protein denoted as connectin and a candidate for the docking protein has been identified as the HA synthase (Klewes et al., 1993).

Thus, the present observation documents the role of the 34 kDa HA-binding protein in HA induced cellular signalling, in addition to the reported involvement of CD44 (Bourguignon et al., 1996; Taber et al., 1996), RHAMM (Hall et al., 1994) and Cdc37 (Grammatikakis et al., 1995).