CHAPTER-I

PURIFICATION, PARTIAL CHARACTERIZATION OF RAT KIDNEY HYALURONIC ACID-BINDING PROTEIN AND ITS LOCALIZATION ON THE CELL SURFACE
4.1 INTRODUCTION

Increasing evidence has implicated macromolecules of the extracellular matrix (ECM) in the regulation of various cellular processes both under normal and pathological conditions [for reviews see the Section 1.1 and Hay, 1981; Yamada and Akiyama, 1984; Hook et al., 1984; lozzo, 1985; Labat-Robert et al., 1990]. In addition to other components, hyaluronate (HA), a large non-sulfated glycosaminoglycan and one of the major constituents of extracellular matrix, influences various aspects of cell behaviour such as adhesion, motility, growth, differentiation and immunogenecity in vitro [for reviews see Toole et al., 1980; Hook et al., 1984; Toole, 1981, 1990; Laurent and Fraser, 1986; Turley et al., 1985, 1991; Evered and Whelan, 1989]. HA has been implicated in regulating these diverse cellular events during the processes of development [Toole et al., 1980, 1984; Toole, 1981, 1982], morphogenesis [Toole and Gross, 1971; Toole, 1976, 1981, 1982; Toole and Underhill, 1983; Toole et al., 1989], tissue remodelling, wound healing and angiogenesis [Toole and Gross, 1971; Weigel et al., 1986, 1989; West and Kumar, 1989] and tumorigenesis [Toole et al., 1979; Toole, 1982; Pauli, 1983; Turley, 1984; lozzo, 1985; Knudson et al., 1984, 1989; Henrich and Hawkes, 1989]. Hyaluronate has been postulated to exert some of its effects on cell behaviour by interacting with its specific binding sites on the cell surface [Underhill and Toole, 1980, 1981; Toole, 1981, 1982; Toole et al., 1984; Hook et al., 1984; Laurent and Fraser, 1986]. Several investigators reported that a group of small-molecular-weight glycoproteins (56 to 102 kDa), which have been isolated from various sources and bind preferentially and non-covalently to HA, are the possible binding sites for this glycosaminoglycan on the cell surface or in the ECM [Delpech and Halavent, 1981; Turley 1982; Turley and Moore 1984; Turley et al., 1987; DSouza and Datta, 1985, 1986a; Underhill et al., 1983, 1985, 1987; Green et al., 1988; Perides et al., 1989; Crossman and Mason, 1990; Gustafson and Forsberg, 1991; Forsberg and Gustafson, 1991]. These HA-binding glycoproteins (HABPs), which include both the non-integral cell surface-associated matrix HA-binding proteins as well as the integral membrane HA-receptors, are structurally distinct from other well-characterized matrix/surface- proteins. The HABP(s) (56 to 70 kDa) described by Turley and her collaborators [Turley, 1982; Turley et al., 1987], from the supernatant culture medium of chick heart fibroblasts and untransformed as well as MSV-transformed mouse 3T3 cells, are structurally related to one another, codistribute with hyaluronate on cell surface [Turley and Torrance, 1985], bind, in addition to HA, matrix polymers such as fibronectin, laminin and collagen [Turley and Moore, 1984], and are functionally
associated with the actin-containing cytoskeleton [Turley et al., 1990]. These HABP(s) are located primarily on the apical surface of locomoting fibroblasts, where they are concentrated in the protrusions and ruffles [Turley and Torrance, 1985] and codistribute with meshwork actin [Turley et al., 1990]. Their expression and localization are regulated correlating with declining cell locomotion [Turley and Auersperg, 1989; Turley et al., 1990]. Underhill et al. [1985, 1987] characterized an 85 kDa receptor for HA, which is an integral membrane glycoprotein present in focal adhesions of SV40-3T3 fibroblasts and BHK cells. This HA-receptor has been implicated in cell adhesion [Underhill et al., 1985], shown to associate with actin [Lacy and Underhill, 1987] and is distinct from other HABP(s). Delpech and Halavent [1981] isolated an ECM-associated 68 kDa HA-binding glycoprotein, hyaluronectin, from human brain, which is localized at the nodes of Ranvier of central and peripheral nerve fibers [Delpech et al., 1982a] as well as in connective tissues particularly in stroma of tumors [Delpech et al., 1982b]. The expression and distribution of this protein is regulated during embryonic development [Delpech and Delppech, 1984]. The abundance of hyaluronectin in the dermis of fetal skin and mesenchymatous stroma reaction of basal cell carcinoma [Delpech et al., 1982b] raises the possibility that this glycoprotein may mediate the effects that HA has in development and tumorigenesis. Earlier from our laboratory, D'Souza and Datta [1985, 1986a] reported the isolation of a 68 kDa HABP from rat brain and liver, which is distinct from the HABP(s) characterized by other investigators. This protein interacts specifically with HA amongst GAGs [D'Souza and Datta, 1986b]. During the course of the present investigation, several more HA-binding proteins or HA-receptors have been reported by many laboratories. For example, recently, Perides et al. [1989] described a matrix-associated 60 kDa glial HA-binding glycoprotein from human brain white matter, which is related to but different from hyaluronectin characterized by Delpech and Halavent [1981]. Crossman and Mason [1990] have, recently, isolated a distinct cell surface-associated hyaluronate-binding glycoprotein of molecular mass 102 kDa (HABP102) from Swarm rat chondrosarcoma, which is not an integral membrane protein. The functions of HABP102 are not yet clearly established. Gustafson and Forsberg [1991] have reported a 60 kDa receptor (an integral membrane protein) for HA on the surface of J774 macrophages. They have suggested that this HA-receptor may mediate the uptake and degradation of HA by macrophages and thus may be essential in regulating the concentration of HA in interstitial fluid as well as the release of this polysaccharide into the blood stream. The receptor for HA on the outer surface of liver endothelial cells is a 100 kDa integral membrane protein, that may
mediate the uptake and degradation of circulating HA by these cells [Forsberg and Gustafson, 1991].

As it is apparent from the literature that in the last decade, although a score of HA-binding proteins or HA-receptors of the different molecular features have been identified in a wide variety of cell types and tissues, so far the functional significance of most of these proteins in the regulation of cell behaviour remains poorly understood. Hence, considering the paucity of such information on HABPs, we have, in the present investigation, purified and partially characterized a novel hyaluronic acid-binding protein (also termed as hyaluronectin) from normal rat kidney with a view to further elucidate its physiological relevance. In order to obtain a biological probe for this protein for its various quantitative, localization and functional studies, polyclonal antibodies were raised against the purified rat kidney HABP. Furthermore, attempts have also been made to (i) study the tissue distribution of kidney-derived HABP, (ii) compare this protein with the HABP isolated from other tissues of rat in order to explore their relationship, (iii) examine the binding characteristics of kidney HABP towards HA, other GAGs and some other structural components of matrix in order to elucidate whether or not this protein can serve as a specific endogenous binding site for HA and whether it has any structural role in the ECM, (iv) examine the renal HABP levels in newborn and aging rats in order to elucidate its role in tissue morphogenesis and development, and (v) localize this protein in human fibroblast culture.

4.2 RESULTS

4.2.1 Purification of hyaluronic acid-binding protein (HABP) from normal rat kidney

All purification steps were carried out at 0-4°C, unless stated otherwise.

Kidneys, collected from normal adult rat, were washed thoroughly in cold 0.01 M phosphate buffer saline (PBS), pH 7.2 and homogenized in two volumes (v/w) of the same buffer by means of a Potter-Elvehjem homogenizer (Remi). The homogenate was centrifuged at 48,000g for 30 min. The pellet was once again homogenized in two volumes (v/w) of 0.2 M glycine-HCl, pH 2.2 and centrifuged at 48,000g for 30 min. The supernatant was neutralized, centrifuged, dialyzed against PBS and then fractionated by 70% saturated ammonium sulfate at 4°C for 30 min. The precipitate obtained was
removed by centrifugation at 10,000g for 30 min. The clear supernatant fraction obtained was dialyzed against PBS, loaded onto an HA-Sepharose 4B affinity column (0.4x15 cm; preparation as described in Section 3.8.1), pre-equilibrated with PBS, and incubated for 45 min. After washing with 20 bed-volumes of PBS and then with 20 bed-volumes of 0.5 M NaCl buffered at pH 7.2 by 0.01 M phosphate buffer, the column was eluted with 0.2 M glycine-HCl, pH 2.2 at a flow rate of 20 ml/h, and the eluate was collected in the fractions of 2 ml each. The protein-containing fractions were determined by measuring their absorbance at 280 nm. The peak fractions were pooled and concentrated. The yield of this purified protein was not poor, since approximately 16-20 μg per mg of total proteins in the extract (supernatant fraction of the 70% ammonium sulfate cut), i.e., approximately 2% of the total protein content in the extract has been recovered.

Although our method of purification is simple and includes only one chromatography step, the HA-binding protein appeared to be homogeneously pure yielding a single band in 7.5% polyacrylamide gel electrophoresis under non-denaturing conditions at pH 8.9 (fig. 4.1a).

### 4.2.2 Characterization of rat kidney HA-binding protein

#### 4.2.2.1 Native molecular weight and subunit composition

The apparent molecular mass of the HABP in native state is 68 kDa as determined by gel filtration chromatography on a pre-calibrated Sephadex G-100 column (Fig. 4.1b) as well as by 4-30% polyacrylamide gradient slab gel electrophoresis under native conditions at pH 8.9 (Fig. 4.1c).

SDS-polyacrylamide gel electrophoretic analysis of the purified HABP on a 12.5% slab gel showed only a single protein band of 34 kDa (Fig. 4.2), thus indicating that the HABP is a homodimer of 34 kDa subunits. Further, this protein does not contain any interchain disulfide bond as it exhibited the same mobility in SDS-PAGE both under reducing (in the presence of β-mercaptoethanol) and nonreducing (in the absence of B-mercaptoethanol) conditions (Fig.4.2).

#### 4.2.2.2 Isoelectric point

The protein appears to be slightly acidic as its isoelectric point is 6 (Fig. 4.3). Two-dimensional gel electrophoretic analysis (by Isoelectrofocusing and SDS-PAGE)
Figure 4.1a. Native polyacrylamide gel electrophoresis of rat kidney HABP. The purified HABP (6 μg) was electrophoresed at pH 8.9 in a 7.5% polyacrylamide slab gel in the absence of SDS at the constant voltage of 80 V as described under Materials and Methods (Section 3.10.1). The protein band was visualized by silver staining [Merril et al., 1981].

Figure 4.1b. Determination of native molecular weight of kidney HABP by exclusion chromatography. Sephadex G-100 column (1.2 x 65.7 cm) was calibrated with BSA, 7 mg (67 kDa); ovalbumin, 7 mg (43 kDa); chymotrypsinogen, 3 mg (25 kDa); and ribonuclease, 10 mg (13.7 kDa). 3 mg of the pure HABP was applied onto the column and the flow rate was maintained at 18 ml/h. Fractions of 2 ml each were collected and analyzed for protein. The molecular mass of the protein was determined from the calibration plot as described in Section 3.11.1.

Figure 4.1c. Analysis of native molecular weight of kidney HABP by native gradient PAGE. — Lane 1 : 10 μg of purified rat kidney HABP; lane 2 : 15 μg of Pharmacia high-molecular- weight marker proteins (67-669 kDa); lane 3 : mixture of Boehringer marker proteins (45-232 kDa, 15 μg); lane 4 : 10 μg of bovine serum albumin (Boehringer); lane 5 : 10 μg of hen egg albumin (Boehringer). Electrophoresis was performed at pH 8.9 in a 4-30% - gradient polyacrylamide slab gel under native conditions (i.e., in the absence of SDS) for 200 volt-hours as described under Materials and Methods (Section 3.11.2.). The protein bands were visualized by Coomassie Brilliant Blue staining. The calibration curve was drawn by plotting the relative migration (Rf) values vs log molecular-weights of the standard proteins. The native molecular mass of the HABP was determined from the calibration curve.
Figure 4.2. Analysis of subunit molecular weight of rat kidney HABP by SDS-PAGE. — Lanes 1 and 2: 4 μg of pure rat kidney HABP in the absence of β-mercaptoethanol (nonreducing conditions) and in the presence of β-mercaptoethanol (reducing conditions), respectively; lane 3: 4 μg of Pharmacia low-molecular-weight marker proteins: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa). Electrophoresis was carried out in a 12.5% polyacrylamide slab gel in the presence of 0.1% SDS using the Laemmli discontinuous buffer system as described under Materials and Methods (Section 3.10.2). The protein bands were visualized by the silver staining method [Merrill et al., 1981]. The HABP band migrated to the same position of 34 kDa both under reducing and nonreducing conditions.

Figure 4.3. Determination of isoelectric point of rat kidney HABP. 1 μg of purified HABP (lane 1) and 1 μg of Pharmacia pi calibration standard proteins (pi 3.5 - 9.3, lane 2) were focused in a precast 5% polyacrylamide pH gradient slab gel (Pharmacia) with a final pH gradient of 3 to 10 as described under Materials and Methods (Section 3.10.3). The protein bands were visualized by Coomassie Brilliant Blue staining.
of 68 kDa HABP purified from rat kidney, brain, liver, heart and plasma is shown in Figure 4.4. The HABP isolated from these tissues (by using same purification protocol) and from plasma exhibited the same pI of approximately 6 and the same subunit size of 34 kDa. These results not only confirm the homogeneous purification of 68 kDa HABP but also indicate that the HABP isolated from different tissues is structurally homologous. The studies conducted on the plasma-derived HABP in relation to tissue-derived 68 kDa HABP have been discussed separately in Chapter III.

4.2.2.3 Glycoprotein nature

In order to investigate whether the 68 kDa HA-binding protein is a glycoprotein, the purified protein was electrophoresed on 12.5% SDS-PAGE and the gel was stained with periodic acid-Schiff (PAS) reagent (as described in Polyacrylamide gel electrophoresis: Laboratory techniques, Pharmacia). The PAS-positive staining of the HABP band suggested that this protein might contain carbohydrate moieties. The glycoprotein nature of HABP was further confirmed by the binding of this protein to Concanavalin A (a lectin which binds to specific carbohydrate moieties, i.e., mannose in glycoconjugates), as shown in Figure 4.5. Moreover, the HABP was also found a sialic acid-containing glycoprotein, since 1.2 μmoles of sialic acid/mg of the pure protein could be detected.

4.2.3 Monospecificity of anti-HABP antibodies

The polyclonal antibodies obtained from the rabbit immunized with purified rat kidney HABP selectively react with the 68 kDa HABP in the tissue extracts and with the purified HABP. As shown in Figure 4.6, with Western blot analysis, it is evident that the anti-kidney HABP antibodies recognize only one protein band at 34 kDa in the glycine extracts of various tissues of rat, such as kidney, brain, liver and heart. Moreover, the migration of this band is identical with the one developed for the purified kidney HABP, thus confirming not only the monospecificity of the antibodies towards 68 kDa HABP but also the occurrence of this protein in various tissues of rat.

4.2.4 Immunological characteristics of HA-binding protein

Western blot analysis (Fig. 4.7a) using anti-rat kidney HABP antibodies revealed that the rat kidney-derived HABP is immunologically identical to the 68 kDa HABP isolated from other tissues of rat, e.g., heart, brain and liver. However, antibodies to the HABP did not cross-react with fibronectin, laminin and collagen type
Figure 4.4. Two-dimensional gel electrophoresis of HASP purified from rat kidney, liver, brain, heart and plasma. For the first dimension, 5 μg of proteins were applied to a 4% polyacrylamide isoelectric focusing tube gel (6 cm) and electrophoresed at 400 V for 12 h over a pH gradient ranging from 5 to 8 (Section 3.10.4). For the second dimension, the tube gel was then placed on top of a 12.5% SDS-polyacrylamide slab gel and SDS-PAGE was carried out at the constant voltage of 80 V to achieve final separation of the proteins as described under Materials and Methods (Section 3.10.2 and 3.10.4). Low-molecular-weight standard proteins (14.4-94 kDa, Pharmacia) were run in the far-left side of the second dimension gel. The protein spots/bands were visualized by the silver staining method [Merril et al., 1981].

Figure 4.5. Binding of Concanavalin A to HASP. — Lane 1: 10 μg of ferritin (negative control); lane 2: 10 μg of pure rat kidney HASP were electrophoresed on 12.5% SDS-PAGE (Section 3.10.2) and then electrophoretically transferred onto nitrocellulose sheets (Section 3.16). After the transfer, the sheets were washed briefly in PBS and incubated for 30 min with 1% periodate-treated BSA in PBS. The blots were then incubated with 50 μg/ml Concanavalin A in PBS containing 1 mM MgCl₂ and 1 mM CaCl₂ for 1 h at RT. The bound Con A was visualized as mentioned under Materials and Methods (Section 3.12).
Figure 4.6. Monospecificity of anti-rat kidney HABP antibodies by Western blot analysis. 5 μg of purified rat kidney HABP (lane 1), and 50 μg of proteins from glycine extract of rat liver (lane 2), kidney (lane 3), heart (lane 4) and brain (lane 5) were subjected to 12.5% SDS-PAGE (Section 3.10.2). Proteins in the gel were electrophoretically transferred onto a nitrocellulose sheet. Blotted proteins were incubated with anti-rat kidney HABP antibodies and the resultant immune complexes were visualized by enzyme-linked immunodetection method using alkaline phosphatase-labeled goat anti-rabbit IgG (secondary antibody) as mentioned under Materials and Methods (Section 3.16). The positions of various molecular-weight standards are shown on the right side of the figure.
Figure 4.7. Determination of immunological characteristics of kidney HABP by Western blot analysis. — a. Cross-reactivity of anti-rat kidney HABP antibodies with HABP from different tissues of rat. 10 μg of pure HABP from rat brain (lane 1), kidney (lane 2), liver (lane 3), and heart (lane 4) were subjected to 12.5% SDS-PAGE (Section 3.10.2). Proteins in the gel were electrophoretically transferred onto nitrocellulose sheets. Blotted proteins were incubated with anti-rat kidney HABP antibodies and the resultant immune complexes were visualized by using the enzyme-linked immunodetection system as mentioned under Materials and Methods (Section 3.16). The positions of various molecular-weight standards are shown on the right side of the figure. — b. Determination of antigenic relationship of rat kidney HABP with other extracellular matrix proteins. 10 μg each of laminin (lane 1), fibronectin (lane 2), collagen type IV (lane 3), purified rat kidney HABP (positive control, lane 4), and BSA (negative control, lane 5), and 50 μg of proteins from rat kidney glycine extract (lane 6) were electrophoresed on a 7.5% SDS-polyacrylamide slab gel (Section 3.10.2). Proteins in the gel were electrophoretically transferred onto nitrocellulose sheets and then tested for their immunoreactivity with anti-rat kidney HABP antibodies by immunoblotting as mentioned under Materials and Methods (Section 3.16).
IV (Fig. 4.7b), indicating that the HABP is antigenically distinct from these well-characterized extracellular matrix proteins. In addition, anti-HABP antibodies did not cross-react with BSA.

4.2.5 Binding characteristics of HABP

4.2.5.1 Specificity towards HA

In order to identify whether the 68 kDa HABP binds specifically to HA only among GAGs, varying concentrations of this protein were allowed to interact with fixed concentration of test compounds, and then its binding activity towards HA was measured quantitatively by ELISA using anti-HABP antibodies (as described in Section 3.18.2 and 3.18.3). As shown in Figure 4.8, the blocking assay clearly demonstrated that the HABP binds specifically to HA but not to other GAGs, e.g., chondroitin sulfate, heparin, and monosaccharides N-acetylglucosamine and D-glucuronic acid. These compounds after preincubation with HABP did not inhibit the binding of HABP to HA, whereas preincubation of HABP with HA specifically blocked its further binding to HA. The specific affinity of HABP towards HA among GAGs was also confirmed with immunodot blot analysis (data not shown).

4.2.5.2 Binding of HABP to matrix-structural proteins

Binding characteristics of HABP towards HA and various structural proteins of extracellular matrix, e.g., laminin, fibronectin and collagen type IV, were studied by two different approaches. Iodinated $[^{125}\text{I}]$HABP was used for binding equilibrium studies. It was evident that $[^{125}\text{I}]$HABP bound not only to HA but also to laminin, fibronectin and collagen type IV (Fig. 4.9). In the presence of equal amounts of protein and biopolymer substrates, binding of $[^{125}\text{I}]$HABP was the greatest to HA, then followed by laminin, fibronectin and collagen type IV. The binding was maximal at 2 h. Moreover, the binding of $[^{125}\text{I}]$HABP to these matrix biopolymers was reduced by 50-60% in the presence of a 20-fold excess of unlabeled HABP, which thus confirms the specificity of the binding. In addition, the ability of HABP to bind to these biopolymers in a saturable manner was investigated by determining binding isotherms through ELISA using anti-HABP antibodies (as described in Section 3.18.2). In order to determine the binding constants of the interactions, standard binding curves were produced using increasing amount of HABP (Fig. 4.10). By increasing the amount of HABP, there was an absolute increase in the binding to the biopolymers. The results of
Figure 4.8. Specificity of HABP binding to HA amongst GAGs by blocking assays. Varying concentrations of kidney HABP, preincubated with tested GAGs, were allowed to bind to HA-coated microliter plates for 2 h at 37°C. The bound HABP was estimated by ELISA using anti-rat kidney HABP antibodies as described under Materials and Methods (Section 3.17 and 3.18.3). HABP (•-•) alone or preincubated with N-acetylglucosamine (N-AcGm), glucuronic acid, chondroitin sulfate or heparin, and (----) with hyaluronic acid. Values represent the mean ± SD of 6 replicates. Even after preincubation of HABP with N-AcGm, glucuronic acid, chondroitin sulfate or heparin, the binding curve of HABP to HA is reproduced exactly in the same manner when binding of HABP to HA was studied without any preincubation of the protein (○-○), whereas preincubation of HABP with HA totally blocked the binding of HABP to HA-coated wells (----).
Figure 4.9. Binding equilibrium of $[^{125}\text{I}]$HABP to HA, laminin, fibronectin and collagen type IV. The binding of $[^{125}\text{I}]$HABP to the matrix biopolymers was examined over time. $[^{125}\text{I}]$HABP (2.2 µg/4.34 x $10^4$ cpm per well, specific activity 1.97 x $10^4$ cpm/µg of protein) was added to biopolymers adsorbed (20 µg/well) to nitrocellulose membranes in the presence of 1% BSA as described under Materials and Methods (Section 3.18.1). The bound $[^{125}\text{I}]$HABP was quantitated in a gamma-counter. The specificity of the binding of $[^{125}\text{I}]$HABP to the biopolymers was tested by measuring the binding in the presence of a 20-fold excess of unlabeled HABP as shown by broken lines. Values represent the mean ± SD of six replicates.
Fig 4.10. Binding isotherms of HABP to HA (●—●), laminin, (△—△), fibronectin (■—■), and collagen type IV (○—○). The kinetics of the HABP binding to these matrix biopolymers was studied by ELISA as mentioned under Materials and Methods (Section 3.18.2). The microtiter plates were coated with 2 μg/50 μL/well solution of HA, laminin, fibronectin or collagen type IV in PBS, pH 7.2 for 12 h at 4°C. The wells were washed with PBST, blocked with 3% BSA in PBST for 2 h at 37°C and then incubated with increasing concentrations of HABP (0.1 to 1.2 μg/50 μL/well) in 1% BSA in PBST for 2 h for 37°C. The wells were washed with PBST and the bound HABP was quantitated by ELISA using anti-rat kidney HABP antibodies (Section 3.17 and 3.18.2). Values represent the mean ± SD of 6 replicates.
this experiment were analysed by (1) a double reciprocal plot of bound vs free HABP (Fig. 4.11) and (2) a Scatchard plot analysis of the binding data [Scatchard, 1949] from Figure 4.1a (Fig. 4.12). The data showed that the interactions had binding constants (Kd) of approximately $10^{-9}$M in the following order: Kd HABP-HA < Kd HABP-Laminin < Kd HABP-fibronectin < Kd HABP-collagen type IV. All these methods of analysis demonstrate complex (i.e., positive cooperative) interactions between HABP and its binding substrates. These observations also indicate that the binding preference of HABP is: HA > laminin > fibronectin > collagen type IV, suggesting the highest affinity towards HA.

4.2.6 Renal HABP levels in newborn and aging rats

To elucidate the organizing role of 68 kDa HABP in tissue morphogenesis, the level of this protein was examined by ELISA in the renal tissues during aging of rats. For quantitation studies, the extracts of renal tissues were prepared by homogenizing the tissues in 0.2 M glycine-HCl, pH 2.2. The supernatants were neutralized and then dialyzed extensively against PBS. The HABP was quantitated in these extracts by ELISA using anti-rat kidney HASP antibodies as mentioned in Materials and Methods (Section 3.17).

As shown in Table 4.1, the renal tissue of newborn rat contains the maximum amount of HABP which decreases concomitantly with the aging of tissues. There is more than a 50% drop in HABP content in adult rat kidney as compared to newborn rat kidney.

4.2.7 Localization of HABP

By indirect immunofluorescence staining using anti-rat kidney HABP antibodies (as described in Section 3.19.2), the localization of 68 kDa HABP was studied both in the confluent nonpermeabilized as well as permeabilized human fetus lung fibroblast cultures (Fig. 4.13). As can be seen in this figure, the HABP is not only distributed uniformly over the surface of fibroblasts (Fig. 4.13a) but also present inside the cells where it occurs predominantly in the perinuclear region (possibly golgi bodies) (Fig. 4.13b). Immunostaining for HABP in the latter region clearly indicates the secretion of this glycoprotein to the outer surfaces of cells or in the extracellular spaces. The association of HABP with the cell surface was further confirmed by treating the cells with 0.2 M urea in DMEM prior to Immunofluorescence staining. Urea
Figure 4.11. Double reciprocal plot analysis of the binding of HABP to HA (●—●), laminin (△—△), and fibronectin (○—○) (a), and collagen type IV (○—○) (b). The binding isotherms presented in Figure 4.10 were analyzed by using the double reciprocal plot method.
Figure 4.12. Scatchard analysis of the binding of HABP to HA (•—•), laminin (Δ—Δ) (a), fibronectin (○—○) (b), and collagen type IV (○—○) (c). Scatchard plot analysis of the binding data from Figure 4.10.
<table>
<thead>
<tr>
<th>Age of rats</th>
<th>µg of HABP/mg of total protein in the tissue extract&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1 day old (new born)</td>
<td>95 ± 4.73</td>
</tr>
<tr>
<td>5 days old</td>
<td>81 ± 4.82</td>
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<tr>
<td>10 days old</td>
<td>68 ± 3.21</td>
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<tr>
<td>20 days old</td>
<td>54 ± 3.85</td>
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<tr>
<td>Adult</td>
<td>43 ± 2.32</td>
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HABP contents of the tissue extracts were assayed by ELISA using anti-rat kidney HABP antibodies as mentioned under Materials and Methods (Section 3.17). <sup>a</sup>The average value of ten different experiments with standard deviation.
Figure 4.13. Indirect immunofluorescent localization of the HABP on nonpermeabilized and permeabilized human fibroblasts. (a) Human fetus lung fibroblasts were subcultured on glass coverslips for 48 h, fixed with 4% paraformaldehyde in PBS, exposed to antibodies to rat kidney-derived HABP and the resultant immune complexes were visualized by FITC-labeled goat anti-rabbit IgG as described under Materials and Methods (Section 3.19.2). The staining for HABP can be observed uniformly over the cell surface. (b) As in (a) but the cultures were permeabilized with methanol and acetone after fixation with paraformaldehyde, and then immunostained for HABP as mentioned above. The staining can be seen inside the cells, particularly, intense staining in the perinuclear region. (c) As in (a) but the fibroblast cultures were washed with 0.2 M urea in DMEM prior to fixation with paraformaldehyde and the immunostaining for HABP. The urea - pretreated cells exhibited highly reduced staining for HABP over their surfaces. (d) Preimmune rabbit serum stained very little of the cells. X 1000.
pretreatment appeared to strip most of the HABP from the cell surface as judged by highly reduced immunostaining for HABP after this treatment (Fig. 4.13c). Immunoblot analysis of serum-free supernatant medium of cultured human fetus lung fibroblasts by anti-HABP antibodies (Fig. 4.14) also demonstrated that the 34 kDa HABP is secreted in the culture medium of these cells and thus provided an evidence for the occurrence of this protein in extracellular spaces. In addition, these observations also reveal that the 68 kDa HABP derived from rat kidney is not species-specific.

4.3 DISCUSSION

Hyaluronic acid-binding proteins (HABPs) are small-molecular-weight glycoproteins which are biochemically characterized by their preferential noncovalent, high-affinity binding towards HA in vitro, and are possible binding sites for this glycosaminoglycan in vivo. They represent a distinct class of extracellular matrix-and cell surface-associated proteins. A number of HABPs have been studied from various tissues and cell types, as described in the introduction.

The present report documents the purification and partial characterization of a novel HABP, which is a 68 kDa glycoprotein derived from normal rat kidney. It is a homodimer of 34 kDa subunit size and lacks interchain disulfide bridge. The protein is slightly acidic having isoelectric point of 6. Moreover, we observed that kidney HABP is immunologically and structurally (molecular weight, pi value and glycoprotein nature) identical to the 68 kDa HABP present in other tissues of rat. These data clearly indicate that kidney-derived 68 kDa HABP is not tissue-specific. However, this protein was found to be antigenically distinct from other well-characterized cell matrix-associated proteins such as fibronectin, collagen and laminin. Furthermore, the HA-binding protein, described in this study, does not seem to be related to any of the known HABPs reported by other investigators, since it has different molecular weight, subunit composition and pi value. Delpech and Halavent [1981] described the extracellular matrix-associated HABP(s) from human brain with the molecular mass in the range of 40 to 100 kDa with a major band at 68 kDa. Turley and her coworkers reported the cell surface-associated extracellular HA-binding, proteins from culture medium of embryonic chick heart fibroblasts having a molecular mass of 60 to 63 kDa and of untransformed as well as MSV-transformed mouse 3T3 cells in the range of 56 to 70 kDa [Turley, 1982; Turley et al., 1987]. Perides et al. [1989] isolated the glial HA-binding protein (GHAP), a matrix-associated 60 kDa glycoprotein, from human brain white matter. GHAP was mainly found in brain and spinal cord white matter and could
Figure 4.14. Evidence for the HABP as secretory protein by Western blot analysis. — Lane 1: Serum-free supernatant culture medium of human fetus lung fibroblasts collected after 48 h of culturing the cells, 50 µg of proteins; lane 2: 5 µg of purified rat kidney HABP (positive control); lane 3: 5 µg of Pharmacia low-molecular-weight marker proteins (14.4 - 94 kDa) were electrophoresed on a 12.5% SDS-polyacrylamide slab gel (Section 3.10.2), and then electrophoretically transferred onto a nitrocellulose sheet. Blotted proteins were incubated with anti-rat kidney HABP antibodies. The resultant immune complexes were then visualized by the enzyme-linked immunodetection method using alkaline phosphatase-labeled goat anti-rabbit IgG as described under Materials and Methods (Section 3.16). The blotted marker proteins lane was cut out from the nitrocellulose sheet and stained with amido black.
not be detected outside the brain and spinal cord, whereas the 68 kDa homodimeric HABP purified from rat kidney, in this study, could be detected in several tissues. The HA-receptor characterized from the plasma membrane fractions of SV 40-3T3 and BHK cells, and of pulmonary macrophages by Underhill and his collaborators [Underhill et al., 1985, 1987; Green et al., 1988] is an integral membrane glycoprotein having a molecular mass of 85 kD and 99.5 kDa, respectively. The HABP, reported by Crossman and Mason [1990], on the surface of rat chondrosarcoma cells is a nonintegral membrane glycoprotein having a molecular mass of 102 kDa. The 60 kDa HA-binding protein and the 100 kDa HA-receptor identified on the outer surfaces of J774 macrophages and of liver endothelial cells, respectively, are intercalated in the plasma membranes and require detergent for extraction [Gustafson and Forsberg, 1991; Forsberg and Gustafson, 1991].

The results presented here clearly show that the 68 kDa HABP binds not only to HA but also to laminin, fibronectin and collagen type IV. However, it does not bind to other GAGs and monosaccharides. In terms of the biopolymers tested in this study, the binding preference of HABP is HA > laminin > fibronectin > collagen type IV, suggesting the highest affinity towards HA. Moreover, HABP binds to these biopolymers with complex (i.e., positive cooperative) interactions. The unusual ability of HABP to bind, in addition to HA, to matrix proteins is consistent with the observation of Turley and Moore [1984] that the 56 to 70 kDa HABP(s) isolated from culture medium of normal and transformed cell lines bind to HA as well as matrix proteins in the following order: HA > fibronectin > collagen IV > laminin, having the highest affinity towards HA. This difference in the binding preference for matrix proteins may be due to the structural differences between the HABP isolated by us and the HA-binding protein(s) characterized by Turley et al. The complex binding interactions of HABP described here are interesting in view of the observations [Goldberg et al., 1984; Underhill et al., 1983, 1985, 1987] that HA binds to endogenous HA-binding sites on cells as well as isolated HA-receptors in a saturable manner. HABP(s) described by Delpech and Halavent [1981], Turley et al. [1987] and Perides et al. [1989] also exhibit a similar kind of binding interaction with HA, thereby supporting our observation. Further, the preferential and saturable, high-affinity binding of 68 kDa HABP to HA in vitro, as observed here, fulfills the normal criteria of binding site-ligand interaction in vivo and therefore strongly suggests that this protein is a possible endogenous binding site for HA.
The ability of 68 kDa HABP to bind, in addition to HA, to various matrix-associated structural proteins raises the possibility that in vivo the HABP in concert with these matrix macromolecules could play an organizing role in structuring the ECM, in tissue morphogenesis and development, as well as in positioning and anchoring the cells in ECM. This suggestion on the possible involvement of the HABP in the process of tissue morphogenesis and development is further supported by the experimental fact that the level of this protein in renal tissue is highest in newborn rat and decreases progressively with the aging of the animal. Earlier, Delpech et al. [1982] had shown that hyaluronectin was abundant in the dermis of fetal skin, and in adult skin it was totally absent except in the hair sheaths and bulbs. The presence of this protein in mesenchymatous tissues was later supported by them [Delpech and Delpech, 1984] showing its higher expression in embryonic mesenchyme. Delpech and Delpech [1984] have also indicated that a considerable modification of HABP occurs after birth in nervous system as well as in mesenchymal tissues of other organs and is present in the adult in a small number of locations. To further support the high contents of HABP in renal tissues of neonatal rats, we add here, that HA-rich extracellular particles have been morphologically associated with the forming cleft of the S-shaped tubule of the newborn rat kidney at the time of this invasion into the cleft [Reeves et al., 1980].

Furthermore, the localization of HABP described here, on the outer surface of confluent nonpermeabilized human fetus lung fibroblasts, is consistent with the previous reports of Turley et al. [Turley and Torrance, 1985; Turley et al., 1985; Auersperg, 1989; Turley et al. 1990] that HABP(s) are spread uniformly over the cell surface in confluent culture and localized in the lamellae, retraction fibers and perinuclear region of actively motile cells. However, the HABP described in this study, in contrast to other integral membrane HA-receptors [Green et al., 1988; Lacy and Underhill, 1987; Underhill et al., 1983, 1985, 1987], is only associated with the cell surface but not intercalated in the plasma membranes, as it is extractable in the absence of detergent and can be stripped off from the cell surface by urea pretreatment. The secretion of 34 kDa HABP in the culture medium of human fetus lung fibroblasts further confirms our observation that the HABP is a cell surface-associated extracellular matrix protein. Moreover, this observation also indicates that 68 kDa HABP derived from rat kidney is not species-specific.

In summary, we have not only purified and partially characterized a novel HABP from normal rat tissues but we have also shown its complex interactions with
matrix polymers. Its localization on the cell surface, in the ECM as well as inside the cell has also been described. The results suggest that the HABP described in this study is distinct from other well-characterized matrix proteins and belongs to the same family of ECM-and cell surface-associated glycoproteins which are able to bind preferentially and with high-affinity to HA, and are possible endogenous binding sites for this polysaccharide.

Moreover, the availability of a purified HA-binding protein and the polyclonal antibodies against it could enable us to further characterize the physiological functions of this protein in various aspects of cell behaviour as described in the following chapters.