Review of Literature
INTRODUCTION

Evolution from unicellular to multicellular organism requires cells, the basic unit of life, to reorganize themselves to form specialized group, the tissue, which performs specific functions. Tissues are not made up solely of cells. A substantial part of their volume is extracellular space, which is largely filled by an intricate network of macromolecules constituting the matrix. This matrix is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them. Variations in the relative amounts of the different types of matrix macromolecules and the way in which they are organized in the extracellular matrix (ECM) give rise to an amazing diversity of forms, each adapted to the functional requirements of the particular tissue. At the interface between an epithelium and connective tissue, the matrix forms a basal lamina, which is important in controlling cell behavior. The vertebrate ECM, once thought to serve mainly as a relatively inert scaffold to stabilize the physical structure of tissues, has a far more active and complex role in regulating the behavior of the cells that contact it, influencing their survival, development, migration, proliferation, shape, and function. The ECM has a correspondingly complex molecular composition. The macromolecules that constitute the ECM are mainly produced locally by cells in the matrix. These cells also help to organize the matrix: the orientation of the cytoskeleton inside the cell can control the orientation of the matrix produced outside. Two main classes of extracellular macromolecules make up the matrix: (1) fibrous proteins, including collagen, elastin, fibronectin, and laminin, which have both structural and adhesive functions, and, (2) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of
proteoglycans. The proteoglycan molecules in connective tissue form a highly hydrated, gel-like “ground substance” in which the fibrous proteins are embedded. The polysaccharide gel resists compressive forces on the matrix while permitting the rapid diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells. The collagen fibers both strengthen and help organize the matrix, and rubber like elastin fibers give it resilience. Finally, many matrix proteins help cells attach in the appropriate locations.

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeating disaccharide units. They are called GAGs because one of the two sugars in the repeating disaccharide is always an amino sugar (N-acetylg glucosamine or N-acetylgalactosamine), which in most cases is sulfated. The second sugar is usually uronic acid (glucuronic or iduronic). Because there are sulfate or carboxyl groups on most of their sugars, GAGs are highly negatively charged. Four main groups of GAGs are distinguished according to their sugars, the type of linkage between the sugars, and the number and location of sulfate groups: (1) Hyaluronan (HA), (2) chondroitin sulfate and dermatan sulfate, (3) heparan sulfate, and (4) keratan sulfate. Moreover, besides associating with one another, GAGs and proteoglycans associate with fibrous matrix proteins such as collagen and with protein meshworks such as the basal lamina, creating extremely complex structures.

Hyaluronan (HA)

The term ‘Grundsubstanz,” was first applied to the amorphous material between cells by the German anatomist, Henle, in 1841, which was better translated as “basic,” “fundamental,” or “primordial” substance. The modern era of ground substance research began in 1928 with the discovery of a “spreading factor” by Francisco Duran-Reynals. He observed that testicular extracts stimulated rapid spread
of materials injected subcutaneously on the backs of shaved rabbits, while simultaneously causing dissolution of the ground substance. The active principal of these extracts was later shown to be the enzyme, hyaluronidase, the class of enzymes that degrade HA. Interestingly, in one of the studies by Duran-Reynals, hyaluronidase-like activity was demonstrated in extracts of human malignancies, particularly from breast cancers and malignant melanoma (Stern 2008). “Ground substance” was subsequently renamed “acid mucopolysaccharides,” a term first proposed by Karl Meyer (Meyer 1938), who also first described HA (Meyer and Palmer, 1934; 1936) as a “polysaccharide acid of high molecular weight” from bovine vitreous that contained a “uronic acid, an amino sugar, and possibly a pentose” but no sulfates. This was the term to designate the hexosamine-containing sugar polymers that occurred in animal tissues alone, as well as when bound to proteins. It is now well established that HA is the predominant “acid mucopolysaccharide” that constitutes “ground substance,” though heparan sulfate is the most abundant GAG on cell surfaces. Chondroitin sulfate, on the other hand, is the major GAG of the matrix of tissues such as cartilage, tendon, and scar.

HA is a ubiquitous high molecular size unbranched polymer that is prominent in vertebrate ECM during embryogenesis, inflammation, in wound healing, whenever there is rapid tissue turnover and repair, and particularly, in neoplasia. HA is a sugar-chain macromolecule in which N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) are linked together by alternating β-1,3 and β-1,4 linkages (Fig. 1). The number of the repeating disaccharide units varies, but can reach at least 25,000 that corresponds to a molecular mass of 10 million Da, and an extended length of 22.5μm. Its unique physicochemical properties, like the capacity to bind large amounts of water and form viscous gels at relatively low concentrations, has been suggested to
provide a pliable matrix for tissue remodeling (Toole, 1997). HA also acts like a filter, facilitating the diffusion of small, but excluding large molecules (Evanko, et al, 2007). In contrast to other glycosaminoglycans, HA does not contain sulfate groups, is not covalently attached to a core protein, but can bind to proteoglycans and other proteins to organize pericellular and ECM.

Despite its apparently simple structure, HA exhibits multiple features depending on its molecular size and its binding molecules. For instance, high molecular weight (HMW) HA forms part of the ECM by linking HA-binding molecules into macromolecular aggregates and regulates a variety of cell behaviors, such as cell adhesion, motility, growth, and differentiation. HA oligosaccharides also regulate such cell behaviors in the different ways by acting on intracellular signaling pathways through interaction with cell surface receptors. Accumulating evidence has demonstrated that the production of HA is increased in cancer malignancies (Toole, 2004); increased serum levels and deposition in tumor tissue are often associated with malignant progression in many cancers, including breast cancer and colorectal cancer (Ponting et al, 1992; Ropponen et al, 1998).

As a polymer in solution, HA is best described on the basis of available hydrodynamic data, as a stiff random coil. In a current prevalent view, the stiffness is attributed to intramolecular hydrogen bonds across the two glycosidic linkages. However it has been argued, that the properties of HA solutions (in the dilute and semi-dilute regime) arise from the presence of specific extended conformational states that promote interchain associations which are cemented by hydrophobic and hydrogen–bond interactions.

**HA Turnover: The Balance between biosynthesis and degradation**

The level of HA varies from tissue to tissue; e.g. in the vitreous of the human eye (0.1-0.4 mg/g wet weight), or in synovial joint fluid (3-
4 mg/ml), or in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml), in hyaline cartilages (~1 mg/g wet weight) or in the pathological matrix that occludes the artery in coronary restenosis (Hascall and Laurent 1997). The largest amount of HA (7-8 g per average adult human, ~50% of the total in the body) is found in skin tissue, where it is present both in the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue) (Tammi et al, 1988). Interestingly, rooster comb, a specialized piece of skin, has even higher amounts of HA (up to 7.5 mg/ml).

Fig. 1: Scheme illustrating the HA structure (A), a predicted structure of mammalian HAS (B), and a proposed secretion process of HA (C) (Itano and Kimata, 2008).

HA levels are greatly enhanced in many pathological conditions including urticaria, the edema associated with wound healing, inflammation and the organ enlargement that occurs after transplantation (Mikecz, et al, 1995; Stern and Csoka, 2000). Myocardial damage following infarction is partially due to the pressure necrosis associated with post-traumatic tissue swelling. Circulating levels of HA rise rapidly in situations such as shock and septicaemia.
in burn patients (Berg et al., 1997). HA level can also be elevated by a number of inflammatory stimuli, including the transformation growth factors (Stair et al., 2002). Such increased levels of HA seem to be a survival mechanism for the organism, providing, together with its vast volume of water-of-hydration, an intravascular volume expander that delays circulatory collapse. HA and its bound water represent an entirely separate compartment, not in equilibrium with other compartments functioning purely as a volume expander. The high level of HA in such settings seems to be a stress response. The dynamic turnover of HA molecules is tightly regulated during embryonic development and homoeostatic processes and is balanced by synthesis and catabolism, thereby maintaining a constant concentration in the tissue.

HA synthases (HASs; EC 2.4.1.212) are glycosyltransferases that polymerize HA. The discovery of three members of the HAS gene family (HAS1, HAS2, and HAS3) has enabled great strides in understanding the unique process of HA biosynthesis and mode of chain elongation. Structurally, all HAS proteins are composed of multiple membrane-spanning regions and large cytoplasmic loops (Fig. 1). Unlike typical glycosyltransferases, the cytoplasmic loop in HAS molecules possesses two active sites, which participate in the transfer of UDP-GlcNAc and UDP-GlcA substrates. All HASs use UDP-sugars in the presence of Mg2+ or Mn2+.

\[ n \text{ UDP-GlcUA } + n \text{ UDP-GlcNAc } \rightarrow 2n \text{ UDP } + (-4\text{GlcUA-\beta1,3-GlcNAc-\β1-})_n \]

Typically, \( n > 10^3-4 \); thus, HASs make HA chains of \( \sim 1-10 \text{ MDa} \). The intrinsic fidelity of the two glycosyltransferases of HAS generates the repeating disaccharide structure. Unlike virtually all other vertebrate glycoconjugates, HA is made as a free glycan, not attached to protein or lipid. HASs do not need a primer for HA synthesis; they all initiate
HA biosynthesis *de novo* with only UDP-GlcNAc, UDP-GlcUA, and Mg$^{2+}$. UDP-sugars are polymerized from cytoplasmic pools by HAS, and the growing HA chain is extruded out of the cell. Characterization of the three HAS isoforms has revealed differences in enzymatic properties, particularly in their ability to form HA matrices and determine product molecular size (Itano *et al.*, 1997). The expression profiles of HAS genes are temporally and spatially regulated during embryogenesis and pathogenesis (Sugiyama *et al.*, 1998, Pienimaki *et al.*, 2001), and divergence in the transcriptional regulation of HAS genes during these processes can be explained to some extent by upstream signaling pathways that are triggered by various growth factors, cytokines, cellular stress, and so on. The dynamic turnover of HA is therefore tightly regulated by altering the expression profiles of HAS isoforms to have different enzymatic properties (Weigel *et al.*, 1997, Itano *et al.*, 2002).

HA size is an important factor governing the ability of HA to alter cell behavior (Toole 2000; Lee and Spicer 2000). In particular, very small HA oligosaccharides (*e.g.* 10–20 sugars) have potent effects in many *in vitro* or *ex vivo* systems, including angiogenesis models. It is generally assumed that small HA oligosaccharides would be derived from large ECM HA, which is degraded inside cells via exo-β-glycosidases and hyaluronidases (Stern, 2003) during local or systemic turnover. However, it is also possible that HASs might be regulated to synthesize very small HA, thus producing small HA oligosaccharides directly. Tissue HA levels vary during embryonic and oocyte development and wound healing (Laurant and Fraser 1992; Fraser *et al.*, 1997). Expression of HAS isoforms or HA levels can be correlated with the proliferation, survival, and metastasis of cancer cells.

In vertebrates, the half-life of HA molecules is short in certain tissues; for instance, HA turnover rates are less than a day in skin
and serum, and normally 2–3 weeks in cartilage. The turnover and removal of HA from the ECM occurs via local catabolism and/or drainage into the lymphatic system for catabolism in regional lymph nodes. The metabolism of circulating HA primarily takes place in the endothelial cells lining the sinusoids of liver. HA catabolism is predominantly regulated by several hyaluronidases, which are classified as endo-β-N-acetylglucosaminidases according to their mammalian hydrolytic mechanisms. HYAL1 is a lysosomal enzyme with an acidic pH optimum and ability to cleave HA into small oligosaccharides. HYAL2 is a glycosylphosphatidylinositol-(GPI-) anchored protein located on the surface of plasma membranes, and is able to degrade high-molecular-mass HA into intermediately sized products of 20 kDa. Since there is little evidence to support extracellular HA depolymerization, local turnover of HA is believed to occur intracellularly within lysosomes via a low pH-responsive hyaluronidase. Numerous studies have shown that receptor-mediated internalization of HA is the primary step for the turnover and catabolism in many tissues, and it is clear that at least a portion of HA is internalized via CD44 and HARE and delivered to lysosomes for degradation (Harada and Takahashi, 2007). HA is cleaved to yield oligosaccharides, which are then sequentially cleaved by β-D-glucuronidase and β-N-acetyl-D-hexosaminidase, resulting in the monosaccharides D-glucuronic acid and N-acetyl-D-glucosamine. These monosaccharides are then released into the cytoplasm for further processing to CO₂, ammonia, acetate and lactate. In the liver, the three latter substances are released from the liver endothelial cells and are metabolised further to urea, H₂O and CO₂ by hepatocytes (Roden et al., 1989).

The degradation of HA can also take place non-enzymatically, by a free radical mechanism, by reducing agents like thiols, ascorbic acid, ferrous or cuprous ions. This degradation is mediated by
molecular oxygen and leads to random destruction of HA to unit monosaccharides (McNeil et al, 1985). Studies have also shown that as compared to sulphated GAGs, HA is more susceptible to these non-enzymatic breakdowns (Moseley et al, 1995).

**Extracellular HA**

HA functions can be broadly divided into (i) organization of the ECM, (ii) formation of a HA coat on the cell surface, (iii) receptor-mediated signaling and (iv) those associated with the intracellular presence of HA. Extracellular HA is found in tissues that are comprised primarily of ECM, for example cartilage, where it is an important structural component of the matrix (Knudson et al, 1999). For an HA rich matrix to form, it is necessary for it to be anchored to the cell surface. In many instances, this is mediated by CD44, but nascent chains of HA that remain attached to the HA synthase (HAS) machinery can also contribute to coat formation (Tammi et al, 1998; Knudson, 1998). A thick HA coat is present around many types of cells in culture, which is removed by *Streptomyces* hyaluronidase, suggesting that HA is the major structural component of the coat. In particular, embryonic mesenchymal cells are rich in surface HA but poor in receptors. Cytotoxic interactions of lymphocytes with synovial fibroblasts and tumour cells are inhibited by the presence of HA-containing coats around target cells (McBride and Bard, 1979). In these cases, removal of the coat with hyaluronidase allows interaction with the lymphocytes, resulting in cytolysis. Retention of HA as a coat at the cell surface allows capture and incorporation of ECM proteins into the immediate vicinity of the cell. This incorporation of proteins is required for the proliferation and migration of certain cell-types. An example of pericellular matrix formation in vivo occurs prior to ovulation during the expansion phase of cumulus cell-oocyte complex
An up-regulation of HA synthesis leads to expansion of the COC by about 20 folds (Salustri et al, 1992).

**Intracellular HA**

There is growing evidence for the presence of HA in the cytoplasm and nuclei of cells in a number of tissues and cultured cells in vivo (Hascall et al, 2004). Experiments on rat vascular tissues have revealed that HA was localized intercellularly in areas characterized by extensive endothelial cell interdigitation, while intracellularly, moderate staining of nuclear heterochromatin were also observed (Eggli and Graber 1995). HA has been shown to accumulate intracellularly in the perinuclear region of aortic smooth muscle cells during premitotic and mitotic stages. Elevated synthesis of HA has been seen during the G2/M stages. Furthermore, HA tends to accumulate at the mitotic spindle suggesting associations with intracellular structure.

The cells also form an extended pericellular matrix at this time that is permissive for cell detachment and rounding, and for mitosis to occur (Hascall et al, 2004). This intracellular HA can be derived from either the extracellular environment or from an as yet unidentified intracellular source and may be involved in nuclear function, chromosomal rearrangement, and other events associated with cell proliferation and motility. Although HA synthases are believed to be located exclusively in the plasma membrane (Laurent and Fraser 1992), it is possible that there is a cytoplasmic enzyme or, more likely, a synthase on an intracellular membrane that is oriented to secrete HA into the cytoplasm or nucleus. It is also conceivable that the HA could be synthesized within a vesicle that is on its way to or from the cell surface but makes its way into the cytoplasm. The functional diversities of HA combined with its intracellular localization lead to the
hypothesis that it may be a component of the nuclear matrix and cytoskeleton.

**HA and Cancer**

It is now widely recognized that HA is dramatically increased in most malignancies. This increases in HA correlates with tumor virulence, and is often used as a prognostic indicator. But such observations were made in number of experimental systems, long before it was appreciated in human cancers. Among the earliest observations on HA in animal tumors, it was demonstrated that the "mucinous substance" associated with Rous sarcomas in the chicken was identical to the material that had been characterized by Karl Meyer (Kabat, 1939). The same material was then demonstrated to be produced in cultures of Rous sarcoma cells (Grossfield, 1962). Following infection of avian cells with the sarcoma virus, there is a five-fold increase in the HA-synthases, the enzymes that synthesize HA (Ishimoto *et al*, 1966), causing a dramatic stimulation of HA deposition. Infection with other oncogenic viruses also caused enormous increases in rates of HA production, as well as abnormal acceleration of cell growth (Hamerman *et al*, 1965). Treatment of chicken embryo fibroblasts with tumor promoters like PMA stimulates HA synthesis (Ullrich *et al*, 1983). The HA isolated from such transformed cells has the ability to stimulate proliferation of growth-retarded, non-transformed cells (Henrich and Hawkes, 1989). The constitutive HA synthesized by non-transformed cells does not possess this property, an ability attributed to the size difference between the two classes of HA polymers (Stern *et al*, 2006). HA was demonstrated in a number of other experimental animal model tumors, including the rat Walker carcinoma (Fiszer *et al*, 1970). The rabbit V2 carcinoma (Toole *et al*, 1979) was one of the earliest studies to demonstrate a direct relationship between HA and invasive tumor
growth. Aggressiveness of other murine tumors were subsequently shown to correlate with HA content (Knudson et al, 1984). Increased levels of HA were shown to correlate with high metastatic potential in variants of mouse mammary carcinoma cells (Kimata et al, 1983). In human mammary cell culture systems, highly aggressive breast cancer cell lines such as MDA-MB-231 synthesize greater amounts of HA than the much less virulent cell line MCF-7. But, the HA synthesized by the breast cancer lines remains cell-associated while normal breast epithelial cells secrete most of their HA into the medium (Chandrasekharan and Davidson, 1979). HA is produced not only by the cancer cells themselves, but production is induced by the tumor cells in their surrounding stromal cells. In human cancers, levels of HA often correlate with prognosis (Anttila et al, 2000). Cancer cell culture systems have facilitated identification of 'factors' that modulate the expression of HA. 17-β-estradiol and growth hormone have been shown to stimulate the production of acid mucopolysaccharides in fetal fibroblasts (Ozello, 1964) Tumor cells also secrete factors that can induce increased synthesis of HA in fibroblasts (Asplund et al, 1993). A similar factor was shown to be present in both fetal serum and the serum of cancer patients (Decker et al, 1989). Some of these tumor-derived factors have been defined (Suzuki et al, 1995), while others have defied explication. Some of these are soluble factors, while others require cell–cell contact (Knudson et al, 1984).

HA accumulation is most conspicuous in malignancies that develop in cells and tissues normally devoid of HA, such as single layered epithelia and their HA-poor connective tissue stroma. The magnitude of the HA accumulation in the malignant epithelium itself (e.g. colon and gastric cancers) or tumor stroma (breast, ovarian, prostate cancers) strongly correlates with an unfavorable prognosis of the patient, i.e. advancement of the malignancy. A completely different
pattern arises from stratified epithelia that normally produce HA and are surrounded by a HA-rich stroma. The cell surface of the tumors like squamous cell carcinomas of skin, mouth, larynx and esophagus, and skin melanoma show abundant HA which tends to get reduced and patchy in the most advanced stages of the tumors, suggesting enhanced turnover. While the assays of human tumors represent snapshots of currently unknown processes and kinetics of HA metabolism, it is obvious that HA accumulation at some stage is an inherent feature in most of the common epithelial malignant tumors (Tammi et al, 2008). HA synthesis correlates with the level of HAS mRNA, suggesting that transcriptional regulation is an important determinant of the net HAS activity (Karvinen et al, 2003). HAS expression is often increased by growth factors like EGF, KGF, PDGF, and both growth factors and their receptors are often overexpressed in cancers. Growth factor exchange between epithelial and stromal cells is common. Cancer (epithelial) cells may stimulate the adjacent stromal cells to produce a new HA rich tissue structure favorable for tumor growth (Edward et al, 2005), and stromal cells secrete factors that enhance cancer cell migration into the new matrix (Karnaub et al, 2007).

HA is connected to the deranged receptor-mediated signaling seen frequently in cancers. HA stimulates ErbB2 signaling in cancer cell lines (Misra et al, 2008). Interestingly, in mice carrying tumors of human breast cancer cells overexpressing ErbB2 receptor, and resistant to the receptor blocker Trastuzumab®, become sensitive to this drug when given together with the HA synthesis inhibitor 4-methylumbelliferone, resulting in stopped tumor growth (Palyi-Krekk et al, 2007). This suggests that HA synthesis somehow protects the receptor and enhances its signaling.

HA is found in abundance on mesenchymal stem cells and their bone marrow niche (Jin et al, 2006). A recent finding indicates that
mesenchymal stem cells from mouse bone marrow migrate into tumors that develop from implanted human breast cancer cells, and the factors secreted by the adjacent mesenchymal stem cells stimulate migration, invasion and metastasis of the cancer cells (Bendall et al, 2007). The abundance of HA in the peritumoral stroma may thus be a sign of the presence of mesenchymal stem cells that seek their ways into injuries and wounds, and stimulate their healing, cancer representing a non-healing wound. HA has been shown to promote anchorage independent growth and the resistance of cancer cells to growth arrest and apoptosis under anchorage independent conditions is dependent on interactions between HA and CD44 (Ghatak et al, 2002).

HA oligomers, on the other hand, can inhibit anchorage independent growth of tumor cells by suppressing the PI-3-kinase/Akt cell survival pathway. HA oligomers competitively displace endogenous polymeric HA from its receptors, replacing endogenous, multivalent, high affinity ligand with a monovalent low affinity ligand (Ghatak et al, 2002). Small fragments or oligosaccharides of HA stimulate endothelial-cell proliferation, motility and tubule formation, and induce angiogenesis in a variety of experimental systems. These oligomers probably interact with CD44 and RHAMM on the surfaces of endothelial cells. HA fragments also stimulate MMP production and promote migration in some cancer cells (Toole, 2004). Thus increased hyaluronidase levels and increased HA degradation might promote tumour progression through the effects of breakdown products on angiogenesis. It has been recently shown that constitutive HA-CD44 interaction stimulates a signaling pathway involving ErbB2, PI3K/AKT, β-catenin, and Cyclooxygenase-2/Prostaglandin E2 in HCA7 colon carcinoma cells.
Fig. 2: Localization and forms of tumor HA (red), and suggested roles in the growth and spreading of cancers derived from simple epithelia.

1. HA is not present in normal simple epithelia, but emerges in epithelial inflammation or injury, presumably to enhance cell proliferation and migration to cover the defect.

2. HA is also upregulated when the epithelial cells undergo malignant transformation. It supports cell proliferation, prevents apoptosis, maintains intercellular space to facilitate nutrient diffusion, and enhances cell locomotion that stimulates invasion.

3. HA synthesis enhances epithelial to mesenchymal transition in the cancer cell phenotype which releases the cells from their epithelial compartment for invasion.

4. Accumulation of HA in the stroma opens the fibrillar matrix for cell migration.

5. A coat of HA on cancer cells shields them from the cytotoxic effects of T-lymphocytes.

6. Free HA, arranged as "cables" in fixed cell preparations, binds tissue macrophages and modulates their activity to favor tumor growth.

7. Fragments of HA (oligosaccharides) stimulate endothelial cell proliferation and budding of new capillaries that allow tumor expansion. (Tammi et al, 2008)

The HA/CD44 activated ErbB2→PI3K/AKT→β-catenin pathway stimulates cell survival/cell proliferation through COX-2 induction in HA-overexpressing HIEC6 cells and HCA7 cells. Perturbation of HA-
CD44 interaction by HA oligomers or CD44-silencing-RNA decreases cyclooxygenase-2 expression and enzyme activity, and inhibition of cyclooxygenase-2 decreases HA production suggesting the possibility of an amplifying positive feedback loop between HA and cyclooxygenase-2 (Misra et al, 2008).

HA and hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC, also called malignant hepatoma) is a primary malignancy (cancer) of the liver. Most cases of HCC are secondary to either a viral infection (hepatitis B or C) or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis). HCC, like any other cancer, develops when there is a mutation to the cellular machinery that causes the cell to replicate at a higher rate and/or results in the cell avoiding apoptosis. In particular, chronic infections of Hepatitis B and/or C can aid the development of HCC by repeatedly causing the body's own immune system to attack the liver cells, some of which are infected by the virus, others merely bystanders. Chronic hepatitis C infection causes HCC through the stage of cirrhosis. In chronic Hepatitis B, however, the integration of the viral genome into infected cells can directly induce a non-cirrhotic liver to develop HCC. Alternatively, repeated consumption of large amounts of ethanol can have a similar effect. Besides, cirrhosis is commonly caused by alcoholism, chronic hepatitis B and chronic hepatitis C. Macroscopically, liver cancer appears as a nodular or infiltrative tumor. The nodular type may be solitary (large mass) or multiple (when developed as a complication of cirrhosis). Tumor nodules are round to oval, grey or green (if the tumor produces bile), well circumscribed but not encapsulated. The diffuse type is poorly circumscribed and infiltrates the portal veins, or the hepatic veins (rarely). Microscopically, there are four architectural and cytological patterns of HCC: fibrolamellar, pseudoglandular (adenoid),
pleomorphic (giant cell) and clear cell. In well differentiated forms, tumor cells resemble hepatocytes, form trabeculae, cords and nests, and may contain bile pigment in cytoplasm. In poorly differentiated forms, malignant epithelial cells are discohesive, pleomorphic, anaplastic, giant. The tumor has a scant stroma and central necrosis because of the poor vascularization.

An intricate relationship exists between HA and liver, as metabolism of circulating HA primarily takes place in the endothelial cells lining liver sinusoids. The endothelial cells also act as the major site for HA degradation (Erickson et al, 1983; Fraser et al, 1985). In many instances, this removal is achieved by endocytic uptake, either within the tissue where it is made or in lymph nodes and the liver (Fraser et al, 1997). The endocytosis of HA is mediated by HA binding proteins like CD44 (Toole, 1997) and HARE (Zhou et al, 2000).

HA is found in blood serum in concentrations <100 μg/L (average 30-40 μg/L in middle-aged persons). The serum level is regulated by the influx of the polysaccharide from the tissues via lymph and its receptor-mediated clearance by liver endothelial cells. Markedly high serum levels are noted in certain liver diseases, especially in patients with cirrhosis, when the clearance is impaired. In these cases serum HA can be used to follow the development of the disease. Serum HA is also a sensitive marker for impending rejection of liver transplants (Laurent et al, 1996). Elevated serum HA is regarded as an indicator for morphological abnormalities and liver and renal diseases (Engstrom-Laurent et al, 1985, Gibson et al, 1992). There is a marked increase in the level of serum HA in disorders like liver cirrhosis and alcoholic liver disease. In alcoholic liver disease, serum HA can be applied as assessment of haemodynamic changes. These high levels of HA in serum could be accredited to impaired clearance of HA in these conditions (Lindqvist, 1997). It has been shown in liver cancers that HA levels are a useful non-invasive index of hepatic fibrosis and
disease severity (Kim et al, 2003). Serum HA is known to be a diagnostic marker of liver fibrosis and cirrhosis in patients with chronic liver disease (Tangkijvanich et al, 2003). The characteristics of glycosaminoglycans (GAGs) in many carcinomas have been reported to be different from those in normal tissues, which can be used as prognostic indices in some cancers. A progressive increase in the content of chondroitin sulfate, low molecular size GAGs, and nonsulfated and disulfated chondroitin sulfate disaccharide units, together with a gradual decrease in heparan sulfate, have been found as the differentiation status of HCC became poorer. A significant increase in HA, which only slightly increased in HCC, was found in intrahepatic cholangiocarcinomas (Lv et al, 2007). Serum concentrations of the 7S fragment of type IV collagen (7S collagen), amino-terminal propeptide of type III procollagen (PIIIP), and HA have been reported to serve as serologic markers of liver fibrosis in hepatitis and cirrhosis (Tsukamoto et al, 2004). Up-regulation of CD44 (HA receptor) isoforms is shown to be associated with poorly differentiated HCC and shortened survival (Endo and Terada, 2000).

HA and Inflammation

Appearance of HA on epithelial cells is a general feature of inflammation (Kemppainen et al, 2005). During inflammation, HA adopts a special conformation, “cables” (Day and de la Motte, 2005; Fig. 2) which immobilizes and deactivates monocytes. Such deactivated macrophages produce proteinases, growth factors and matrix molecules that clear the inflammation and start healing of the wound. Tumor inflammatory cells are often deactivated, in an immunologically suppressed state, i.e. unable to destroy the cancer cells (Ben-Baruch 2006), and HA oligosaccharides were recently shown to promote the deactivation of tumor macrophages (Kuang et al, 2007). These findings may indicate a novel function for tumor-
associated HA: attracting, binding and deactivating leukocytes potentially lethal to the tumor cells, and instead leading the tissue into a state of chronic wound healing process, favorable for tumor growth and invasion. High molecular mass HA on tumor cells may also prevent firm attachment of leukocytes on cancer cell surface (del Fresno et al, 2005), necessary for a proper immune reaction.

Recently, it has been reported that HAS overexpressing cells have unique plasma membrane extensions, which form the skeleton of an extensive HA coat (Kultti et al, 2006). Similar structures were also reported to be present in tumor-derived fibroblasts which secrete large amounts of HA and bind monocytes. These HAS-induced extensions with their HA cover might also act as local immunological shields either via mechanical basis or via immunomodulatory actions.

**HA and Immunological Response**

GAG's like HA, chondroitin sulfate A (CSA), polysaccharides chains of proteoglycans, often accumulated at the inflammatory area may play an important role in regulating the functions of lymphoid and myeloid cells. HA, heparin sulfate, dermatan sulfate and stimulate invitro growth of murine magakaryocyte progenitors. HA can accumulate on mouse B cells and induce lymphocyte aggregation, tyrosine phosphorylation, enhance the surface expression of MHC class II, CD40, CD54, CD80, CD86 in dendritic cells, and trigger cell adhesion molecular expression in murine kidney tubular epithelial cells (Yang et al, 2002). HA fragments and low molecular weight HA has been found to induce macrophages to produce various cytokines and chemokines and inducible nitric oxide synthase. CD44v3-, v6- and v9-, isoforms expressed by human dendritic cells have been shown to mediate cell adhesion to HA and induce DC maturation and secretion of cytokines. HA, in the presence of GM-CSF has been shown to rapidly upregulate the expression of antigen presenting and
co-stimulatory molecules on immature dendritic cells, augment the activation of NF-κB and promote protein phosphorylation (Termeer et al., 2002). There might be a possibility that HA and CSA in combination with GM-CSF can induce an effective immunity against tumors in tumor microenvironment (Yang et al., 2002).

**HA and Cellular Signaling**

HA is overproduced in many types of tumors, and in some, HA levels are prognostic for malignant progression. HA interacts with cell surfaces by binding to cell surface receptors such as CD44 and RHAMM (receptor for HA mediated motility), to induce transduction of a range of intracellular signals, either directly or by activating other receptors. HA can also be retained on the cell surface by sustained transmembrane interactions with its synthases. Retention of HA on cell surface can generate a voluminous pericellular matrix that incorporates several other HA binding molecules. Interactions of HA with CD44 and RHAMM lead to numerous cellular responses, including those that involve tyrosine kinases, protein kinase C, focal adhesion kinase (FAK), phosphotidylinositol 3-kinase (PI3-kinase), mitogen-activated protein kinase, nuclear factor-κB and RAS, as well as cytoskeletal components. In general, the interactions of HA with CD44 and RHAMM are of obvious physiological importance, and their normal activities seem to be disrupted in cancer cells. The other ligands of CD44 and RHAMM, compound this complexity. The interactions of HA with these receptors, particularly with CD44, regulate two specific cellular functions that are especially important for tumorigenesis, cell survival and ERBB-family signaling.

All CD44 isoforms contain a HA-binding site in their extracellular domain, and thereby serve as a major cell surface receptor for HA. CD44 is expressed in both normal and tumor stem cells (displaying unique ability to initiate normal and/or tumor cell-specific properties) and CD44 has been suggested as one of the important surface
markers for both normal stem cells and cancer stem cells. The fact that both CD44 and HA are overexpressed at sites of tumor attachment and HA binding to CD44 stimulates a variety of tumor cell-specific functions and tumor progression suggests that HA–CD44 interaction is a critical requirement for tumor progression.

Different HA-dependent and CD44-specific signaling pathways during oncogenesis and tumor progression are depicted below in Fig. 3.

Fig. 3: A current model illustrating HA-dependent and CD44-specific signaling pathways during oncogenesis and tumor progression (Bourguignon, 2008).
Ankyrin-based signaling pathways

(a) HA binding to CD44 stimulates ankyrin-mediated cytoskeleton rearrangement required for cell adhesion. (b) HA–CD44 interaction also promotes recruitment of IP3 receptor (located in the intracellular Ca2+ storage organelles) into CD44 and caveolin-1/cholesterol-containing lipid rafts. This event is required for the formation of functional IP3 receptor Ca2+ channels (presumably via conformational changes and/or IP3 receptor tetramerization), and the onset of internal Ca2+ release leading to CaMKII (Ca2+/calmodulin-dependent kinase-II) activation and filamin phosphorylation as well as cytoskeleton-mediated tumor cell migration.

RhoA-specific signaling pathways

(a) HA–CD44 binding is tightly coupled with LARG in a complex which can induce LARG-mediated RhoA activation. HA/CD44 and LARG-activated RhoA then stimulates PLCγ-mediated IP3 production and IP3 receptor-triggered intracellular Ca2+ mobilization resulting in CaMKII activation. CaMKII then phosphorylates the cytoskeletal protein, filamin, leading to cytoskeleton reorganization and tumor cell migration. (B-1) The binding of HA–CD44 also induces activation of ROK which, in turn, phosphorylates myosin phosphatase and myosin light chain, thereby activating myosin adenosine triphosphatase (ATPase) and generating actomyosin-mediated membrane motility and cell migration. (B-2) The binding of HA–CD44 stimulates ROK phosphorylation of NHE1. Most importantly, the phosphorylation of NHE1 by ROK promotes Na+-H+ exchange activity, intracellular/lysosomal pH changes and extracellular acidification leading to an activation of low pH-dependent ECM degradation enzymes required for ECM modification and tumor cell invasion. (B-3) HA–CD44 interaction is tightly coupled with p115RhoGEF in a complex which can up-regulate RhoA signaling and ROK activity. Activated ROK then phosphorylates certain cellular proteins including
the linker molecule, Gab-1. Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI3-kinase to CD44 and activates certain isoforms of PI3-kinase to convert PtdIns (4, 5) P2 to PtdIns (3,4,5)P3 leading to AKT activation and tumor cell survival required for breast tumor progression. (B-4) HA binding to CD44 promotes ROK phosphorylation of CD44 which, in turn, recruits ankyrin from the cytosol to the membrane leading to ankyrin–Tiam1 interaction and Rac1 activation as well as cytoskeleton-mediated cancer progression.

Rac1-specific signaling pathways

The binding of HA–CD44 promotes Tiam1/Vav2-mediated Rac1 activation and downstream effector functions (e.g., PAK (a) and IQGAPI (b)) leading to actin assembly and cytoskeleton reorganization and cancer progression.

Cdc42-specific signaling pathways

(D-a) The binding of HA–CD44 promotes Cdc42–CD44 association and PAK1 phosphorylation/activation and PAK1–filamin complex formation leading to cytoskeleton activation and tumor cell migration and invasion. (D-b) HA-activated CD44 is tightly coupled with IQGAP1 in a complex which interacts with both Cdc42 and ERK2. The CD44–IQGAP1–Cdc42 complex is capable of binding to F-actin required for cytoskeletal function and tumor cell migration. At the same time, the association of CD44–IQGAP1 with ERK2 promotes ERK2 phosphorylation and ERK2 activity which, in turn, phosphorylates Elk-1 and ERα. Most importantly, phosphorylation of Elk-1/ERα by IQGAP1-linked ERK2 enhances transcriptional activation.

All these oncogenic signaling events and tumor cell-specific behaviors are known to play a pivotal role in promoting tumor progression.
Hyaluronon : A Regulator of Chemoresistance

The possibility that HA might influence drug resistance was suggested in the finding that hyaluronidase treatment enhances the action of various chemotherapeutic agents, especially when used locally (Baumgartner et al, 1998). The relation between HA and multidrug resistance was also studied in multicellular mammary tumor cell spheroids, known to be enriched in therapy-resistant stem-like cells; dispersion of these spheroids with hyaluronidase reverses drug resistance (St Croix et al, 1998).

Increased HA production was found to stimulate drug resistance in drug-sensitive cancer cells, whereas disruption of endogenous HA-induced signaling, suppresses cell resistance to several drugs, including doxorubicin, taxol, vincristine, and methotrexate (Misra et al, 2003). Several studies show that HA and CD44 promote drug resistance in a variety of cancer cell types, including breast, lung and head and neck carcinomas, and lymphoma (Misra et al, 2005). Although the anti-apoptotic effect of HA is likely to contribute to these phenomena, HA–CD44 interactions also regulate expression of drug transporters, including P-glycoprotein, MRP2 (Misra et al, 2005) and BCRP (Gilg et al, 2008).

Activation of the MDR1 upstream promoter, associated with P-glycoprotein over-expression, has been shown to correlate with metastases to the lymph nodes in breast carcinoma cells (Raguz et al, 2004). In accordance with the role of CD44 in malignant cell behavior and metastases, it was shown by confocal microscopic co-localization and fluorescence resonance energy transfer (FRET) studies in NIH3T3 cells that P-glycoprotein is closely associated with CD44 and other components of plasma membrane lipid microdomains, commonly known as lipid rafts (Bacso et al, 2004) Interestingly, P-glycoprotein was found to be anchored to the cytoskeleton. CD44 is known to bind to the actin cytoskeleton through ERM-family proteins or ankyrin
CD44 has been shown to present in close molecular vicinity (<10 nm) to P-glycoprotein and may be one of the proteins responsible for the cytoskeletal association of this transporter. Furthermore, raft localization of P-glycoprotein seems to be of functional importance since cholesterol depletion results in strong inhibition of transporter activity (Bacso et al., 2004). In addition, a study comparing multidrug resistant cell lines of breast, oral, and ovarian origin that overexpress P-glycoprotein with their respective P-glycoprotein-negative, drug-sensitive, parental cell lines, demonstrated a positive correlation in the expression of CD44 and P-glycoprotein. The two were found to co-immunoprecipitate, and drugs that interfered with the function of P-glycoprotein also interfered with cell motility and invasion, both hallmarks of CD44 receptor activity (Miletti-Gonzalez et al., 2005). It has also been observed that CD44 co-localizes in the plasma membrane of cancer cells with the transporters, P-glycoprotein and BCRP, and that treatment of the cells with HA oligomers rapidly induces internalization of the transporters and CD44 into the cell. Interestingly, it has been noted that drugs that interfere with P-glycoprotein can also affect localization of CD44 on the cell membrane and promote CD44 capping, and therefore might act via inhibition of actin polymerization (Miletti-Gonzalez et al., 2005).

Emmprin (Extracellular-matrix metalloproteinase inducer; also known as CD147 or basigin), a cell surface glycoprotein of the immunoglobulin-like superfamily, has been shown to stimulate the production of HA. Emmprin is expressed at high levels in many types of malignant tumors and its levels on the tumor cells have been shown to correlate with MDR expression. It has been shown to induce drug resistance in a HA-dependent manner (Toole, 2004).

Constitutive interaction of HA with CD44 has been shown to regulate assembly and activation of ErbB2 containing signaling complex, which stimulates PI3-kinase activity in multidrug resistant
MCF-7/Adr human breast carcinoma cells. HA, PI3-kinase and ErbB2 form a positive feedback loop that strongly amplifies MDR1 expression and regulates drug resistance in these cells (Misra et al, 2005).

It has been recently reported that HA binding to MCF-7 cells and human ovarian tumor cells (SK-OV-3.ipi cells) promotes Nanog (embryonic stem cell transcription factor) protein association with CD44 followed by Nanog activation and the expression of pluripotent stem cell regulators (e.g., Rex1 and Sox2). Nanog also forms a complex with Stat-3 in the nucleus leading to Stat-3-specific transcriptional activation and MDR1 gene expression. It was further observed that HA-CD44 interaction induces ankyrin (a cytoskeletal protein) binding to MDR1 resulting in the efflux of chemotherapeutic drugs e.g., Doxorubicin and Paclitaxel, in these tumor cells (Bourguignon et al, 2008). All these events can contribute to tumor cell growth and multidrug resistance in both breast and ovarian cancer cells.

**HA Binding proteins or Hyaladherins**

Such diversity in the functions of HA, a molecule with a simple structure, results from the large number of HA-binding proteins (also termed hyaladherins), which exhibit significant differences in their tissue expression, cellular localization, specificity, affinity and regulation.

The hyaladherins include matrix HA binding proteins, cell-surface HA binding proteins, intracellular HA-binding proteins and enzymatic HA binding proteins, all exhibiting specific affinity towards HA and elucidating its functional diversity (Knudson and Knudson, 1993). A representation of a few hyaladherins is compiled in Table 1.

Though numerous hyaladherins have been identified, their exact mechanism of interaction with HA still remains unsolved. One group of hyaladherins possesses a common structural domain of ~100 amino acids termed as “Link module”, which is involved in HA binding
(Day, 2000), while the other group possesses a B-(X₇)-B motif, where B is either arginine or lysine and X₇ contains a stretch of 7 amino acids, none of which are acidic and each harbours at least one basic amino acid (Yang et al, 1994).

<table>
<thead>
<tr>
<th>Hyaladherins</th>
<th>Mol mass</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Link protein</td>
<td>45-50 kDa</td>
<td>Oegema et al, 1975</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>220 kDa</td>
<td>Barry et al, 1993</td>
</tr>
<tr>
<td>Versican</td>
<td>400 kDa</td>
<td>LeBaron et al, 1992</td>
</tr>
<tr>
<td>Neurocan</td>
<td>245 kDa</td>
<td>Margolis et al, 1996</td>
</tr>
<tr>
<td>Brevican/BEHAB</td>
<td>145 kDa</td>
<td>Jaworski et al, 1994</td>
</tr>
<tr>
<td>GHAP</td>
<td>54-76 kDa</td>
<td>Perides et al, 1991</td>
</tr>
<tr>
<td>TSG-6</td>
<td>39 and 120 kDa</td>
<td>Lee et al, 1992</td>
</tr>
<tr>
<td>SPACR</td>
<td>150 kDa</td>
<td>Acharya et al. 1998</td>
</tr>
<tr>
<td>HABP102</td>
<td>102 kDa</td>
<td>Crossman and Mason, 1990</td>
</tr>
<tr>
<td>IoT1</td>
<td>180 kDa</td>
<td>Bost et al, 1998</td>
</tr>
<tr>
<td>HABP1/P32/gClqR</td>
<td>68 kDa</td>
<td>D’Souza and Datta, 1985</td>
</tr>
<tr>
<td>BRAL.1</td>
<td>38 kDa</td>
<td>Hirakawa et al, 2000</td>
</tr>
<tr>
<td>SPACRCAN</td>
<td>400 kDa</td>
<td>Acharya et al. 2000</td>
</tr>
<tr>
<td><strong>Cell Surface</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>80-160 kDa</td>
<td>Culty et al, 1992</td>
</tr>
<tr>
<td>CD38</td>
<td>46 kDa</td>
<td>Nishina et al, 1994</td>
</tr>
<tr>
<td>RHAMM</td>
<td>52-125 kDa</td>
<td>Hardwick et al, 1992</td>
</tr>
<tr>
<td>HABP1/P32/gClqR</td>
<td>68 kDa</td>
<td>D’Souza and Datta, 1985</td>
</tr>
<tr>
<td>HARE</td>
<td>175 kDa</td>
<td>Zhou et al. 2000</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>60 kDa</td>
<td>Banerji et al, 1999</td>
</tr>
<tr>
<td>Layilin</td>
<td>55 kDa</td>
<td>Bono et al, 2001</td>
</tr>
<tr>
<td>Stabilin-1&amp;2</td>
<td>220-300 kDa</td>
<td>Politz et al, 2002</td>
</tr>
</tbody>
</table>
Table 1: The 'Hyaladherin' family. Representation of a few hyaladherins originating from different sources exhibiting diverse cellular localization.

<table>
<thead>
<tr>
<th>Intracellular</th>
<th>Grammatikakis et al, 1995</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc37</td>
<td>29.3 kDa</td>
</tr>
<tr>
<td>HABP1/P32/gC1qR</td>
<td>68 kDa</td>
</tr>
<tr>
<td>IHABP</td>
<td>95 kDa</td>
</tr>
<tr>
<td>IHABP4</td>
<td>~ 55 kDa</td>
</tr>
<tr>
<td>D'Souza and Datta, 1985</td>
<td></td>
</tr>
<tr>
<td>Hofmann et al, 1998</td>
<td></td>
</tr>
<tr>
<td>Huang et al, 2000</td>
<td></td>
</tr>
</tbody>
</table>

The Link Module Superfamily

The Link module, also referred to as a proteoglycan tandem repeat or PTR (Kohda et al, 1996), was first identified in the link protein isolated from cartilage. The structure of link module consists of two α-helices and two triple-stranded anti-parallel β-sheets. The link protein is comprised of an immunoglobulin domain and two contiguous Link modules, and this structural arrangement is also found in the G1 domains of aggrecan, versican, neurocan, and brevican (Day and Prestwich, 2002). These proteoglycans form huge, link protein-stabilized complexes with HA, providing the load-bearing function in articular cartilage, giving elasticity to blood vessels and contributing to structural integrity of tissues like skin and brain (Watanabe et al, 1997). Members of the link module superfamily include link protein (Oegema et al, 1975), aggrecan (Barry et al, 1993), versican (LeBaron et al, 1992), neurocan (Margolis et al, 1996), Brevican/BEHAB (Jaworski et al, 1994), TSG-6 (Lee et al, 1992), BRAL1 (Hirakawa et al, 2000), CD44 (Culty et al, 1992), LYVE-1 (Prevo et al, 2001) and Stabilin-1 (Politz et al, 2002). TSG-6 contains a single link module, which is sufficient for high affinity interaction with HA, and is secreted in response to inflammatory stimuli (Kahmann et al, 2000); both link protein and aggrecan have a pair of contiguous Link modules in their HA-binding regions (Watanabe et al, 1997). In
comparison, CD44 possesses a HA-binding domain of ~160 amino acids comprised of a Link module with N- and C-terminal extensions that are essential for folding and functional activity. The size of the binding domain appears to correlate broadly with the length of HA recognized.

**The B-(X)\textsubscript{7}-B motif**

Apart from the members of the link module superfamily, many hyaladherins have been reported, which though unrelated to each other at the primary sequence level, bind HA. The commonality among these proteins is a B-(X)\textsubscript{7}-B motif, which is thought to be essential for HA binding (Yang et al., 1994). These proteins can bind HA even under denatured and reduced conditions, suggesting that the three-dimensional structure may not be an important criterion for binding. Some members of this family include RHAMM (Hardwick et al., 1992), Cdc37 (Grammatikakis et al., 1995), inter-\(\alpha\)-trypsin inhibitor (\(\alpha\)I) (Bost et al., 1998), plasma HA-binding protein (Choi-Miura et al., 1996) and fibroblast HA-binding protein (Deb and Datta, 1996).

The presence of hyaladherins inside cells is not surprising giving the increasing evidence for intracellular HA. Three HA-binding proteins, in addition to RHAMM are reported to have intracellular address: a) HABP1/p32/gC1qR, which was originally co-purified with pre-mRNA splicing factor, SF2 and was subsequently shown to be a HA-binding protein (Krainer et al., 1991; Deb and Datta, 1996). This protein was also detected on the surface of some cells, e.g. transformed fibroblasts; b) IHABP4, which has a possible role in HA trafficking and intracellular signaling (Huang et al., 2000); and c) cdc37, a vertebrate homologue of yeast and Drosophila, which may be involved in cell cycle functions (Grammatikakis et al., 1995).

In recent years, significant advances have been made in the identification of new hyaladherins and of HA-protein interactions.
Determination of the three-dimensional structures of different types of HA-binding domains and their ligand complexes is clearly essential to understand the molecular mechanisms underlying the diverse biology of this important glycosaminoglycan (Day and Prestwich, 2002).

**HA Binding Protein 1 (HABP1)**

In 1985, D'Souza and Datta reported the presence of a naturally occurring HA binding protein in rat liver, which they purified using HA affinity chromatography and identified it as a glycoprotein containing sialic acid. Biochemical analysis revealed that this protein to be rich in glycine and glutamic acid and distinct from other HA binding proteins such as fibronectin and link protein that are also rich in glycine (D'Souza and Datta, 1986 a,b). The protein was later found to be ubiquitously present in other mammalian organs, including liver, kidney, spleen, brain and testis. It was later isolated from rat kidney, using HA affinity chromatography and shown to have a native molecular weight of 68 kDa, generating sub-units of 34 kDa on SDS-PAGE (Gupta et al, 1991). This led to the nomenclature of this protein as 34 kDa HABP. Polyclonal antibodies raised against this protein showed it to be antigenically distinct from other ECM proteins.

**Homologues of human HABP1/p32/gC1qR**

HABP1 is conserved eukaryotic protein ubiquitously present from yeast to mammals. HABP1 pseudogene is reported to present universally in all animal species but not in plants (Sengupta et al, 2004). The homologues of HABP1 have been reported from yeast, mouse, rat, *C. elegans*, *S. cerevisiae*, chicken and *Trypanosoma brucei*. The common feature in all HABP1 homologues is the conserved nature of polar amino acid residues. In almost all the homologues ratio of positively charged amino acid to negatively charged residues is same.
and is always less than one. A brief overview of HABP1 homologues is given in the Table 2.

<table>
<thead>
<tr>
<th>HABP1/p32/gC1qR homologue</th>
<th>Reported Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mam33p: <em>Saccharomyces cerevisiae</em> homologue</td>
<td>Soluble protein located in mitochondria of <em>S. cerevisiae</em>. HABP1/gC1qR/p32 exhibits 53% similarity and 26% identity to Mam33p. Mam33p may be involved in the sorting of Cytochrome b2 to the inner mitochondrial membrane space</td>
<td>Seytter <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>F59A2.3: a <em>Caenorhabditis elegans</em> homologue</td>
<td>Uncharacterized mitochondrial matrix protein</td>
<td>Hamasaki <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>gC1qBP: murine homologue</td>
<td>89.9% homology with HABP1. Function not known</td>
<td>Lim <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>P22: <em>Trypanosoma brucei</em> homologue</td>
<td>p22 is speculated to be a regulatory factor in <em>T. brucei</em> mitochondrial gene expression by modulating the RNA binding properties of RBP16</td>
<td>Hayman <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>38 kDa myosin binding protein: Chicken homologue</td>
<td>Shows 90% homology to HABP1. Under in <em>vitro</em> conditions it can bind to de-phosphorylated myosin and assist in myosin assembly to form filament.</td>
<td>Okagaki <em>et al.</em>, 2001</td>
</tr>
</tbody>
</table>

Table 2: HABP1 homologues and their putative functions
Cloning and characterization of the 34kDa HABP

Studies on rat histiocytoma unveiled the involvement of this protein in cell adhesion and solid tumour formation (Gupta and Datta, 1991). Presence of HABP was ascertained in cell membrane fractions of rat brain, liver and ascitic fibrosarcoma cultures, substantiating its claim of being a bonafide cell surface receptor for HA. Investigating cultures of neonatal cardiac myoblasts and fibroblasts revealed an enhanced level of 34 kDa HABP in fibroblasts as compared to myoblasts and showed a gradual decline in its level during the progression of myoblast differentiation (Ranganathan and Datta, 1995).

Further analysis revealed that the proteins purified from myoblasts and fibroblasts were identical in terms of their molecular weight and biochemical properties. The antibodies raised against the purified protein were used to immunoscreen λgt11 cDNA expression library of human skin fibroblast and a partial cDNA clone encoding the protein was isolated and characterized. The internal polypeptide sequence (83 residues) of the purified protein was found to be identical to the predicted protein sequence derived from the cDNA, thus confirming the clone. This 34 kDa HABP was given the accession ID: 9786126 and named HABP1 by the Hugo Nomenclature Committee of GDB. The cDNA sequence of HABP1 shows complete identity with p32, a protein co-purified with the human pre-mRNA splicing factor SF2 (Krainer et al, 1991) and gC1qR, receptor for the globular head of complement sub-component 1q (Deb and Datta, 1996; Das et al, 1998). Henceforth, in the following text, the 34 kDa HABP has been referred to either as HABP1 or p32 or gC1qR.

The HABP1 gene encoding HABP1 was mapped at chromosome 17p12-p13 by fluorescence in situ hybridization (FISH) analysis. This chromosomal localization shows 99.5% similarity (from base 928 to base 1163) with STS WI-9242, an STS flanking marker of human
chromosome 17 (Majumdar and Datta, 1998). Following this finding, an extensive analysis for the gene and the promoter was undertaken. The human as well as the mouse HABP1 genes have since been cloned and characterised. They possess a similar exon/intron organisation (Lim et al., 1998). The 6 kb gene encoding HABP1 comprises of 6 exons and 5 introns (Fig. 4). Hybridisation of the full length HABP1 cDNA probe to human genomic DNA digests indicate a simple pattern suggestive of a single copy of the gene in the human genome (Ghebrehiwet and Peerschke, 1998).

The 7.8 kb human HABP1 (C1qBP) gene containing 6 exons and 5 introns was cloned and the 1.3 kb DNA fragment at the 5' flanking region revealed the presence of multiple TATA, CCAAT and Sp1 binding sites (Tye et al., 2001). Luciferase reporter assay in cell lines demonstrated the ubiquitous expression of the promoter. Subsequent 5' deletion analysis confined the promoter elements to within 400 bp upstream of the translation start site. Since the removal of the 8 bp consensus TATATATA at -399/-406 and CCAAT at -410/-414 did not significantly affect the transcription efficiency of the promoter, a GC rich sequence downstream of the TATA box is presumably important for the HABP1/C1qBP promoter activity. One of the seven GC rich sequences in this region bind specifically to PANC-1 nuclear extracts and gel-shift assay established the binding of the transcription factor Sp1 to this GC rich sequence.

Primer extension studies mapped three major transcription start regions, the farthest one being 496 bp upstream of the first ATG codon and in close proximity of Sp1 binding site. The open reading frame of HABP1/p32/gC1qR encodes a pro-protein of 282 amino acid residues (Honore et al., 1993) which after post translational cleavage of first 73 amino acid residues generates the mature protein of 209 amino acid residues. The mature protein corresponds to 23.7 kDa but migrates at 34 kDa on SDS-PAGE due to high ratio of polar to
hydrophobic amino acid residues. The mature protein is preceded by a 60 residue-long hydrophobic stretch containing five cysteines, which in turn is preceded by a 13 residues long leader peptide, which contains the mitochondrial targeting signal sequence. The precise role of these 60 residues immediately preceding the mature protein has not yet been determined.

![Genomic DNA](image)

**Fig. 4:** A schematic representation of the genomic organization of HABP1. The scheme shows the position of the exons and introns in the HABP1 gene. The exons are labeled 1-6, while the introns are labeled I-V. The cDNA generated from the gene is shown below and the tentative size of the gene and the cDNA are marked alongside.

However, it has been predicted to play a role in cellular translocation. The mature protein has a calculated \( pI \) of 4.15 suggesting its acidic nature. There are 28 glutamic acid, 20 aspartic acid, 16 lysine, 5 histidine, and 4 arginine residues present in 209 residues long mature protein. In contrast, the first 73 residue long stretch of the pre-pro-protein does not have any glutamic acid, aspartic acid, lysine or histidine residues, but does have 11 arginine, 9 proline and 5 Cys residues (Ghebrehiwet et al, 1994). In absolute contrast, the mature protein (residues 74 to 282) possesses 28 Glu, 20
Asp, 16 Lys, 5 His, 4 Arg and a lone Cys (Ghebrehiwet et al., 1994; Deb and Datta, 1996). The 15 amino acids immediately preceding the mature protein probably contain the signal sequence for mitochondrial targeting, with the protein getting cleaved between the serine and Leucine residues. The 60 amino acid residues preceding the mature protein are highly hydrophobic and their exact function is not well defined (Ghebrehiwet and Peerschke, 1998).

In silico analysis of functional motifs in HABP1

Biochemical studies with the recombinant protein and subsequent search analysis of HABP1 sequence provided information on the probable motifs present in the protein. These motifs include those for ligands interacting with HABP1 and the sites for various cellular modifications like phosphorylation and glycosylation. The minimal motif required for the binding of HABP1 to HA is referred to as the B-(X)7-B motif, where B is either R or K and X7 is a stretch of 7 non-acidic amino acid residues in between (Yang et al., 1994). The motif 119KLVRKVAGEK128 represents the HA binding motif in mature HABP1. This motif however contains an extra glutamic acid residue, but the crystal structure reveals that the accessibility of Glutamic acid is very low, as it forms a salt-bridge with Arg246. This effectively generates the B-(X)7-B motif at the binding site.

Motif search analysis also unveiled the presence of a potential tyrosine sulfation site (125DCHY*PEDEV129) and three N-glycosylation sites (109WELELN*GTEA118, 131VTFNIN*NSIPPTFD144 and 218EWKDTN*YTLNT228). A proline-directed 160PELTSTP166 sequence, enables HABP1 to act as a substrate for protein kinases like extracellular signal-regulated kinase (ERK) (Alvarez et al., 1991) and cdc2 family (Kemp and Pearson, 1990). HABP1 has one phosphorylation site for protein kinase C (202DIFS*IREVS208) and five casein kinase II phosphorylation sites (74LHT*DGDKAFVD84, 200ESDIFS*IREV204, 208EVSFQS*TGESEWKD221, 226EVSFQS*TGESEWKD227)
246RGVDNT*FADEVEL269 and 256LVELST*ALEHQEY1264), ranking it among multi-site-phosphorylated proteins (Deb and Datta, 1996). The presence of the 246RGVD249 sequence might assist in the membrane localization of the protein, as it is closely related to the consensus integrin binding RGD sequence (Hynes, 1992). Though the protein does not possess a consensus bipartite sequence, which would direct it to the nucleus, two basic amino acid rich putative nuclear localization signals (NLS) 94RKIQKHK100 and 118AKLVRK123 have been identified in HABP1 which explains the localization of the protein in the nucleus (Brokstad et al., 2001).

Crystal structure of HABP1

The crystal structure of HABP1/p32/gC1qR shows it to be a trimer with a doughnut shaped quaternary structure and a non-crystallographic three-fold symmetry, without any distinct domains (Jiang et al., 1999). The trimer has an asymmetric charge distribution along its surface. While one side has highly negatively charged residues, the other side possesses positive polarity. Clearly, this polarity in charge distribution has functional implications.

Each monomer in the trimer consists of seven consecutive β-strands, designated β1 to β7, forming a highly twisted anti-parallel β-sheet, with β1 nearly perpendicular to β7. The β-strands are flanked by one N-terminal (αA) and two C-terminal (αB and αC) α-helices. All the three helices are located on the same side of the β-sheet. The N-terminal helix αA does not contact the β-sheet within the monomer, but forms an anti-parallel coiled-coil with the C-terminal portion of αC of the adjacent sub-unit. This coiled-coil interaction is required for the homo-oligomerization of HABP1. The helix αB and the N-terminal portion of helix αC make extensive hydrophobic contacts with the β-sheet, thus stabilizing the trimer. The structure of HABP1 is hitherto unknown for other proteins, thus making it a structurally unique protein.
The trimer has an outer diameter of approximately 75 Å, an inner channel of approximately 20 Å, and a thickness of about 30 Å. Though the channel is about 20 Å in diameter, the loops connecting β6 and β7 partially cover the channel, reducing its effective diameter to about 10 Å. The channel wall is formed by β-sheets from all the three subunits. Due to the high degree of twisting, β-strands from adjacent monomers do not form contiguous β-sheets. Instead, the residues located at the tip of β1-turn-β2 and the loop connecting the β3 and β4 interact with β7 of the adjacent molecule.

The HA-binding motif has been mapped to amino acids 119-128 of HABP1, which corresponds to a region between β2 and β3, with a loop in between. HABP1 does not have the canonical HA-binding motif, as the presence of Glu-127 disrupts the B-(X)7-B motif. This residue, though conserved across species, forms a salt-bridge with Arg-246, another conserved residue. This renders the glutamic acid inaccessible to the surface and generates the typical HA-binding motif. Presence of counter ions in the molecular environment reduces the electrostatic repulsion by screening the charges and hence affecting the three dimensional structure and thermodynamic stability of HABP1 due to the existence of more compact structure under acidic pH or at high ionic strength at neutral pH (Jha et al., 2004).

Among all the tested ligands only HA-HABP1 interaction is found to be tightly regulated with change in pH and ion concentration suggesting this interaction to be highly significant in terms of the biological functions. Moreover, HABP1 exhibits structural flexibility which is influenced by the ionic environment under in vitro conditions near physiological pH which may play an important role in its binding towards different ligands (Jha et al., 2003). However it is also observed that the N- and C- termini truncated variants of HABP1 with truncated α-helices do not undergo oligomerisation but their binding affinities for HA remains comparable to that of HABP1 (Sengupta et al.,
The coiled coil region αA and αC forms extensive intermolecular contacts: αA packs with the anti-parallel αB of an adjacent monomer and the C-terminal region of αC packs against the back of the β-sheet. Most of these interactions are hydrophobic in nature.

**Fig. 5: The crystal structure of HABP1.** The asymmetric charge distribution along the faces of HABP1 is shown in panel A and B. Blue and red represent regions of positive and negative electrostatic potential respectively. Panel C shows the schematic diagram of the trimer, while panel D shows a monomer (Jiang et al, 1999).

In summary, the overall architecture of the trimer can be visualised as if the β-sheet forms a hyperboloid shaped spool with α-helices wrapped around it.

The crystal structure has elucidated on some unexplainable results. Though HABP1 does not have any signal sequence for cell surface localization or transport to extracellular space, its presence on the extracellular surface is intriguing. The crystal structure suggested that as extracellular signals lead to change in the divalent cation concentrations, it may in turn stimulate the transport of HABP1 to the cell surface from where can be eventually secreted to the extracellular space. This hypothesis supports the observations of HABP1 being released from transformed or activated lymphocytes and macrophages (Gupta and Datta, 1991) into the culture media. Therefore, when normal physiological conditions are perturbed, the release of HABP1 into the serum may be promoted.
The crystal structure of HABP1/p32 sheds light on newer aspects of HABP1 function by predicting its possible involvement in numerous cellular processes and helped in understanding its functional diversity and sub-cellular localization pattern.

**Subcellular localization**

HABP1 is a multifunctional protein that interacts with a wide range of biomolecules residing at different subcellular locations. Considerable controversy has surrounded the localization of HABP1 since its cDNA sequence does not predict a traditional membrane-anchoring domain, and has a probable mitochondrial targeting sequence. HABP1 has been found to be present in a number of cellular compartments including the mitochondria, nucleus and cytoplasm where it interacts with diverse range of other proteins.

Though the complete cDNA sequence of HABP1, corresponding to the pro-protein, shows the presence of only a mitochondrial localization signal (Dedio et al, 1998), immunofluorescence and sub-cellular fractionation studies have detected this protein or its homologues in the mitochondrial matrix in yeast (Seytter et al. 1998) and mammals (Muta et al. 1997); cell surface (Gupta and Datta, 1991); cytoplasm (Dedio et al. 1998) and nucleus (Simos and Georgatos, 1994) of cultured cell lines.

Primary sequence of HABP1 has demonstrated a distinct mitochondrial localization signal at the N-terminal part of the pro-protein. This signal comprises of an amphipathic helix with basic residues (Muta et al. 1997). Transient expression system in PLC cells has demonstrated that the cDNA encoding full length protein, localizes to the mitochondria, while the cDNA encoding the mature form of HABP1, targets it to the cytosol (Muta et al, 1997). This clearly shows that the mitochondrial localization signal exists only in the first 73 amino acids. Furthermore, the yeast homologue of HABP1 (Yeast p30), is located in the mitochondrial matrix and plays an important role in
oxidative phosphorylation. The acidic-surface of HABP1 is rich in aspartic and glutamic acid residues. This enables it to serve as a high capacity divalent cation storage protein and modulate the mitochondrial matrix cation concentration. Another interesting feature of HABP1 is its high negative charge density and asymmetric charge distribution, which suggests a possible mode of association with the inner mitochondrial membrane. The negatively charged side of HABP1 may potentially bind to the inner mitochondrial membrane in the presence of divalent metal ions. The presence of HABP1 in the inner mitochondrial membrane could permit the central channel in the trimer to function as a protein import channel (Jiang et al, 1999).

A substantial number of cellular molecules interacting with HABP1, like C1q (Ghebrehiwet et al, 1994), HK (Dedio and Muller-Ester, 1996; Herwald et al, 1996; Joseph et al, 1996; Lim et al, 1998), Vitronectin (Lim et al, 1996; Lim et al, 1998), HA (Deb and Datta, 1996; Gupta et al, 1991), Fibrinogen (Lu et al, 1999) and Factor XII (Joseph et al, 1996; Lim et al, 1998;) occur at extra-mitochondrial sites. For HABP1 to interact with these ligands it should be either localized on the extracellular side of the plasma membrane or in the ECM.

Gupta and Datta (1991) and Deb and Datta (1996) have shown that HABP1 interacts with HA and plays an important role in tumour cell adhesion. Furthermore, Gupta et al (1991) have shown the cell surface localization of HABP1 in fibroblast cells using immunoflorescence techniques and also established its secretory nature. The cell surface localization and its role in C1q-mediated responses have been demonstrated by (Ghebrehiwet et al, 1997). They have also proposed that HABP1 mediates anchoring of cC1q-R to cell surface signaling in a metal dependent fashion and such interaction might lead to C1q-mediated responses. It has been reported that HABP1 binds to several serum proteins, and thus plays a key role in
blood clotting (Peerschke et al, 1998) and fibrin polymerisation (Lu et al, 1999).

Immunolocalization studies using tagged HABP1 have shown that the protein is localized mainly to the nucleus and cytosol (van Leeuwen and O'Hare, 2001). Interaction of HABP1 with viral proteins also targets it to the nucleus (Matthews and Russell, 1998).

In a comprehensive study using immunogold microscopy, strong reactivity of the protein was shown in zymogen granules, condensing vacuoles, endoplasmic reticulum and on the cell surface of pancreatic acinar cells, on the cell surface of microvascular endothelial cells in pancreas and kidney, on the cell surface and nuclei of splenic lymphocytes, and acrosomes of developing spermatids in testes (Soltys et al, 2000). These results indicate that HABP1 is primarily a mitochondrial protein that may also localize outside mitochondria in certain cells and tissues. The diverse subcellular localization of HABP1, coupled to its various interacting proteins suggest that it could be a component of the trafficking pathway connecting the nucleus, mitochondria and cytoplasm and the export pathway to the cell surface (van Leeuwen and O'Hare, 2001).

Though, enough evidence exists for mitochondrial matrix localization of HABP1, the explicit function of the protein in mitochondria and other sub-cellular sites is still unclear.

**HABP1 in pathogenic infection**

There are numerous reports showing the interaction of HABP1 with various parasitic, viral and bacterial proteins indicating the specific role of HABP1 in infection.

It has been recently demonstrated that *P. falciparum* infected RBCs (IBRC) use the 32-kDa human protein gC1qR/HABP1/p32 as a receptor to bind to human endothelial cells, including brain microvascular endothelial cells. It was also shown that *P. falciparum*
Bacterial and Viral interacting proteins | Implications of the interactions | References
--- | --- | ---
*Staphylococcus aureus* Protein A | Potential role in *S. aureus* pathogenesis and a novel mechanism for *S. aureus* localization to sites of vascular injury and thrombosis | Nguyen *et al.*, 2000
Rubella Virus Capsid Protein | Interaction with p32 plays a role in the regulation of nucleocapsid assembly and/or virus-host interactions | Beatch and Hobman, 2000
Epstein Barr virus nuclear antigen-1 (EBNA-1) | Interaction between EBNA-1 and p32/HABP1/gC1qR may promote ori-P dependent DNA replication in the EBV infected cell during the S-phase of the cell cycle | Chen *et al.*, 1998
Adenovirus core protein V | The interaction with p32 helps to deliver the viral genome to the nucleus | Matthews and Russell, 1998
HCV virus core protein | HCV core/gC1qR induced immunosuppression might play a critical role in the establishment of a persistent HCV infection | Yao *et al.*, 2001
HVS-ORF 73 of Herpes virus saimiri | ORF 73 can function as a regulator of gene expression and p32 is involved in ORF 73-dependent transcriptional activation | Hall *et al.*, 2002
Herpes Simplex Virus Type 1 IE63 protein | Possible role of HABP1/p32 in splicing inhibition as well as host cell shut-off, which could facilitate splicing-independent nuclear export of the viral transcripts. | Bryant *et al.*, 2000
Human Immunodeficiency Virus Type-1 Rev and Tat | These interactions might play important role in HIV replication | Yu *et al.*, 1995a,b; Berro *et al.*, 2006
Hepatitis B virus protein 22 | The interaction plays a role in splicing control | Laine *et al.*, 2006
Cytomegalovirus kinase pUL97 | p32 might recruit pUL97 to induce dissolution of the nuclear lamina thereby facilitating the nuclear export of viral capsids | Marschall *et al.*, 2005

**Table 3:** Bacterial and viral HABP1/p32/gC1qR interacting proteins. The table shows the various bacterial and viral proteins which interact with HABP1/p32/gC1qR and the possible implications of these interactions in pathological infection
IRBCs can bind to gC1qR/HABP1/p32 on platelets to form clumps. Thus gC1qR/HABP1/p32 was identified as a novel host receptor that is used for both adhesion to vascular endothelium and platelet mediated clumping (Biswas et al, 2007).

Numerous viral and bacterial proteins are known to interact with HABP1 thus establishing its role in infection. A brief review of the interacting viral and bacterial proteins is given above in Table 3.

**HABP1 interacting proteins at the cell surface**

*Globular head domain of the complement subcomponent 1q, gC1q*

Originally isolated from the Raji cells, the receptor for the globular head domain of the complement subcomponent 1q was reported to be identical to p32, a splicing factor 2 associated protein (Ghebrehiwet, et al, 1994). The binding was reported to be specific and saturable with an affinity of approximately 200 nM under physiological ionic strength and 50-100nM when sub-physiological (90nM) conditions were used. Recombinant or tissue gC1qR/HABP1/p32 was found to bind to both denatured as well as non-denatured C1q and the binding was predominantly through the A-chain and moderately to the C-chain of C1q. gC1q-R/HABP1 is found to bind with arginine residues at the positions 162 and 163 of the A-chain, which is identical or overlapping with the IgG binding site. In addition, it has also been shown that gC1qR/HABP1 inhibits complement activation by binding to C1q and thus preventing the immune complexes from binding to the globular heads of C1q. First 18 amino acid residues from N-terminus of mature gC1q-R/HABP1 (76-93) have been reported to contain a major binding site for C1q (Ghebrehiwet, et al, 1994).
Calreticulin or cC1qR

Calreticulin (CR), a homologue of cC1qR, is an acidic protein residing on the cell membrane. CR is a calcium-binding protein, being associated with nearly all the cellular compartments. Extensive studies have presumed CR to be a mannoside lectin. CR has been shown to be involved in a nuclear export pathway of the glucocorticoid receptor (Holaska et al, 2002). It binds to the collagen like "stalk" of C1q and also interacts with HABP1/gC1qR. Binding sites for cC1qR are located within residues 76-93 and residues 204-218 of HABP1 (Ghebrehiwet et al, 1997). It has been observed that cC1qR and HABP1/gC1qR often co-elute during purification. The biological responses accomplished through cC1qR are complex in nature and believed to interplay between cC1qR, gC1qR and some hitherto unidentified proteins.

Fibrinogen

Cell surface HABP1/p32/gC1qR has been shown to interact with a number of plasma proteins. One such protein is fibrinogen and this interaction can be effectively inhibited by excess soluble fibrinogen or polyclonal antibodies directed against gC1qR or fibrinogen. Furthermore, HABP1/gC1qR has also been shown to inhibit fibrin polymerization in a dose-dependent manner, being a stronger inhibitor than fibrinogen. However, when added together, the effect of HABP1/gC1qR and soluble fibrinogen were additive in nature. This interaction between HABP1/p32/gC1qR and fibrinogen is speculated to aid in the modulation of fibrin formation during injuries and inflammations (Lu et al, 1999).

High molecular weight kininogen and factor XII

High molecular weight kininogen (HK) is a plasma protein that is cleaved by plasma kallikrein to liberate the vasoactive peptide bradykinin. HK and factor XII are known to bind to human umbilical vein endothelial cells (HUVEC) in a zinc-dependent and saturable
manner. The interaction of Factor XII and high molecular weight kininogen (HK) with human umbilical vein endothelial cells (HUVEC) involves at least two proteins. The heavy chain of HK binds to cytokeratin 1, while the light chain binds to HABP1/gC1qR. Factor XII also binds to HABP1/gC1qR (Joseph et al, 1996) and competes with HK for binding. The use of monoclonal antibodies reveal that the interactive site for Factor XII and HK are distinct from that of C1q. Residues 76-93 have been found to contain a major binding site for C1q, while residues 204-218 contain binding sites for both HK and factor XII. Antibody to HABP1/gC1qR inhibits HK binding to endothelial cells by 72%. The competition of Factor XII with HK in binding to HABP1/gC1qR suggest that an endothelial binding protein mediates the assembly of critical components of the kinin-generating pathway on the surface of endothelial cells, thereby linking the early events of kinin formation and complement activation (Ghebrehiwet and Peerschke, 1998).

**Vitronectin**

HABP1/p32/gC1qR has been shown to bind specifically to the heparin-binding multimeric form of vitronectin and the ternary complex comprising vitronectin-thrombin-antithrombin. However, it did not bind to the monomeric or uncomplexed form of vitronectin in plasma (Lim et al, 1996). In order to identify the functional domains of HABP1/gC1qR, required for binding to vitronectin, a N-terminal truncated variant (possessing residues 96-282) of HABP1/gC1qR was generated. Studies with this variant showed that C1q and vitronectin did not compete for binding to HABP1/gC1qR and that the major part of the binding site for vitronectin lies in the N-terminal region of the protein. This ascertains HABP1/gC1qR as a novel vitronectin-binding protein, which might participate in the clearance of vitronectin-containing complexes or cooperate with vitronectin in the inhibition of complement-mediated cytolysis (Lim et al, 1996).
**Tumor homing peptide, Lyp-1**

Lymphatic vessels are an important conduit for the spread of solid tumors, and their abundance in and around tumors correlates with propensity to tumor metastasis. LyP-1, a cyclic nonapeptide that specifically recognizes lymphatic vessels in certain tumors, provides a marker for these vessels and also binds to tumor cells, offering the potential to selectively target both tumor lymphatics and tumor cells. Although the mechanism of Lyp-1 mediated antitumor effect is unknown, but LyP-1 seems to be cytotoxic against tumor cells undergoing stress because its accumulation coincides with hypoxic areas in tumors and tumor starvation enhances its binding and internalization in cultured tumor cells. It has been recently reported that mitochondrial/cell-surface protein, p32/HABP1/gC1qR, is the receptor for a tumor-homing peptide, LyP-1, which specifically recognizes an epitope in tumor lymphatics and tumor cells in certain cancers. The tumor specificity of the peptide and anti-p32 antibodies was found to be the result of higher expression of total p32 and the propensity of malignant and tumor-associated cells to express p32 at the cell surface. It was also found that some tumors contain a p32-positive subpopulation of macrophages/myeloid cells that are closely associated with tumor lymphatics (Fogal et al, 2008).

These findings have established p32, particularly its cell-surface-expressed form, as a new marker of tumor cells and tumor-associated macrophages/myeloid cells in hypoxic/metabolically deprived areas of tumors. Its unique localization in tumors and its relative tumor specificity may make p32 a useful target in tumor diagnosis and therapy.

**Matrix metalloproteinase MT1-MMP**

Membrane type-1 metalloprotease (MT1-MMP) is a key enzyme in cell locomotion and is present on the leading edge of the migrating cells.
MT-1 MMP has been reported to associate with multifunctional, ubiquitously expressed mature gC1qR, via its cytoplasmic tail (Rozanov et al, 2002). gC1qR directly associates with MT1-MMP and is susceptible to MT1-MMP proteolysis. It was found that there are two distinct gC1qR binding sites in the MT1-MMP molecule, the first in the extracellular part and the second in the intracellular CT sequence of the protease. The transient association between gC1qR and cytoplasmic tail of MT1-MMP are likely to be involved in the mechanisms regulating the presentation of the protease at the tumor cell surface.

**HABP1 interacting proteins in nucleus**

**pre mRNA splicing factor (SF2/ASF)**

Alternate splicing factor or splicing factor 2 (SF2/ASF) is a member of the Serine Arginine (SR) family of splicing factors, which stimulate constitutive splicing and regulate alternative RNA splicing either in positive or negative fashion, depending on their binding site on the pre-mRNA. HABP1/p32/gC1qR was isolated, cloned and sequenced as a protein co-purified with splicing factor ASF/SF2 during its purification from HeLa cell nuclear extracts (Krainer et al, 1991). Later on, it was reported that p32 interacts with ASF/SF2 and SRp30c, another member of the SR protein family and inhibits ASF/SF2 function, both as a splicing enhancer and splicing repressor. P32 inhibits the binding capacity of ASF/SF2 and, hence, its function in initiation of pre-spliceosome formation. Also, p32 interaction with ASF/SF2 blocks ASF/SF2 phosphorylation, a post-translational modification which is required for ASF/SF2-mediated protein-protein interaction during spliceosome assembly. However, p32 does not block SRp30c function (Petersen-Mahrt et al, 1999). Therefore, it has been postulated that p32 can regulate ASF/SF2 RNA binding and
phosphorylation. The report placed HABP1/p32/gC1qR in the group of proteins, which control RNA splicing by sequestering an essential RNA splicing factor into an inhibitory complex (Peterson-Mahart et al, 1999).

**Lamin B Receptor (p58)**

The lamin B receptor (p58) is an inner nuclear membrane protein that forms an *in vivo* complex with the nuclear lamins, a nuclear envelope kinase, and two other nuclear proteins with apparent M(r) of 18,000 Da (p18) and 34,000 Da (p34). The protein of 34 kDa was isolated by partial dissociation of the immunoaffinity-purified p58 protein complex. Determination of the N-terminal amino acid sequence of purified p34 revealed its homology with p32. The identity between p34 and p32 was further established by showing that antibodies raised against N- and C-terminal peptides of p32 cross-react with purified p34. As the amino acid sequence of p58 contains an arginine-serine-rich (RS-rich) region similar to the RS-rich region found in SF2, it was postulated that these domains provide binding sites for p34 and that this protein may be a linker between the nuclear membrane and intranuclear spliceosomal substructures (Simos and Geogatos, 1994).

**Transcription factor IIB (TFIIB)**

Studies with HIV-1 Tat identified HABP1/p32 as an interacting protein, giving it the name TAP (Yu et al, 1995a). A simultaneous study showed that TAP activation domain interacts with the cellular protein TFIIB, a general transcription factor. TAP has a strong transcriptional activation region at the C-terminal, which binds HIV-1 Tat *in vitro* and *in vivo* (Yu et al, 1995a). Interestingly, TFIIB shares its binding domain with Tat (Yu et al, 1995b), but they may not bind the same amino acid sequences. The interacting sites for Tat and TFIIB map within the TAP C-terminal region, containing the TAP activation
domain. TAP exhibits typical properties of a co-activator and like VP16, possesses a strong acidic domain that can interact directly with TFIIB (Lin et al, 1991; Yu et al, 1995a). Thus, TAP has been suggested to function as a cellular co-activator, bridging Tat to the general transcriptional machinery (Yu et al, 1995b).

**CCAAT Binding Factor (CBF/NF-γ)**

The mammalian CCAAT-binding factor (CBF), also called nuclear factor Y (NF-Y), consists of three different subunits, CBF-A, CBF-B and CBF-C. These subunits together form a complex with promoter DNA containing the CCAAT motif, which is present in the promoters of many mammalian genes. Mutations in the CCAAT motifs prevent CBF binding to these sites and decrease the transcriptional activity. The CBF binding site is one of the most common motifs present in cell cycle regulated promoters, endoplasmic stress responsive promoters and some tissue specific promoters such as major histocompatibility complex class II, type I collagen promoters. In an attempt to understand the role of CBF in transcription, CBF complex assembled *in vivo* from human HeLa cell extracts was purified using immunoaffinity resins. Mass spectrometry identified human protein p32/HABP1/gC1qR to be present in the purified CBF complex. A 96 amino acid segment of CBF-B containing the C terminal DNA binding region was reported to be sufficient to form CBF-B/p32 complex but p32 cannot bind to DNA by itself. It was demonstrated in an *in vitro* transcription reaction, that reconstitution of p32 specifically inhibits CBF-mediated transcription activation. Based on these observations, p32 was identified as a novel and specific co-repressor of CBF-mediated transcription (Chattopadhyay et al, 2004).
HABP1 interacting proteins in cytoplasm

**Short Mitochondrial ARF (SmARF)**

The alternative reading frame (ARF) mRNA encodes two pro-death proteins, the nucleolar p19 ARF and a shorter mitochondrial isoform named sm ARF. The p19 ARF can inhibit cell growth by causing cell cycle arrest or type 1 apoptotic death while, smARF induces type II autophagic cell death. It has been reported that steady state levels of smARF are regulated through its interaction with mitochondrial p32. The p32 protein interacts physically with both murine and human smARF forms and colocalizes with these short isoforms to the mitochondria. Knocking down p32 significantly reduced the steady state levels of smARF by increasing its turnover. As a consequence, the ability of ectopically expressed smARF to induce autophagy and to cause mitochondrial membrane dissipation was significantly reduced (Reef et al, 2007). Thus, the interaction with p32 provides a means of specifically regulating the expression of the autophagic inducer, smARF.

**Alpha 1B-adrenergic receptor**

Alpha 1B-adrenergic receptor (α1B-AR) is a cytoplasmic protein, whose physiology can be altered drastically by mutations at the C-terminal (Parker et al, 1995). *In vitro* and *in vivo* studies identified HABP1/gC1qR as an interacting protein of α1B-AR. HABP1/gC1qR binds to the C-terminal region of α1B-AR, spanning residues 344-516, with the mature form binding more efficiently than the pro-protein form. Mutational analysis identified ten arginine-rich amino acids (369-378) as the site of interaction. The interaction was confirmed by specific co-immunoprecipitation reaction. HABP1/gC1qR regulates the cellular localization and expression of α1B-AR. When expressed alone, α1B-AR is mainly localized in the cell membrane, while HABP1/gC1q-R localizes in the cytoplasm. However, when co-expressed, most of the
α1B-AR translocates to the cytoplasm along with HABP1/gC1qR, with a notable down regulation of α1B-AR expression (Xu et al, 1999). Thus, the expression level and cellular localization of α1B-AR is governed through its interaction with HABP1/gC1qR.

**Cytochrome b₂**

Cytochrome b₂, a soluble component of the respiratory chain, resides in the mitochondrial inter-membrane space. It is synthesized in the cytosol of *Saccharomyces cerevisiae* as a precursor with a bipartite pre-sequence. The yeast homologue of HABP1, Mam33p forms a homo-oligomer and binds to the sorting signal of cytochrome b₂ that directs the protein to the inter-membrane space. However, studies on MAM33 null strains did not show a significant difference in the levels of cytochrome b₂ in the mitochondria. Consequently, it has been suggested that Mam33p is not essential for cytochrome b₂ sorting and acidic Mam33p has been attributed to interact in a non-specific manner with the positively charged sorting peptide of cytochrome b₂ (Seytter et al, 1997).

**Protein Kinase C μ**

The protein kinases C (PKC) are a family of intracellular Ser/Thr specific kinases, implicated in signal transduction regulating numerous biological responses. Yeast two-hybrid analysis, precipitation assays using glutathione S-transferase fusion proteins, and reciprocal coimmunoprecipitation studies have identified HABP1/p32/gC1qR as a PKC binding protein. p32 interacts with PKCμ in a compartment specific manner, as it can be co-immunoprecipitated mainly from the particulate and not from the soluble fraction, despite the presence of p32 in both fractions. Although p32 binds to the kinase domain of PKCμ, it does not serve as its substrate. It is speculated that HABP1/p32 is a part of an intracellular receptor that restricts PKCμ at an intracellular
compartment such as mitochondria and modulates its kinase activity (Storz et al, 2000).

**γ-Amino Butyric Acid (GABA<sub>A</sub>) Receptor**

GABA<sub>A</sub> receptors are the major inhibitory neuronal ion channels in mammalian brain. They are members of ligand gated ion channel superfamily that included nicotinic acetylcholine, glycine, and serotonin type 3 receptors. GABA<sub>A</sub> receptors are drug targets for a range of clinically important substances like benzodiazepines and barbiturates. HABP1/gC1qR/p32 was found to be co-immunoprecipitated along with GABA<sub>A</sub> receptors, using monoclonal antibodies against the α1 subunit (Schaerer et al, 2001). Using the yeast two-hybrid system it was found that all β subunits, but not α1 and γ2 subunits bind to gC1q-R. A stretch of 15 amino acids (aa 399-413) containing 7 positively charged residues was identified to be responsible for binding with HABP1/p32/gC1qR. This region contains residue Ser-410, which is a protein kinase substrate, and it is known that phosphorylation of this residue leads to an alteration in receptor activity. The presence of functionally relevant Ser-410 within the interacting site suggests a modulatory role of gC1qR either in biosynthesis or the mature receptor (Schaerer et al, 2001).

**Hrk/DP5**

Hrk/DP5 is one of the mammalian BH-3 only proteins implicated in a variety of physiological and pathological apoptosis, yet the molecular mechanism involved in Hrk-mediated apoptosis remains poorly understood. In an attempt to identify cellular proteins participating in Hrk-mediated apoptosis, yeast two hybrid screening for Hrk interacting proteins was conducted and p32 was identified. In vitro binding assay, co-immunoprecipitation as well as immunocytochemical analysis verified specific interaction and
colocalisation of Hrk and p32, both of which depended on the presence of highly conserved C-terminal region of p32. Importantly, Hrk-induced apoptosis was suppressed by the expression of p32 mutants lacking the N-terminal mitochondrial signal sequence, p32(74-282) and the conserved C-terminal region, p32(1-221), which are expected to inhibit binding of Hrk competitively to the endogenous p32 protein and disrupt the channel function of p32 respectively. Furthermore, small interfering RNA-mediated knockdown of p32 conferred protection against Hrk-induced apoptosis. Altogether these results suggest that p32 may be a key molecule that links Hrk to mitochondria and is critically involved in the regulation of Hrk mediated apoptosis (Sunayama et al, 2004).

**Functional diversity of HABP1**

Several studies from our laboratory have addressed the role of HABP1 in diverse cellular processes like cellular signaling, sperm egg interactions, ovulation, apoptosis, muscle cell differentiation, and as a receptor for *P. falciparum* IRBC.

The revelation of HABP1 as a phosphoprotein (Babu et al, 1991) initiated studies in understanding the relationship between its phosphorylation and HA-binding. It was later demonstrated that phosphorylation of HABP1 is regulated by PMA, calyculin A and Ca\(^{2+}\) ionophore in mammalian cell lines (Rao et al, 1997). The interaction of HABP1 with HA enhanced its phosphorylation, which could be inhibited by anti-HABP1-antibodies, validating the role of this protein as a mediator in HA induced signal transduction (Rao et al, 1997).

Since HA is one of the main carbohydrates involved in sperm-egg interaction, the role of HABP1 in the reproductive system was examined. Ranganathan et al (1994, 1995) reported its presence on spermatozoa and showed the differential localization of this protein on the sperm head, mid-piece and tail of different organisms. Later, it was seen that
this protein is present in lower concentrations in teratozoospermic and asthenozoospermic patients in comparison to the normozoospermic spermatozoa, with enhanced phosphorylation in fertile spermatozoa as compared to the lower level of phosphorylation in infertile spermatozoa, thus assigning a marker status of the protein in assessing human fertility (Ranganathan, 1995). The declining pattern of HABP1 during the epididymal maturation was also observed. Pre-treatment of sperms with anti-HABP1-antibodies inhibited sperm-oolemmal adherence. Examination of the expression of HABP1 in adult rat testis during spermatogenesis revealed the presence of a pro-protein form of HABP1, specifically in the testis (Bharadwaj et al., 2002). Further, enhanced phosphorylation of this protein in motile sperms was also demonstrated. This report clearly demonstrated the involvement of HABP1 in sperm maturation, motility and fertilisation processes. Investigation of HA mediated signal transduction events in the spermatozoa showed that HA mediates sperm motility by enhancing the phosphorylation of HABP1.

HABP1 is expressed in a stage specific manner in the testicular tubules and shows specific binding to ZP3, the sperm receptor in zona pellucida, which is involved in primary sperm-egg binding (Ghosh and Datta, 2003). All these amply demonstrate that HABP1 is an important protein in the whole process of fertilization. Along with this, it has also been observed that HABP1 level in sperm samples of infertile patients reveal a substantial decline, and a direct correlation could be drawn between sperm motility and HABP1 expression (Ghosh et al., 2002). So, this protein could be used to serve as a prognostic marker for male infertility and can also be used in the analysis of testicular biopsies where spermatogenic arrest has occurred. HABP1 pro-protein accumulation has been reported during spermatogenesis, specifically in the pachytene spermatocytes and round spermatids, which suggests that HABP1 might play a role in the meiotic and the
postmeiotic stages of spermatogenesis and that this accumulation might be further regulated by the important phenomenon of proteolysis (Thakur et al, 2006).

HABP1 has been shown to be involved in stabilizing HA crosslinking during COC expansion. HABP1 expressed in rat COCs (having excess HA synthesis) during maturation facilitates the formation of the HA matrix in the extracellular space around the oocyte with cumulus expansion during maturation. The level of HABP1 increases markedly during ovulation after gonadotropin stimulation, and the overexpression was seen in mural granulosa cells, expanding cumulus cells and follicular fluid (Thakur and Datta, 2008).

Studies from our laboratory have shown that HABP1 is a substrate for MAP kinase and phosphorylation of endogenous HABP1 was observed following treatment of J774 cells with PMA. It was also observed that upon mitogenic stimulation, HABP1 translocated to the nucleus. This nuclear translocation was simultaneous with that of ERK, suggesting the requirement of ERK activation for translocating HABP1 into nucleus. These studies established HABP1 as a substrate for ERK and a part of the MAP kinase cascade (Majumdar et al, 2002).

Constitutive expression of HABP1 in normal fibroblast cell line has shown to inhibit its growth, formation of autophagic vacuoles, and induction of apoptosis at 60 hours without media change (Meenakshi et al, 2003). Upon constitutive overexpression of HABP1 in fibroblast cell line F111, HABP1 has been shown to get accumulated in the mitochondria which leads to the generation of reactive oxygen species (ROS), mitochondrial dysfunction and ultimately apoptosis (Roy Chowdhury et al, 2008).

HABP1 expression in Schizosaccharomyces pombe has been shown to induce growth inhibition, morphological abnormalities like elongation, multinucleation and aberrant cell septum formation in several strains of S. pombe, implicating its role in cell cycle
progression and cytokinesis. It was speculated that HABP1 induces morphological changes and modulates the cell cycle by interacting with proteins like CDC 25 through its N-terminal a-helix (Mallick and Datta, 2005).

The expression of HABP1/p32/gC1qR has been shown to be upregulated in cisplatin treated HeLa cells. The number of apoptotic cells reduced significantly if the expression of HABP1/p32/gC1qR was knocked down, using short interfering RNA specific to the HABP1/p32/gC1qR transcript, prior to cisplatin suggesting its possible involvement in the apoptotic process (Kamal and Datta, 2006).

It is intriguing how a single protein can interact with such a diverse array of proteins to carry out distinct functions. It is known that the interactions of biomolecules are generally controlled by the dynamic nature of energetically viable conformational ensembles adopted by them under different conditions. HABP1 has been shown to exist in different oligomeric forms under various in vitro conditions in solution, although the molecule is predominantly trimeric in anaerobic conditions at 150 mM salt concentration. The expanded structure of HABP1 is similar to molten globule-like state under low ionic environment. At a low ionic environment HABP1 exists in an expanded molten globule-like state, which attains a globular conformation above 50 mM of salt concentrations. The gradual increase in ionic strength (150 mM to 1 M) results in a simultaneous decrease in shape and size of the molecule with increased secondary structural content. Neutralization of charges on HABP1 by counter ions seems to promote the transition from disordered to ordered structure.

This global change in the shape of the molecule seems to originate from the different conformations that the molecule adapts under the influence of changes in ionic strength. The asymmetric
charge distribution along the faces of HABP1 may force the molecule to exist in marginally stable, expanded conformations at 10–25 mM ionic strength. This marginally stable expanded conformation attains a compact stable structure at 150–600 mM, which further becomes more compact at higher salt concentration (600 mM to 1 M). The structural plasticity of HABP1 may play an important role in regulating its binding toward different ligands. Under the conditions of 10 mM ionic strength, where HABP1 exists in a loosely held trimeric structure, it fails to bind HA. However, it shows appreciable binding with other reported ligands/interacting molecules, namely Clq and clustered mannose. This fact suggests that binding of HABP1 toward different ligands is governed by its structural state. The binding of HABP1 toward HA shows a drastic change from 0 mM of NaCl (no binding) to 20 mM of NaCl (substantial binding), where HABP1 gains structure to some extent. Further increase in HA binding with increasing salt concentration up to 150 mM suggests the improvement of binding due to the better structural fit of the HABP1 for its ligand HA (Jha et al, 2003). The transitions from loosely held trimeric structure to compact ordered structure might be linked with one of its reported functions as a molecular chaperon.

HABP1 is known to regulate the kinase activity of almost all isoforms of protein kinase C at 50 mM ionic strength under reducing conditions without being their substrate like a molecular chaperon. In this context these structural transitions are important because of the fact that the accessible hydrophobic surface is the key element for chaperon-like action. HABP1 may belong to the group of eukaryote proteins, which remain only partially structured under physiological conditions and execute numerous functions consistent with the physico-chemical microenvironment of the molecule. The asymmetric charge distribution along the faces of HABP1 in association with cations and pH in the molecular environment play critical roles in
maintaining its physical stability and three-dimensional structure at secondary, tertiary, and quaternary levels in solution (Jha et al, 2004). These factors govern its structural transitions from disordered to ordered and regulate its affinity toward HA. The electrostatic repulsion, together with an accessible hydrophobic core of HABP1 and the consequent dynamics because of its intrinsic flexibility imparts a repertoire of ligand recognition specificity and biological functions to this protein.