RAPID HYALURONAN UPTAKE IS ASSOCIATED WITH
ENHANCED MOTILITY AND TRANSFORMATION:
IMPLICATIONS FOR AN INTRACELLULAR MODE OF ACTION

by

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A thesis submitted in conformity with the requirements
for the Degree Master of Science,
Graduate Department of Anatomy and Cell Biology
University of Toronto

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0-612-45877-6
Rapid Hyaluronan Uptake is Associated with Enhanced Motility and Transformation: Implications for an Intracellular Mode of Action

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ABSTRACT

Transformed fibroblasts rapidly internalize Texas Red labeled hyaluronan (TR-HA), and target it to cell nuclei, processes and the perinuclear region. TR-HA uptake is blocked by: excess polymeric HA, HA-dodecasaccharide, colchicine, BrefeldinA, and by digestion of TR-HA with hyaluronidase. Uptake is dose dependent, saturable and strongly enhanced upon cell attachment, monolayer wounding and transformation. TR-HA nuclear targeting requires a minimum of 30 HA-saccharides, suggesting the existence of novel hyaladherins. Enhanced uptake correlates with increased CD44 and RHAMM expression and both CD44 antibodies and RHAMM peptides alter uptake. Interestingly, MEK inhibition also affects uptake. Connexin43 overexpression, regulated by RHAMM expression, enhances HA uptake and targeting to cell processes. In vitro, HA enhances DNaseI-DNA interaction, suggesting nuclear HA’s potential influence on gene expression. Roles for intracellular hyaluronan in cell function are suggested by a correlation of cell process HA targeting with increased motility, after oncogene transformation, monolayer wounding, or phorbol ester treatment.
I would like to express the deepest admiration and thanks to my parents who are a continual source of love and support and to Chad for his love, patience and constant encouragement, and it is to them that I dedicate this thesis.
ACKNOWLEDGMENTS

This thesis would not have been completed without help and support from numerous co-workers and friends.

I would first like to thank my supervisor Dr. Eva Turley, for providing me with the opportunity to do this work, and for her guidance and support throughout this study. I am likewise indebted to our collaborators Dr. Glen Prestwich and Michael Ziebell of the University of Utah for their assistance in developing and analyzing the TR-HA conjugate, Dr. Markku Tammi and Dr. Raija Tammi of the University of Kuopio, Finland for providing me with purified and labelled HA oligosaccharides, Dr. Jim Nagy of the University of Manitoba for providing the connexin43 transfected glioma cells and Bruce Lynn of the University of Manitoba for his patience during our attempts at live uptake, and his instruction on image analysis. I further wish to express my sincere gratitude to my committee members Dr. Michael Opas and Dr. Sean Egan for their time and knowledge.

I am also indebted to all the members of Dr. Turley’s research group, past and present. My thanks extend especially to Frouz Paiwand and Rene Harrison who have been such great benchmates, confidants and friends during this formative experience. I would also like to express my thanks to Jingbo A, Shiwen Zhang, Arian Khandani, Jie Lu, FuSheng Wang, Joycelyn Entwistle and Judy Edwards for their support as both friends and colleagues.

This study was performed at The Hospital for Sick Children in the Department of Cardiovascular Research under the aegis of the Department of Anatomy and Cell Biology of the University of Toronto during the years 1997 – 1999 with financial support from an MRC studentship and Hyal Pharmaceutical Corporation.
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LIST OF ABBREVIATIONS

aa                 Amino acid(s)
BSA                Bovine serum albumen
CD44s              Standard CD44 isoform
CD44v              Variant CD44 isoform
cDNA               Complimentary DNA
CPC                Cetylpyridinium chloride
CS                 Chondroitin sulphate
Da                 Dalton
DMEM               Dulbecco’s modified eagle’s medium
DMSO               Dimethyl sulfoxide
ECM                Extracellular matrix
EDTA               Ethylenediaminetetraacetate
ERK                Extracellular signal regulated protein kinase
FACS               Fluorescence activated cell sorter (used in flow cytometry)
FCS                Fetal calf serum
FITC               Fluorescein isothiocyanate
GAG                Glycosaminoglycan
GPI                Glycosylphosphatidyl inositol
HA                 Hyaluronan, hyaluronic acid, hyaluronate
HA#                Hyaluronic acid oligosaccharide of “#” monosaccharide units
HABP               Hyaluronan binding protein(s)
HARLEC             Hyaluronan receptor for liver endothelial cells
HAS                Hyaluronan synthase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HS</td>
<td>heparin sulphate</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase(s) or 1000bp</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton or 1000da</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen associated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen associated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>rER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RHAMM</td>
<td>Receptor for hyaluronan mediated motility</td>
</tr>
<tr>
<td>RHAMMΔ1-5</td>
<td>RHAMM exons 1-5 deleted (old nomenclature RHAMMv4)</td>
</tr>
<tr>
<td>RHAMM exon9</td>
<td>old nomenclature = exon4</td>
</tr>
<tr>
<td>RITC</td>
<td>Rhodamine isothiocyanate</td>
</tr>
<tr>
<td>TR</td>
<td>Texas red</td>
</tr>
<tr>
<td>TR-HA</td>
<td>Texas red - hyaluronan conjugate</td>
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CHAPTER I

INTRODUCTION
Hyaluronan (HA) was first described as a polysaccharide isolated from the vitreous humour in 1934 (Meyer et al. 1934). It contained uronic acid and Meyer named the polysaccharide hyaluronic acid from *hyalos* (glassy, vitreous) and uronic acid. However at physiological pH all carboxyl groups on the uronic acid residues are dissociated and the polysaccharide has therefore been named sodium hyaluronate when sodium is the counter ion. It is often difficult to specify the counter ion, for example in a tissue, and Balzacs *et al.* (1989) therefore suggested that the name hyaluronan (HA) should be used.

Hyaluronan is a molecule of paradoxes and contrasts. On the one hand, it is a homopolymer of simple disaccharide units endlessly repeated, present in some tissues (such as the cockscomb and vitreous humor) in large amounts and performing very simple mechanical jobs as a space filler or lubricant (in synovial joints). On the other hand, HA takes part in cell surface phenomena of great specificity when used at very low dilution in the presence of specific receptors.

Interest in HA has increased dramatically since 1980 when major clinical applications in ophthalmology and in the treatment of joint disease were introduced. Since then, HA has found multiple applications in drug delivery and surgery. HA has been found to enhance absorption of drugs and proteins through mucus tissues (Morimoto *et al.* 1991). When combined with HA, the efficacy of many agents (such as anti-inflammatory drugs) are strengthened (Goa and Benfield 1996; Miller *et al.* 1997). HA has found important applications in the field of viscosurgery, viscosupplementation and wound healing (Davidson *et al.* 1991; Juhlin *et al.* 1997).
In reproductive medicine, HA is used to improve retention of the mobility of cryopreserved and thawed spermatozoa (Sbracia et al. 1997).

HA is a ubiquitous compound and there is no known genetic disease in which HA is not synthesized. HA may therefore be of fundamental importance in the animal organism and mutations causing defects in HA synthesis may be lethal. HA is found in virtually every species in the animal kingdom, in every tissue in the human body as well as the capsule of certain microorganisms. Moreover the HA repeating disaccharide is identical in all tissues and species and consequently HA is never recognized as immunologically foreign (Fraser and Laurent 1997).

1.1. Biochemical Properties of HA

1.1.1. Structure

The polysaccharide HA is a negatively charged linear polymer with the structure 1-β-4 D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4) (Figure 1.1.) that can have a molecular mass of up to several million daltons. The relative molecular weight of HA varies dramatically depending upon its source. For example, the HA isolated from human synovial fluid is about 7000kDa, from human blood serum it is approximately 200kDa, and from human urine it may be as small as 4-12kDa (Fraser and Laurent 1996).

Studies on conformation of HA by X-ray diffraction and spectroscopy indicate that the molecule can take up helical conformations stabilized by hydrogen bonds that run in parallel with the chain axis (Scott and Tigwell 1978; Scott 1989). The polymer consequently takes up a stiffened helical configuration which gives the
molecule an overall expanded coil structure in solution. High molecular weight (HMW) forms of HA can have a diameter in the order of 500nm (Laurent 1989).

Along the HA molecule there are clusters of adjacent CH groups forming patches of a highly hydrophobic character. These hydrophobic patches are repeated at regular intervals on alternate sides of the molecule (Scott 1989). The alternation of clustered hydrophobic and hydrophilic segments in HA facilitates its interactions with biological membranes, hydrophobic proteins (e.g.: link protein) and also in HA self-aggregation (Scott 1989; Scott et al. 1992).

Intramolecular interactions of HA in solution dampen interactions with other molecules, and promote its neutral space filling and molecule passing roles (Scott 1998). HA networks are more easily formed by HMW HA. Welsh et al. (1980) showed that an admixture of LMW HA dramatically alter the rheologic properties of HMW HA which these authors tentatively suggest is an aggregation breaking effect.
1.1.2. Family of glycosaminoglycans

HA is one of a family of connective tissue heteropolysaccharides containing hexosamine, collectively called glycosaminoglycans (GAG). Other prominent GAGs include heparin sulphate (HS), heparin, chondroitin sulphate (CS), dermatan sulphate (DS), and keratin sulphate (reviewed in Sames 1994; Soldani and Romagnoli 1991).

HA is the simplest GAG, and is structurally unique in at least three respects. Firstly it is the only GAG which does not exist covalently linked to a core protein (i.e. as proteoglycan). Secondly it has no sulphate content. Thirdly, its linear polymers can reach a much greater relative molecular mass. For example, HA in joint fluid can be up to several million daltons in size, while proteoglycans are commonly less than 30kDa (Fraser 1994).

1.1.3. Synthesis of HA

Eukaryotic HA synthases are localized to the inner leaflet of the plasma membrane where they extrude HA directly into the extracellular space (Prehm 1989; Weigel et al. 1997). HA chains are elongated at the reducing end by alternate transfer of sugar residues from the substrates UDP-glucuronic acid and UDP-N-acetylglucosamine (Prehm 1983a,b). This occurs inside the plasma membrane, and the chain (with the non-reducing end ahead) is extruded into the pericellular space, while the growing HA chain remains bound to the enzyme (Prehm 1984; Heldin et al. 1993). The mechanism for release of HA extracellularly is not known. HA
chain release typically occurs after >20,000 monosaccharides >4 x 10^6Da) have been assembled. HA extrusion to the extracellular milieu is presently viewed as a process requiring the enzyme to form a pore or channel-like pocket to guide the hydrophilic HA chain through the lipid layer (see Figure I.2). Extrusion of the growing chain into the extracellular space allows unconstrained growth of the HA polymer. This mechanism of synthesis and release is in contrast to the smaller GAGs which are synthesized in the Golgi compartment and released by exocytosis (Hascall et al. 1998).

Figure I.2. Extrusion model for HA synthesis (modified from Prehm et al. 1983)
There are three HA synthase (HAS) genes in eukaryotes (Itano and Kimata 1996; Spicer et al. 1996; Fulop et al. 1997; Spicer et al. 1997a). In contrast with the low levels of identity between HAS within a species (HAS1/HAS2, 55% identity; HAS1/HAS3, 57% identity; HAS2/HAS3, 71% identity), the sequence between individual human and mouse HAS orthologues is highly conserved (identity 96%) (Spicer et al. 1997b; Weigel et al. 1997). These sequence characteristics suggest a conservation of the functionally important residues of HAS during evolution and the differences of action of the three HAS proteins (Spicer et al. 1997b; Weigel et al. 1997). Localization of the three HAS genes on different chromosomes and the appearance of HA throughout the vertebrate class suggest that this gene family is ancient and the isozymes appeared by duplication early in the evolution of vertebrates (Spicer et al. 1997b). Recently, Spicer and Nguyen (1999) demonstrated that HAS-2 deficient embryos were deficient in HA and had severe developmental defects, including a failure to form the endocardial cushions of the heart (Spicer and Nguyen 1999). HAS-1 and HAS-3 mutant mice have yet to be reported.

The HAS protein has four transmembrane domains and one or more amphipathic helices that may be partially embedded in the membrane (Watanbe et al. 1996; Spicer et al. 1997a). Translocation of an HA chain across the bilayer by such a small protein is difficult to envision. It has recently been shown that streptococcal HA synthases do not oligomerize, but require cardiolipin molecules for maximal activity and stability (Tlapak-Simmons et al. 1998). Thus it has been
proposed that a collection of cardiolipin molecules may function with HAS proteins to create or maintain a pore-like structure (Tlapak-Simmons et al. 1998).

It seems likely that most cells can synthesize HA at some point in their natural life cycle and most of all during periods of rapid growth (Fraser and Laurent 1989; Fraser et al. 1994). The activity of hyaluronan synthase correlates with a variety of cellular parameters including cell growth, transformation and differentiation (Prehm et al. 1980), metastasis (Toole et al. 1979), cell migration (Chen et al. 1989; Schor et al. 1989; Ellis et al. 1992; Ellis et al. 1996) and mitosis (Moscatelli and Rubin 1975; Brecht et al. 1986). HA synthesis has been proposed to increase during active division and decline at confluence, with fluctuations during the cell cycle peaking at mitosis (Brecht et al. 1986; Matuoka et al. 1987; Yoneda et al. 1988). HA extrusion onto the cell surface just prior to mitosis may create a hydrated microenvironment which promotes partial detachment and rounding of dividing cells (Brecht et al. 1986; Hall et al. 1994; Koochekpour et al. 1995).

HA synthesis is responsive to environmental factors such as hormones (glucocorticoids, thyroid and sex hormones), vitamins (vitamin C and retinoic acid), growth factors (EGF, PDGF, TGFβ, FGF, IGF-1) and cytokines (IL-1, IL-6 and TNF) which can directly activate HA synthesis (Heldin et al. 1989; Honda et al. 1989; Sampson et al. 1992; Tammi et al. 1994; Ellis and Schor 1995; Tirone et al. 1997). Many of these factors, especially the growth factors, can act in autocrine or paracrine modes, and are frequently related to matrix changes in neoplasia and inflammation. Furthermore, cycloheximide and protein kinase inhibitors have been
shown to inhibit synthesis, indicating that transcription as well as phosphorylation is required for activation (Klewes and Prehm 1994).

1.1.4. HA degradation

Circulating levels of HA in human serum are normally ~30-40ng/ml (Laurent et al. 1995). This is due to a rapid turnover of HA in the bloodstream that results in a mean half-life for HA of only 2.5 to 5.0 minutes in healthy adults (Fraser and Laurent 1989; Laurent et al. 1996). Of the 10-100mg/day entering the bloodstream the majority (about 90%) goes to the liver, while the remainder goes to the kidney (Laurent et al. 1987) and spleen (Bentsen et al. 1986).

Studies with separated liver cells (Smedsrod et al. 1984) have shown that sinusoidal liver endothelial cells are primarily responsible for HA uptake. Metabolic degradation of HA is principally intracellular and relies on receptor mediated uptake (Eriksson et al. 1983; Smedsrod et al. 1984; Fraser et al. 1985; Laurent and Fraser 1986; Raja et al. 1988). Endocytic degradation in the liver is thought to be mediated by HARLEC an HA receptor complex of liver endothelial cells which has been isolated from rat sinusoidal liver endothelial cells as. This complex contains three proteins of 100, 166, and 175kDa proteins (Gustafson 1996; Yannariello-Brown et al. 1996). HARLEC also recognizes chondroitin sulphate (CS) and dermatan sulphate (DS). Thus CS given intravenously into rats prior to intravenous HA can inhibit the uptake of HA by the liver (Gustafson and Bjorkman 1997).
Abnormal HA accumulation has been noted in a variety of fibrotic disorders (Whiteside and Buckingham 1989). Examples include scleroderma (Freitas et al. 1996), hypertrophic scarring (Savage and Swann 1985) and fibrosis of liver and lung (Frebourg et al. 1986; Bjermer et al. 1989). Because HA accumulation in fibrotic disorders, understanding how HA is removed from connective tissues has long been of interest (Fraser and Laurent 1989; Roden et al. 1989). Elevation of serum HA levels also occurs in rheumatoid arthritis, liver cirrhosis and various malignancies (Engstrom-Laurent 1989; Coppes 1993; Laurent et al. 1996).

HA is turned over locally within cells and tissues, particularly during embryogenesis, tumourigenesis and upon injury. This process occurs in embryonic tissues during development and also in remodeling tissues (Fraser and Laurent 1989). Numerous cell types including human synovial cells (Truppe et al. 1977), SV40-transformed 3T3 fibroblasts (Culty et al., 1992), embryonic myocardial cells (Bernanke and Orkin 1984), macrophages (Culty et al. 1992; Gustafson and Forsberg 1991) and human breast cancer cell lines (Culty et al. 1994) have been shown to degrade HA.

Studies both on cultured cells and on developing tissues suggest that the mechanism of HA degradation may require internalization/endocytosis before degradation. Degradation of HA is thought to occur in lysosomes because the hyaluronidase is an acidic pH requiring enzyme (Orkin and Toole 1980; Orkin et al. 1982; Bernanke and Orkin 1984). Indeed, inhibitors of lysosomal acidification such as ammonium chloride and chloroquine block HA degradation (Alston-Smith et al. 1992; Culty et al. 1992).
HA degradation in macrophages fibroblasts (Culty et al. 1992), chondrocytes (Hua et al. 1993) and several tumour cell lines (Culty et al. 1994), requires CD44. Underhill and coworkers (Underhill et al. 1993; Pavasant et al. 1994) have shown that CD44 mediated removal of HA occurs at several stages of development. For instance, during lung development, macrophages expressing CD44 increase in number in parallel with a marked decrease in interstitial HA concentration; injection of a blocking antibody against CD44 into newborn mice was shown to cause an increase in lung HA relative to controls (Underhill et al. 1993). In embryonic skin, newly forming hair follicles are associated with condensing mesoderm; this process of mesodermal condensation is also associated with lung HA degradation and elevation of CD44 (Underhill et al. 1993). During bone development, lacunae surrounding hypertrophic chondrocytes are highly enriched in HA (Pavasant et al. 1994) and swelling pressure exerted by this HA contributes to expansion of lacunae as bone growth occurs (Pavasant et al. 1996). Subsequently, in the zone of erosion, the HA within these lacunae has been proposed to be removed via CD44-mediated endocytosis (Pavasant et al. 1994). Upon injury, metabolism of HA has been observed to modify the outcome of a wound (Burns et al. 1997). Likewise, in neoplasia HA deposition exceeds degradation (Auvinen et al. 1997; Ropponen et al. 1998).

Under specific conditions HA degradation may also occur extracellularly. For example, HA within the cartilage ECM becomes partially depolymerized, most likely due to the action of free radicals (Baker et al. 1989; Ng et al. 1992). Free radicals can be generated by ionizing radiation and are also present at wound sites
(Forschi et al. 1990). Such initial depolymerization of HMW HA may an initial step which facilitates HA catabolism. For instance, McGuire et al. (1987) demonstrated that myocardial cells bind, internalize and degrade partially depolymerized HA more efficiently than HMW native HA. Thus partial extracellular together with endocytosis results in the complete degradation of the HA (Hua et al. 1993).

1.2. Functions of HA

Through its complex interactions with matrix components and cells, HA has multiple roles in biology. These biological roles range from mechanical functions in the extracellular matrix to regulation of cellular behavior. HA affects many biologic properties including wound healing (Chen and Abatangelo 1999), angiogenesis (Rooney et al. 1995), embryonic morphogenesis (Toole 1997), tumour metastasis (Bourguignon et al. 1997), inflammation (McKee et al. 1996), cell adhesion (Hall et al. 1994; Klein et al. 1996) cell migration (Chen et al. 1989; Melrose et al. 1996) and proliferation (Wiig et al. 1996; Bertrand et al. 1997).

1.2.1. Physicochemical functions of HA

HA function has long been attributed to its physical and chemical properties and its interaction with other macromolecules. Because of its water attracting properties, HA plays a role in the osmotic homeostasis of the extracellular space (Comper and Laurent 1978; Comper and Zamparo 1990). HA meshworks also exhibit macromolecular exclusion and thus can regulate the distribution and transport
of plasma proteins in the tissues such that small molecules move freely in the network, while larger particles become immobilized (Scott et al. 1989). This steric exclusion property of HA are thought to serve to protect cells by hindering the approach of particles that would otherwise cause damage (Presti and Scott 1989) and serve as a barrier against the spread of infectious agents (Laurent and Fraser 1992). The rheological properties of HA solutions showing both viscoelasticity and shear dependence have been related to its lubricating functions in joints and other tissues (Balzacs and Delinger 1993).

1.2.2. HA binding proteins: the hyaladherins

Although HA is not covalently linked to proteins like other GAGs, several extracellular intracellular and plasma membrane proteins bind to it with high affinity (Figure I.3.). These HA binding proteins, or hyaladherins, are widely distributed in the body and have diverse functions (Toole 1997). Extracellular and cell surface hyaladherins are important in modifying matrix assembly, cell-matrix and cell-cell interactions. Besides their extracellular structural and organizational functions, of particular interest is the discovery of hyaladherins that exist within cells.

Cellular hyaladherins can be grouped into at least two classes of proteins. CD44 is a prototype of the type I transmembrane HA receptor. This protein is predominantly present at the cell surface but can be shed into the extracellular space. CD44 binds to HA via a complex site known as the link module (Kohda et al. 1996). RHAMM is a prototype of cell-associated hyaladherins that occur at
Figure I.3. Hyaluronan binding proteins – the Hyaladherins (modified from Paiwand et al. 1999)

multiple cellular loci, including the cell surface, cytoplasm and nucleus. This group, including RHAMM, cdc37, and p68 are characterized by a lack transmembrane domain, signal sequence or link module. HA binds to this class of hyaladherins through short, basic amino acid motifs (Yang et al. 1994; Kohda et al. 1996).

I.2.2.1. CD44

CD44 was first described as a cell surface molecule of T-lymphocytes, granulocytes, and cortical thymocytes (Dalchau et al. 1980). This protein was then rediscovered as the phagocytic glycoprotein 1 (Pgp-1) (Mackay et al. 1988), and
GP90 Hermes (Goldstein et al. 1989). CD44 is now known to be a widely-expressed receptor for HA (Underhill et al. 1987; Aruffo et al. 1990).

CD44 consists of an external domain, highly conserved transmembrane domains and cytoplasmic domains (Naor et al. 1997). There are 20 known isoforms of CD44 which are created through alternative splicing of 10 variant exons (reviewed in Ponta et al. 1998). The smallest isoform, known as CD44s, lacks all ten variant exons. CD44s is the most abundant isoform in vivo. Additional post-translational modifications of CD44 include phosphorylation and palmitoylation in the cytoplasmic domain, N- and O- linked glycosylation and addition of GAGs in the external domain (Lesley et al. 1993). Dimerization of CD44 has been implicated in enhanced binding of HA can occur naturally with CD44v4v7 (Sleeman et al. 1997), can be induced experimentally with certain divalent antibodies to CD44 (Lesley et al. 1993), or can be induced by constructing CD44 with cysteine in the intramembranous domain that forms disulfide cross links (Perschl et al. 1995).

Interestingly, not all CD44-expressing cells are able to bind HA. This property can be acquired or can occur transiently (Hyman et al. 1991). The ability of HA to bind to CD44 is regulated by both protein conformation, rather like integrin activation, and by glycosylation patterns. Thus, CD44 can be stimulated to bind HA by phorbol esters, anti-CD44 antibodies, or deglycosylation (Sherman et al. 1994). Blocking anti-CD44 mAbs studies suggest that topography of the CD44 epitopes and their orientation toward the HA binding site determine the ability of antibodies to interfere with HA binding (Lesley et al. 1993; Zheng et al. 1995).
Clustering of CD44 proteins, which is dependent upon cytoskeletal proteins, also seems important to its ability to bind HA (Lokeshwar et al. 1994). Certain cells, including some B and T cell lines, appear constitutively able to bind HA. However, further studies are required to define the molecular mechanisms that result in CD44/HA interactions as well as to assess the impact that these interactions have on cell behaviour.

CD44 is a multifunctional receptor involved in cell-cell and cell-ECM adhesion, i.e. cell motility, trafficking, lymph node homing, lymphocyte activation, presentation of chemokines and growth factors to traveling cells, and transmission of these growth signals (Lesley et al. 1993). As well, CD44 participates in the endocytic uptake and intracellular degradation of HA (Culty et al. 1992; Hua et al. 1993), and transmission of signals mediating hematopoiesis and apoptosis (Ayrolidi et al. 1995; Henke et al. 1996) that are relevant to tumour progression and wound repair. CD44 is constitutively expressed in regions of active cell growth (Mackay et al. 1994).

CD44 expressed on tumour cells and host tissue stromal hyaluronan can enhance growth and invasiveness of certain tumours. Disruption of CD44-HA interaction by soluble recombinant CD44 has been recently shown to inhibit tumour formation by lymphoma and melanoma cells transfected with CD44 (Zeng et al. 1998). CD44 is expressed on many types of cancer cells, such as malignant gliomas (Koochekpour et al. 1995) human breast cancer cells (Culty et al. 1994), colon cancer cells (Ropponen et al. 1998) ovarian cancer cells (Catterall et al. 1997) and basal cell carcinomas (Baum et al. 1996). A diverse set of CD44 receptor isoforms
is found on breast cancer cell lines, not only among different cell lines but also within individual cell lines (Culty et al. 1994). All cell lines investigated can bind HA. Interestingly HA binding and CD44 expression is positively correlated with invasive potential of breast ovarian and colon cancer cell lines (Culty et al. 1994; Catterall et al. 1997; Ropponen et al. 1998). Disruption of the CD44 function on mammary carcinoma cells induces apoptosis (Yu et al. 1997). In basal cell carcinoma expression of CD44s is found to be down regulated, while the expression of the CD44v6 isoform is increased (Baum et al. 1996). This variant of the receptor seems to be linked to cellular functions, while the absence of the CD44s accounted for the noninvasive character of this type of tumour.

The interaction of HA with the CD44 receptor is also involved in inflammation (Lesley et al. 1997), and liver cirrhosis (Rockey et al. 1998). Synovial fibroblasts isolated from patients with osteo- or rheumatoid arthritis were shown to exhibit complex splicing variations in their CD44 expression (Croft et al. 1997). Noninflamed tissue samples showed no such splicing events. It was found that rheumatoid tissue contained higher levels of expressed CD44, while osteoarthritic tissue samples showed greater variety in their CD44 expression. The interaction of HA fragments with the CD44 receptor on macrophages in inflamed tissues can induce the expression of inflammatory genes of the chemokine family (IL-1β, TNF-α and IGF-1; Noble et al. 1993; McKee et al. 1996) and nitric-oxide synthase through a nuclear factor kappa beta (NFκB) dependent mechanism (Noble et al. 1996; McKee et al. 1997). A similar effect of HA fragments on nitric oxide synthase was observed in liver endothelial cells and Kupffer cells (Rockey et al.
It is proposed that the various interactions of CD44 with HA fragments might sustain or increase the process of inflammation.

An extensive body of evidence exists suggesting that CD44 is involved in angiogenesis (Goshen et al. 1996; Trochon et al. 1996; Deed et al. 1997; Griffioen et al. 1997; Ozer et al. 1997). Endothelial cells of solid tumour vasculature have been found to display an enhanced expression of CD44, an upregulation that could be induced by angiogenic factors such as FGF and VEGF (Griffioen et al. 1997). Further, it has been demonstrated that LMW oligosaccharides HA (HA8-50) can induce angiogenesis (West et al. 1985), while HMW HA in the ECM has been shown to inhibit angiogenesis (Dvorak et al. 1987; West and Kumar 1989). Both should bind to cell surface receptors but how they have different effects on cells in comparison to HA oligosaccharides is unclear.

CD44 interactions with angiogenic oligosaccharides of HA have also been found to upregulate the expression of early-response genes such as c-fos, c-jun, junB, Krox-20 or Krox-24 in endothelial cells (Deed et al. 1997). The products of these genes are known to act as transcription factors for genes encoding extracellular proteinases, which are additional factors that promote endothelial cell migration and thus angiogenesis.

1.2.2.2. RHAMM and related proteins

RHAMM is a member of a group of cell-associated hyaladherins including p68 and cdc37 that localize to several subcellular loci and that perform multiple functions in regulating cell motility and cell cycle (Turley et al. 1982, 1994;
Grammatikakis et al. 1995; Deb and Datta 1996; Mohapatra et al. 1996; Toole et al. 1997; Wang et al. 1998). For instance, cell surface forms of RHAMM are transiently expressed in most cells but are nevertheless key to regulating cell motility as determined by antibody blocking experiments (Samuel et al. 1993; Hall et al. 1994, 1995, Pilarski et al. 1994; Turley et al. 1994; Savani et al. 1995a, 1995b; Nagy et al. 1995; Masellis-Smith et al. 1996; Delpech et al. 1997; Zhang et al. 1998). Intracellular forms of this class of hyaladherins, including RHAMM, bind to and chaperone signaling molecules involved in regulating cell cycle and cell motility (Grammatikakis et al. 1995; Mohapatra et al. 1996; Kimura et al. 1997). These types of hyaladherins may also perform functions within the nucleus. Such hyaladherins typically lack a link module for binding HA but rather utilize short sequences encoding basic amino acid motifs (Yang et al. 1994) which are required for cell motility and proliferation (Samuel et al. 1993; Sherman et al. 1994). Even though they are present on the cell surface (Samuel et al. 1993; Sherman et al. 1994; Grammatikakis et al. 1995; Kimura et al. 1997; Crainie et al. 1999) this class of hyaladherins is also characterized by an absence of both signal sequences and transmembrane domains. Therefore, the molecular basis for their subcellular distribution is not yet clear. Based upon their modular and dynamic subcellular location and the different mechanisms by which they bind to HA, these proteins likely regulate cell motility and cell cycle in a manner that is fundamentally distinct from the more well characterized HA receptor, CD44.

Surface RHAMM regulates signals generated by both HA (Turley 1982; Entwistle et al. 1996; Toole et al. 1997) and growth factors such as PDGF (Zhang et
al. 1998) and TGFβ (Samuel et al. 1993). The ability of HA to signal motility via RHAMM implies that the HA binding domains are also necessary for signal transduction. Interestingly, the HA binding domains of intracellular forms of RHAMM are required for activation of erk kinase by mutant active ras and by growth factors that activate ras, such as PDGF (Zhang et al. 1998).

Signals from HA/cell surface RHAMM interactions are associated with activation of src (Hall et al. 1996), focal adhesion turnover (Hall et al. 1994) and a reduction in the tyrosine phosphorylation focal adhesion kinase (FAK) (Hall et al. 1995). RHAMM-mediated activation of src is required for HA/RHAMM-regulated fibroblast motility and both locomotion of these cells and activation of src by HA can be blocked with anti- RHAMM antibodies (Hall et al. 1996). However, cells transfected with either a constitutively active form of src or v-src no longer require cell surface RHAMM (as detected by antibody blocking) for signaling motility, indicating that src is downstream of cell surface RHAMM. Nevertheless, v-src-induced disassembly of focal adhesions cannot occur in the absence of cellular expression of RHAMM (Hall et al. 1996). This, although not confirmed experimentally, is most easily interpreted by proposing that intracellular isoforms of RHAMM are required for src-generated effects on the cytoskeleton and is consistent with the ability of a RHAMM isoform to co-immunoprecipitate with src (Hall et al. 1996).

In addition to its effect on src signaling, RHAMM isoforms encoding exon 9, appear under certain conditions (e.g. subconfluence) to control the erk kinase cascade through ras (Zhang et al. 1998). Mutations of domains of intracellular
RHAMM isoforms block signaling through ras (Hall et al. 1995) and activation of erk (Zhang et al. 1998). Specifically, RHAMMΔ1-5 (73 kD) interacts specifically with erk 1 kinase and its upstream activator MEK in transformed cells (Zhang et al. 1998; Paiwand et al. in preparation) and its overexpression constitutively activates this cascade. Thus, at least one form of RHAMM, in a manner possibly analogous to cdc37 (Kimura et al. 1997, Silverstein et al. 1998), directly associates with kinases that regulate transformation, proliferation and motility (Zhang et al. 1998). Importantly, a RHAMM isoform of the same molecular weight predicted by the RHAMMΔ1-5 cDNA (e.g. 70-73 kDa) is uniquely upregulated after wounding of smooth muscle cell monolayers (Savani et al. 1995). Furthermore, manipulation of RHAMMΔ1-5 expression or function alters the ability of PDGF, a key growth factor in response-to-injury processes, to activate signaling cascades (Zhang et al. 1998) and modify actin assembly (Hall et al. 1994, Chang et al. 1997). The role of erk in these functions remains to be investigated.

A recently characterized intracellular HA binding protein encodes a 29.3kDa chicken homologue protein homologous to cdc37, an essential cell cycle regulatory factor previously characterized genetically in yeast and Drosophila (Grammatikakis et al. 1995). These findings suggest a role for HA in cell division control. Cdc37 is associated with Cdk4 through HSP90, further suggesting that cdc37 may regulate the mammalian cell cycle through direct effect on Cdk4 (and thus the G1S boundary) (Dai et al. 1996). Further, raf and src are known to directly interact with HSP90/p50-cdc37 complexes (Perdew et al. 1997).
A recently characterized 34kDa HABP is a new member of the cellular hyaladherins as demonstrated in a wide variety of cell lines. This protein has been found to form a homodimer of 68kDa (p68) that binds specifically to HA (Deb and Datta 1996). P68 has been shown to be highly phosphorylated in transformed fibroblasts compared to normal fibroblasts, and phosphorylation was enhanced in the presence of HA, PMA and calyculin-A (Rao et al. 1997). The phosphorylated form of p68 is shown on the cell surface and can be detected in serum free medium (Rao et al. 1997). P68 has a 100% homology with gClq, the complement protein (Ghebrehiwet et al. 1994) and has been demonstrated to be the human counterpart of murine YL2 (Luo et al. 1994). Thus it has been proposed to modulate the function of Rev which is expressed at the early stage of HIV1 and act as viral transactivator for the replication of HIV1 (Das et al. 1997). Interestingly, the cDNA sequence of p68 has complete homology with the cDNA sequence of a protein P-32, co-purified with the human pre-mRNA splicing factor SF2 (Krainer et al. 1991).

I.2.2.3. Hyaluronan and tumourigenesis

It has been clear for years that the HA content of tumours is often elevated (Knudson et al. 1989), which is consistent with the raised concentrations of HA in serum of cancer patients (Kumar et al. 1989). Although increased HA synthesis is not a universal characteristic of tumours, there seems to be an overall tendency for transformed cells to exhibit higher levels of HA production (Kimata et al. 1983; Turley and Tretiak 1985).
In cell culture many cell types have reduced capacities to synthesize HA even when derived from tumours enriched in HA (reviewed in Knudson et al. 1989). These can nevertheless stimulate HA synthesis by normal fibroblasts. Production of tumour associated HA occurs via tumour-stromal cell interactions. Also some human tumour cells also possess unoccupied high affinity cell surface binding sites for HA which may allow tumour cells to interact directly with HA enriched ECM. This may allow them to use HA as a support for adhesion and locomotion and guide them into surrounding stroma.

Takeuchi et al. (1976) reported that the HA content of malignant breast lesions was higher than that of normal tissue. Bertrand et al. (1992) confirmed the finding and extended it by showing that the increase was more marked in the peripheral invasive areas of the tumour than in the central parts. Histochemical staining localized HA over-expression in the malignant stroma, whereas the tumour epithelium was HA negative (de la Torre et al. 1993). Further, Auvinen et al. (1997) have demonstrated HA expression was restricted to the stromal connective tissue in benign lesions, while in malignant breast tumours the intensity of stromal HA staining was significantly stronger and was also detected in the cell membranes and cytoplasm of adenocarcinoma cells. Victor et al. (1999) recently showed that the passage of human breast cancer cells from the primary state to the metastatic state was characterized by a dramatic increase of HA and hyaluronidase production and expression of HA-binding sites at the invasion areas. Thus, both synthesis and degradation of HA presumably support tumour invasion and growth.
It has recently been shown that invasive breast adenocarcinoma has a significantly higher level of hyaluronidase activity, correlating with its invasive potential (Madan et al. 1999). This hyaluronidase can disrupt basement membrane integrity and produce an angiogenic response that has been implicated in tumour invasiveness and metastasis (Madan et al. 1999). In contrast, HYAL1, a widely expressed hyaluronidase in somatic tissues (Frost et al. 1997) has been proposed as a candidate tumour suppressor gene as it is found at the site of a common tumour suppressor locus (Csoka et al. 1998). This is a locus frequently deleted either as a loss of heterozygocity or homozygous deletion in a number of human malignancies, particularly tobacco-related lesions of the mouth, head and neck, and of the lung (Killary et al. 1992; Buchhagen 1996). HYAL-1 activity is absent or strongly suppressed in squamous cell carcinoma cell lines compared to normal keratinocyte controls (Frost et al. in preparation).

In a recent study, HA intensity was shown to be stronger in high grade colorectal tumours (Ropponen et al. 1998) and to be prognostic of poor outcome. The abnormal expression of HA in the neoplastic colon epithelial cells is suggested to provide a distinct advantage for invasive growth and metastasis.

Itano et al. (1999) have recently implicated HA in tumourigenesis through selection of mouse mammary carcinoma cell lines mutant deficient in HA. These mutants show loss of HA production and loss of pericellular HA coats, and more important, decreased metastatic ability. Further, transfection with the HA synthase HAS-1 can rescue these mutants (Itano et al. 1999). In a related study, human HT1080 cells were transfected with HAS2, which caused the cells to grow more
rapidly and produce tumours that were 2-4 times larger than control (Kosaki et al. 1999), providing more direct evidence for HA’s role in tumorigenicity.

1.2.2.4. Hyaluronan and wound healing

As wound healing progresses in vivo there are changes in the composition of the extracellular matrix. Initially, a wound contains a fibrin-rich matrix, followed by a HA-rich matrix (Bertolami and Donoff 1978). HA is then removed by hyaluronidases and then there is a deposition of a fine soft temporary reticular collagen, called Type III, which is in turn replaced by fibers of Type I collagen. The latter is the hallmark of a mature scar. In fetal wounds there is no scarring. It is tempting to speculate that the high concentrations of HA which persist in fetal wounds (Longaker et al. 1989; 1990; 1991; Mast et al. 1992) may in part underlie scar free healing.

HA also functions in wound repair as a regulator of cell motility. This may be mediated through both its physicochemical properties as well as through direct interactions with cells. In the former, HA appears to provide an open, hydrated matrix that permits cell migration (Toole et al. 1997), whereas in the latter, HA:cell interactions via cell surface hyaladherins actively promote migration. RHAMM in particular forms links with several protein kinases associated with cell locomotion, for e.g., ERK, p125Fak and pp60c-src (Hall et al. 1994; Hall et al. 1996; Wang et al. 1998). Kobayashi and Terao (1997) have shown a dose-dependent increase of the proinflammatory cytokines TNF-α, IL-1β and IL-8 production by human uterine fibroblasts at HA concentrations of 10µg/ml to 1mg/ml via a CD44 mediated
mechanism, which may also contribute to cell motility as well as impact on the progress of wound healing.

Consistent with the model that scarless wound healing is due in part to HA, many reports have attested to the ability of exogenous HA to produce beneficial wound healing outcome (reviewed in Chen and Abatangelo 1999). In animal experiments, topically applied HA has been shown to accelerate skin wound healing in rats (Abatangelo et al. 1983; Cabera et al. 1995) and hamsters (King et al. 1991), and to enhance repair of tympanic membranes (Hellstrom and Laurent 1987), and corneal epithelial wounds (Nakamura et al. 1992). In chronic wounds such as venous leg ulcers, HA application has been shown to promote healing (Falanga et al. 1996). These results and the observation that intrauterine treatment of fetal wounds with hyaluronidase converted the healing from a fetal type to the scarring model observed ex utero (Mast et al. 1992; West et al. 1997) are consistent with a persistence of HA as one necessary feature for scarless healing to take place.

1.3. Rationale of this study

In addition to its role in the ECM and at cell surfaces, evidence is growing that HA is also present in the cytoplasm and nuclei of cells in a number of tissues in vivo (Margolis et al. 1976; Furukawa and Terayama 1977; 1979; Londono and Bendayan 1988; Ripellino et al. 1989; Kan 1990; Eggli and Graber 1995). Furthermore, recent studies have shown that there are a number of intracellular hyaladherins that may be important in regulation of the cell cycle or in gene transcription (Grammatikakis et al. 1995; Deb and Datta 1996; Hofmann et al.)
1998; Zhang et al. 1998). For example, intracellular forms of the hyaluronan receptor, RHAMM, have been shown to regulate erk kinase activity (Hofmann et al. 1998; Zhang et al. 1998), and a vertebrate homologue of the cell cycle control protein cdc37 was recently cloned and found to bind HA (Grammatikakis et al. 1995). Thus we postulate that intracellular HA may be targeted to sites relevant to intracellular hyaladherin expression by a novel mechanism, there to exert effects relevant to diverse cell processes. To investigate this we have created a fluorescent conjugate of HA which can be used to determine the mechanism of transport and potential functions of intracellular HA.
CHAPTER II

MATERIALS AND METHODS
## II.1. Cell Culture

Murine 10T1/2 parental, ras-transformed (C3) (Egan et al. 1987) or vRHAMMAΔ1-5-transfected cells (LR21; Hall et al. 1995); parental and connexin43 transfected glioma cells (provided by J. Nagy, Winnipeg, MB); and human breast cancer MDA-MB-231 and MCF-7 cell lines purchased from American Type Culture Collection (Rockwell, MD) were all maintained at 37°C in 5% CO₂ on 100mm plastic tissue culture dishes (Nunclon) in DMEM (GibcoBRL) supplemented with 10% (v/v) fetal calf serum (FCS) (Intergen) and 10mM HEPES (Sigma) (growth medium), pH 7.3. Cells were routinely subcultured using 0.25% trypsin/1mM EDTA in HBSS (GIBCO BRL) from 80% confluent cultures and passaged at a 1:10 dilution. Transfected cells were routinely selected using 900μg/ml geneticin (Gibco BRL). All cells were used under passage 25. Frozen stocks of cells were prepared by resuspending the cells in freezing medium (70% DMEM, 20% FCS, 10% DMSO), and then stored under liquid nitrogen.

## II.2. Conjugation of HA with Texas Red

HA (Hyal Pharmaceutical Corp., Mississauga, Ontario) suspended in 20mM MES (Sigma), pH 4.5, with 30% ethanol, was mixed with three-fold excess EDCI (Aldrich) as per the number of HA disaccharides. Texas red hydrazide (Molecular Probes) dissolved in DMF (Aldrich) at a molar ratio of probe to disaccharide of 1:10 was added and the mixture was shaken overnight at room temperature. Unconjugated material was then removed with dialysis in 10 000 MWCO
membranes (Pierce) against 75mM NaCl and 40% ethanol for 6 days at 4°C in the dark. The product (see Figure II.1) was lyophilized for storage. Analysis of success of conjugation was conducted with gel permeation chromatography using refractive index and UV absorption to determine purity and the molecular size range of the bioconjugate (see Appendix 2). The molecular weight of the HA conjugated to the probe was shown to be 100kDa and the success of conjugation was shown to be approximately one molecule of Texas Red per fifteen hyaluronan disaccharides (see Appendix 2 – Figure VII.1).

**Figure II.1.** Conjugation of hyaluronan to Texas Red hydrazide
II.3. Treatment of cells with TR-HA and analysis of resultant intracellular fluorescence

Cells were seeded in DMEM (Gibco BRL) supplemented with 10% FCS (Intergen) at 60% confluence on 25mm sterile glass coverslips (VWR) in 35mm tissue culture plates (Nunclon) [except cells plated for oligosaccharide treatments, which were plated on 13mm glass coverslips (VWR) plated in 24 well plates (Falcon)]. After 8 h at 37°C the culture medium was aspirated, cells were rinsed and medium was replaced with serum-free DMEM containing 4 μg/ml transferrin (Human, Gibco BRL) 4μg/ml insulin (Bovine; Gibco BRL) and 10mM Hepes (Sigma) (defined medium), for 12 h at 37°C. Where indicated cells were pretreated with 50μg/ml BrefeldinA (Molecular Probes) for 30 minutes; 10mM colchicine (Sigma) for 30 minutes; 100nM phorbol 12-myristate 13-acetate (PMA; Calbiochem) for up to 4 h; or 50μg/ml MEK inhibitor (PD098059; Oncogene Science; dissolved in DMSO; Alessi et al. 1995) for 2 hours; at 37°C and 5% CO₂ in defined media. The cells were then gently exposed to 100μg/ml (unless concentration otherwise indicated) TR-HA conjugate in 1 ml of (37°C) defined medium, wrapped in foil incubated at 37°C with 5% CO₂ for 10 minutes (unless incubation time otherwise indicated). The cells were then rinsed twice in cold 5X PBS to prevent release of bound hyaluronate during washing (Underhill and Toole 1980; McGuire et al. 1987), and fixed in a solution of 2% paraformaldehyde and 1% cetylpyridinium chloride (CPC), which precipitates glycosaminoglycans, in a 0.1 M Na-phosphate buffer pH 7.4 for 10 minutes at room temperature. Coverslips were then washed three times
for 5 minutes each in 1XPBS (2.7mM KCl; 1.1mM KH₂PO₄; 128mM NaCl; 8.1mM Na₂HPO₄; pH 7.4), mounted with elvanol (PVA 15%, glycerin 30%), and viewed on a Zeiss Axiophot 100 confocal microscope using the 63X objective (unless otherwise specified). To ensure that confocal images represented internalized TR-HA, some cultures were exposed to TR-HA for 10 minutes, washed, then digested with *Streptomyces* hyaluronidase (1 IU/ml at 37°C for 1 h to remove TR-HA remaining on the cell surface that might interfere with confocal images), prior to viewing.

Images represent a z-axis slice through the center of a cell interior. Analysis was done with UTHSCA Image Tool version 1.28 (University of Texas Health Sciences Centre in San Antonio, Texas) using zero thresholding of equal central sections taken from the perinuclear areas of cells. Staining of cells with different cell thicknesses were equilibrated by dividing mean fluorescent intensity by the intensity of the FITC-Dextran counterstain (10,000MW lysine fixable; Molecular Probes). Laser settings were constant for all images compared.

**II.3.1. Digestion of TR-HA with hyaluronidase**

Digestion of 150µg of TR-labeled HA prior to addition to cells was carried out in acetate buffer pH 5.0, using 15 TRU of *Streptomyces* hyaluronidase (Sigma) for 24 h at 37°C in a proteinase inhibitor buffer (25mg/ml ovomucoid; 1.0mg/ml pepstatin A; 18.6mg/ml iodoacetic acid; 37mg/ml EDTA; 17.4 mg/ml PMSF). The mixture was heat inactivated at 56°C for 30 minutes to destroy enzyme activity. Control incubations (minus the hyaluronidase) were carried out under the same conditions. Digestion products were used to treat cells as in II.3.
II.3.2. Competition with unlabeled HA and HA oligosaccharides

To assess specificity of uptake, cultures were also exposed to 80µg/ml TR-labeled HA combined with excess (4mg/ml; i.e. 50X excess) unlabeled HA (Hyal Pharmaceutical, Mississauga, Ontario) or HA oligosaccharide (HA4, HA8, HA10, HA12; provided by R. Tammi, Kuopio, Finland). Cells were treated and fixed as described above in II.3.

II.3.3. Treatment of cells with Texas Red hydrazide probe alone

Cells were treated with 6.67µg/ml TR-hydrazide probe (equivalent amount of probe for 100µg/ml TR-HA given the determined ratio of 1 HA disaccharide per 15 Texas red molecule success of conjugation – see Figure VII.1.) followed by fixation and analysis as above in II.3.

II.3.4. Treatment of cells with Syto16

Cells were treated simultaneously with 5µM Syto16 live cell nucleic acid stain (Molecular Probes) and with 100µg/ml TR-HA for 10 minutes at 37°C and fixed and analyzed as above in II.3.

II.3.5. TR-HA concentration curves

vRHAMM1-5 transfected, ras transfected and parental 10T1/2 murine cells, and MDA-MB-231 and MCF-7 breast cancer cells were exposed to 0, 0.01, 0.05, 0.1, 0.2, 0.5, 0.75, 1.25, 2.0 and 3.0mg/ml TR-HA, and 1mg/ml FITC-dextran
(10,000MW lysine fixable; Molecular Probes) for 15 minutes at 37°C followed by fixation and analysis as above in II.3. Confocal laser settings were kept constant throughout analysis, on a low, medium and high setting (contrast = 153; 202; 264) for Texas Red, and on a single laser for FITC-dextran, and different lasers were equilibrated upon analysis.

II.3.6. TR-HA treatment of cells over timecourses

Cells were exposed to 150μg/ml TR-HA for 1 minutes, 15 minutes, 45 minutes, 2 hours, 6 hours and 20 hours at 37°C and 5% CO₂. All samples were incubated with 1mg/ml FITC-dextran (10,000MW lysine fixable; Mol Probes) 10 minutes prior to fixation as above in II.3.

II.3.7. Prebinding of peptides to TR-HA

150μg TR-HA was preincubated with 1mg/ml of a peptide that mimics the HA binding domain I of RHAMM (peptideRNA423-432, Yang et al. 1994) or 1mg/ml of a scrambled peptide of domain I which has been shown not to bind to HA (Yang et al. 1994), for 2 h at 37°C prior to cell treatment as in II.3.

II.3.8. Anti-CD44 and Anti-RHAMM antibody blocking

Live cells were preincubated for 30 minutes at 37°C and 5% CO₂ with 50μg/ml with, anti-CD44 mAbs: KM201 (R&D Systems) or Hermes1 (Endogen); or anti-RHAMMexon9 pAb, or IgG (Sigma) diluted in defined media prior to addition of 50μg/ml TR-HA for 5 minutes at 37°C and 5% CO₂, followed by fixation and
analysis as in II.3. (note: KM201 and RHAMM<sup>exon9</sup> antibodies were dialyzed exhaustively to remove sodium azide in 10,000MWCO membranes; Pierce).

**II.3.9. AMAC labeled HA-oligosaccharide treatment of cells**

Cells were treated with 1.67mg/ml AMAC labeled hyaluronan oligosaccharides of discrete length (HA8, HA12, HA26, HA30; provided by R. Tammi, Kuopio, Finland), for 10 minutes at 37°C, and fixed and analyzed for fluorescence as described in II.3.

**II.4. Wounding assays**

100% confluent 10T1/2 cell monolayers growing in DMEM (Gibco BRL) supplemented with 10% FCS (Intergen) were scratch wounded, rinsed with fresh DMEM/10%FCS and allowed to recover for up to 24 hours before TR-HA uptake and analysis was performed as in II.3. Trypan blue (Gibco BRL) addition was used to exclude areas of cells which sustained unrepaired injury.

**II.5. Immunofluorescence**

10T1/2 cells were seeded at 70% confluence, onto 25mm glass coverslips and allowed to attach for 24 hours in growth medium, washed and placed in defined medium for 24 hours. Cells were rinsed 5x in 1XPBS (pH 7.3) and fixed with freshly prepared 3% paraformaldehyde (Sigma) in PBS, for 10 minutes at RT, washed 5x for 5 minutes with 1XPBS. Cells were permeabilized for 2 minutes with 0.2% Triton X-100 (VWR) in PBS, and washed 5x for 5 minutes with 1XPBS. Cells
were then blocked for 1 hour at RT in 3% BSA (Sigma) in PBS washed and
incubated with 1°Abs: anti-RHAMM R10.1 pAb or anti-caveolin mAb
(Transduction Laboratories) at 1:50 in 1% BSA (Sigma) in PBS, at 37°C for 1 hour.
Cells were then washed 5x for 5 minutes in 1XPBS and incubated with 2°
antibodies: goat anti-mouse-RITC (Jackson) and goat anti-rabbit-FITC (Jackson) for
1 hour at 37°C in the dark, then washed 5x in 1XPBS. Coverslips were then washed
in xylene, dried on paper towels, and mounted using “anti-fade” media (10mM p-
phenylenediamine, 118mM Tris-HCl, 90% glycerol, pH 7.4) and sealed. Cells were
visualized with a Nikon confocal laser scanning microscope.

II.6. Flow cytometry (FACS analysis)

70% confluent 10T1/2 cells, which had been in defined medium for 24 hours
previously, were incubated with 2.0mM EDTA (ethylene-diaminetetraacetic acid) in
HBSS (Hank’s balanced salt solution, Gibco), with 10mM Hepes, for 5-6 minutes,
gently harvested with FACS buffer (10% FCS in HBSS with 20mM Hepes), and
pelleted by centrifugation at 1500rpm for 10 minutes. Cells were then resuspended in
FACS buffer. An aliquot of the cell suspension was stained with trypan blue (0.4%
Sigma) and cellular viability was determined to be above 90% using a
hemacytometer and trypan blue exclusion. Samples of $3 \times 10^5$ cells were treated in
suspension with 0.1μg/ml HA (Healon) over a time course of 0, 0.5, 1, 3, 5, 10, 15,
20, 25, 30, 60 minutes, followed by wash and centrifugation at 4°C, and
resuspension in 0.5ml of wash solution (HBSS; 10% FCS; 10mM Hepes) at 4°C.
Cells were then incubated with 1:100 dilution of 1° Ab: anti-RHAMM R10.1 pAb,
or IgG alone, for 45 minutes at 4°C, washed by centrifugation 2x, and resuspended in wash solution. Cells were then incubated with 2° Ab: Goat anti-RHAMM-cy3 for 45 minutes at 4°C, washed 2x, and resuspended in 1% paraformaldehyde (Sigma). Cells were scanned using a coulter electronics EPICS 754 flow cytometer for surface RHAMM expression. All tests were duplicated 3x.

II.7. DNaseI digestions

Genomic DNA was isolated from ras-transformed cell monolayers (as shown in Current Protocols). Briefly cells were suspended with trypsin (Sigma), washed 2x in HBSS, and resuspended in lysis buffer with proteinase K and incubated with shaking at 50°C for 18 hours, then extracted. Precipitate was recovered with glass rod and resuspended in 10mM Tris pH 8.0. Extraction was repeated with RNase treatment.

Each sample consisted of: 0.24μg genomic DNA, 5μl of 2x DNA binding buffer and either 2.5μl (10μg or 50μg) of HA or chondroitin sulfate or water. Samples were incubated together at RT for 2 hours with shaking. Then DNaseI (0.0, 0.6, 1.8, 3.0, 4.2, 5.4, 6.6, or 7.8 x 10^{-4} units respectively) was added to each sample and incubated for a further 30 minutes. DNaseI was inactivated by heating at 65°C for 15 minutes with 5mM EDTA. Samples were loaded onto a 1% agarose gel.
CHAPTER III

RESULTS
III.1. Texas Red labeled hyaluronan (TR-HA) is rapidly taken up into ras-transformed cells and targeted to cell processes and the nucleus.

Optical sectioning of cells, from which cell surface-bound TR-HA has been removed with hyaluronidase, shows an intracellular location of the TR-HA within 10 minutes after treatment of subconfluent monolayers of ras-transformed cells (Figure III.1.). TR-HA rapidly accumulates within cell lamellae and nuclei (Figure III.1.). Some general cytoplasmic and striking perinuclear accumulation is also observed (Figure III.1.). TR-HA within the nucleus is seen in the same confocal plane as a live cell nucleic acid stain, confirming its location in the nucleus (Figure III.2.)

*Streptomyces* hyaluronidase-digested TR-HA (Figure III.3a.) or TR-HA mixed with excess unlabeled HA (Figure III.3b.) block of the TR-HA uptake by cells, indicating that unlabeled HA competes effectively for the uptake of TR-HA and that the uptake requires larger than hexa- saccharide units of HA respectively. Cells treated with Texas-red hydrazide alone (Figure III.3c.) show no staining.

III.2. TR-HA uptake is dose and time dependent and increases with cellular transformation.

TR-HA concentration curves indicate that uptake of TR-HA is dose dependent until an apparent saturation point is reached (Figure III.4A.). Of the five cell lines examined, the highest levels of intracellular HA accumulation occur
in ras and RHAMM transformed and as compared to their parental 10T1/2 cells (Figure III.4.A.). Further, overall HA uptake is enhanced in invasive human breast epithelial cell line MDA-MB-231 cells as compared to the non-invasive MCF-7 breast cancer cell lines (Figure III.4.A.) (note, MDA-231 contains mutant active ras, while MCF-7 does not). When these cell lines are examined over a 20 hour timecourse (Figure III.4.B.) it is seen that TR-HA uptake achieves its highest levels within the first hour of incubation and decreases thereafter. Confocal images illustrate differences in both TR-HA intensity and intracellular distribution amongst the five cell lines (Figure III.4.C.). RHAMM and ras transformed, as well as MDA-231 invasive breast cancer cells accumulate significant amounts of intracellular TR-HA, further, HA appears to be targeted strongly to the tips of cell processes and lamellae of these cells (Figure III.4.C.). Interestingly, non-transformed 10T1/2 and non-invasive MCF-7 (which do not express mutant active ras) cell lines display weaker TR-HA accumulation and more diffuse intracellular distributions.

III.3. Specific HA oligosaccharides inhibit TR-HA uptake

Simultaneous treatment of cells with TR-HA and excess amounts of different sizes of HA-oligosaccharides were used to determine the minimum effective length of oligosaccharide which would interfere with cellular uptake of the TR-HA probe. Cells treated with HA oligosaccharides composed of ten monosaccharides (HA10) or fewer appear to have no significant effect upon TR-HA uptake (Figure III.5.).
However, cells treated with excess HA oligosaccharide of 12 monosaccharides (HA12) show a significant overall decrease in TR-HA intracellular intensity (Figure III.5.), indicating that the HA12 is the minimum effective length of oligosaccharide that is able to effectively compete with full length TR-HA for uptake. However the degree of blocking with HA12 does not achieve the level of inhibition shown for competition with excess unlabeled HA (Figure III.5.A.g).

III.4. Thirty HA-saccharide minimum length is required for effective targeting of HA to the nucleus.

In order to determine the minimum size of HA necessary to achieve cellular uptake and intracellular targeting to different cellular compartments, different sizes of HA oligosaccharides were fluorescently labeled such that their intracellular accumulation could be monitored. It appears that HA8 and HA12 are internalized by cells at extremely low levels, if at all, while HA26 is internalized and intracellularly distributed into discrete perinuclear vesicles. Interestingly, HA30 is internalized and distributed into both perinuclear structures and to the nucleus (Figure III.6). Thus it would appear that a minimum of 30 HA monosaccharides is necessary for nuclear targeting.

III.5. Anti-CD44 antibodies affect TR-HA uptake

The KM-201 mAb blocks the binding of HA to CD44 in murine (Culty et al. 1992) hamster (Miyake et al. 1990) and human cells (Thomas et al. 1992). TR-HA
uptake into cells significantly decreases in both RHAMM transfected, and 10T1/2 cells following preincubation with KM201 mAb (Figure III.7.A.B.).


Immunofluorescent staining of serum starved 10T1/2 cells shows that RHAMM and caveolin co-localize (Figure III.8a.). Within 5 minutes after HA addition RHAMM leaves the caveolin compartment and the two no longer colocalize (Figure III.8.b.). Further, flow cytometry shows that HA induces a rapid fluctuation in display of cell surface RHAMM expression during the first 15 minutes of HA treatment.

III.7. RHAMM peptides block TR-HA targeting to the nucleus.

Preincubation of TR-HA with peptides that mimic the HA binding domains of RHAMM (Yang et al. 1994) causes a dramatic decrease in the degree of nuclear accumulation of TR-HA, while targeting of TR-HA to the cytosol, perinuclear area and cell processes appears unaffected (Figure III.9.a.). This effect appears to require the HA binding capability of the peptide, since pretreatment of TR-HA with scrambled peptides made of these domains which lack the ability to bind HA (Yang et al. 1994) will target HA to all intracellular compartments including the nucleus (Figure III.9.b.).
III.8. TR-HA uptake is unaffected by blocking with RHAMM exon 9 antibody.

In an attempt to determine the role of RHAMM in TR-HA uptake we preincubated cells with anti RHAMM exon 9 pAb prior to treatment with TR-HA. Intracellular accumulation and subcellular targeting within RHAMMΔ1-5 and parental 10T1/2 cell cell lines was unaffected by the presence of the antibody (Figure III.10.).

III.9. HA uptake varies depending upon length of cell plating time and positively correlates with functional cellular RHAMM levels.

Image analysis of relative cellular intensities indicates that when cells are subcultured and allowed to attach for only 2 hours, RHAMM transfected and parental 10T1/2 cells appear to take up TR-HA equally, but by 12 hours after attachment the capability of RHAMM transfected cells to take up HA significantly exceeds their parental 10T1/2 counterparts (Figure III.11.). 24 hours after subculture RHAMM transfected cells are still maintaining their high levels of TR-HA uptake, while parental 10T1/2 cells have dropped to 1/2 their original intensity - the same basal level at which another set of RHAMM transfectants, those with mutated HA binding domains, have been displaying intracellular TR-HA uptake at all time points (Figure III.11.).
III.10. Connexin43 transfected cells show increased TR-HA uptake and targeting to the tips of the cell processes.

Treatment of connexin43 transfected glioma cells with TR-HA results in cellular uptake to the cytoplasm, nucleus and notably to the tips of the cell processes (Figure III.12.a.), whereas parental glioma cells do not target HA to the tips of cell processes (Figure III.12.b.). When these same cells are plated at confluence and treated with TR-HA it is seen that intracellular TR-HA intensity is much higher in connexin43 transfected glioma cells (Figure III.12.c.), than in their parental glioma counterparts (Figure III.12.d.).

III.11. TR-HA uptake is decreased upon MEK inhibition

Treatment of breast cancer cells with a specific inhibitor for MEK (PD098059; dissolved in DMSO; Alessi et al. 1995) prior to TR-HA treatment show that TR-HA uptake is dramatically reduced upon MEK inhibition (Figure III.13.A. a-c), as compared to cells treated with DMSO alone (Figure III.13.A. d-f). Image analysis indicates that reduction in fluorescent intensities is significant (Figure III.13.B.).

III.12. HA is targeted to the cell processes upon phorbol ester treatment of parental 10T1/2 cells.

10T1/2 cells treated with TR-HA for 10 minutes show diffuse cytoplasmic and nuclear staining (Figure III.14.a.). Following 45 minutes with phorbol ester
10T1/2 cells exhibit an intracellular accumulation pattern of TR-HA in the cytoplasm that resembles ras-transformed cells in that TR-HA accumulation occurs at the tips of cell processes (Figure III.14.b). Furthermore, treatment of cells with PMA for 4 h appears to enhance TR-HA uptake in a dramatic cytoplasmic and lamellar pattern (Figure III.14.c.).

III.13. TR-HA shows enhanced uptake and targeting to the tips of the cell processes at the wound edge in parental 10T1/2 cells.

Scratch wounding of 10T1/2 cell monolayers shows that TR-HA accumulation is enhanced at the wound edge and that cells at the wound edge show HA targeting to the tips of cell processes (Figure III.15.A.B.). Comparison of cells 30 minutes and 24 hours post wounding indicates that TR-HA uptake enhancement is most pronounced immediately after wounding and declines with time (Figure III.15.C.).
**FigureIII.1.** Confocal optical sectioning of ras-transformed cells exposed to 100μg/ml TR-HA. Consecutive optical sections (beginning from the basal surface of the cell and proceeding at 0.5μm increments in the z-axis towards the apical surface of the cell) show TR-HA in the cell processes (indicated by arrow), perinuclear area and nucleus of a ras-transformed 10T1/2 fibroblast 10 minutes after its addition to the culture medium. Bar = 10 μm.
Figure III.2. Cells treated simultaneously with 100μg/ml TR-HA and 5μM Syto16 live cell nucleic acid stain for 10 minutes at 37°C. (a) Dual fluorescence image of Syto16 and TR-HA shows staining of the two probes in the same z-axis plane of confocal imaging, though there is no signal colocalization. (b) TR-HA image of a. shows fluorescence in the nucleus and perinuclearly. (c) Syto-16 image of a. shows fluorescence in the nucleus. (d) Phase contrast image of the cell. Bar = 5μm.
Figure III.3. (a) 100μg/ml TR-HA digested with Streptomyces hyaluronidase prior to its addition to cells, (b) 100μg/ml TR-HA incubated simultaneously with 4mg/ml unlabeled HA. Both treatments abolish cellular uptake of TR-HA. (c) Cells treated with an equivalent amount of TR-hydrazide probe show no staining. All incubations were for 15 minutes at 37°C Bar =10μm.
**Figure III.4.** A. The amount of uptake of TR-HA by cells is proportional to the concentration of TR-HA applied, until an apparent saturation point is reached (at ~2mg/ml), at which the amount of uptake does not increase any further. Five cell lines are shown here, and it appears that ras and RHAMM transformed cell lines internalize HA to a greater extent than their parental 10T1/2 counterparts. As well, HA uptake is enhanced in invasive MDA-MB-231 breast cancer cells compared to their non-invasive MCF-7 counterparts. n=50. B. After an initial peak in intensity at ~1hr of TR-HA incubation, levels of internalized HA decline over a ~20hr timecourse. RHAMMA1-5 transfected fibroblasts display the greatest HA uptake over the timecourse. n=50. C. Confocal images of the five cell lines used in A and B show (a) RHAMMA1-5 transfected 10T1/2 fibroblasts (b) ras-transfected 10T1/2 fibroblasts (c) parental 10T1/2 fibroblasts (d) MCF-7 human breast cancer cells (e) MDA-231 human breast cancer cells; treated with 10μg/ml TR-HA for 15 minutes at 37°C. (a) Ras and (b) RHAMM transfected fibroblasts and (e) MDA-231 human breast cancer cells show TR-HA accumulation in cell processes, nucleus and a striking amount perinuclearly. (c) Parental 10T1/2 fibroblasts show TR-HA accumulation diffusely in the nucleus and in the cytoplasm. (d) MCF-7 cells show weak TR-HA signal across the entire cell. Bar = 10μm.
A. 

B. 

C.
Figure III.5. A. Confocal images of RHAMM transfected cells treated with 4mg/ml excess HA-oligosaccharide: (a) none, (b) HA4, (c) HA6, (d) HA8, (e) HA10, (f) HA12, or (g) 100kDa HA; at the time of TR-HA addition show an overall decrease in TR-HA intracellular intensity in treatments which included oligosaccharides composed of (f) 12 saccharide units, though inhibition does not achieve the level of inhibition obtained using (g) full length (100kD) unlabeled HA. Bar = 10μm. B. Analysis of cellular intensities in A. n=30. Bars represent SEM.
Figure III.6. A. RHAMM transfected cells treated for 10 minutes at 37°C with 1.67mg/ml AMAC labeled oligosaccharides of (a) HA8, (b) HA12, (c) HA26, and (d) HA30 (monosaccharide units of HA). HA8 and HA12 do not appear to be taken up into cells to any significant degree. HA26 is intracellularly distributed into discrete perinuclear vesicular structures, whereas HA30 appears to be the minimum effective size of HA that shows targeting to the nucleus. (a’-d’) show phase-contrast images of the cells. Bar = 10μm. B. Image analysis of (n=40) cells shows mean fluorescent intensity of n=40 cells of each sample. Bars indicate SEM. Line indicates degree of autofluorescence in this experiment.
Figure III.7. A. Preincubation of (a) RHAMM transfected fibroblasts and (b) parental 10T1/2 fibroblasts with anti-murineCD44 mAb KM201 (50μg/ml), which has been previously shown to block HA binding to CD44 (Culty et al. 1992; Hua et al. 1993), appears to significantly decrease TR-HA internalization in both cell lines as compared to preincubation of (c) RHAMM transfected fibroblasts and (d) parental 10T1/2 fibroblasts with IgG. B. Image analysis of experiments in A. n=60.
Figure III.8. (a) 10T1/2 cells serum starved for 24 hours show colocalization (yellow) between caveolin (green fluorescence) and RHAMM (red fluorescence).

(b) Addition of HA (0.1μg/ml) for 5 minutes caused a redistribution of RHAMM, and a reduction of its colocalization with RHAMM over the cell body. Bars = 10μm.

(c) Flow cytometry of 10T1/2 cells serum starved 24 hours prior to treatment with HA (0.1μg/ml) for up to 1 hour, and stained for RHAMM show that cell surface RHAMM intensity fluctuates rapidly within the first 15 minutes after HA addition. Blocking with RHAMM fusion peptide could ablate fluctuation (data not shown).

Error bars = SEM.
Figure III.9. RHAMMAΔ1-5 transfected cells treated for 10 minutes with 100μg/ml TR-HA that has been preincubated for 2 hours with: (a) 1mg/ml of a peptide mimicking an HA binding domain of RHAMM will traffic TR-HA to the cell processes and perinuclear area but no TR-HA signal is evident within the nucleus; (b) 1mg/ml of a scrambled peptide that is unable to bind HA show distribution of HA to all intracellular compartments. Arrows indicate cell processes. Bar = 10μm. n = 50.
Figure III.10. A. Confocal images of parental (a, c, and e) and RHAMM-transformed (b, d, and f) 10T1/2 fibroblasts preincubated for 30 minutes with: (a+b) 0μg/ml, (c+d) 10μg/ml and (e+f) 50ug/ml, of anti-RHAMM antibody prior to TR-HA incubation. Intracellular accumulation of TR-HA appears unaffected by antibody treatments. Bars =25μm. B. Image analysis of cellular intensities of A. (n=60). Error bars indicate SEM.
Figure III.11. RHAMMΔ1-5 transfected, mutant RHAMMΔ1-5 (unable to bind HA) transfected, and parental 10T1/2 fibroblasts were subcultured and allowed to attach for 2, 12 or 24 hours prior to TR-HA treatment (150μg/ml; 10 minutes). Cells were analyzed for fluorescent uptake of TR-HA. While RHAMMΔ1-5 transfected and parental 10T1/2 levels of uptake are initially equal, by 12 hours after plating RHAMMΔ1-5 transfectants clearly have more intracellular TR-HA. 24 hours after plating, parental 10T1/2 levels of uptake decrease until they are equal to HA uptake of the transfectants mutated in their HA binding domains, which have had significantly lower TR-HA intracellular staining at all three plating times. n=60. Error bars represent SEM.
Figure III.12. TR-HA (100µg/ml; 10 minutes) treated (a) connexin43 transfected glioma cells display nuclear and cytoplasmic HA accumulation as well as high levels of TR-HA targeted to the tips of the cell processes, while (b) parental glioma cells do not show HA targeted to the tips of cell processes. (c) These same connexin43 transfected cells plated at confluence show greater overall TR-HA uptake than their (d) parental glioma counterparts plated at confluence. Bars = 10µm.
Figure III.13. A. Human breast cancer cell lines: (a,d) MDA-MB-231, (b,e) MCF-7 and (c,f) MCF-10 neo 2T treated (a-c) with 50μM MEK inhibitor PD098059 or DMSO alone (d-f) for 2 hours prior to treatment with TR-HA (100μg/ml; 10 minutes). Bar=10μm. B. Analysis of mean fluorescent cell intensities indicates that all three cell lines display reduced TR-HA uptake in the presence of MEK inhibitor. Bars represent SEM. n=30.
Figure III.14. Confocal analysis of (a) 10T1/2 parental fibroblasts treated with TR-HA (100µg/ml; 10 minutes) shows TR-HA distributed to the nucleus and diffusely present in the cytoplasm. (b) 10T1/2 fibroblasts treated with 100nM PMA for 45 minutes prior to TR-HA treatment show TR-HA localized at the edge of cell processes and increased in the perinuclear area, although nuclear staining remains unchanged. (c) 10T1/2 fibroblasts treated with 100nM PMA for 4 h prior to TR-HA addition show dramatically enhanced uptake of TR-HA into cell processes. Bar = 10µm.
Figure III.15.  A. 10T1/2 cell monolayer scratch wounded and treated with TR-HA 30 minutes post wounding shows TR-HA strongly in the nucleus, perinuclear area and cell processes of cells at the wound edge. Those cells that are further from the wound edge display much weaker TR-HA staining. Bright outline indicates cells at the wound edge. Arrows indicate cell processes. Image captured with 63x objective.

B. Analysis of cell intensities in different layers of the 30 minute post-wounded culture indicates that TR-HA uptake significantly increases with proximity to the wound edge. n=30. Bars indicate SEM.  

C. Confocal images captured with the 40x objective show the wound edge of 10T1/2 parental fibroblast cultures. While enhanced uptake at the wound edge can be seen clearly at (a) 30 minutes post wounding, the contrast is less vivid (b) at 24 hours post wounding, though cells at the wound edge do clearly demonstrate increased intracellular TR-HA.
CHAPTER IV

DISCUSSION
Whereas intracellular HA degradation in the lysosomes has been extensively reviewed in many studies (Truppe et al. 1977; Orkin and Toole 1980; Orkin et al. 1982; Bernanke and Orkin 1984; Alston-Smith et al. 1992; Culty et al. 1992; Hua et al. 1993; Culty et al. 1994), few studies have focussed upon the modes by which hyaluronan may target to alternate intracellular sites (Collis et al. 1998; Evanko and Wight in press). This is interesting, since hyaluronan has been localized to many such alternate subcellular sites including the nucleus (Margolis et al. 1976; Furukawa and Terayama 1977; 1979; Castejon and Castejon 1987; Ripellino et al. 1988; Kan 1990; Eggli and Graber 1995; Evanko and Wight in press), rER and golgi (Kan 1990), caveolae (Eggli and Graber 1995), mitochondria (Kan 1990; Eggli and Graber 1995) and membrane ruffles (Evanko and Wight in press). Since the HA synthases are present only at the cell membrane and are thought to extrude newly synthesized HA directly into the extracellular space (Prehm et al. 1989; Weigel et al. 1997), presumably mechanisms must exist to deliver HA back into these intracellular compartments. These intracellular sites have also been shown to contain intracellular hyaluronan binding proteins which have a number of significant roles in cell signaling. We begin to delineate some aspects of a novel HA internalization process, and its cellular mechanisms in this study.
IV.1. The phenomenon of rapid HA uptake

Fluorochrome-tagged HA can be rapidly taken up by the transformed cell and accumulate within subcellular compartments, such as cell processes (Figure III.1) and the nucleus (Figure III.2), which have been shown to contain intracellular hyaladherins (Frost et al. 1990; Grammatikakis et al. 1995; Deb and Datta 1996; Entwistle et al. 1996; Zhang et al. 1998). The TR-HA uptake process is HA specific since it is blocked by predigestion of the TR-HA probe with hyaluronidases (Figure III.3.a.), is effectively competed by excess unlabeled HA (Figure III.3.b.), and since TR hydrazide alone is not taken up by cells (Figure III.3.c.).

The presence of nuclear HA is consistent with histological studies in which endogenous HA has been localized to nucleoli, nuclear clefts, heterochromatin and to areas of condensed chromatin in the nuclear periphery of oocytes, cumulus cells, and smooth muscle cells using nanogold labeled hyaladherins (Kan 1990; Eggli and Graber 1995; Evanko and Wight in press) and in biochemical studies of rat liver and brain nuclei purified by cell fractionation (Margolis et al. 1976; Furukawa and Terayama 1977; 1979). Our observations that exogenous HA can rapidly target to the nuclear compartment are supported by laser scanning confocal microscopy, which resolves the fluorescent signal in adjacent 1.0μm thick optical sections (Figure III.1.), and demonstrates that TR-HA staining is present in the same focal plane as a nucleic acid specific stain, confirming its nuclear localization (Figure III.2.).
The process appears to be dose dependent and saturable in at least five cell lines as demonstrated by concentration curves (Figure III.4.A.). This indicates that it is dependent upon some limiting factor, such as receptor sites. Examination of TR-HA uptake over a 20 hour timecourse demonstrates the rapidness of this process, as the greatest amounts of intracellular HA are seen within 1 hour of TR-HA incubation (Figure III.4.B.).

**IV.2. Mechanisms of HA uptake**

**IV.2.1. The potential role of cell surface receptors in TR-HA uptake**

TR-HA uptake was decreased in the presence of excess HA oligosaccharides of 12 monosaccharides in length (HA12) (Figure III.5.). It remains unclear whether HA12 is able to enter the cell itself (Figure III.6.), and thus HA12 may be interfering with TR-HA uptake through direct competition through cellular internalization (and consequent involvement of intracellular sites) as full length unlabeled HA does, or by statically occupying binding sites in the HA uptake machinery at the cell surface. One candidate for HA12 binding is CD44, since it has been showed previously to require a minimum binding site of HA6 (Culty et al. 1992; Hua et al. 1993). Tammi et al. (1998) have more recently showed that displacement of HA pericellular matrices from CD44 in vivo requires HA10 and they postulate that tight HA binding necessitates dimerization of CD44 or its cooperative interaction with other cell surface molecules. Our result of HA12 being required to
inhibit internalization of HA (Figure III.5.) is consistent with a complex process potentially mediated by aggregates of CD44, or the involvement multiple forms of cell surface receptors. The inhibition of TR-HA achieved using HA12 was incomplete, as evidenced by the much greater degree of blocking achieved using unlabelled HA (Figure III.5.). Thus, a significant amount of HA uptake is still occurring in the presence of HA12. It is likely that larger oligos would be more effective at blocking HA internalization, further suggesting the complexity of the cell surface/ intracellular protein binding interactions at work here.

Interestingly, it appears that nuclear targeting of HA requires a minimum of 30 monosaccharides (Figure III.6.). Since no single protein has been shown to require such a large number of HA saccharides for binding it would appear that if protein binding is important for nuclear entry (as implicated by Figure III.9.), then either a complex of proteins, or a novel hyaladherin is required for nuclear trafficking of HA.

HA uptake to the lysosomal degradation pathway has been shown to be mediated by a clathrin dependent process (Alston-Smith et al. 1992) which involves CD44 (Culty et al. 1992; Hua et al. 1993), as determined by antibody blocking. In this study we treated cells with two different anti-CD44 antibodies prior to exposure to exogenous TR-HA. It would appear that CD44 plays a role in the rapid uptake of HA into 10T1/2 cells, since the KM201 antibody, which has previously been shown to block CD44-HA binding (Culty et al. 1992; Thompson et al. 1992; Hua et al. 1993), was able to significantly inhibit TR-HA uptake in both transformed and non-
transformed cells (Figure III.7). It should be noted that the block was not a complete one, which is consistent with the involvement of other molecules. It would appear that CD44 is involved in targeting HA to multiple subcellular compartments independent of the previously reported degradative pathway.

A role for the another HA receptor, RHAMM, is also suggested by dramatic alterations of RHAMM cytoplasmic and cell surface distributions upon HA treatment (Figure III.8.) and by the ability of RHAMM peptides to block HA uptake to the nucleus (Figure III.9). The redistribution of RHAMM to the cell periphery, and indeed to the cell surface (Figure III.8.c) as shown by flow cytometry, upon exogenous HA administration however, is interesting, because it occurs rapidly, as does HA uptake. Ultrastructural analyses have demonstrated that HA is associated with caveolae (Egglie and Graber 1995), and these results would collectively implicate pinocytosis as a potential mode of TR-HA uptake worthy of further study. CD44 is potentially found associated with the caveolae as well, since CD44 has been demonstrated to have a strong association with GPI domains (Ilangumaran et al. 1997). Further, RHAMM and CD44 have been shown to colocalize and coimmunoprecipitate in human breast cancer cells (Paiwand et al., in preparation), and could act cooperatively to mediate HA uptake.

Although RHAMM HA-binding peptides blocked nuclear uptake of TR-HA (Figure III.9.), pretreatment of cells with anti-RHAMM antibodies proved inconclusive (Figure III.10.). These results suggest that the HA binding property and not other domains shown to be necessary for cell motility, which these
antibodies target, are required for uptake. RHAMM therefore may perform multiple functions at the cell surface to mediate cell locomotion.

Cellular RHAMM levels have been shown to vary depending upon time in culture. This prompted an examination of the relationship between cellular RHAMM levels, and relative TR-HA uptake. **Figure III.11.** shows that TR-HA uptake is at its highest in RHAMMΔ1-5 transfected cells at 12 hours, this correlates nicely with previous results using flow cytometry which indicate that RHAMM expression on the cell surface is highest upon 12 hours of plating (Chang et al. in preparation). Further, among the three cell lines used in these experiments, TR-HA uptake is shown to positively correlate with functional (i.e. RHAMM which is capable of binding HA) cellular RHAMM levels at all timepoints (**Figure III.11.**).

RHAMM has been proposed to regulate gap junctional intercellular communication through connexin43 (Nagy et al. 1995), and a potential role for gap junctional transport in TR-HA trafficking was investigated using connexin43 transfected glioma cells. At confluence it can be seen that connexin43 transfected cells show a greater amount of intracellular TR-HA than their parental glioma cells and individual connexin43 transfectants appear to target TR-HA to the tips of cell processes, while their parental glioma cells do not (**Figure III.12.**). These results are consistent with a role for gap junctions in TR-HA targeting, but it is not possible to determine if this is a specific effect resulting from functionally competent gap junctions between cells or hemigap junctions (Ebihara et al. 1995) at the plasma membrane, or one reflecting other factors which are upregulated upon connexin43
transfection. Future experiments are needed to determine the role of connexin43 in TR-HA uptake.

In a recent study HA treatment of rat embryonic cell lines, under similar conditions of confluence, plating time, and HA concentrations/times as those used in this study, has been shown to rapidly activate tyrosine phosphorylation of cellular proteins and MAP kinase in a time and dose dependent manner, and subsequently stimulate cell growth (Serbulea et al. 1999). This HA dependent activation of MAP kinase was strongly suppressed by dominant negative ras expression (Serbulea et al. 1999). These results suggest that the ras-MAP kinase pathway is activated by HA and may play an important role in HA dependent signaling (Serbulea et al. 1999). Our lab has demonstrated that the HA binding domains of intracellular RHAMM are required for interaction in MAP kinase signaling (Zhang et al. 1998), and these results collectively suggest a role for intracellular HA in the MAP kinase cascade. Conversely MEK inhibition with PD098059, which prevents the activation of MEK by Raf in this cascade (Alessi et al. 1995), appears to inhibit TR-HA internalization (Figure III.13.), and would suggest that HA internalization is somehow dependent upon MAP kinase signaling. This effect may be due to multiple effects of the MEK inhibitor, including hyperpolymerization of microtubules (Harrison and Turley in preparation) with consequent interference with vesicle mediated transport of HA along microtubules (see Appendix1 – Figure VI.1.). MEK inhibition also causes decreased cell motility (Paiwand et al., in preparation), which may have far reaching implications in decreasing levels of overall cellular activity and HA uptake.
IV.2. Potential roles for intracellular HA targeting

IV.2.1. Potential role of HA in the nucleus

Precedent for regulatory roles of nuclear GAGs come from studies showing that transient nuclear heparin sulfate is involved in growth control and transcriptional regulation (Fedarko and Conrad 1986; Fedarko et al. 1989; Ishihara and Conrad 1989; Busch et al. 1992). Exogenous addition of heparin to cells has been shown to arrest the cell cycle in G1 (Fedarko et al. 1989), stimulate/inhibit DNA synthesis in normal/tumor cells (Furukawa and Bhavanandan 1982), compete for binding with histones (Furukawa and Bhavanandan 1983; Scheerer et al. 1989), suppress the expression and DNA binding actions of AP-1 (Au et al. 1994) and accumulate at the nuclear membrane (Fedarko and Conrad 1986). Though multiple GAGs including HA, CS, HS and heparin have all been reported to be associated with chromatin in nuclei (Furukawa and Terayama 1977), heparin has so far received more attention for possible direct regulatory roles.

We show here a novel import of exogenous HA into the nucleus, and its distribution appears ordered (Figures III.1.–III.2.). Other studies have reported the presence of endogenous HA in the nucleus (Margolis et al. 1976; Furukawa and Terayama 1977; 1979; Castejon and Castejon 1987; Ripellino et al. 1988; Kan 1990; Eggli and Graber 1995; Evanko and Wight in press). Within the nucleus HA has been found associated with nucleoli (Kan 1990; Evanko and Wight in press), heterochromatin (Eggli and Graber 1995), fine filaments within the nuclei (Evanko
and Wight in press) and in nuclear clefts and surrounding condensed chromatin during mitosis (Evanko and Wight in press). GAGs have been found associated with DNA in loops, many in aggregates or cycles resembling the pore complexes, which suggests evidence for a structural role for GAGs in the nucleus (Engelhardt et al. 1982). As well, nuclear GAGs appear increased during liver regeneration (Furukawa and Terayama 1977). The functional importance of these findings is not known.

The recent report of a cytoplasmic HA binding protein p68 which has been co-purified with the splicing factor SF2 (Deb and Datta 1996), as well as the existence of other hyaladherins in the nucleus including chick cell cycle homologue cdc37 (Grammatikakis et al. 1995), and RHAMM, all indicate the potential for a multifaceted control of cell function by HA from within the nucleus.

Aside from its protein interaction, HA has the potential to exert influence in the nucleus through basic biochemical and structural modalities. HA is a calcium chelator and could potentially be involved in regulating nuclear calcium levels, which control a variety of nuclear functions, including gene transcription, DNA synthesis, DNA repair and nuclear envelope breakdown (Santella and Carafoli 1997). The nucleus is a dynamic structure, with chromosome movements, and cycles of condensation and decondensation (Lamond and Earnshaw 1998). The hydrating and basic physicochemical properties of HA suggest that HA may be involved in facilitating transport of small molecules within the nuclear interior just as it does in the ECM.
How or whether HA might interact with nuclear pores remains unclear, but a growing body of research on nuclear import of glycoconjugates suggests that when proteins are substituted with 25 GlcNAc residues (linked in β1-4 fashion), they are permitted to be transported from the cytosol to the nucleus (Duverger et al. 1993; 1995; 1996). This form of nuclear transport has been shown to use the nuclear pore in a novel fashion which does not compete for cofactors associated with traditional NLS dependent protein import (Duverger et al. 1995). It is possible that this form of transport could be responsible for mediating the novel nuclear translocation of HA seen in this study.

The size of HA present in both the nucleus and cytoplasm remains open for speculation. Recently identified GPI linked cell surface hyaluronidases (Chan et al. 1999), and potential hyaluronidases within the cell may act to cleave HA as it is being transported into the cell. Localization of HA in the nucleus using a biotinylated aggrecan probe (Eggli and Graber 1995; Evanko and Wight in press) demonstrates that the HA within the nucleus must be at least HA10, since aggrecan HABP requires at least a decasaccharide of HA to recognize and bind HA (Hascall and Hinegard 1974).

Surprisingly, preliminary evidence has identified the nucleus as the predominant cellular localization of the newly characterized hyaluronidase HYAL1 in normal epithelium both in vitro and in vivo (Frost et al. in preparation). HYAL1 is a candidate tumor suppressor gene (Frost et al. 1997), as it is found at the site of a common tumor suppressor locus (Killary et al. 1992; Buchhagen 1996). The
putative localization of HYAL1 to the nucleus in normal epithelia has given insight into possible mechanisms of how HYAL1 might function as a tumor suppressor gene. In addition, HYAL-1 is unable to remove cell surface HA but may rather play a functional role in the nucleus to remove nuclear GAGs that may regulate cellular proliferation, differentiation, and or apoptosis (Frost et al. in preparation).

IV.3.2. Potential roles of HA uptake in transformation

HA production and metabolism are often altered during tumorigenesis (Knudson and Knudson 1993) and a causal role for this modification has recently been suggested by a study showing that HA accumulation around and within colorectal tumor cells is prognostic of poor outcome (Ropponen et al. 1998). Consistent with the notion that HA directly regulates cell behaviour, study of HA binding proteins termed hyaladherins has indicated that HA contributes to the control of cell cycle and cell motility (Entwistle et al. 1996; Mohapatra et al. 1996; Wang et al. 1998; Zhang et al. 1998). In spite of the importance of these observations, the molecular mechanism by which HA directs these processes are not clear.

HA uptake was observed in five cell lines and levels of cellular internalization were shown to be highest in cells expressing the highest levels of RHAMM, ras, CD44 and erk, as shown in both cells and invasive breast cancer cells compared to their non transformed counterparts (Figure III.4.). This trend correlates with both increased motility and proliferation in these cell lines.
HA oligosaccharides have been shown to significantly reduce proliferation and migration in response to PDGF (Evanko et al. 1999), and to inhibit tumor formation in subcutaneous melanoma cells (Zeng et al. 1998). These effects may be mediated through blocking accumulation of HA in intracellular compartments relevant to tumorigenesis.

The potential uses of covalently modified HA for selectively targeting and accumulating in aggressive cancer cells are manifold in both diagnostics and treatment. One recent study used HA linked to sodium butyrate to target HA to breast cancer cells and found that cells could internalize both HA and the covalently linked compound, which resulted in inhibition of cellular growth rates (Coradini et al. 1999).

IV.3.3. Potential roles of intracellular HA in cell motility

The ability of HA microinjected into cells that do not aggressively take up HA or target it into the cell processes, to stimulate cell motility provides the first direct evidence of an effect of intracellular HA on cell behaviour (Collis et al. 1998). Results in this study further suggest that the uptake and intracellular targeting of HA to cell processes is controlled by ras and PKC signaling pathways. 10T1/2 cells which are not transformed or treated with PKC do not take up HA aggressively, and do not target HA to the tips of cell processes (Figure III.14.a.). These same cells will not respond to exogenous administration of HA with increased motility - as ras and RHAMM transformed cells do (Collis et al. 1998). However, when 10T1/2 cells
are treated with phorbol ester, exogenous HA is targeted to the tips of cell processes (Figure III.14.b.), concomitant with manifold increased motility (Collis et al. 1998).

The ability of HA to be directed into 10T1/2 cell processes after acute phorbol ester treatments suggest an involvement of protein phosphorylation by protein kinase C in this HA uptake. This protein serine/threonine kinase has previously been implicated in cell motility (Liu et al. 1997) and its kinase activity has also previously been linked to release of HA from the cell (Klewes and Prehm 1994; Thiebot et al. 1999), which may involve analogous mechanisms to uptake noted here.

The dramatic alterations in TR-HA subcellular targeting upon phorbol ester treatment (Figure III.14) may be mediated through PKC and ras dependent activation of microtubule-dependent particle motility (Alexandrova et al. 1993). PMA treatment has been shown to target transferrin receptors to cellular compartments which do not participate in normal routes of receptor traffic, or to otherwise inaccessible compartments along new membrane traffic pathways (Schonhorn et al. 1995). It would likewise be interesting to examine the effects of PKC inhibitors upon this process.

Further evidence for intracellular HA upregulation upon onset of motility is shown in wounded monolayers, where TR-HA is shown to accumulate intracellularly at the wound edge (Figure III.15.A.B.). This effect is most dramatically enhanced within 30 minutes post-wounding (Figure III.15.C.), concomitant with increased RHAMM expression during early wound responses (Savani et al. 1995b). Further,
wounding is shown to promote subcellular targeting of HA to the tips of cell processes concomitant with their increasing motility (Figure III.15.A.).

Previous reports have demonstrated HA accumulation at wound sites, both in culture and in vivo (Gerdin and Hallgren 1997; Chen and Abatangelo 1999), but the possibility that HA’s therapeutic activity at such sites is being mediated intracellularly is a novel one.

IV.4. Conclusions

We note here that the rapid uptake of fluorochrome-tagged HA into cells appears to be independent of the traditional receptor-mediated endocytic pathway, since uptake is acute, is incompletely blocked by CD44 antibodies, and results in the accumulation within cell processes, the nucleus, and the perinuclear area. The localization of HA in the nucleus requires an interaction with subcellular proteins, since exposing cells to peptides that mimic HA binding motifs of RHAMM block this targeting and results in accumulation of HA only within the cytoplasm (Figure III.9). HA binding motifs (Yang et al. 1994) are found in RHAMM, cdc37, and p68, which are all potential candidates involved in the uptake of HA. Our results also suggest the targeting of HA to cell processes involves a mechanism distinct from nuclear targeting.

Collectively, our results suggest that both HA/cell surface receptor and intracellular HA/protein interactions may be involved in regulation of cell behaviour.
A multifaceted role of intracellular HA is suggested by its localization in the nucleus, previous reports of HA binding to nuclear proteins (Furukawa and Terayama 1977; 1979) and the possibility that a hyaluronan binding protein may interact with RNA splicing machinery (Deb and Datta 1996). Our results provide the first preliminary evidence of the ability of exogenously added HA to accumulate within multiple subcellular compartments, and to directly affect cell motility. Further analysis is required to determine the precise role(s) of HA within the cell, to define the mechanisms involved in this uptake and accumulation process and to determine the signaling processes that intracellular HA might regulate. Clearly, study in this field is only just beginning, and more work is necessary to elucidate the mechanisms mediating HA traffic, and the role(s) of HA inside the cell.
CHAPTER V

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VI.1. Results

VI.1.1. Colchicine treatment prevents nuclear uptake of TR-HA

To examine the potential role of the microtubules in subcellular trafficking of HA, cells were pretreated with colchicine to disrupt their microtubule networks, then exposed to TR-HA. Cells pretreated with colchicine display TR-HA staining diffusely throughout the nucleus and cell processes, while nuclear targeting of TR-HA is ablated and perinuclear accumulation is decreased (Figure VI.1.).

VI.1.2. Brefeldin A pretreatment blocks uptake of TR-HA by 10T1/2 parental cells.

In order to assess the potential role of vesicle trafficking in TR-HA transport, treatment with Brefeldin A were used. Pretreatment of ras transformed and parental 10T1/2 cells with Brefeldin A prior to addition of TR-HA caused a dramatic decrease in the intracellular accumulation of TR-HA in all cellular compartments (Figure VI.2.).

VI.1.3. HA alters the ability of DNaseI to interact with DNA

DNase digestion of genomic DNA is enhanced in the presence of HA as evidenced by increased smear length (corresponding to fragmented DNA) across an agarose gel (Figure VI.3.). Another glycosaminoglycan, chondroitin sulphate has no effect on DNA degradation, indicating that the process is HA specific (Figure VI.3.). This phenomenon would appear to suggest that HA may promote interactions
between protein and DNA on a wider basis, and suggests a fundamental role for HA in the nucleus.
**Figure VI.1.** RHAMMAΔ1-5 transfected cells treated with 10mM colchicine for 30 minutes prior to addition of TR-HA (100μg/ml) and further incubation for (a) 10 minutes or (b) 30 minutes show no TR-HA trafficking to the nucleus, in contrast to non-colchicine treated cells at (c) 10 minutes and (d) 30 minutes TR-HA. Bars = 10μm.
Figure VI.2. (a,b) Parental and (c,d) ras-transformed fibroblasts pretreated with (b+d) 50μg/ml Brefeldin A for 30 minutes prior to TR-HA treatment (100μg/ml; 10 minutes) show greatly decreased levels of TR-HA uptake compared to cells not pretreated (a+c). Bar = 10μm.
**Figure VI.3.** Lanes 1-8 are loaded with genomic DNA degraded by increasing concentrations (0.0 - 7.8 x 10^4 units) of DNaseI. Prebinding of DNA with 10μg (lanes 9-16) or 50μg (lanes 17-24) HA results in increased DNA degradation by DNaseI (as visualized by increased smear length). 10μg chondroitin sulfate (lanes 25-28) had no effect on DNA degradation by DNaseI. Samples loaded on a 1% agarose gel.
VI.2. Discussion

VI.2.1. Cytosolic factors in the uptake process

Cells which have had their microtubule networks disrupted by colchicine have dramatically reduced transport of TR-HA to the nuclear and perinuclear compartments, while entry of TR-HA into the cytoplasm and cell processes appears unaffected (Figure VI.1.). This predicts a form of microtubule dependent transport of HA across the cytoplasm, which is involved in targeting HA to sites relevant for nuclear entry. Microtubules have been previously shown to direct a multistep process of caveolae cycling between the Golgi and plasma membrane, and this transport is interrupted by microtubule disruption using nocodazole (Conrad et al. 1995). Therefore we propose that at least part of the TR-HA transport could be occurring via this microtubule mediated transport of caveolae.

In previous studies, colchicine has been demonstrated to decrease levels of cellular HA, potentially impinging upon HA synthesis (Brecht et al. 1986). Perhaps, without an intact microtubule network, levels of HA accumulate at cellular and intracellular sites which act to downregulate HA synthesis. Interestingly, addition of HA has recently been shown to cause an increase in intracellular concentrations of tubulin in human cells (Greco et al. 1998), and these results have implications for the role of HA in cell division.

Vesicular transport within the cell is facilitated by recruitment of cytosolic coat proteins that promote the budding of transport vesicles (Kreis and Pepperkok 1994). Brefeldin A inhibits recruitment of these coat proteins and formation of transport vesicles. Brefeldin A has been shown to interfere with vesicular transport
causing disassembly and collapse of Golgi components back to the ER (Hidalgo et al. 1992). Disruption of vesicular transport with Brefeldin A causes a decrease in overall intracellular accumulation of TR-HA (Figure VI.2.), implicating vesicular mediated transport at both the plasma membrane, and in cytoplasmic trafficking of HA. These effects are also consistent with a microtubule mediated vesicular mode of TR-HA transport (perhaps associated with caveolae), to the perinuclear/nuclear compartments (see Figure VI.1.).

VI.2.2. Potential influence of HA on DNA - protein interactions

HA has been found associated with the chromatin fraction of the nucleus (Furukawa and Terayama 1977; 1979; Eggli and Graber 1995), and previous work in our lab has demonstrated that HA is capable of binding to genomic DNA (Zhao et al. in preparation). We show that HA is able to increase the effectiveness of DNaseI interactions with DNA, resulting in increased degradation of DNA (Figure VI.3.). At this time it is not possible to speculate whether this specific ability of HA to alter protein-DNA interactions is efficacious within cell nuclei, but it does present us with an interesting model for HA activity. Likewise, in the cytoplasm, it is possible that HA influences protein-protein interactions. The HA binding domains of RHAMM are also required for RHAMM-erk binding to occur (Zhang et al. 1998). It is possible that HA can compete with erk for these binding sites, and thereby influence downstream events. Peptides which mimic HA's ability to bind these domain have been developed and are being used to investigate this possibility (Ziebell et al. in preparation).
Figure VI.4. Model of potential uptake mechanisms and intracellular functions of HA (see next page): HA uptake is potentially mediated by multiple processes:

1) Clathrin mediated HA uptake and targeting to lysosomes involves CD44 (Alston-Smith et al. 1992). 2) Caveolae mediated pinocytic HA uptake may involve RHAMM, CD44, and p68 proteins. This form of transport utilizes microtubules to traffic vesicles to and from the golgi/ER perinuclear compartment. (3) The HA synthase complex may at times direct HA cytoplasmically. (4) HA may interact with the phospholipids of the cell membrane to form pores or channels (Pasquali-Ronchetti et al. 1997), and thus enter the cell cytoplasm in a non energy dependent fashion. Once internalized HA occurs notably at tips of cell processes upon onset of motility as produced by cell transformation and PKC treatments. Intracellular HA of larger than HA30 also accumulates in cell nuclei. The presence of cytoplasmic and intranuclear hyaladherins provide additional functional targets for intracellular HA.
1. Degradative Endocytic Pathway

2. Pinocytosis

3. HA synthase complexes

4. HA as a pore former

Intranuclear Hyaladherins:
- RHAMM
- cdc37
- p66

Hydrating and structural roles

HYAL-1
CHAPTER VII

APPENDIX II. - HYALURONAN DETECTION
VII.1. Existing probes for hyaluronan

The analytical techniques for HA detection have been continuously refined. For many years it was necessary to purify the polysaccharide so that its sugar constituents, glucuronic acid and glucosamine could be measured by colour reactions. A classical technique was the carbazole method for uronic acids. These analyses required at least 100μg of HA. With the discovery of specific microbial enzymes that degrade HA into unsaturated disaccharides, it became possible to design techniques in which a few micrograms of polymer were sufficient. The third generation came in 1980. These make use of proteins with specific affinity for HA and can be used as antibodies in ELISA-like assays to detect as little as 1ng of polysaccharide.

More recently a technique for the histolocalization of HA (Ripellino et al. 1985) that utilizes these highly specific HA binding peptides has become widely used. However, even this HABP procedure may seriously underestimate the HA content of tissues fixed in a routine manner (Lin et al. 1997). Other GAGs are fixed as a result of their covalent binding to proteoglycan core proteins, however the free form of HA apparently diffuses slowly into the fixation solution during the process. Although CPC improves the preservation of HA in such samples, total HA cannot be accounted for (Lin et al. 1997).

VII.2. The Texas Red – hyaluronan conjugate

Four groups on the HA molecule are available for chemical modification: the carboxylate, the acetamido, the reducing end-group and the hydroxy groups.
Modification of HA with ethyl (N,N-dimethylaminopropyl)carbodiimide (EDCI) has been done since 1986 (Kuo et al. 1990). The productions of glucuronamides requires the activation of the carboxylic group which can be accomplished using a water soluble carbodiimide such as EDCI as the condensing agent.

Advantages include 1) preparation of HA derivatives under mild, aqueous conditions which preserves the structural integrity and molecular size range of HA, also 2) homogenous modified HA materials can be easily obtained by removal of all side products and unreacted reagents during dialysis and 3) all new HA derivatives based on this bishydrazide technology are chemically well-characterized (Prestwich et al. 1998).

TR-HA can be prepared directly from HA and Texas Red sulphonylhydrazide (TR-HA used in this study has been analyzed to have 1 in 15 disaccharides successfully labeled with TR – Figure VII.1.). This gives maximal sensitivity while preserving the minimum decasaccharide recognition unit of HA for HABDs. These probes offer advantages over reducing end-modified HA derivatives (Raja et al. 1988; Yannariello-Brown et al. 1996), in that multiple probe moieties can be included per HA molecule, and the probes are positioned in the internal milieu of HA.

Future directions in the chemical modification of HA include development of covalent attachments of therapeutic drugs to HA (Band et al. 1998). Attachment of HA to proteins, short peptides, anti-cancer agents, anti inflammatory or anti infective drugs or cell adhesion and growth promoting ligands provides the potential for drug
delivery through exploitation of HA recognition and receptor-mediated endocytosis for specific uptake by and delivery to target cells (Vercruysse and Prestwich 1998).

Figure VII.1. HPLC analysis of TR-HA conjugate. Analysis of success of conjugation of TR-HA: the (a) refractive and (b) absorptive (at 590nm) indices overlap at 19 minutes elution time in a single peak indicating the sample is pure. (c) Molecular weight of the HA is shown to be approximately 100kDa through comparison to other HAs of known molecular weights. To measure success of conjugation (d) comparative absorbance of the TR-HA on a TR standard curve shows that approximately 1 in 15 HA dissaccharides has been successfully labeled.