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HA Receptors: Regulators of Signalling to the Cytoskeleton

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Abstract Hyaluronan (HA) is a ubiquitous component of the extracellular matrix (ECM) and occurs transiently in both the cell nucleus and cytoplasm. It has been shown to promote cell motility, adhesion, and proliferation and thus it has an important role in such processes as morphogenesis, wound repair, inflammation, and metastasis. These processes require massive cell movement and tissue reorganization and are always accompanied by elevated levels of HA. Many of the effects of HA are mediated through cell surface receptors, three of which have been molecularly characterized, namely CD44, RHAMM, and ICAM-1. Binding of the HA ligand to its receptors triggers signal transduction events which, in concert with other ECM and cytoskeletal components, can direct cell trafficking during physiological and pathological events. The HA mediated signals are transmitted, at least in part, by the activation of protein phosphorylation cascades, cytokine release, and the stimulation of cell cycle proteins. A variety of extracellular signals regulate the expression of both HA and the receptors necessitating that HA-receptor signalling is a tightly controlled process. Regulated production of soluble forms of the receptors, alternately spliced cell surface isoforms, and glycosylation variants of these receptors can dramatically modulate HA binding, ligand specificity, and stimulation of the signalling pathway. When these processes are deregulated cell behaviour becomes uncontrolled leading to developmental abnormalities, abnormal physiological responses, and tumorigenesis. The elucidation of the molecular mechanisms regulating HA-mediated events will not only contribute greatly to our understanding of a variety of disease processes but will also offer many new avenues of therapeutic intervention.

Key words: hyaluronan receptors, CD44, RHAMM, ICAM-1, signal transduction, cell migration

Hyaluronan (HA) is a heteropolysaccharide comprised of a variable number of repeating units of D-glucoronic acid and N-acetyl glucosamine in a β-4 linkage and can have a molecular weight of up to several million daltons. It belongs to the glycosaminoglycan (GAG) family; other prominent members of this group include heparan sulphate, chondroitin sulphate, dermatan sulphate, and heparin. The structure and function of the GAGs have been carefully and comprehensively reviewed [Jackson et al., 1991]. Hyaluronan is a ubiquitous component of the extracellular matrix and was historically thought to play only a structural role in maintaining the ECM architecture. A wealth of research later demonstrated that HA was a key component in promoting cell motility, adhesion, and proliferation processes. These events are mediated mainly through three HA cell surface receptors, namely CD44 [Underhill et al., 1987; Stamenkovic et al., 1989; Aruffo et al., 1990; Lesley et al., 1990; Miyake et al., 1990], RHAMM [Turley et al., 1991; Hardwick et al., 1992; Yang et al., 1993], and ICAM-1 [McCourt et al., 1994]. The molecular characterization of these receptors, the identification of a wide array of isoforms, and the partial elucidation of the HA-mediated signalling pathways have contributed greatly to an understanding of the physiological roles of this polymer. In addition to the cell surface receptors, intracellular HA binding proteins have been described and this observation together with a reported intracellular location of HA, point to additional novel mechanisms by which HA may regulate cell behaviour. Several previous reports have documented that intracellular GAGs can potently affect cell proliferation by altering the expression of nuclear transcription factors such as c-fos, c-jun, and c-myc [Fedarko et al., 1989; Pukac et al., 1990; Busch et al., 1992]. This bipartate location predicts that a dynamic equilibrium may exist between extracellular and intracellular GAGs/receptors that ultimately regu-

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late cell behaviour by controlling the signal transduction pathways of the cell. This review will therefore describe the structure and expression of the HA receptors described to date, then examine their roles in signal transduction.

HA CELL SURFACE RECEPTORS AND THEIR EXPRESSION

CD44 was the first of the cell surface HA receptors to be identified [Underhill et al., 1987; Lesley et al., 1990; Culty et al., 1990]. It is a broadly distributed cell surface glycoprotein and can be found on hematopoietic cells, fibroblasts, and numerous tumour cells. It has a diverse role in cell-cell and cell-matrix interactions such as lymphocyte recirculation, prothymocyte homing, lymphocyte activation, cell aggregation, and cytokine release [Haynes et al., 1989, 1991; Lesley et al., 1993]. The gene encoding CD44 has twenty exons, ten of which can be alternately spliced to produce a vast array of isoforms, some of which are cell or tissue specific [see Herrlich et al., 1993]. The CD44 gene encodes both a leader sequence and a transmembrane domain [Stamenkovic et al., 1989; Zhou et al., 1989], and the protein has two HA binding domains in the extracellular region and one in the cytoplasmic tail [Yang et al., 1994]. The most abundant form of CD44 on lymphocytes has a 37 kDa protein core which undergoes extensive N- and O-linked glycosylation and occurs as an 85–95 kDa protein. This widely distributed isoform, namely CD44s (hemopoietic or standard form), does not include any of the ten variant exons. Variant forms (CD44v) have a more restricted expression, and can be identified within epithelial cells, keratinocytes, activated T cells, and at specific stages of tumour development and metastasis [Stamenkovic et al., 1991; Gunthert et al., 1991; Sy et al., 1991]. CD44 has not been directly implicated in transformation; however, certain isoforms of CD44 rarely seen in normal tissue become highly expressed in tumour cells. These variants have been linked to specific stages in the metastatic process, such that overexpression of CD44 in non-metastatic tumor cells can induce a metastatic phenotype [Gunthert et al., 1991]. While overexpression of CD44 normally has no effect on transformation or metastasis, there is one noteworthy exception. The transfection of a Burkitt lymphoma cell line with CD44s resulted in lung colonization when injected into nude mice [Sy et al., 1991]. The diverse functions of CD44 are likely due to its role in cell adhesion and indeed, the adhesion potential and role of CD44 in metastasis is associated with its affinity for HA [Aruffo et al., 1990; Miyake et al., 1990; Lesley et al., 1992]. The HA mediated CD44 signalling pathways impact on the integrity of the cytoskeleton and can stimulate release of cytokines (Fig. 1). Mutation of the HA binding domains in CD44 has been shown to completely ablate its physiological responses. In addition to HA, CD44 also recognizes other ligands such as fibronectin and collagen [Jalkanen and Jalkanen, 1992], mucosal addressin [Picker et al., 1989], the chondroitin sulfate modified invariant chain of class II MHC [Naujokas et al., 1993], and the high molecular weight proteoglycan, serglycin [Toyama-Sorimachi et al., 1995].

RHAMM, the second cell surface receptor to be characterized, has emerged as a critical regulator of cell motility, focal adhesion turnover and is transforming when overexpressed [Hardwick et al., 1992; Hall et al., 1994, 1995; Entwistle et al., 1995]. It is present in almost all cell types including fibroblasts, smooth muscle cells, macrophages, T lymphocytes, ras-transformed cells, and breast carcinoma cells [see Turley, 1992]. The expression of RHAMM is very low in quiescent cells but becomes markedly upregulated during cell migration, following cytokine stimulation and in transformed cells [Hardwick et al., 1992; Samuel et al., 1993]. Interestingly, RHAMM belongs to a group of cell surface receptors which possess neither a signal peptide nor a transmembrane domain. Other members of this group include the high affinity elastin/laminin receptors and certain animal lectins. It has been proposed that these proteins are transported to the surface by other carrier proteins and associate with the cell surface via integral docking proteins. Hyaluronan synthase has been identified as a potential docking molecule for RHAMM [Klewes et al., 1993]. Alternately, RHAMM may be prove to be inserted into the membrane via a glycolipid tail. The RHAMM gene is comprised of 14 exons, 3 of which have been shown to be alternately spliced [Entwistle et al., 1995]. The encoded protein has two HA binding domains located in the carboxy terminal region. RHAMM has nine potential sites of N-glycosylation and like CD44 its potential for alternate splicing and post translational modification gives rise to numerous tissue and

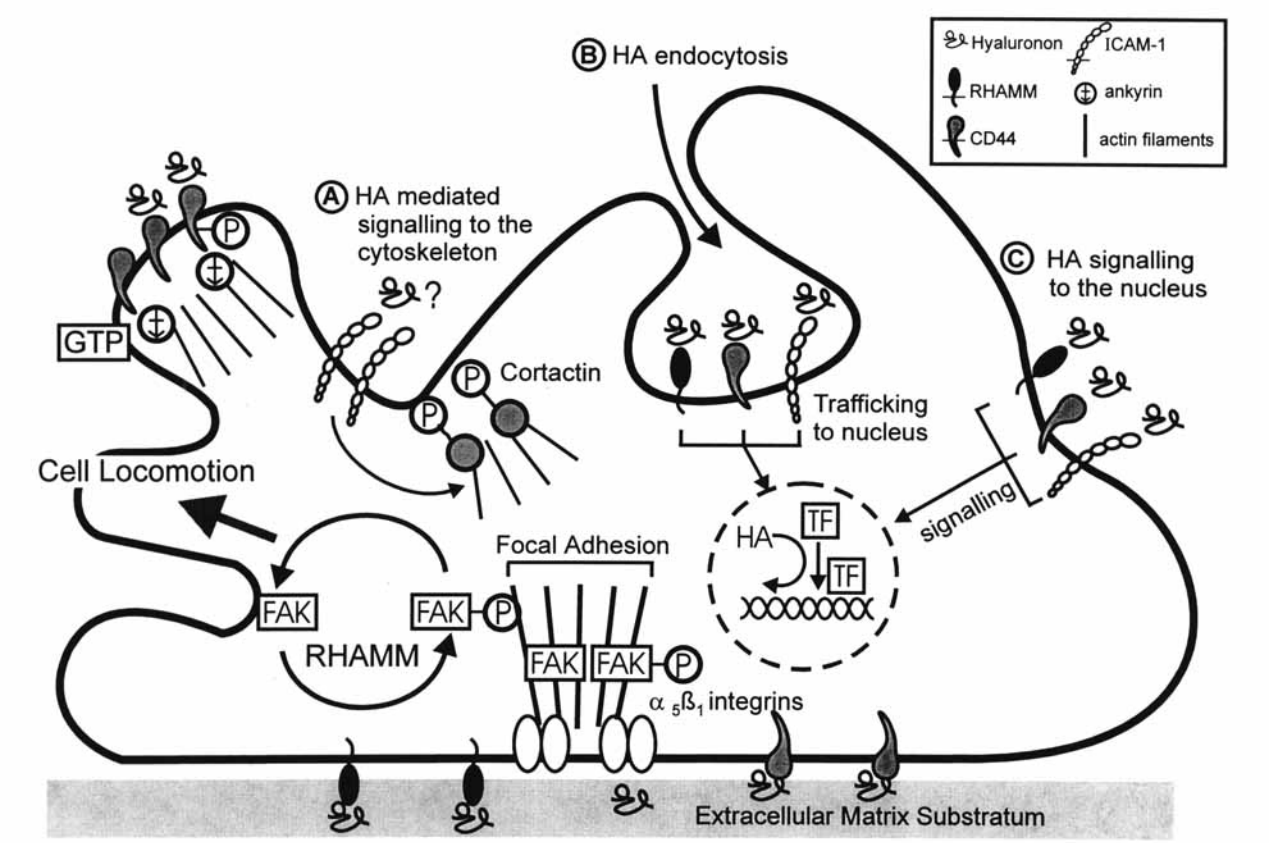

Fig. 1. Hyaluronan binding to the cell surface receptors CD44, RHAMM, and ICAM-1 results in (**A**) signal transduction to the cytoskeleton, (**B**) endocytosis and trafficking to the nucleus, and (**C**) signal transduction to the nucleus regulating transcription (see the text for references).

species specific protein species. The most commonly expressed form of RHAMM in untransformed murine tissue is a 70 kDa protein (RHAMM1). A 73 kDa RHAMM1v4 variant is also detected which is expressed at very low levels, however, its expression is markedly elevated in transformed cells and it is itself transforming when overexpressed in murine fibroblasts [Entwistle et al., 1995; Hall et al., 1995]. In addition, the injection of cells overexpressing this variant resulted in tumour formation when injected subcutaneously into mice and lung colonization after tail vein injection [Hall et al., 1995]. The hyaluronan:RHAMM signal transduction regulating cell motility and focal adhesion turnover is likely to be key to RHAMM's oncogenic role. Again, like CD44, mutation of the HA binding domains in RHAMM will completely abolish its functional roles [Yang et al., 1993]. The HA:RHAMM mediated signal cascade has been shown to be a prerequisite for initiating and maintaining *ras*-transformation [Hall et al., 1995]. The overexpression of a dominant negative form of the RHAMM receptor (i.e., one in which the HA binding domains are non functional) or the expression of antisense RHAMM resulted in the reversion of the *ras*-transformed phenotype. The reversion has been linked to the RHAMM mediated phosphorylation events involving $pp125^{FAK}$, the kinase present in focal adhesions described in the next section. The results indicate that FAK dephosphorylation acts downstream of ras and is required for transformation by this oncogene. Thus the effect of both RHAMM and CD44 in tumorigenesis is linked to both alternate splicing and hence alternate signal transduction pathways (Fig. 1).

ICAM-1, the intercellular adhesion molecule-1, has been demonstrated to be a third cell surface receptor for hyaluronan [McCourt et al., 1994]. ICAM-1 is a specific ligand for the β_2 integrins, LFA-1 (lymphocyte function-associated Ag-1) found on all types of leukocytes and the macrophage associated Mac-1. It is a glycosylated protein of 80–114 kDa which has a core polypeptide

of 55 kDa. It has an important role in leukocyte trafficking and cell-cell adherance, it is critical in the immunologic response and displays tissue specific and cytokine specific expression. It is expressed on epithelial cells, macrophages, and smooth muscle cells but generally at low level. Its expression is dramatically increased during the inflammatory response and by the cytokines IL-1 and TNF-α and IFN-γ [Dustin et al., 1986]. The gene comprises 7 exons, encoding a signal peptide, 5 Ig-like domains, a transmembrane domain [Voraberger et al., 1991], and at least 2 HA binding domains. Many of the adhesive functions of ICAM-1 are likely associated with HA binding. It has been shown that the predominant HA-binding protein on liver endothelial cells (LEC) is ICAM-1 [McCourt et al., 1994]. The major site for elimination of HA from the bloodstream is via receptor mediated endocytosis by LEC (Fig. 1B). It remains to be determined whether other cell types, expressing this receptor, are implicated in HA internalization and/or HA-mediated signal transduction. It is noteworthy that CD44 has also been reported to internalize HA, some of which can be trafficked to the nucleus or degraded (Fig. 1B).

SIGNAL TRANSDUCTION AND CYTOSKELETAL ASSOCIATION

The induction or cessation of cell migration and development of anchorage independent growth requires complex ECM-cell signalling that involves cell adhesion, de-adhesion, and cytoskeletal reorganization. HA-mediated RHAMM signalling induces a rapid, transient protein tyrosine kinase phosphorylation followed by a net dephosphorylation, events which culminate in a twofold increase in locomotion in fibroblasts [Hall et al., 1994]. This phosphorylation event affects several proteins and anti-phosphotyrosine staining demonstrates the post translational modification is mainly confined to focal adhesions (FA) in extending lamellae. Focal adhesions are points of adhesive contact between the cell and the ECM. Focal adhesions contain transmembrane receptors such as the β1 integrins which attach extracellularly to the ECM substratum and intracellularly to cytoskeletal components such as talin, vinculin, tensin, and actin filaments. In addition, they contain several kinases, including focal adhesion kinase ($pp125^{FAK}$), protein kinase C, and the tyrosine kinase oncoproteins c-src and c-abl. The phosphorylation of FAK appears to permit FA assembly, a process which is integrin dependent, while conversely, the disassembly of focal adhesions involves the dephosphorylation of FAK, an event that is integrin independent and apparently RHAMM dependent (Fig. 1A). Non-motile and anchorage dependant cells have stable FAs and are firmly locked onto the ECM substratum. Motile cells however exhibit FA turnover, which permits amoeboid movement across the ECM and anchorage independant proliferation via the undulating assembly and disassembly of FAs. Interestingly and consistent with its effect on locomotion and proliferation, RHAMM regulates the transient phosphorylation and net dephosphorylation of FAK (Table I). Disassembly of FAs also requires a protein tyrosine kinase that associates with FAK and is regulated by RHAMM. Whatever the mechanism, the evidence that RHAMM is required for turnover of focal adhesions is further established by the observation that cells in which RHAMM is ablated by antisense expression, exhibit large, stable FAs (Table I). Also, the expression of a dominant suppressor mutant of RHAMM (i.e., one in which the HA binding domains are mutated) prevents the above signalling events, reverts ras induced transformation, and results in cells with large FAs and low motility rates (Table I, Fig. 2) [Hall et al., 1995]. The involvement of HA:RHAMM in the turnover of focal adhesions may well be the basis for the role of RHAMM in tumorigenesis and this possibility predicts that this receptor will also play an important role in embryogenesis inflammation and wound repair.

The binding of HA to CD44 plays a crucial role in CD44 mediated cell-cell, cell-matrix adhesive interactions, and in signal transduction. These CD44 mediated events often involve post-transcriptional modifications of CD44, such as serine phosphorylation [Kalomiris and Bourguignon, 1989], palmitoylation [Bourguignon et al., 1991], and GTP binding [Lokeshwar and Bourguignon, 1992] that affect cytoskeletal association (Fig. 1A). Indeed, the integrity of the cytoskeleton has been reported to influence CD44:HA adhesion [Bourguignon et al., 1993], although there are some contradictions in the literature [Murakami et al., 1994]. In T-lymphocytes, HA:CD44 interactions signal a rapid increase in intracellular Ca^{2+} followed by a redistribution of CD44 into capped structures. The capped CD44 binds directly to ankyrin, a membrane cytoskeletal component which accumulates intracellularly under the structures (Fig.

TABLE I. The Effect of HA:RHAMM on Cell Motility, Signal Transduction, and Focal Adhesions*

Cell type	RHAMM expression	Motility rate	Signal transduction	Focal adhesions
10T½ fibroblasts	+	+	–	Stable focal adhesions
H-*ras*-10T½ fibrosarcoma	++++	+++	HA stimulates transient tyrosine phosphorylation followed by net dephosphorylation of several proteins including pp125^{FAK}	Focal adhesion turnover
RHAMM 1v4-transformed 10T½ fibroblasts	+++++	+++	ND	Focal adhesion turnover
Dominant negative RHAMM/H-*ras* 10T½	++++ No HA binding	+	Ablation of signal transduction; uniform FAK phosphorylation	Stable focal adhesions
Antisense RHAMM/H-*ras*-10T½ fibroblasts	–	±	ND	Large stable focal adhesions

*ND: not determined. RHAMM expression reflects relative amounts of RHAMM protein in cell lysates and on cell surface. Motility rate is expressed as relative random locomotion for 100 cells [see Hall et al., 1994, 1995] (Yang et al., manuscript submitted).

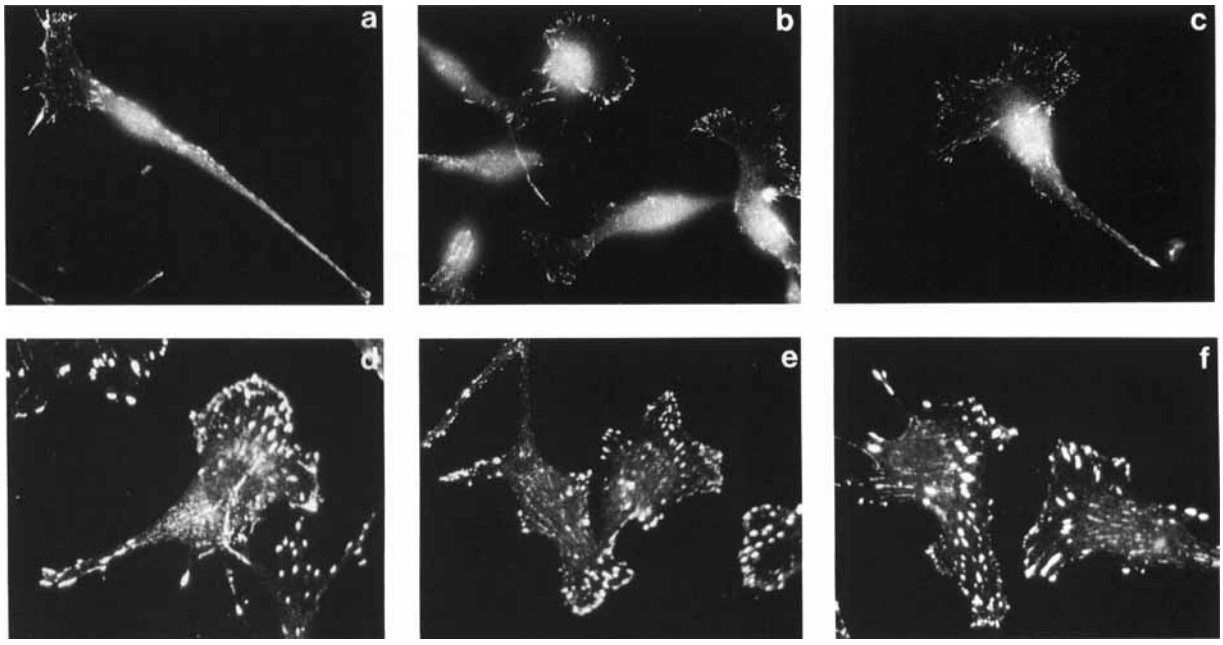

Fig. 2. RHAMM regulates cell locomotion and focal adhesion turnover. *Ras*-transformed 10T½ fibroblasts (**a–c**) or the same fibroblasts expressing a dominant suppressor mutant of RHAMM (**d–f**) were stained for the focal adhesion marker vinculin using indirect immunofluorescence [Hall et al., 1995]. The *ras*-transformed fibroblasts express RHAMM, bind HA, and show vinculim staining in podosomes (a–c) characteristic of locomoting fibroblasts. The same cells overexpressing an HA binding mutant of RHAMM, do not bind HA, and have larger, stable focal adhesions (d–f) resulting in a low motility rate.

1A) [Bourguignon et al., 1993]. Furthermore, colocalization of surface CD44 and intracellular ankyrin occur when cells attach to HA-coated plates indicating that soluble HA-mediated receptor capping resembles cell adhesion processes in these cells. Both microfilament disruptors such as cytochalasin D and inhibitors of calcium signalling, including Ca^{2+} channel blockers, Ca^{2+} chelators, and calmodulin inhibitors, significantly inhibit CD44 capping and adhesion

events [Bourguignon et al., 1993]. This suggests that the actomyosin contractile system regulated by Ca^{2+}/calmodulin is required for HA-mediated functions. In addition, deletion of the ankyrin binding domain of CD44 drastically reduces HA-binding and HA-mediated cell adhesion [Lokeshwar et al., 1994]. This suggests that membrane-associated ankyrin is directly involved in HA:CD44 adhesion and in linking CD44 to the cytoskeleton.

HA:CD44 interactions also cause the release of cytokines, IL-1β, TNF-α plus the associated protein synthesis of IGF-1 which has been shown to be TNF-α dependent (Fig. 1C). Since HA is often elevated in concert with these cytokines, the fibroproliferative action, ascribed to TNF-α in a wound response, may act through the production of IGF-1 by allowing competent cells to move through G_1. This signalling pathway could represent a step where excessive fibroproliferation in wound repair could be abrogated.

Like RHAMM and CD44, ICAM-1 has also been found to be associated with intracellular signalling events and the cytoskeleton. Association of ICAM-1 with the actin containing cytoskeleton has been shown to induce its distribution in microvilli and the uropod region on adherent COS cells and transformed B-cells, respectively [Carpen et al., 1992]. The actin binding protein α-actinin has been shown to associate with ICAM-1's cytoplasmic domain and to be codistributed suggesting anchorage between ICAM-1 and the cytoskeleton. Interestingly, there was no correlation in distribution between ICAM-1 and other cytoskeletal proteins such as talin, tensin, and vinculin [Carpen et al., 1992]. In addition, the activation of ICAM-1 by antibody crosslinking induces the tyrosine phosphorylation of several proteins coincidentally with src activation. The actin-binding protein cortactin, a $pp60^{src}$ substrate was identified as a substrate of this pathway (Fig. 1A) [Durieu and Trautmann et al., 1994]. While involvement of HA in these processes has not yet been demonstrated, the association of ICAM-1 with the cytoskeleton suggests yet another mechanism by which HA could influence cell adhesion, shape, and migration.

REGULATION OF HA BINDING CAPABILITY OF RECEPTORS

The lack of sequence homology between the HA-binding proteins described indicates that they are linked only by their common ability to bind HA. Although the specific HA binding sequence is variable, a common hyaluronan-binding motif has been identified ($B[X_7]B$), where B is arginine or lysine, X is any non acidic amino acid and there is at least one additional basic amino acid within or adjacent to the basic motif [Yang et al., 1993, 1994]. It is noteworthy that many HA binding proteins have affinity for other GAGs and indeed other ligands. Thus the HA binding domains of RHAMM will bind both hyaluronan and heparin but not chondroitin sulphate or dermatan sulphate [Yang et al., 1994]. The transforming RHAMM1v4 isoform appears to be more HA responsive than the non-transforming RHAMM1 and it is presently unknown whether conformational changes are conferring a greater affinity for HA. CD44 has three HA binding domains, two in the extracellular portion and one in the cytoplasmic tail [Yang et al., 1994]. CD44 however binds to both hyaluronan and chondroitin sulfate when present within the cell membrane but will also recognise chondroitin sulphate when in solution. The regulation of HA binding to ICAM-1 is not yet clear, however, it has been reported that ICAM-1 is alternately spliced and certain isoforms do not bind HA (Aruffo, personal communication). The newly isolated chick Cdc37 homologue possesses one HA binding domain and will also recognize chondroitin sulphate and heparin [Grammatikakis et al., 1995]. Interestingly, the hyaluronan binding domains of many of the extracellular matrix proteins, such as link protein, appear to be specific for hyaluronan.

Sequences other than the HA binding domains have been shown to be involved with ligand binding. In CD44 the HA binding domains alone will not permit ligand binding; the presence of the loop structure and also a very specific glycosylation pattern are required. Although almost all lymphoid cells express CD44s, only a few have affinity for HA. The affinity for HA may be determined by isoform-ligand specificity, protein conformation, posttranslational modification, or the interaction with neighbouring cells. The activation of lymphocytes leads to an induction of HA binding and in monocytes HA stimulation results in the release of IL-1β, TNF-α transcription, and IGF protein synthesis. This indicates that although the cell surface receptors can bind HA they may be modified to become inactive which represents a potentially

important mode of regulation. Interestingly, soluble forms of both CD44 and RHAMM inhibit proliferation and migration and may also block signalling. RHAMM becomes increasingly shed from the cell surface as cell confluence is reached. Removal of this soluble RHAMM results in stimulation of cell motility (Entwistle et al., in preparation).

INTRACELLULAR HA BINDING PROTEINS

Extracellular HA-binding proteins have been extensively reviewed in the literature and include link protein, versican and aggrecan and hyaluronectin and will not be a focus of this paper. However, a newly described cDNA encoding the intracellular chick Cdc37 homologue [Grammatikakis et al., 1995] is the first report of an intracellular HA binding protein. This cDNA encodes a protein of 29 kDa, has an HA binding motif in the carboxy terminal region and, as expected, possesses neither signal peptide nor transmembrane domain. The protein is highly homologous with the central coding domain of the Drosophila cell cycle protein Cdc37 [Cutforth and Rubin, 1994] and has a more limited homology to yeast Cdc37 [Ferguson et al., 1986]. Cdc37 is thought to mediate cell cycle progression by influencing the active state of p34 (Cdc2) kinase [Reed, 1992; Boschelli, 1993; Cutforth and Rubin, 1994]. It is noteworthy that RHAMM, which also occurs intracellularly, has recently been shown to regulate the expression of Cdc2 [Mohapatra et al., 1996]. Interestingly, a cell surface protein having common epitopes with the chick Cdc37 has also been identified [Grammatikakis et al., 1995] and indeed many cell responses elicited by adding anti-chick Cdc37 antibody indicate the existence of a cell surface isoform. This would indicate that the chick Cdc37, like RHAMM, has both an intracellular and cell surface counterpart.

ROLE OF INTRACELLULAR FORMS OF HA RECEPTORS

Many representatives of the GAG family have been reported in the cell nucleus and cytoplasm including HA, chondroitin sulphate, dermatan sulphate, and heparan sulphate. This intracellular location is likely due to the internalization of GAGs and subsequent trafficking to the nucleus (Fig. 1B). Intracellular heparin has been reported to suppress mitogen induced entry of non-transformed cells through the G_0/G_1 phase or the G_1 to S phase by arresting them early in the cell cycle [Wright et al., 1989; Pukac et al., 1990; Busch et al., 1992]. The effects of heparin on cell proliferation have been correlated with alterations in the expression of trans-activating factors such as c-fos, c-jun, and c-myc. These results clearly indicate a regulatory role for heparin in altering transcription events involved in cell cycle progression. A role for hyaluronan in intracellular events has not been elucidated. However, it is intriging that RHAMM and Cdc37 occur intracellularly and that several regulatory proteins such as gelsolin and PKC exhibit well-defined HA binding motifs.

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