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A ménage à trois in B cells

Robbert van der Voort
Hepatocyte growth factor, Met, and CD44

A ménage à trois in B cells
Cover: Cluster of lymphocytes and a follicular dendritic cell stained for heparan sulfate proteoglycans (green), and for the FDC marker DRC-1 (red). Co-expression appears yellow.

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A ménage à trois in B cells

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Chapter 1

General introduction
Introduction

The immune system serves to protect organisms against pathogens such as bacteria, viruses, and parasites. Based on functional grounds, it can be divided in two branches: the innate and the adaptive immune system. The innate immune system is constitutional and acts as a first line of defence. It involves soluble factors, natural killer cells, and phagocytes, and does not improve on repeated contact with the same pathogenic factor (antigen [Ag]). The adaptive immune system, by contrast, will evoke a highly specific immune reaction. In addition, it will develop an immunological memory which will provide a quicker and enhanced immune response upon restimulation with the Ag. The most important players during an adaptive immune response are the T and B lymphocytes. While T lymphocytes are mainly involved in the destruction of infected cells, and in the regulation of the immune response, B cells are the producers of immunoglobulins (Igs). These Igs specifically recognize and bind Ag, leading to the destruction of the organisms or cells that express the Ag. Most B cells express Igs at their cell surface in a complex with several other proteins, the B cell antigen receptor (BCR). However, if B cells are activated by antigen, and differentiate into plasma cells, they no longer express BCRs, but secrete the Igs instead. Igs are composed of the unique combination of

![Diagram of Immunoglobulin (Ig)](image)

**Figure 1.** Schematic representation of an immunoglobulin (Ig). Two Ig heavy (IgH) chains pair with two Ig light (IgL) chains to form two identical antigen-binding sites. The constant (C\textsubscript{H} or C\textsubscript{L}) and variable (V\textsubscript{H} or V\textsubscript{L}) regions, the complementarity determining regions (CDRs), the framework regions (FRs), the hinge region (H), the antigen binding site, and the sulphate bridges (SS) are shown.
two identical heavy (IgH) and two identical light chains (IgL) (Fig. 1). Both the IgH and IgL chains consist of constant (C\textsubscript{H} or C\textsubscript{L}) and variable (V\textsubscript{H} or V\textsubscript{L}) regions (Fig. 1). While C regions mediate the expression at the cell membrane, the binding of complement, and the interaction with Ig-receptors on other cells, V regions interact with the Ag. The recognition of Ag is mainly mediated by three hypervariable domains of the V regions, the complementarity determining regions (CDRs) (Fig. 1). These CDRs are separated by less variable framework regions (FRs) which maintain the stability of the V regions (Fig. 1).

**B cell development**

Primary B cell development takes place in the bone marrow. Here, (committed) lymphoid progenitors differentiate into immature B cells. An important process during early B cell development is the rearrangement of the variable (V), diversity, and joining (J) gene segments of the IgH gene locus (Tonegawa, 1983; Alt et al., 1987). This will allow the B cell to express the pre-BCR, which is composed of the rearranged IgH chain complexed to a surrogate IgL chain (Melchers et al., 1994). Successful expression of the pre-BCR is indispensable for survival of the B cell. B cells lacking a pre-BCR, or expressing an aberrant pre-BCR, will die by apoptosis (Melchers, 1999). Signaling via the pre-BCR will induce rearrangement of the V and J gene segment of the IgL gene locus (Ten Boekel et al., 1995; Constantinescu and Schlissel, 1997). Adequate rearrangement, and successful complexation with the IgH chain, are required for B cell survival, and allows the B cell to express a BCR of the IgM isotype (Melchers et al., 1994).

The IgM-expressing B cell will now leave the bone marrow, and travel via the blood to secondary lymphoid organs, i.e. the spleen, lymph nodes, and mucosal-associated lymphoid tissue (MALT). In these organs, the B cells may be confronted with native Ag that is recognized by their cell surface-expressed immunoglobulins which form the B cell antigen receptor (BCR). In case of recognition, the B cells will internalize and process the Ag. At the border of the T cell area and the B cell area (follicle), the B cells will now present Ag-derived peptides on major histocompatibility complex (MHC) class II molecules to antigen receptors (TCRs) expressed by T helper cells that have been activated by dendritic cells (Lindhout et al., 1997; MacLennan et al., 1997; Steinman et al., 1997; Banchereau and Steinman, 1998; Garside et al., 1998). Upon recognition of the right peptide/MHC combination, the T cell will provide the B cell with co-stimulatory signals which are indispensable for further differentiation. These signals have been shown to critically depend on the interaction between CD40 and CD40L, and between CD28 and CD86 (Foy et al., 1994; Gray et al., 1994; Renshaw et al., 1994; Facchetti et al., 1995; Han et al., 1995; Ferguson et al., 1998).
Chapter 1

1996). The activated B cell can now develop into an antibody-producing plasma cell, or respond with a series of events known as the germinal center reaction (Fig. 2) (MacLennan, 1994, 1997; Nossal, 1994; Kelsoe et al., 1995; Kosco-Vilbois et al., 1997; Lindhout et al., 1997; Liu and Arpin, 1997). This process, which will lead to affinity maturation of the humoral immune response, is initiated by the migration of activated B cells into the primary follicle of the secondary lymphoid organ (Liu et al., 1991; Jacob et al., 1991a). Here, the activated B cells will initiate the development of a germinal center (GC). On histological grounds, GC are subdivided into a dark- and a light zone (Fig. 2) (Liu et al., 1992). In the GC dark zone, the B cells, which have become centroblasts, undergo rapid clonal expansion and start to somatically hypermutate the variable regions of their Ig genes (Berek et al., 1991; Jacob et al., 1991b; Küppers et al., 1993; McHeyzer-Williams et al., 1993; Pascual et al., 1994; Kelsoe, 1996). Camacho et al. (1998) demonstrated that in mice, clonal expansion and somatic hypermutation take place in the GC light zone as well. After expansion, and IgV hypermutation, the centroblasts migrate to the basal part of the germinal center light zone and differentiate into centrocytes. In the light zone the centrocytes will start to express their mutated slgs, and re-encounter their Ag in the form of immune complexes presented by follicular dendritic cells (FDCs) (Gray et al., 1984; Liu et al., 1992, 1996b; Caux et al., 1995; Lindhout et al., 1997; Liu and Arpin, 1997; Banchereau and Steinman, 1998). During this process, the apoptosis-sensitive centrocytes will become affinity selected (Liu et al., 1989; Tew et al., 1990; Hardie et al., 1993; Lindhout et al., 1997). Centrocytes expressing slgs with a high affinity for the Ag will survive, while centrocytes with low affinity slgs, and autoreactive centrocytes, will die by apoptosis. It has been demonstrated that strong adhesion, involving the adhesion molecules LFA-1 - ICAM-1, and α4β1 - VCAM-1 (see section Adhesion molecules and their ligands), is required for an effective interaction between the centrocyte and the FDC in vitro (Koopman et al., 1991, 1994; Lindhout et al., 1993). After their rescue from apoptosis, the centrocytes will retrieve and process some of the Ag, and present Ag-derived peptides in the context of MHC class II proteins to Ag-specific T cells present in the apical light zone of the GC. If these T cells, which have previously been activated by dendritic cells (Grouard et al., 1996), recognize the peptide-loaded MHC, they will provide the B cells with co-stimulatory signals via CD40-CD40L interaction and cytokine production (Lederman et al., 1992; Fuller et al., 1993; Casamayor-Palleja et al., 1995; Zheng et al., 1996). In addition, CD40-CD40L binding will downregulate the expression of the apoptosis-inducing FasL protein on the T cells (Rathmell et al., 1996). The stimulatory signals will eventually lead to Ig isotype switching and differentiation of the centrocytes into either memory B cells or Ig-secreting plasma cells (Kraal et al., 1982; Arpin et al., 1995; Liu et al., 1996a; Malisam et al., 1996). Finally, the
fully differentiated Ag-specific B cells will receive signals that mediate their export from the lymphoid tissues.

**Figure 2.** Schematic representation of the consecutive developmental steps that take place during the T cell-dependent B cell differentiation in secondary lymphoid organs. See the text for details. B, B cell; FDC, follicular dendritic cell; T, T cell.

### Adhesion and migration of B cells

Adhesion and migration are essential for the differentiation and functioning of B cells, since these cells have to migrate between different lymphoid tissues, and have to interact with many cell types. Due to the large number of possible migration pathways and cellular interactions, the adhesion and migration of B cells have to be tightly regulated. The cells of the immune system are equipped with a number of molecules that perform this task. While adhesion molecules and their ligands are involved in the adhesion and migration of B cells, several cytokines, in particular members of the chemokine family, attract B cells and in this way determine the direction of their migration. In the next sections these molecules will be discussed.
Adhesion molecules and their ligands

Selectins
The selectin family consists of three members: L-selectin, E-selectin and P-selectin (Table I) (for review see Lasky, 1992, 1995; Bevilacqua 1993; Rosen et al., 1994; McEver et al., 1995; Tedder et al., 1995; Rossiter et al., 1997). Structurally, they are characterized by the presence of an N-terminal lectin domain, an epidermal growth factor-like domain, a variable number of tandem consensus repeats homologues to complement-binding domains, and a transmembrane and short cytoplasmic domain (Fig. 3) (Bevilacqua et al., 1989; Johnston et al., 1989; Lasky et al., 1989; Siegelman et al., 1989; Tedder et al., 1989). Whereas E-selectin is expressed by cytokine-activated endothelial cells, P-selectin is present in the Weibel-Palade bodies of endothelial cells and in α-granules of platelets (Hsu-Lin et al., 1984; Stenberg et al., 1985; Pober et al., 1986; Bevilacqua et al., 1987; Bonfanti et al., 1989; Johnston et al., 1989; Leeuwenberg et al., 1989; McEver et al., 1989). Upon cytokine-activation P-selectin becomes rapidly, but transiently, expressed on the cell-surface (Hsu-Lin et al., 1984; McEver et al., 1989). In contrast to E- and P-selectin, L-selectin is expressed on lymphocytes and myeloid cells (Gallatin et al., 1983; Tedder et al., 1989, 1990; Bowen et al., 1990).

In the presence of calcium ions, selectins bind to sugar moieties present on glycoproteins (sialomucins) expressed on the surface of leukocytes or endothelial

Figure 3. Schematic representation of the four major families of adhesion molecules (not drawn to scale) involved in leukocyte adhesion and migration. See the text for details.
Table I. The selectin family of adhesion molecules.

| Name       | CD | Expression       | Ligand(s)                              | References                                    |
|------------|----|------------------|----------------------------------------|-----------------------------------------------|
| L-selectin | CD62L | leukocytes      | GlyCAM-1, CD34, MAAdCAM-1, PSGL-1, PCLP-1 | Lasky et al., 1992; Baumhueter et al., 1993; Berg et al., 1993; Guyer et al., 1996; Spertini et al., 1996; Tu et al., 1996; Sassetti et al., 1998 |
| E-selectin | CD62E | activated        | ESL-1, PSGL-1; CLA                      | Berg et al., 1991; Levinovitz et al., 1993; Walcheck et al., 1993; Alon et al., 1994; Asa et al., 1995; Steegmaler et al., 1995 |
| P-selectin | CD62P | activated        | PSGL-1                                 | Moore et al., 1992; Sako et al., 1993; Alon et al., 1994 |

| Name       | CD | Expression       | Ligand(s)                              | References                                    |
|------------|----|------------------|----------------------------------------|-----------------------------------------------|
| L-selectin | CD62L | leukocytes      | GlyCAM-1, CD34, MAAdCAM-1, PSGL-1, PCLP-1 | Lasky et al., 1992; Baumhueter et al., 1993; Berg et al., 1993; Guyer et al., 1996; Spertini et al., 1996; Tu et al., 1996; Sassetti et al., 1998 |
| E-selectin | CD62E | activated        | ESL-1, PSGL-1; CLA                      | Berg et al., 1991; Levinovitz et al., 1993; Walcheck et al., 1993; Alon et al., 1994; Asa et al., 1995; Steegmaler et al., 1995 |
| P-selectin | CD62P | activated        | PSGL-1                                 | Moore et al., 1992; Sako et al., 1993; Alon et al., 1994 |

1. CD, cluster of differentiation; CLA, cutaneous lymphocyte-associated antigen; E-selectin, endothelial selectin; ESL, E-selectin ligand; GlyCAM, glycosylation-dependent cell adhesion molecule; L-selectin, leukocyte selectin; MAAdCAM, mucosal addressin cell adhesion molecule; PCLP, podocalyxin-like protein; P-selectin, platelet selectin; PSGL, P-selectin glycoprotein ligand.

cells (Lasky, 1992, 1995; Nelson et al., 1995). Sialic acid, fucose, and sulfate have been demonstrated to be essential components of most of the carbohydrate structures recognized by the selectins (Brandley et al., 1990; Lowe et al., 1990; Walz et al., 1990). These structures have been shown to be closely related to the tetrasaccharides sialyl Lewis x (sLeX) and sialyl Lewis a (sLea) (Lasky, 1995). The carbohydrate ligands for the selectins are linked to several mucin-like molecules. For instance, L-selectin binds to saccharides attached to CD34, or to saccharides of the secreted glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), while all three selectins are able to interact with sugars on P-selectin glycoprotein ligand 1 (PSGL-1) (Table I) (Lasky et al., 1992; Moore et al., 1992; Baumhueter et al., 1993; Sako et al., 1993; Asa et al., 1995; Puri et al., 1995; Guyer et al., 1996; Spertini et al., 1996; Tu et al., 1996). Moreover, E-selectin interacts also with E-selectin ligand 1 (ESL-1), and with the cutaneous lymphocyte antigen (CLA) expressed by skin-homing T cells (Berg et al., 1991; Levinovitz et al., 1993; Walcheck et al., 1993; Asa et al., 1995; Steegmaler et al., 1995).

Selectins and their ligands are mainly involved in tethering (bouncing) and rolling of leukocytes on the endothelium (see also the section Lymphocyte
Table II. Immunoglobulin superfamily members involved in leukocyte adhesion

| Name      | CD     | Expression                        | Ligand(s)         | Reference(s) |
|-----------|--------|-----------------------------------|-------------------|--------------|
| ICAM-1    | CD54   | leukocytes, endothelium, dendrite cells | αLβ2, αMβ2, CD13  | Makgoba et al., 1988; Staunton et al., 1988; Diamond et al., 1990; Staunton et al., 1989 |
| ICAM-2    | CD102  | leukocytes, endothelium            | αLβ2              | Fawcett et al., 1992; Vareux et al., 1992; Gentenbeek et al., 2000 |
| ICAM-3    | CD50   | leukocytes                         | αLβ2 DC-SIGN      |              |
| ICAM-4    | nd     | erythrocytes                       | αLβ2              | Batly et al., 1995 |
| ICAM-5    | nd     | telencephalic neurons               | αLβ2              | Tian et al., 1997; Morano et al., 1997 |
| VCAM-1    | CD106  | activated endothelium, dendrite cells | α4β1              | Oshorn et al., 1989; Elices et al., 1990 |
| MAdCAM-1  | nd     | mucosal endothelium, mucosal dendrite cells | α4β7; L-selectin | Briskin et al., 1993; Berg et al., 1993; Berlin et al., 1993; Shyn et al., 1996; Szabo et al., 1997 |
| PECAM-1   | CD31   | leukocytes, endothelium            | PECAM-1, αβ3, GAGs | Albida et al., 1991; Maller et al., 1992; DeLisser et al., 1993 |
| ALCAM     | CD166  | activated T cells, B cells, monocytes | ALCAM, CD6        | Uchida et al., 1997; Bowen et al., 1995 |

ALCAM, activated leukocyte cell adhesion molecule; CD, cluster of differentiation; GAG, glycosaminoglycan; ICAM, intercellular cell adhesion molecule; LFA, lymphocyte function-associated antigen; L-selectin, leukocyte selectin; MAdCAM, mucosal addressin cell adhesion molecule; nd, not determined; PECAM, platelet-endothelial cell adhesion molecule; VCAM, vascular cell adhesion molecule.

extravasation: a multistep process). Several characteristics make these molecules well suited for these loose and reversible interactions. For example, the bonds between selectins and their carbohydrate ligands have low association and dissociation constants. Moreover, the high concentration of L-selectin and other tethering receptors, e.g. PSGL-1, on the tips of the lymphocyte’s microvilli enhances the efficiency of the brief interactions with their ligands (von Andrian et al., 1995). Finally, the rapid cleavage of L-selectin from the cell-surface upon
cross-linking, ensures the reversibility of the binding process (Palecanda et al., 1992).

**Immunoglobulin superfamily**

The members of the immunoglobulin superfamily (IgSF) are characterized by the presence of so-called immunoglobulin (Ig) domains. These Ig-domains are composed of about 100 amino acids folded in a loop by disulfide bonds (Fig. 3). Beside well-known family members like the IgGs themselves, the T cell receptor, CD3, and the major histocompatibility complexes, this family also contains several proteins involved in cell adhesion (Table II). IgSF adhesion molecules can be expressed by a broad array of cell types (Table II) (for review see Wang and Springer, 1998). For instance, intercellular cell adhesion molecule 1 (ICAM-1) can be expressed by leukocytes as well as by activated endothelial cells, dendritic cells, and epithelial cells (Dustin et al., 1986). Often the level of expression is regulated by inflammatory mediators, e.g. both IL-1 and interferon γ induce the the expression of ICAM-1 on endothelial cells (Dustin et al., 1986).

Many of the IgSF adhesion molecules are involved in the interaction between leukocytes and endothelial cells. During most of these interactions the IgSF members bind to activated integrins (Table II). Since these interactions are often of high affinity, they are mainly involved in the tight adhesion of leukocytes to the endothelium (see also the section Lymphocyte extravasation: a multistep process). Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is an exceptionally member in this respect, because this molecule binds both L-selectin via carbohydrate moieties, and the integrin α4β7 via Ig-domains (Berg et al., 1993; Berlin et al., 1993).

**Integrins**

The integrin family is composed of related type I transmembrane proteins (for review see Hynes, 1992; Schwartz et al., 1995; Shimizu et al., 1999). These proteins are heterodimers consisting of an α chain non-covalently associated with a β chain (Fig. 3). So far 17 α and 8 β subunits have been characterised in humans (Shimizu et al., 1999). Since most, if not all, β subunits can associate with several different partner subunits, more than 20 different heterodimers have been detected at present. Based on their common β chains, eight integrin subfamilies have been defined. Some of these subfamilies, e.g. the β1 subfamily, are widely expressed. Members of the β2 subfamily are only expressed by leukocytes (Table III). B cells are able to express the integrins α4β1, α5β1, αLβ2, and α4β7 (Freedman et al., 1990; Koopman et al., 1991; Roldan et al., 1992; Arroyo et al., 1996; Drillenburg
Table III. The major integrins involved in leukocyte adhesion

| Name | Synonym(s) | CD | Expression | Ligand(s) | Reference(s) |
|------|------------|----|------------|-----------|--------------|
| α1β1 | VLA-1      | CD49a/CD29 | broad       | laminin, collagen | Goodman et al., 1991 |
| α2β1 | VLA-2      | CD49b/CD29 | broad       | laminin, collagen | Ilies et al., 1989 |
| α3β1 | VLA-3      | CD49c/CD29 | broad       | laminin, collagen, fibronectin, epiligrin | Carter et al., 1994; Lakada et al., 1991 |
| α4β1 | VLA-4      | CD49d/CD29 | broad       | fibronectin, VCAM-1 | Ilies et al., 1990 |
| α5β1 | VLA-5      | CD49e/CD29 | broad       | fibronectin | Pytel et al., 1985 |
| α6β1 | VLA-6      | CD49f/CD29 | broad       | laminin | Soumenberg et al., 1988 |
| α5β2 | LFA-1      | CD11a/CD18 | leukocytes  | ICAM-1, 2, 3, 4, 5 | Martin et al., 1985; Makgoba et al., 1997; Stuover et al., 1989; Lawett et al., 1989; Bailey et al., 1998; Tian et al., 1999 |
| αMβ2 | Mac-1, CR3 | CD11b/CD18 | monocytes, macrophages, granulocytes, lymphocytes | ICAM-1, 2, fibrinogen, tC3b, Factor 
X, glycoprotein lba, CD23, fibrinogen, iC3b, CD23 | Alluri and Edgerton, 1988; Wright et al., 1988; Diamond et al., 1990; Leen et al., Hench et al., 1995; Xie et al., 1998; Simon et al., 2000; Macklin et al., 1985; Myones et al., 1988; Liske et al., 1991; Hench et al., 1991; Leen et al., Hench et al., 1995 |
| αXβ2 | p150, 95   | CD11c/CD18 | macrophages, monocytes, granulocytes | MAdCAM-1, fibronectin, VCAM-1 | Ruege et al., 1992; Berlin et al., 1993, 1995 |
| α4β7 | LPAM-1     | CD49d/nd  | lymphocytes | MAdCAM-1, fibronectin, VCAM-1 | Cepek et al., 1994 |
| α1Eβ7 | HML-1      | CD103/nd  | lymphocytes | E cadherin | |

1. CD, cluster of differentiation; E cadherin, epithelial cadherin; HMLI, human mucosal lymphocyte; ICAM, intercellular cell adhesion molecule; LFA, lymphocyte function-associated antigen; LPAM, lymphocyte Peyer’s patch adhesion molecule; Mac, macrophage; MAdCAM, mucosal addressin cell
adhesion molecule; nd, not determined; VCAM, vascular cell adhesion molecule; VLA, very late activation antigen.

et al., 1997). However, their expression is dependent on the differentiation state and site of origin of the B cell (Horst et al., 1991; Shimizu et al., 1999; Butcher and Picker, 1996; Butcher et al., 1999).

Most integrins can bind to several different ligands (Table III). Many of these ligands are molecules of the extracellular matrix, e.g. fibronectin and laminin, while others are cell surface-expressed members of the immunoglobulin superfamily, e.g. ICAM-1 and VCAM-1 (see Immunoglobulin superfamily). In order to bind their ligand, integrins require metabolic energy, the presence of extracellular Ca^{2+} and/or other divalent cations like Mg^{2+} or Mn^{2+} (Hynes, 1992; Schwartz et al., 1995; Shimizu et al., 1999). Generally, leukocyte integrins reside in a resting state and have to be activated before they can bind their ligand. This activation may involve enhanced affinity due to a conformational change of the integrin, and/or enhanced avidity resulting from the recruitment of integrin molecules in adhesion complexes (Shimizu et al., 1999). Many studies have indicated that activation of signaling molecules, and alterations in the organization of the cytoskeleton are involved in integrin-dependent adhesion (Schwartz et al., 1995; Hemler, 1998; Giancotti and Ruoslahti, 1999). The mechanisms involved in these (transient) processes are as yet not completely clear. However, it has been demonstrated that activation of several cell surface receptors, including the TCR, MHC class II, and CD44, leads to activation of integrins (Dustin and Springer, 1989; van Kooyk et al., 1989; Koopman et al., 1990; Mourad et al., 1990; Alexander et al., 1993; Vermotdesroches et al., 1995). In addition, cytokines, in particular the chemokines, have been shown to induce integrin-dependent adhesion (Schwartz et al., 1995; Baggioolini, 1997, 1998) (see also the section Regulation of B cell adhesion and migration). Hence, intracellular signals evoked by cell surface-expressed receptors can lead to integrin activation, a process known as inside-out signaling (Hynes, 1992; Schwartz et al., 1995). Alternatively, outside-in signaling, i.e. stimulation of integrins via external factors, can lead to signals that influence many biological processes like adhesion, proliferation, cytokine production, and apoptosis (Hynes, 1992; Koopman et al., 1992, 1994; Schwartz et al., 1995; van Kooyk et al., 1998; Shimizu et al., 1999).

**CD44**

CD44 represents a large family of cell-surface expressed glycoproteins encoded by one gene which consists of 19 exons (Screaton et al., 1992; Lesley et al., 1993) (Figs. 3 and 4). Through alternative RNA-splicing, involving at least 10 exons,
many different CD4 isoforms can be generated (Goldstein et al., 1989; Dougherty et al., 1991; Günthert et al., 1991; Stamenkovic et al., 1989, 1991; Jackson et al., 1992; Tölö et al., 1993). In addition to alternative splicing, post-translational modifications contribute to the diversity of the CD44 family. These modifications can result in isoforms modified with N- and/or O-linked glycans, and/or glucosaminoglycan (GAG) chains like chondroitin sulfate, heparan sulfate and dermatan sulfate (Jalkanen et al., 1988; Brown et al., 1991; Jackson et al., 1995; Takahashi et al., 1996). Members of the CD44 family can be expressed by almost all cell types (Lesley et al., 1993). Most cells express the 90 kDa "standard" (CD44s) CD44 isoform. This protein is the smallest CD44 isoform and does not contain any of the alternative domains. However, several cell types, including activated lymphocytes and endothelial cells, epithelial cells, and tumor cells, also express CD44 splice variants with molecular weights of up to 200 kDa (Pals et al., 1989b; Dougherty et al., 1991; Günthert et al., 1991; Hofmann et al., 1991; Stamenkovic et al., 1991; Arch et al., 1992; Jackson et al., 1992; Heider et al., 1993; Koopman et al., 1993; Fox et al., 1994; Mackay et al., 1994; Griffioen et al., 1997).

Through alternative splicing of exon 18, CD44 proteins can have a short (3 amino acids) or long (70 amino acids) cytoplasmic tail (Fig. 4) (Goldstein et al., 1989; Stamenkovic et al., 1989; Sercaton et al., 1992). CD44 isoforms with the long cytoplasmic tail have been shown to interact with several components of the cytoskeleton, including actin, ankyrin, and the linker proteins ezrin, radixin, and moesin (Tarone et al., 1984; Lacy and Underhill, 1987; Kalomiris and Bourguignon, 1988; Isacke, 1994; Tsukita et al., 1994). The association between CD44 proteins and the cytoskeleton has been suggested to regulate the binding between CD44 and its extracellular ligand hyaluronic acid (HA, see below) (Lesley et al., 1992; Thomas et al., 1992). In addition, cross-linking of CD44, or binding to HA, have been demonstrated to induce signal transduction resulting in, for instance, proliferation, adhesion or cytokine production (Heet et al., 1989; Shimizu et al., 1989; Koopman et al., 1990; Rothman et al., 1991). As yet, not much is known about the signal transduction pathways activated by CD44. However, in T cells CD44-induced signal transduction was shown to involve the Src family tyrosine kinases lek and fyn (Taher et al., 1996; Illangumaran et al., 1998). Recently, Ras, PKC, and IκB were demonstrated to be involved in the activation of NF-κB induced by the binding of CD44 to HA (Fitzgerald et al., 2000).

The CD44 family has been implicated in a number of important physiological and pathological processes (Lesley et al., 1993). For instance, CD44 isoforms play a role in migration, lymphocyte activation, hematopoiesis, development, and autoimmune diseases (Jalkanen et al., 1986, 1987; Koopman et al., 1990; Miyake et al., 1990a; Haynes et al., 1991; Camp et al., 1993; Wheatley
Figure 4. Schematic representation of the CD44 gene and proteins it encodes. As a result of alternative mRNA splicing both the extracellular domain and the cytoplasmic tail can vary in size. The alternatively spliced exons are indicated by open boxes. The human v1 exon contains a stop codon. All (putative) glycosylation sites of the CD44 protein are indicated: O-linked glycosylation sites (open circles); N-linked glycosylation sites (closed circles); chondroitin sulphate-attachment sites (open diamonds); heparan sulphate (HS)-attachment site (rod). In addition, the hyaluronic acid-binding sites (black lines), the disulfide bonds (S-S), the ankyrin-binding site (---), the ezrin-binding site (gray line), the phosphorylation sites (P), and the putative interaction site for the src-family kinase p56\textsuperscript{Lck} are indicated.
Moreover, experimental and clinical data suggest that several CD44 isoforms are involved in tumorigenesis and metastasis (Herrlich et al., 1993; Naor et al., 1997; Drillenburg and Pals, 2000; Wielenga et al., 2000). For instance, CD44s was shown to enhance the migration of melanoma cells in vitro, as well as the local tumor formation and metastatic capacity of lymphoma cells in nude mice (Sy et al., 1991, 1992; Thomas et al., 1992). Interaction of CD44s with HA (see below) was shown to be important in these tumor models (Thomas et al., 1992; Bartolazzi et al., 1994). In other studies, expression of CD44 splice variants containing the domain encoded by exon v6 was shown to confer a metastatic potential to rat pancreatic carcinoma cells (Güntert et al., 1991). Several clinical studies demonstrated that enhanced expression of CD44 by non-Hodgkin's lymphomas is associated with tumor dissemination and an unfavorable prognosis (Pals et al., 1989a; Horst et al., 1990; Jalkanen et al., 1991; Stauder et al., 1995; Drillenburg and Pals, 2000). Furthermore, high expression of CD44 proteins by colorectal carcinomas was reported to be correlated with a more malignant tumor phenotype and with tumor-related death (Wielenga et al., 1993, 1998; Mulder et al., 1994, 1995; Orzechowski et al., 1995; Imazeki et al., 1996; Yamaguchi et al., 1996).

One extensively studied interaction is that between CD44, mainly CD44s, and the proteoglycan hyaluronic acid (HA) (Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990b; Lesley et al., 1990, 1993; Kincade et al., 1997; Siegelman et al., 1999). HA is a major component of the extracellular matrix (ECM) and plays a role in cell migration and differentiation (Knudson and Knudson, 1993). The N-terminal CD44 domain, which is homologues to the HA-binding domains of the cartilage link and core proteins, mediates the binding to HA (Peach et al., 1993). Although some cell lines and tumor cells bind HA constitutively, CD44 on most normal cells needs to be activated before it can mediate adhesion to HA (Lesley et al., 1990, 1994; Murakami et al., 1990; Lesley and Hyman, 1992; Liao et al., 1993; DeGrendele et al., 1997b; Maita et al., 1998). So far, three distinct mechanisms have been shown to regulate CD44 – HA interaction: First, differences in glycosylation of CD44 can result in cell-type-dependent differences in HA binding. For instance, the presence of GAG chains, or N- or O-linked saccharides on CD44 proteins regulates their ability to bind HA (Katoh et al., 1995; Bartolazzi et al., 1996; Takahashi et al., 1996; English et al., 1998; Skelton et al., 1998). Also, sialylation of saccharides attached to CD44 was shown to be an important factor (Katoh et al., 1999). Furthermore, the proinflammatory cytokine tumor necrosis factor α (TNF-α) was demonstrated to convert CD44 from its inactive, nonbinding form to its active form by inducing sulfation of CD44, resulting in enhanced binding to HA and to vascular endothelial cells (Maiti et al., 1998). Second, alternative splicing of CD44 mRNA was shown to influence the affinity for HA.
presumably by inducing conformational changes or by attachment of saccharides to de novo expressed CD44 domains (Stamenkovic et al., 1991; Bennett et al., 1995a; this thesis). Third, clustering of CD44 was shown to increase HA binding capacity (Sieeman et al., 1996). Although some reports suggest that sequences in the cytoplasmic tail and/or transmembrane domain of CD44 are also required for the binding to HA, this issue remains controversial (He et al., 1992; Lesley et al., 1992, 2000; Thomas et al., 1992; Isacke, 1994; Lokeshwar et al., 1994; Perschl et al., 1995; Puré et al., 1995; Uff et al., 1995; Liu and Sy, 1996; Leg and Isacke, 1998; Liu et al., 1998).

Functional studies indicate that triggering of the T cell receptor induces CD44-dependent rolling of T cells on purified or endothelial-expressed HA (DeGrendele et al., 1996, 1997b; Siegelman et al., 1999) (see also the section Lymphocyte extravasation: a multistep process). In animal models this interaction was shown to play an important role in the extravasation of activated T cells into sites of inflammation (DeGrendele et al., 1997a).

In addition to HA, fibronectin, collagen, laminin, the cytokine osteopontin, and the proteoglycan serglycin were also reported to bind to CD44 (Wayner and Carter, 1987; Jalkanen and Jalkanen, 1992; Faassen et al., 1992, 1993; Toyama-Sorimachi et al., 1995; Weber et al., 1996; Smith et al., 1999). As yet it is not known which specific CD44 isoforms bind these molecules and what the functional consequence of their interaction is.

An interesting interaction is that between CD44 and “heparin-binding” cytokines. Tanaka et al. (1993b) demonstrated that purified CD44 molecules were able to bind the chemotactic cytokine (chemokine) macrophage inflammatory protein 3α (MIP-3α) and to present it to T cells. Presentation of MIP-3α resulted in enhanced integrin-dependent adhesion of the T cells and was shown to be HS-dependent. Also fibroblast growth factor 2 (FGF-2) and heparin-binding epidermal growth factor (HB-EGF) were reported to bind to CD44 proteins in a heparan sulfate (HS)-dependent way (Bennett et al., 1995b). Moreover, binding and presentation of FGF-8 by HS-modified CD44 (CD44-HS) may be involved in limb bud formation during vertebrate development (Sherman et al., 1998). It was shown that only CD44 isoforms expressing the domain encoded by exon v3 can be modified with HS and hence are able to bind cytokines like FGF-2 and HB-EGF (Bennett et al., 1995b; Jackson et al., 1995; this thesis). Covalent attachment of HS occurs at an evolutionary conserved SGSG consensus motif present in the CD44v3 domain (Bourdon et al., 1987; Greenfield et al., 1999). The presence of several specific amino acids flanking the SGSG motif has been shown to be essential for the modification with HS (Greenfield et al., 1999). In addition to the attachment of HS, all CD44 isoforms can be modified with the GAG chondroitin sulfate (CS) (Jalkanen et al., 1988). Modification with CS takes place at the SGSG site in the v3 domain, but also at an SG site present in the domain encoded by the constant CD44
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Interestingly, CS attached to CD44 mediates the binding of IFN-γ and probably some of its biological functions, including the suppression of cell proliferation, and the induction of major histocompatibility complex II antigens on vascular smooth muscle cells. The chemokine RANTES (regulated upon activation, normally T cell expressed and secreted) was shown to bind both CS and HS attached to recombinant CD44 molecules, resulting in enhanced T cell activation (Wolff et al., 1999).

Surprisingly, despite the broad expression and functionality of the CD44 family, Schmits and colleagues (1997) observed only minor defects in mice carrying a disrupted CD44 gene. Although these mice could not express any CD44 isoforms, they were born in a Mendelian ratio and showed no obvious developmental abnormalities. More detailed analysis demonstrated minor impairments in hematopoietic progenitor distribution and an exaggerated granuloma formation during infection. An independent study using CD44-deficient mice showed that two small T cell subsets, CD4⁺CD25⁺ and CD8⁺CD25⁺, are lost in the thymus (Protin et al., 1999). Therefore, the authors hypothesized that CD44 isoforms are involved in the recirculation and/or survival of these T cell subsets. In addition, a study using mice bearing a keratinocyte-specific antisense CD44 transgene demonstrated that these mice suffered from a disrupted HA metabolism in their skin and an impaired proliferation of their keratinocytes (Kaya et al., 1997). These latter results indicate that for investigating the functions of the CD44 family, the generation of conditional knock-out mice is desirable.

Lymphocyte extravasation: a multistep process

Lymphocytes entering lymphoid tissues via the blood have to migrate between the endothelial cells lining the vessel wall, a process that generally takes place at specialised sites of the blood circulation known as high endothelial venules (HEVs). HEVs are located within secondary lymphoid organs, but often also develop at sites of inflammation (Pals et al., 1989c; Kraal and Mebius, 1997). HEVs are lined by cuboid endothelial cells which are well equipped with adhesion molecules to facilitate lymphocyte transmigration. As shown in Fig. 5, lymphocyte extravasation is a multistep process (Butcher, 1991; Springer, 1994). During the first step leukocytes tether and subsequently roll on the endothelial cells of post-capillary venules. These events are transient and reversible, and mainly involve the (weak) interaction between selectins and their cognate carbohydrate ligands (sialomucins). Rolling slows the leukocyte down, and allows it to sample the environment for activating and/or chemotactic factors. If these factors, predominantly chemokines, are indeed present on the endothelial cells, this leads to
the activation of the leukocyte, the second step. There is now increasing evidence that the activating chemokines are bound and presented by heparan sulfate proteoglycans (HSPGs) (see below) expressed by the endothelial cells. Chemokine presentation would highly increase the number of chemokine – receptor interactions, and consequently increase the chance to activate the leukocyte (Tanaka et al., 1993a). Activation of the leukocyte results in step three: strong and sustained adhesion, predominantly caused by the activation of integrins which now bind to members of the immunoglobulin superfamily (IgSF) expressed by the endothelial cells. The last step involves the transendothelial migration of the leukocyte. It has been suggested that the IgSF-member PECAM-1/CD31 (see section Adhesion molecules and their ligands – Immunoglobulin superfamily) plays an important role during this process (Muller et al., 1993; Wakelin et al., 1996).
Interestingly, the extravasation of lymphocytes is often tissue-specific, a phenomenon known as ‘homing’ (Springer, 1994; Butcher and Picker, 1996; Salmi and Jalkanen, 1997; Butcher et al., 1999). Homing of lymphocytes is mediated by adhesion molecules, the “vascular addressins”, expressed on endothelial cells present in specific regions of the body. So far, specific homing to the skin, gastrointestinal tract, central nervous system and peripheral lymph nodes have been described. For instance, the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) mediates the binding of gut-homing lymphocytes by interacting with the integrin α4β7 (Berlin et al., 1993).

Regulation of B cell adhesion and migration

Chemokines

The chemokines are a group of small secreted cytokines with chemotactic capacity (Baggiolini, 1997, 1998; Mantovani, 1999; Zlotnik and Yoshie, 2000). In addition, chemokines are involved in proliferation, apoptosis, and angiogenesis (D’Souza and Harden, 1996; Baggiolini, 1997, 1998; Berger et al., 1999). Chemokines are being secreted by a wide variety of cell types, including lymphocytes, dendritic

Table IV. Chemokine receptors expressed on B cells.

| Receptor | B cell subpopulations | Ligands(s) |
|----------|-----------------------|------------|
| CCR2     | naive and activated B cells | MCP-1, 2, 3, 4, 5 |
| CCR5     | immature, naive and activated B cells | RANTES, MIP-1α, 1β |
| CCR6     | naive and activated B cells | MIP-3α |
| CCR7     | immature, naive and activated B cells | MIP-3β, SLC |
| CXCR2    | naive and activated B cells | IL-8, GROα, β, γ, NAP-2, ENA-78, GCP-2, LIX |
| CXCR4    | immature, naive and activated B cells | SDF-1 |
| CXCR5    | naive and activated B cells | BCA-1 |

BCA, B cell attracting chemokine; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; ENA, epithelial neutrophil activating protein; GCP, granulocyte chemotactic protein; GRO, growth related oncogene; IL, interleukin; LIX, lipopolysaccharide induced CXC chemokine; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NAP, neutrophil activating protein; RANTES, regulated upon activation, normally T cell expressed and secreted; SDF, stromal cell derived factor; SLC, secondary lymphoid tissue chemokine.
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cells and stromal cells. To date, almost 50 chemokines have been characterized in
man (Mantovani, 1999; Zlotnik and Yoshie, 2000). Chemokines are at least 20% homologues to each other and have been divided into 4 subgroups defined on the
basis of a conserved cysteine motif near the N-terminus: C, CC, CXC, or CX3C.
Chemokines can also be subdivided into homeostatic chemokines and inflammatory chemokines (Mantovani, 1999; Lindhout et al., 1999; Zlotnik and
Yoshie, 2000). Generally, homeostatic chemokines regulate the homing of lymphocytes to lymphoid organs/microenvironments, while inflammatory
chemokines are involved in the transient recruitment of leukocytes to sites of
inflammation.
All chemokines bind and activate seven-transmembrane-spanning receptors
expressed by many cells of the immune system, including all white blood cells, and
dendritic cells (Baggiolini, 1997, 1998; Lindhout et al., 1999; Mantovani, 1999).
Binding of a chemokine to its receptor induces G protein-dependent signal
transduction, resulting in rearrangement of the cytoskeleton, and in integrin
activation, ultimately leading to chemotaxis or other biological responses (Ben-
Baruch et al., 1995; Bokoch, 1995; Kuan et al., 1996; Laudanna et al., 1996).
B cells can express several chemokine receptors including CCR7, CXCR4,
and CXCR5 (see Table IV). CCR7 and its ligand macrophage inflammatory protein
3β (MIP-3β) attract B and T cells, but not monocytes or granulocytes (Kim et al.,
1998; Ngo et al., 1998). A second agonist for CCR7 is secondary lymphoid tissue
chemokine (SLC). SLC is expressed by endothelial cells of HEVs and by stromal
cells in the T cell areas of secondary lymphoid organs (Gunn et al., 1998b; Nagira
et al., 1998). Both human and murine SLC are potent attractants for T cells and B
cells (Gunn et al., 1998b; Nagira et al., 1997). Nagira et al. (1998) demonstrated
that SLC is able to induce the transendothelial migration of large numbers of B
cells and T cells in vitro. Remarkably, it was shown that rolling of T cells on HEVs
of murine Peyer’s patches is dependent on SLC/CCR7, while rolling of B cells is
not (Warnock et al., 2000). Mice deficient in CCR7 demonstrate a disturbed
microarchitecture of all secondary lymphoid organs, probably as a result of
impaired entry and retention of B cells, T cells, and dendritic cells (Förster et al.,
1999). The deranged architecture of lymphoid organs in CCR7-deficient mice
resulted in delayed humoral responses against DNP-KLH, and in absent contact
sensitivity or delayed type hypersensitivity reactions.
CXCR4 and its ligand stromal cell-derived factor 1 (SDF-1) have also been
described to be important for B cell functioning. SDF-1 was originally identified as
a pre-B cell growth-stimulating factor (Nagasawa et al., 1994). Subsequently, it
was demonstrated that it also acts as a chemoattractant for both lymphocytes and
monocytes and that it attracts naive and memory B cells, but not germinal center B
cells (Bleul et al., 1996, 1998; D’Apuzzo et al., 1997). Interestingly, by promoting
CXCR4 internalization, activation of the B cell antigen receptor (BCR) inhibits
SDF-1-induced chemotaxis (Guinamard et al., 1999). In addition to mediating B cell migration, CXCR4 and/or SDF-1 are involved in B lymphopoiesis and myelopoiesis, angiogenesis, and cardiac and cerebellar development (Nagasawa et al., 1996; Nagasawa et al., 1996; Oberlin et al., 1996; Ma et al., 1998, 1999; Tachibana et al., 1998; Zou et al., 1998).

A chemokine receptor, highly expressed in murine B cells and Burkitt's lymphomas, was cloned by Dobner et al. (1992). Disruption of the murine gene encoding this receptor, CXCR5, resulted in a severely impaired migration of B cells to follicles of the spleen and Peyer's patches, and in the absence of inguinal lymph nodes (Förster et al., 1996). However, despite the disturbed architecture of their lymphoid organs, these mice demonstrate normal humoral immune responses and Ig affinity maturation upon immunization with T cell-dependent antigens (Förster et al., 1996; Voigt et al., 2000). Recently, a B cell-homing chemokine, called B cell attracting chemokine 1 (BCA-1) in humans and B lymphocyte chemoattractant (BLC) in mice, was shown to be an agonist for CXCR5 (Gunn et al., 1998a; Legler et al., 1998). BLC is highly expressed in the follicles of secondary lymphoid organs and attracts B cells and to a much lesser degree T cells and macrophages (Gunn et al., 1998a). BCA-1 was shown to induce chemotaxis of B cells, but not activated T cells, monocytes, or neutrophils (Legler et al., 1998).

**Hepatocyte growth factor**

Hepatocyte growth factor (HGF), also known as scatter factor (SF), induces growth, motility, and morphogenesis of target epithelial and endothelial cells by binding to the receptor tyrosine kinase Met (Stoker et al., 1987; Nakamura et al., 1989; Bottaro et al., 1991; Montesano et al., 1991; Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Many studies suggest important roles for HGF in tumor growth, invasion, and metastasis (Weidner et al., 1990; Giordano et al., 1993; Rong et al., 1994). In addition, HGF was shown to be involved in hematopoiesis, adhesion of neutrophils, and in the migration of T cells (Kmiecik et al., 1992; Adams et al., 1994; Galimi et al., 1994; Nishino et al., 1995; Takai et al., 1997; Mine et al., 1998; Weimar et al., 1998). In Chapter 6 of this thesis an extensive overview of the structure, expression, and functions of HGF and Met is given. Data demonstrating a function for HGF/Met in B cell adhesion and differentiation will be presented and discussed in Chapter 3. In addition, the binding and presentation of HGF by heparan sulfate-modified CD44 to Met are shown and discussed in Chapters 4 and 5.
General introduction

Heparan sulfate proteoglycans

The function of many cytokines, including the chemokines, can be modified by heparan sulfate proteoglycans (HSPGs). HSPGs form a subgroup of the family of proteoglycans, proteins that contain complex carbohydrate chains, called glycosaminoglycans (GAGs) (Kjellen and Lindahl, 1991). The GAG heparan sulfate (HS) is composed of a tetrasaccharide linkage unit covalently attached to a serine present in the core protein, followed by repeats of uronic acid (either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)) and D-glucosamine (GlcN) (Fig. 6). The GlcN units of HS can either be N-sulfated or N-acetylated (Fig. 6). Because of differences in the number and position of N-linked and O-linked sulfates, HS chains are often very heterogeneous (Fig. 6) (Kjellén and Lindahl, 1991; Lindahl et al., 1998; Bernfield et al., 1999). Additional HSPG variability is given by the large number of core proteins to which HS chains can be attached.

HSPGs are widespread throughout mammalian tissues both as cell surface molecules, e.g. the syndecans, glypicans, several CD44 isoforms, and as extracellular matrix (ECM) components, e.g. perlecan (Bernfield et al., 1992, 1999; David, 1993; Jackson et al., 1995; Iozzo, 1998). A number of cell biological and genetic studies have recently provided compelling evidence for an in vivo role of cell-surface HSPGs in growth control and morphogenesis in fruitflies, mice and humans (Selleck, 1998; Bernfield et al., 1999). For instance, proteoglycans in Drosophila bind the secreted glycoprotein wingless, and promote wingless-induced signal transduction (Reichsman et al., 1996; Binari et al., 1997). Moreover, mutation of a Drosophila glypican, or of enzymes required for the biosynthesis of HSPGs, lead to major developmental defects (Jackson et al., 1997; Lin et al., 1999). Deletion of the murine gene encoding the sulfation enzyme HS 2-sulfotransferase, resulted in developmental defects of the kidney, eye, and skeleton (Bullock et al., 1998). These data indicate the importance of correct HS synthesis and expression for murine development. In man, mutations in GPC3, the glypican-3 gene, cause the Simpson-Golabi-Behmel syndrome, which is characterized by both pre- and postnatal overgrowth, a distinct facial appearance, and a diverse spectrum of other developmental defects (Pilia et al., 1996). This suggest that glypican-3 plays an important role in growth control during development. In addition to these functions in development, HSPGs have been implicated in cell adhesion and migration, angiogenesis, and in the regulation of blood coagulation (Jackson et al., 1991; Kjellén and Lindahl, 1991; Iozzo, 1998). During these processes, HSPGs bind ligands via their core protein, but more often via their HS chain(s). For instance, many cytokines, e.g. fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), and most, if not all, chemokines can bind to heparin, a highly sulfated GAG homologues to HS (Kjellén and Lindahl,
Figure 6. Schematized structure of a (short) heparan sulfate chain attached to a core protein. See the text for details. Modified from Bernfield et al., 1992.

1991; Nelson et al., 1995). The binding of cytokines to HS chains has been shown to be highly specific and depends on the chemical composition and structure of the HS chains (Ishihara et al., 1993; Maccarana et al., 1993; Lyon et al., 1994; Guimond and Turnbull, 1999). Cytokine/HS interactions may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of their biological function (Ruoslahti and Yamaguchi, 1991; Schlessinger et al., 1995). This functional importance is illustrated by fibroblast growth factor 2 (FGF-2), whose binding to its signal-transducing receptor and consequent biological effects is dependent on its interaction with cell-surface HSPGs (Rapraeger et al., 1991; Yayon et al., 1991). HS chains may bind and oligomerize FGFs, thereby promoting FGF receptor (FGFR) cross-linking and subsequent activation (Spivak-Kroizman et al., 1994; Schlessinger et al., 1995; DiGabriele et al., 1998). The structural model of a recently crystallized dimer of FGF-2 bound to a variant of FGFR1 suggested that heparin- (or HS-) induced dimerization represents the minimal structural unit required for activation of FGFRs (Plotnikov et al., 1999). Interestingly, this model also suggests that a heparin dodecasaccharide interacts with heparin-binding regions in both FGF-2 and FGFR1. An alternative function for HS is, that it induces conformational changes in FGFs, which might be necessary to bind to FGFRs (Yayon et al., 1991). In addition, HS might protect...
FGFs from degradation (Gospodarowicz and Chen, 1986; Saksela et al., 1988; Damon et al., 1989).

Most studies concerning the expression and function of cell-surface HSPGs have focussed on epithelial cells and fibroblasts, but these molecules presumably also play important roles in the immune system. For instance, a vast number of cytokines, involved in lymphoid tissue homeostasis or inflammation bind to HS/heparin. These cytokines, which include chemokines, as well as interleukins and hematopoietic growth factors, e.g. interleukin (IL) 3, IL-8, granulocyte-monocyte colony stimulating factor (GM-CSF), and HGF, thus can be potentially immobilized by HSPGs (Gordon et al., 1987; Roberts et al., 1988; Webb et al., 1993; Lyon et al., 1994; Jackson, 1997; Gupta et al., 2000). Moreover, HSPGs expressed on the luminal surface of endothelial cells have been shown to bind chemokines produced at sites of inflammation (Tanaka et al., 1998), preventing their immediate dilution by the blood stream. Presentation of HSPG-bound chemokines, e.g. macrophage inflammatory protein (MIP) 1β and IL-8, to leukocytes plays a crucial role in activating the leukocyte integrins that mediate stable adhesion to and transmigration across the vessel wall (Rot, 1992; Tanaka et al., 1993a, 1993b, 1996). Chemokines and other heparin-binding cytokines do not exclusively act at the endothelial-blood interface. They also play key roles in the regulation of lymphocyte trafficking within lymphoid tissues and are involved in the control of lymphocyte growth, differentiation and survival (see also section Regulation of B cell adhesion and migration - Chemokines). This suggests that cell-surface HSPGs on cells of the immune system, such as lymphocytes and antigen-presenting cells, might also be involved in the regulation of cytokine responsiveness.

As yet, not much is known about the expression of HSPGs by B cells. In mice, precursor B cells present in the bone marrow, and plasma cells in interstitial matrices, have been shown to express the HSPG syndecan-1 (Sanderson et al., 1989). In vitro, expression of syndecan-1 was shown to mediate the binding of B cells to collagen (Sanderson et al., 1992) Also human plasma cells, in addition to myeloma cells, and Reed-Sternberg cells of classical Hodgkin’s disease, express syndecan-1 (Ridley et al., 1993; Wijdenes et al., 1996; Carbone et al., 1997; Carbone et al., 1997). Ectopic expression of syndecan-1 in a human B cell line confers adhesion and spreading on thrombospondin and fibronectin (Lebakken and Rapraeger, 1996). However, this binding was shown to be mediated by the syndecan-1 core protein and not by HS chains. Also syndecan-4 has been shown to be expressed by murine B cells, with the interesting exception of Ig isotype switched B cells (Yamashita et al., 1999). So far the functional consequence of HS expressed by B cells is largely unknown, although HS has been reported to mediate IL-7-dependent B lymphopoiesis in vitro (Borghesi et al., 1999).
Chapter 1

Aim of this study

The studies described in this thesis investigate the involvement of two types of proteins, *i.e.* the CD44 family of adhesion molecules, and the hepatocyte growth factor (HGF) receptor Met, in the regulation of B cell activation and adhesion.

The molecular structure of distinct members of the CD44 family of adhesion molecules differs greatly as a result of alternative mRNA splicing and differential glycosylation. These molecules might bind to different ligands, or to the same ligand with distinctive affinity. In Chapter 2, the capacity of several alternatively spliced CD44 isoforms to bind hyaluronic acid, a major CD44 ligand, was explored.

HGF and its receptor tyrosine kinase Met are involved in mitogenesis, motogenesis, and morphogenesis of epithelial and endothelial cells. Moreover, HGF plays a role in hematopoiesis, and in the adhesion and migration of T cells and neutrophils. In Chapter 3, we investigated the role of HGF/Met in B cell functioning. It describes the expression of Met on freshly isolated tonsillar B cells, and the secretion of HGF by cells present in lymphoid organs. In addition, we describe the effect of HGF on the adhesion of B cells to the extracellular matrix molecule fibronectin, and to vascular cell adhesion molecule 1 (VCAM-1), a molecule expressed by endothelial and follicular dendritic cells.

The action of many cytokines, including HGF, is regulated through their binding to heparan sulfate proteoglycans. CD44 isoforms containing a domain encoded by the alternatively expressed exon v3 can be modified with heparan sulfate (HS). Therefore, we investigated the influence of HS-modified CD44 (CD44-HS) on HGF functioning. Data concerning HGF-induced signal transduction in B cells expressing Met in combination with CD44-HS are described in Chapters 4 and 5. Chapter 5 furthermore describes the delicately regulated expression of HSPGs, in particular CD44-HS, on tonsillar B cells.

Chapter 6 comprises a review on the involvement of the HGF-Met pathway during development, tumorigenesis, and B cell differentiation.

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Chapter 2

Binding of cell-surface expressed CD44 to hyaluronate is dependent on splicing and cell type.

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SUMMARY

CD44 is a major cell-surface receptor for hyaluronate (HA). By alternative RNA-splicing a large number of CD44 variants is generated. To explore the role of CD44 splicing in the regulation of cell binding to HA, three different isoforms of CD44 were transfected in the CD44 negative B cell lymphoma line Namalwa and in the fibroblastoid cell line COS7. We observed that whereas the standard form of CD44 (CD44s) mediated adhesion of Namalwa to HA, Namalwa transfected with CD44v3-10 or CD44v8-10 was unable to bind to either immobilized or soluble HA. After stimulation of CD44 with an activating anti-CD44 mAb or with phorbol ester, the binding of CD44s to HA was 5- to 10-fold higher than that of the other two isoforms. By contrast, COS7 cells transfected with CD44s, CD44v8-10, or CD44v 3-10 bound equally effective to HA. Hence, in addition to alternative splicing, cell type determines CD44 binding to HA.

INTRODUCTION

CD44 is a broadly distributed family of cell surface glycoproteins that has been implicated in a number of important biological processes including lymphocyte homing, T cell activation, hematopoiesis, and tumor metastasis (1-11). The CD44 gene consists of 20 exons. Due to alternative RNA-splicing involving at least 10 exons encoding domains of the extracellular portion of the molecule, a large number of CD44 splice variants is generated (8, 12-15).

Although unique functional properties have been attributed to certain CD44 splice variants, the precise effects of particular inserted domains on the ligand binding specificity of the molecule have remained unclear. The standard (hematopoietic) form of CD44 has been shown to function as a receptor for hyaluronate (HA) (15-18). Cell surface HA receptors are believed to regulate many aspects of cell behaviour such as cell differentiation, cell migration and tumor metastasis (19). Interestingly, Stamenkovic and colleagues have reported that CD44E, a major CD44 splice variant on epithelium containing a 132 amino acid insert encoded by exons CD44v8-10, is unable to bind this ligand (16). This suggested that CD44 splicing might regulate cell adhesion to HA. This finding has remained controversial, however, since studies by He et al. (20) suggested that the murine homologue of CD44v8-10 (CD44E) is capable of mediating binding to HA. Furthermore, in a recent paper Dougherty et al. (21) reported that CD44v8-10 cloned from the human myelomonocytic leukemia line KG1a, binds avidly and specifically to HA when transfected in COS7 cells. The above controversy prompted us to further explore the role of alternative splicing of CD44 in the regulation of HA binding.
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**Figure 1.** Schematic representation of CD44 DNA (A) and the CD44s, CD44v8-10, and CD44v3-10 cDNAs (B) used for transfection.

**MATERIALS AND METHODS**

*Monoclonal antibodies (mAbs).* The anti-CD44 mAbs used were NKI-P1 (3), Hermes-1 (2), and Hermes-3 (2) (kindly provided by Drs. C.G. Figdor, Nijmegen and E.C. Butcher, Stanford) all reactive with epitopes on the constant portion of the extracellular domain of CD44.

*CD44 cDNAs.* CD44s, CD44v8-10, and CD44v3-10 cDNAs were cloned as EcoRI-BglII-PCR fragments from HPKII cells into EcoRI and BamHI cloning sites of the expression vector pAD-CMV2. The sequences of the inserts were identical to the CD44 sequences published by Screaton *et al.* (12). A schematic representation of the CD44 gene and the cDNAs is given in Fig. 1.

*Cell cultures and transfections.* Stable Namalwa (ATCC, Rockville, MD) CD44 transfectants were obtained by electroporation-mediated co-transfection of CD44 cDNAs and pSVneo plasmid containing the bacterial neomycin resistance gene driven by a SV40 promotor. Cells with surface expression of CD44 were identified by indirect immunofluorescence using the CD44 mAb NKI-P1 and cloned by single cell sorting using a Becton Dickinson FACStar plus flow cytometer (Mountain View, CA). At least two
Figure 2. Expression of CD44 on untransfected Namalwa cells and on Namalwa cells transfected with CD44s, CD44v8-10, and CD44v3-10. CD44 expression was analysed by FACS using mAb NKI-P1 against a standard CD44 epitope.

Independent transfectants expressing each CD44 variant were used. The SV40-transformed simian fibroblastoid cell line COS7 (ATCC) was transiently transfected with the different CD44 cDNAs by electroporation and then incubated for 72 h in DMEM culture medium to allow replication and expression.

**Immunofluorescence.** Surface expression of CD44 on Namalwa and COS transfectants was analysed by flow cytometry (FACScan, Becton Dickinson) using indirect immunofluorescence (13). The fluorescein-tagged hyaluronic acid (HA-FITC) (22) was a generous gift of Dr. P. Heldin (Dept. Of Medical and Physiological Chemistry, University of Uppsala, Sweden). HA-FITC binding to CD44 transfectants was performed as described by Lesley et al. (23).

**Cell adhesion to immobilized hyaluronate.** To study the adhesion of cells to HA, wells of a 96-wells-plate (Costar, Cambridge, MA) were coated with 0.001-5 mg/ml rooster
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![Graph showing percentage of Namalwa cells transfected with different CD44 splice variants bound to immobilized HA before (-) or after treatment with the stimulating anti-CD44 mAb NKI-P1 or with PMA. Mean +/- standard deviation of 4 experiments.]

**Figure 3.** Percentage of Namalwa cells transfected with different CD44 splice variants bound to immobilized HA before (-) or after treatment with the stimulating anti-CD44 mAb NKI-P1 or with PMA. Mean +/- standard deviation of 4 experiments.

comb HA (Sigma, St Louis, MO) at 4°C overnight followed by 1 h at 37°C. Aspecific binding was blocked by incubating the wells with 4% BSA/RPMI 1640 at 37°C for 1 h. Subsequently, cells (100 ml, 1 x 10^6 cells/ml) were added to the wells and incubated for 1 h at 37°C in 5% CO2 in air. In some cases, cells were incubated with mAbs (for 1 h on ice) or PMA (50 ng/ml, for 18 h at 37°C in 5% CO2 in air), before adding the cells to the HA coated wells. After binding to HA, the wells were washed 6 times with 1% BSA/RPMI. The adherent cells were scored using a quantitative determination MTT assay (Boehringer, Mannheim, Germany) (24).

**RESULTS AND DISCUSSION**

*Namalwa cells transfected with different CD44 variants show differential binding to hyaluronan.* cDNAs of CD44s, CD44v8-10, and CD44v3-10 (Fig.1) were transfected into Namalwa cells, which in accordance with previous reports (9, 25), were negative for CD44 at both the protein (Fig. 2) and RNA level (data not
CD44 positive cells were cloned and several independent clones were established for each CD44 variant. Fig. 2 shows the approximately equal levels of expression of CD44 on a panel of representative CD44s, CD44v8-10, and CD44v3-10 transfectants.

To assess whether modification of the extracellular domain of CD44 by alternative splicing plays a role in regulating the HA receptor function of CD44, the binding of the various CD44 Namalwa transfectants to plastic immobilized and soluble HA was measured. Untransfected and mock transfected Namalwa cells, which lack CD44, were unable to bind to coated HA (Fig. 3). However, transfection of CD44s resulted in a significant adhesion of Namalwa to HA. This adhesion was completely blocked by Hermes-1, a mAb that is known to interfere with CD44-HA binding, confirming the direct role of CD44 in the binding (not shown). By contrast, CD44v8-10 or CD44v3-10, did not support adhesion of Namalwa cells to HA. Like immobilized HA, hyaluronate in solution (FITC-labeled) also bound to CD44s but hardly or not to CD44v8-10 and CD44v3-10 (Fig. 4).

Previous studies of CD44-HA interaction have shown that CD44 can exist...
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CD44 isoforms expressed by COS7 cells show identical hyaluronate binding. Both CD44s and CD44v8-10 transfected into the Simian monkey fibroblastoid cell line COS7 have been reported to mediate HA binding (21). We studied the interaction of CD44s, CD44v8-10, and CD44v3-10 transfected COS7 cells with HA-FITC (Fig. 5). FACS double staining with mAb NKI-P1 against CD44 and HA-FITC clearly showed that the transfected COS7 bound on average...
approximately 10-fold the amount of HA-FITC of the non-transfected cells. Interestingly, this binding was similar for cells transfected with all three CD44 cDNAs. Hence, unlike in Namalwa cells, CD44v8-10, and CD44v3-10 variants transfected to COS7 cells bind HA effectively.

The molecular basis for the huge differences in HA affinity of the CD44 isoforms when expressed on Namalwa and for the cell type specific character of this difference in affinity is presently unknown. Possible mechanisms include conformational changes at the HA binding site of CD44 induced by insertion of the alternatively spliced domains, differences in receptor clustering on the cell surface, and interactions with other cell surface molecules. Also, the observed differences in affinity for HA might be caused by differential association with the cytoskeleton, which in turn might be regulated by phosphorylation of cytoplasmic serine residues. Indeed, it has recently been shown that serine phosphorylation of CD44 regulates HA binding (26). In conclusion, our data provide further evidence that CD44 mediated cell adhesion to HA is subject to complex regulation. This regulation involves alternative splicing as well as other unknown cell type-specific factors, and presumably allows for functional activity of CD44 at the appropriate time and location.

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Chapter 3

Paracrine regulation of germinal center B cell adhesion through the c-Met - hepatocyte growth factor/scatter factor pathway.

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SUMMARY

T cell-dependent humoral immune responses are initiated by the activation of naive B cells in the T cell areas of the secondary lymphoid tissues. This primary B cell activation leads to migration of germinal center (GC) cell precursors into B cell follicles where they engage follicular dendritic cells (FDC) and T cells, and differentiate into memory B cells or plasma cells. Both B cell migration and interaction with FDC critically depend on integrin-mediated adhesion. To date, the physiological regulators of this adhesion were unknown. In the present report, we have identified the c-Met-encoded receptor tyrosine kinase and its ligand, the growth and motility factor hepatocyte growth factor/scatter factor (HGF/SF), as a novel paracrine signaling pathway regulating B cell adhesion. We observed that c-Met is predominantly expressed on CD38⁺CD77⁺ tonsillar B cells localized in the dark zone of the GC (centroblasts). On tonsil B cells, ligation of CD40 by CD40-ligand, induces a transient strong upregulation of expression of the c-Met tyrosine kinase. Stimulation of c-Met with HGF/SF leads to receptor phosphorylation and, in addition, to enhanced integrin-mediated adhesion of B cells to both VCAM-1 and fibronectin. Importantly, the c-Met ligand HGF/SF is produced at high levels by tonsillar stromal cells thus providing signals for the regulation of adhesion and migration within the lymphoid microenvironment.

INTRODUCTION

Antigen-specific B cell differentiation, the process by which naive B cells develop into memory cells or plasma cells, requires multiple interactions of B cells with other cells, such as T cells and follicular dendritic cells (FDC), and with the extracellular matrix (ECM), that take place within distinct microenvironmental compartments of the lymphoid tissues (1-6). After their initial activation in the extrafollicular T cell (paracortical) area, germinal center (GC) founder cells migrate into B cell follicles where they initiate the formation of GCs (7, 8). Once in the GC, the B cells first pass the dark zone where they undergo rapid clonal expansion and somatic hypermutation in their IgV genes (9-13). Mutated B cells then progress to centrocytes and move to the basal light zone of the GC. Here they reencounter antigen, presented as low levels of immune complexes on FDC, and undergo affinity selection (14-16). Whereas low-affinity mutants and autoreactive mutants die by apoptosis, high-affinity mutants internalize antigen and process it on their migration pathway to the apical light and outer zones of the GC. In these areas, the affinity-selected B cells present antigen to antigen-specific GC T cells (17-19). Cognate T-B interaction results in expansion and Ig isotype switching of high-affinity B cells (20, 21), that mature into memory B cells or plasma cells and
receive signals mediating their export from the lymphoid organ (1). Adhesion regulation, particularly regulation of lymphocyte integrin function, is believed to be fundamental to the control of cell migration and microenvironmental homing during this B cell differentiation process (22, 23).

Integrins are a widespread family of heterodimeric (αβ) transmembrane glycoproteins that can function as cell-ECM and cell-cell adhesion receptors (for review see reference 24). In the immune system they are involved at multiple levels, including interaction of lymphoid precursors with stromal cells during lymphopoiesis, lymphocyte homing, and antigen presentation. Importantly, adhesion receptors of the integrin family have recently been implicated in B cell differentiation. Integrins, specifically α4β1, were shown to be involved in adhesion and terminal differentiation of precursors during B-lymphopoiesis in the bone marrow in vitro (25, 26), and in B cell adhesion to FDC during GC reactions (27-29). In vivo experiments with α4null chimeric mice, confirmed a key role for this integrin in early B cell development (30). Together, these studies indicate that integrin mediated adhesion plays an important role in the control of several steps of B cell development, including migration and adhesion during antigen-specific differentiation. However, the physiological regulators of lymphocyte integrin activity during B cell differentiation remain unknown.

In a survey of the molecular pathways that might regulate B cell adhesion, we explored the possible role of the c-Met-encoded receptor tyrosine kinase and its ligand hepatocyte growth factor/scatter factor (HGF/SF). The HGF/SF - c-Met pathway has been shown to regulate growth, motility and morphogenesis of epithelial and endothelial cells (31-36), which requires tight regulation of adhesion and de-adhesion. Furthermore, this pathway mediates invasion and migration of tumor cells (37-39), a process reminiscent of lymphocyte migration. Here, we identify the HGF/SF - c-Met pathway as a novel molecular pathway in antigen-specific B cell differentiation, which is involved in the regulation of integrin-mediated B cell adhesion.

**MATERIALS AND METHODS**

**Antibodies.** Mouse monoclonal antibodies used were anti-c-Met, DO24 (IgG2a) (Upstate Biotechnology, Lake Placid, NY); anti-CD38, OKT-10 (IgGl) (American Type Culture Collection [ATCC], Rockville, MD); FITC-conjugated anti-CD38, HIT2 (IgGl) (Caltag Laboratories, Burlingame, CA); biotin-conjugated anti-CD38, HIT2 (IgGl) (Caltag); anti-β1 integrin (CD29), 4B4 (IgGl) (Coulter Immunology, Hialeah, FL); anti-α4 integrin (CD49d), IHP2/1 (IgGl) (Immunotech, Marseille, France); anti-α5 integrin (CD49e), SAM-1 (IgG2b) (40) (a gift from A. Sonnenberg, NKI, Amsterdam, The Netherlands); anti-α4β7, Act-1 (IgGl) (41) (a gift from A. Lazarovits, University of
Western Ontario, London, Canada; anti-ICAM-1 (CD54), RR1/1 (IgG1) (42) (a gift from T. Springer, Harvard University, Boston, MA); anti-HGF/SF, 24612.111 (IgG1) (R&D Systems, Abingdon, UK); anti-CD3, OKT-3 (IgG2a) (ATCC); anti-DRC-1, R4/23 (IgM) (DAKO, Glostrup, Denmark); anti-CD19, HD37 (IgG1) (DAKO); and anti-phosphotyrosine, PY-20 (IgG2b) (Affinity, Nottingham, UK). Polyclonal antibodies used were rabbit anti-e-Met, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-HGF/SF (R&D Systems); FITC-conjugated rabbit anti-IgD (DAKO); RPE-conjugated goat anti-mouse (Southern Biotechnology, Birmingham, AL); AP-conjugated goat anti-mouse (total, IgG1, or IgG2a) (Southern Biotechnology); biotin-conjugated rabbit anti-mouse (DAKO); biotin-conjugated goat anti-mouse (DAKO); biotin-conjugated rabbit anti-goat (Vector Laboratories, Burlingame, CA); HRP-conjugated goat anti-rabbit (DAKO); and HRP-conjugated goat anti-rabbit (DAKO). In addition we used a rat monoclonal anti-CD77, 38.13 (IgM) (43) (provided by J. Wiels, Institute Gustave-Roussy, Villejuif, France); and RPE-Cy5-conjugated streptavidin (DAKO).

**Cell lines.** The epidermoid carcinoma cell line A431, the lung fibroblast cell line MRC-5, and the B cell lines Raji, Namalwa, Daudi, Ramos, JY, and Nalm-6 were obtained from ATCC and cultured in RPMI 1640 (Gibco BRL/Life Technologies, Paisley, UK) supplemented with 10% FCS (Integro, Zaandam, The Netherlands). The Burkitt's lymphoma line EB-B (44) was provided by R. Jefferis (University of Birmingham, Edgbaston, UK) and cultured in 10% FCS/RPMI 1640.

**B cell isolation and culturing.** B cells were isolated as described previously (29). Total B cell fractions were >97% pure as determined by FACS analysis.

B cells were cultured in Iscove's medium (Gibco BRL/Life Technologies) containing 10% FCS, 0.5% BSA, 50 μg/ml human transferrin (Sigma, Bornem, Belgium) and 5 μg/ml bovine pancreas insulin (Sigma). Some media were supplemented with 50 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma).

For CD40 ligation, B cells were cultured on irradiated (7,000 rad) CD40L transfected or, as a control, wild-type 1 cells (45) (provided by J. Banchereau, Schering Plough, Dardilly, France). In specific experiments, culture media were supplemented with either pansorbin cells of *Staphylococcus aureus* strain Cowan I (0.0025%; Calbiochem Novabiochem, La Jolla, CA), rabbit anti-human Ig-coated beads (2 μg/ml) (BioRad Laboratories, Hercules, CA), recombinant human IL-2 (100 U/ml) (Eurocetus, Amsterdam, The Netherlands), recombinant human IL-4 (100 U/ml) (Genzyme Diagnostics, Cambridge, MA), or recombinant human IL-6 (1,000 U/ml) (CLB, Amsterdam, The Netherlands).

**T cell isolation and culturing.** Tonsillar T cells were isolated as described for the B cell isolation, except that after the second Ficoll-Isopaque density gradient centrifugation the pellet was collected, washed, and resuspended in shock medium. The remaining B cells were removed by using a MACS magnetic cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-CD19. The T cell fraction was >98% pure as determined by FACS analysis.
Stromal cell isolation and culturing. Tonsillar stromal cells were isolated as described (46). The cells were cultured in 100-mm petri dishes (Costar, Cambridge, MA) containing 10% FCS/RPMI 1640. After 4 d nonadherent cells were removed.

FDC isolation and culturing. FDC were isolated as described (29). The cells were cultured in Iscove’s medium containing 10% Fetal Clone I serum (HyClone Laboratories, Logan, UT). These FDC-enriched cell cultures contained 10-15% DRC-1 positive cells.

Transfections. c-Met transfected Namalwa cells (Nam™) were obtained by electroporating Namalwa cells with the eukaryotic expression plasmid pA7l d containing full-length c-Met cDNA (a gift from G. Hartmann and E. Gherardi, University of Cambridge, Cambridge, UK). After 2 d in culture, transfecants were selected in culture medium containing 250 µg/ml hygromycin (Sigma). c-Met positive cells were sub-cloned by using a FACStarPlus flow cytometer (Becton Dickinson, Mountain View, CA).

Immunoprecipitation and Western blot analysis. For analysis of tyrosine phosphorylation of the c-Met protein, cells were incubated overnight in serum-free RPMI 1640. Nam™ or EB4B cells were incubated in serum-free RPMI 1640 in the presence or absence of 200 ng/ml HGF/SF (R&D Systems). After 5 min at 37°C the cells were solubilized in ice-cold 2X lysis buffer containing 20 mM Tris-HCl (pH 8), 250 mM NaCl, 20% glycerol, 2% NP-40, 20 µg/ml aprotinin (Sigma), 20 µg/ml leupeptin (Sigma), 4 mM sodium orthovanadate (Sigma), 10 mM EDTA, and 10 mM NaF. After 1 h at 4°C the insoluble nuclear material was removed by centrifugation at 1 X 10^4 g at 4°C for 20 min after which the supernatant was pre-cleared with protein A-Sepharose CL-4B (Pharmacia Biotech) for 45 min at 4°C. c-Met was precipitated with rabbit anti-c-Met coupled to protein A-Sepharose at 4°C for at least 2 h. The immune complexes were washed with lysis buffer and diluted in Laemmli sample buffer containing final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol (BioRad Laboratories), and 0.001% bromophenol blue. After boiling for 5 min, the samples were subjected to 8% SDS-PAGE. Western blotting was performed as described previously (48).

For analysis of c-Met in total cell lysates, cells were lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, 2 mM EDTA, and 5 mM NaF for 1 h at 4°C. After centrifugation at 1 X 10^3 g and 4°C for 20 min, the supernatant was diluted in Laemmli sample buffer, boiled for 5 min and subjected to 8% SDS-PAGE. Western blotting was performed as described previously (48).

FACS analysis. Expression of c-Met on tonsillar B cell subpopulations was studied using a triple staining technique (47). Staining was measured by using a FACSCalibur flow cytometer (Becton Dickinson).

Immunohistochemistry. Expression of c-Met in tonsillar tissue was analysed by single and double staining. For single staining cryostat tonsil sections were fixed in acetone for 10 min, washed in PBS and pre-incubated with 10% normal goat serum (Sera Lab, Sussex, UK) in PBS for 15 min. After incubating with the primary antibody for 1 h, endogenous peroxidases were blocked with 0.1% NaN₃, 0.3% H₂O₂, PBS for 10 min.
Subsequently, the sections were stained with biotin-conjugated rabbit anti-mouse for 30 min, followed by an incubation with HRP-conjugated avidin-biotin complex for 30 min. Substrate was developed with 3,3-aminophenylcarbazole (Sigma) for ~10 min. Tissue sections were counterstained with Haematoxylin (Merck, Darmstadt, Germany).

Double staining was performed as described for the single staining, except that a cocktail of primary antibodies was used, which was detected by either a cocktail of AP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit, or a cocktail of AP-conjugated goat anti-mouse IgG2a and HRP-conjugated goat anti-mouse IgG1. The second color was developed with Fast Blue BB (Sigma) for about 10 min.

**Adhesion assays.** 96-well flat-bottom plates (Costar) were coated overnight with 5 μg/ml human fibronectin (CLB) or 0.2 μg/ml recombinant human sVCAM-1 (R&D Systems) at 4°C. After blocking the plates with 4% BSA, RPMI 1640 (2 h at 37°C), B cells that had been pre-incubated with HGF/SF (R&D Systems), in the presence or absence of monoclonal antibodies, for 30 min at 37°C, were added. Then, the plates were centrifuged (3 min 800 rpm, no brake) and incubated at 37°C for 25 min. After washing the wells, the bound cells were fixed with 10% neutral buffered formalin solution (Sigma) and stained with Giemsa (Merck). Bound cells were quantified by using a color CCD camera (Sony) and NIH Image 1.60 software on an Apple Quadra 840AV.

**c-Met ELISA.** 96-well ELISA plates (Costar) were coated overnight with mouse anti-HGF/SF immunoglobulins at 4°C. Then, the plates were washed and blocked with 4% BSA. PBS for 1 h at 37°C. Next, the wells were incubated with culture supernatants or with a HGF/SF concentration series for 2 h at 37°C, followed by an incubation with goat anti-c-Met immunoglobulins for 1 h at 37°C. Subsequently, the wells were incubated with biotin-conjugated rabbit anti-goat immunoglobulins for 60 min at 37°C followed by HRP-conjugated avidin-biotin complex (DAKO) for 1 h at 37°C. Substrate was developed with 2,4-diaminobenzidine (Fluka; Chemica, Buchs, Switzerland) in 50 mM KH₂PO₄, 50 mM Na₂HPO₄·2H₂O (pH 5.4) containing H₂O₂. The reaction was stopped with 1 N H₂SO₄ and the results were analysed at 492 nm using a microplate reader (BioRad Laboratories).

**RNA Isolation and RT-PCR.** Total RNA was isolated with RNAsol (Cinna/Biotech Laboratories, Houston, TX) according to manufacturer’s description. First-strand cDNA synthesis was performed on total RNA by a standard reverse transcription reaction, using Moloney leukemia virus reverse transcriptase (Gibco BRL/Life Technologies) and dNTPs (Pharmacia Biotech). PCR was performed with Taq DNA Polymerase (Gibco BRL/Life Technologies), 200 μM dNTPs (Pharmacia Biotech) and 1.5 mM MgCl₂ in 1X PCR Buffer (both Gibco BRL/Life Technologies). Primers used were HGF-1 (5'- CGACAGTGTTTCCCCTTCTCG-3') in combination with HGF-3 (5'- GGTGGGTGCAGACACAC-3'), or 5'B2M (5'-ATCCAGGTACTCCAAAGATT-3') in combination with 3'B2M (5'-CATGTCTCGATCCCACTTAAC-3'). PCR was started with a 5 min denaturation step at 95°C, after which amplification was performed in 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min and elongation at 72°C for 2 min.
The c-Met pathway in B cell differentiation

Figure 1. c-Met expression on human B cells. (A) PMA induces c-Met expression on tonsillar B cells. (B) Several B cell lines constitutively express c-Met. A431 (positive control) is an epidermoid carcinoma cell line. Raji, EB4B and Namalwa (Nam'') are Burkitt’s lymphoma cell lines. Nam'' are c-Met transfected Namalwa cells. In both A and B the Western blot of the cell lysates was stained with anti-c-Met. The c-Met precursor (pre c-Met) and c-Met β chain (c-Met (β)) are indicated.

RESULTS

The c-Met receptor tyrosine kinase is expressed by activated human tonsillar B cells as well as by several B cell lines. Expression of c-Met by human tonsillar B cells and by a panel of B cell lines was assessed by Western blotting and by FACS analysis. On Western blot, c-Met expression was hardly detectable in freshly isolated tonsillar B cells, but we observed a strong induction of c-Met (and the c-Met precursor [pre c-Met]) upon stimulation with the phorbol-ester PMA (Fig. 1A). Furthermore, constitutive expression of c-Met was found in the Burkitt’s lymphoma cell lines Raji and EB4B, but not in the Burkitt’s lymphoma cell line Namalwa (Fig. 1B) nor in the B cell lines Daudi, Ramos, JY, or Nalm-6 (data not shown). As positive controls, c-Met expression of the epidermoid carcinoma cell line A431 and of Namalwa cells stably transfected with c-Met (Nam’') are shown (Fig. 1B).

The c-Met receptor on B cells is functional. The above findings clearly show that B cells can express c-Met and, hence, might potentially be triggered via the HGF/SF - c-Met pathway. To demonstrate that the c-Met receptor on B cells can indeed be functionally activated by HGF/SF, we studied c-Met receptor phosphorylation on tyrosine residues in response to HGF/SF. As is shown in Fig. 2,
Figure 2. Tyrosine phosphorylation of c-Met on B cells in response to HGF/SF. c-Met on EB4B and Nam\textsuperscript{met} B cells becomes phosphorylated on tyrosine residues upon triggering with HGF/SF. c-Met was precipitated with anti-c-Met antibodies and the Western blot was consecutively stained with anti-phosphotyrosine- or with anti-c-Met antibodies. The c-Met precursor (pre c-Met) and c-Met β chain are indicated.

HGF/SF stimulation of EB4B cells as well as of Nam\textsuperscript{met} B cells resulted in an enhanced tyrosine phosphorylation of c-Met. This indicates that the HGF/SF - c-Met pathway on B cells is capable of signaling.

\textit{c-Met receptor expression on human tonsillar B cell subsets.} To investigate whether c-Met induction is a physiological phenomenon, that occurs also during antigen-specific B cell differentiation \textit{in vivo}, we assessed the expression of c-Met on human tonsillar B cell subsets using FACS triple staining. The subsets studied, recently defined by Pascual \textit{et al.} (13), were: the naive B cell subset, IgD\textsuperscript{+} CD38\textsuperscript{−} (Bm1-2); two GC B cell subsets, IgD CD38\textsuperscript{−} CD77\textsuperscript{+} centroblasts (Bm3), and IgD CD38\textsuperscript{+} CD77 centrocytes (Bm4); and an IgD CD38 memory B cell subset (Bm5). Fig. 3 shows that c-Met is expressed by CD38\textsuperscript{−} CD77 centroblasts (Bm3) and by a part of the CD38\textsuperscript{+} CD77 subset. This finding is supported by immunohistochemical studies on frozen sections of human tonsillar tissue: as is shown in Fig. 4, c-Met is predominantly expressed by lymphocytes within the dark zone of the GC, which contains rapidly dividing centroblasts and low numbers of FDC. These results mean that c-Met induction \textit{in vivo}, occurs in GC-cells at a pre-selection stage, \textit{i.e.}, cells that have recently been recruited by antigen plus antigen-specific T lymphocytes in the T cell-rich extrafollicular microenvironment.

\textit{CD40 ligation induces a transient expression of c-Met on tonsillar B cells.} Ligation of the B cell antigen receptor (BCR) and CD40 plays a key role in the initiation of a T cell dependent B cell response and initiates the GC reaction (1, 49-51). In view of the expression of c-Met on centroblasts, \textit{i.e.}, on recent GC
immigrants, we hypothesized that these receptors might also regulate c-Met expression. To address this hypothesis, the biological conditions for B cell activation were mimicked in vitro. Tonsillar B cells were cultured on CD40 ligand (CD40L) transfected L cells or, as a control, on wild-type L cells, in the presence or absence of BCR stimuli (anti-Ig antibodies or *Staphylococcus aureus* Cowans strain I [SAC]). As is shown in Fig. 5A, concurrent ligation of CD40 and the BCR induced a strong transient induction of c-Met in human tonsillar B cells, peaking at 48 h. Single triggering of CD40 also strongly induced c-Met (Fig. 5C) but single ligation of the BCR did not induce c-Met expression above control levels (untransfected L cells and medium alone) (Fig. 5B and D). In approximately half of the experiments, concurrent CD40 and BCR stimulation resulted in a c-Met induction that was stronger than after CD40 ligation alone, suggesting synergy between the CD40 and BCR pathways. Stimulation by various cytokines including IL-2, IL-4, and IL-6 did not induce c-Met (data not shown).

These data clearly identify CD40-CD40L as a major pathway for induction of c-Met in B cells.

*HGF/SF induces integrin-mediated adhesion of c-Met-positive B cells to VCAM-1 and fibronectin.* Cell motility and morphogenesis, major functions of the

**Figure 3.** Expression of c-Met on tonsillar B cell subsets. *(A)* Germinal center (CD38<sup>+</sup>) B cells express the c-Met tyrosine kinase, while naïve (IgD<sup>−</sup>CD38<sup>−</sup>) and memory (IgD<sup>−</sup>CD38<sup>+</sup>) tonsillar B cells are c-Met negative. Tonsillar B cells were triple-stained with anti-CD38, anti-IgD, and either anti-c-Met (solid line) or control antibodies (dotted line). *(B)* c-Met is expressed on centroblasts (CD38<sup>+</sup>CD77<sup>+</sup>) and on a subset of CD38<sup>−</sup>CD77<sup>−</sup> GC cells. Tonsillar B cells were triple stained with anti-CD38, anti-CD77, and either anti-c-Met (solid line) or control antibodies (dotted line).
c-Met - HGF/SF pathway, are dependent on tightly controlled cell adhesion. This prompted us to study whether the c-Met - HGF/SF pathway might regulate B cell adhesion. Since c-Met-positive B cells represent a subset of tonsillar B cells that cannot be readily purified by negative selection procedures, we addressed this
Figure 5. Induction of c-Met expression on tonsillar B cells by CD40 and BCR ligation. c-Met expression by tonsillar B cells cultured on (A) CD40L-transfected L cells plus anti-Ig antibodies; (B) wild-type L cells; (C) CD40L-transfected L cells; or (D) wild-type L cells plus anti-Ig antibodies. Western blots of the cell lysates were stained with anti-c-Met antibodies. The c-Met precursor (pre c-Met) and the c-Met β chain (c-Met (β)) are indicated. In the absence of CD40 stimulation (B and D), no viable B cells were recovered at 96 and 192 h.

question by using Namalwa cells transfected with c-met cDNA (Nam<sup>met</sup>). The expression of c-Met in this B cell lymphoma line and the wild-type control (Nam<sup>wt</sup>) are shown in Fig. 1B. We observed that HGF/SF induces a strongly augmented adhesion to both vascular cell adhesion molecule 1 (VCAM-1) and fibronectin of c-met transfected Namalwa B cells (Nam<sup>met</sup>) (Fig. 6A and B). This effect of HGF/SF on B cell adhesion was dose dependent and was not observed upon stimulation of wild-type Namalwa cells (Nam<sup>wt</sup>). An increased adhesion in response to HGF/SF to both VCAM-1 and fibronectin was also observed with the Burkitt’s lymphoma cell lines Raji and EB4B (data not shown).

To identify the adhesion receptors on Nam<sup>met</sup> responsible for enhanced VCAM-1 and fibronectin binding, antibody blocking experiments were performed. Nam<sup>met</sup> expresses α4β1 and α4β7, which both are receptors for VCAM-1 and for an alternatively spliced segment (CS-1) of fibronectin, but expresses no detectable
level of the fibronectin receptor α5β1 (data not shown). We observed that adhesion
to VCAM-1 and fibronectin was completely blocked by mAbs against both the α4
and β1 integrin chain (Fig. 6C and D). Since c-Met stimulation by HGF/SF did not
lead to increased α4β1 expression (and also did not upregulate or induce α4β7 or
α5β1) (data not shown), these results indicate that the c-Met - HGF/SF pathway
enhances B cell adhesiveness through activation of the α4β1 integrin.

**Tonsillar stromal cells produce high levels of HGF/SF.** Alltogether, the above
data strongly favor a functional role of the c-Met -HGF/SF pathway in B cell
differentiation, namely in the regulation of B cell adhesiveness. However,
obviously, an *in vivo* biological role in this process would require the availability
of HGF/SF within the lymphoid tissue microenvironment. This prompted us to *(a)*
assay the production of HGF/SF by primary cultures of various tonsillar cell

![Figure 6. The HGF/SF - c-Met pathway regulates 4β1 integrin-mediated adhesion to
sVCAM-1 and fibronectin. (A) Effect of HGF/SF on the binding of c-Met transfected
(Nammet) and control (Namwt) B cells to sVCAM-1. (B) Effect of HGF/SF on the binding of
Nammet and Namwt B cells to fibronectin. (C) Effect of anti-β1 (4B4), anti- 4 (HP2/1), and
anti- 4β7 (Act-1) integrin antibodies on the binding of Nammet B cells to sVCAM-1. (D)
Effect of anti-β1 (4B4), anti- 4 (HP2/1), and anti- 4β7 (Act-1) integrin antibodies on the
binding of Nammet B cells to fibronectin. Cells were pre-incubated with HGF/SF in the
presence or absence of anti-integrin monoclonal antibodies. The results are expressed as
relative (compared with the control cells not incubated with HGF/SF) adhesion. Error bars
represent the standard deviation of triplicate wells. 76
Figure 7. Expression of HGF/SF protein and mRNA by tonsillar stromal cells and lymphocytes. (A) HGF/SF secretion by cultured T cell, B cells, stromal cells, and FDC enriched tonsillar cells. ELISAs were performed to determine HGF/SF concentrations in the culture media (lower limit of detection 400 pg/ml). T and B cells were stimulated as indicated. (B) Expression of HGF/SF mRNA in T cells, B cells, tonsillar stromal cells, and, as a positive control, in the lung fibroblast cell line MRC-5. The RT-PCR was performed on total RNA, a plasmid containing full-length human HGF/SF cDNA (pHGF/SF), or on water. Primers used were HGF/SF-specific or, as a control, β2-microglobulin specific.

populations, and (b) study the expression of HGF/SF mRNA within these cell populations by RT-PCR. Determinations of HGF/SF production by ELISA demonstrate that high levels of HGF/SF are produced by primary cultures of tonsillar stromal cells, including cultures of FDC-enriched tonsillar cell subfractions (Fig. 7A). By contrast, tonsillar T- or B-lymphocytes, cultured in the presence or absence of various mitogenic stimuli, did not produce detectable levels of HGF/SF. Consistent with these results, in RT-PCR studies HGF/SF mRNA was exclusively detectable in tonsillar stromal cells (Fig. 7B).

**DISCUSSION**

The products of proto-oncogenes are important regulatory molecules that exert a wide range of effects on basic cellular functions such as the control of cell growth and differentiation. The c-met proto-oncogene product is a receptor tyrosine kinase (52, 53) that binds HGF/SF, a mesenchymally derived cytokine with pleiotropic biological effects on proliferation, cell motility and morphogenesis of epithelial, endothelial, and myogenic cells (31-36, 54). More recently, the c-Met - HGF/SF pathway has also been implicated in the proliferation and differentiation of early hematopoietic progenitor cells (55-58) and in monocyte-macrophage
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differentiation (59). Here, we demonstrate that the e-Met - HGF/SF pathway is also operative during T cell dependent B cell differentiation, where it is involved in the control of lymphocyte integrin function on B cells, a process regulating adhesion and homing of B cells within the lymphoid microenvironment.

We observed that stimulation of tonsillar B cells with phorbol ester PMA leads to a rapid e-Met induction (Fig. 1A). Induction of e-Met expression upon protein kinase C (PKC) stimulation by phorbol ester has previously also been reported in epithelial cell lines (60). In addition, we observed constitutive expression of e-Met on several B cell lymphoma lines (Fig. 1B). The e-Met receptor on these B cell lines is signaling competent, as triggering by HGF/SF resulted in enhanced tyrosine phosphorylation of e-Met (Fig. 2). These findings present the first direct evidence for expression of a functional e-Met receptor on B lymphocytes. Indirect evidence for a role of e-Met in B cells has previously been provided by Delaney et al. (61), who demonstrated that HGF/SF enhances immunoglobulin production by murine B cells. However, as e-Met expression was not studied and whole splenocyte cultures were used, indirect effects of HGF/SF were not ruled out. For T cells, Shaw and colleagues reported that HGF/SF stimulated the adhesion and migration of the memory subset. However, these target cells appeared not to express e-Met (62).

One of the key findings of our study is that concurrent CD40 and BCR ligation induces a strong transient expression of e-Met on B cells in vitro (Fig. 5). Presumably, BCR and CD40 mediated signals are also instrumental in the physiological induction of e-Met. This is suggested by the fact that e-Met is expressed in vivo on a subset of tonsillar centroblasts (CD38 CD77+) (Figs. 3 and 4). Centroblasts are the offspring of B cells that have recently been activated at extrafollicular sites by antigen plus accessory signals provided by antigen-specific T cells (11). These signals critically involve CD40/CD40L interactions: patients with x-linked hyper-IgM syndrome (due to mutated and consequently defective CD40L) do not develop GC and blocking of the CD40/CD40L pathway in mice leads to complete inhibition of GC reactions (49-51, 63, 64). Our results strongly suggest that e-Met induction is directly linked to the initiation of the B cell immune response. Indeed, we observed that dual ligation of CD40 and the BCR also induces e-Met on naive (IgD CD38-) B cells (our own unpublished observation).

As both the migratory and morphogenetic responses to HGF/SF are critically dependent on cell adhesion, could regulation of the e-Met-HGF/SF pathway also have a regulatory role in B cell adhesion? This idea is supported by the finding that HGF/SF augments adhesion of e-Met transfected Namalwa B cells as well as the e-Met expressing B cell lines Raji and EB4B to VCAM-1 and fibronectin (Fig. 6 and data not shown). This HGF/SF induced adhesion was mediated through activation of the integrin α4β1. Previous studies from our own and from other laboratories have shown an important role for α4β1 in GC formation (27-29). In the GC, α4β1
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mediates B cell adhesion to VCAM-1 on FDC, an interaction that regulates the formation of the microenvironment required for the affinity selection of GC B cells. Apart from establishing physical contact between B cells and FDC, α4β1 presumably contributes directly to the B cell selection process itself, as signaling through the α4β1-VCAM-1 pathway costimulates rescue of GC B cells from apoptosis (6, 29, 47). Furthermore, α4β1 also regulates cell adhesion to fibronectin (65), an important substrate for cell migration.

The above data strongly favor a functional role of the c-Met-HGF/SF pathway in B cell differentiation, namely in the regulation of B cell adhesiveness. Obviously, however, an in vivo biological role in this process requires the availability of HGF/SF within the lymphoid microenvironment. Interestingly, we indeed observed production of high levels of HGF/SF as well as expression of HGF/SF mRNA by tonsillar stromal cells. In contrast, tonsillar T- or B-lymphocytes were HGF/SF negative (Fig. 7).

Adhesion regulation is believed to be fundamental to the control of cell migration and microenvironmental homing during lymphocyte differentiation. This migration and homing within tissues, like recruitment from the blood, presumably is determined by an organized display of adhesive ligands and regulatory factors, specific for a given microenvironment (23). Thusfar, most studies on the regulation of integrin-mediated adhesion have focussed on cytokines of the chemokine family (66). Chemokines, which bind to G protein linked 7-transmembrane serpentine receptors (67, 68), have been shown to mediate chemotaxis and rapid functional activation of leukocyte integrins on myeloid cells, macrophages, and lymphocytes (69-73). HGF/SF belongs to the family of plasminogen-related growth factors (74), that also includes macrophage stimulating protein. These molecules, that are structurally unrelated to the chemokines, have the basic domain organization and mechanism of activation of the blood proteinase plasminogen, i.e., they are characterized by the presence of a kringle domain(s), an activation domain, and a serine proteinase domain. Our present data strongly support a physiological role of HGF/SF in the regulation of B cell adhesiveness, microenvironmental homing and in the morphogenesis of the GC. Local production of HGF/SF has been demonstrated surrounding blood vessels in inflammation (75, 76), and in this paper we demonstrate that HGF/SF is produced by FDC-enriched cell populations. In view of the pleiotropic effects of HGF/SF on other cell types, HGF/SF may have additional, as yet unknown, roles in antigen-specific B cell differentiation. In particular, cross-talk between integrins and c-Met-signaling pathways triggered by FDC might contribute to B cell survival (6, 29, 47). Interestingly, like the chemokines, HGF/SF has a high affinity for heparin, which is present on cell surfaces and in the ECM in the form of heparan sulphate proteoglycans. This heparan sulphate binding limits diffusion, thus allowing the development of
chemotactic gradients and the localization of proadhesive activity to the appropriate lymphoid microenvironment (77).

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Chapter 4

Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met.

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SUMMARY

CD44 has been implicated in tumor progression and metastasis, but the mechanisms(s) involved are as yet poorly understood. Recent studies have shown that CD44 isoforms containing the alternatively spliced exon v3 carry heparan sulfate side chains and are able to bind heparin-binding growth factors. In the present study, we have explored the possibility of a physical and functional interaction between CD44 and hepatocyte growth factor/scatter factor (HGF/SF), the ligand of the receptor tyrosine kinase c-Met. The HGF/SF-c-Met pathway mediates cell growth and motility and has been implicated in tumor invasion and metastasis. We demonstrate that a CD44v3 splice variant efficiently binds HGF/SF via its heparan sulfate-side chain. To address the functional relevance of this interaction Namalwa Burkitt's lymphoma cells were stably co-transfected with c-Met and either CD44v3 or the isoform CD44s, which lacks heparan sulfate. We show that, as compared to CD44s, CD44v3 promotes: (i) HGF/SF-induced phosphorylation of c-Met, (ii) phosphorylation of several downstream proteins, and (iii) activation of the MAP kinases ERK1 and 2. By heparitinase treatment and the use of a mutant HGF/SF with greatly decreased affinity for heparan sulfate, we show that the enhancement of c-Met signal transduction induced by CD44v3 was critically dependent on heparan sulfate moieties. Our results identify heparan sulfate-modified CD44 (CD44-HS) as a functional co-receptor for HGF/SF which promotes signaling through the receptor tyrosine kinase c-Met, presumably by concentrating and presenting HGF/SF. As both CD44-HS and c-Met are over expressed on several types of tumors, we propose that the observed functional collaboration might be instrumental in promoting tumor growth and metastasis.

INTRODUCTION

The CD44 family of cell surface glycoproteins is broadly expressed by cells of epithelial, mesenchymal, and hematopoietic origin and is involved in cell-matrix adhesion, hematopoiesis, and lymphocyte homing and activation (1). Furthermore, a large body of experimental and clinical studies support a role for CD44 in tumor progression and metastasis (2-4). The CD44 gene consists of 19 exons (5). Due to alternative splicing, which involves at least 10 exons encoding domains of the extracellular portion of the CD44 molecule, a large number of CD44 isoforms is generated (6-10). Posttranslational modification generates further diversity, yielding both N- and O-linked glycan forms of CD44 in addition to proteoglycan variants containing chondroitin-, keratan-, or heparan sulfate (11-14). The expression pattern of these CD44 variants is tissue-specific. On lymphocytes the short 80-90 kDa standard form of CD44 (CD44s) is most abundant, while larger
variants (CD44v) predominate on some normal and neoplastic epithelia and are also found on activated lymphocytes and on malignant lymphomas (15-19). This selective expression suggests specific biological functions for the various splice variants, but at present, these are poorly defined. Similarly, the mechanism(s) through which CD44 functions in tumorigenesis is not known.

An obstacle towards understanding the functions of the CD44 family is the limited knowledge of its molecular partners. The cytoplasmic tail of the CD44 molecule has been shown to interact with the actin cytoskeleton via ankyrin and proteins of the ERM-family, and is associated with Src-family tyrosine kinases (20-23). This suggests a role in signaling as well as in the regulation of cell shape and motility. Although several potential CD44 ligands have been identified, the only interaction of the extracellular domain of CD44 that has been extensively studied is that with hyaluronate. CD44s acts as a major receptor for this glycosaminoglycan which is highly abundant in mesenchymal tissues and is believed to play a role in cell migration and differentiation (24, 25).

A novel and potentially highly significant function of CD44 is its ability to interact with heparin-binding growth factors (26, 27). These growth factors bind to a HS side chain attached to the evolutionary conserved consensus motif SGSG encoded by exon v3 (13, 27). Heparan sulfate proteoglycans (HSPGs) are believed to play an important regulatory role in cell growth and motility by binding growth factors and by presenting these factors to their high affinity receptors. This process has been particularly well explored for the fibroblast growth factors 1 and 2 (FGF-1 and 2). For these factors, binding to HSPGs has been shown to be required for their biological function, presumably by promoting FGF dimerization required for efficient receptor cross-linking and activation (28-32).

In the present study, we explored the physical and functional interaction between heparan sulfate modified forms of CD44 (CD44-HS) and hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF is a heparin-binding growth factor (33) that induces growth, motility, and morphogenesis of target epithelial and endothelial cells by binding to the receptor tyrosine kinase c-Met (34, 35). In addition, recently HGF/SF was shown to be involved in hematopoiesis, and lymphocyte adhesion and migration (36-42). Apart from these physiological functions, there is ample evidence for a key role of the HGF/SF - c-Met pathway in tumor growth, invasion and metastasis. For example, HGF/SF induces epithelial cells to invade collagen matrices in vitro, and NIH 3T3 cells co-transfected with c-met and HGF/SF acquire an invasive and metastatic phenotype (43-45). Furthermore, in HGF/SF transgenic mice, tumors develop in many different tissues including mammary glands, skeletal muscles and melanocytes (46). In human cancer, both HGF/SF and c-Met are often over expressed, and in hereditary renal cancer germline mutations in the c-met gene have recently been reported (47-52). Here, we show that CD44-HS strongly promotes signal transduction through the
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HGF/SF - c-Met pathway, which is demonstrated to occur in a heparan sulfate-dependent fashion.

MATERIALS AND METHODS

**Antibodies.** Mouse monoclonal antibodies (mAbs) used were anti-pan CD44, NKI-P1 (IgG1) (53) and Hermes-3 (IgG2a) (54) (a gift from S. Jalkanen, University of Turku, Turku, Finland); anti-HGF/SF: 24612.111 (IgG1; R&D Systems, Abingdon, United Kingdom); anti-heparan sulfate, 10E-4 (IgM) (55); anti-desaturated uronate from heparitinase treated heparan sulfate ("AHS stub"), 3G10 (IgG2b) (55); anti-phosphotyrosine, PY-20 (IgG2b) (Affiniti, Nottingham, UK); and IgG1 and IgM control antibodies (ICN, Zoetermeer, The Netherlands). Polyclonal antibodies used were rabbit anti-c-Met, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (New England Biolabs, Beverly, MA); rabbit anti-ERK1 (C-16) and anti-ERK2 (C-14) (Santa Cruz Biotechnology); RPL-conjugate goat anti-mouse (Southern Biotechnology, Birmingham, AL); FITC-conjugated rabbit antiamouse (DAKO, Glostrup, Denmark); HRP-conjugated rabbit anti-mouse (DAKO); and HRP-conjugated goat anti-rabbit (DAKO).

**Cell lines and transfectants.** The Burkitt's lymphoma cell line Namalwa was purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in RPMI 1640 (Life Technologies, Breda, The Netherlands) supplemented with 10% Fetal Clone 1 serum (HyClone Laboratories, Logan, UT), 10% FCS (Integro, Zaandam, The Netherlands), 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (all Life Technologies). Namalwa cells transfected with CD44s (Nam-S), CD44v-8-10 (Nam-V8) or CD44v-3-10 (Nam-V3) were described previously (56). A second transfection of Namalwa cells, expressing either CD44s (Nam SM) or CD44v-3-10 (Nam V3M), with c-Met was performed as described (41).

**Purification of wild type and mutant HGF/SF.** The construction of pVL1393 vectors (Pharmingen, San Diego, CA) containing wild type or mutant HGF/SF (IIP1) cDNA was described elsewhere (57). HGF/SF (wild type and HPI) was produced in a Baeutovirus system as described previously (58). In brief, Sf 9 insect cells were transduced with an amplified virus stock and after 3 days media were pooled and analysed for scattering activity in the MDCK dissociation assay (59). Then, HGF/SF was purified with Ni-NTA resin from the QIAexpress system (Qiagen, Hilden, Germany). HGF/SF concentrations were measured by ELISA as described previously (41). In addition, HGF/SF (wt and HPI) was analysed by Western blotting using goat anti-HGF/SF.

**Enzyme treatments.** For enzymatic cleavage of glycosaminoglycans, cells were treated with either heparitinase (Flaebacterium heparinum, EC 4.2.2.8, ICN Biomedicals, Aurora, OH) or chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4, Boehringer Mannheim).
CD44 promotes c-Met activation

Almere, The Netherlands) in PBS at 37°C for the periods indicated. Enzyme treatments were followed by FACS analysis or immunoprecipitation.

**FACS analysis.** For FACS analysis cells were blocked with 10% pooled human serum (CLB, Amsterdam, The Netherlands), 1% BSA (Fraction V) (Sigma, Bornem, Belgium) in PBS at 4°C for 15 min and washed with FACS buffer (1% BSA in PBS), respectively. Then, the cells were incubated with the primary antibodies for 1 h, washed, and incubated with the secondary antibody for 30 min. Incubations were in FACS buffer at 4°C, and cells were analyzed by using a FACScan (Becton Dickinson, Mountain View, CA).

For binding of recombinant human HGF/SF (wild type or HP1) R & D Systems or our own product, cells were incubated with this protein (18 nM or as indicated) for 1 h prior to the antibody incubations. This step was followed by washing with FACS buffer.

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation was performed as described (41). The only modifications were that, for precipitation of CD44, cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 1 mM sodium orthovanadate (Sigma), 2 mM EDTA, and 5 mM NaF. For precipitation of c-Met, cells were lysed in 100 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 1% NP-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 2 mM sodium orthovanadate (Sigma), 5 mM EDTA, and 5 mM NaF.

Western blotting of immunoprecipitates and total cell lysates was essentially performed as described previously (23). A single modification was that, for analysis of phosphorylated proteins, membranes were blocked and stained in 2% BSA, 20 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween-20 (Sigma). Films were scanned with an Eagle Eye II video system (Stratagene, La Jolla, CA) and band intensities were determined with ONE-Dscan software (Stratagene). c-Met phosphorylation was expressed as the ratio of phosphorylated c-Met to c-Met precipitated.

For analysis of phosphorylation of the ERK1 and 2 MAP kinases, after the indicated treatments, 5 x 10⁵ cells were directly lysed in sample buffer and analysed by 10% SDS-PAGE and blotted. Equal loading was confirmed by Ponceau S staining of the blot. The part of the blot below 50 kD was stained with anti-phospho-MAPK antiserum, the upper part with anti-phosphotyrosine PY-20. Primary antibodies were detected by HRP-conjugated goat anti-rabbit and HRP-conjugated rabbit anti-mouse, respectively. Identification of the ERKs was confirmed by staining with anti-ERK1 or anti-ERK2.

**RESULTS**

**Binding of HGF/SF to CD44 isoforms.** Binding of HGF/SF to different CD44 isoforms was assessed by using a panel of Namalwa Burkitt’s lymphoma cell lines.
Figure 1. A. Schematic representation of the CD44 gene, and the CD44v3-10, CD44v8-10, and CD44s cDNAs used for transfection. Solid boxes represent constant exons while open boxes represent alternative exons. Note that, due to a stop codon, the variable exon 1 (v1) is not translated in the human. UT, untranslated region; EC, extracellular constant region; EV, extracellular variable region; TM, transmembrane region; CT, cytoplasmic region. B. Binding of HGF/SF to CD44 Namalwa transfectants. Using a FACS flow cytometer, one clone of mock transfected (Neo) Namalwa cells, and two independent clones of CD44s, CD44v8-10 or CD44v3-10-transfected Namalwa cells were analysed for their binding of HGF/SF. Bound HGF/SF was detected with mouse anti-HGF/SF followed by RPE-conjugated goat anti-mouse.
Table I. Surface expression of CD44 on Namalwa transfectants.

| CD44 isoform     | clone | MFI  | % positive cells |
|------------------|-------|------|-----------------|
| none (Neo)       | A     | 4    | 3               |
|                  | B     | 83   | 94              |
| CD44s            | A     | 96   | 97              |
| CD44v8-10        | A     | 126  | 84              |
|                  | B     | 142  | 88              |
| CD44v3-10        | A     | 137  | 88              |
|                  | B     | 82   | 87              |

Mean fluorescence intensity after staining with the anti-pan CD44 mAb NKI-P1 followed by FITC-conjugated rabbit anti-mouse.

CD44 promotes c-Met activation.

Stably transfected with cDNAs encoding either CD44s, CD44v8-10 or CD44v3-10 (Fig. 1A) (56). Prior to transfection, the cells were negative for CD44 and c-Met expression at both the protein and mRNA level (data not shown). All transfectants used for HGF/SF binding studies expressed comparable levels of CD44 (Table I). HGF/SF binding to the CD44 transfectants was measured by FACS analysis using an anti-HGF/SF mAb, an approach that avoids chemical modification of the ligand. As shown in Fig. 1B, CD44 negative control cells as well as CD44s and CD44v8-10 transfectants showed a low saturable binding of HGF/SF. In contrast, cells expressing CD44v3-10 bound much larger quantities of HGF/SF. These results suggest that CD44v3-10 contains (a) binding site(s) for HGF/SF.

Binding of HGF/SF to CD44 is heparan sulfate-dependent. We next conducted a series of experiments aimed at determining the role of HS-side chains in the binding of HGF/SF. First, the presence of total HS on the different transfectants was assessed by FACS analysis using the HS-specific mAb 10E4 (Fig. 2A), and the mAb 3G10 (Fig. 2B) which recognizes the ΔHS-stubs remaining on HSPG core proteins after treatment with heparitinase (55). Both figures show that cells transfected with CD44v3-10 express approximately 20-fold higher levels of HS compared to those transfected with other CD44 isoforms. Next, we investigated the presence of HS on CD44 itself. This was done by using mAb 3G10. With this mAb, a single major HS band was detected in Western blots of CD44 precipitates from the CD44v3-10 cells, but not from the other transfectants (Fig. 2C). Staining the blot with an anti-pan CD44 mAb demonstrated that this band corresponded to CD44v3-10 (Fig. 2C).

To assess the role of HS in the interaction between HGF/SF and CD44v3-10, we studied the effect of heparitinase treatment and performed binding studies.
Figure 2. Presence of heparan sulfate on CD44 isoforms. A. Heparan sulfate expressed on representative mock, CD44s, CD44v8-10, or CD44v3-10 Namalwa transfectants that were treated with either PBS (filled histogram), 25 mU/ml heparitinase (solid line), or 25 mU/ml chondroitinase ABC (dotted line) at 37°C for 3 h. Heparan sulfate was detected by FACS analysis using the mAb 10E4, followed by RPE-conjugated goat anti-mouse. B. A similar FACS analysis as shown in A, but with the use of mAb 3G10 which recognizes HSPG core proteins after treatment with heparitinase. C. Western blot of CD44 immunoprecipitates. CD44 was precipitated from CD44 Namalwa transfectants using the anti-pan CD44 mAb Hermes-3. Precipitates were then treated with either PBS (-), 200 mU/ml heparitinase (HT), or 1 U/ml chondroitinase ABC (CH) at 37°C for 2 h. The Western blot was stained with the anti-pan CD44 mAb Hermes-3 (upper panel), stripped, and re-stained with the mAb 3G10 (lower panel) which recognizes ΔHS-stubs after treatment of HS with heparitinase.
with HP1, a HGF/SF mutant which has a greatly decreased (more than 50-fold) affinity for heparan sulfate and heparin (57). As shown in Fig. 3A, heparitinase treatment resulted in a near complete loss of HGF/SF binding, while treatment with chondroitinase ABC had no effect. The essential role of HS-moieties on CD44v3-10 in HGF/SF binding was further confirmed by the observation that HP1 did not bind to CD44v3-10 (Fig. 3B). These data demonstrate that CD44v3-10 is a heparan sulfate modified CD44 isoform (CD44-HS), that binds HGF/SF via its HS side chain.

**CD44-HS promotes c-Met activation.** To explore the functional impact of HGF/SF bound to CD44-HS on the c-Met signaling pathway, we generated double transfectants expressing c-Met in combination with either CD44v3-10 or CD44s. We selected stable transfectants expressing equal amounts of c-Met to be used in the subsequent studies (Fig. 4). Using these cell lines, we assessed in the first instance HGF/SF induced c-Met phosphorylation. As shown in Fig. 5, triggering with HGF/SF led to a vast and rapid increase in the phosphorylation of c-Met on tyrosine residues in the cells expressing CD44v3-10. By contrast, phosphorylation

Figure 3. The role of heparan sulfate in the binding of HGF/SF to CD44 Namalwa transfectants. A, FACS analysis to detect HGF/SF bound to CD44 Namalwa transfectants that were treated with either PBS, 10 mU/ml heparitinase, or 50 mU/ml chondroitinase ABC at 37°C for 2 h prior to incubation with 18 nM HGF/SF at 4°C for 1 h. B, FACS analysis of wild type or mutated (HP1) HGF/SF bound to CD44 Namalwa transfectants. HGF/SFs were detected with mouse anti-HGF/SF followed by RPE-conjugated goat anti-mouse. Results are expressed as relative mean fluorescence intensity (MFI) (as compared with PBS treated mock transfectants). Error bars represent the standard deviation from three independent experiments.
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Figure 4. Expression of c-Met in CD44 or CD44/c-Met Namalwa transfecants. CD44s and CD44v3-10 Namalwa transfecants with or without c-Met were lysed and analysed for the expression of c-Met by Western blotting. The Western blot was stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit. The epidermoid carcinoma cell line A431 was used as a positive control. The c-Met precursor (pre c-Met) and β-chain (c-Met (β)) are indicated.

of c-Met was only weakly increased in the cells with CD44s (Fig. 5) and was absent in the parental cell line (data not shown), confirming the lack of endogenous c-Met in these cells. The dose-response studies demonstrated that CD44v3-10 promotes c-Met phosphorylation over a broad dose range (Fig. 5A) with an approximately 7-fold relative increase at plateau level. The time curve (Fig. 5B) showed that phosphorylation was maximal between 2 and 10 min after addition of the growth factor and declined thereafter. Moreover, this strong enhancing effect of CD44v3-10 on c-Met phosphorylation was dependent on HS moieties since it was lost upon heparitinase treatment (Fig. 6A). The importance of HS for HGF/SF signaling was further strengthened by studies using the HGF/SF heparin-binding domain mutant HP1. This mutant induced an equal (weak) phosphorylation of c-Met in both the CD44v3-10 and CD44s transfecants (Fig. 6B). Thus, these data suggest that CD44v3-10 binds HGF/SF via its HS side chains and then presents it to the high affinity receptor c-Met.

**CD44-HS promotes downstream signaling through c-Met in a heparan sulfate-dependent fashion.** The pivotal role of CD44-HS in promoting the action of HGF/SF was further supported by analysing the cell lysates of HGF/SF-stimulated cells for tyrosine phosphorylated proteins. We observed tyrosine phosphorylation of several substrates, the two most prominent phosphoproteins of unknown identity are found at 115-125 kD. A minor phosphoprotein is found at 145 kDa which likely represents c-Met (Fig. 6C). In addition, several smaller
Figure 5. CD44v3-10 strongly promotes c-Met activation. A. Dose-kinetics of the tyrosine phosphorylation of c-Met in CD44v3-10/c-Met and CD44s/c-Met double transfectants. Transfectants were stimulated with increasing concentrations HGF/SF for 10 min at 37°C. c-Met was immunoprecipitated with rabbit anti-c-Met and the Western blot was stained with the anti-phosphotyrosine mAb PY-20 followed by HRP-conjugated rabbit anti-mouse (upper panel). Then, the blot was stripped and re-stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). The ratios of tyrosine-phosphorylated c-Met to precipitated c-Met, as determined by densitometric scanning of the blots, are shown in a diagram. B. Time-kinetics of the tyrosine phosphorylation of c-Met in CD44v3-10/c-Met and CD44s/c-Met double transfectants that were stimulated with 2.2 nM HGF/SF for increasing periods at 37°C. c-Met was precipitated and analysed as in (A). The ratios of tyrosine-phosphorylated c-Met to precipitated c-Met, as determined by densitometric scanning of the blots, are shown in a diagram. The c-Met precursor (pre c-Met) and β-chain (c-Met (β)) are indicated. Several independent clones were tested and gave comparable results.

Phosphoproteins of unknown origin were observed (not shown) including a 42 kD phosphoprotein which may represent the p42 ERK2 MAP kinase.

In order to establish whether signal transduction by c-Met is potentiated by the HS moieties on CD44v3-10, we further investigated the activation of
Figure 6. HGF/SF binding to heparan sulfate moieties on CD44v3-10 potentiates signal transduction through c-Met. A. CD44v3-10/c-Met (v3) and CD44s/c-Met (s) double transfectants were treated with 10 mU/ml heparitinase at 37°C for 3.5 h. and subsequently incubated in the presence or absence of 2.2 nM HGF/SF. Then, c-Met was precipitated with rabbit anti-c-Met and the Western blot was stained with anti-phosphotyrosine (PY-20) followed by HRP-conjugated rabbit antimouse (upper panel). Next, the blot was stripped and stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). The c-Met precursor (pre c-Met) and β-chain (c-Met (β)) are indicated. B. CD44v3-10 does not promote c-Met phosphorylation by a HGF/SF heparin-binding domain mutant. CD44s/c-Met (s) and CD44v3-10/c-Met (v3) double transfectants were incubated in the presence or absence 2.2 nM wild type HGF/SF or with the heparin-binding domain mutant HGF/SF (HP1) for 10 min at 37°C. Then, c-Met was precipitated with rabbit anti-c-Met and the Western blot was stained with anti-phosphotyrosine (PY-20) followed by HRP-conjugated rabbit antimouse (upper panel). Next, the blot was stripped and re-stained with rabbit anti-c-Met followed by HRP-conjugated goat anti-rabbit (lower panel). C. Western blot from total cell lysates from equal numbers of the cells described in (A). The upper part of the blot was stained with the anti-phosphotyrosine mAb PY-20, followed by HRP-conjugated rabbit anti-mouse. The lower part of the same blot was stained with anti-phospho-MAPK antibody, followed by HRP-conjugated goat anti-rabbit. The arrows indicate a phosphorylated protein at 145 kDa and two major phosphoproteins at 115-125 kDa (upper panel), and the phosphorylated ERK1 and ERK2 MAP kinases (lower panel). Several independent clones were tested and gave comparable results.
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downstream targets of c-Met signaling. Since HGF/SF has been shown to activate the ERK MAP kinases in MDCK, HT29 and A549 cells (60-64), we assessed whether HGF/SF is also able to induce MAP kinase activation in Namalwa B cells. For this purpose we used an antibody recognizing only the active, phosphorylated, form of the ERK 1 and 2 (p44 and p42) MAP kinases. As shown in Fig. 6C, HGF/SF treatment results in phosphorylation of the MAP kinases ERK1 and 2 in Namalwa transfectants expressing c-Met. The phosphorylation of the ERK2 MAP kinase upon HGF stimulation of the cells was also confirmed by MAP kinase gel-shift analysis. We observed stronger phosphorylation of ERK1 and 2 in the CD44v3-10 expressing cells as compared to the CD44s expressing cells (Fig. 6C, bottom panel). Moreover, heparitinase treatment resulted in a decrease of HGF/SF-induced ERK phosphorylation in the CD44v3-10 cells, resulting in a level of ERK phosphorylation that is similar to the level of HGF/SF-induced ERK phosphorylation in CD44s transfectants. HGF/SF-induced phosphorylation of the ERKs in CD44s transfectants remained unaffected by heparitinase treatment. Taken together, our data demonstrate that signal transduction elicited by HGF/SF-induced c-Met activation is strongly promoted by CD44-HS, and depends on the presence of the HS moiety on CD44-HS.

DISCUSSION

We observed that cells transfected with CD44v3-10 efficiently bind HGF/SF (Fig. 1) and that this CD44 isoform is decorated with HS moieties (Fig. 2). By contrast, transfectants that express CD44s or CD44v8-10, CD44 isoforms which are not modified with HS (Fig. 2), were not able to bind HGF/SF above background (parental) levels (Fig. 1). This selective HS-modification of CD44v3-10 is in line with the recent study by Jackson et al. (13) which demonstrated that HS side chains bind to CD44 at the SGS G motif encoded by exon v3. Indeed, we demonstrated that the interaction of HGF/SF with CD44v3-10 is HS-dependent. Binding was completely abrogated by heparitinase treatment, and HP1, a HGF/SF mutant with greatly decreased affinity for heparan sulfate and heparin (57), failed to bind CD44v3-10 (Fig. 3). Interestingly, it has been demonstrated that specific chemical modifications of HS side chains on proteoglycans appear to regulate their affinity for selected heparin-binding growth factors, including HGF/SF and FGF-2, and hence determine growth factor binding specificity (65-69). This suggests that the HS moiety covalently attached to CD44v3-10 contains specific binding sites for HGF/SF.

The key finding of our study is that CD44-HS has a major functional effect on HGF/SF-induced signal transduction. Expression of CD44-HS at the cell surface led to a vast increase in HGF/SF induced phosphorylation of c-Met on
tyrosine residues (Fig. 5). Furthermore, it resulted in a strong tyrosine phosphorylation of two as yet unidentified 115-125 kDa proteins that were hardly phosphorylated in the absence of CD44-HS (Fig. 6C). One of these proteins might represent p110/115-Grb2 associated binder (Gab)-1, an adaptor protein that has recently been found to associate with the multifunctional docking site of c-Met (70). Alternatively, the observed bands might be p120-Cbl and/or p125-FAK. Both protein tyrosine kinases participate in signal transduction via receptor protein tyrosine kinases and integrins (71, 72). This is particularly interesting given our previous results that HGF/SF-stimulation of Namalwa Burkitt’s lymphoma cells results in enhanced integrin α4β1-mediated adhesion (41) and the recent observation that Cbl is involved in integrin activation and spreading of

Figure 7. Model for the presentation of HGF/SF to c-Met. A. HGF/SF molecules, which are largely monomers, only weakly activate the c-Met pathway in (tumor) cells that lack cell surface expression of CD44-HS. B. By upregulating CD44-HS, (tumor) cells acquire a greatly increased sensitivity to HGF/SF, which might result in a growth and motogenic/metastatic advantage. Presumably, CD44 acts by concentrating HGF/SF at the cell surface and by presenting HGF/SF to c-Met. This presentation may involve ligand multimerization by HS side chains, resulting in increased c-Met dimerization. Alternatively, HGF/SF - CD44-HS interaction might lead to a conformational changes of the c-Met receptor promoting signal transduction.
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macrophages (73). Furthermore, Cbl was recently reported to be required for efficient cellular transformation through the Tpr-Met oncoprotein (74). In addition to the 120-125 kDa proteins, we demonstrated for the first time that HGF/SF induces phosphorylation of the MAP kinases ERK1 and 2 in B cells (Fig. 6C). Even more intriguing was the observation that CD44-HS promoted the HGF/SF-induced phosphorylation of ERK1 and 2. ERK1 and 2 are intermediates in signaling pathways linking extracellular signals to gene transcription in the nucleus and have been implicated in a wide variety of biological responses including cell proliferation. Interestingly, several recent studies have implicated the ERKs in integrin activation (75) as well as in HGF/SF-induced motility (i.e. scattering), and tubulogenesis of the epithelial Madin-Darby canine kidney cell line (60, 62, 63). Because of our previous data concerning the involvement of HGF/SF in integrin-mediated adhesion of B cells (41), we are currently investigating the possible role of the ERKs in B cell adhesion and migration.

We demonstrated that the enhancing effects of CD44-HS on signal transduction via c-Met were critically dependent on the interaction of HGF/SF with the HS moieties on CD44-HS, as they were not observed after heparitinase treatment, or when the cells were triggered with the heparin-binding domain HGF/SF mutant HP1 (Fig. 6). Importantly, the specific effects of the heparitinase treatment and the mutations in HP1 on HGF/SF-induced signal transduction in the CD44v3-10 expressing cells as compared to the CD44s cells demonstrates that the difference in HGF/SF-elicted responses in these cells is not due to any possible clonal variation in these stable cell lines. We speculate that CD44-HS promotes the action of HGF/SF through concentration of HGF/SF on the cell surface and by presenting it to the high affinity receptor c-Met (Fig. 7). Similar mechanisms were proposed for the role of high and low affinity receptors in FGF functioning (32, 76, 77). In addition, CD44-HS might also protect HGF/SF from proteolytic degradation as endothelial cell-derived HS was shown to do for FGF-2 (78).

It should be noted, that, apart from growth factor presentation, CD44 may have additional functions in HGF/SF - c-Met mediated signaling. For example, CD44 might recruit molecular partners into a multi-molecular complex with c-Met. This possibility is suggested by the fact that two recently identified cytoplasmic molecules associated with CD44 have also been implicated in c-Met signaling. First, studies by Ponzetto et al. (64) have shown that c-Met is a substrate for Src-family tyrosine kinases, while our own studies have revealed a physical and functional association between CD44 and Src-family member p56k (23). Second, studies by Jiang et al. (79) and Crepaldi et al. (80) have demonstrated that HGF/SF stimulates the tyrosine phosphorylation of the ERM-protein ezrin. As reported by Tsukita et al. (22), ERM-proteins serve as molecular linkers between CD44 at the cell surface and the actin cytoskeleton. This interaction is believed to be involved in the regulation of cell shape and motility.
We propose that collaboration between CD44-HS and growth factor receptors, *viz.* c-Met, as shown in our present study, might be an important factor in tumor growth and metastasis. By over expressing CD44-HS, tumor cells would acquire a strongly increased sensitivity to HGF/SF mediated growth signals, leading to a growth advantage and promoting metastasis (Fig. 7). This hypothesis is supported by the fact that c-Met and HGF/SF are (over)expressed in conjunction with CD44 in several types of tumors. In colorectal cancer, for example, c-Met is frequently over expressed (48, 49, 81), while HGF/SF is expressed within the tumor tissue microenvironment. Interestingly, in these tumors CD44 splice variants, including variants decorated with HS, are often over expressed and predict metastatic spread and tumor related death (82, 83). A similar scenario may hold for breast cancer and non-Hodgkin's lymphoma, as in these tumor types over expression of CD44v3 as well as c-Met has also been reported (19, 42, 51, 84).

In conclusion, we demonstrated that through binding and presenting HGF/SF, CD44-HS promotes signal transduction via the receptor tyrosine kinase c-Met. Consequently, over expression of CD44-HS might give tumor cells a growth and metastatic advantage and, in this way, might influence disease outcome.

**FOOTNOTES**

1. M. Spaargaren and G.J.T. Zwartkruis, unpublished observation.
2. V.J.M. Wielenga, unpublished observation.

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Chapter 5

Regulation of cytokine signaling by B cell antigen receptor and CD40 controlled expression of heparan sulfate proteoglycans.

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Chapter 5

SUMMARY

Recently, biochemical, cell biological, and genetic studies have converged to reveal that integral membrane heparan sulfate proteoglycans (HSPGs) are critical regulators of growth and differentiation of epithelial- and connective tissues. Since a large number of cytokines involved in lymphoid tissue homeostasis or inflammation contain potential heparan sulfate-binding domains, HSPGs presumably also play important roles in the regulation of the immune response. In the present report, we explored the expression, regulation and function of HSPGs on B lymphocytes. We demonstrate that activation of the B cell antigen receptor (BCR) and/or CD40, induces a strong transient expression of HSPGs on human tonsillar B cells. By means of these HSPGs, the activated B cells can bind hepatocyte growth factor (HGF), a cytokine that regulates integrin-mediated B cell adhesion and migration. This interaction with HGF is highly selective since the HSPGs did not bind the chemokine stromal-derived factor 1α (SDF-1α), even though the affinities of HGF and SDF-1α for heparin are similar. On the activated B cells, we observed induction of a specific HSPG isoform of CD44 (CD44-HS), but not of other HSPGs such as syndecans or glypican-1. Interestingly, the expression of CD44-HS on B cells strongly promotes HGF-induced signaling, resulting in a heparan sulfate-dependent enhanced phosphorylation of Met, the receptor tyrosine kinase for HGF, as well as downstream signaling molecules including the Grb2 associated binder 1 (Gab1) and Akt/protein kinase B (PKB). Our results demonstrate that the BCR and CD40 control the expression of HSPGs, specifically CD44-HS. These HSPGs act as functional co-receptors which selectively promote cytokine signaling in B cells, suggesting a dynamic role for HSPGs in antigen-specific B cell differentiation.

INTRODUCTION

Proteoglycans are proteins that are covalently linked to sulfated glycosaminoglycan (GAG) chains composed of repeating disaccharide units (1). These molecules, which are widespread throughout mammalian tissues as extracellular matrix (ECM) components and membrane-bound molecules, have been implicated in several important biological processes including cell adhesion and migration, angiogenesis, tissue morphogenesis, and regulation of blood coagulation (1-3). In these processes, proteoglycans are believed to function as scaffold structures, designed to accommodate proteins through non-covalent binding to their GAG chains. In particular heparan sulfate proteoglycans (HSPGs) have been shown to function as versatile protein co-receptors. Their ligand-binding sites reside within discrete sulfated domains formed by complex, cell-specific, chemical modifications
HSPGs regulate cytokine signaling in B cells

of the heparan sulfate (HS) disaccharide repeat (4). Binding of proteins, including growth factors/cytokines, to HS chains may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of biological function (5, 6). This functional importance is illustrated by fibroblast growth factor 2 (FGF-2), whose binding to its signal-transducing receptors and consequent biological effects are critically dependent on its interaction with cell-surface HSPGs (7, 8). Recently, a number of cell biological and genetic studies have provided compelling evidence for an in vivo role of cell-surface HSPGs in growth control and morphogenesis in Drosophila, mice and humans (9-14).

Most studies concerning the expression and function of cell-surface HSPGs have focussed on epithelial cells and fibroblasts, but these molecules presumably also play important roles in the immune system. A vast number of cytokines involved in lymphoid tissue homeostasis or inflammation bind to heparin, a GAG structurally related to HS. These cytokines, which include chemokines, as well as interleukins and hematopoietic growth factors, e.g. interleukin (IL) 3, IL-8, granulocyte-monocyte colony stimulating factor (GM-CSF), and HGF (15-20), can thus be potentially immobilized by HSPGs. HSPGs expressed on the luminal surface of endothelial cells have been shown to bind chemokines produced at sites of inflammation (21), thereby preventing their immediate dilution by the blood stream. Presentation of HSPG-bound chemokines, e.g. macrophage inflammatory protein (MIP) 1β and IL-8, to leukocytes plays a crucial role in activating the leukocyte integrins that mediate stable adhesion to and transmigration across the vessel wall (22, 23). However, chemokines and other heparin-binding cytokines do not exclusively act at the endothelial-blood interface. They also play key roles in the regulation of lymphocyte trafficking within lymphoid tissues and are involved in the control of lymphocyte growth, differentiation and survival (24). This suggests that cell-surface HSPGs on cells of the immune system, such as lymphocytes and antigen-presenting cells, might also be involved in the regulation of cytokine responsiveness. To explore this hypothesis, we have studied the expression, identity, regulation and function of HSPGs on human tonsillar B cells. We show that ligation of the BCR or CD40, two key receptors in the initiation of antigen-specific B cell differentiation (25-29), induces a strong upregulation of cell-surface HSPGs, specifically of CD44-HS. These HSPGs enable B cells to selectively bind hepatocyte growth factor (HGF), a growth factor that induces integrin-dependent adhesion and migration of B cells (30, 31). Moreover, we show that CD44-HS strongly potentiates HGF-induced signaling in B cells.
MATERIALS AND METHODS

**Antibodies.** Mouse monoclonal antibodies used were anti-pan CD44, Hermes-3 (IgG2a) (32); anti-CD44v3, 3G5 (IgG2b) (R&D Systems, Abingdon, UK); anti-heparan sulfate, 10E4 (IgM) (Seikagaku, Tokyo, Japan); anti-desaturated uronate from heparitinase treated heparan sulfate (anti-ΔHS-stub), 3G10 (IgG2b) (Seikagaku); anti-HGF, 24612.111 (IgG1) (R&D Systems); anti-Met, D024 (IgG2a) (Upstate Biotechnology, Lake Placid, NY); anti-CXCR4, 12G5 (IgG2a) (Pharmingen, San Diego, CA); anti-syndecan-1, 1D4 (IgG1) (CLB, Amsterdam, The Netherlands); anti-syndecan-2, 10H4 (IgG1) (33); anti-syndecan-4, 8G3 (IgG1) (34); anti-glypican-1, S1 (IgG1) (35); anti-phosphotyrosine, PY-20 (IgG2b) (Affiniti, Nottingham, UK) and IgG1, IgG2a, IgG2b and IgM control antibodies (ICNN Zoetermeer, The Netherlands). Polyclonal antibodies used were rabbit anti Met, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-Ga b1 (Upstate Biotechnology); rabbit anti-SDF-1α (Pepro Tech, Rocky Hill, NJ); rabbit anti-Akt (H-136) (Santa Cruz Biotechnology); phospho-specific rabbit anti-Akt (Ser 473) (New England Biolabs, Hitech, UK); RPE-conjugated anti-mouse (Southern Biotechnology Associates, Birmingham, AL); biotin-conjugated swine anti-rabbit (DAKO); HRP-conjugated goat anti-rabbit (DAKO), and HRP-conjugated rabbit anti-mouse (DAKO). In addition we used RPE-conjugated streptavidin (DAKO).

**B cell isolation and culturing.** B cells were isolated from human tonsils as described previously (36). Total B cell fractions were >97% pure as determined by FACS analysis. B cells were cultured in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all Gibco BRL/Life Technologies, Breda, The Netherlands). Some media were supplemented with either 50 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma), 0.002% Staphylococcus aureus strain Cowan I (SAC) (Calbiochem Novabiochem, La Jolla, CA), 1 μg/ml Immunobeads with covalently bound rabbit anti-human Ig (Irvine Scientific, Santa Ana, CA), 100 U/ml IL-2 (Eurocetus, Amsterdam, The Netherlands), 100 U/ml IL-4 (Genzyme Diagnostics, Cambridge, MA), 0.5 ng/ml IL-6 (CLB), or 25 ng/ml IL-10 (Genzyme Diagnostics).

For CD40 ligation, B cells were cultured on irradiated (7000 rad) CD40L (CD154)-transfected or, as a control, wild-type L cells (37).

**Cell lines and transfectants.** The Burkitt’s lymphoma cell line Namalwa was purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in RPMI 1640 (Gibco BRL/Life Technologies) supplemented with 10% Fetal Clone 1 serum (HyClone Laboratories, Logan, UT), 10% FCS (Integro, Zaandam, The Netherlands), 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (all Gibco BRL/Life Technologies). The Namalwa cell lines transfected with CD44s (Nam-SM) or CD44v3-10 (Nam-V3M) were described previously (30, 38).

**Enzyme treatments.** For enzymatic cleavage of HS, cells or tissue sections were treated with 10 mU/ml heparitinase (Flavobacterium heparinum, EC 4.2.2.8, ICN Biomedicals, Aurora, OH) in RPMI 1640 (Gibco BRL/Life Technologies) at 37°C for 3 h.
The cleavage of HS by heparitinase was determined by the loss of cell-surface expressed HS (mAb 10E4), and the simultaneous gain of HS-stub expression (mAb 3G10). Chondroitinase treatment was used as a specificity control.

**FACS analysis.** FACS analyses using a single or triple staining technique were described previously (39, 40). For cytokine binding assays, cells were incubated with saturating concentrations (20 nM) of recombinant human HGF or recombinant human SDF-1α (both R&D Systems), in PBS for 1 h. prior to the antibody incubations. This step was followed by washing with FACS buffer. For blocking studies, cells were incubated with 125 nM recombinant human FGF-2 or recombinant human SDF-1α (both R&D Systems) in PBS at 4°C for 1 h. prior to the incubation with HGF.

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation and Western blotting were performed as described (39). For the immunodepletion experiments, the lysates were immunoprecipitated twice and the lysate remaining after the second immunodepletion and the immunoprecipitate obtained during the first immunoprecipitation were analysed by Western-blotting.

**RESULTS**

**Expression and Regulation of HSPGs on Human B Cells.** We investigated the expression of HSPGs on the cell-surface of resting and activated human tonsillar B cells by means of FACS analysis, employing mAb 10E4, against an epitope on HS-chains. In addition, we used mAb 3G10, recognizing the ΔHS-stubs that remain present on HSPG-core proteins after heparitinase treatment. HSPGs were hardly detectable on freshly isolated tonsillar B cells. However, upon stimulation of these cells with the phorbol ester PMA, we observed a strong induction of HSPGs (data not shown). This observation prompted us to explore whether HSPGs can also be induced by physiological B cell activators. Since engagement of the BCR and CD40 plays a key role in the initiation of T cell-dependent B cell responses and in the formation of germinal centers (GC) (25-29), we assessed whether activation via these receptors also leads to HSPG upregulation. Tonsillar B cells were cultured on CD40 ligand (CD40L) transfected L cells or, as a control, on wild-type L-cells, in the presence or absence of BCR stimuli (anti-Ig antibodies or *Staphylococcus aureus* Cowans strain I [SAC]). As is shown in Fig. 1, concurrent ligation of CD40 and the BCR induced a strong induction of HSPGs on the B cells. Single triggering of either the BCR or CD40 also led to enhanced HSPG expression, although the HSPG levels were lower than those obtained upon dual receptor ligation. In contrast, stimulation by various cytokines including IL-2, IL-4, IL-6, and IL-10 did not lead to a significant induction of HSPGs (data not shown). These data identify activation via the BCR and CD40 as major signals for the induction of HSPG expression on tonsillar B cells.
**HSPGs on Activated B Cells Selectively Bind HGF.** HSPGs are capable of highly selective cytokine binding and presentation (4). To explore the cytokine-binding ability and specificity of the HSPGs expressed on activated B cells, we tested their capacity to bind two distinct cytokines with established heparin-binding capacity, i.e. HGF and stromal cell-derived factor (SDF) 1α (18, 41). Although they are structurally unrelated, HGF belongs to the plasminogen-related growth factor family (42) and SDF-1α is a chemokine (43), these cytokines have both been implicated in the regulation of B cell adhesion and migration (30, 31, 41, 43). In agreement with the data presented in Fig. 1, culturing of tonsillar B cells on CD40L transfected, but not on wild-type, L-cells, led to a strong induction of HS (Fig. 2A). In parallel, these B cells acquired a vast capacity to bind HGF (Fig. 2A). This HGF binding was largely dependent on HS, since over 80% was lost after heparitinase.

![Graph](image)

**Figure 1.** Activation via the BCR and CD40 induces strong expression of HSPGs on B cells. Tonsillar B cells were cultured on CD40L-transfected L cells or wild-type L cells, in the presence or absence of BCR stimuli, i.e. anti-Ig immunobeads (Ig) or *Staphylococcus aureus* Cowans strain I (SAC), for 0, 24, or 48 h, and analysed by FACS. Expression of HSPGs is given as the mean fluorescence intensity (MFI) of the anti-ΔHS-stub staining after heparitinase treatment minus its staining before treatment.
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Figure 2. HSPGs on activated B cells bind HGF but not SDF-1α. A, CD40 stimulation induced HS expression and HGF binding, but not SDF-1 binding. Tonsillar B cells were analyzed by FACS for HS expression and for their capacity to bind HGF or SDF-1α after being cultured on CD40L-transfected L cells or wild-type L cells for 48 h. To determine the involvement of HS, the cells were analyzed after control or heparitinase treatment. Expression of HS is given as the MFI after staining with anti-HS mAb 10E4 minus staining with an isotype-matched control mAb. Binding of HGF or SDF-1α is given as the MFI of cells that were incubated with one of the cytokines, washed, and stained with a cytokine-specific Ab, minus the MFI of identically stained control cells. B, Effect of CD40 stimulation on Met and CXCR4 expression. Expression of the receptor for HGF, Met, and the receptor for SDF-1α, CXCR4, on unstimulated or CD40L-stimulated tonsillar B cells was analysed by FACS. Expression of Met or CXCR4 is given as the MFI after staining with receptor-specific mAbs, minus the MFI after staining with an isotype-matched control mAb.

treatment of the B cells (Fig. 2A). The HGF that remained present on the cells after heparitinase-treatment most probably was bound to its receptor tyrosine kinase Met, as Met was also induced by CD40 ligation (Fig. 2B). In contrast to HGF, binding of SDF-1α to the B cells was completely independent of HS: The HSPG low B cells that were cultured on wild-type L cells had a much greater SDF-1α binding capacity than the HSPG high B cells cultured on CD40L-transfected L cells (Fig. 2A). Moreover, heparitinase treatment did not have any effect on SDF-1α binding. The differences in SDF-1α binding between resting and activated cells were directly related to differential expression of CXCR4, the high affinity receptor.
Figure 3. CD40 stimulation induces expression of CD44-HS on tonsillar B cells. A, CD44-HS, but not syndecan-1, is induced by CD40 stimulation. Expression of syndecan-1, or CD44v3 by tonsillar B cells cultured on CD40L-transfected L cells, or wild-type L cells for 0, 24, or 48 h, was analyzed by FACS. Expressions are given as the MFI after staining with anti-syndecan-1 or anti-CD44v3, minus the staining with isotype-matched control mAbs. B, CD44v3 isoform(s) on CD40-activated B cells are decorated with HS. CD44 was immunoprecipitated from tonsillar B cells that had been cultured on wild-type L cells or CD40L-transfected L cells for 48 h, and immunoblotted with anti-pan CD44 (left panel), or with anti-AHS-stub (right panel). To allow the detection of AHS-stubs, the cells had been treated with heparitinase before immunoprecipitation. Namalwa B cells transfected with the HSPG CD44v3-10 (Nam-V3M) were used as a positive control.
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Figure 4. HGF, but not SDF-1α, binds to CD44v3-10 (CD44-HS). A, Namalwa B cells stably transfected with CD44v3-10 (Nam-V3M) express HS, whereas those transfected with CD44s (Nam-SM) do not. B, Namalwa B cells stably transfected with CD44v3-10 (Nam-V3M) express Met and CXCR4, and show HS-dependent binding of HGF but not of SDF-1α. Nam-V3M cells were analyzed by FACS for their expression of HS, Met, or CXCR4. In addition, their capacity to bind HGF or SDF-1α is shown. To determine the involvement of HS, the cells were analyzed after control or heparitinase treatment. Expression of HS, Met, or CXCR4 is given as the MFI after staining with specific mAbs minus the MFI after staining with an isotype-matched control mAb. Binding of HGF or SDF-1α is given as the MFI of cells that were incubated with one of the cytokines, washed, and stained with a cytokine-specific Ab, minus the MFI of identically stained control cells. C, HGF binding to CD44v3-10 is cross-blocked by FGF-2 but not by SDF-1α. HGF binding to Namalwa CD44v3-10 transfectants was analyzed by FACS as described above. Prior to the incubation with HGF, the cells were incubated with a range of concentrations of either FGF-2 or SDF-1α.
for SDF-1α: Expression of this receptor strongly decreased as a result of CD40 ligation (Fig. 2B).

**Activated B Cells Express HSPG forms of CD44.** The above data show that, upon their activation, B cells acquire cell-surface expressed HS-chains that are capable of selective growth factor binding. This may be the consequence of either upregulation of proteoglycan core protein(s) or upregulation or activation of the enzymes involved in HS-synthesis. To address this issue and to identify the proteoglycan core proteins carrying the HS-chains, we employed mAbs against a panel of defined proteoglycan core proteins, i.e., the syndecans-1, -2, and -4, glypican-1, and CD44. As is shown in Fig. 3A, B cell activation enhanced expression of CD44 splice variants containing epitopes encoded by exon v3, which can be decorated with HS (39, 44). By contrast, no basal expression, nor induction of the distinct syndecans or glypican-1 was observed after B cell activation via CD40 and/or the BCR (Fig. 3A, and data not shown).

To ensure that the CD44 isoforms expressed by activated tonsillar B cells are indeed decorated with HS-chains, CD44 was immunoprecipitated from resting and activated B cells and the immunoprecipitates were analysed on Western blot for the presence of CD44 and HS. Whereas both unstimulated and CD40L-stimulated B cells expressed the 90 kDa ‘standard’ isoform of CD44 (CD44s) (Fig. 3B), activation via CD40, in addition, induced expression of a 200 kDa CD44 isoform (Fig. 3B). Upon re-staining the blot, only this 200 kDa CD44 isoform was found to be modified with HS. By its size, the CD44-HS isoform on activated B cells resembles the HS-modified CD44v3-10 isoform expressed on Namalwa cells (Fig. 3B), suggesting that they are the products of similar or identical transcripts.

Since we demonstrated in Fig. 2 that the HSPGs which were induced upon B cell activation specifically bound HGF but not SDF-1α, we investigated the ability of CD44-HS to bind HGF and SDF-1α. For this purpose we employed Namalwa B cells transfected with either the CD44-HS isoform CD44v3-10, or the isoform CD44s, which does not contain an HS-attachment site (44). Indeed, as shown in Fig. 4A, did only the Namalwa cells transfected with CD44v3-10 express HS. Moreover, in contrast to HGF, SDF-1α did not bind to these cells in a HS-dependent manner (Fig. 4B). Furthermore, in contrast to the heparin-binding growth factor FGF-2, SDF-1α did not compete with HGF for binding to the cells, even at concentrations exceeding those of FGF-2 by more than a factor of ten (Fig. 4C). Taken together, these results indicate that ligation of CD40 on tonsillar B cells induces the expression of CD44-HS, most likely the CD44v3-10 isoform. This CD44 isoform is capable of selectively recruiting HGF to the B cell surface.
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Figure 5. CD44-HS promotes HGF/Met signaling in B cells in a HS-dependent fashion. A, CD44-HS promotes HGF-induced tyrosine phosphorylation of Met. Namalwa B cells, stably transfectected with either CD44v3-10 (v3), which is HS-decorated, or CD44s (s), were treated with heparitinase, as indicated, and subsequently stimulated with HGF. Anti-Met immunoprecipitates were immunoblotted with anti-phosphotyrosine (PY-20) (upper panel), or with anti-Met (lower panel). B, CD44-HS promotes HGF-induced tyrosine phosphorylation of proteins downstream of Met. An immunoblot of total cell lysates prepared from cells treated as described in A, was stained with anti-phosphotyrosine. The arrow indicates (a) highly phosphorylated protein(s) at 115-120 kDa. C, HGF-induced Gab1 phosphorylation is enhanced in the presence of CD44-HS. An immunoblot of immunodepleted Namalwa CD44v3-10 cell lysates (left panels) or their corresponding immunoprecipitates (right panels) is shown. Immunoprecipitation was performed with anti-Gab1 or with control mAbs (C) as indicated. The blots were stained with anti-phosphotyrosine (upper panels) or with anti-Gab1 (lower panels). The arrowhead indicates a band probably representing co-immunoprecipitated Met, while the thin and thick arrow indicate an unidentified protein of about 115 kDa, and Gab1, respectively. D, CD44-HS promotes HGF-induced activation of Akt/PKB. Control or heparitinase-treated cells were incubated with HGF for 0, 2, or 10 min as indicated. Total cell lysates from the transfectants described in A were immunoblotted with anti-phospho-Akt (upper panel), or anti-Akt (lower panel).
Chapter 5

Cell Surface HSPGs Regulate Met Signaling in B Cells. The above data suggest a role for HSPGs, specifically CD44-HS, in the regulation of HGF/Met signaling. To address this hypothesis, we employed Met positive Namalwa B cells transfected with either a CD44-HS isoform (CD44v3-10), or, as a control, with a CD44 isoform which can not be decorated with HS (CD44s) (39). Upon HGF stimulation a strong phosphorylation of Met was induced in the cells expressing CD44-HS, whereas phosphorylation in the cells expressing CD44s was weak (Fig. 5A). HS-moieties decorating CD44 were responsible for the strongly enhanced Met phosphorylation in the cells carrying CD44-HS, as heparitinase treatment reduced the HGF induced phosphorylation to the control level observed in the cells expressing CD44s (which lack HS) (Fig. 5A). The strong potentiation of Met signaling by HS on the B cell surface was not only present at the level of receptor phosphorylation: As is show in Fig. 5B, upon HGF stimulation a broad band representing (at least) two bands (Fig. 5C). The lower of these bands was identified as Grb2-associated binder 1 (Gab1) (Fig. 5C), an adapter protein that can associate with the cytoplasmic docking site of Met (45). Indeed, in anti-Gab1 immunoprecipitates, we detected a phosphorylated protein of about 145 kDa which probably represents Met (Fig. 5C). In addition to Gab1 phosphorylation, a strongly enhanced activation of Akt, also referred to as protein kinase B (PKB), was found upon HGF-stimulation in cells expressing CD44-HS as compared to cells expressing CD44s (Fig. 5D). Again, enhanced Akt activation was HS-dependent, as it could be abrogated by heparitinase treatment of the cells expressing CD44-HS but not CD44s (Fig. 5D). Taken together, these data demonstrate that CD44-HS is capable of regulating HGF/Met signaling in B cells in a HS-dependent fashion.

DISCUSSION

Heparan sulfate proteoglycans are involved in regulating the growth, migration, and differentiation of epithelial cells and fibroblasts (1, 3, 7-9). During these processes HSPGs immobilize and oligomerize cytokines and present them to their high affinity receptors (5, 46-48). In this way, HSPGs create niches in the microenvironment and regulate cytokine responses. Since a large number of cytokines involved in lymphoid tissue homeostasis or inflammation contain potential heparan sulfate-binding sites, HSPGs presumably also play important roles in the regulation of the immune response. However, the expression and
function of HSPGs on the cell surface of lymphocytes, as well as within the ECM of the lymphoid tissues, has thusfar remained largely unexplored. In the present study, we investigated the regulation and function of HSPGs on human B cells. We demonstrate that expression of HSPGs on human B cells is dynamic and that HSPGs are capable of selective cytokine binding and regulation of cytokine-induced signaling.

We observed that freshly isolated tonsillar B cells express low levels of HSPGs but that single or concurrent ligation of the BCR and CD40 induced a strong expression of HSPGs on the B cell surface (Fig. 1). These observations for the first time show that B cell triggering by physiological stimuli has a profound effect on their HSPG expression and suggests that B cells use cell surface HSPGs as a means to control their cytokine-binding capacity and responsiveness. To explore this hypothesis, we analyzed the binding of the cytokines HGF and SDF-1α to HSPGs on activated B cells. These cytokines were selected since they bind heparin, a heavily sulfated heparan sulfate proteoglycan, with similar affinities (41, 49, 50). Moreover, although HGF and SDF-1α are structurally unrelated, they have both been implicated in the regulation of B cell adhesion and migration (30, 31, 51, 52). HGF is a 90 kDa cytokine, which induces complex responses in target cells, e.g. stimulation of motility, growth and morphogenesis, by binding to the receptor tyrosine kinase Met (53-58). HGF is essential for vertebrate development, since knock-out of the HGF or Met genes is lethal and causes abnormal development of the liver and placenta and disrupt the migration of myogenic precursors to the limb buds (59-61). Other studies suggest important roles for HGF in tissue regeneration and in tumor growth, invasion, and metastasis (53, 62-64). The CXC chemokine SDF-1 was originally identified as a pre-B cell growth stimulating factor (65) and, more recently, has been implicated in a variety of processes, including hematopoiesis, cerebellar and vascular development, and cardiogenesis, by activating its receptor CXCR4 (66-69). SDF-1α is a potent chemoattractant for hematopoietic progenitors, and induces migration of naive and memory, but not germinal center, B cells (43, 52, 69, 70).

We observed that the inducibly expressed HSPGs on CD40-activated B cells are capable of binding large quantities of HGF, but not SDF-1α. Instead, B cell activation resulted in a strongly decreased binding of SDF-1α to the B cells (Fig. 2A). This could be explained by the observation that CXCR4, the SDF-1α receptor, was down-regulated in response to CD40 triggering, in analogy to BCR-induced down-regulation of CXCR4 (71) (Fig. 2B). The finding that HS-moieties on B cells do not bind SDF-1α was further corroborated by our observation that SDF-1α, unlike the heparin-binding cytokine FGF-2, does not compete with HGF for HSPG-binding (Fig. 4C). Hence, upon their activation via the BCR and CD40, B cells do not only gain expression of the HGF receptor Met (30) (Fig. 2B), but in addition acquire the appropriate HSPGs, i.e. HSPGs with the capacity to bind large
quantities of HGF. Thus, unlike interactions with heparin, the interaction between cytokines and the natural HSPGs that are induced during B cell activation appear to be highly selective, suggesting that HSPG contribute an additional level of specificity to B cell-cytokine interactions and may co-regulate B cell differentiation. Selectivity of HSPGs-protein interactions has also been observed in other biological systems, including blood coagulation and embryonic development (2-4, 9). It is determined by the structural modifications of the HS-chains, which take place within the Golgi complex, as well as by the nature of the core protein (4, 18).

HSPGs consist of HS-chains covalently attached to a core protein. Ligation of CD40 resulted in a strong induction of cell-surface expressed CD44 splice variants containing the domain encoded by exon v3 (Fig. 3A). This domain contains a consensus motif for HS attachment (39, 44), and we indeed confirmed that CD44 isoforms on activated B cells are decorated with HS (Fig. 3B). The relative molecular mass of the HSPG form of CD44 on activated B cells was indistinguishable from that of a CD44v3-10 isoform expressed by Namalwa cells, suggesting that they are products of similar or identical transcripts (Fig. 3B). Although we cannot exclude a contribution of other (unknown) core proteins, our findings identify CD44 as an important cell surface HSPG on activated B cells. As of yet, data on the expression and function of HSPGs in lymphocytes are scarce. The HSPG syndecan-1 can be expressed by human plasma cells, myeloma cells, and Reed-Sternberg cells of classical Hodgkin’s disease (72-74), and syndecan-4 expression has been demonstrated in mouse B cell (75). Apart from a possible role in growth factor presentation, analogous to that observed for CD44-HS in our present study, syndecan-1 and -4 may be important mediators of cell-cell adhesion since their transfection to B lymphoblastoid cell lines results in cell spreading and aggregation (76, 77).

Incubation of HGF with heparin or HS-derived oligosaccharides has been reported to promote phosphorylation of the HGF receptor Met (15). This prompted us to explore the impact of CD44-HS expression on HGF-induced signal transduction. Interestingly, we observed that the autophosphorylation of Met, as well as the phosphorylation of the kinase Akt/PKB, and of two proteins of 110-120 kDa is strongly promoted by expression of CD44-HS at the B cell surface (Fig. 5). Immunodepletion experiments indicated that the smaller of the two proteins represents Grb2-associated binder 1 (Gab1), an adapter protein which can associate with Met (45) (Fig. 5C). The other hyperphosphorylated protein might represent p120-Cbl, a protein tyrosine kinase that participates in signal transduction via receptor tyrosine kinases, and that has implicated in the regulation of integrin activation (78, 79). This is of particular interest, since stimulation of B cells with HGF results in enhanced integrin dependent adhesion (see below).
Our observations suggest a scenario in which B cells, upon their activation by antigen and T cells, become insensitive to the migration promoting activity of SDF-1α as a result of down-regulation of CXCR4. At the same time they acquire the receptor tyrosine kinase Met (30) as well as HSPG, viz. CD44-HS, which allow them to selectively recruit HGF to the B cell surface, resulting in efficient HGF/Met signaling. We have previously shown that HGF is produced by follicular dendritic cells (FDC) and enhances integrin-dependent adhesion of B cells to fibronectin and VCAM-1. Hence, activation of the HGF/Met pathway may strengthen B cell adhesion, specifically to FDC, which is mediated by α4β1 – VCAM-1 (80). Interestingly, apart from establishing physical contact, outside-in signaling via α4β1 presumably contributes to the B cell selection process in the germinal center by inhibiting apoptosis of B cells (36, 40). Since both integrin engagement and Met stimulation may lead to activation of Akt/PKB, a pathway reported to suppress apoptosis (81), it will be of interest to explore the collaborative effects of integrin and Met signaling on B cell survival.

In conclusion, our data demonstrate that the BCR and CD40 control the expression of HSPGs, specifically CD44-HS, on B cells. By selectively binding HGF to the B cell surface, these HSPGs act as functional co-receptors for HGF promoting signaling through Met, which suggests a role for these HSPGs in the regulation of antigen-specific B cell differentiation.

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Chapter 6

The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation.

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I. INTRODUCTION

Hepatocyte growth factor/Scatter factor (HGF), originally described as a strong mitogen for hepatocytes (Michalopoulos et al., 1984; Nakamura et al., 1984; Russell et al., 1984a, 1984b), is a multifunctional cytokine with a domain structure and a proteolytic mechanism of activation similar to that of the blood serine protease plasminogen. Unlike plasminogen, however, HGF is devoid of protease activity but has pleiotropic effects on target cells, including stimulation of growth, motility, and morphogenesis. All known biological effects of HGF are transduced via a single receptor, i.e., Met, the product of the Met proto-oncogene. The Met protein is a receptor tyrosine kinase and is the prototype of a distinct subfamily, also encompassing Ron and Sea. Upon ligand binding, Met interacts with several cytoplasmic target proteins resulting in activation of a number of distinct signaling cascades including the Ras/MAP kinase and PI3-K/PKB pathways. In addition to binding Met, HGF has a high affinity for heparin and heparan sulfate. Heparan sulfate is present on the cell surface and in the ECM, in the form of heparan sulfate proteoglycans (HSPGs). By binding HGF, HSPGs function as co-regulators of Met signaling.

Genetic studies in mice have indicated that HGF is indispensable for mammalian development, as mutations of the HGF or Met genes cause abnormal development of the liver and placenta, and disrupt the migration of myogenic precursors into the limb bud. Other studies have provided evidence for important roles of HGF in angiogenesis, and in the three dimensional organization of kidney
tubular cells and various glandular structures, e.g. mammary glands. Apart from mediating these physiological functions, the HGF/Met pathway is also believed to play a key role in tumor growth, invasion, and metastasis. For example, Met was originally isolated as the product of a human oncogene Tpr-Met and Met and/or HGF overexpression have been reported in several human tumors. The tumorigenicity of HGF/Met signaling has been confirmed in transgenic mouse models, which develop tumors in many different tissues. In human hereditary papillary renal carcinomas, potentially activating Met mutations are found.

In this review, we discuss the structure, signal transduction, and physiological functions of the HGF/Met pathway, as well as its role in tumorigenesis. Furthermore, we highlight recent studies which indicate a role for the HGF/Met pathway in antigen-specific B cell differentiation and B cell neoplasia.

II. STRUCTURE AND FUNCTION OF HGF AND MET

A. Structure of HGF and Met

1. Structure of HGF

Hepatocyte growth factor/scatter factor (HGF) was independently identified by groups working in two different fields of research. In 1984, a factor present in serum of partially hepatectomized rats and in rat platelet lysates, was found to have a strong mitogenic effect on hepatocytes (Michalopoulos et al., 1984; Nakamura et al., 1984; Russel et al., 1984a, 1984b). Hence, this factor was designated hepatocyte growth factor (HGF). Almost simultaneously, Stoker and Perryman (1985) identified a molecule secreted by fibroblasts, which causes dissociation or "scattering" of epithelial cell colonies, and was thus named scatter factor (SF). Subsequent structural and functional studies showed HGF and SF to be identical (Gherardi and Stoker 1990; Weidner et al., 1990, 1991; Furlong et al., 1991; Konishi et al., 1991; Naldini et al., 1991c; Rubin et al., 1991).

The mature HGF protein has a relative molecular mass of 90 kDa under non-reducing conditions and is a heterodimer composed of an α-subunit of 60 kDa and a β-subunit of 30 kDa linked together by a disulfide interchain bridge (Nakamura et al., 1987, 1989; Weidner et al., 1990; Rubin et al., 1991). Due to differential glycosylation, two β-chains, of ~34 kDa and ~32 kDa, respectively, can generally be detected. HGF is secreted as a biologically inactive monomer which is activated through proteolytic cleavage (Naka et al., 1992). Several proteases have been shown to be able to activate HGF. These include urokinase-type (uPA) and
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Figure 1. Schematic representation of the HGF protein. HGF is a secreted glycoprotein composed of a 60 kDa α-chain and a 30 kDa β-chain linked by a disulfide bridge. The α-chain contains an NH$_2$-terminal domain with a hairpin loop (HL), and 4 kringle domains (K1-4). The β-chain is homologous to the protease domain of plasminogen, but has no catalytic activity due to the lack of several essential amino acids.

tissue-type (tPA) plasminogen activator, proteases known to function in blood clotting and extracellular matrix (ECM) breakdown, blood-coagulating factor XIIa, and two new serine proteases, i.e. HGF activator and HGF converting enzyme (Naldini et al., 1992; Mars et al., 1993; Miyazawa et al., 1993; Mizuno et al., 1994; Shimomura et al., 1995). Recently, a negative regulator of HGF activation was identified, underscoring the complexity of this activation process (Shimomura et al., 1997).

The full length human HGF cDNA encodes a protein of 728 amino acids (Fig. 1) (Nakamura et al., 1989). Its amino acid sequence predicts translation as a precursor protein, which becomes activated by proteolytic cleavage at an Arg-Val cleavage site. This cleavage results in the above mentioned α and β-chains. Furthermore, the cDNA sequence contains 4 putative N-linked glycosylation sites. Interestingly, significant homology was found between HGF and plasminogen. Like this serine protease, the α-chain of HGF has 4 kringle domains, structures that play a role in protein-protein interaction. The β-chain shows high homology with the catalytic domain of plasminogen, but, due to the lack of 2 crucial amino acids from the active site, HGF has no proteolytic activity.
Several structurally different HGF transcripts were shown to exist. For instance, in cultured human fibroblasts, Northern blotting revealed 3 HGF mRNAs of 6, 3 and 1.5 kb, respectively (Chan et al., 1991; Miyazawa et al., 1991b; Rubin et al., 1991; Weidner et al., 1991). Molecular cloning and Northern blotting indicated that the 6 and 3 kb messages emanated from differential polyadenylation (Weidner et al., 1991). The 1.5 kb mRNA represents a splice variant encoding the N-terminal domain of HGF in combination with the first 2 kringle domains (Chan et al., 1991; Miyazawa et al., 1991b). This variant, NK2, behaves as an HGF antagonist (Chan et al., 1991). The subsequently described one kringle domain variant, NK1, functions as a partial HGF agonist (Cioce et al., 1996; Jakubczak et al., 1998). In addition to these two variants, a putative splice variant containing a deletion of 15 nucleotides in the first kringle domain has been described (Rubin et al., 1991; Weidner et al., 1991). This deletion results in a change of the biological activity of HGF, presumably caused by a change in its tertiary structure (Shima et al., 1994).

Genomic studies have revealed that human HGF is encoded by a single gene localized on the long arm of chromosome 7, band 21.1 (Weidner et al., 1991; Saccone et al., 1992). The gene spans about 70 kbp of DNA and contains 18 exons.
Figure 3. Schematic representation of the receptor tyrosine kinase Met. The receptor is composed of two disulfide-linked chains: a 50 kDa α-chain and a 145 kDa β-chain. The β-chain contains the tyrosine kinase domain (TK) and a ‘docking site’ (DS) which interacts with signaling molecules.

(Miyazawa et al., 1991a). The promoter region contains a number of regulatory sequences, including a TATA-like element, an IL-6 responsive element, and a potential binding site for nuclear factor-IL-6, a regulator of IL-6 expression. Also, wild-type, but not mutant, p53 was shown to activate the HGF promoter (Metcalf et al., 1997).

Han and colleagues (1991) identified a gene which shared about 50% sequence homology with HGF. The molecule was designated hepatocyte growth factor-like protein (HGFL), but was subsequently shown to be identical to macrophage stimulating protein (MSP) (Yoshimura et al., 1993), a molecule involved in macrophage chemotaxis and in phagocytosis (Skeel et al., 1991). Structural analysis suggests that HGF and MSP, together with plasminogen and apolipoprotein (a) have evolved from a common ancestral gene (Fig. 2) (Donate et al., 1994).
2. Structure of Met, the high affinity receptor for HGF

Met, the receptor for HGF, was originally identified as the product of an oncogene (Cooper et al., 1984). This oncogene, TPR-Met, results from a chromosomal translocation, fusing the sequence encoding the intracellular domain of Met to that of Tpr (Park et al., 1986), a protein with unknown function. Tpr-Met functions as a constitutively active homodimer with a strong transforming capacity (Cooper et al., 1984; Gonzatti-Haces et al., 1988). Molecular cloning of the 8 kb Met proto-oncogene indicated that this molecule is a cell-surface tyrosine kinase receptor for growth factors (Dean et al., 1985; Park et al., 1987; Rodrigues et al., 1991), whereas functional studies revealed that HGF is the ligand of Met (Bottaro et al., 1991; Naldini et al., 1991b; Rubin et al., 1991).

The Met protein is synthesized as a single-chain 170 kDa precursor. After synthesis, the molecule is cleaved and rearranged into a 190 kDa heterodimer linked by a disulfide bridge (Fig. 3) (Giordano et al., 1989a, 1989b). Komada et al. (1993) demonstrated that Met can be cleaved by furin, but that endoproteolytic processing is not essential for HGF-induced signal transduction. The Met heterodimer is composed of a 50 kDa α-subunit, and an 145 kDa β-subunit (Giordano et al., 1989a). The cytoplasmic tail of the β-chain contains the tyrosine kinase domain and a ‘docking site’, which interacts with multiple signaling molecules (Ponzetto et al., 1994). Both functional domains will be discussed in more detail in Section II.C.

The identification of Tpr-Met resulted in the assignment of the human Met gene to chromosome 7, band q31 (Cooper et al., 1984; Dean et al., 1985; Lin et al., 1996). The gene spans more than 110 kbp and contains 21 exons (Duh et al., 1997; Lin et al., 1998; Liu, 1998) The sequence of the Met promoter region revealed a number of binding sites for regulatory elements, including AP1, AP2, NF-κB, and, like the HGF gene, IL-6RE (Liu, 1998). Recently, wild-type, but not mutant p53, was shown to enhance the activity of the Met promoter (Seol et al., 1999), as had been shown before for the HGF gene.

Two receptor tyrosine kinases related to Met, i.e. Sea (Huff et al., 1993) and Ron (Ronsin et al., 1993), have been identified. MSP was shown to be the ligand for Ron (Gaudino et al., 1994; Wang et al., 1994), whereas Sea remains, as yet, an orphan receptor. In addition, Met shows homology with the putative receptor tyrosine kinase stem cell-derived tyrosine kinase (STK) and with the SEX family of transmembrane proteins (Iwama et al., 1994; Maestrini et al., 1996).
3. Low affinity receptors for HGF

Apart from binding to Met, HGF also binds to heparan sulfate proteoglycans (HSPGs). These interactions, which appear to play an important role in the regulation of HGF activity, will be discussed in Section II.D.

B. Expression and functions of HGF and Met

1. Introduction

The receptor tyrosine kinase Met is prominently expressed on a wide variety of epithelial cells, whereas its ligand, HGF, is expressed by stromal cells. This reciprocal expression pattern points to their important role in epithelial-mesenchymal interactions underlying branching morphogenesis and tubulogenesis during development of organs such as lungs, kidney and mammary glands. Over the past few years, it has become clear, however, that HGF and Met are also involved in a plethora of other biological processes. In the next paragraphs we will give an overview of the well established expression pattern and functions of HGF and MET, as well as of those attributed more recently. It places HGF and Met in the center of developmental processes, leading to a proper organization not only of epithelial tissues, but also of muscle, endothelium, and the nervous and haematopoietic systems.

2. Expression pattern during amphibian, avian and mammalian development

During embryogenesis of the tadpole Xenopus laevis, Met is present as early as in the gastrula stage and remains expressed at high levels throughout neurulation (Aoki et al., 1996). Sites of expression include the foregut region, tailbud mesenchyme, and, in neurulating embryos, neural tissues. HGF expression becomes apparent later, from the neurula stage onwards. The spatiotemporal expression pattern of both HGF and Met point to multifarious roles in amphibian organogenesis. This has been shown more specifically by use of dominant-negative Met constructs, introduced into fertilized Xenopus eggs. Embryos thus treated fail to develop a normal liver, whereas organogenesis of the gut and early kidney are greatly impaired (Aoki et al., 1997). Hence, in Xenopus embryos, a functional HGF-Met system is involved in early organogenesis, especially of organs derived from the primitive gut.

Interference with the HGF-Met system during early chick embryo development leads to abnormal axis formation, underscoring its determining role.
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during avian development, especially in neural induction and limb bud elevation (Stern et al., 1990). **HGF**, but not **MET**, is expressed in the mesoderm of the limb bud and in the central core region of mandibular arch and maxillary processes at stages 17 to 24 of development (Myokai et al., 1995; Théry et al., 1995). During limb bud extension, **HGF** is expressed in the mesenchyme and becomes later confined to the ventral and subapical mesenchyme of the limb bud, suggesting that HGF production in the limb bud is involved in the induction and maintenance of apical ectoderm during limb bud development (Myokai et al., 1995).

During embryonic development of rodents, HGF is prominently expressed in a multitude of tissues, mainly at sites where epithelial/mesenchymal interactions determine organogenesis (Iyer et al., 1990). In gastrulating mouse embryos, the expression of **HGF** and **Met** overlaps. Initially, the two genes are expressed in the endoderm and in the mesoderm along the rostro-intermediate part of the primitive streak and, later, in the node and in the notochord. Neither **HGF** nor **Met** is expressed in the ectodermal layer throughout gastrulation (Sonnenberg et al., 1993; Adermarcher et al., 1996). During early organogenesis, overlapping expression of **HGF** and **Met** is found in the heart, condensing somites, and neural crest cells. However, a second and distinct pattern of expression, characterized by the presence of the ligand in mesenchymal tissues and the receptor in the surrounding ectoderm, is seen in the bronchial arches and in the limb buds. At E13, only this second pattern of expression is observed in differentiated somites and several major organs, such as the lungs, the liver, and the gut (Adermarcher et al., 1996). The expression of the **HGF** and **Met** genes throughout embryogenesis suggests a shift from an autocrine to a paracrine signaling system. Halfway gestation, HGF is present in renal collecting tubes of the kidney, in the liver, in esophageal and skin squamous epithelium and in bronchial epithelium (Defrances et al., 1992; Lee et al., 1993). HGF is also detected in brain, somites, haematopoietic cells, and chondrocytes (Defrances et al., 1992).

Similar patterns of **Met** and **HGF** expression are found along human embryonic development. From the 5th week of gestation onwards, placental tissue highly expresses **HGF** and **Met**. HGF is secreted by amniotic epithelium, the placental villi and the villous core mesenchyme, whereas Met is present on the trophoblast and vascular endothelium (Kauma et al., 1997; Somerset et al., 1998; Wolf et al., 1991). A human pathological condition, known as intra-uterine growth restriction, is associated with an underdeveloped placenta and could be linked to a decreased secretion of HGF by the villous stromal cells (Somerset et al., 1998). The absolute dependence of placenta maturation on HGF has been unequivocally shown in **HGF** null mutant mouse embryos, whose placenta fail to develop properly and which die in utero (Schmidt et al., 1995; Uehara et al., 1995). From week 6-13 of gestation, when major organogenesis takes place, **HGF** and **Met** are co-expressed in liver, metanephric kidney, intestine, lung, gall bladder and spleen.
(Kolatsi-Joannou et al., 1997; Wang et al., 1994b). In the digestive tract of 7-8 week old embryos, Met is localized in epithelia of the liver, pancreas, esophagus, stomach, the small and large intestine, and in smooth muscle layers, whereas HGF becomes concentrated in mesenchymal tissue and smooth muscle (Kermorgant et al., 1997). Interestingly, HGF expression has also been shown in epithelial tissues in the interval from week 9-17 of gestation, particularly in the crypt region of the small intestine, keratinizing epithelium of the tongue, skin and esophagus (Wang et al., 1994b).

In conclusion, HGF and Met are highly conserved molecules in a wide range of species, not only structurally (see Section II.A), but also with respect to their particular role during embryogenesis. In the next paragraphs some specific

Figure 4. Scattering of MDCK cells induced by HGF. A, HGF treatment leads to dissociation of the islands and to migration of the cells. B, MDCK cells grown in the absence of HGF form islands.
functions of HGF and Met will be discussed. We will focus on branching morphogenesis, muscle development, angiogenesis and neuronal development.

3. Mesenchymal-epithelial interaction and branching morphogenesis

HGF induces scattering of epithelial cells in vitro (Stoker et al., 1987; Uehara and Kitamura, 1992) through activation of Met (Weidner et al., 1993) (Fig. 4). This effect can be mimicked by a constitutively active mutant of Met (Jeffers et al., 1998a), suggesting that activation of Met is sufficient in this process. Once activated, Met can in turn activate PI3K and the Ras-MAPK pathway (Boccaccio et al., 1998; Potempa and Ridley, 1998). Furthermore, enzymes involved in ECM proteolysis (e.g. uPA) are activated (Pepper et al., 1992). Partial ECM proteolysis may increase cell motility by diminishing adhesion properties of epithelial cells towards matrix components. Although scattering in vitro can hardly be considered as its physiological function, the phenomenon per se reflects the first phase of epithelial morphogenesis (by activation of MET) through mesenchymal induction (secretion of HGF), underlying the complex, but coordinated formation of branched organs, such as the lungs, the kidney and mammary gland (Sonnenberg et al., 1993).

Tubular differentiation can be induced under “ECM conditions”, i.e. when epithelial cells are cultured in a 3-dimensional ECM-like environment. Thus, kidney epithelial cells, treated with HGF, form tubules resembling those emanating during kidney organogenesis in early embryonic development (Boccaccio et al., 1998; Cantley et al., 1994; Liu et al., 1998a; Sachs et al., 1996). Ezrin, a member of the ERM family of membrane to cytoskeleton linkers (reviewed in Tsukita and Yonemura, 1997), and a substrate of MET, is involved in the cytoskeletal reorganization associated with tubulogenesis (Crepaldi et al., 1997). Embryonic mesenchymal kidney cells undergo a mesenchymal to epithelial transition, which is accelerated by HGF (Karp et al., 1994). This conversion mimics developmental processes in the metanephros in vivo, where mesenchymal specialization is induced by the ingrowth of a branching ureteric bud and is in accordance with expression patterns of Met and HGF during development (Santos et al., 1994; Woolf et al., 1995).

Surprisingly, kidney epithelial cells derived from Met null mutant mouse embryos, and hence unresponsive to HGF, were able to form tubular structures in vitro and to express epithelial-specific markers after treatment with epidermal growth factor (EGF) (Kjelsberg et al., 1997). An intact HGF-Met pathway may thus not be necessary for kidney development, although it can play an auxiliary role. This opens the possibility that HGF may be involved in kidney epithelial regeneration, rather than embryonic kidney development. Indeed, following renal
injury. HGF expression is elevated (Horie et al., 1994; Igawa et al., 1993; Liu et al., 1999). Moreover, transgenic mice, overexpressing HGF in the kidney die of renal failure, associated with the stimulation of the HGF-Met autocrine pathway (Takayama et al., 1997a). In these mice, kidney pathology is not apparent at birth, but rather develops progressively.

In epithelial cells derived from another branched, lumen forming organ, the mammary gland, HGF treatment leads to the formation of branches and structures resembling mammary gland ducts when cultured in a 3-dimensional matrix (Berdichevsky et al., 1994; Brinkmann et al., 1995; Niemann et al., 1998; Soriano et al., 1995; Yang et al., 1995). In accordance with its role in mesenchymal-epithelial interaction in the mammary gland, Met expression is confined to the epithelial cells lining the mammary ducts, whereas HGF is produced by mammary gland fibroblasts (Niranjan et al., 1995; Tsarfaty et al., 1992; Wang et al., 1994a; Yang et al., 1995). During pregnancy, HGF and Met transcripts are progressively reduced to background levels during lactation, and increase during the phase of involution to pre-pregnancy levels. The reduction in HGF and Met expression corresponds to periods in which functions other than tubulogenesis predominate in the mammary gland: alveolar budding and milk protein synthesis (Pepper et al., 1995). Indeed, treating mammary gland cultures with the milk production inducing hormone prolactine sharply reduces Met transcript levels (Pepper et al., 1995).

In the developing lung, HGF is expressed in the mesenchyme and Met in the pulmonary epithelium (Ohmichi et al., 1998). Alveolar type II cells, when cultured in the presence of HGF, are induced to proliferate (Mason et al., 1994; Shiratori et al., 1995), whereas tracheal epithelial cells are driven to differentiate into a polarized cell type (Shen et al., 1997). HGF also proved to be a mitogen for bronchial epithelial cells (Singh-Kaw et al., 1995) and furthermore to facilitate the organotypic rearrangement of cultured E15 mouse lung epithelial cells (Sato and Takahashi, 1997) and branching morphogenesis in organ cultures (Ohmichi et al., 1998). Apart from the function in pulmonary development, HGF can act as growth factor in vivo for alveolar type II cells after lung injury and can thus add to the restoration of epithelial integrity (Panos et al., 1996; Yanagita et al., 1993). Its tissue distribution in the developing lung, together with its proliferation and differentiation-stimulating effects, renders HGF a paracrine growth factor in lung development and regeneration.

Pancreatic epithelial cells, as well as pancreas carcinoma cells, are induced to proliferate and differentiate by HGF, forming tubular structures composed of a lumen, lined by polarized epithelial cells (Brinkmann et al., 1995). These cells have characteristics of pancreas ductal epithelia, including apical microvilli (Jeffers et al., 1996a) and the appearance of characteristic markers of normal ductal cells (Vila et al., 1995). HGF further influences pancreatic islet formation and β-cell differentiation, leading to the secretion of insulin (Otonkoski et al., 1994, 1996).
HGF has initially been described as a mitogenic factor for cultured hepatocytes (Michalopoulos et al., 1984; Nakamura et al., 1984) (see also Section II.A) and it has been implicated in embryonic hepatic development. In the liver, HGF is expressed in Ito cells, whereas Met transcripts are strongly expressed by hepatocytes (Hu et al., 1993). After chemical or mechanical liver injury HGF levels sharply increase, leading to a strong hepatocyte proliferation (Horimoto et al., 1995; Hu et al., 1993). Livers from transgenic mice with liver-specific overexpression of HGF are twice the size of livers of control animals and they regenerate much faster after partial hepatectomy (Sakata et al., 1996; Shiota et al., 1994). Apart from their placental phenotype described above, HGF null mutant mouse embryos fail to develop a fully functional liver (Schmidt et al., 1995), demonstrating that the presence of HGF is an absolute requirement during liver organogenesis. In rats with an experimental liver cirrhosis the administration of HGF through autologous gene transfer was shown to have a beneficial effect on overall survival (Ueki et al., 1999). Thus, HGF acts as a paracrine factor for hepatocyte proliferation and differentiation, both during embryonic liver development, as well as during post-trauma regeneration.

HGF and Met are furthermore involved in the proliferation and migration of a wide variety of epithelial cells, and in the morphogenesis of epithelial tissue. In colon epithelial cells, a complete epithelial developmental program is enrolled upon treatment with HGF, including apical/basal polarization and the formation of crypt-like structures (Brinkmann et al., 1995). Prostate stromal cells produce HGF in vivo (Kasai et al., 1996) and prostate epithelial cells, grown in the presence of HGF proliferate and develop tubular structures reminiscent of those found in the prostate (Brinkmann et al., 1995). Other implications of HGF-Met include the development of bone (especially of cartilage) (Blanquaert et al., 1999; Grumbles et al., 1996; Takebayashi et al., 1995), teeth (Tabata et al., 1996), the (male and female) reproductive tract (Depuydt et al., 1996; Naz et al., 1994; Parrott and Skinner, 1998), thyroid (Schulte et al., 1998; Trovato et al., 1998), and the regulation of hair growth (Jindo et al., 1994, 1998; Shimaoka et al., 1995).

During epithelial wound healing, many cellular programs that play a role during embryonic development are re-activated. The HGF-Met axis has been implicated in epithelial wound healing of various epithelia, including gastric, intestinal and corneal epithelia (Nusrat et al., 1994; Takahashi et al., 1995a, b; Wilson et al., 1999).

In conclusion, HGF and Met are involved in tissue-specific programs of differentiation in a wide variety of lumen-forming organs, leading to the formation of contiguous, polarized epithelial cell layers and, depending on the type of tissue, tubulogenesis and branching.
Chapter 6

4. Development of the nervous system

As described above, HGF and Met are already expressed in the developing central and peripheral nervous system (CNS and PNS), but they remain present during adulthood. Embryonic prospective chick neural plate explants, when treated with HGF, differentiate into cells with a neuronal morphology, and start to express neuronal markers (Streit et al., 1995), whereas in transgenic mice that ectopically express HGF, cells of the neural crest lineage become appropriately targeted (Takayama et al., 1996). Thus, HGF is involved in neural induction, as well as in later stages of neuronal development, when neural cells adopt a migratory phenotype. In the mammalian CNS, Met is abundantly expressed in the neurons of the hippocampus, cerebral cortex, septum, amygdala, pons, olfactory bulb, medulla and spinal cord (Achim et al., 1997; Honda et al., 1995; Jung et al., 1994; Thewke and Seeds, 1999; Wong et al., 1997). During embryogenesis, HGF expression seems to be confined to prospective target cells for the outgrowing neurites. HGF is therefore considered as a chemoattractant, e.g. for spinal motoneurons. Furthermore, HGF has been identified as a survival factor for these neurons, and is secreted by their target tissue, muscle, during later stages of development (Ebens et al., 1996; Yamamoto et al., 1997). HGF has been shown to act synergistically with another neurotrophic factor, ciliary neurotrophic factor (CNTF), in motoneuron survival (Wong et al., 1997). During adulthood, HGF remains expressed in the CNS, where it is found, apart from its localization in neurons, in a/o central glial, ependymal cells, and cells lining the choroid plexus (Honda et al., 1995; Jung et al., 1994). In cultured sympathetic neurons, which express both HGF and Met throughout development, HGF acts as an autocrine axonal outgrowth-stimulating factor, and not as a survival factor (Maina et al., 1998; Yang et al., 1998). However, in the precursor cells of the sympathetic neurons, the sympathetic neuroblasts, HGF does have a stimulating effect on cellular survival, pointing to a shift in the dependence upon HGF from a survival factor to an outgrowth-stimulating factor (Maina et al., 1998). Transgenic mice expressing dominant negative Met fail to develop a complete set of sensory innervating connections (Maina et al., 1997). In cultured dorsal root ganglia of these mice, which contain predominantly sensory neurons, HGF acts synergistically with nerve growth factor (NGF) in axonal outgrowth (Maina et al., 1997). Neurotrophic effects of HGF have also been reported in mesencephalic dopaminergic neurons from neocortical explants in vitro (Hamanoue et al., 1996). In these explants, HGF is mainly expressed in microglia, suggesting a role in CNS development.

Thus, depending on the spatiotemporal distribution pattern and the type of neurons involved, HGF may act as a neural inducer, a neuronal survival factor, or an axonal guidance factor.
5. Angiogenesis

HGF is a potent in vitro motility-stimulating factor for endothelial cells under 2-dimensional culture conditions (Rosen et al., 1990), whereas in 3-dimensional collagen matrices, endothelial cells can be induced by HGF to adapt an elongated phenotype, or to even form tubular, vessel-like structures (Bussolino et al., 1992; Grant et al., 1993). Abundant and genuine angiogenesis in vivo is observed when rabbit cornea is treated with HGF (Bussolino et al., 1992). Accordingly, after implantation into mice, tumor cells that express both Met and HGF, expand much faster than cells that do not secrete HGF and they constitute larger tumors. This coincides with increased and abundant microvascularization of the HGF-secreting tumors (Lamszus et al., 1997; Laterra et al., 1997). Blood vessel endothelial cells express Met on their plasma membrane (Bussolino et al., 1992), but it is not clear whether HGF-induced angiogenesis is a direct consequence of increased endothelial cell motility and proliferation. HGF can also enlarge the expression of vascular endothelial growth factor (VEGF) in gastric epithelial cells and could thus be responsible for neovascularization in gastric tumors (Takahashi et al., 1997). On the other hand, VEGF induction has been described in endothelial smooth muscle cells after HGF treatment, where it may interact synergistically with HGF in angiogenesis (Van Belle et al., 1998). Finally, HGF has been described to induce platelet-activating factor in macrophages that are in the vicinity of the site of neovascularization (Camussi et al., 1997). HGF thus increases proliferation and migration of endothelial cells and may engender angiogenesis directly, or indirectly via VEGF and platelet-activating factor.

6. Muscle development

During embryonic muscle development, HGF secreted by limb bud mesenchymal cells induces migration of Met-expressing myogenic precursor cells from the somites (Bladt et al., 1995; Yang et al., 1996). Met signaling is essential for the detachment of the myogenic precursor cells and the subsequent migration into the limb bud and diaphragm (Brand-Saberi et al., 1996). In Met null mutant mouse embryos, myogenic precursor cells remain in the dermomyotome and consequently, the limb bud and diaphragm are not colonized, leading to the absence of skeletal muscles in the limb and diaphragm (Bladt et al., 1995; Dietrich et al., 1999). In contrast, development of the axial skeletal muscles proceeds in the absence of Met signaling. Ectopic HGF expression leads to aberrant muscle development as shown in chick embryos, where additional limb buds had been induced by the ectopic application of fibroblast growth factor (FGF). Here, myogenic precursor cells colonize this newly formed limb bud, through chemo-
attraction towards HGF (Heymann et al., 1996), whereas in transgenic mice that inappropriately express HGF, ectopic muscle formation occurs (Takayama et al., 1996).

Met and HGF mRNA are present in immature neonatal rat skeletal muscle, but in adult skeletal muscle their levels are below detection limits. After muscle damage, both HGF and Met expression is upregulated in the regenerating muscle (Jennische et al., 1993; Anastasi et al., 1997; Tatsumi et al., 1998). In a cultured undifferentiated myoblast cell line both genes are also co-expressed, pointing to the existence of an autocrine pathway in the regulation of cell proliferation (Anastasi et al., 1997). It appears that HGF expression is developmentally regulated in skeletal muscle and transiently re-expressed during muscle regeneration. The latter process may involve the concerted activation of quiescent satellite cells to proliferate (Allen et al., 1995), while at the same time their differentiation is inhibited (Gal-Levi et al., 1998).

HGF and Met are also expressed in progenitor cells of the cardiomyocytes and may play a role in cardiomyogenic differentiation and heart organogenesis (Rappolee et al., 1996; Song et al., 1999).

HGF is hence an inducer of myogenic migration during embryonic development and of satellite cell proliferation during muscle regeneration. Contrarily to the mutually exclusive expression pattern generally found in mesenchymal/epithelial tissues, myoblast proliferation may be regulated by HGF-Met in an autocrine fashion.

7. Haematopoiesis

The HGF/Met pathway has also been implicated in haematopoiesis. Both HGF and Met are expressed in the yolksac of the chicken embryo (Théry et al., 1995), and in the human and rodent fetal liver, primordial sites of haematopoiesis (Selden et al., 1990; Hu et al., 1993). Within the adult haematopoietic microenvironment, the bone marrow, Met is expressed by a subset of haematopoietic precursor cells (HPC), whereas HGF is expressed by stromal cells, suggesting that HGF functions as a paracrine growth factor (Kmiecik et al., 1992; Galimi et al., 1994; Takai et al., 1997; Weimar et al., 1998). Indeed, it was shown that HGF promotes differentiation and proliferation of HPC induced by other haematopoietic growth factors. In the presence of IL-3, HGF stimulates the formation from CD34+ progenitors of burst forming units erythroid, as well as colony forming units granulocyte erythroid macrophage, but not of colony forming units granulocyte monocyte (Galimi et al., 1994; Takai et al., 1997). In the presence of stem cell factor, an even stronger synergistic effect is obtained (Galimi et al., 1994; Weimar
**Figure 5.** A schematic representation of the most relevant signaling pathways activated by HGF. For reasons of clarity both relevant autophosphorylation sites of Met, *i.e.* Y1349 and Y1356, are depicted twice. Furthermore, the Met-associating proteins, except for Grb2, are able to interact with either autophosphorylation site. Crossed shapes represent adaptor or docking proteins without catalytic activity, squares represent kinases, circles represent GTPases, octagons represent guanine nucleotide dissociation stimulators, and pentagons represent transcription factors. The solid arrows indicate a direct activation, whereas the dotted arrows indicate activation via known or unknown intermediate proteins or phospholipid metabolites, and the blunted arrows indicate a direct inhibition. Although only Met is depicted, most signaling pathways also apply to the oncoprotein Tpr-Met. See text for further details.
et al., 1998). Apart from effects on growth and differentiation, HGF stimulation of CD34+ cells leads to integrin activation and adhesion to fibronectin. This adhesive interaction prolonged survival of haematopoietic cells in culture (Weimar et al., 1998). Taken together, these data indicate that the HGF/Met pathway is involved in the regulation of the proliferation, differentiation and survival of haematopoietic progenitors.

C. Signal transduction by Met

1. Introduction

Like in most other receptor tyrosine kinases, the activation of the kinase domain of Met is believed to depend upon receptor dimerization or oligomerization, resulting in intermolecular transphosphorylation. This process of di- or oligomerization may be facilitated by the action of HSPGs, as discussed in Section II.D. Upon stimulation by HGF, the C-terminus of the β-chain of Met is strongly tyrosine phosphorylated (Bottaro et al., 1991; Naldini et al., 1991a,b). The autophosphorylation of the tyrosine residues Y1349 and Y1356 of Met, as well as the equivalent residues Y482 and Y489 of the oncoprotein Tpr-Met, are critical for most biological responses (Ponzetto et al., 1994, 1996; Zhu et al., 1994; Fixman et al., 1995). These tyrosine residues serve as a multistart docking site for several proteins, including Gab1, Grb2, phosphatidylinositol 3-kinase (PI3-K), phospholipase C (PLCγ), Src, Shc, SHP-2 and STAT3 (Fig. 5). Except for Gab1, which has a unique Met-binding domain (Weidner et al., 1996; see, however, discussion below), these proteins interact with Met via their SH2 domains: Grb2 specifically to Y1356, the other proteins to both Y1349 and Y1356 (Ponzetto et al., 1993, 1994, 1996; Pelicci et al., 1995; Nguyen et al., 1997). Here we will discuss the nature and function of the different Met-associating signaling molecules. Furthermore, the signaling pathways activating Met, and their biological function, will be discussed.

2. The role of Grb2 and signal transduction via Ras

One of the signaling molecules that associates directly with Met upon HGF stimulation is Grb2 (Ponzetto et al., 1994). Grb2 is an adapter protein consisting of one SH2 and two SH3 domains. SH2 domains are involved in binding to phosphorylated tyrosine residues, whereas SH3 domains bind to proline-rich regions. By means of its SH3 domain, Grb2 is constitutively associated with Sos, an exchange factor for Ras. The Grb2-Sos complex is recruited by receptor tyrosine
kinases, via their autophosphorylated tyrosine residues, to the plasma membrane where Ras is localized. As a consequence, Ras becomes activated (Fig. 5). After HGF-induced autophosphorylation, Met also associates with the Shc adapter protein (Pelicci et al., 1995). Interestingly, upon phosphorylation, Shc is also able to associate with Grb2 (Pelicci et al., 1995). Thus, HGF stimulation can trigger the Ras-pathway by both direct and Shc-mediated association of the Grb2-Sos complex to Met (Fig. 5).

Activation of Ras has been implicated in a wide variety of cellular responses including cytoskeletal reorganization, adhesion, proliferation, differentiation, and apoptosis. The first identified and best characterized effector molecule for Ras is the serine/threonine kinase Raf1, which phosphorylates and activates MEK, resulting in the phosphorylation and activation of MAP kinase (Campbell et al., 1998; Vojtek and Der, 1998) (Fig. 5). Among the substrates for MAP kinase are the transcription factors Elk-1 and Ets-2 involved in ternary complex formation at serum response elements. Upon activation, these transcription factors regulate expression of immediate early genes, such as c-fos, eventually leading to cell proliferation (Wasylyk et al., 1998). Besides Raf, several additional effector molecules for Ras have been identified. These include PI3-K (Rodriguez-Viciana et al., 1994), which will be discussed below, and RalGDS (Spaargaren and Bischoff, 1994), an exchange factor for Ral (Albright et al., 1993; Feig et al., 1996) (Fig. 5). Ral has been implicated in Ras-dependent proliferation, gene expression, phospholipase D activation and transformation (Wolthuis et al., 1999), however, no studies have been conducted yet to investigate its involvement in Met signal transduction and functional responses.

In initial studies using mutants of Met, it was shown that Y1356, the Grb2 binding site, is required for scattering and branching tubulogenesis of MDCK cells, whereas the equivalent residue Y489 of Tpr-Met is required for cell proliferation and transformation (Zhu et al., 1994; Fixman et al., 1995). However, these mutations also reduced binding of other Met associating proteins (Ponzetto et al., 1993, 1994; Pelicci et al., 1995). Using a more sophisticated mutant that selectively fails to bind Grb2 only, it was shown that Grb2 association by Met is required for HGF-induced branching tubulogenesis of MDCK cells, but not for scattering (Fournier et al., 1996; Ponzetto et al., 1996; Royal et al., 1997). Similarly, whereas Grb2 binding by Tpr-Met is not required for motility, it is required for induction of transformation and invasion, in vitro, as well as metastasis and tumorigenicity, in vivo (Fixman et al., 1996; Ponzetto et al., 1996; Giordano et al., 1997; Jeffers et al., 1998b; Bardelli et al., 1999). Intriguingly, however, Grb2 binding to Met is dispensable for transformation, metastasis and tumorigenicity, when Met is activated by either a point mutation or by autocrine HGF stimulation (Jeffers et al., 1998b).
Interestingly, it has been shown that expression of N17-Ras, a dominant negative mutant of Ras, abolishes HGF-induced cell scattering (Hartmann et al., 1994; Ridley et al., 1995). Since scattering does not require a Grb2 binding site, this suggests that HGF/Met activates Ras by a Grb2-independent mechanism. Indeed, a recent study by Tulasne et al. (1999), shows that a mutant of Met, which lacks four major autophosphorylation tyrosine residues (including Y1349 and 1356 which constitute the multisubstrate docking site), despite its loss of Grb2 binding ability, is still able to induce Ras activation. Moreover, the scattering response, which was clearly not affected by these mutations, was abolished upon treatment with the specific pharmacological inhibitor PD98059 of the Ras-MAP kinase pathway intermediate MEK (Tulasne et al., 1999). Using either this MEK-inhibitor or constitutively active or dominant negative mutants of Ras or MAP kinase, it was concluded that the activation of the Ras-MAPK pathway is required but not sufficient for HGF-induced scattering (Ridley et al., 1995; Potempa and Ridley 1998; Herrera 1998; Khwaja et al., 1998; Tanimura et al., 1998; Tulasne et al., 1999), and for tubulogenesis of MDCK cells (Khwaja et al., 1998). In conclusion, Grb2 and the Ras-MAP kinase pathway appear to play an important regulatory role in a variety of responses elicited by HGF/Met, including mitogenesis, motogenesis and morphogenesis, as well as in Tpr-Met-induced transformation, invasion, metastasis and tumorigenicity.

3. The role of Gab1 and signal transduction via PI3-K

Another important substrate for Met is the docking protein Gab1 (Weidner et al., 1996). In vitro, Gab1 interacts directly with Met via a proline rich binding domain (Weidner et al., 1996), but it has been concluded that the interaction of Gab1 with Met and Tpr-Met in vivo is mediated by Grb2 (Nguyen et al., 1997; Bardelli et al., 1997; Fixman et al., 1997). Gab1, which contains a PH-domain as well as a proline-rich region, and can become heavily tyrosine phosphorylated, has the ability to directly associate with several signaling molecules such as Grb2, PI3-K, PLCγ and SHP2 (Holgado-Madruga et al., 1996).

Overexpression of Gab1 partially mimics the action of HGF, as it results in tubulogenesis of mammary epithelial cells (Niemann et al., 1998), as well as in enhanced MAP kinase activity, cell scattering and tubulogenesis of MDCK cells (Weidner et al., 1996). The HGF responses in MDCK cells could be abrogated by overexpressing the Met binding domain of Gab1 (Weidner et al., 1996). In NIH3T3 fibroblasts, however, Holgado-Madruga et al. (1996) did not observe enhanced MAP kinase activity or activation upon overexpression of Gab1. Moreover, a recent study shows that Met with mutations of the multisubstrate docking site, which abolish recruitment of Gab1, as well as Grb2. She and PI3-K, although
indeed impaired in the induction of morphogenesis, is still able to activate the Ras-
MAP kinase pathway and to induce MEK-dependent scattering (Tulasne et al.,
1999). Finally, the transforming potential of Tpr-Met mutants correlates with their
ability to induce tyrosine phosphorylation of Gab1 (Bardelli et al., 1997; Fixman
et al., 1997). Taken together, these findings convincingly demonstrate the
involvement of Gab1 in Met-induced morphogenesis (Weidner et al., 1996;
Nguyen et al., 1997; Niemann et al., 1998; Maroun et al., 1999; Tulasne et al.,
1999), and suggest a role for Gab1 in transformation by Tpr-Met

One of the first molecules that was shown to become associated with Met
upon HGF stimulation was PI3-K (Graziani et al., 1991; Ponzetto et al., 1993).
This interaction of PI3-K with Met may enhance PI3-K activity and/or localize PI3-
K in the proximity of its substrate (Ponzetto et al., 1993). PI3-K is composed of a
p85 adapter subunit, which contains the Met interacting SH2 domain, and a p110
catalytic subunit. PI3-K is able to phosphorylate PIP2 in order to produce PIP3.
PIP3 in its turn can bind to the PH domain of target proteins, resulting in their
translocation, membrane localization and, indirectly, in their activation. Among the
PH-domain-containing effector molecules of PI3-K is the kinase Akt/PKB
(Burgering and Coffer, 1995), which, upon membrane localization, is
phosphorylated and activated by PDK1 (Stokoe et al., 1997; Stephens et al., 1998)
(Fig. 5). Downstream effector molecules for the PI3-K-regulated kinase PKB
include the Bcl-2 family member Bad, which can exert pro-apoptotic activity by
interacting with Bcl-2 (Datta et al., 1997; del Peso et al., 1997); glycogen synthase
kinase 3 (GSK3), involved in regulation of glycogen synthesis and, as discussed
below, in phosphorylation of β-catenin (Cross et al., 1995); p70S6K, involved in
regulation of protein synthesis and gene expression (Proud, 1996); and the forkhead
transcription factor AFX (Kops et al., 1999). The function of these effector
molecules in Met signal transduction has not yet been investigated.

Besides the ability of PI3-K to directly interact with Met, two additional
mechanisms may account for Met-induced PI3-K activation (Fig. 5). Firstly, the
p85 subunit of PI3-K was also found to associate with Gab1 (Holgado-Madruga et
al., 1996), and, at least in cells overexpressing both Met and Gab1, more PI3-K
activity is associated with Gab1 than with Met (Maroun et al., 1999). Interestingly,
besides being able to associate with PI3-K, Gab1 requires PI3-K activity and an
intact PH domain for proper localization and induction of morphogenesis (Maroun
et al., 1999). Secondly, PI3-K has been identified as an effector molecule for Ras,
as Ras has the ability to directly interact with the p110 catalytic subunit of PI3-K
(Rodriguez-Viciana et al., 1994). To what extent these three different mechanisms
contribute to HGF-induced PI3-K activation remains to be established. However,
Ras-mediated PI3-K activation has been implicated in HGF-induced adherens
junction disassembly in MDCK cells (Potempa and Ridley, 1998).
By means of either specific pharmacological inhibitors such as Wortmannin and LY294002, or by expression of dominant negative or constitutively active mutants of PI3-K, its function in Met signaling has been extensively studied. These studies revealed a prominent regulatory role for PI3-K in Met-induced mitogenesis, motility and morphogenesis. (Royal and Park. 1995; Rahimi et al., 1996; Royal et al., 1997; Potempa and Ridley, 1998; Khwaja et al., 1998). Activation of PI3-K has been reported to be required and sufficient for tubulogenesis, and required for scattering (Royal and Park, 1995; Khwaja et al., 1998; Potempa and Ridley, 1998). Interestingly, however, mutation of the multisubstrate docking site of Met, which results in the loss of PI3-K and Gab1 association with Met upon HGF stimulation, does not abrogate HGF-induced scattering or Ras activation (Tulasne et al., 1999). This indicates that Ras-mediated, rather than direct Met-induced or Gab1-mediated, activation of PI3-K is required for HGF-induced scattering.

Whether PI3-K activation alone is also sufficient for HGF-induced scattering of MDCK cells is still a matter of debate. On the one hand Potempa and Ridley (1998) reported that neither expression of an active mutant of PI3-K, nor the combined expression of PI3-K with active Raf or MEK, was sufficient for adherens junction disassembly, a prerequisite for scattering. On the other hand, Khwaja et al. (1998) reported that expression of an active mutant of PI3-K is sufficient to induce scattering, provided a basal level of MAP kinase activity is present, however, expression of active Rac or PKB is not sufficient to induce scattering. Based upon these observation, both studies suggested the requirement of an additional (novel) mitogenic pathway for HGF-induced scattering, either downstream of Ras, other than PI3-K or Raf (Potempa and Ridley, 1998), or downstream of PI3-K, other than PKB or Rac (Khwaja et al., 1998). Noteworthy, a recent study also suggested the existence of an additional mitogenic signaling pathway, as NK2, the truncated HGF isoform as described in Section II.A, despite its ability to induce both PI3-K and MAP kinase activation, as well as a mitogenic response, is unable to induce a mitogenic response in breast epithelial cells (Day et al., 1999). In agreement with the data from Khwaja et al. (1998), it has been reported that expression of active PI3-K disrupts the polarized tubular growth of well-differentiated mammary epithelial cells, resulting in enhanced motility and invasion (Keely et al., 1997). Taken together, PI3-K activation is required, and may also be sufficient, for HGF-induced scattering.

PI3-K has also been implicated in the responses elicited by Tpr-Met. A mutant of Tpr-Met, which preferentially binds PI3-K over Grb2, although still able to elicit cell motility, is unable to induce transformation, invasion and metastasis (Bardelli et al., 1999). However, a mutant of Tpr-Met which selectively binds Grb2 only, is also impaired in its ability to induce invasion and metastasis (Giordano et al., 1997). This could, however, be overcome by expression of constitutively active
PI3-K (Bardelli et al., 1999). Thus, these data indicate that simultaneous activation of the Ras and PI3-K pathway is required and sufficient for full invasive and metastatic activity of Tpr-Met. In conclusion, the PI3-K pathway is an important regulatory pathway in HGF/Met-induced mitogenesis, motogenesis and morphogenesis, as well as in Tpr-Met-induced motility, invasion and metastasis.

4. The role of signal transduction via Rho-family GTPases and β-catenin

Members of the Rho subfamily of Ras-related GTPases, as well as Ras itself, have been implicated in HGF-induced cytoskeletal reorganization, cell scattering and tubulogenesis. Initially, mainly based upon studies in fibroblasts, cdc42, Rac and Rho were shown to regulate the formation of filopodia, lamellipodia, and stress fibers, respectively (Hall, 1998; van Aelst and D’Souza-Schorey, 1997). Evidence was presented indicating that cdc42 may function upstream from Rac, which in turn may function upstream from Rho (Zigmond, 1996). Furthermore, it was shown that Ras can induce PI3-K-dependent activation of Rac (Rodriguez-Viciana et al., 1997), which may be mediated by the PH domain-containing exchange factor for Rac, Tiam (Michiels et al., 1995). More recently, these Rho-family GTPases were shown to be involved in the regulation of a wide variety of cellular functions, including regulation of membrane trafficking, transcriptional activation and cell growth control (Hall, 1998; van Aelst and D’Souza-Schorey, 1997). Several effector molecules for Rho-family GTPases have been identified, including the Rho effectors ROK and ROCK, involved in stress fiber formation, and the Rac effector PAK, involved in JNK activation and cytoskeletal organization (van Aelst and D’Souza-Schorey, 1997).

With respect to HGF/Met signaling, it has been reported that the activation of Rho is required for HGF-induced membrane ruffling and cell motility in keratinocytes (Takaishi et al., 1994; Nishiyama et al., 1994). Furthermore, HGF-induced actin reorganization, membrane ruffling, spreading and scattering of MDCK cells was reported to require activation of Ras and Rac (Hartmann et al., 1994, Ridley et al., 1995, Potempa and Ridley, 1998), but not of Rho (Ridley et al., 1995). Interestingly, recent studies revealed that Rac and Rho are involved in intercellular E-cadherin-mediated adhesions in epithelial cells. In MDCK cells and keratinocytes the basal cadherin-mediated cell-cell adhesion was inhibited by the dominant negative mutant N17-Rac and by inhibition of Rho (Braga et al., 1997; Takaishi et al., 1997). Furthermore, expression of constitutively active V12-Rac enhanced cadherin-mediated cell-cell adhesion in MDCK cells (Takaishi et al., 1997).

In agreement with the stimulatory effect of V12-Rac on cell-cell adhesion, overexpression of either V12-Rac or Tiam1 inhibits HGF-induced scattering of
MDCK cells (Hordijk et al., 1997). Furthermore, V12-Rac and Haml suppressed the scattered appearance and invasion of fibroblast-like Ras-transformed MDCK3 cells. This was shown to be due to restoration of E-cadherin-mediated cell-cell adhesion, as a consequence of enhanced levels of β-catenin and E-cadherin at intercellular junctions (Hordijk et al., 1997) (Fig. 5). In contrast, however, in well-differentiated mammary epithelial cells expression of active mutants of Rac, cdc42 or PI3-K disrupts their polarized tubular growth, and rather promotes their motility and invasion (Keely et al., 1997). Most likely, these apparent contradictory results reflect the delicate balance between the Rac-dependent regulation of cell-cell adhesion, cell-matrix adhesion, and matrix-dependent cell migration (Sander et al., 1998). Finally, expression of N17-Rac in MDCK cells prevents HGF-induced and Ras-mediated dispersal of β-catenin and E-cadherin, adherens junction disassembly and, as mentioned before, scattering (Potempa and Ridley, 1998). Since tyrosine phosphorylation of β-catenin has been implicated in the dissociation of the cadherin/β-catenin complex from the actin cytoskeleton, it is noteworthy that, although this was not observed in MDCK cells (Potempa and Ridley, 1998), β-catenin as well as plakoglobin (γ-catenin) become phosphorylated on tyrosine residues upon HGF stimulation of HT29 colon adenocarcinoma cells (Shibamoto et al., 1994). In conclusion, Rac, Rho and β-catenin play an important regulatory role in cell-cell adhesion and HGF-induced cytoskeletal organization and motogenesis.

Besides its involvement in cell-cell adhesion, β-catenin has also been implicated in the regulation of gene transcription. β-Catenin can form mutually exclusive complexes with either cadherins or with APC, the tumor suppressor gene product that is mutated in colon carcinoma (Rubinfeld et al., 1993). GSK3, which can be phosphorylated and inactivated by the PI3-K-dependent PKB, can in turn phosphorylate APC and β-catenin on serine residues (Rubinfeld et al., 1996; Cross et al., 1995) (Fig. 5). As a consequence of this phosphorylation, β-catenin is degraded (Morin et al., 1997). However, upon phosphorylation and thus inhibition of GSK3, free β-catenin will accumulate, translocate to the nucleus and interact with the transcriptional regulator T cell factor/lymphocyte enhancer-binding factor (TCF/LEF-1), thereby inducing expression of TCF/LEF-1 target genes (Behrens et al., 1996; Molenaar et al., 1996) (Fig. 5). Interestingly, HGF stimulation of mouse mammary cells was reported to result in a decrease in GSK3 activity, the nuclear accumulation of β-catenin, and the activation of TCF/LEF-1 (Papkoff and Aikawa, 1998). Thus, via direct or indirect phosphorylation of β-catenin on either tyrosine or serine residues. Met appears to be able to regulate cell-cell adhesion as well as gene expression.

Expression of N17-Rac has also been shown to inhibit both JNK activation and transformation by Tpr-Met (Rodrigues et al., 1997). Based upon this observation it was suggested that activation of the JNK pathway, which is mediated by the sequential activation of PI3-K and Rac (Cosso et al., 1995; Minden et al.,

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1995) (Fig. 5), is essential for transformation by the Tpr-Met oncoprotein (Rodrigues et al., 1997). JNK in turn is able to phosphorylate a number of transcription factors, including the immediate early gene c-jun. Noteworthy, both GTPases Rac and Rho have also been implicated in regulation of the transcriptional activity of the Serum response factor (SRF) (Hill et al., 1995). In conclusion, Rac, Rho and β-catenin play an important regulatory role in HGF/Met-induced mitogenesis and motogenesis, whereas Rac is also involved in Tpr-Met-induced transformation.

4. The role of additional signaling molecules

An additional Met associating protein is PLCγ (Ponzetto et al., 1994). PLCγ mediates the production of IP3, which results in enhanced calcium release from intracellular stores, and diacylglycerol, which activates of PKC. Indeed, PKC has been implicated in Met signaling in a variety of cell types (Santos et al., 1993; Adachi et al., 1996; Dunsmore et al., 1996; Laping et al., 1998; Machide et al., 1998). Furthermore, both PKC and calcium have also been implicated in the negative regulation of Met signaling, by phosphorylation of residue S985 of Met, resulting in decreased kinase activity (Gandino et al., 1990, 1991, 1994). Using a pharmacological inhibitor, PLCγ has been implicated in the chemotactic response elicited by stimulation of a chimeric PDGF-Met receptor molecule expressed in renal epithelial cells (Derman et al., 1996).

HGF also activates STAT3 (Schaper et al., 1997), and stimulates recruitment of STAT3 to the autophosphorylated Y1356 of Met (Boccaccio et al., 1998). Upon phosphorylation, the STAT proteins can dimerize and translocate to the nucleus, where they act as transcription factors controlling the promoter activity of target genes. Inhibition of STAT-mediated transcription prevents HGF-induced tubulogenesis, whereas scattering and proliferation were unaffected (Boccaccio et al., 1998).

Furthermore, the Src tyrosine kinase was shown to directly associate with Met (Ponzetto et al., 1994). This association with Met and activation of Src was shown to play a critical role in carcinoma cell motility (Rahimi et al., 1998), and in HGF-induced phosphorylation of FAK (Chen et al., 1998). With respect to another Met associating protein, the tyrosine phosphatase SHP-2 (Nguyen et al., 1997), no functional data are available yet. Recent data indicate that SHP-2 can also be indirectly recruited to Met via Gab1 (Maroun et al., 1999).

In addition, BAG-1, a cell death suppressor gene product that binds the anti-apoptotic proto-oncogene product Bel-2 in a cooperative fashion (Takayama et al., 1995), interacts with Met. This interaction was independent of phosphorylation of either Y1349 or Y1356 of Met (Bardelli et al., 1996), and may very well be
mediated by the molecular chaperone Hsp70 (Takayama et al., 1997c). Overexpression of BAG-1 enhances the anti-apoptotic effect of HGF on liver progenitor cells (Bardelli et al., 1996).

Finally, it is noteworthy that ezrin, a member of the ERM protein family involved in membrane-cytoskeleton interactions, is a substrate for Met in vitro and also becomes phosphorylated on tyrosine residues in vivo. Both a truncated and a tyrosine mutated variant of ezrin impair the motogenic and morphogenic response of epithelial kidney cells to HGF (Crepaldi et al., 1997). Furthermore, as will be discussed in more detail in the next Section, the observations that the HSPG CD44 can directly interact with ezrin (Tsukita et al., 1994), and can bind and present HGF to Met (van der Voort et al., 1999; Taher et al. 1999), adds an extra dimension to the role of ezrin in Met-signaling.

D. Heparan sulfate proteoglycans and HGF/Met function

Besides Met, heparan sulfate proteoglycans (HSPGs) have been identified as a second class of HGF-binding sites on various cell types. These binding have a much lower affinity than the Met receptor, but they are considerably more numerous (10-1000 fold) (Higuchi et al., 1991; Tajima et al., 1992).

HSPGs are proteins that carry one or more covalently linked heparan sulfate chains. They are widespread throughout mammalian tissues both as cell surface molecules, e.g. the syndecans, glypicans, and CD44-HS, and as ECM components, e.g. perlecan. HSPGs have been implicated in several important biological processes including cell adhesion and migration, angiogenesis, tissue morphogenesis, and regulation of blood coagulation. In these processes, they are believed to function as scaffold structures, designed to accommodate proteins through non covalent binding to their heparan sulfate (HS)-chains (reviewed in Schlessinger et al., 1995; Lindahl et al., 1998). The ligand-binding sites reside within discrete sulfated domains formed by complex, cell-specific modifications to the HS disaccharide repeat. Binding of proteins, including many growth factors and cytokines, e.g. FGFs, VEGF, HB-EGF, IL-3, IL-7, GM-CSF, and certain chemokines, to HS-chains may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of their biological function. This functional importance is illustrated by fibroblast growth factor 2 (FGF-2), whose binding to its signal-transducing receptor and consequent biological effects is critically dependent on its interaction with cell-surface HSPGs (Rapraeger et al., 1991; Yayon et al., 1991; Schlessinger et al., 1995). Furthermore, a number of cell biological and genetic studies have recently provided compelling evidence for an in vivo role of cell-surface HSPGs in growth.
control and morphogenesis in *Drosophila*, mice and humans (reviewed by Selleck, 1998).

The modular structure of HGF has facilitated the identification of the domains responsible for binding to Met and heparin/HS. By using deletion mutants of HGF and examining their binding ability to immobilized heparin, Mizuno et al. (1994) identified the hairpin loop of the amino-terminal domain and the second kringle domain as sites essential for heparin binding. The same domains are also critical for Met binding and signaling (Matsumoto et al., 1991; Hartmann et al., 1992; Lokker et al., 1992; Okigaki et al., 1992). In order to dissect the binding sites for Met and heparin/HS in HGF, the groups of Gherardi and Blundell generated three-dimensional models of the individual HGF domains to help to design specific mutants (Donate et al., 1994). Based on the X-ray structures of antithrombin- (Carrell et al., 1994) and FGF-heparin complexes (Faham et al., 1996), they predicted the heparin-binding sites to contain clusters of positively charged residues which make electrostatic contact with negatively charged groups in HS-chains. Indeed, three such clusters were identified on the surface of HGF; two in the hairpin loop and one in the kringle 2 domain (Donate et al., 1994). By introducing specific mutations at these sites, it was confirmed that these residues play a key role in heparin-binding (Hartmann et al., 1998). A study by Chirgadze et al. (1999) has recently reported the crystal structure of NK1, a natural splice variant of HGF with agonistic activity, consisting of the N- and first kringle-domains (see Section II.A). It was shown that NK1 assembles as an asymmetric homodimer in which the N-domain of one partner interacts with the kringle domain of the other. Short heparin fragments (14-mer) effectively dimerized NK1 in solution, suggesting that heparan sulfate chains expressed on cells or in the ECM may stabilize the NK1 dimers *in vivo*.

Although HS-chains are composed of a linear array of disaccharide units consisting of alternating hexuronic acid (L-iduronic acid (IdoA) or D-glucuronic acid) and D-glucosamine, there is evidence that they are capable of highly specific protein binding. Variations in O-sulfation pattern, hexuronate composition, and length of the sulfated segments determine this specificity (Lindahl et al., 1998). The first specific binding domain identified, was a pentasaccharide which binds with high affinity to antithrombin III (AT III), a serine protease inhibitor. This sequence induces a conformational change in AT III and accelerates its binding to factor Xa and thrombin, and in this way promotes the anticoagulant action of AT III (Lindahl et al., 1984). Lyon et al. (1994) and Ashikari et al. (1995) have analyzed the structural basis of the interaction between HGF and HSPGs. Both studies indicate that high affinity HGF-binding requires oligosaccharides with a minimum length of 8-12 units containing 6-O-sulfated GlcNSO residues, which may be flanked by IdoA(SO₃) units (Ashikari et al., 1995). Interestingly, this
Different isoforms of CD44 can promote Met signaling by distinct mechanisms. CD44v3-10 promotes Met signaling by heparan sulfate-mediated presentation of HGF to Met, whereas CD44 can also promote Met signaling as a consequence of adhesion to HA. See text for further details.

Structural specificity for binding to HS differs radically from that of FGF-2 (Maccarana et al., 1993), illustrating the importance of structural diversity of the HS-chain in selective growth factor binding.

Whereas HSPGs have been shown to be crucial for FGF interaction with its receptor, and thus for FGF functioning, their role in HGF/Met interaction is, as yet, less well defined. Binding to heparin/HS does not appear to affect the affinity of full-length HGF for the Met receptor, but it increases receptor phosphorylation and mitogenicity on rat hepatocytes (Zioncheck et al., 1995; Schwall et al., 1996). In contrast, Met binding and mitogenicity of NK1 has been reported to require HSPGs (Schwall et al., 1996; Sakata et al., 1997). Recently, we have shown that HGF binds to a HSPG splice variant of CD44 (CD44-HS) expressed on B cells (van der Voort et al., 1999). This binding strongly promotes the HGF-induced tyrosine
phosphorylation of Met as well as phosphorylation of several substrates (Fig. 6) (see also Section III.C). Taken together, these in vitro studies indicate that HSPGs may play an important regulatory role in HGF/Met signaling.

Interaction of HGF with HSPG could modulate Met-signaling via several mechanisms (Fig. 6). Firstly, as already mentioned, HSPGs may promote dimerization of HGF, thereby promoting receptor cross-linking and tyrosine kinase activity (Chirgadze et al., 1999). Secondly, by inducing a conformational change HSPGs may influence the affinity of HGF for Met, as has been demonstrated for the NK1 splice variant (Sakata et al., 1997). Thirdly, HGF may mediate colocalization of HSPGs and Met, which may bring relevant intracellular signaling molecules in the proximity of each other. For example, we have shown that the cytoplasmic tail of CD44 interacts physically and functionally with Src-family protein tyrosine kinases (Taher et al., 1996), which have also been implicated in Met signaling (Ponzetto et al., 1994). The above-mentioned processes may involve the formation of a ternary complex between HGF, Met, and a HSPG. In case of ternary-complex-formation between CD44-HS, HGF, and Met, Src kinases associated with the cytoplasmic tail of CD44, might be recruited into the complex (Taher et al., 1999). This may facilitate their activation by Met. Similarly, the ERM family member ezrin, which is also associated with CD44 and a downstream target for Met, may also be assembled into the complex. Ezrin acts as a linker between the intracellular domain of CD44 and the actin-based cytoskeleton (Tsukita et al., 1994), and has been shown to be involved in HGF-induced cell migration (Crepaldi et al., 1997). By recruiting ezrin, CD44-HS might thus contribute to the regulation of cell adhesion and migration.

The preceding data suggest an important physiological role for HSPG in the regulation of HGF function. To directly address this issue, Hartmann et al. (1998) performed in vivo studies comparing wild-type HGF with HGF mutants with a strongly (50 fold) reduced affinity for heparin. Mutant HGF showed a delayed clearance from the blood, and interestingly, induced a higher DNA synthesis in normal mouse liver. Based on these findings, the authors suggest a role for HSPGs in promoting the internalization and degradation of HGF in vivo. Although no further in vivo data are as yet available, it is tempting speculate on other physiological roles for HSPGs. HSPGs may help to localize HGF to specific cells or ECM components within the microenvironment and may be required for the establishment of a chemotactic gradient. Examples are the migration of myogenic precursor cells to the limb bud during embryogenesis, which is critically dependent on HGF and Met, and possibly, the migration B cells within the germinal center. Furthermore, membrane and matrix HSPG may also protect HGF from proteolytic degradation (Saksela et al., 1988). Further studies are needed to explore these possible roles of HSPG.
In Section II.A.2, we briefly discussed the oncogenic potential of the Tpr-Met chimera. In this chimera, the intracellular domain of Met is fused to Tpr, resulting in a constitutively active homodimer with transforming capacity (Cooper et al., 1984; Park et al., 1986; Gonzatti-Haces et al., 1988). A vast body of clinical and experimental data shows that, apart from Tpr-Met, also the Met proto-oncogene and HGF play a pivotal role in tumorigenesis. For instance, overexpression and high levels of autophosphorylation of Met have been found in human tumor cell lines (Fig. 7) (Tempest et al., 1988; Giordano et al., 1989a; Ponzetto et al., 1991; Kuniyasu et al., 1992). Often this overexpression is caused by gene amplification (Giordano et al., 1989a; Ponzetto et al., 1991; Kuniyasu et al., 1992). Interestingly, however, transfection of tumor cells with activated ras and ret oncogenes also causes Met overexpression and enhances HGF-dependent invasion (Ivan et al., 1997; Webb et al., 1998). Furthermore, Met overexpression can be induced by HGF itself, as well as by a number of other cytokines, including EGF, IL-1, and IL-6 (Chen et al., 1997). These data indicate that both oncogenes and cytokines present in the tumor microenvironment can induce Met expression and thereby promote tumorigenesis.

In addition to Met overexpression, overproduction of HGF can also occur in tumors. For instance, it was demonstrated that tumor cells can release factors, e.g. IL-1, FGF-2 or PDGF, which stimulate neighboring fibroblasts to secrete HGF (Fig. 7) (Rosen et al., 1994a, 1994b; Nakamura et al., 1997). Alternatively, some tumor cell lines were shown to express both HGF and Met, suggesting the presence of an autocrine loop (Fig. 7) (Naidu et al., 1994; Moriyama et al., 1995; Tuck et al., 1996; Trusolino et al., 1998). Aberrant expression and activation of the HGF/Met pathway are not only present in tumor cell lines, but also in many native human tumors. Overexpression of Met was detected in carcinomas of the stomach, liver, colon, pancreas, lung, and thyroid gland (Di Renzo et al., 1991, 1992, 1995a, 1995b; Prat et al., 1991; Kuniyasu et al., 1992; Liu et al., 1992; Boix et al., 1994; Kurukawa et al., 1995; Ueki et al., 1997). For colorectal, liver, thyroid, and brain cancer, it was shown to be correlated with disease progression (Di Renzo et al., 1995b; Belfiore et al., 1997; Koochekpour et al., 1997; Ueki et al., 1997). Similarly, overexpression of HGF was also reported in human cancer, e.g. in tumors of the pancreas and in gliomas (Furukawa et al., 1995; Koochekpour et al., 1997; Lamszus et al., 1998). Importantly, in patients with breast or non-small cell lung cancer, expression of HGF was shown to be a strong and independent predictor of recurrence and tumor-related death (Yamashita et al., 1994; Siegfried et al., 1997).
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Figure 7. The HGF/MET pathway in tumor growth, invasion and metastasis. Uncontrolled MET-signaling can be caused by receptor overexpression, illegitimate autocrine or paracrine stimulation, activating mutations, and translocation and fusion with Tpr. In addition, uncontrolled MET-signaling may also result from overexpression of HSPG. As a consequence of enhanced MET activity, tumor cells may increase their growth rate and become resistant to apoptosis, resulting in a growth and/or survival advantage. Furthermore, MET activation may lead to cytoskeletal reorganization, and integrin activation, as well as to activation of proteolytic systems involved in ECM degradation, resulting in an increased invasive and metastatic capacity. HGF production, either by fibroblasts in the tumor stroma or by the tumor cells, may stimulate angiogenesis.

Weidner and colleagues (1990) demonstrated that HGF induces invasion of carcinoma cell lines into collagen gels. Similar effects of HGF were subsequently reported for many other tumor cell lines, including mammary, colon and squamous cell carcinoma, and melanoma lines (Jiang et al., 1993; Matsumoto et al., 1994;
Rosen et al., 1994b; Hendrix et al., 1998). In accordance with these findings, Date et al. (1998) showed that a 4-kringle-containing HGF antagonist (NK4) inhibits HGF-induced tumor invasion. Interestingly, Jiang et al. (1993) reported that, while colon carcinoma cells became more motile in the presence of HGF, their growth was inhibited. Similar observations were made by Giordano et al. (1993) in NIH 3T3 fibroblasts. A recent report suggests that HGF-induced growth suppression can be caused by the induction of oxidative stress (Arakaki et al., 1999).

At least two mechanisms may be involved in the promotion of invasiveness by the HGF/Met pathway. First, HGF may induce invasion and metastasis by causing cytoskeletal rearrangement and by activating adhesion molecules (Fig. 7). For example, HGF was shown to induce tyrosine phosphorylation of molecules involved in the assembly of focal adhesions, resulting in cell spreading and migration (Matsumoto et al., 1994) (see also Sections II.B and C). In addition, we and others have shown that HGF induces activation of integrins and consequent adhesion and migration (van der Voort et al., 1997; Weimar et al., 1997; Trusolino et al., 1998). Secondly, activation of Met may lead to an enhanced degradation of the ECM by invasive cells (Fig. 7). Met activation by HGF increases the expression of urokinase-type plasminogen (uPA) and its receptor (Pepper et al., 1992; Rosen et al., 1994b; Jeffers et al., 1996b), molecules known to play a role in ECM proteolysis.

Studies using in vivo models confirm the involvement of HGF and Met in tumorigenesis. Autocrine stimulation of Met transfected NIH 3T3 cells with HGF enhanced the tumorigenic and metastatic capacity of these cells in nude mice (Rong et al., 1992, 1994). Similar findings were reported for SK-LMS-1 human leiomyosarcoma cells and for mouse mammary tumor cells (Rosen et al., 1994b; Jeffers et al., 1996b; Lamszus et al., 1997). In the leiomyosarcoma model, HGF/Met signaling was shown to increase the expression of both uPA and its receptor uPAR, suggesting a role for this proteolysis network in promoting invasiveness and metastasis. In the mouse mammary tumor model as well as in a glioma model, stimulation of angiogenesis by HGF appeared to play a key role (Fig. 7) (Lamszus et al., 1997; Laterra et al., 1997).

Studies in transgenic mice corroborate the role of HGF and Met in tumorigenesis. HGF transgenic mice were shown to develop a broad array of primary tumors and metastases of mesenchymal as well as epithelial origin, including malignant melanoma, fibrosarcoma, and mammary carcinoma (Takayama et al., 1997b; Otsuka et al., 1998). Many of these tumors arose from abnormally developed tissues, suggesting a functional link between HGF-dependent morphogenesis and tumorigenesis. Since most neoplasms, melanomas in particular, demonstrated overexpression and enhanced activation of Met, autocrine signaling via Met was thought to be a major cause of tumorigenesis.
Recently, Met has been implicated in the genesis of hereditary papillary renal carcinomas (HPRC). Schmidt et al. (1997) showed that missense mutations in the Met gene are present in the germline of affected members of HPRC families. Remarkably, affected individuals often have a duplication of the chromosome bearing the mutated Met allele (Fischer et al., 1998; Zhuang et al., 1998). Similar mutations were also found in a subset of sporadic papillary renal carcinomas and of childhood hepatocellular carcinomas (Zhuang et al., 1998; Park et al., 1999). Importantly, NIH 3T3 fibroblasts transfected with Met constructs containing the mutations detected in HPRC, are transforming in vitro and tumorigenic in vivo (Jeffers et al., 1997). Taken together, these data strongly suggest that the Met mutants expressed in HPRC initiate tumorigenesis.

III. HGF/MET IN B CELL DEVELOPMENT AND NEOPLASIA

A. HGF/Met in antigen-specific B cell differentiation

Interestingly, recent studies from our laboratory have provided evidence for a role of the HGF/Met pathway in the immune system, i.e. in the regulation of antigen-specific B cell differentiation (van der Voort et al., 1997, 1999; Pals et al., 1998; Taher et al., 1999). During this process, naive B cells develop into memory cells or plasma cells. This requires multiple interactions of B cells with other cells, such as T cells and follicular dendritic cells (FDC), and with the ECM, which take place within distinct microenvironmental compartments of the lymphoid tissues (Fig. 8) (MacLennan, 1994; Nossal, 1994; Thorbecke et al., 1994; Liu et al., 1996a; Rajewsky, 1996; Lindhout et al., 1997). After their initial activation in the extrafollicular T cell (paracortical) area, germinal center (GC) founder cells migrate into B cell follicles where they initiate the formation of GCs (Liu et al., 1991; Jacob et al., 1991a). Once in the GC, the B cells first pass the dark zone where they undergo rapid clonal expansion and somatic hypermutation in their IgV genes (Berek et al., 1991; Jacob et al., 1991b; Küppers et al., 1993; McHeyzer-Williams et al., 1993; Pascual et al., 1994). Mutated B cells then progress to centrocytes and move to the basal light zone of the GC. Here they reencounter antigen, presented as low levels of immune complexes on FDC, and undergo affinity selection (Tew et al., 1990; Hardie et al., 1993; MacLennan, 1994). Whereas low affinity mutants and autoreactive mutants die by apoptosis, high affinity mutants internalize antigen and process it on their migration pathway to the apical light and outer-zones of the GC. In these areas, the affinity selected B cells present antigen to antigen-specific GC T cells (Fuller et al., 1993; Casamayor-Palleja et al., 1995; Zheng et al., 1996). Cognate T-B interaction results in expansion and Ig-isotype switching of high affinity B cells (Kraal et al., 1982; Liu
et al., 1996b), that mature into memory B cells or plasma cells and receive signals mediating their export from the lymphoid organ (MacLennan, 1994).

We observed that stimulation of human tonsillar B cells by phorbol ester and, more importantly, by concurrent CD40 and B cell receptor (BCR) ligation, leads to a rapid transient Met induction (van der Voort et al., 1997). Presumably, BCR- and CD40-mediated signals are also instrumental in the physiological induction of Met, as Met is expressed in vivo on tonsillar centroblasts (CD38-CD77+), which are the offspring of B cells that have recently been activated at extrafollicular sites by antigen plus T cells signals (MacLennan, 1994). These activating signals critically involve CD40/CD40L interactions: Patients with the X-linked hyper-IgM syndrome (due to mutated and consequently defective CD40L) do not develop GC and blocking of the CD40/CD40L pathway in mice leads to complete inhibition of GC reactions (Banchereau et al., 1994; Foy et al., 1994; Han et al., 1995; Kawabe et al., 1994; Facchetti et al., 1995). Our findings thus link Met induction to the initiation of the B cell immune response.

![Figure 8](image)

**Figure 8.** Schematic representation of the T cell-dependent B cell differentiation in secondary lymphoid organs. See text for further details. B, B cell; FDC, follicular dendritic cell; T, T cell.
Adhesion regulation, particularly regulation of lymphocyte integrin function, is believed to be fundamental to the control of cell migration and microenvironmental homing during B cell differentiation (Koopman and Pals, 1992; Butcher and Picker, 1996). In functional studies, we observed that HGF augments adhesion of Met positive B cells lines to VCAM-1 and fibronectin by activating the integrin α4β1 (van der Voort et al., 1997). Similar effects of HGF on integrin-mediated adhesion of B cells to fibronectin were also reported by Weimar et al. (1997). The physiological relevance of these findings is strongly supported by our observation that HGF is produced by stromal cells and FDC (van der Voort et al., 1997; and our unpublished observations). During B cell differentiation the integrin α4β1 mediates B cell adhesion to FDC (Freedman et al., 1990; Koopman et al., 1991, 1994), an interaction that regulates the formation of the microenvironment required for the affinity selection of GC B cells. Apart from establishing physical contact between B cells and FDC, α4β1 presumably contributes directly to the B cell selection process itself, as signaling through the α4β1/VCAM-1 pathway costimulates rescue of GC B cell from apoptosis (Koopman et al., 1994, 1997). Furthermore, α4β1 also regulates cell adhesion to fibronectin (Wayner et al., 1989), an important substrate for cell migration. Interestingly, Weimar et al. (1997) reported that HGF indeed stimulates B cell migration on fibronectin.

In view of the pleiotropic effects of HGF on many cell types, it is possible that, HGF may have other, as yet unknown, roles in antigen-specific B cell differentiation in addition to adhesion regulation. For example, as will be discussed in Section III.B. Met signaling might promote B cell proliferation and survival.

Preliminary results from in vivo studies support the involvement of the HGF/Met pathway in antigen-specific B cell differentiation. We explored this role by a molecular genetic approach using Met knock-out mice. Since homozygous Met knock-out mice die in utero at around day E15.5 (Bladt et al., 1995), the immune function of these mice can not be studied directly. To circumvent this problem, we reconstituted RAG-2−/−IL-2R−/− mice with fetal liver cells from MET+ mice or control littermates. After i.v. injection, the haematopoietic stem cells present in the fetal liver migrate to the bone marrow, and regenerate B- and T-cell populations. Thus far, analysis of the B and T-cell compartments, the organization of the lymphoid organs, and of the baseline levels of immunoglobulin, demonstrated no significant difference between MET+ and control mice. Interestingly, however, the immune response against the T cell-dependent antigen TNP-KLH was reduced in the MET+ mice. Furthermore, in the spleens of immunized MET+ mice, we observed a reduction in the number of plasma cells, the Ig-secreting population of B cells.
B. Met signaling in B cells

Although most data on Met-signaling have been obtained in epithelial cells, and hence do not necessarily apply to B cells, we have recently been able to demonstrate HGF/Met-induced phosphorylation and/or activation in B cells of at least several key signaling molecules such as Ras, MAP kinase, PI3-K, PKB and Gab1 (Fig. 5) (our unpublished observations). Here we will outline the putative roles of these molecules in B cell development.

As discussed above, the HGF/Met pathway is implicated in integrin regulation in B cells (van der Voort et al., 1997; Weimar et al., 1997). Several different signaling pathways have been implicated in inside-out signaling to integrins. Key regulatory proteins in these pathways appear to be PI3-K and different Ras-like GTPases (Howe et al., 1998; Kolanus and Seed, 1997; Hughes and Palla, 1998). PI3-K is involved in the activation of integrins in leukocytes (Shimizu et al., 1995), T cells (Zell et al., 1998) and platelets (Zhang et al., 1996a), whereas activated R-ras increases integrin activity in myeloid cells (Zhang et al., 1996b) and epithelial cells (Keely et al., 1999). Since R-ras is able to bind the same effector molecules as Ras, including PI3-K (Spaargaren et al., 1994; Spaargaren and Bischoff, 1994; Marte et al., 1997), integrin activation by R-ras may involve the activation of PI3-K.

In contrast to the stimulatory effect of R-ras, activated H-ras and Raf were found to inhibit activity of platelet integrins expressed in CHO cells (Hughes et al., 1997). Interestingly, R-ras appears to function as an antagonist of Ras-suppressed integrin activity in this cell system (Sethi et al., 1999). However, unlike its inhibitory effects on the activation of platelet-integrins, active Ras promotes TCR-triggered integrin-mediated T cell adhesion to ICAM-1 (O'Rourke et al., 1998). These data imply that, dependent on the specific cell system studied, Ras may either inhibit or promote integrin activity. In addition to Ras and R-ras, the GTPase Rho has also been implicated in integrin activation, specifically in chemoattractant-induced integrin activation in lymphocytes and neutrophils (Laudanna et al., 1996). Since HGF stimulation of Met in B cells results in activation of PI3-K as well as the Ras-MAP kinase pathway (van der Voort et al., 1999) (our unpublished observations), and Rho has been implicated in Met signaling in epithelial cells (Takaishi et al., 1994, Nishiyama et al., 1994), it is likely that these proteins also play an important role in the regulation of integrin activity by HGF in B cells.

Recent studies have revealed several points of convergence between B cell antigen receptor (BCR) and HGF/Met signaling. Firstly, the prominent Met substrate Gab1 was recently shown to become phosphorylated, and associated with Grb2, PI3-K, Shc and SHP-2, upon BCR triggering (Ingham et al., 1998; Nishida et al., 1999) (our unpublished observations). Secondly, PI3-K, which is activated upon stimulation of B cells by HGF, has a prominent role in BCR-signaling and B
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cell development: targeted disruption of the gene encoding the p85 subunit of PI3-K arrests B cell development at the pro-B cell stage, resulting in decreased immunoglobulin production (Fruman et al., 1999; Suzuki et al., 1999), similar to that in X-linked hypoglobulinemia. Thirdly, STAT3, which becomes phosphorylated and translocates to the nucleus upon stimulation by HGF (Boccaccio et al., 1998), also plays a role in BCR-mediated signaling (Karras et al., 1997). Finally, we observed that HGF activates the Ras-MAPK pathway in B cells. This presumably constitutes an important transcription regulatory and proliferative signal for Met expressing GC B cells, which are in the process of undergoing rapid clonal expansion and selection (Lindhout et al., 1997; Tarlinton, 1998b). Ras is also involved in BCR-signaling and B cell development. Expression of dominant negative Ras arrests development at a very early stage, prior to formation of the pre-B cell receptor (Iritani et al., 1997). Furthermore, activated Ras causes progression of RAG1-deficient pro-B cells to pre-B cells and to cells with characteristics of the more mature GC B cells (Shaw et al., 1999).

Successful B cell selection in the GC requires tight regulation of cell survival. Interestingly, several studies indicate that the HGF/Met pathway may generate survival signals. HGF can rescue MDCK cells from apoptosis (Frisch and Francis, 1994), and inhibits apoptosis induced by staurosporin or DNA damaging agents of liver progenitor and carcinoma cells (Bardelli et al., 1996; Fan et al., 1998; Liu et al., 1998b). In addition, overexpression of an active Met mutant renders hepatocytes resistant to anoikis and staurosporin-induced apoptosis (Amicone et al., 1997). Given the ability of Met to interact with the anti-apoptotic BAG-1 upon HGF stimulation (Bardelli et al., 1996), it is interesting to note that BAG-1 has been reported to play a role in survival and proliferation of the IL-3-dependent B cell line Ba/F3 (Clevenger et al., 1997). Furthermore, we have found that Met can activate PKB, in a PI3-K-dependent fashion, in B cells (our unpublished observation). PKB is able to phosphorylate Bad, a Bcl-2 antagonist expressed in GC B cells (Ghia et al., 1998), and thereby may suppresses the pro-apoptotic activity of Bad (Datta et al., 1997; Mok et al., 1999) (Fig. 5). Taken together, these data suggest that Met may play an important role in the regulation of apoptosis in the GC B cells, and thus in the process of affinity selection, which is critical for antigen-specific B cell differentiation.

Finally, regulation of GC B cell migration is important for the antigen-specific B cell differentiation (Tarlinton, 1998a; Pals et al., 1998). Several chemokines have been shown to be involved in this process, including SDF-1 , which is produced by reticulum cells surrounding the GC and acts via the G protein-coupled receptor CXCR4 (Bleul et al., 1996, 1998). A recent study has reported the ability of the BCR to arrest SDF-1α-induced migration (Bleul et al., 1998). Interestingly, it was shown that this arrest was caused by PKC-mediated CXCR4 downregulation (Guinamard et al., 1999). Since HGF stimulation also
results in activation of PKC. This mechanism may provide a means to arrest migration of Met expressing B cells in the GC.

C. Heparan sulfate proteoglycans on B cells promote Met signaling

We have recently obtained evidence that HSPGs expressed on the cell-surface of specific B cell subsets may play an important role in regulating Met signaling. Several human B cell subpopulations, including plasma cells and memory B cells, express HSPGs (van der Voort et al., 2000). Interestingly, this HSPG expression was greatly enhanced by activation of B cells with the phorbol ester PMA, and more importantly, by ligating the co-stimulatory molecule CD40. An even stronger induction of HSPG was obtained after simultaneous ligation of CD40 and the BCR, signals which also induce expression of Met (van der Voort et al., 1997). Since CD40 and the BCR play key roles during the T cell-dependent B cell differentiation (see Section III.A), these data suggest that Met and HSPGs act in concert during this biological process. Indeed, we observed that, upon activation, B cells acquire the capacity to bind large amounts of HGF via HS-moieties. CD44 isoforms carrying HS-chains (CD44-HS) are the major proteoglycan core proteins on these activated B cells, which did not express the core proteins of syndecan-1, 2, 4, or glypican. However, others have shown that human as well as murine plasma cells express syndecan-1 (Sandersen et al., 1989), whereas syndecan-4 was recently shown to be expressed by murine immature and mature B cells (Yamashita et al., 1999).

By using Burkitt's lymphoma cells transfected with either CD44-HS or a CD44 isoform lacking HS (CD44s), we demonstrated that CD44-HS strongly promotes signal transduction via Met, including Met phosphorylation, phosphorylation of Gab-1, activation of the MAP kinases ERK1/2, and phosphorylation of PKB. Taken together, our results identify HSPGs, specifically CD44-HS, as functional co-receptors for HGF promoting signal transduction through Met (Fig. 6). We hypothesize that, via concentration and presentation of HGF, HSPGs regulate the biological activity of the HGF/Met pathway in B cells.

D. The HGF/Met pathway in B cell neoplasia

The HGF/Met pathway presumably is not only involved in normal B cell differentiation, but also in the development and progression of B cell neoplasia. Met is constitutively expressed by several Burkitt's lymphoma cell lines, including Raji, BJAB, and EB4B (Jücker et al., 1994; van der Voort et al., 1997, Weimar et al., 1997), as well as by a subset of native Burkitt's lymphomas (Weimar et al.,
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1997; our unpublished observations). On these tumor cells, which represent the malignant counterparts of GC centroblasts, HGF induces Met phosphorylation, as well as activation of downstream signaling molecules including MAP kinases and PKB (van der Voort et al., 1997, 1999; Taher et al., 1999; our unpublished observations) (see also Section III.B). Furthermore, HGF stimulation of Met positive Burkitt's lymphoma cells enhances α4β1 and α5β1-mediated adhesion to fibronectin, collagen and VCAM-1, and promotes their invasion into fibroblast monolayers (van der Voort et al., 1997; Weimar et al., 1997). Since HGF is produced by follicular dendritic cells and lymphoid stromal cells (van der Voort et al., 1997) (our unpublished observation), paracrine stimulation of Burkitt's lymphoma cells by HGF most likely takes place within the lymphoid microenvironment, promoting tumor growth and/or survival. HGF/Met signaling may stimulate survival via at least two distinct routes. As a direct consequence of HGF stimulation, Met may become associated with the anti-apoptotic protein BAG-1 (Bardelli et al., 1996) (see also Section III.B). At the same time, HGF stimulation may down-modulate the activity of the Bcl-2 antagonist Bad (Datta et al., 1997). Alternatively, activation of integrins by HGF/Met signaling may prevent tumor cell death by anoikis. This scenario is supported by our previous observation that integrin mediated adhesion to FDC presents a strong anti-apoptotic signal for GC B cells (Koopman et al., 1994, 1997).

Although precise data concerning the expression of Met and HGF in different subtypes of malignant lymphoma are at present not available, a study by Weimar and colleagues (1997) indicates that Met expression is not confined to Burkitt's lymphoma. In 8 out of 11 follicle center cell lymphomas, and in some cases of large B cell lymphoma, Met expression was observed. Furthermore, in approximately half of the cases of Hodgkin's disease Met expression was found in Hodgkin's/Reed-Sternberg (RS) cells, which presumably represent "crippled" GC B cells (Braeuninger et al., 1997). Interestingly, Met expression in RS cells was strongly correlated with the presence of Epstein-Barr virus (EBV), suggesting a role for EBV in Met regulation (Weimar et al., 1997). Although recent studies from our laboratory do not confirm the high percentage of Met positive cases among follicle center cell lymphomas, we did find Met expression in large B cell lymphomas (our unpublished observations).

HGF has also been identified as a potential growth factor for multiple myeloma (MM), a neoplasm of terminally differentiated B cell (i.e. plasma cells). By screening myeloma supernatants for their ability to inhibit the activity of transforming growth factor-β (TGF-β), Borset and colleagues (1996c) isolated an antagonist. This protein was identified as HGF, and was produced by all five myeloma cell lines tested. Interestingly, in four of these cell lines Met was also expressed, suggesting the existence of an autocrine HGF/Met loop. Indeed, in the human myeloma cell line JIN-3, Met was found to be constitutively

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phosphorylated and could be dephosphorylated by anti-HGF antibodies (Borset et al., 1996b). These findings were further extended by analyzing MM cells freshly isolated from patients. In all seven cases studied, co-expression of HGF and Met was observed on MM cells isolated from the bone marrow (Borset et al., 1996a). Recently, the Nordic Myeloma Study Group reported the HGF serum levels of over 400 MM patients. In approximately half of these patients elevated HGF levels were present; these patients had an unfavorable prognosis and poor response to melphalan/prednisone treatment (Seidel et al., 1998).

In conclusion, while its precise role needs to be elucidated, the above data suggests that deregulated HGF/Met signaling may contribute to the development and progression of specific subtypes of B cell neoplasia, including Burkitt's lymphoma, large B cell lymphoma, and multiple myeloma.

IV. SUMMARY

This review summarizes the structure, signal transduction and physiological functions of the HGF/Met pathway, as well as its role in tumor growth, invasion, and metastasis. Moreover, it highlights recent studies indicating a role for the HGF/Met pathway in antigen-specific B cell development and B cell neoplasia.

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Chapter 7

Summary and conclusions

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Summary and conclusions

The generation of an effective immune response depends on the capacity of lymphocytes to interact with other cells and with the extracellular matrix (ECM). These interactions are achieved through the action of adhesion molecules and their ligands. Many different adhesion molecules have now been characterized, however, each type of lymphocyte expresses its own limited set of adhesion molecules, thereby creating cell and tissue-specific migration pathways.

Most adhesion molecules need to be activated before they can bind their ligand(s). Others need a special chemical composition, achieved through, for instance, RNA splicing or glycosylation, in order to bind their ligand. The studies described in this thesis characterize several mechanisms which regulate the binding of two families of adhesion molecules, i.e. CD44 and the integrins, to their ligand(s).

Chapter 2 describes two mechanisms which regulate the adhesive capacity CD44. CD44 represents a family of adhesion molecules encoded by one gene. Through alternative RNA splicing and differential glycosylation, many different CD44 isoforms exist. Several of these isoforms have been described to bind the extracellular matrix carbohydrate hyaluronate (HA). We observed that the capacity to bind HA depends both on the presence of specific alternatively expressed CD44 domains, and on the cell-type which expresses the CD44 molecule. The latter is very likely the consequence of cell-specific glycosylation. These data suggest that through differential splicing and glycosylation of CD44, cells can regulate their affinity for HA.

A new signaling pathway which regulates the activation of the integrin family of adhesion molecules is described in Chapter 3. This pathway becomes activated through the binding of hepatocyte growth factor (HGF) to its receptor tyrosine kinase Met. It is shown that a specific subset of B lymphocytes, the germinal center (GC) cells, express Met. This exclusive expression on GC cells suggests that HGF and Met have an important function during the GC reaction. This complex T cell-dependent process generates memory lymphocytes, and plasma cells producing antibodies with a high affinity. Indeed, we observed that activation of CD40 and the B cell antigen receptor (BCR), reactions which are important during the GC reaction, induced a strong transient expression of Met on B lymphocytes. In addition, we demonstrate that Met becomes activated upon binding of HGF, which results in enhanced integrin-mediated adhesion of B cells to the adhesion molecule vascular adhesion molecule 1 (VCAM-1), and to the ECM protein fibronectin. Since we also demonstrated that HGF is secreted by stromal cells and follicular dendritic cells, cells present in or near the GC, these data strongly suggest that the HGF-Met pathway plays an important role during the GC reaction.
Heparan sulfate proteoglycans (HSPGs) are believed to play an important role in the regulation of the activity of many cytokines. CD44 isoforms containing the domain encoded by the alternatively spliced exon v3 can be modified with heparan sulfate (HS), and consequently are HSPGs. The data presented in Chapters 4 and 5 show that HGF-induced signal transduction is strongly promoted when B cells express CD44-HS. First, Chapter 4 demonstrates that CD44-HS efficiently binds HGF via its HS-side chain. In addition, we show that, as compared to a CD44 isoform without HS, CD44-HS promotes: (i) HGF-induced phosphorylation of Met, and (ii) phosphorylation of several downstream proteins, including the MAP kinases ERK1 and 2. Heparitinase treatment, and the use of a mutant HGF with greatly reduced affinity for HS, demonstrates that the enhancement of Met signal transduction induced by CD44-HS is critically dependent on HS moieties. Furthermore, Chapter 5 describes the delicately regulated expression and function of HSPGs, in particular CD44-HS, on tonsillar B cells. It demonstrates that activation of the BCR and/or CD40, induces a strong...
Chapter 7

transient expression of HSPGs on human tonsillar B cells. By means of these HSPGs, the activated B cells bind relatively large amounts of HGF. This interaction with HGF is highly selective since the HSPGs on activated B cells do not bind the chemokine stromal cell-derived factor 1α (SDF-1α), even though the affinities of HGF and SDF-1α for heparin are similar. On the activated B cells, we observed induction of CD44-HS, but not of other HSPGs. Confirming the data presented in Chapter 4, the expression of CD44-HS strongly promoted HGF-induced signaling, resulting in a HS-dependent enhanced phosphorylation of Met, as well as downstream signaling molecules including the Grb2 associated binder 1 (Gab1) and Akt/protein kinase B (PKB). These results identify HSPGs, in particular CD44-HS, as functional co-receptors for HGF which promote signaling through Met. We hypothesize that CD44-HS concentrates HGF at the cell surface and presents it to Met, thereby creating a ménage à trois between HGF, Met and CD44 (see Figure).

This interaction will promote the activation of Met, and consequently lead to enhanced signal transduction (see Figure). Preliminary data suggest that next to extracellular cross-talk between CD44-HS and Met, CD44-HS can also stimulate Met via intracellular cross-talk between signaling molecules (see Figure). Presentation of cytokines by CD44-HS (or other HSPGs) likely serves an important function for B cell biology. However, when deranged, for instance by overexpression of CD44-HS as found in a subset of malignant lymphoma, it may contribute to a malignant phenotype by providing a growth and/or motility advantage.

Chapter 6 provides an overview of the structure and functions of the multifunctional cytokine HGF and its receptor Met. The chapter presents and discusses classical and new data concerning the role of HGF and Met in development, tumorigenesis, and B cell differentiation.

Taken together, the data presented in this thesis demonstrate that CD44-dependent adhesion of B cells is regulated by splicing and glycosylation of CD44, while integrin-dependent adhesion of B cells can be regulated by activation of the HGF – Met pathway. In addition, they suggest a role for HGF/Met in T cell-dependent immune responses. Moreover, the data show that HS-modified CD44 isoforms promote signaling through the HGF – Met pathway. Since several lymphoid as well as non-lymphoid tumors express both Met and CD44, the data presented in this thesis suggest that the regulation of adhesion by the ménage à trois of HGF, Met, and CD44, is involved in tumor growth and metastasis as well. We have recently demonstrated that expression of CD44-HS indeed promotes HGF-induced signal transduction in colorectal cancer cells (Wielenga et al., 2000).
Wielenga V., R. van der Voort, T.E.I. Taher, J. Smit, C. van Krimpen, F.A. Beuling, M. Spaargaren, and S.T. Pals. (2000) Co-expression of e-Met and heparan sulfate proteoglycan forms of CD44 in colorectal cancer. *Am. J. Pathol.*, in press.
Samenvatting en conclusies

Het teweegbrengen van een effectieve immuunrespons hangt af van het vermogen van lymfocyten om een interactie aan te gaan met andere cellen of met de extracellulaire matrix (ECM). Adhesiemoleculen en hun liganden zijn verantwoordelijk voor deze interacties. Hoewel het immuunsysteem over een groot aantal adhesiemoleculen beschikt, brengt elke lymfocyt zijn eigen (beperkte) set adhesiemoleculen tot expressie. Hierdoor migreren lymfocyten door het organisme via een aantal cell- en weefselspecifieke migratieroutes.

De meeste adhesiemoleculen moeten geactiveerd worden voordat zijn hun ligand(en) kunnen binden. Andere moeten een specifieke chemische structuur hebben, bijvoorbeeld door RNA-splicing of glycosylering verkregen. De studies beschreven in dit proefschrift karakteriseren verschillende mechanismen die de binding reguleren van twee families van adhesiemoleculen, te weten CD44 en de integrines, aan hun liganden.

**Hoofdstuk 2** beschrijft twee mechanismen die de adhesiecapaciteit van CD44 reguleren. CD44 vertegenwoordigt een familie van adhesiemoleculen gecodeerd door één gen. Door alternatieve RNA-splicing en differentiële glycosylering bestaan er veel verschillende CD44 isoformen. Beschreven is, dat een aantal van deze isoformen bindt aan de extracellulaire matrix koolhydraat hyaluronan (HA). Wij lieten zien, dat de capaciteit om HA te binden afhing van de aanwezigheid van specifieke, alternatief tot expressie komende, CD44 domeinen, en van het celtype welke de CD44 moleculen tot expressie bracht. Het laatste is zeer waarschijnlijk het gevolg van cel-specifieke glycosylering. Deze data suggereren dat, door middel van differentiële splicing en glycosylering van CD44, cellen hun affiniteit voor HA reguleren.

Een nieuwe signaleringsroute die de activiteit van de integrine adhesiemoleculen reguleert is beschreven in **Hoofdstuk 3**. Deze route wordt geactiveerd door binding van hepatocyte growth factor (HGF) aan zijn receptor tyrosine kinase Met. Hoofdstuk 3 beschrijft, dat een specifieke subgroep van B lymfocyten, de kiemcentrumcellen, Met tot expressie brengen. De exclusieve expressie op kiemcentrumcellen suggereert, dat HGF en Met een belangrijke functie hebben tijdens de kiemcentrumreactie. Dit complexe T cel-afhankelijke proces genereert memory lymfocyten, en plasma cellen, cellen die antilichamen met een hoge affiniteit produceren. Inderdaad observeerden wij, dat activatie van CD40 en de B cel antigen receptor (BCR), processen van groot belang tijdens de kiemcentrumreactie, een sterke tijdelijke expressie van Met op B lymfocyten induceerde. Tevens demonstreerden wij, dat Met geactiveerd wordt tijdens de binding van HGF. Dit resultert in een versterkte integrine-gemedieerde adhesie van B cellen aan het adhesie molecuul vascular adhesion molecule 1 (VCAM-1) en
Samenvatting en conclusies

Model voor de presentatie van HGF aan Met. Het ontstaan van een complex tussen HGF, Met en CD44-HS versterkt de activatie van verschillende signaleringsroutes, zoals degene die integrines activeren. Deze signaaltransductie wordt geacht celadhésie, migratie, proliferatie, en/of differentiatie te bevorderen.

aan het ECM-eiwit fibronectine. Aangezien wij ook aantoonden, dat HGF door stromale cellen en folliculaire dendritische cellen, cellen aanwezig in of nabij het kiemcentrum, gecreëerd wordt, suggereren deze data dat de HGF - Met route een belangrijke rol speelt tijdens de kiemcentrumreactie.

Heparan suïfaat proteoglycanen (HSPG) worden geacht de activiteit van veel cytokines te reguleren. CD44 isoformen die het alternatieve domein v3 bevatten kunnen gemodificeerd zijn met heparan suïfaat (HS), en zijn dan ook HSPG. De data gepresenteerd in de Hoofdstukken 4 en 5 laten zien, dat HGF-geïnduceerde signaaltransductie is versterkt als deze B cellen CD44-HS tot expressie brengen. Ten eerste toont Hoofdstuk 4 aan, dat CD44-HS via zijn HS-keten efficiënt HGF bindt. Tevens lieten wij zien dat, in vergelijking met een CD44 isoform zonder HS, CD44-HS: (i) HGF-geïnduceerde fosforylering van Met, en (ii) fosforylering van verscheidene andere signaleringseiwitten, inclusief de MAP kinases ERK1 en -2, versterkt. Heparitinase-behandeling en het gebruik van een
mutant HGF met sterk gereduceerde affiniteit voor HS, toonden aan dat de, door CD44-HS geïnduceerde, versterking van de signaaltransductie door Met, afhankelijk is van HS. Daarnaast beschrijft Hoofdstuk 5 de delicaat gereguleerde expressie en functie van HSPG. CD44-HS in het bijzonder, op B cellen. Het laat zien, dat activatie van de BCR en/of CD40 een sterke tijdelijke expressie van HSPG op humane B cellen induceert. Door middel van deze HSPG binden de geactiveerde B cellen relatief grote hoeveelheden HGF. Deze interactie met HGF is zeer selectief, omdat de HSPG op geactiveerde B cellen met de chemokine stromal cell-derived factor 1α (SDF-1α) binden, terwijl HGF en SDF-1α een vergelijkbare affiniteit voor heparine hebben. Op de geactiveerde B cellen ontdekten wij een verhoogde expressie van CD44-HS, maar niet van andere HSPG. In overeenstemming met de data gepresenteerd in Hoofdstuk 4, versterkte de expressie van CD44-HS de HGF-geïnduceerde signalering. Dit resulteerde in een HS-afhankelijke toenam e in fosforlyering van Met, alsmede van additionele signaleringsmoleculen zoals de Grb2 associated binder (Gab1) en Akt/protein kinase B (PKB). Deze resultaten tonen aan, dat HSPG, in het bijzonder CD44-HS, als coreceptoren voor HGF kunnen functioneren. Volgens onze hypothese concentreert CD44-HS HGF op het celoppervlak en presenteert het HGF vervolgens aan Met. Daarbij ontstaat een ménage à trois tussen HGF, Met en CD44 (zie Figuur). Deze interactie zal de activatie van Met versterken en vervolgens leiden tot een toegenomen signaltransductie (zie Figuur). Voorlopige data suggereren dat, naast extracellulaire signalering tussen CD44-HS en Met, CD44-HS ook Met kan stimuleren via intracellulaire signalering (zie Figuur). Presentatie van cytokinen door CD44-HS (of andere HSPG) is waarschijnlijk van groot belang voor B cel biologie. Echter, indien ontregeld, bij voorbeeld door overexpressie van CD44-HS, zoals vastgesteld op een subgroep van maligne lymfomen, zou dit door het verschaffen van een groei- en/of motilitieitsvoordeel, tot een maligne fenotype kunnen bijdragen.

Hoofdstuk 6 geeft een overzicht van destructuur en functies van de multifunctionele cytokine HGF en zijn receptor Met. Het hoofdstuk presenteert en bediscussieert klassieke en nieuwe data omtrent de rol van HGF en Met in ontwikkeling, tumorgenese en B cel differentiatie.

Concluderend mogen wij vaststellen dat de data beschreven in dit proefschrift, aantonen dat CD44-afhankelijke adhesie van B cellen gereguleerd wordt door splicing en glycosylereing van CD44, terwijl integrine-afhankelijke adhesie van B cellen gereguleerd kan worden door activatie van de HGF – Met route. Tevens suggereren onze resultaten, dat HGF/Met betrokken is bij T cel-afhankelijke immuunrespons. Daarnaast tonen de data aan, dat HS-gemodificeerd CD44 de signalering via de HGF – Met route versterkt. Omdat verscheidene lymfoïde alsook niet-lymfoïde tumoren zowel Met als CD44 tot expressie brengen, suggereren de in dit proefschrift beschreven data dat de
regulatie van adhesie door de ménage à trois tussen HGF, Met en CD44 ook betrokken is bij tumorgroei en metastasering. Wij hebben recent aangetoond, dat expressie van CD44-HS inderdaad HGF-geïnduceerde signaaltransductie in colorectale tumorcellen bevordert (Wielenga et al., 2000).

Wielenga V., R. van der Voort, T.E.I. Taher, L. Smit, C. van Krimpen, E.A. Beuling, M. Spaargaren, and S.T. Pals. (2000) Co-expression of c-Met and heparan sulfate proteoglycan forms of CD44 in colorectal cancer. *Am. J. Pathol.*, in press.
Appendices

Abbreviations
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## Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| Ag           | antigen     |
| BCA          | B cell attracting chemokine |
| BCR          | B cell antigen receptor |
| BSA          | bovine serum albumin |
| CCR          | CC chemokine receptor |
| CD           | cluster of differentiation |
| CD40L        | CD40 ligand |
| CD44E        | epithelial CD44 isoform |
| CD44H        | hematopoietic CD44 isoform |
| CD44-HS      | HS-modified CD44 |
| CD44s        | CD44 standard isoform |
| CD44v        | CD44 variant isoform |
| CDR          | complementarity determining region |
| CLA          | cutaneous lymphocyte-associated antigen |
| CXCR         | CXC chemokine receptor |
| ECM          | extracellular matrix |
| LLAM         | endothelial leukocyte adhesion molecule |
| E-selectin   | endothelial selectin |
| ESL          | E-selectin ligand |
| FACS         | fluorescence activated cell sorter |
| FDC          | follicular dendritic cell |
| FGF          | fibroblast growth factor |
| FGFR         | fibroblast growth factor receptor |
| FITC         | fluorescein isothiocyanate |
| FR           | framework region |
| GAG          | glycosaminoglycan |
| GC           | germineral center |
| GlyCAM       | glycosylation-dependent cell adhesion molecule |
| GM-CSF       | granulocyte-macrophage colony stimulating factor |
| GMP          | granule membrane protein |
| HA           | hyaluronic acid |
| HB-E:GF      | heparin-binding epidermal growth factor |
| HEV          | high endothelial venule |
| HGF          | hepatocyte growth factor |
| HIV          | immunodeficiency virus |
| HS           | heparan sulfate |
| HSPG         | heparan sulfate proteoglycan |
| ICAM         | intercellular cell adhesion molecule |
| IFN-γ        | interferon γ |
| Abbreviation | Full Name |
|--------------|-----------|
| Ig           | immunoglobulin |
| IgH          | Ig heavy chain |
| IgL          | Ig light chain |
| IgSF         | immunoglobulin superfamily |
| IgV          | immunoglobulin variable region |
| IL           | interleukin |
| LFA          | lymphocyte function-associated antigen |
| L-selectin   | leukocyte selectin |
| LT           | lymphotoxin |
| mAb          | monoclonal antibody |
| MAdCAM       | mucosal addressin cell adhesion molecule |
| MALT         | mucosal-associated lymphoid tissue |
| MHC          | major histocompatibility complex |
| MIP          | macrophage inflammatory protein |
| NK           | natural killer |
| PCLP         | podocalyxin-like protein |
| PADGEM       | platelet activation-granule external membrane protein |
| PE           | phycoerythrin |
| PKC          | protein kinase C |
| PMA          | phorbol 12-myristate 13-acetate |
| PNAd         | peripheral lymph node addressin |
| P-selectin   | platelet selectin |
| PSGL         | P-selectin glycoprotein ligand |
| RANTES       | regulated upon activation, normally T cell expressed and secreted |
| RPE          | R- phycoerythrin |
| SAC          | Staphylococcus aureus strain Cowan 1 |
| SDF          | stromal cell-derived factor |
| SF           | scatter factor |
| SLC          | secondary lymphoid tissue chemokine |
| TCR          | T cell antigen receptor |
| TNF          | tumor necrosis factor |
| sIg          | surface immunoglobulin |
| sLeX         | sialyl Lewis X |
| VAP          | vascular adhesion protein |
| VCAM         | vascular cell adhesion molecule |
| VEGF         | vascular endothelial growth factor |
| VLA          | very late antigen |
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Curriculum vitae

Robbert van der Voort werd geboren op 26 augustus 1969 te Amsterdam. In 1988 slaagde hij voor het eindexamen Voorbereidend Wetenschappelijk Onderwijs en startte datzelfde jaar met de studie Biologie aan de Universiteit van Amsterdam. Tijdens deze opleiding vervulde hij stages aan de afdelingen Antropogenetica (Dr J.M.N. Hoovers en Prof. dr A. Westerveld), en Pathologie (Dr E. Manten-Horst, Dr F. van den Berg en Prof. dr S.T. Pals) van het Academisch Medisch Centrum te Amsterdam. Tevens liep hij een jaar stage bij de afdeling Pathologie van de Stanford Universiteit in de Verenigde Staten (Dr M.J. Briskin, Dr L.M. McEvoy, Dr J. Thompson en Prof. dr E.C. Butcher). Na zijn afstuderen eind 1993, werkte hij een paar maanden op vrijwillige basis op de afdeling Pathologie van het Academisch Medisch Centrum te Amsterdam. In 1994 begon hij op diezelfde afdeling als Assistent in Opleiding, begeleidt door Prof. dr S.T. Pals, met een promotieonderzoek. De resultaten van dat werk staan in dit proefschrift beschreven. Sinds eind 1999 werkt Robbert als post-doc op de afdeling Tumorimmunologie (Dr G.J. Adema en Prof. dr C.G. Figdor) van het Universitair Medisch Centrum St Radboud te Nijmegen.
Appendices

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