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CD44: a multitude of isoforms with diverse functions

Ursula Günthert

Basel Institute for Immunology
Grenzacherstrasse 487
CH-4005 Basel, Switzerland

1. Introduction.

The CD44 transmembrane glycoprotein of 90 kD has been known for more than ten years under such diverse designations as lymphocyte homing receptor gp90^{Hemes}, phagocytic glycoprotein Pgp-1, extracellular matrix receptor III (ECMR^{III}) and hyaluronate receptor H-CAM (see reviews by Haynes et al., 1989 and 1991). Studies with monoclonal antibodies revealed similarity, and most likely identity among these molecules (Omary et al., 1988; Gallatin et al., 1989; Picker et al., 1989; Aruffo et al., 1990; Miyake et al., 1990; Culty et al., 1990). When the human, baboon and murine cDNA sequences were established identity was confirmed. However, the cDNA sequence codes only for about 360 amino acids, revealing a just 37 kD encompassing protein core (Stamenkovic et al., 1989; Goldstein et al., 1989; Idzerda et al., 1989; Nottenburg et al., 1989; Zhou et al., 1989; Wolffe et al., 1990). This protein core is highly glycosylated by N- and O-linked sugars to yield a 85 to 90 kD form and is sometimes additionally linked to chondroitin sulfate side chains to produce a 180 - 200 kD form (Jalkanen et al., 1988; Stamenkovic et al., 1989).

Concomitant with the diverse names for CD44, the description of functions was as diverse: CD44 molecules were described to participate in cell-cell and cell-matrix interactions such as lymphocyte recirculation and prothymocyte homing, hematopoiesis, lymphocyte and monocyte activation, cell migration and metastasis (reviewed in Haynes et al., 1989 and 1991).

It seemed rather unlikely that all these functions were associated with one and the same molecule, though differences in the posttranslational modification may as well modulate the adhesive properties (Brown et al., 1991). Thus, the description of new extracellular regions led to the assumption that the multitude of functions may be attributed to the various isoforms (Günthert et al., 1991; Brown et al., 1991; Hofmann et al., 1991; He et al., 1992; Matsumura and Tarin, 1992).

The aim of this review article is to describe the ever growing family of isoforms and their organization and to discuss possible functional implications.

2. Genomic organization of the CD44 gene.

The CD44 gene locus is on chromosome 2 in the mouse genome and on chromosome 11p13 in humans (Goodfellow et al., 1982; Forsberg et al., 1989). In humans, the CD44 locus is close to a region causing suppression of metastasis for certain tumor types and the catalase gene (11p11.2 - 13: Ichikawa et al., 1992) and in the mouse the CD44 locus is linked to the genetic control of susceptibility to colon carcinoma, *Sec-1* (Moen et al., 1992).

Recently, most of the genomic structure of the human CD44 gene had been established (Screaton et al., 1992). Over a length of about 60 kb, at least 20 exons are distributed (Figure 1). 10 of these encode sequences for the standard form of CD44 (exons 1 to 5 and 16 to 20). Between exons 5 and 16, at least 10 further exons are localized, which are subjected to alternative splicing (exons 6 to 15). 9 of these 10 variant exons have now been identified by sequencing the genomic DNA (Screaton et al., 1992; Cooper et al., 1992), but 8 of them had been identified previously by RT PCR analysis of CD44 variants expressing cell lines (Hofmann et al., 1991).

Although exon 6, the first of the alternatively spliced exon (designated 1v thus), has not been described in the genomic sequence, the authors indicate preliminary evidence for further exons within this large region of at least 9 kb (Screaton et al., 1992). So far, there is no evidence for more than one additional variant exon in this region, as judged by RT PCR, resulting in a total of 10 alternatively spliced exons in the extracellular region between exons 5 and 16 (Figure 1).

The pancreatic rat cell line BSp73ASML (Matzku et al., 1983), was the original source of the alternatively spliced regions (Günthert et al., 1991). Isolation of the respective sequences from human and murine origin confirmed the existence of 10 additional exons in the extracellular part of CD44 (see Figure 2; unpublished results). The exon borders have previously been postulated due to their existence in various combinations of RNA from human, rat and murine carcinoma cell lines (see Figure 3).

In the cytoplasmic region, variations have also been reported giving rise to either 70 or 3 amino acids lengths (Stamenkovic et al., 1989; Goldstein and Butcher, 1990). Determination of the genomic sequence revealed the existence of both the described cytoplasmic and 3' untranslated regions; the shorter form being encoded by exon 19 and the longer one by exon 20 (Screaton et al., 1992). Predominantly exon 20 is expressed, less than 1% of the short version, which is generated by a stop codon in exon 19 is expressed in B lymphocytes (Goldstein and Butcher, 1990).

An approximately 3 kb long untranslated 3' region was detected in the rat CD44 mRNA with four polyadenylation sites, generating lengths of 1.5; 2.0; 2.9 and 4.3 kb for the standard RNAs (Günthert et al., 1991). In humans three polyadenylation sites are present and in usage, giving rise to RNAs of 1.5; 2.2 and 4.5 kb length (Goldstein et al., 1989; Stamenkovic et al., 1991; Harn et al., 1991). In the mouse there is evidence for four polyadenylation sites, which generate RNAs of 1.6; 3.2; 4.0 and 5.2 kb (Wolffé et al., 1990).

3. Sequence comparison of the alternatively spliced exons, encoding extracellular regions.

Between exons 5 and 16 (see Figure 1), near the membrane proximal region of the standard or hematopoietic form of CD44 (CD44S or CD44H), additional sequences of varying lengths have been detected in rats, humans and mice (Günthert et al., 1991; Stamenkovic et al., 1991; Hofmann et al., 1991; Brown et al., 1991; Dougherty et al., 1991; Cooper et al., 1992; Jackson et al., 1992; He et al., 1992; Screaton et al., 1992). While the 5' region of the standard form (exons 1 to 4) and the 3' region (exons 17, 18, and 20; exon 19 encodes the 3' untranslated region of the short cytoplasmic form in humans) are highly conserved among the three species with 82% and 87% identity, the flanking regions (exons 5 and 16) are less well conserved with only 54% and 35% identity, respectively (Günthert et al., 1991).

By RT PCR cDNA sequences were amplified from a rat pancreatic carcinoma line, BSp73ASML (Matzku et al., 1983), a human large cell lung carcinoma line, LCLC97 (Bepler et al., 1988), human immortalized keratinocyte lines, HaCaT (Boukamp et al., 1988) and HPKII (Dürst et al., 1987), a murine squamous cell carcinoma line, KLN205 (ATCC #CRL1453), and a murine colon carcinoma line, CMT93 (ATCC #CCL223), followed by DNA sequencing (Figure 3).

From the rat, exons 3v to 10v had already been described, as well as the human sequences from the same region (Günthert et al., 1991; Hofmann et al., 1991). Recently, also the murine sequences from exons 4v to 10v were determined (He et al., 1992), which differ at two positions from the one determined by us (positions 341 and 354 in Figure 2). Some cell line specific differences have been observed between CMT93 and KLN205 sequences, which usually occurred in the third position of the codon and thus either did not affect the translation or led to conservative amino acid changes (e.g. positions 130, 154, 158, 165 and 368 in Figure 2).

The identity between the murine and the rat amino acid sequences from exons 1v to 10v is as high as 90%. Still about 65% of the murine and the human or the rat and the human sequences, respectively, are identical. Most of the potential O-linked glycosylation sites are conserved among the species, as well as the chondroitin sulfate linkage positions, whereas the N-linked glycosylation sites are not so well conserved (Figure 2).

Data base searches did not reveal obvious homology of the variant exons 1v to 10v to any known other protein species.

4. The occurrence and composition of CD44 splice variants.

The highly metastatic rat pancreas carcinoma line BSp73ASML (Matzku et al., 1983) was the source of the first CD44 variant sequences (Günthert et al., 1991). A monoclonal antibody (mAb1.1ASML) with specificity for the metastatic BSp73ASML cells, which did not recognize antigens on the nonmetastatic line BSp73AS (Matzku et al., 1989), was utilized to isolate a cDNA sequence from a bacterial cDNA expression library (Günthert et al., 1991). This cDNA sequence turned out to be inserted at position 783 into the rat CD44 standard sequence; the continuity of the total sequence being confirmed by RNase protection analyses (Günthert et al., 1991). Among the at least ten different splice variants produced in BSp73ASML cells, two were of predominance: exons 4v-5v-6v-7v and 6v-7v (#10 and #11 in Figure 3). These two variants however did not contain exon 16 (or 6s) from the 3' standard region (Günthert et al., 1991).

The so-called epithelial form of CD44, CD44E, contains exons 8v-9v-10v (#5 in Figure 3; Stamenkovic et al., 1991; Brown et al., 1991; Dougherty et al., 1991). Variations of these exons were detected in the murine colon carcinoma line CMT93 (#1 - #5 in Figure 3); exon 10v can even appear in a smaller version, due to an exon internal splice site (at position 390 of the murine CD44 sequence: #2 in Figure 3; unpublished results).

Longer forms, having in addition to the "epithelial" exons different combinations with exons 4v, 5v, 6v, and 7v were detected not only in murine carcinoma lines (KLN205 and CMT93), but also in early developmental stages of murine embryos (#6 - #9 in Figure 3: U.G. and M.V. Wiles, unpublished results). Similar variants, namely # 4, #5, #7 and #9 have recently been isolated in P. Kincade's lab from the carcinoma line KLN205 (He et al., 1992).

In the human colon carcinoma line HT29 (ATCC #HTB38) and the large cell lung carcinoma line LCLC97 (Bepler et al., 1988), not only the epithelial variant CD44E is expressed, but in addition to this form, varying combinations of exons 3v to 10v are being synthesized (#13 - #15 in Figure 3; Hofmann et al., 1991). The immortalized keratinocytes, HaCaT (Boukamp et al., 1988) and HPKII (Dürst et al., 1987), predominantly synthesize long isoforms containing contiguously exons 3v to 10v, 2v to 10v or 1v to 10v (Hofmann et al., 1991 and unpublished results). Another exon-internal splice site occurs at position 93 of exon 3v in humans (see Figure 2 and Hofmann et al., 1991; Screaton et al., 1992).

Thus, so far 17 different combinations of the alternatively spliced exons 1v to 10v have been detected in human, rat and murine cell lines (Figure 3).

Besides the already mentioned variations concerning exon 6s (= exon 16) and the alterations in the cytoplasmic region, involving exons 9s and 10s (= exons 19 and 20), exon-internal splice sites add to the complexity of the CD44 variants. Two of these exon-internal splice sites have been described above to be present in exons 3v and 10v, another one has been detected in exon 5s (amino acid position 192 of the CD44S sequence: Shtivelman and Bishop, 1991).

In summary, variations of the CD44 molecule are not only due to alternative splicing of the exons 1v to 10v, but the flanking regions (exons 5s and 6s), usually present in the standard molecule, may also be involved in this process. This eminent potential to combine at least 13 peptide units in the

extracellular region, which are all encoded by one genomic sequence, makes CD44 one of the most variable surface molecules.

Similarly complex splicing patterns have been detected for the transcripts of fibronectin and the neural cell adhesion molecule N-CAM (Norton and Hynes, 1990; Reyes et al., 1991). How the process of alternative splicing is regulated has not been elucidated in detail (Maniatis, 1991). The small nuclear ribonucleoprotein particle U1 seems to be involved in the regulation of differential binding of the spliceosomes (Kuo et al., 1991). The splicing machinery has to differentiate between splice donor and acceptor sites in the introns flanking the standard and variant exons in a developmentally and physiologically regulated manner.

The amount of CD44 transcript synthesized varies tremendously in different cell types, e.g. transcription is downregulated in neuroblastoma cell lines and upregulated in melanoma and osteosarcoma cell lines (Shtivelman and Bishop, 1991). Several proteins bind to the CD44 promoter, the composition of which is altered during cellular transformation (Mai et al., in prep.).

5. Functional implications of the CD44 isoforms.

Several functions have been attributed to the CD44 standard molecule, like binding to high endothelial venules (HEV; Jalkanen et al., 1988), binding to collagen and fibronectin (Carter and Wayner, 1988; Jalkanen and Jalkanen, 1992) and binding to hyaluronic acid (HA; Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990).

The interaction between CD44S and HEV can be blocked with mAb Hermes-3 (Jalkanen et al., 1987), which recognizes an epitope in the membrane proximal region near the insertion site for exons 1v to 10v (Goldstein et al., 1989).

Whereas the location of collagen interaction has not been described, binding to fibronectin is mediated via the chondroitin sulfate side chains, thus only the 180 - 200kD CD44 form is able to bind to this ligand (Jalkanen and Jalkanen, 1992).

Binding to hyaluronic acid has initially been postulated due to the homology of the amino terminal CD44 region to the HA binding domains of cartilage proteoglycan core and link proteins (Stamenkovic et al., 1989; Goldstein et al., 1989). Hyaluronic acid is a polysaccharide of high molecular mass located in the extracellular matrix (ECM). Various physiological functions have been attributed to HA, like immobilizing water in the ECM, and involvement in cell proliferation, migration, and differentiation (Laurent and Fraser, 1992). The ability of CD44 to bind HA adds it to the family of HA binding proteins, including apart from the proteoglycan core and link protein, aggrecan, versican and TSG-6, a novel TNF α -inducible HA binding protein (Hardingham and Fosang, 1992; Lee et al., 1992).

The binding to HA mediates cellular aggregation, cytokine release and lymphocyte activation (Haynes et al., 1989). In human melanoma cells the CD44S molecule is involved in hematogenous spread of the tumor cells (Birch et al., 1991). Highly malignant melanomas exhibit increased expression of CD44S, but do not express any CD44 variants (unpublished results). cDNA transfections of human lymphoma cell lines (Namalwa) led to enhanced local tumor formation and metastatic spread only when the CD44S molecule was overexpressed, not when the transfectants expressing the epithelial variant were analyzed (Sy et al., 1991 and 1992).

Although almost all lymphoid cells express CD44S, only a minority has an affinity for HA (Lesley et al., 1992). The affinity of CD44 for HA may be influenced by conformational changes, interaction with neighbouring cell surface molecules to form heterodimers, by homodimerization or the formation of larger complexes. Activation of lymphocytes leads to an induction of HA binding (Murakami et al., 1990 and 1991; Lesley et al., 1992).

It seems rather likely that the binding functions described for the standard form of CD44 are conserved in the various isoforms, predicting that the insertion of the new regions does not interfere with the active sites and does not completely rearrange the conformation. Several reports however indicated that only CD44S is able to bind HA, while the epithelial form of CD44, CD44E, has lost the affinity for HA (Stamenkovic et al., 1991; Sy et al., 1991 and 1992; Thomas et al., 1992). Contradictory results were obtained with murine CD44 isoforms exhibiting the same affinity to HA as the standard molecule (He et al., 1992). The authors conclude that the amino terminal portion of CD44 is sufficient for HA binding and that loss of this function is not necessarily a consequence of insertion of additional exons in the membrane proximal region (He et al., 1992).

The longer cytoplasmic domain (exon 10s) is phosphorylated by a serine/threonine kinase and has been shown to interact with the cytoskeleton (Carter and Wayner, 1988). A potential relationship between these two events was reported (Camp et al., 1991). However, in a recent publication, it was demonstrated that serines 323 and 325 of the standard CD44 sequence are the kinase targets, but that these phosphorylations do not regulate membrane localization and cytoskeletal interactions (Neame and Isacke, 1992).

So far, no ligand has been identified for the region encompassing variant exons 1v to 10v. A hydrophilicity plot of the total CD44 molecule denotes concentration of hydrophilic areas predominantly in the variant exons 1v to 9v (Figure 4). Another hydrophilic center is located in exon 5s, whereas the signal peptide in exon 1s and the transmembrane domain in exon 8s are highly hydrophobic. Since hydrophilic regions of a protein are usually facing outwards, these regions are possible candidates for interaction of peptide stretches with ligands. Non-peptide binding to ligands is most probably another mode of interaction for the variant region, because there are several potential N- and O-glycosylation sites, most of them are flanking the hydrophilic centers (e.g. in exons 1v, 3v, 5v, 6v, 8v and 9v; Figure 4). Interaction of the standard CD44 molecule to fibronectin is known to be mediated by the chondroitin sulfate chains of exon 7s (Jalkanen and Jalkanen, 1992). Exon 3v contains in addition two conserved chondroitin sulfate linkage positions which are possibly also involved in fibronectin binding.

The only functional assays so far concerning the variant exons 1v to 10v have been performed with mAb1.1ASML (Matzku et al., 1989), which recognizes an epitope at the 5' end of exon 6v in the rat (Hofmann et al., 1991). Concomittant application of mAb1.1ASML (i.p.) with the highly metastatic cell line BSp73ASML (s.c.) into syngeneic rats considerably blocked metastasis formation in the lungs (Reber et al., 1990). cDNA transfections of variants #10 or #11 (see Figure 3) into the nonmetastatic cell line BSp73AS conferred metastatic potential to these transfectants in the spontaneous metastasis protocol (Günthert et al., 1991). Metastasis formation of these transfectants can be dramatically blocked with mAb1.1ASML, when given (i.v.) prior to lymph node colonization (Seiter et al., 1993). It is likely that mAb1.1ASML interferes with interactions between exon 6v and a not yet identified ligand in the draining lymph node or the lung (Seiter et al., 1993).

During normal developmental and regenerative processes, inflammation and wound healing, as well as in metastasis formation, massive cell movements in a highly directed order take place (Nicolson, 1987; Ruiz and Imhof, 1992; Van Roy and Mareel, 1992; Hart and Saini, 1992; Keller et al., 1993). The CD44 variant isoforms are expressed during these processes and they are most likely not only involved in metastasis formation (Günthert et al., 1991; Hofmann et al., 1991; Arch et al., 1992; Seiter et al., 1993; unpublished results). Since metastatic tumor cells mimick embryonic cells, activated T- and B-lymphocytes, and highly regenerative cells, like keratinocytes and cells from the base of the colon crypts, the underlying receptor ligand-interaction may be very similar in all these necessary contacts (Alho and Underhill, 1989; Hofmann et al., 1991; Brown et al., 1991; Arch et al., 1992; Thomas et al., 1992).

For a better analysis of these malignant and non-malignant processes, we generated antibodies directed against the human variant CD44 region (Mackay et al., in prep.). With these mAb a panel of lymphoid and non-lymphoid human tissues was screened. Expression of variants #1 to #5 (see Figure 3) was detected in most of the epithelial layers (Figure 5; Mackay et al., in prep.). Variants containing exons 5v and 6v are however only scarcely expressed in non-malignant tissues, e.g. in the basal layer of the skin, the base of the colon crypts, the basal layer of the bronchial epithelium, the squamous epithelium of the oesophagus, the basal layer of the mammary gland ducts and the tonsillary epithelium (Mackay et al., in prep.).

Expression of exon 6v containing CD44 variants is not only upregulated in highly metastatic rat cell lines (Günthert et al., 1991), but also correlates with malignancy in colon and breast carcinomas (Matsumura and Tarin, 1992; Heider et al., 1993).

Variant specific CD44 mAb may be valuable tools for the development of a sensitive diagnosis for malignant processes. Thus, knowledge of how tumor cells mimick non-malignant processes by analysing the multifarious CD44 molecule may even help to devise new strategies in tumor prevention (Frost and Levin, 1992).

While the exact mechanisms by which CD44 isoforms participate in embryogenesis, tumor progression and lymphocyte migration (in inflammation) still need to be clarified, it is tempting to speculate that the variant regions play a major role in these processes. A common feature of these processes is the ability of the CD44V molecules carrying cells to migrate to specific sites. This may be

mediated via CD44V by contacting ECM molecules, degrading components from the ECM, inducing factors that regulate signalling, by specific interactions between the variant region(s) and/or organ-specific ligands on endothelia. Once these ligands are identified which interact with the various isoforms, these issues may be addressed.

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Figure Legends.

Figure 1: Organization of the genomic structure of the CD44 gene. A linear map indicates the exon nomenclature and the approximate lengths of the introns, according to Screaton et al. (1992). Exon #6, the first of the variant exons, has been added to the published map. Numbering of the exons refers to their presence in the standard or the variant region: exons 1 to 5 encode the 5' standard region (= exons 1s to 5s); exons 6 to 15 encode the variant exons 1v to 10v; and exons 16 to 20 encode the 3' standard region (= exons 6s to 10s).

Figure 2: Amino acid composition of the human, rat and murine variant CD44 regions. The symbols refer to
 O-glycosylation sites;
 N-glycosylation sites; chondroitin sulfate side chains; exon borders.

Figure 3: Compilation of the so far isolated CD44 variant molecules. The number of amino acids for each exon refers to the human sequence. The arrow heads point to additional splice sites, either within the standard region, or in the variant exons 3v and 10v.

Figure 4: Hydrophilicity plot of the total rat variant CD44 molecule. The plot structure was established according to Chou and Fasman (1974). Rounded ends denote hydrophilic areas, sharp ends indicate hydrophobic areas. The filled arrow heads indicate the borders between the standard and the variant sequences, the open arrow heads denote additional splice sites within the standard region. The other symbols refer to:
 N-glycosylation sites; O-glycosylation sites; chondroitin sulfate side chains. Numbering of the exons refers to the numbering indicated in Figure 1.

Figure 5: Detection of variant CD44 antigens in human skin. mAb against standard CD44 (25.32), exon 9v (11.24), exon 6v (11.31) and exon 4v (11.10) were used to stain skin sections. While mAb 25.32 stains epithelial and stromal cells, mAb 11.24 and 11.31 reactions are strictly limited to the epithelial layers, mAb 11.10 only detects the basal layer of the skin (upper left corner). Detection of mAb binding was performed with APAAP (Dako).

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