A Di-hydrophobic Leu-Val Motif Regulates the Basolateral Localization of CD44 in Polarized Madin-Darby Canine Kidney Epithelial Cells*

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Both in vivo and in vitro the distribution of the resident plasma membrane adhesion protein, CD44, is restricted to the basolateral domain of polarized epithelial cells, suggesting a role in interepithelial interactions. To determine how this localization might be regulated, a range of CD44 cytoplasmic domain mutations were generated and a minimal 5 amino acid sequence, His330-Leu-Val-Asn-Lys334, was identified which when deleted results in expression of CD44 on the apical microvillar membrane. Further mutagenesis throughout this region pinpointed a critical di-hydrophobic motif, Leu331/Val332. The ability of wild type but not mutant CD44 cytoplasmic domains to redirect an apically targeted protein, placental alkaline phosphatase, to the basolateral plasma membrane demonstrates that this sequence can function as a dominant localization signal. This His330-Lys334 sequence is spatially separate from other CD44 regulatory elements and as discussed here, a comparison with known basolateral sorting sequences identified in other transmembrane proteins suggests that a distinct mechanism operates to retain resident plasma membrane proteins in their correct plasma membrane subdomains.

CD44 was originally identified as an abundant 80–100-kDa transmembrane glycoprotein on a variety of cell types. In recent years, much of the interest in CD44 has stemmed from experiments demonstrating that it is a principle receptor for the important extracellular matrix glycosaminoglycan, hyaluronan, and that inappropriate expression can result in increased tumor growth and metastasis (reviewed in Refs. 1 and 2). However, although enhanced levels of CD44 expression and/or alterations in CD44 splicing are frequently found in epithelial tumors, little is known about the function and regulation of this adhesion protein in epithelial cells. In stratified epithelia such as the skin, abundant CD44 is found in the basal and spinous layers co-localized with its ligand, hyaluronan (3–5), and an interaction between CD44 and hyaluronan mediates cell:cell adhesion between keratinocytes (6, 7). A different picture emerges in simple epithelia such as that in the gastrointestinal tract, where CD44 is found only on the dividing cells within the crypts of Lieberkuhn. In these cells abundant CD44 is found on the lateral plasma membranes, but no intercellular hyaluronan is detected (3). This raises the possibility that in simple epithelia CD44 may have a role in hyaluronan-independent intercellular interactions.

Polarized epithelia have two compositionally and morphologically distinct cell surface domains reflecting their disparate functions (reviewed in Refs. 8 and 9). The upper apical microvillar surface provides a protective role by acting as a barrier between the external and internal environments and it contains the necessary transporters for the uptake of small molecules. The basolateral surface is involved in cell:cell and cell:matrix adhesion and also contains plasma membrane proteins involved in signal transduction and nutrient uptake. Generation and maintenance of these two domains is required for the correct functioning of polarized cells and involves the continuous sorting and correct plasma membrane insertion of newly synthesized proteins. In addition, the ability of mature proteins presented at the plasma membrane to be endo- and transcytosed must be strictly regulated (reviewed in Refs. 10–13). In the studies described here we have employed the well characterized MDCK cell line which can be grown to confluence to generate a functional polarized monolayer. In these cells CD44 is localized exclusively to the lateral plasma membrane (14) supporting the in vivo data for a role of CD44 in interepithelial interactions. As has been demonstrated for a number of other transmembrane receptors (15–17), the basolateral localization of CD44 is regulated by the cytoplasmic domain in that the truncation of this domain results in a tailless (T-1) molecule which is localized to the apical microvillar membrane (14). However, unlike these well characterized transmembrane receptors, CD44 is a long lived resident plasma membrane protein that is not subject to rapid endo- or transcytosis (14, 18–22). This manuscript describes experiments identifying a specific sequence motif within the CD44 cytoplasmic domain that regulates the correct localization of this protein in polarized epithelial cells. These results provide insight into the regulation of this important adhesion protein which has been strongly implicated to play a role in epithelial disease progression. Furthermore, these studies provide clues as to the mechanisms controlling the trafficking and stabilization of resident membrane proteins in polarized cells.

MATERIALS AND METHODS

Generation of CD44 Cytoplasmic Domain Mutants—The isolation of a human CD44 cDNA clone containing none of the alternatively spliced exons (23), the generation of the T- CD44, and the insertion of these cDNA clones into pUC19 vector, pSELECT mutagenesis vector, and pSRα-neo eukaryotic expression vector have been described previously (14, 24). To generate the ΔSer316-Thr345 cytoplasmic domain deletion, a previous mutant Ala316 CD44 (24) in the pSRα-neo expression vector

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1 The abbreviations used are: T-, tailless; bp, base pair(s); WT, wild type; MDCK, Madin-Darby canine kidney; mAb, monoclonal antibody; PLAP, placental alkaline phosphatase; TGN, trans-Golgi network.
was employed. Generation of the Ala^316 CD44 mutant created a novel PvuII restriction site. pSR9-neo/Ala^316 CD44 was digested with BstXI, and the linearized vector and a 411-base pair (bp) BstXI fragment were isolated and purified. The 411-bp fragment was then restricted with PvuII to give 255-, 90-, and 66-bp fragments. Linearized vector along with the 255- and 66-bp fragments were ligated together. The loss of the 90-bp fragment results in a deletion of 30 amino acids from Ala^316–Thr^325 inclusive.

To generate the ΔGly^306-Pro^315 mutant, the same restriction strategy as described above was employed. Following the PvuII digestion the 255-bp fragment was discarded and the 90- and 66-bp fragments were purified. pUC19/WT CD44 was restricted with MboI producing a 256-bp fragment, which was isolated and purified. This 256-bp fragment was then treated with the Klenow fragment of DNA polymerase I and dNTPs to fill in overhanging ends and then restricted with BstXI generating two fragments of 196 and 60 bp. The 196-bp fragment was retained and ligated along with the 90- and 66-bp PvuII fragments into the BstXI linearized pSR9-neo/Ala^316 CD44 vector to generate a 20-amino-acid deletion from Gly^296–Pro^315.

The following oligonucleotides (see Table I) were used for loop out or point mutagenesis with WT CD44 in the pSELECT vector (Promega) as a template. C15 for ΔGly^307–Ser^323, C14 for ΔLys^324–Val^329, C12 for ΔHis^330–Ser^337, C11 for ΔGlu^338–Thr^342, C21 for ΔHis^330–Lys^334, C22 for ΔGln^335–Ser^337, C19 for Ala^330, C25 for Ala^322, C11 for Ala^330–Ala^332, Ala^333, and Ala^334 mutants were generated by a second round polymerase chain reaction mutagenesis strategy, using C24, C18, and C20 mutagenic oligonucleotides, respectively, as primers. The first round was primed with C18, C20 or C24, and C4, the template used was pUC19/CD44. The product was purified, denatured by heating, and used with primer C3 in a second round polymerase chain reaction using the same template. The product was then isolated, purified, Klenow-treated, and ligated into end-polished BamHI restricted pSR9-neo expression vector. The identity of all mutant clones was confirmed by restriction endonuclease digestion and DNA sequencing.

Expression and Analysis of CD44 Cytoplasmic Domain Mutants and PLAP/CD44 Chimeras in MDCK Cells—CD44 cytoplasmic domain mutants in the pSR9-neo vector were transfected into MDCK cells by electroporation and clonal lines expressing human CD44 selected and maintained as described previously (14). PLAP/CD44 chimeras were electroporated into MDCK cells, and protein expression in G418-resistant mixed populations was detected using a rabbit anti-PLAP antisemum (Dako). MDCK cells were cultured as confluent polarized monolayers on Transwell filters, the integrity of the tight junctions was tested and the distribution of CD44 mutants and PLAP/CD44 chimeras determined by confocal microscopy and cell surface biotinylation as described previously (14) using the anti-human CD44 mAb, E1/2, or the anti-PLAP antisemum, respectively. The percentage of proteins in bands from the autoradiograms was quantitated by scanning densitometry with a UVP scanner in conjunction with a gel analysis program for Windows 3.1.

### RESULTS

**Polarized Expression of Cytoplasmic Deletion Mutants of CD44**—CD44 is subject to extensive alternative splicing due to the variable insertion of 10 exons into a single extracellular membrane proximal site (23, 26). Although there are potentially numerous CD44 isoforms all of these proteins contain a common intracellular domain. In all of the experiments described here, a CD44 clone encoding the human CD44H isoform that contains none of these variant exons was employed. To identify a potential basolateral determinant contained within the CD44 cytoplasmic domain, a series of internal cytoplasmic deletion mutants were generated (Fig. 1), and single cell clones of MDCK cells expressing mutant proteins were isolated. Clonal cell lines were cultured as functional polarized monolayers on Transwell filters and the distribution of transfected human CD44 examined by confocal microscopy using mAb E1/2, which is specific for human CD44 (Fig. 2). A mutant containing a deletion in the membrane proximal part of the cytoplasmic domain, ΔGly^306-Pro^315 CD44, had a distribution indistinguishable from WT CD44 in that essentially all of the protein was tightly restricted to the lateral plasma membrane (Fig. 2, a and c; Ref. 14). In contrast, removal of the adjacent downstream 30 amino acids (ΔSer^316–Thr^342) resulted in a mutant protein that was localized to the apical plasma membrane with a proportion observed intracellularly (Fig. 2, b and d). This distribution was reminiscent of that found with a truncated T- CD44 in which only three membrane proximal arginine residues remain in the cytoplasmic tail (Fig. 1; Refs. 14 and 24). By generating smaller deletions within this Ser^316–Thr^345 30-amino-acid segment, it was demonstrated that removal of an 8-amino-acid sequence (ΔHis^330–Ser^337) gave a mutant protein with a T- CD44 localization pattern (Fig. 2g), whereas removal of residues upstream (ΔGln^335–Ser^337, Fig. 2e) ΔLys^324–Val^329, Fig. 2f) or downstream (ΔGlu^338–Thr^345, Fig. 2h) generated mutant proteins with a WT CD44 localization pattern. A comparison of the His^330–Ser^337 amino acid sequence between the eight species sequenced to date (22, 52) revealed that residues His^330–Lys^334 are completely conserved.
Glu335 is changed to a glycine in bovine CD44, Ser336 is changed to proline in a number of species, and Ser337 is changed to threonine in the rat. Consequently two further deletions were generated in this region, DHis330–Lys334 and DGlu335–Ser337. By confocal microscopy, the DGlu335–Ser337 mutant behaved like WT CD44 (Fig. 2j), whereas the DHis330–Lys334 mutant resembled a T-CD44 (Fig. 2i).

To confirm and quantify the distribution of these CD44 cytoplasmic deletion proteins in epithelial cells, clonal MDCK cell transfectants were cultured on duplicate Transwell filters, the integrity of the monolayer was assessed by [3H]inulin testing, and then cells were surface-biotinylated from either the apical or basolateral side. In agreement with the confocal data, it was found that in those mutants with a WT CD44 localization pattern, less than 13% of the CD44 was accessible to biotinylation on the apical plasma membrane, and conversely, in those mutants whose cell surface localization resembled T-CD44, greater than 88% of the CD44 could be labeled by apical biotinylation (Fig. 3).

Basolateral Localization of CD44 Requires a Di-hydrophobic Amino Acid Motif—To further characterize the sequence required for the basolateral localization of CD44, amino acids His330 to Lys334 were changed individually to alanine residues (Fig. 4a), and clonal cell lines expressing these alanine mutations were examined by confocal microscopy (Fig. 4b). Mutation of His330, Asn333, or Lys334 to alanine did not alter the distribution of CD44. By contrast, mutation of Leu331 generated a mutant protein that was expressed on the apical plasma membrane, and mutation of Val332 resulted in the majority of the protein being localized to the lateral surfaces of the cells, with a residual proportion observed at the apical surface. Unsurprisingly, the double mutation of Leu331/Val332 to Ala331/Ala332 produced a mutant with a T- phenotype. Biochemical analysis of these point mutants (Fig. 4c) substantiated the confocal microscopy data in that less than 5% of His330 mutant and greater than 95% of the Leu331 and Leu331/Val332 mutants are detected on the apical plasma membrane. The Val332 mutant is the only CD44 mutation described here that clearly exhibits a mixed phenotype with 30% of the protein detected apically and 70% detected basolaterally.

The Cytoplasmic Domain of CD44 Contains a Dominant Basolateral Localization Signal—In order to determine whether the cytoplasmic domain of CD44 contained a dominant determinant, a chimeric protein was generated that contained the extracellular domain of PLAP and the transmembrane and cytoplasmic domains of human WT CD44. Normally PLAP is found as a glycosylphosphatidylinositol-linked glycoprotein on the apical plasma membrane (28). PLAP 489 is a truncated form of PLAP that is not glycosylphosphatidylinositol-tailed (29) and when expressed in MDCK cells is secreted apically (16). Attachment of the transmembrane and cytoplasmic domains of WT CD44 to PLAP 489 (Fig. 5A) resulted in PLAP being redirected to the lateral plasma membrane (Fig. 5B) such that less than 5% of the chimera was accessible to apical biotinylation (Fig. 5C). To demonstrate that this dominant effect was due to a specific determinant in the cytoplasmic domain of CD44, chimeras were generated that either lacked amino acids His330–Ser337 or contained the Ala331/Ala332 mutation. Morphological and biochemical analysis showed that these mutant cytoplasmic domains were incapable of redirecting PLAP, resulting in the mutant chimeras being predominantly expressed at the apical surface (Fig. 5).

DISCUSSION

Both the endogenous canine CD44 and transfected WT human CD44 are localized to the basolateral plasma membrane of polarized MDCK epithelial cells. This distribution could result from (a) sorting of CD44 in the trans-Golgi network (TGN) followed by direct delivery to the basolateral membrane, (b) delivery of CD44 to the apical plasma membrane followed by rapid and efficient transcytosis to the basolateral plasma membrane, or (c) nonspecific delivery to both plasma membranes followed by stabilization of CD44 at the basolateral membrane and rapid degradation/transcytosis of apical CD44. This latter pathway is unlikely to operate in polarized cells, as we demonstrate here that the transmembrane/cytoplasmic domain of CD44 is capable of redirecting an apically targeted protein. This indicates that CD44 encodes a dominant sorting signal.

Mutational analysis of a number of basolaterally localized cell surface receptors has demonstrated the presence of domi-
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Characteristically these overlapping signals contain an aromatic (usually tyrosine)-X-X-large hydrophobic motif. Secondary structure analysis suggests that such motifs can form β-turns that presumably interact with intracellular machinery (reviewed in Ref. 34). In some cases the overlapping basolateral sorting motifs and endocytosis motifs can be distinguished by mutagenesis. For example endocytosis of lysosomal acid phosphatase is critically dependent on the tyrosine residue in the YRHV motif, but basolateral sorting remains efficient if this residue is substituted for a phenylalanine (35). This suggests that the same motif interacts with different sets of cellular machinery which have individual sequence and secondary structure requirements. A second group of basolateral sorting determinants as identified in the low density lipoprotein receptor (33), transferrin receptor (17), and polymeric immunoglobulin receptor (16) are functionally and spatially separate from endocytosis determinants. There is no clear sequence consensus within this second group of targeting signals, although interestingly, soluble peptide representing the polymeric immunoglobulin receptor basolateral signal has been shown to adopt a β-type turn in solution (36).

In this study we have identified a minimal 5 amino acid basolateral localization motif within the CD44 cytoplasmic domain. In principle this motif could function either as a TGN sorting signal for the direct delivery of CD44 to the basolateral membrane or as an internalization motif required for the transcytosis of apically expressed CD44. Our experiments do not distinguish between these two possibilities; however, a transcytosis signal is considered unlikely for the following reasons. Transcytosis would require CD44 to be recruited into the coated pits, and yet in studies by Bretscher et al. (18), it was demonstrated that CD44 (H63 in their nomenclature) is excluded from these plasma membrane subdomains. Supporting data for the stable residency of CD44 on the plasma membrane

![Figure 4. Point mutations within the HLVNK basolateral localization sequence define critical amino acids.](image-url)

A series of alanine mutations were generated in the HLVNK-LVS sequence as described under "Materials and Methods" (A). Clonal lines of MDCK cells expressing alanine point mutants were generated, and the distribution of transfected protein was examined by confocal microscopy. B shows vertical xz sections taken through polarized monolayers of transfected MDCK cells cultured on Transwell filters where human CD44 has been detected using mAb E1/2 as described in the legend to Fig. 2. Bar = 10 μm. C shows biotinylation of CD44 on the apical and basolateral membranes as described in the legend to Fig. 3 and the percent of transfected CD44 detected on the apical plasma membrane. The three molecular size markers represent 116, 84, and 58 kDa. ND = not done.
comes from metabolic labeling of CD44 in MDCK cells, demonstrating that it has a long (>24 h) half-life in these cells (14) and that labeled anti-CD44 mAbs remain bound to CD44 on the cell surface for >100 h without becoming internalized (19–21). Finally, antibody labeling of permeabilized cells indicates that there is not a significant pool of intracellular CD44 (14, 24). In addition there is no precedent for resident basolateral trans-membrane proteins being routed via the apical plasma mem-brane in mature polarized monolayers such as those examined here. Together, these data suggest that the His330–Lys334 motif functions to sort CD44 in the TGN for subsequent delivery to the basolateral plasma membrane. Unfortunately, the long lived nature of CD44 combined with its extensive post-translational glycosylation prevents the necessary metabolic pulse labeling combined with cell surface biotinylation which would be required to directly test this. As a consequence, the sequence motif identified in these experiments cannot be unambiguously classified as a sorting sequence, and we therefore refer to it as a basolateral localization motif.

The His330–Lys334 basolateral localization motif has no sequence similarity with the well characterized basolateral sorting signals described above. Alanine scan mutagenesis of this region reveals a critical dependence on the leucine residue (Leu331) and partial dependence on the neighboring valine residue (Val332). Recently there have been several reports demonstrating that Leu-Z di-hydrophobic motifs (where Z is usually Leu, Ile, or Val) can play a important role intracellular protein targeting (reviewed in Ref. 37). In all of these cases, this LZ motif is associated with the endosomal targeting of proteins from the TGN or plasma membrane. The only exception is the Fc receptor, FcRII-B2, which has a critical dependence on an LL motif for basolateral sorting in epithelial cells (38, 39); however, unlike CD44 this motif also mediates recruitment of FcRII-B2 into the endocytic pathway. Moreover, both internalization and sorting of FcRII-B2 is partially dependent on an upstream Y residue in the context of YSLL. The CD44 cytoplasmic domain does not contain any tyrosine residues, and its single phenylalanine residue is not conserved between species (reviewed in Ref. 22). The only other aromatic amino acid within the CD44 cytoplasmic domain is His330 that lies adjacent to the Leu331/Val332 motif, and mutation of this histidine has no effect on CD44 localization (see Fig. 4).

It is not known what secondary structure the CD44 LV di-hydrophobic motif forms. Sandoval et al. (40) have demonstrated that a critical L1 motif within the cytoplasmic tail of the lysosomal protein, LIMP II, is held in an extended configuration and does not adopt a β-turn. Thus it remains to be determined whether the basolateral sorting cellular machinery which recognizes β-turn motifs could also interact with di-hydrophobic motifs, such as those found in FcRII-B2 and CD44. The second issue raised from these studies is how the cellular machinery distinguishes between basolaterally sorted proteins such as FcRII-B2 that are readily endocytosed and those such as CD44 that remain as resident plasma membrane proteins. In this respect, studies on the vesicular stomatitis virus glycoprotein are of interest. Vesicular stomatitis virus glycoprotein utilizes a classic Y-X-X-aliphatic motif, YTDI, to mediate its basolateral sorting in MDCK cells, but this glycoprotein remains expressed at the plasma membrane and is not endocy-tosed (41, 42). If common machinery is used for basolateral sorting, then resident basolateral proteins such as vesicular stomatitis virus glycoprotein and CD44 must either be missing additional components necessary for efficient endocytosis and/or be subject to retention mechanisms that would prevent recruitment into the coated pits.

The cytoplasmic tail of CD44 is highly conserved between species (reviewed in Ref. 22) and, as demonstrated here, is necessary for the correct plasma membrane localization of this molecule. In addition, this intracellular domain of CD44 can regulate ligand binding as cells expressing truncated CD44 mutants exhibit reduced or abolished ability to bind hyaluro-nan (43–46). Although there is dispute in the literature as to whether ligand binding is regulated by a discrete domain, there is general agreement that truncation of the CD44 cytoplasmic domain upstream of the basolateral localization sequence identified here does not alter its ability to associate with hyaluronan (44, 46–48). Similarly, this basolateral localization sequence appears to be functionally and spatially distinct from the transmembrane domain of CD44 that regulates the Triton X-100 solubility of this receptor (14, 49, 53) and from the two phosphorylation sites, Ser323 and Ser325 (24). There are reports in the literature that di-hydrophobic motifs might function in
the context of upstream phosphorylation events, for example to enhance the internalization of CD4 (50). However, mutation of the Ser$^{232}$ and Ser$^{325}$ phosphorylation sites does not disrupt the basolateral localization of CD44 (24) nor reduce the half-life of the protein (data not shown). This WT distribution of the CD44 phosphorylation mutants also strengthens the suggestion that CD44 is not rapidly transcytosed from the apical membrane given that at least in some cases transcytosis is known to be a phosphorylation dependent mechanism (51). To understand how these different domains of CD44 regulate function, it will be necessary to identify both cytoplasmic and membrane components with which these individual receptor domains interact. Finally, the lateral plasma membrane distribution of CD44 in simple epithelial cells suggests a role for CD44 in interepithelial interactions. There is increasing evidence that this interaction may operate in a hyaluronan-independent manner, and therefore identification of novel ligands will be crucial in elucidating mechanisms controlling this important transmembrane receptor.

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