Molecular Cloning of a Dendritic Cell-associated Transmembrane Protein, DC-HIL, That Promotes RGD-dependent Adhesion of Endothelial Cells through Recognition of Heparan Sulfate Proteoglycans*

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We isolated a novel molecule (DC-HIL) expressed abundantly by the XS52 dendritic cell (DC) line and epidermal Langerhans cells, but minimally by other cell lines. DC-HIL is a type I transmembrane protein that contains a heparin-binding motif and an integrin-recognition motif, RGD, in its extracellular domain (ECD). A soluble fusion protein (DC-HIL-Fc) of the ECD and an immunoglobulin Fc bound to the surface of an endothelial cell line (SVEC). This binding induced adhesion of SVEC to its immobilized form. Sulfated polysaccharides (e.g. heparin and fucoidan) inhibited binding of soluble DC-HIL-Fc and adhesion of SVEC. By contrast, an integrin inhibitor (RGDS tetramer) had no effect on binding to SVEC, but prevented adhesion of SVEC. This differential RGD requirement was confirmed by the finding that DC-HIL-Fc mutant lacking the RGD motif can bind to SVEC but is unable to induce adhesion of SVEC. Furthermore, DC-HIL appears to recognize directly these sulfated polysaccharides. These results suggest that DC-HIL binds to SVEC by recognizing heparan sulfate proteoglycans on endothelial cells, thereby inducing adhesion of SVEC in an RGD-dependent manner. We propose that DC-HIL serves as a DC-associated, heparan sulfate proteoglycan-dependent integrin ligand, which may be involved in transendothelial migration of DC.

Dendritic cells (DCs) are a member of antigen-presenting cells (APC) family, which are characterized morphologically by the extension of long, lamellar dendrites (1). DC are distinguished from other APC (e.g. macrophages and B cells) by an unsurpassed potency in presenting antigens to naive T cells, thereby most efficiently initiating primary T cell-mediated immune responses (1). DC are widely distributed but comprise only a minuscule fraction (typically <5%) of the total cell population of a given peripheral tissue. After capturing antigens, tissue DC undergo maturation, losing endocytic capacity but acquiring increased immunostimulatory capacity (2). During maturation, DC migrate from peripheral tissues to T cell areas of secondary lymphoid organs where they activate T cells (2–4). Thus, DC migration is a critical step in initiating antigen-specific immune responses.

Several adhesion molecules (e.g. cutaneous lymphocyte-associated antigen (5), lymphocyte function-associated antigen-1/CD11a (6), CD11b (7), CD44 (7), E-cadherin (8), and α5 integrins (9)) regulate the migration of DC through the entire life cycle. For example, blood-circulating precursors of Langerhans cells (LC), which are DC that reside in epidermis, attach to the blood vessel wall prior to initiating transendothelial migration into the epidermis where they develop into APC with phenotypes distinct from other members of the DC family. The LC homing and anchoring in the epidermis are probably controlled by lymphocyte-associated antigen (5) and E-cadherin (8), respectively. Conversely, after antigenic stimulation, LC dissociate from surrounding keratinocytes by down-regulating E-cadherin expression (8, 10), then adhere via α5 integrins to the basement membrane (9) with subsequent passage into the dermis, where they re-enter the afferent lymphatics or blood vessels. This endothelial migration is essential for completion of the DC life cycle. Despite this importance, very little is known about the molecular mechanisms underlying the adhesion of DC to endothelial cells.

Study on the unique properties of DC at molecular levels has been hampered by the paucity of DC within tissues and the relative difficulty in maintaining their viability ex vivo. To overcome this problem, we have previously established long-lived DC lines, designated XS series, derived from the skin of newborn BALB/c mice (11). These XS cells retain many important features of LC, including their morphology, surface phenotype (B7–2, major histocompatibility class II, CD11b, ICAM-1, CD49d/e, and E-cadherin), responsiveness to cytokines, expression of cytokines and their receptors, and potent APC function (11, 12). Having these cell lines, our goal is to illuminate the molecular properties that distinguish DC/LC from other APC, and our principal strategy has been to identify and define genes and gene products expressed selectively by DC and LC. We have previously isolated five novel molecules using a subtractive cDNA cloning method in which the cDNA library prepared from the XS52 DC line was subtracted with...
mRNA isolated from the J774 macrophage line. Two of these molecules, termed dectin-1 and dectin-2, are expressed selectively by XS52 DC (13). They are type II transmembrane glycoproteins that belong to the Ca2+-dependent (C-type) lectin superfamily and may serve as costimulatory molecules that are required for efficient activation of T cells by DC. We now focus on the third gene, originally designated as 2B4, which encodes a type I transmembrane protein that can function as a ligand for integrins most likely through recognition of heparan sulfate proteoglycans (HSPG) abundantly expressed on endothelial cell surfaces (14). Based on its expression pattern and function, we have renamed this molecule DC-associated, HSPG-dependent integrin ligand (DC-HIL). Here we report its structural, biochemical, and functional properties and discuss its potential roles in DC-endothelial cell adhesion.

**EXPERIMENTAL PROCEDURES**

**Animals**—Female BALB/c mice (6–10 weeks old) were purchased from Harlan (Indianapolis, IN) and housed in the pathogen-free facility of the Animal Resource Center at The University of Texas Southwestern Medical Center. To study tissue distributions and Langerhans cell (LC)-specific expression of DC-HIL mRNA, skin and other tissues were excised from mice sacrificed by overdose of methoxyflurane inhalation.

**Cell Lines—**XS52 is a long term DC line established from the epidermis of BALB/c mouse (12). These cells were maintained and expanded in complete RPMI 1640 supplemented with mouse recombinant granulocyte/macrophage-colony stimulating factor (1 ng/ml) and NS47 fibroblast culture supernatant (10% v/v) as a source of colony-stimulating factor (12). J774 and Raw macrophages, D558 myeloma cells, BW5147 thymoma cells, HD1 (Th1) and D10 (Th2) T cells, SVEC mouse vascular endothelial cells, and COS-1 cells were purchased from the American Tissue Type Collection (ATCC, Rockville, MD). Pam 212 keratinocytes, NS47 fibroblasts, and 7-17 dendritic epidermal T cells (DETC) were kindly supplied by Dr. Akira Takashima (UT Southwestern Medical Center). The original clone 2B4 was one of the 50 clones selected in the above manner. The DNA sequences of both sense and antisense strands were determined by Tag dye deoxy termination cycle sequencing on a DNA sequencer (model 373A, Applied Biosystems, Foster City, CA) at 9,000 bases per min. Partial amino acid sequence was confirmed by fast protein sequencing and mass spectrometry. For RT-PCR, total RNAs (10 μg/lane) from mouse organs were isolated from cell lines or mRNA (5 μg/lane) from mouse tissues excised from mice sacrificed by overdose of methoxyflurane inhalation.

**Isolation of DC-HIL cDNA Clone—**A DC-specific cDNA library was constructed by subtracting the cDNA library prepared from the XS52 DC with excess amounts of mRNAs isolated from the J774 macrophage. Twelve thousand independent clones from this library were screened by colony hybridization, slot blotting, and Northern blotting for the genes expressed by the XS52 DC but only minimally or not at all by J774 macrophages (13, 18). The original clone 2B4 was one of the 50 clones selected in the above manner. The DNA sequences of both sense and antisense strands were determined by Tag dye deoxy termination cycle sequencing on a DNA sequencer (model 373A, Applied Biosystems, Foster City, CA).

**Northern Blotting** and **RT-PCR Analyses**—Northern blotting was performed as described previously (15). Briefly, total RNAs (10 μg/lane) isolated from cell lines or mRNA (5 μg/lane) from mouse organs were run on a vertical agarose gel, transferred onto a nylon membrane, and hybridized with the 32P-labeled cDNA probe for DC-HIL, glyceraldehyde-3-phosphate dehydrogenase, or β-actin.

For RT-PCR, total RNAs were isolated from epidermal cells that were treated with complement and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) and enriched for LC by centrifugation over Histopaque (1.083, Sigma Chemical Co., St. Louis, MO) as described previously (15–17). In some experiments, LC were depleted from this cell population using anti-1A monoclonal antibody (mAb) plus complement treatment as described before (16). For RT-PCR, total RNAs were isolated from epidermal cells that were treated with complement alone or complement plus anti-1A Ab. After conversion of the RNAs (1 μg) to cDNA forms by reverse transcriptase (Life Technologies, Inc., Rockville, MD), an aliquot (typically 5%) was used for PCR amplification as described below (19) by using the following primer sets: 5’-CGACTCCGGCTCTCCTTCAACT-3’ and 5’-CACAGCCCTCAGCGACACAGCAGC-3’ for DC-HIL, 5’-TACAAGCTCG- CAGATGAAACAAACA-3’ and 5’-TGGAAGGAGCATTAGAAAGCT C-3’ for IL-1β, or 5’-GTGTCGCGCTTCTAGAAGCTT-3’ and 5’-TTGATGCACCCAGATTCTT-3’ for β-actin. Followed by 30-cycle amplification, PCR products were separated on 1.5% agarose gel, transferred onto a nylon membrane, and hybridized with 32P-labeled cDNA probe for DC-HIL, IL-1β, or β-actin.

**Immunoblotting of DC-HIL Protein—**Anti-DC-HIL peptide Ab was generated by immunizing rabbits with a synthetic 20-aa peptide of C-GHEQYPNHMREHNLQR(GWS (Alpha Diagnostics Int., San Antonio, TX) (the amino-terminal cysteine was attached for thiol coupling) corresponding to aa 30–48 in the ECD of DC-HIL. After three rounds of immunization, serum was collected and subjected to affinity purification of peptide-specific Ab using the same 20-mer peptide as described above (15).

Whole cell extracts were prepared from several different cell lines by lysis with 0.3% Triton X-100 in Dulbecco’s PBS (–) for 15 min, followed by centrifugation for 20 min at 10,000 g. The full-length cDNA for DC-HIL was enzymatically excised from clone 2B4 and inserted into a mammalian expression vector pcMV5 (a gift from Dr. D. Russel, UT Southwestern Medical Center). COS-1 cells were transfected with the expression vector (pcMV5-DC-HIL) or an empty vector using FuGene 6 (Roche Diagnostics, Indianapolis, IN), cultured for 3 days, and the whole cell lysate was prepared as described above. The samples were separated by 4–15% SDS-PAGE, transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA), and then blotted with 1 μg/ml purified rabbit anti-DC-HIL or control rabbit IgG. After washing, the membrane was further blotted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) and developed with ECL system (Amersham Pharma- ceutic Biotech, Piscatway, NY).

**Sublocalization of DC-HIL—**XS52 DC were incubated in 0.25 μm su- crose for 10 min at 4 °C. The pellet after centrifugation was resus- pended and homogenized in 10 ml HEPES (pH 7.5) by 5–10 strokes with a Dounce homogenizer (G-Tec, Princeton, NJ) and then centrifuged for 10 min at 10,000 g. The resulting supernatant was assayed for soluble and membrane fractions by further centrifugation for 50,000 × g at 4 °C. These samples were subjected to immunoblotting for determining localization of DC-HIL proteins.

For experiments in which cell surface expression of DC-HIL was assessed, XS52 DC and COS-1 cells transfected either with pcMV5-DC-HIL or a luciferase-expressing vector (pGL3-Control, Promega, Madison, WI) were surface-biotinylated by incubation with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 60 min at 4 °C. After washing, cell lysates were prepared using 0.3% Triton X-100 and incubated with streptavidin-conjugated agarose beads (Sigma) by gently rocking overnight at 4 °C. Following centrifugation, the supernatant was collected as the streptavidin-unbound fraction, and the beads were washed extensively with PBS and then eluted by boiling in Laemmi’s sample buffer (streptavidin-bound fraction). DC-HIL or luciferase in each fraction was detected by immunoblotting with the respective Ab.

**N-Glycosidase Treatment—**The whole XS52 DC lysate was boiled at 95 °C for 5 min with 1% SDS, and then incubated with or without 5 units/ml N-glycosidase (Roche Diagnostics) in the presence of 1% Nonidet P-40 (SDS at final concentration of 0.2%) overnight at 37 °C. Samples were analyzed for molecular weights of DC-HIL by SDS-PAGE followed by immunoblotting.

**Immunocytochemistry—**Visualization of DC-HIL proteins in XS52 DC was performed using anti-DC-HIL Ab and a peroxidase-based immunostaining kit (LSAB, Dako, Carpenteria, CA) according to manu- facturer recommendations. Briefly, XS52 DC cultured on a coverslip were air-dried and subjected to the following sequential treatment: 15 min fixation in 4% paraformaldehyde, 20 min permeabilization in 0.5% Triton X-100 at room temperature, and 30 min blocking in 10% normal goat serum. Cells were incubated with anti-DC-HIL Ab (1 μg/ml) in the presence of 0.5% saponin, and followed by color development using HRP and 3-amino-9-ethylcarbazole substrate. Subsequently, the specimens were counter-stained by hematoxylin and mounted onto slides. Images were taken by slide films using an Olympus BH-2 microscope; these were digitized and image contrast was adjusted using Photoshop (Adobe, San Jose, CA).

**Preparation of DC-HIL-Fc Fusion Protein—**DC-HIL-Fc protein, consisting of, from the amino terminus, the extracellular domain (ECD) of DC-HIL and a Fc region of human IgG, (hlgFcF, was produced in COS-1 cells. The DNA fragment encoding the ECD (aa 34–511) was PCR-amplified with primers containing HindIII and XbaI sites at the 5’ and 3’ ends, respectively. Using these sites, the PCR product was ligated in-frame to the coding sequence of the Fc region sequence (789 bp), which had been cloned into pSecTagB vector (Invitrogen, Carlsbad, CA) at XbaI and Apol sites in the 5’ and 3’ ends (pSTB-DC-HIL-Fc). An expression vector for Dectin-1-Fc was constructed by replacement of the 3’ non-coding region in pSTB-DC-HIL-Fc with the ECD of Dectin-1 (aa 71–215) (15). We have reported previously that the Dectin-1 receptor protein, tagged with the amino-terminal histidine binds selectively to T cells (15), whereas the C-terminal Fc fusion proteins lack the binding activity. The Fc-encoding expression vector was constructed by insertion of the DNA fragment encoding hlgFcF into the pSecTagA vector using XbaI and Apol sites. These expression vectors were introduced into COS-1 cells by FuGene 6 (Roche Diagnostics). At 96 h after transfection,
Identification of DC-HIL

RESULTS

Cloning of DC-HIL cDNA and Its Deduced Amino Acid Structure—We employed a subtractive cDNA cloning strategy to isolate genes expressed by XSS52 DC but not by J774 macrophage. Fifty clones were selected and were searched for their identities in the GenBank® data base (updated in 1997). Five novel genes were identified, one of which was designated 2B4 (the original clone name).

The clone 2B4 contained a cDNA insert of 2279 bp, including the largest open reading frame (nucleotides 91–1815) with sequences that matched Kozak consensus sequences (20). The open reading frame encoded a putative polypeptide, termed DC-HIL, consisting of 574 amino acids (aa) with features typical of type I integral membrane proteins (Fig. 1). DC-HIL consists of a leader sequence (aa 1–19), a long extracellular domain (aa 20–592), a transmembrane domain (aa 593–612), and a cytoplasmic domain. Putative functional motifs identified were a heparin-binding domain (BBXB, shown by the # symbol), RGD motifs (boxed), putative N-glycosylation sites (arrowsheads), a proline-rich region (boxed), an immunoreceptor tyrosine-based activation motif (ITAM, underlined), and a lysosomal targeting signal (asterisks).

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MESLCGLYFILLLAGLPLAAKRFVRDOLGHQHEPNHHRE 40
HQLRGRVSSANDEWEHLYPVRGRGKWDSGEGVRGA 80
VLTSDDPLVGSNITFVNVLYPFRQKEDANGNYKVENK 120
RDLNGLTVSDHLYNTAGADGWDGETSRSOHLRFPDRR 160
PFPRFWQKWSFYVVFHTLOYKFLGCRSARSINTYN 200
LTAGOVMEYTVFRGGRAYPISKVKDYYITDQIPVFY 240
TMQSNRDFSLTDRLPIYFVDLHIQDSHLNDSAIS 280
WKNFQGNTGLYSNHHLTNHYLVGNTFLNLTVQTV 320
GCQPSPPSSTRPSSPSTPSPPSPLTLSPTSPSLMPTGY 360
KSMELSISNENCRIRGYFRATITIEGIVELSIDMIA 400
DPVPMTPOANSRLDFFTVCTGCAPTMEACTISDPTCQIA 440
QRNVCSPVAVDGLCLSSVRRAFNSGTYCVNTLDDAIL 480
ALTLSISIPKDPDSLAVYNGLISGCLAVLVTMI 520
ŁŁYKKHKAYKPĮIGNPCTRNYKGGKLSSLVİHKSAPFCR 560
QEKDPLLQDKPRTL 574

FIG. 1. Deduced amino acid sequence and putative domain organization of DC-HIL. Deduced amino acid sequence of DC-HIL is shown and segmented, from the amino terminus, into a putative leader sequence (underlined), an extracellular domain, a transmembrane domain (boxed), and a cytoplasmic domain. Putative functional motifs found are a heparin-binding motif (BBXB, shown by the # symbol), RGD motifs (boxed), putative N-glycosylation sites (arrowsheads), a proline-rich region (boxed), an immunoreceptor tyrosine-based activation motif (ITAM, underlined), and a lysosomal targeting signal (asterisks).
In a homology search, the aa sequence of DC-HIL had identity to mouse nmb (GenBank accession number: AJ251685) with 99.4% (by the Clustal method analyzed with the Lasergene Program, DNA Star, Madison, WI) containing four substitutions of aa residues. The human nmb (GenBank accession number: NM002510), a polypeptide that has been reported to be expressed preferentially by lowly metastatic melanoma cells (25), had homology of 71.1% to DC-HIL (Fig. 2). In addition, DC-HIL showed homology with rat osteoactivin, for which the GenBank data annotates the abundant expression in osteopetrotic bones (88.3%); QNR-71, which is responsible for melanin production in quail neuroretina cells (26) (48%); and pMEL17s melanoma markers (27) (24%). Less homology was identified with lysosome-associated membrane protein (LAMP) family members (11–13%), such as hLAMP-1 and -2 (28, 29), mCD68 (30), and hDC-LAMP (31). These structural characteristics led us to postulate that DC-HIL is a type I transmembrane protein that is heavily glycosylated, is involved in heparin binding, promotes integrin-mediated cell adhesion, and is capable of transducing tyrosine-based signaling.

Cell and Tissue Distributions of DC-HIL mRNA—Northern blot analysis showed DC-HIL mRNA (2.9 kb) to be expressed at relatively high levels by XS52 DC, but only minimally by macrophages J774 and Raw (Fig. 3A). Importantly, DC-HIL mRNA was undetectable in other tested cell lines, including γδ T cells (7-17 DETC), two αβ T cells (HDK-1 and D10), B cell hybridoma clones (5C5), keratinocytes (Pam 212), and dermal fibroblasts (NS01). As noted in Fig. 3B, DC-HIL mRNA was detected in greatest quantities in bone marrow and adipose tissues, in modest levels in thymus, and at low levels in skin. Although mRNA was not detectable by Northern analysis in lymph node and spleen, RT-PCR demonstrated its expression in lymph node (data not shown). Tissue distribution of DC-HIL mRNA does not well correlate with that of DC, suggesting that the expression may be restricted to certain subpopulations of DC. The expression of DC-HIL mRNA in skin was consistent with its establishment from skin-derived XS52 DC (12), which resemble skin resident DC, i.e. LC in many features (11, 12).

We then assessed by RT-PCR the source of DC-HIL mRNA expression in epidermis (Fig. 3C). DC-HIL mRNA was detected in epidermal cell suspensions freshly prepared from BALB/c mice. Importantly, depletion of LC (Ia⁺) by treatment with anti-Ia mAb plus complement abrogated most of the signal for DC-HIL. The absence of PCR signals for IL-1α, which is known to be specific for LC in mouse epidermis (32, 33), shows complete depletion of LC. This corroborates our observations that DC-HIL mRNA was detected by Northern blot in the LC-like XS52 cells, but was totally absent from cells derived from other epidermal populations (i.e. Pam 212 keratinocytes and the 7-17 epidermal γδ T cells) (Fig. 3A). On the other hand, the incomplete abrogation of DC-HIL mRNA by the depletion suggests a different minor source for this signal in epidermis, possibly melanocytes, because DC-HIL mRNA was detected in several murine melanoma lines by RT-PCR (data not shown). Collectively, our data suggest that DC-HIL mRNA is expressed constitutively and preferentially by XS52 DC and epidermal LC.
Identification of DC-HIL Protein—The DC-HIL protein was characterized biochemically by immunoblotting with affinity-purified rabbit Ab Raised against a synthetic peptide corresponding to aa 30–48 of DC-HIL. This Ab detected under reducing conditions a broad band migrating between 90 and 110 kDa in COS-1 cells that was transected with full-length cDNA for DC-HIL but not with the empty vector (Fig. 4A). The same Ab recognized in XS52 DC lysates two bands of 95 and 125 kDa. Consistent with Northern blot data (Fig. 3A), DC-HIL protein was expressed preferentially by XS52 DC, detectable in J774 macrophage and B16 melanoma cells, but undetectable in J558 myeloma and BWS5147 thymoma cells (Fig. 4B). The lower two bands visible in COS-1 transfectants, XS52 DC, and other cell lines were also detected by control rabbit IgG (data not shown). The estimated molecular size of the DC-HIL protein was considerably larger than that predicted from the full-length amino acid sequence (67 kDa), a discrepancy probably due to glycosylation, because DC-HIL contains 11 putative N-glycosylation sites (Fig. 1). Indeed, N-glycosidase treatment of XS52 DC lysate reduced significantly the molecular size of two bands (95 and 125 kDa) (Fig. 4C). By using this recombinant protein, together with control proteins consisting of Dectin-1 and hIgG1Fc (Fc) alone, we were able to test binding of DC-HIL-Fc to the cell surface by flow unrecognizable in the streptavidin-bound fraction (Fig. 5C), validating the efficacy of our system.

The intracellular localization was further examined by immunocytochemistry. When XS52 DC were membrane-permeabilized with saponin, most DC-HIL accumulated in a single, large perinuclear vesicle and also in small vesicles scattered toward the periphery (Fig. 6). This reactivity was significantly reduced when cells were stained in the absence of saponin (data not shown). Together, these results suggest that DC-HIL mostly localize in the cytoplasm, but is present at lower levels on the surface of XS52 DC.

Binding of Soluble DC-HIL-Fc to Cell Lines—To study the function of DC-HIL on DC, we prepared a soluble Fc fusion protein, DC-HIL-Fc. DC-HIL-Fc was produced in COS-1 cells as a disulfide-linked homodimer (data not shown), which was highly purified by affinity chromatography with protein A agarose, and detected as a single band by SDS-PAGE and subsequent CBB staining (Fig. 7A). By using this recombinant protein, together with control proteins consisting of Dectin-1 ECD and hlgG1Fc (Dectin-1-Fc) or hlgG1Fc (Fc) alone, we were able to test binding of DC-HIL-Fc to the cell surface by flow
immunoblotting. An aliquot (equivalent to 1 × 10^6 cells) was separately recovered and tested for the amounts of DC-HIL by the supernatant (cytosol fraction) and the pellet (membrane fraction) after centrifugation for 45 min, and the supernatant (streptavidin-unbound fraction, shown by "U") and the eluate from the beads (streptavidin-bound fraction, "B") were immunblotted for DC-HIL. The sample proteins loaded for streptavidin-unbound (C) and bound (D) were equivalent to 1 and 3 × 10^6 cells, respectively. C, the efficacy of the surface biotinylation method was confirmed by immunoblotting for recombinant DC-HIL (left) and luciferase (Luc), used as an intracellular control protein, right in COS-1 cells transfected with the corresponding cDNAs. The sample proteins loaded for streptavidin-unbound (U) and bound fractions (B) were the same as those in B. The data shown are representative of three independent experiments.

Fig. 6. Immunostaining of DC-HIL in XS52 DC. XS52 DC were fixed with 4% paraformaldehyde and stained with anti-DC-HIL Ab in the presence of 0.5% saponin. Stained XS cells shown in the inset at magnification × 40, one of which was enlarged to show that DC-HIL is responsible for the binding of soluble DC-HIL-Fc. Inhibition studies showed that synthetic RGD-containing peptide (1 mM RGDS) fails to inhibit binding on SVEC (Fig. 7C), whereas blocking was reduced significantly by 1 μg/ml heparin (Fig. 7C). Further experiments demonstrated that other highly sulfated polysaccharides such as dextran sulfate (500 kDa) and fucoidan were also inhibitory in a dose-dependent manner, whereas the less sulfated polysaccharide, chondroitin sulfate A, and a nonsulfated polysaccharide mannan were much less or not inhibitory at all (Fig. 7D). These results suggest that HSPG, but not RGD, is involved in the binding of DC-HIL ECD to SVEC. We then tested whether DC-HIL binds directly to heparin or fucoidan. DC-HIL-Fc showed dose-dependent binding to cells coated with either heparin-BSA or fucoidan (Fig. 8A). Binding to heparin was inhibited by heparin, fucoidan, and dextran sulfate, but less so by chondroitin sulfate A or mannan (Fig. 8B). On the other hand, binding to fucoidan was not inhibited by heparin (Fig. 8C). These data suggest that a class of binding sites on DC-HIL is shared by heparin, fucoidan, and dextran sulfate, and a second class not recognized by heparin. Thus, DC-HIL binds to HSPG probably via multiple sites, including a heparin-binding motif. Recognition of HSPG on EC by DC-HIL was supported by the findings that just preincubation of SVEC with these inhibitors did not affect binding of DC-HIL-Fc (Fig. 8D), and that pretreatment with chlorate, a reversible inhibitor of glycosaminoglycan sulfation, significantly reduced binding (Fig. 8E). It is therefore highly likely that DC-HIL binds to SVEC through recognition of HSPG on the surface of SVEC.

Adhesion of SVEC to Immobilized DC-HIL-Fc—The presence of the RGD motif in the ECD of DC-HIL led us to test the possibility that binding of DC-HIL-Fc to SVEC leads to cell adhesion. We employed an in vitro adhesion assay in which cells were allowed to adhere to the recombinant proteins-immobilized plates. Typically 50–60% of input SVEC adhered to plates coated with DC-HIL-Fc in a dose-dependent (Fig. 9A) and time-dependent manner (Fig. 9B), but they adhered to Dectin-1-Fc or Fc-coated plates only negligibly (data not shown). Maximal adhesion of SVEC was observed at 45- to 60-min incubation and at 37 °C temperature. Importantly, an RGDS peptide but not control peptide RPKP inhibited adhesion of SVEC in a dose-dependent manner, with maximum inhibition (90%) at 0.5 mM (Fig. 9C). Moreover, chelation of divalent cations by EDTA, which interferes with the integrin subunit assembly (34, 35), also inhibited adhesion dose-dependently. These data strongly suggest that SVEC adhere to immobilized DC-HIL-Fc through recognition of its RGD sequence by some members of integrin family, which are expressed on EC and play pivotal roles in the adhesion to leukocytes (36, 37). Of interest was that the adhesion of SVEC was also inhibited by most of the sulfated polysaccharides that could inhibit soluble DC-HIL-Fc binding on SVEC (Fig. 7D). On the other hand, adhesion of SVEC to vitronectin, a well known extracellular matrix component containing RGD sequence as well as heparin-binding motifs (38, 39), was blocked completely by RGDS peptide but not affected by any of these sulfated polysaccharides (data not shown). Thus DC-HIL-mediated adhesion is more dependent on HSPG recognition than vitronectin.

Heparin inhibited binding of soluble DC-HIL-Fc to SVEC, and blocked adhesion of SVEC to immobilized DC-HIL-Fc. However, RGDS had no effect on binding of soluble protein to SVEC, but prevented cell adhesion to immobilized DC-HIL-Fc. Therefore, we investigated whether DC-HIL-Fc interaction...
with sulfated polysaccharides was needed prior to integrin-mediated adhesion. Notably, BW5147 thymoma cells, which lacked the ability to capture DC-HIL-Fc (Fig. 7B) did not adhere to immobilized DC-HIL-Fc (Fig. 10). On the other hand, BW5147 cells showed constitutive adhesion to vitronectin, which was comparable to those of SVEC and Queens (Fig. 10), indicating a good correlation between cell binding and cell adhesive capacities of DC-HIL. Moreover, SVEC once adhered to immobilized DC-HIL-Fc were no longer removable by incubation with sulfated polysaccharides (data not shown). These data suggest that RGD-dependent cell adhesion is preceded by interaction of this molecule with HSPG.

Binding and Adhesion Profiles of RGD-deficient DC-HIL-Fc Mutant—To more definitively demonstrate involvement of RGD sequence in adhesion of SVEC to immobilized DC-HIL-Fc, we generated RGD-deficient DC-HIL-Fc, termed RAA mutant, by altering RGD to RAA using site-directed mutagenesis. This strategy has successfully documented the critical role of RGD in the interaction of several different molecules with integrins (40–42). RAA mutant DC-HIL-Fc was produced in COS-1 cells and purified as highly as the wild-type was (Fig. 11A). When tested for its binding ability to SVEC, the RAA mutant showed an almost identical binding profile to the wild-type, including the inhibition by heparin (Fig. 11B). In sharp contrast, when tested for the adhesive activity, the mutant was unable to induce adhesion of SVEC (Fig. 11C). Thus, these results clearly demonstrate that the RGD-integrin interactions are indispensable for the adhesion of SVEC to immobilized DC-HIL-Fc but not for the binding of soluble DC-HIL-Fc to SVEC.

**DISCUSSION**

In searching for DC-specific genes that provide unique properties of DC, a number of DC-specific or -associated, novel molecules have been identified, e.g. DC-SIGN (43), Langerin (44), and DC-LAMP (31). In fact, functional studies on these molecules demonstrated that they are involved in establishing distinct properties of DC. In addition to dectin-1 and dectin-2, we added to the current list of DC-associated molecules a new member, termed DC-HIL, that may contribute to unique mechanisms for transendothelial migration of DC.

DC-HIL showed significant homologies with melanoma/melanosome-associated membrane proteins and lower degree with members of the LAMP family (Fig. 2). In addition, its structural and biochemical features are also in accordance with these membrane protein families, which include: 1) a proline-rich hinge region containing numerous O-glycans, 2) highly N-glycosylated forms, 3) lysosomal targeting motifs, 4) a tyrosine-based signaling motif in a cytoplasmic tail, 5) high accumulation in a perinuclear region (Fig. 6) (28, 29), and 6) relatively low expression on the cell surface (Fig. 5) (45–47). Because DC do not have melanosomes, we speculate that DC-HIL may be a newly identified member of the LAMP family and may play roles in the intracellular events.

In this study we focused on characterizing the function of the ECD rather than the intracellular domain of DC-HIL. The ECD contains an RGD motif and a heparin-binding motif, both of which are frequently found in adhesion molecules, leading us to hypothesize a role as an adhesion molecule. Using the recom-
**Identification of DC-HIL**

**FIG. 8.** DC-HIL-Fc recognizes heparin and fucoidan. DC-HIL-Fc was assessed for binding capacity to heparin and fucoidan. 

*Panel A.* Dose-dependent binding. Varying concentrations of DC-HIL-Fc were incubated for 60 min at room temperature in the ELISA plates precoated with 100 μg/ml heparin-BSA or fucoidan overnight. After extensive washing, the plates were sequentially incubated with biotin-conjugated anti-human Fcγ and with HRP-conjugated streptavidin. After adding TMB substrate, the amounts of DC-HIL-Fc bound to the plates were measured by absorbance at 450 nm.  

*Panel B.* Inhibition of binding. Binding of DC-HIL-Fc (1 μg/ml) to immobilized heparin (B) or fucoidan (C) was performed in the presence of heparin (H), fucoidan (F), dextran sulfate (500 kDa) (D), chondroitin sulfate A (C), or mannan (M) at the increasing concentrations. 

*Panel C.* Blocking of DC-HIL-Fc-binding to SVEC by pretreatment with heparin. SVEC were treated with (closed histogram) or without (open histogram) 1 μg/ml heparin, washed, and then examined for binding of DC-HIL-Fc by flow cytometric analysis. E, pretreatment with sodium chlorate. SVEC were treated with (closed histogram) or without (open histogram) 30 mM sodium chlorate for 24 h prior to the binding assays. Dotted histograms show the binding of Dectin-1-Fc.

**FIG. 9.** SVEC adhere to immobilized DC-HIL-Fc. A, dose-dependent adhesion. [3H]Thymidine-pulsed SVEC (2 × 10⁶ cells/well) were cultured for 60 min at 37 °C in 96-well plates precoated with different concentrations of DC-HIL-Fc or control fusion proteins. After washing, the adherent cells were lysed and measured for the radioactivity, and shown as percent cpm relative to total input cpm. 

*Panel B.* Time course of SVEC adhesion. In the same adhesion assays, the adhesive activity was measured at the various time points after incubation of SVEC in the wells precoated with 10 μg/ml DC-HIL-Fc. 

*Panel C.* Blocking of SVEC adhesion by polysaccharides. SVEC were incubated for 60 min in the wells precoated with 10 μg/ml DC-HIL-Fc in the presence of sulfated or nonsulfated polysaccharides at the 10-fold increasing concentrations. Inhibitory activity is shown by percentage of [3H] cpm obtained in the absence of inhibitors. 

*Panel D.* Blocking with inhibitors for integrin-mediated adhesion. Increasing doses of inhibitors (an RGDS tetramer, an RPKP control tetramer, and EDTA) were added to the same adhesion assays. Note that adhesion of SVEC to control fusion proteins typically showed as little as 1%. Data shown are representative of five independent experiments.
Identification of DC-HIL

8133

The interaction of DC-HIL and HSPG on EC induces an RGD-dependent cell adhesion, two possible molecular models are considered: First, the interaction activates integrins expressed on SVEC, resulting in recognition of the RGD. Second, the interaction induces conformational changes of DC-HIL, thereby exposing the cryptic RGD sequence, as suggested for von Willebrand factor and thrombospondin (48, 49). Further studies will be required for determining which model accounts for the DC-HIL-induced cell adhesion.

This working model shares some features with the two-step adhesion cascade for neutrophil/EC interactions, in which the primary adhesion involves three members of selectin family and their oligosaccharide ligands. Subsequently, secondary adhesion involves β2 (CD18) integrins on neutrophils and immunoglobulin gene superfamily counter-receptors on EC, primarily ICAM-1 (37). However, DC-HIL-mediated adhesion differs from this two-step model in that DC-HIL serves also as one of the counter-receptors for integrins. In this respect, DC-HIL is distinguishable from other adhesion molecules on the cell surface: e.g. Thy-1, Ly-5, and NCAM are all capable of interacting with heparin (50–52), whereas they do not function as ligands for integrins. This feature of DC-HIL may exemplify that DC display not only common but also unique mechanisms for their transendothelial migration.

Heparan sulfate, as products of the enzymatic degradation of HSPG, is rapidly released from cell surface and extracellular matrices under conditions of inflammation and tissue damages (53, 54). Recently, this heparan sulfate has been demonstrated to induce phenotypic as well as functional maturation of DC characterized by enhanced surface expression of major histocompatibility class II, CD80 (B7–1), and CD86 (B7–2), lowered rate of antigen uptake, and increased allostimulatory capacity (55). Although it is not defined how heparan sulfate induces maturation signals to DC, direct binding of heparan sulfate to its putative receptors on DC is thought to be one of the most possible mechanisms. Because a toll-like receptor, which is expressed on DC, binds to peptidoglycan, including lipopolysaccharide and heparan sulfate (56, 57), this receptor is proposed to be a candidate responsible for inducing maturation signals. It is thus tempting to propose that DC-HIL is a second candidate for such a receptor, which may transduce maturation signals to DC via the residues ITAM on its cytoplasmic tail.

We were unable to block adhesion of XS52 DC to SVEC by pretreatment of SVEC with DC-HIL-Fc even at 75 μg/ml (data not shown). Because our anti-DC-HIL antibody does not recognize proteins expressed on the cell surface of XS52 DC (probably the epitope for the Ab is cryptic in the molecule), we have not examined blocking of SVEC adhesion by Ab. Successful blocking may require antibodies reactive with the DC-HIL proteins on cell surface. It thus remains uncertain to which extent DC-HIL contributes to the DC/EC adhesion under physiological conditions. Nevertheless, DC-HIL is potentially involved in endothelial adhesion of DC, and its preferential expression of the protein in DC suggests that it plays critical roles in the unique mechanisms for DC migration.

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