Proteomic Analysis of DC-SIGN on Dendritic Cells Detects Tetramers Required for Ligand Binding but No Association with CD4*

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DC-SIGN (dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin) or CD209 is a type II transmembrane protein and one of several C-type lectin receptors expressed by dendritic cell subsets, which bind to high mannose glycoproteins promoting their endocytosis and potential degradation. DC-SIGN also mediates attachment of HIV to dendritic cells and binding to this receptor can subsequently lead to endocytosis or enhancement of CD4/CCR5-dependent infection. The latter was proposed to be facilitated by an interaction between DC-SIGN and CD4. Endocytosis of HIV virions does not necessarily lead to their complete degradation. A proportion of the virions remain infective and can be later presented to T cells mediating their infection in trans. Previously, the extracellular domain of recombinant DC-SIGN has been shown to assemble as tetramers and in the current study we use a short range covalent cross-linker and show that DC-SIGN exists as tetramers on the surface of immature monocyte-derived dendritic cells. There was no evidence of direct binding between DC-SIGN and CD4 either by cross-linking or by fluorescence resonance energy transfer measurements suggesting that there is no constitutive association of the majority of these proteins in the membrane. Importantly we also show that the tetrameric complexes, in contrast to DC-SIGN monomers, bind with high affinity to high mannose glycoproteins such as mannan or HIV gp120 suggesting that such an assembly is required for high affinity binding of glycoproteins to DC-SIGN, providing the first direct evidence that DC-SIGN tetramers are essential for high affinity interactions with pathogens like HIV.

Dendritic cells (DCs)† and their subsets are potent antigen-presenting cells functioning at the interface between the adaptive and innate immune system (1, 2), which recognize and internalize pathogens and subsequently activate T cells with pathogen-derived peptides. To internalize pathogens, DCs express a series of pathogen recognition receptors such as Toll-like receptors that recognize lipoproteins, lipopolysaccharide, or bacterial DNA commonly found on various bacteria. C-type (calcium-dependent) lectin receptors (CLRs) are also expressed and these bind to conserved oligosaccharides that are commonly found on the surface glycoproteins of viruses. CLRs expressed by DCs include the mannose receptor (CD206), DEC-205 (CD205), Langerin (CD207), and DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN; CD209) (3). These receptors differ not only in their expression on various subsets of DCs and other tissues, but they also recognize different oligosaccharides thus discriminating between different ligands (3, 4).

DC-SIGN is a 44-kDa type II transmembrane protein that consists of a carbohydrate recognition domain, a neck domain involved in oligomerization, a transmembrane domain, and a cytoplasmic tail mediating interactions with the endocytosis machinery important for ligand internalization (4–6). DC-SIGN binds and internalizes several viruses such as HIV, Ebola virus, cytomegalovirus, Dengue virus, and hepatitis C virus (7–10) though other receptors are also involved (2). Other non-viral pathogens can also interact with DC-SIGN (11). Many of these viruses, however, have evolved a mechanism leading to their escape from lysosomal degradation and allowing them either to infect DCs or to hide inside the cells followed by transfer to and infection of T cells when these cells are being activated by the mature DC. In mature monocyte-derived DCs (mMDDCs) it has been shown that HIV binding to DC-SIGN and subsequent internalization into the DC does not lead to the complete degradation and part of it is protected and subsequently infects T cells (12, 13). The mechanism of this protection is poorly understood (11, 14) but it has been suggested that it is an HIV-induced change in endocytic routing (11, 13). Alternatively, a minor proportion of HIV is transferred to the CD4/CCR5-mediated infection pathway. A detailed knowledge of the DC-SIGN interaction with glycoproteins is thus crucial for the understanding of DC-SIGN-induced routing of internalized ligands and involves both the selectivity for individual sugar structures as well as the stoichiometry of the DC-SIGN-ligand complex including any DC-SIGN-associated proteins in the cell membrane.

Crystal structural data have revealed that DC-SIGN binds to internal tri-mannose glycosylation structures present in N-binding protein; PMA, phorbol 12-myristate 13-acetate; HIV, human immunodeficiency virus; DTT, dithiothreitol.

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1 The abbreviations used are: DC, dendritic cell; CLR, C-type (calcium-dependent) lectin receptor; DC-SIGN, dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin; MDDC, monocyte-derived dendritic cell; MS, mass spectrometry; DSS, disuccinimidyl suberate; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; FRET, fluorescence resonance energy transfer; MBP, mannose-
linked high mannose oligosaccharides. The receptor makes contact with the three adjacent mannose residues at an internal branched structure but fails to bind to the core tri-mannose motif in complex oligosaccharides because of steric interference resulting from different anomeric linkages (15). This observation explained the preference of DC-SIGN for high mannose carbohydrate structures and is in contrast to binding characteristics of other CLR such as the mannose receptor, which has been suggested to bind to single terminal mannose residues (11). Based on the oligomerization of its extracellular domain it was suggested that DC-SIGN forms tetramers and that this oligomerization enhances the affinity for neoglycoproteins (5). Support for this is provided by electron microscopy detection of clusters of DC-SIGN residing in rafts on the surface of immature monocyte-derived DCs (iMDDCs) (16). It is therefore proposed that the interaction between DC-SIGN and glycoproteins involves several molecules of DC-SIGN binding to differential sugar moieties on the glycoprotein spaced at appropriate distances determined by the DC-SIGN oligomerization (5). However, tetramer formation on the cell surface has not yet been proven. Furthermore, it is still unclear whether DC-SIGN associates with any other proteins and how this could influence ligand binding. This is especially important as the mechanism of cis infection of DCs by HIV suggests a possible association between CD4/CCR5 and DC-SIGN in the plasma membrane and such an association has been shown by colocalization on alveolar macrophages but the authors were unable to co-immunoprecipitate those molecules in transiently transfected cells (17).

Here we report the use of novel “proteomics” techniques based on mass spectrometry (MS) along with fluorescence resonance energy transfer (FRET) measurements to investigate lateral protein associations of native DC-SIGN on iMDDCs. We have reported recently the versatility of those tools in determining lateral membrane protein associations on lymphoid cells (18). In this article, we use cross-linking, immunopurification, and Western blotting to show that DC-SIGN assembles into oligomers of very high apparent molecular weight on the cell-surface of iMDDCs. Mass spectrometry analysis of the purified complexes identified them as homo-oligomers of DC-SIGN and cross-linking at different concentrations of the cross-linker suggests that they are tetramers. However, CD4 could not be detected in the complexes. Furthermore FRET measurements and cross-linking followed by co-immunoprecipitation also did not detect any CD4/DC-SIGN association. We also show for the first time that the DC-SIGN complexes bind immobilized mannann (a yeast-derived polysaccharide) as well as gp120 with high affinity whereas DC-SIGN monomers are not bound. This confirms the formation of DC-SIGN tetramers on immature iMDDCs, shows that they do not associate with other proteins and that the oligomerization mediated by the lateral organization in the membrane is required for high affinity ligand binding.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Unless otherwise specified, all reagents were obtained from Sigma. Immature MDDCs were prepared from blood monocytes as described previously (2). The T lymphoblastoid cell line CemT4 and the CD4-negative lymphoblastoid derivative cell line A2.01 were obtained from the National Institutes of Health AIDS reagents and reference program (Rockville, MD). OKT4 hybridoma cells were obtained from the American Type Tissue Collection. Q4120 hybridoma cells were a generous gift from Dr. Quentin Sattentau (Centre d’Immunologie de Marseille-Luminy, Marseille, France), and WM82 hybridoma cells were donated by Dr. Tony Hennicker (Westmead Hospital, Westmead, NSW, Australia). The DC-SIGN specific monoclonal antibody 120507 was obtained from R&D Systems (Minneapolis, MN), and the rabbit polyclonal antibody against DC-SIGN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HIV-1 gp120 (Clade E, 3234, used for co-immunoprecipitations) was obtained from the National Institutes of Health AIDS Research and Reference Program. Sheep polyclonal antibody raised against gp120 (Lot DV-012) had been obtained from the Center for Biologicals Evaluation and Research (FDA, Bethesda, MD). Mouse IgG1 was from Sigma. The horseradish peroxidase-labeled secondary antibody (donkey anti-rabbit) was purchased from Amersham Biosciences (Castle Hill, NSW, Australia). Disuccinimidyl suberate (DSS), dithiobis(succinimidylpropionate) (DSP), were from Pierce, protein G-Sepharose and Cy3 monoreactive NHS ester from Amersham Biosciences. 6-(Fluorescein-5-carboxamido)-hexanoic acid was from Molecular Probes (Eugene, OR) and PMA was purchased from Sigma-Aldrich.

Cross-linking and Cell Lysate Preparation—Cells were washed twice with cross-link buffer (CLB) (10 mM HEPES, pH 8.0, 140 mM NaCl, 1 mM MgCl2, 0.1 mM EGTA, 0.02% (v/v) NaN3) and resuspended in CLB at 5 × 10^6 cells/ml. DSS or DSP were dissolved in Me2SO at 25 mM or at lower concentrations when indicated and added to the suspension (20 μM per 1 ml suspension). Cross-linking was carried out for 30 min at room temperature followed by quenching of the cross-linker, which was done by adding TBS, pH 7.5, pelleting the cells, and resuspending them in TBS, pH 7.5 for 15 min. Cells were pelleted again and lysed in CLB containing 1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, and 0.1% (v/v) SDS for 1 h at 4 °C. Insoluble debris was pelleted for 10 min at 10 000 × g and resuspended by 100 000 × g for 10 min and cleared lysates were then incubated with 10 mM CaCl2. For the preparation of a mock lysate, Me2SO was added without dissolved DSS. For direct analysis, lysate was then supplemented with 5 × SDS sample buffer, and proteins were separated on an 8–16% gradient gel or a 5% gel, Western-blotted, and DC-SIGN was detected with a polyclonal antibody.

To generate CemT4 and A2.01 lysates, cells were harvested, washed twice with CLB, and lysed in CLB containing 1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, and 0.1% (v/v) SDS for 1 h at 4 °C. Insoluble debris was pelleted by centrifugation at 10 000 × g for 10 min at 4 °C, and the supernatant was used for immunoprecipitations.

Immunoprecipitations and Co-immunoprecipitations—Affinity beads were generated by coupling the monoclonal antibodies Q4120 (anti-C4D) and the anti-DC-SIGN antibody as well as mouse IgG1 to cyanogen bromide-activated Sepharose according to the manufacturer’s instructions. For precipitation of DC-SIGN complexes, 0.5 ml lysates were precipitated with 2.5 μg of anti-DC-SIGN antibody bound to 20 μl of protein G beads. For detecting cross-linking of the CD4 and DC-SIGN receptors, 0.5 ml lysates were precipitated with 20 μl of Q4120 beads (specific for CD4) or 20 μl of anti-DC-SIGN beads. For co-immunoprecipitating gp120 and DC-SIGN, 10 μl of anti-gp120 serum-bound to 20 μl of protein G beads were incubated with 1 ml of lysate. All incubations were performed for 1 h at room temperature. For co-immunoprecipitations, 2.5 μg of gp120 was incubated with the lysate for 1 h at room temperature prior to addition to the antibody bound to the beads. For precipitations with mannann beads the beads were washed with HBS, pH 7.5, 10 mM CaCl2, and 0.1% (v/v) Triton X-100 and then incubated with the lysate.

After binding the beads were washed 5 × with HBS, pH 7.5, 10 mM CaCl2, and 0.1% (v/v) Triton X-100 and resuspended in 1 × SDS sample buffer. If indicated, DTT was added to a concentration of 50 mM for reduction of the disulfide bonds. Proteins were separated on an 8–16% gradient gel and again Western-blotted. DC-SIGN and CD4 were detected using polyclonal antibodies.

Affinity Purification of DC-SIGN Complexes—Lysate from about 9 × 10^7 iMDDCs that had been treated with DSS was first passed over ~100 μl of IgG1-Sepharose to absorb nonspecific binding proteins and subsequently passed over ~100 μl of anti-DC-SIGN beads (containing ~100–150 μg of immobilized antibody). The beads were then washed with TBS, pH 8.0 containing 0.1% (v/v) Triton X-100. Complexes were eluted by boiling the beads for 5 min in 1 × SDS sample buffer.

SDS-PAGE and Protein Identification Using Mass Spectrometry—The eluate (50 μl) was loaded onto a 8–16% gradient gel (Gradipore, Frencha Forest, NSW), and proteins were separated with a constant current of 25 mA per gel. Proteins were stained with Coomassie Brilliant Blue as described previously (18). Excised gel slices were washed, subjected to in-gel digest, and the tryptic peptides were extracted and purified as described elsewhere (18).

Electrospray ionization mass spectrometry (ESI-MS) analyses were carried out using a Quadrupole-Time-of-flight (Q-Tof 2) instrument (Micromass/Waters, Manchester, UK) in nanoelectrospray mode. Typically 5–8 μl of sample were loaded into a coated glass capillary (Pro- tana, Odense, Denmark). The mass spectrometry data acquisition was performed as described in our previous studies (18, 19), and all multi-charged ions that were clearly distinguishable from the background mass were excluded.
**DC-SIGN Associating Proteins on Dendritic Cells**

**Detection of high molecular mass oligomers of DC-SIGN after cross-linking of cell surface molecules.** Immature MDDCs were subjected to cross-linking with DSS where indicated. Cells were either directly lysed in SDS sample buffer (whole MDDCs) or in a detergent-containing lysis buffer. Insoluble debris was pelleted and the cleared lysate supplemented with SDS sample buffer. DC-SIGN was also precipitated out of cleared lysate using a murine antibody bound to protein G beads and eluted by boiling in SDS-PAGE sample buffer (DC-SIGN precipitation). Proteins were separated on an 8–16% gradient gel without prior reduction and transferred to a nitrocellulose membrane. DC-SIGN was detected with a rabbit polyclonal antibody and a horseradish peroxidase-conjugated secondary antibody. The band at ~250 kDa in the DSS sample from precipitation with anti-DC-SIGN antibody (and similar bands in later figures) is the murine anti-DC-SIGN antibody that is weakly recognized by the anti-rabbit secondary antibody.

were selected for subsequent fragmentation and analyses (in so-called tandem mass spectrometry or MS/MS experiments). Spectra were interpreted and matched using the MASCOT search engine (www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) and checked manually if the score obtained was below 50.

**FRET Measurements on CD4-DC-SIGN Association**—The monoclonal antibodies Q4120, OKT4, WM82, and anti-DC-SIGN were labeled with either fluorescein or Cy3 according to the manufacturer's instructions. Immature MDDCs were labeled similarly as described for flow cytometry (18) without fixing the cells. Cells were stained with unconjugated CD4 and DC-SIGN antibody (blank sample), with unconjugated DC-SIGN antibody and fluorescein conjugated CD4 antibody (donor sample), with Cy3-conjugated DC-SIGN antibody and unconjugated CD4 antibody (acceptor sample) and Cy3-conjugated DC-SIGN antibody and fluorescein-conjugated CD4 antibody (FRET sample). Flow cytometry analysis was carried out using a FACSCalibur (BD Biosciences) with a 488 nm laser. Cells were gated to exclude debris and dead cells and mean fluorescence intensities were used for analysis. Two fluorescence intensities were collected, FL1 at 530 nm and FL2 at 585 nm. All samples were stored on ice until analyzed. Cells were subsequently incubated at 37 °C for a given time and then analyzed again. For cell stimulation, 100 ng/ml PMA was added at the same time to each sample before the incubation period. Nothing was added to mock-treated samples. Energy transfer efficiencies were determined as donor fluorescence quenching and calculated using the formula in Equation 1.

$$E = \frac{[(FL1_{\text{donor}} - FL1_{\text{FRET}})/(FL1_{\text{donor}}) - FL1_{\text{acceptor}}]}{100\%}$$  

(Eq. 1)

**RESULTS**

**Complexes of High Molecular Weight-containing DC-SIGN Can Be Detected after Cross-linking**—Because DC-SIGN was reported to form tetramers based on the properties of the recombinantly expressed extracellular domain, we investigated here, whether DC-SIGN is engaged in lateral protein interactions on the surface of iMDDCs. Cells were treated with DSS or subjected to a mock treatment (see above), and DC-SIGN was visualized after PAGE separation and Western blotting using a DC-SIGN-specific polyclonal antibody. Fig. 1 shows the detection of DC-SIGN in lysates and immunoprecipitates from mock or DSS-treated cells. In a whole cell lysate from mock-treated iMDDCs, DC-SIGN shows an apparent molecular mass of around 50 kDa, which is a good approximation to the theoretical molecular mass of 44 kDa from PAGE results alone. DC-SIGN present in a clarified lysate from mock-treated cells shows a similar molecular mass to that in untreated cells, whereas DC-SIGN present in the lysate from cells treated with DSS has an apparent molecular mass of more than 300 kDa. This indicates that DC-SIGN was cross-linked into large complexes. Immunoprecipitation of DC-SIGN with a murine monoclonal antibody confirms that the detected species are monomers and large complexes of DC-SIGN. The band at ~250 kDa in the DSS sample from precipitation with anti-DC-SIGN antibody (and similar bands in later figures) is the murine anti-DC-SIGN antibody that is weakly recognized by the anti-rabbit secondary antibody.

The Complexes are Homo-oligomers of DC-SIGN—To investigate whether the identified large complexes of DC-SIGN are homo-oligomers or associations with other molecules, complexes were affinity-purified from ~9 × 10^7 iMDDCs after DSS treatment, separated on a polyacrylamide gel and visualized with Coomassie Brilliant Blue. Purification with IgG1 Sepharose prior to the DC-SIGN purification was included as a control for nonspecific binding. Fig. 2 shows the visualized proteins from the DC-SIGN and the control purification. A band of high molecular mass can be seen in the lane containing the purified DC-SIGN complexes whereas a band of around 250 kDa can be seen in the control lane.

Mass spectrometry analysis was carried out on the excised bands to determine their protein composition. Table I shows the peptides detected in the sample derived from the in-gel digest from the visible DC-SIGN complex. Using the sensitive nano-ESI-MS and nano-ESI-MS-MS-techniques, ten tryptic peptides from DC-SIGN were detected whereas no other protein was identified showing that the complexes are most likely homo-oligomers of DC-SIGN. Further, no ions that could represent cross-linked peptides could be detected. The band in the control purification was identified as murine IgG1 (data not shown) suggesting that some antibody eluted from the beads during boiling of the sample. The intensity of the antibody-derived band was subject to run-to-run differences and was weaker in subsequent control purifications. Further, in some DC-SIGN purifications confirming the isolation of complexes...
containing only DC-SIGN, IgG-derived peptides could be detected suggesting that the intensity of the antibody-derived band varies from sample to sample (data not shown).

The High Molecular Weight Complexes Are Tetramers—In order to deduce the oligomerization state of the complexes, iMDDCs were subjected to cross-linking with different concentrations of DSS. Cells were then lysed and the cleared lysate separated on 8–16% gradient gels as well as on 5% gels in order to increase the resolution in the high molecular weight region of the gel. Fig. 3 shows representative DC-SIGN oligomers detected in the cleared lysate after cells had been treated with increasing concentrations of DSS. It can be seen in Fig. 3A that the amount of monomeric DC-SIGN gradually decreases and that bands of high apparent molecular weight appear with increasing concentrations of the cross-linker. Fig. 3B shows resolution of the high molecular mass bands into three individual bands indicating that they consist of DC-SIGN dimers, trimers and tetramers. Dimers are detected at an apparent molecular mass of around 100 kDa slightly below a nonspecific band. Trimer bands migrate at a molecular mass of around 150 kDa whereas the tetramer bands are detected slightly below 250 kDa. In each case the migration is slightly above the predicted molecular mass, which is probably a consequence of some globularity being retained through the cross-linking that interferes with the normal migration of the proteins through the gel. A similar effect can be seen in Fig. 2, where the antibody of a molecular mass of 150 kDa migrates at an apparent molecular mass of 250 kDa. This is probably because of the globularity of the antibody that is retained through the intact interchain disulfide bridges.

The Tetramers Bind to Mannan and HIV-1 gp120—It has been proposed that DC-SIGN tetramerization is required for high affinity ligand binding. An increased affinity of the extracellular domain of recombinant DC-SIGN for mannoylated BSA was detected when the neck region required for oligomerization was present (5). To directly investigate whether HIV gp120 as well as yeast-derived mannann binds with higher affinity to DC-SIGN tetramers than to monomers on dendritic cells, we conducted co-immunoprecipitation experiments between the molecules. Fig. 4 shows the co-immunoprecipitation of DC-SIGN tetramers (arrow) with HIV gp120 whereas DC-SIGN monomers, detected by precipitation with a murine anti-DC-SIGN antibody, were not detected in Fig. 4B. In Fig. 4A nonspecific bands from contaminating antibody obscured in Fig. 4A at ~50 kDa where the gel had not been reduced. This shows that the complexes bound to HIV gp120 with an affinity that is sufficiently high to allow for co-immunoprecipitation and is higher than the affinity between gp120 and DC-SIGN monomers. Bands derived from nonspecific recognition of the anti-gp120 antibody can be seen at ~50 kDa in Fig. 4A and at 150–250 kDa in Fig. 4B because of the large amount of antibody present in the sample. Fig. 5 similarly shows precipitation of DC-SIGN complexes with mannan beads, whereas no DC-SIGN monomers are precipitated. Complexes were not detected, involving binding by Sepharose, itself indicating that the binding occurs predominantly between mannan and DC-SIGN.

FRET Measurements Do Not Detect an Association between CD4 and DC-SIGN—To further examine the proposed association between CD4 and DC-SIGN (despite the absence of peptides from other proteins in the cross-linked complexes), we conducted FRET measurements between CD4 and DC-SIGN on the surface of iMDDCs using similar techniques to our previous study (18). Fig. 6 shows energy transfer efficiency measured between CD4 and DC-SIGN using two different anti-CD4 antibodies as donor molecules. Energy transfer efficiencies between CD4 and the transferrin receptor, with or without stimulation with PMA, are also included as a positive control for a protein association (18). Furthermore, the energy transfer between the two CD4 epitopes Q4120 and OKT4 was included to serve as a positive control for FRET measurements on iMDDCs. These data show that no significant energy transfer (above 5–7%, Refs. 20 and 21) could be detected between CD4 and DC-SIGN. However there was significant FRET between the two adjoining CD4 epitopes Q4120 and ORT4 on iMDDCs as well as between CD4 and the transferrin receptor (positive control) after PMA stimulation on CemT4 cells. Those cells express higher levels of CD4 than iMDDCs. Because energy transfer is measured as the proportion of donor fluorescence that could be transferred to an acceptor molecule, it is independent of the level of donor expression (20, 21). Nevertheless a low donor expression amplifies measurement variations, as reflected in the values obtained between the CD4 epitopes Q4120 and ORT4. However, a detectable FRET requires that the donor molecules are associated with a high number of acceptor molecules so that a significant portion of fluorescence can be transferred. Redundant acceptor molecules not engaged in the interaction do not alter the measured energy transfer. Therefore, generally the partner that is lower in abundance (here CD4) is selected as a donor molecule (20, 21). As the detected energy transfer is independent of CD4 expression, the lower level of CD4 on iMDDCs cannot account for the lack of energy transfer from CD4 to DC-SIGN. However the absence of a detectable CD4-TFR interaction is caused by the very low expression of TFR on iMDDCs, which impedes detection of a CD4-TFR interaction in those settings. Nevertheless it is pos-

### Table 1

| Precursor mass | Peptide Sequence | Position | MASCOT SCORE |
|---------------|-----------------|----------|--------------|
| 818.9         | 1635.8          | SAEQQNFLQLQSSR 296–309 | 87 |
| 784.9         | 1567.9          | LQLQLGELEEELQR 9–21 | 58 |
| 760.9         | 1519.8          | QQEEQETLQQLK 225–246 | 32 |
| 753.4         | 1504.8          | M*QEIYQELTR 166–175 | 70 |
| 663.8         | 1325.6          | MQEIYQELTR 166–175 | 18 |
| 655.8         | 1309.6          | QQEIYQELTR 189–198 | 37 |
| 654.3         | 1306.7          | QQEIYQELTR 120–129 | 52 |
| 646.3         | 1291.7          | VSQSSGQEQSR 63–73 | 53 |
| 528.3         | 1054.6          | EVGAQLLTVK 286–295 | 47 |
| 457.3         | 912.5           | AAVGEPPEK 109–117 | 60 |

*a* Experimentally determined *m/z* value.  
*b* Theoretical uncharged monoisotopic peptide mass.  
*M* indicates oxidized methionine.  
*d* Protein produces multiple peptides of the same sequence, only the first position is shown.
Overall, observed patterns are representative for more than three different experiments. Two independent experiments and the different onset of detection of multimers suggest some variability between experiments; however, the overall observed pattern is representative for more than three different experiments.

Detection of DC-SIGN oligomers after cross-linking was performed with different concentrations of DSS. Immature MDDCs were subjected to cross-linking DSS at different concentrations as indicated. Cleared lysates were prepared and directly separated on an 8–16% gradient gel (A) or a 5% gel (B). DC-SIGN complexes were detected after Western transfer with a polyclonal antibody. A and B are derived from two independent experiments and the different onset of detection of multimers suggests some variability between experiments; however, the overall observed pattern is representative for more than three different experiments.

Co-immunoprecipitation studies between the two molecules were also conducted after previous studies showed cross-linking of CD4 with the transferrin receptor on unstimulated CemT4 cells (18). To minimize contamination of blots with antibody-derived nonspecific bands, the antibodies employed for precipitating the complexes were directed linked to Sepharose. To determine whether any faint bands in the samples were derived from the antibody heavy chain, control experiments were performed using the A2.01 cell line, which does not express CD4 or DC-SIGN. Precipitation of CD4 from the CD4-positive CemT4 cell line was included as a positive control. DSP was used as a cross-linker as it can be reductively cleaved allowing concentration of the proteins as monomers thus facilitating their detection. Fig. 7A shows detection of CD4 by Western blot analysis after immunoprecipitation of lysate from CD4-negative (A2.01) and CD4-positive (CemT4) cells, and iMDDCs subjected to mock treatment (−) or cross-linking (+) with beads specific for CD4 or DC-SIGN. CD4 can be detected in samples derived from iMDDCs and CemT4 cells precipitated with anti-CD4 beads but not in precipitations from CD4-negative cells and in samples derived from iMDDCs precipitated with anti-DC-SIGN beads. Similarly, Fig. 7B shows the detection of DC-SIGN in the same samples except from the CD4-positive molecules were also conducted after previous studies showed cross-linking of CD4 with the transferrin receptor on unstimulated CemT4 cells (18). To minimize contamination of blots with antibody-derived nonspecific bands, the antibodies employed for precipitating the complexes were directed linked to Sepharose. To determine whether any faint bands in the samples were derived from the antibody heavy chain, control experiments were performed using the A2.01 cell line, which does not express CD4 or DC-SIGN. Precipitation of CD4 from the CD4-positive CemT4 cell line was included as a positive control. DSP was used as a cross-linker as it can be reductively cleaved allowing concentration of the proteins as monomers thus facilitating their detection. Fig. 7A shows detection of CD4 by Western blot analysis after immunoprecipitation of lysate from CD4-negative (A2.01) and CD4-positive (CemT4) cells, and iMDDCs subjected to mock treatment (−) or cross-linking (+) with beads specific for CD4 or DC-SIGN. CD4 can be detected in samples derived from iMDDCs and CemT4 cells precipitated with anti-CD4 beads but not in precipitations from CD4-negative cells and in samples derived from iMDDCs precipitated with anti-DC-SIGN beads. Similarly, Fig. 7B shows the detection of DC-SIGN in the same samples except from the CD4-positive


**DISCUSSION**

Interactions between DCs and pathogens such as HIV are a key and probably the first step in the immune recognition of the pathogens and initiation of the subsequent cell-mediated response to control them. Alternatively, HIV may use DCs to obtain access to its major cellular targets, CD4+ T lymphocytes. The complex formation between CLRAs on dermal (or epidermal) DCs such as DC-SIGN and HIV-1 gp120 may be the first contact between the virus and its new host and it is therefore crucial to understand all protein interactions involved in binding and entry of the virus including receptor oligomerization and receptor-associated proteins.

Such lateral membrane protein interactions on intact cells are difficult to analyze, especially on a cell type such as iMDDCs. Binding affinities are often too weak to withstand the conditions required for protein solubilization (see our previous study, Ref. 18, for a more detailed discussion). Until now, the oligomerization state of DC-SIGN has been addressed using cross-linking of the recombinantly expressed extracellular domain (5), which showed tetramer formation of the molecule. However, the properties of extracellular fragments outside the natural membrane environment do not always correlate with the true cell surface arrangement of proteins. For example, CD4 was shown to crystallize as dimers interfacing in domain D4 (22) whereas other studies suggested disulfide bridged dimers mediated by domain D2 (23). Using electron microscopy, clusters of DC-SIGN have been observed recently on MDDCs but their stoichiometry could not be defined (16). We have described previously the efficient use of cross-linking/mass spectrometry to determine lateral protein interactions of CD4 including interactions in which only a fraction of CD4 was involved (18). Here we showed that DC-SIGN assembles into complexes on the surface of iMDDCs and that no other proteins were detected in those interactions, especially CD4 or the mannose receptor, which is the other CLR expressed by DCs. DC-SIGN has been shown to colocalize with CD4 and CCR5 on alveolar macrophages, but direct association could not be verified by co-immunoprecipitation from transfected cell lines (17). Because such an association could facilitate cis infection, we further investigated a possible interaction with CD4 using FRET measurements between the two molecules as well as co-immunoprecipitation studies with or without preceding cross-linking but could not detect an association between the two molecules. Contradictory evidence has been reported on the effects of DC-SIGN expression on entry of HIV into dendritic cells. Lee et al. (17) demonstrated enhancement whereas Nobile et al. (24) demonstrated reduced entry of HIV supposedly because DC-SIGN competes with CD4 for the binding of HIV. The expression level of DC-SIGN seems critical for whether HIV is bound and targeted to the endolysosomal pathway or toward the infectious pathway via CD4/CCR5. A high expression of DC-SIGN could mean that despite a possible association with CD4, most DC-SIGN would not be close to CD4 and bound viruses therefore targeted to the endolysosomal pathway (24). However, as Nobile et al. (24) suggest, a different location of CD4 and DC-SIGN may contribute to the limited access of HIV to CD4 at low DC-SIGN levels. Our findings that DC-SIGN and CD4 are spatially distant on iMDDCs support such a model, as HIV would be captured and internalized by DC-SIGN without gaining access to CD4 required for fusion and infection. However our findings do not necessarily exclude association of a minor fraction of either molecule (below the detection limit of our studies) with the other receptor. To facilitate cis infection, a CD4/DC-SIGN interaction could also be induced by binding of a ligand such as gp120 similar to the gp120-induced association of CD4 and CXCR4, which do not interact constitutively. It is also possible that the interaction is a looser association of the two molecules at a distance beyond the range of the cross-linker or energy transfer and this would explain the reported colocalization of the proteins (17), without direct physical association (17).

Using cross-linking at various concentrations of DSS we showed evidence that the complexes are indeed tetramers as has been suggested previously from studies with the DC-SIGN extracellular domain (5). This provides confirmation of the mechanism of ligand recognition of DC-SIGN on cells as first described by Mitchell et al. (5) supporting their hypothesis that DC-SIGN assembles as dimers on the surface of DCs mediated by the neck region of the molecule. The so defined arrangement of four carbohydrate recognition domains at a prede-
Complexes were eluted by boiling in SDS-PAGE sample buffer containing 50 mM DTT for reduction of disulfide bonds and cleavage of complexes were precipitated with CD4-specific Q4120 beads and DC-SIGN-containing complexes were precipitated with DC-SIGN-specific beads. Complexes were eluted by boiling in SDS-PAGE sample buffer containing 50 mM DTT for reduction of disulfide bonds and cleavage of complexes were precipitated with CD4-specific Q4120 beads and DC-SIGN-containing complexes were precipitated with DC-SIGN-specific beads. Complexes were eluted by boiling in SDS-PAGE sample buffer containing 50 mM DTT for reduction of disulfide bonds and cleavage of the cross-linked complexes into monomers. Proteins were resolved via SDS-PAGE and transferred to a nitrocellulose membrane. A, detection of CD4 with the poly T4–5 antibody and an anti-rabbit horseradish peroxidase-conjugated secondary antibody. CD4 is detected in the CD4-positive CemT4 cells and in iMDDCs when CD4-specific beads were used for immunoprecipitation but not when DC-SIGN was precipitated. B, detection of DC-SIGN with a rabbit polyclonal antibody and an anti-rabbit horseradish peroxidase-conjugated secondary antibody. DC-SIGN is only detected in iMDDCs after precipitation with beads specific for DC-SIGN but not with the CD4-specific Q4120 beads, suggesting that the two proteins are not efficiently cross-linked. Bands at high molecular mass in the lane derived from cross-linked iMDDCs precipitated and detected for DC-SIGN are the cross-linked oligomers, which were incompletely reduced and cleaved by DTT.

Oligomerization, however, conserves the spatial arrangement of the tetrameric complexes, which are then preserved in the presence of SDS. The cross-linked complexes retain their ability to bind mannann and gp120 with high affinity whereas the solubilized monomers fail to bind the glycoproteins with sufficient affinity to be co-immunoprecipitated and detected via Western blot. This demonstrates that the assembly of DC-SIGN into tetramers in the membrane environment and in our experiments conserved by cross-linking is required for high affinity binding of pathogens by DC-SIGN.

Our studies thus provide evidence for a binding mechanism involving a DC-SIGN tetramer binding to glycoproteins carrying probably four individual high mannose carbohydrate residues spaced at a defined distance. This mechanism is distinct to the mechanism by which other lectins such as the mannose-binding protein (MBP) bind their ligand as discussed by Mitchell et al. (5). Each MBP binds to individual mannose residues on the surface of the glycoprotein whereas the assembly of MBP into trimers allows for recognition of broad mannose-containing structures with high affinity. In contrast, DC-SIGN requires a specific arrangement of carbohydrates on its ligands, which explains its selectivity.

Further studies will also attempt co-precipitation of HIV-1 gp120 with immobilized DC-SIGN tetramers and examine gp120 trimer and direct viral particle binding to DC-SIGN tetramers to assess whether X4 or R5; T-tropic or M-tropic viral strains are bound with different affinities by DC-SIGN. Mass spectrometry studies will be conducted to search for cross-linked peptides from interchain cross-links to provide spatial information on the residues that are involved in cross-linking.

Thus this is the first study of the DC-SIGN interactions on the surface of dendritic cells (or any cell type). As the formation of tetramers significantly influences the binding affinity for ligands like HIV, attempts to design molecules to block these interactions must take into account the oligomeric nature of this protein. These quaternary interactions may also provide a paradigm for high affinity gp120 binding to other C-type lectin receptors.

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REFERENCES

1. Banchereau, J., and Steinman, R. M. (1998) Nature 392, 245–252
2. Turville, S. G., Cameron, P. U., Handley, A., Lin, G., Pithmann, S., Doms, R. W., and Cunningham, A. L. (2002) Nat. Immunology 3, 975–983
3. Figdor, C. G., van Kooyk, Y., and Adema, G. J. (2002) Nat. Rev. Immunology 2, 77–84
4. Frison, N., Taylor, M. R., Silleux, E., Boucher, M.-T., Mayer, R., Monsigny, M., Drickamer, K., and Roche, A.-C. (2003) J. Biol. Chem. 278, 39292–39299
5. Mitchell, D. A., Fadden, A. J., and Drickamer, K. (2001) J. Biol. Chem. 276, 28939–28945
6. Geijtenbeek, T. B. H., Kwon, D. S., Torensma, R., van Vliet, S. J., van Kuip, G., van de Wouw, F., Honkoop, J. C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
7. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
8. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
9. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
10. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
11. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
12. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
13. Halary, F., Amara, A., Lortat-Jacob, H., Meserelle, M., Delaunay, T., Houles, C., Fieschi, F., Arentzen-Seisdedos, F., Moreau, J. F., and Dechanet-Merville, J. (2002) Immunity 17, 653–664
9. Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier J. L., and Arenzana-Seisdedos, F. (2003) *Embo Rep.* 4, 1–6
10. Pohlmann, S., Zhang, J., Baribaud, F., Chen, Z., Leslie, G. J., Lin, G., Granelli-Piperno, A., Doms, R. W., Rice, C. M., and McKeating, J. A. (2003) *J. Virol.* 77, 4070–4080
11. van Kooyk, Y., and Geijtenbeek, T. B. (2003) *Nat. Rev. Immunol.* 3, 697–709
12. Moris, A., Nobile, C., Buseyene, F., Perrot, F., Abastado, J.-P., and Schwartz, O. (2004) *Blood* 103, 2648–2654
13. Turville, S. G., Santos, J. J., Frank, I., Cameron, P. U., Wilkinson, J., Miranda-Saksena, M., Dable, J., Stossel, H., Rosmani, N., Piatak, M., Lifson, J. D., Pope, M., and Cunningham, A. L. (2003) *Blood* 103, 2170–2179
14. Kwon, D. S., Gregorio, G., Bitton, N., Hendrickson, W. A., and Littman, D. R. (2002) *Science* 294, 533–536
15. Feinberg, H., Mitchell, D. A., Drickamer, K., and Weis, W. I. (2001) *Science* 294, 2163–2166
16. Cambi, A., de Lange, P., van Maarseveen, N. M., Nijhuis, M., Joosten, B., van Dijk, E. M. H. P., de Bakker, B. I., Fransen, J. A. M., Bovee-Geurts, P. H. M., van Leeuwen, F. N., Van Hult, N. F., and Figdor, C. G. (2004) *J. Cell Biol.* 164, 145–155
17. Lee, B., Leslie, G., Soilleux, E., O’Doherty, U., Baik, S., Levron, E., Flummerfelt, K., Swiggard, W., Coleman, N., Malim, M., and Doms, R. W. (2001) *J. Virol.* 75, 12028–12038
18. Bernhard, O. K., Sheil, M. M., and Cunningham, A. L. (2004) *Biochemistry* 43, 256–264
19. Bernhard, O. K., Burgess, J. A., Hochgrebe, T., Sheil, M. M., and Cunningham, A. L. (2003) *Proteomics* 3, 139–146
20. Szilékos, J., Nagy, P., Sebestyen, Z., Damjanovich, S., Park, J. W., and Matys, L. (2002) *Rev. Mol. Biotechnol.* 82, 251–266
21. Kenworthy, A. K., Petranova, N., and Edidin, M. (2000) *Mol. Biol. Cell* 11, 1645–1655
22. Wu, H., Kwong, P. D., and Hendrickson, W. A. (1997) *Nature* 387, 527–530
23. Matthias, L. J., Yan, P. T. W., Jiang, X.-M., Vandegraaff, N., Li, P., Poubhourios, P., Donoghue, N., and Hogg, P. J. (2002) *Nat. Immunol.* 3, 727–732
24. Nobile, C., Moris, A., Porrot, F., Sol-Foulon, N., and Schwartz, O. (2003) *J. Virol.* 77, 5313–5323
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