Lateral Membrane Protein Associations of CD4 in Lymphoid Cells Detected by Cross-Linking and Mass Spectrometry†

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ABSTRACT: Interactions of membrane proteins are important in various aspects of cell function. However, weak membrane protein−protein interactions are difficult to study using techniques such as co-immunoprecipitations. CD4 is a cell surface protein involved in T cell activation and the binding of the human immunodeficiency virus to HIV target cells. Here we report the use of cross-linking followed by affinity purification of CD4 in combination with mass spectrometry for identification of proteins that are in the proximity of CD4. Besides the components of the CD4 receptor complex, CD4 and lck, we have identified by tandem mass spectrometry 17 tryptic peptides from transferrin receptor CD71, three peptides from protein phosphatase CD45, and one peptide from 4F2 lymphocyte activation antigen CD98. The efficiency of the cross-linking did not correlate with the level of cell surface expression of the detected molecules, excluding a possible bias of the cross-linking toward the most abundant cell surface molecules. Whereas the association of CD4 with CD45 has been reported, the associations with CD71 and CD98 have not been previously described. We used small-scale immunoprecipitation after cross-linking in combination with fluorescence resonance energy transfer (FRET) measurements to investigate the association between CD4 and CD71. Our data show that CD71 self-associates on the cell surface, that a small fraction of CD4 can be detected by copurifying it with CD71 after cross-linking, and that the level of association between CD4 and CD71 significantly increases after phorbol 12-myristate 13-acetate-induced endocytosis of CD4. This suggests that a small fraction of CD4 associates with clusters of CD71. As both molecules undergo endocytic recycling, the association and cross-linking result from their clustering in the same pit and/or vesicle. The CD4−CD98 association probably results from nonspecific cross-linking.

Mass spectrometry-based identification of the components of purified protein complexes has become one of the most powerful technologies for high-throughput detection of protein interactions (*1*). In this strategy, complexes are purified either directly with immunoaffinity chromatography (*2*) or after introduction of a tagged bait protein into the cells followed by affinity tag-mediated purification (*3*). Alternatively, the bait protein is expressed recombinantly, and binding partners are pulled down from cell lysates (*4*). The purified complexes are usually then separated by PAGE,[1] and tryptic peptides obtained from in-gel digests are identified using MS and MS/MS (*1*). Successful studies employing such strategies have included the yeast interaction map (*5*) and characterization of the brain *N*-methyl-D-aspartate receptor complex (*6*). However, to be detected with such a strategy, a protein interaction needs to be sufficiently strong to withstand dissociation during the cell lysis and/or affinity purification steps. Strong protein interactions that occur, for example, between antibodies and their antigens are characterized by dissociation constants in the low nano- to picomolar range. For CD4 and the monoclonal antibody IOT4a, K_D values between 38 nM (*7*) and 0.5 nM (*8*) have been measured. Weaker (but not less important) protein interactions have K_D values that are much lower than this. For example, the interaction of CD4 and the adaptor protein AP-2, which mediates its endocytosis, has a K_D value of only 90 μM (*9*). Such weak protein complexes are likely to dissociate during the cell lysis and/or affinity purification steps and may not be detected. Detection of similar weak interactions between membrane proteins could provide important information about the function of those proteins (*10*).

CD4 is a 55 kDa transmembrane protein mainly expressed on cells of the immune system (*11*, *12*). It functions as a coreceptor during T cell activation (*13*), as a chemotactic

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[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MS, mass spectrometry; FRET, fluorescence resonance energy transfer; FITC, fluorescein isothiocyanate; PMA, phorbol 12-myristate 13-acetate; TCEP, tris(2-carboxyethylphosphine) hydrochloride; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DSP, dithiobis(succinimidyl propionate); IL, interleukin; HIV, human immunodeficiency virus; HLA, human leukocyte antigen.

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receptor for IL-16 (*14*), and as a receptor for the human immunodeficiency virus (*15*, *16*). It exerts its functions in T cell activation through association with the protein tyrosine kinase lck (*17*). Apart from lck, CD4 has been found to associate with a range of other molecules, including CD45, a transmembrane tyrosine phosphatase (*18*). We have previously described a method aimed at the identification of CD4-associated proteins in T lymphoblastoid cells (*19*). We showed the versatility of the method by identifying CD4 along with its associated kinase lck. However, several functionally unrelated proteins were also present, which we attributed to the promotion of nonspecific binding and the dissociation of weak protein associations in the presence of detergents.

Chemical cross-linking has become a useful tool for the study of protein complexes, including, for example, determination of the oligomerization state of proteins (*20*). More sophisticated approaches combined with mass spectrometry are aimed at the identification of the individual cross-linked peptides to draw conclusions about spatial arrangements in a protein complex (*21*). To determine whether a peptide connected to a cross-linker has arisen from cross-linking of the protein with itself or with another protein, Simpson et al. (*22*) have delineated an approach to distinguishing peptides derived from inter- and intramolecular cross-linking of proteins using differentially isotope-labeled proteins. Cross-linking in conjunction with mass spectrometry has also been used to detect novel protein interactions of a given protein. It has been used to cross-link the components of complexes bound to affinity beads. After cleavage of the cross-linker, proteins that were cross-linked to the protein of interest can be identified by PAGE and MS analysis (*23*). Another strategy used cross-linking to connect associated proteins in purified endosomes. The protein under investigation, apolipoprotein B, was then purified along with the proteins that were cross-linked to it. Using SDS−PAGE and liquid chromatography−mass spectrometry (LC−MS), Rashid and co-workers (*24*) identified an impressive number of proteins that are possibly linked to the function of apolipoprotein B.

In this paper, we report the use of cross-linking followed by affinity purification and mass spectrometry analysis for the identification of proteins that associate with the CD4 receptor in the cell membrane. Figure 1 shows schematically the strategy for identifying associating proteins. After purifying cross-linked complexes, we were able to identify the three transmembrane proteins CD45, CD71, and CD98 along with CD4, indicating that those proteins are close to CD4 on the cell surface. Using co-immunoprecipitation and fluorescence resonance energy transfer (FRET), we found evidence that at least some CD4 associates with (clusters of) CD71 and that the level of this association increases upon treatment of the cells with phorbol 12-myristate 13-acetate (PMA). We provide an explanation for this based on the endocytic properties of the molecules.

EXPERIMENTAL PROCEDURES

Materials. The PolyT4-5 antibody was a generous gift from R. Sweet (Smith-Kline Beecham, King of Prussia, PA). The Q4210, Q425, and WM82 antibody was purified from hybridoma medium. Q4120 and Q425 hybridoma cells had been obtained from Q. Sattentau (Centre d'Immunologie de Marseille-Luminy, Marseille, France), and WM82 hybridoma cells were a generous gift from T. Hennicker (Westmead Hospital). IgG1-FITC and IgG2a-FITC were from BD-Pharmingen (San Diego, CA). The polyclonal anti-CD71 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Lymphoblastoid cell line CemT4 was from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Cells were maintained in RF-10 medium (CSL, Parkville, Victoria, Australia). Mouse IgG2b was from ICN Biochemicals (Seven Hills, New South Wales, Australia). TCEP and DSP were from Pierce (Rockford, IL). Protein G−Sepharose beads, Sephadex G-25, anti-rabbit horseradish peroxidase-conjugated antibody, and Cy3 monoreactive NHS ester were all obtained from Amersham-Pharmacia (Uppsala, Sweden). PMA was purchased from Sigma-Aldrich (St. Louis, MO). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI), and 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester was obtained from Molecular Probes (Eugene, OR).

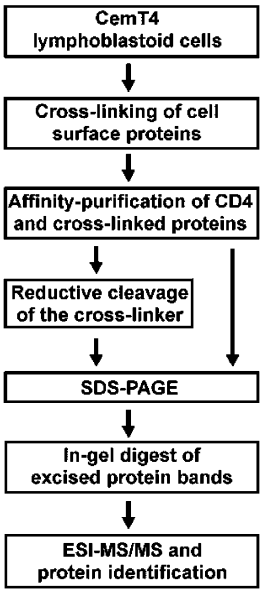

FIGURE 1: Strategy for identifying proteins that come in close contact with CD4 on the cell surface. See Experimental Procedures for details.

Cross-Linking and Cell Lysate Preparation. Cells were washed twice with buffer 1 [10 mM HEPES (pH 8.0), 140 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 0.02% (w/v) NaN_3] and then resuspended in buffer 1 at a density of 5×10^6 cells/mL. DSP in dimethyl sulfoxide was added to the cell suspension (2 mL of a 25 mM/100 mL suspension). Cross-linking was carried out for 30 min at room temperature unless otherwise indicated. Unreacted cross-linker was quenched by adding Tris-buffered saline (TBS) [25 mM Tris (pH 7.5), 140 mM NaCl, and 3 mM KCl] and resuspending the cells in TBS, followed by a 15 min incubation at room temperature. Cells were then pelleted and lysed in lysis buffer [buffer 1, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl suflate, and 1% (v/v) nonident P-40] at a concentration of 10^7 cells/mL. After 60 min at 4 °C, insoluble debris was pelleted and the supernatant passed through a 0.22 μm syringe-driven filter unit (Millipore, Bedford, MA). The lysate was then supplemented with 10 mM $CaCl_2$ because the Q425 binding of CD4 is dependent on Ca^{2+}. For

preparation of mock cross-linked cell lysates, dimethyl sulfoxide was added without dissolved cross-linker.

Immunoprecipitations and Affinity Purification. CELL lysate (1 mL) was precipitated by incubation at 4 °C for 1 h with 2.5 μg of the respective antibody and 20 μL of protein G−Sepharose beads. The beads were washed exhaustively with HBS [HEPES-buffered saline, 10 mM HEPES with 140 mM NaCl (pH 7.5)] containing 10 mM $CaCl_2$ and 0.1% (v/v) Triton X-100. Bound protein was eluted into SDS−PAGE sample buffer containing 25 mM TCEP by incubation for 5 min at 95 °C. TCEP was omitted when indicated. For affinity chromatography, Q425 beads were equilibrated with HBS (pH 7.5) containing 10 mM $CaCl_2$, 1% (v/v) Triton X-100, and 1% (v/v) NP-40 followed by application of the lysate as described previously (*25*). Beads were washed with TBS (pH 8.0) containing 10 mM $CaCl_2$, 1% (v/v) Triton X-100, and 1% nonident P-40 (NP-40) and then with TBS (pH 8.0) containing 1% (v/v) Triton X-100 and 1% (v/v) NP-40. Complexes were eluted with TBS (pH 8.0) containing 10 mM EDTA, 0.1% (v/v) Triton X-100, and 0.1% (v/v) NP-40.

SDS−PAGE, Western Blotting, and Coomassie Brilliant Blue Staining. Immunoprecipitation- or affinity chromatography-derived samples were separated by polyacrylamide gel electrophoresis on an 8 to 16% gradient gel (Gradipore, Frenchs Forest, New South Wales, Australia). Protein gels for mass spectrometry analysis were stained with Coomassie Brilliant Blue as described previously (*25*) and destained in 40% (v/v) methanol and 10% (v/v) acetic acid. Protein gels for Western blotting experiments were subjected to electrophoretic transfer of the proteins to nitrocellulose membranes. After being blocked with 5% milk, the membranes were incubated for 1 h with either PolyT4-5 antibody against CD4 or with a polyclonal antibody against CD71 and then for 1 h with anti-rabbit horseradish peroxidase-conjugated antibody. Blots were developed using the Western lightening detection kit (Perkin-Elmer, Boston, MA).

In-Gel Digest and Peptide Extraction. Gel slices were excised from the Coomassie-stained gels, dehydrated with methanol for 5 min, and rehydrated with 30% methanol for 5 min. Slices were washed twice with water and then three times for 10 min each with 100 mM ammonium bicarbonate and 30% (v/v) acetonitrile. Gel slices were then crushed into fragments, washed with 30% (v/v) acetonitrile, and dehydrated for 5 min with 100% ethanol. Fragments were dried for 1 h in a vacuum; 50−80 μL of a trypsin solution (5 ng/μL sequencing-grade modified trypsin in a 25 mM ammonium bicarbonate solution) was added, and the protein hydrolysis was carried out at 37 °C overnight. Proteins were extracted with a 50% acetonitrile, 5% formic acid extraction solution. The sample was concentrated, and acetonitrile was evaporated using a "Speed-Vac" centrifuge with moderate heating. Peptides were purified for MS analysis using "ZipTips" (Millipore) according to the manufacturer's recommendations. Samples were acidified before purification by addition of 15 μL of 5% formic acid, and trifluoroacetic acid was replaced with 5% formic acid throughout the purification.

Mass Spectrometry Analysis. Electrospray mass spectrometry (ESI-MS) analyses were carried out using a Q-Tof 2 instrument (Micromass, Manchester, U.K.) in nano-electrospray mode. Typically, 5 μL of the sample was loaded into a coated glass capillary (Protana, Odense, Denmark). The ESI parameters were as follows: capillary voltage of 800−1000 V, cone voltage of 30−40 V, desolvation temperature of 40 °C, and desolvation gas flow rate of 50−100 L/h. Peptides were detected by acquisition of the *m*/*z* range from 50 to 2000 using a 2.4 s integration time and a 0.1 s delay. The instrument was calibrated with a solution of 100 fM [Glu^1]fibrinopeptide B in 40% acetonitrile and 2.5% formic acid. Precursor ions were selected for fragmentation within a mass window of 4 *m*/*z*. MS/MS spectra were collected using collision cell voltages from 25 to 45 V with argon as the collision gas. Typically, all multiply charged signals that could be easily distinguished from the background were subjected for fragmentation. Exceptions were made for signals that were known to be peptides derived from CD4 which were sometimes omitted after several CD4-derived peptides had been identified. Also, a targeted ion approach was sometimes used when a multiply charged signal that could not be clearly distinguished from the background was subjected to fragmentation because the presence of a peptide ion at the *m*/*z* was expected.

Protein Identification. Spectra were interpreted and matched using either the MASCOT search engine (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) or manual *de novo* sequencing. Manual sequencing was also used to confirm peptide matches obtained with MASCOT and was carried out similarly as described previously (*19*).

Labeling of Antibodies with Fluorophores. Antibodies were labeled with either 6-(fluorescein-5-carboxamido)-hexanoic acid, succinimidyl ester or Cy3 according to the manufacturer's instructions. Labeled antibodies were separated from unconjugated dyes using Sephadex G-25 gel filtration. The concentration of the antibody and the degree of labeling were determined spectrophotometrically according to the manufacturer's instructions.

Flow Cytometry Measurements and FRET Measurements. Flow cytometry measurements were conducted as described previously (*26*) without fixation of the cells. For FRET analysis, cells were labeled in a manner similar to that for flow cytometry analysis. Cells were labeled with unconjugated WM82 antibody or fluorescein (donor)- or Cy3 (acceptor)-conjugated antibody to determine the control values. For FRET measurements on CD71 self-association, cells were labeled with a mixture of fluorescein- and Cy3-conjugated WM82 with a WM82-fluorescein/WM82-Cy3 ratio of 1/3, 1/1, or 3/1. Flow cytometry measurements were carried out using a FACSVantage SE DiVa apparatus from Becton Dickinson (San Diego, CA). Three fluorescence intensities were collected, two with excitation at 488 nm and detection at 530 and 585 nm. The third fluorescence intensity was collected with excitation at 514 nm and detection at 585 nm. No compensation was used. Cells were gated to exclude debris and dead cells, and both mean and median fluorescence intensities were used for analysis. To eliminate the influence of cellular autofluorescence, test values were obtained using the equation test value = mean fluorescence intensity obtained with the conjugated antibody − mean fluorescence obtained with the unconjugated antibody. Energy transfer efficiencies were calculated from the test values using the formulas published elsewhere (*27*). *E* values were excluded when the value obtained from the median

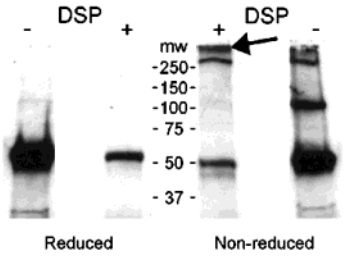

FIGURE 2: Western blot showing CD4 purification with the Q425 antibody with or without preceding cross-linking of cell surface proteins. CD4 was purified from CemT4 lysates with the Q425 antibody after the cells were cross-linked with DSP (+) or not (−). CD4 was detected with PolyT4-5 antibody, and high-molecular mass complexes are denoted with an arrow. Reduction of the complexes before SDS−PAGE, where indicated, produces monomeric CD4 because of the cleavage of the cross-linker. The difference in the intensity of the CD4 bands detected with or without cross-linking arises from inactivation of antibody binding epitopes on CD4 by the cross-linker.

fluorescence intensities differed from the value obtained from the mean fluorescence intensities by more than 50%.

For FRET measurements of CD4−CD71 association after PMA treatment, cells were labeled in a manner similar to that for flow cytometry analysis. Four samples were prepared for each measurement, and cells were stained with unconjugated WM82 and Q4120 antibody (blank sample), with unconjugated WM82 antibody and fluorescein-conjugated Q4120 antibody (donor sample), with Cy3-conjugated WM82 antibody and unconjugated Q4120 antibody (acceptor sample), and Cy3-conjugated WM82 antibody and fluorescein-conjugated Q4120 antibody (FRET sample). Flow cytometry analysis was carried out using a FACScalibur apparatus (Becton Dickinson) 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 they were analyzed; subsequently, 100 ng/mL PMA was added to each sample, and cells were incubated at 37 °C for 8 min followed by a second analysis. Nothing was added to mock treated samples. Energy transfer efficiencies were determined as donor fluorescence quenching and calculated using the formula $E = \{[FL1(donor) - FL1(FRET)]/[FL1(donor) - FL1(blank)]\} \times 100\%$. Mean fluorescence intensities for the blank samples were subtracted from the mean fluorescence intensities obtained from the donor and acceptor samples when investigating the cell surface expression of CD4 and CD71.

RESULTS

Cross-Linking of Cell Surface Molecules and Purification of Cross-Linked CD4 Complexes. We used dithiobis(succinimidyl propionate) (DSP), a cleavable, amine-reactive homobifunctional cross-linker with a 12 Å linker, to cross-link cell surface molecules that are closely associated. To investigate whether cross-linked complexes that include CD4 could be affinity purified with the Q425 antibody, we conducted small-scale immunoprecipitations and detected the presence of CD4 using PolyT4-5 antiserum. Figure 2 shows a Western blot of affinity-purified CD4 from cells that had or had not been cross-linked with DSP. The purified CD4 complexes were left either nonreduced or reduced so that cross-linked proteins were released as monomers. The data indicate that a significant part of the CD4 that is purified with Q425 is incorporated in complexes with higher molecular masses if cell surface molecules had been cross-linked. Without cross-linking, the large majority of CD4 is present in the monomeric form. The complexes are observed to disappear upon reduction. CD4 dimers and higher oligomers as detected in the nonreduced DSP− lane have been described previously (*25*) and are disulfide-bridged dimers and oligomers with undetermined physiological function.

Mass Spectrometry Identification of the Proteins Cross-Linked to CD4. The purification results suggested that part of CD4 is indeed cross-linked to complexes with a sufficiently high molecular mass so that the complexes only just migrate into the very top section of the gel. To investigate other molecules that were present in those complexes, they were affinity purified from 10^9 cells and separated by SDS−PAGE followed by Coomassie Brilliant Blue staining. We observed a very faint band at the very top section of the gel (data not shown). Because the employed MS strategy can detect proteins even in sub-Coomassie stainable amounts (O. Bernhard, unpublished results), we dissected the region above 150 kDa into slices and subjected them to our protein identification strategy. We were able to identify three transmembrane proteins CD45, CD71 (transferrin receptor), and CD98 (4F2 lymphocyte antigen) along with the CD4 receptor complex CD4 and lck. To test for reproducibility, we carried out a total of three independent experiments. Whereas in the first experiments, the complexes were left in their high-molecular mass form, experiments 2 and 3 used TCEP to cleave the cross-linked complexes into their monomeric constituents so that individual proteins migrated according to their molecular mass. In the latter two experiments, the gel was dissected into individual slices from the very top to ~15 kDa. To determine any effect of temperature on the composition of the cross-linked complexes, experiment 3 involved cross-linking carried out at 4 °C. Table 1 lists the proteins that were identified in the cross-linked complexes in the three experiments. It also shows how many peptides were identified by MS/MS in each individual experiment. Peptides from CD71 and CD45 were identified in each experiment, whereas the peptide derived from CD98 was observed only weakly in experiments 2 and 3. The second experiment had less abundant peptide ions. Figure 3 shows a MS/MS spectrum of a tryptic peptide from CD45 representative of MS/MS spectra, leading to the identification of the constituents of the CD4 receptor complex, CD4 and lck, along with the three transmembrane proteins CD45, CD71, and CD98. Two peptides derived from annexin II were identified in the third experiment. None of the recorded MS/MS spectra showed evidence that it was derived from a DSP-linked peptide, providing information about the epitope of the protein interaction.

A Fraction of CD4 Was Copurified with CD71 Only after Cell Surface Molecules Were Cross-Linked. Small-scale immunoprecipitations were conducted to investigate the specificity of the detection of CD71 along with CD4. Figure 4 shows detection of CD4 by Western blot analysis after immunoprecipitation with WM82 antibody specific for CD71, Q425 antibody specific for CD4, or IgG2b isotype control. CD4 was detected copurifying with CD71 only after

Table 1: Peptides and Proteins Identified via MS/MS in the Cross-Linked Sample

protein	molecular mass (kDa)	SWISS-PROT accession number	no. of peptides detected via MS/MS			
			experiment 1	experiment 2	experiment 3	total
CD45	144	P08575	3	1	2	3
transferrin receptor CD71	84	P02786	14	2	15	17
4F2 antigen CD98	60	P08195	1	(1)[a]	(1)[a]	1
p56Lck	58	P06239	(1)[a]	0	8	8
CD4 T cell surface glycoprotein	55	P01730	9	1	4[b]	10
annexin II	38	P07355	0	0	2	2

[a] Peptides were only detected in low abundance using a targeted ion approach. [b] Several ions corresponding to typical CD4-derived peptides were present but not subjected to MS/MS.

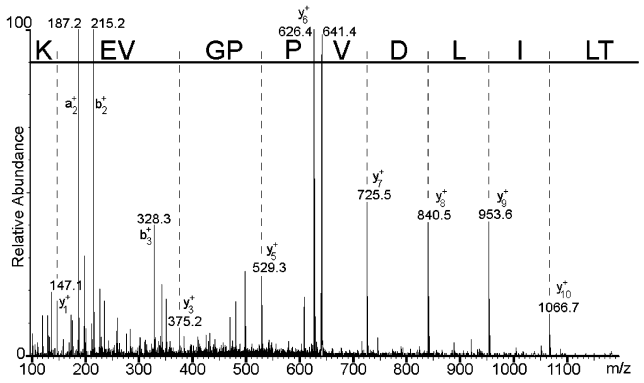

FIGURE 3: Identification of proteins that were cross-linked to CD4 via tandem MS spectrometry. A representative tandem MS spectrum from a CD45-derived peptide with a precursor *m*/*z* of 641.0 (2+, peptide mass of 1280.9) is shown. Similar spectra were obtained for the other proteins.

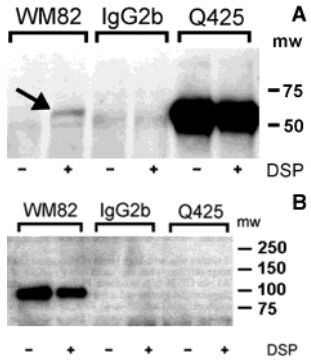

FIGURE 4: Western Blot detection of CD4 and CD71 purified with antibodies for CD71 (WM82) or for CD4 (Q425) or with IgG2b (isotype control) with or without cross-linking. CemT4 cells were either subjected to cell surface cross-linking with DSP (+) or not (−). Complexes were precipitated with either WM82 antibody against CD71, with Q425 against CD4, or with an isotype control (IgG2b) for the WM82 antibody. The cross-linker was reductively cleaved, and proteins were separated by SDS−PAGE. (A) CD4 was detected with the PolyT4-5 antibody. The arrow denotes a small amount of CD4 that was copurified with CD71 only after cell surface molecules had been cross-linked. (B) Reverse blot against CD71 with a rabbit polyclonal antibody.

the cell surface molecules had been cross-linked. The CD4-specific band was persistently observed in three independent experiments. No CD4 could be detected when the IgG2b isotype control experiment was carried out. No CD4 could be detected in similar experiments using anti-CD98 and anti-CD45 antibodies (data not shown). The reverse blot shows detection of CD71 after precipitation with the anti-CD71 antibody WM82. No CD71 could be detected in the precipitation with Q425 presumably because of the sensitivity of the polyclonal anti-CD71 antibody which does not recognize its antigen as well as the highly sensitive PolyT4-5 antibody.

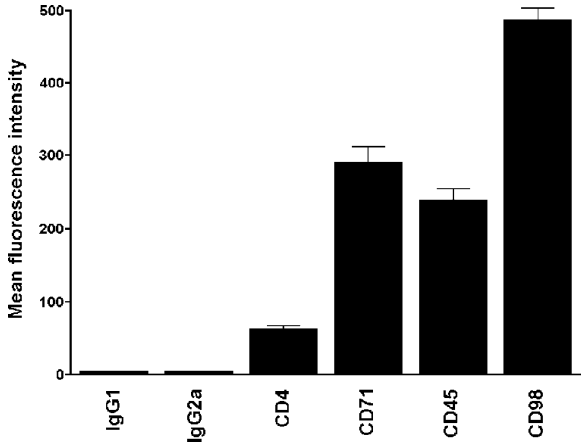

FIGURE 5: Cell surface expression of the identified proteins on CemT4 cells. Shown are the mean fluorescence intensities of three or four flow cytometry experiments using fluorescein-labeled antibodies Q4120 (CD4), WM82 (CD71), anti-CD45, and anti-CD98. FITC-labeled IgG1 and IgG2a were used as isotype controls.

Cell Surface Expression of CD4, CD71, CD45, and CD98 Does Not Correlate with Cross-Linking Efficiency. To evaluate a possible correlation between the degree of cell surface expression of the CD45, CD71, and CD98 molecules and the efficiency of cross-linking, the level of cell surface expression of those molecules was determined using standard flow cytometry procedures. Figure 5 shows that of the three molecules cross-linked to CD4, CD98, is the most abundant on the surface of CemT4 cells followed by CD71 and CD45. CD4 is the least abundant. A protein that is cross-linked more effectively with CD4 will be present in the gel in a greater amount. Even though protein abundance in the gel and peptide abundance as detected with mass spectrometry cannot be correlated quantitatively, it is obvious that CD98, which was only represented by one low abundant peptide throughout the study, is less abundant in the gel than CD71, of which 17 peptides had been detected in sometimes very high abundance. Consequently, the efficiency with which those proteins were cross-linked to CD4 is not correlated with the level of cell surface expression of those proteins: CD98 was the most abundant protein on the cell surface, but only one CD98-derived peptide was detected per experiment.

FRET Experiments Detect Clusters of CD71 Molecules on CemT4 Cells. Fluorescence resonance energy transfer (FRET) technology has become a versatile tool for the study of protein−protein interactions especially because it can be used for membrane protein interactions on the surface of living cells. FRET is a physical phenomenon that involves

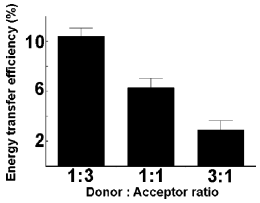

FIGURE 6: FRET detection of clustering of the CD71 molecule. Energy transfer efficiencies were measured on CemT4 cells that were labeled with different ratios of fluorescein- and Cy3-labeled WM82 antibodies. Energy transfer efficiencies are shown for different ratios of donor-labeled antibodies to acceptor-labeled antibodies.

energy transfer from an excited donor molecule (e.g., fluorescein) to a suitable acceptor molecule (e.g., Cy3 or rhodamine). The transfer efficiency is highly dependent on distance and does not take place if the donor and acceptor are separated by more than 5−10 nm, thus acting as a molecular ruler to investigate the proximity between cell surface proteins. FRET is measured as energy transfer efficiency (*E*), which is the percentage of excited donor molecules that transfer their energy to an acceptor molecule. An *E* value of more than 5−7% indicates a protein interaction (*18*, *27*). Here we measure FRET using flow cytometry and fluorescein- and Cy3-labeled monoclonal antibodies as described extensively in the literature (*10*, *28*−*31*).

To investigate if CD71 forms clusters on CemT4 cells, as has been reported for other cell types (*28*), we conducted FRET measurements using the CD71 specific WM82 antibody labeled with either fluorescein or Cy3. Figure 6 shows energy transfer efficiencies measured for CemT4 cells that were labeled with mixtures of fluorescein- and Cy3-labeled WM82 antibodies employing different donor-to-acceptor ratios. Significant FRET efficiencies could be detected that vary with the donor-to-acceptor ratio. Experiments using WM82 as the acceptor in combination with anti-CD4 antibodies as donor molecules yielded FRET efficiencies of <5% (see below), showing that the energy transfer efficiencies observed between CD71 molecules are not a result of the high acceptor surface concentration. This behavior is characteristic for FRET determination of receptor clustering (*29*). However, it indicates no strong association between CD4 and CD71, suggesting only a small fraction of CD4 associates with CD71.

PMA Treatment of Cells Induces an Association between CD4 and CD71. The cell activator PMA is known to induce CD4 endocytosis in lymphoid cells (*32*). To further investigate whether the association between CD4 and CD71 is mediated through their endocytic properties, we investigated cell surface expression of CD4 and CD71 as well as energy transfer efficiencies between CD4 and CD71 before and after treatment with PMA or a mock treatment. Figure 7 shows expression of CD4 and CD71 before and after activation, showing a reduction in the level of CD4 detection in both samples with the reduction being slightly stronger when cells were treated with PMA. The level of expression of CD71 is similarly reduced after PMA and mock treatment. Figure 8 shows the energy transfer efficiency between CD4 and CD71 before and after activation, and it can clearly be seen that only after PMA treatment can a significant association between CD4 and CD71 be detected.

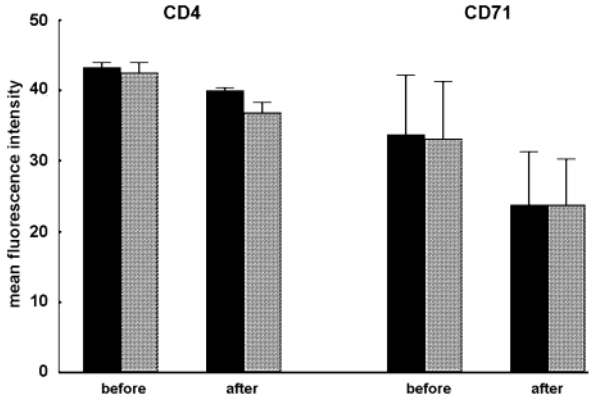

FIGURE 7: Cell surface expression of CD4 and CD71 before and after treatment for 8 min with PMA (gray bars) or mock treatment (black bars) was assessed using the fluorescein-conjugated Q4120 antibody and detection at 530 nm or the Cy3-conjugated WM82 antibody and detection at 585 nm. Shown are the mean fluorescence intensities of three independent experiments.

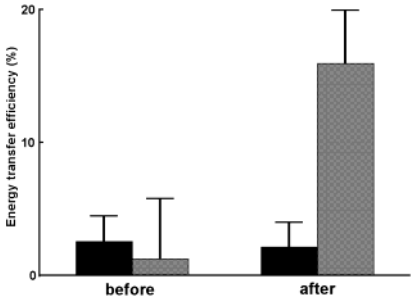

FIGURE 8: Energy transfer between CD4 and CD71 before and after treatment for 8 min with PMA (gray bars) or mock treatment (black bars) was assessed using fluorescein-conjugated Q4120 and Cy3-conjugated WM82 antibody. Shown are the mean energy transfer efficiencies of three independent experiments.

DISCUSSION

Membrane proteins have very important functions, for example, in cell signaling, in growth inhibition, and as virus receptors. However, membrane proteins are intrinsically difficult to study because, for example, detergents are required for solubilization of the proteins which, in turn, can interfere with their interactions. Further, their function is more subtly controlled by the membrane environment in which they reside. Participation in, or exclusion from, lipid rafts has an influence on protein function (*33*), as does the conformation and other proteins that permanently or transiently associate with the membrane (*10*). Detecting proteins that reside in the proximity of a protein of interest can provide crucial information about how the function of a protein is influenced by those proteins. Standard methods such as immunoprecipitation are not always suited for detecting such associating proteins as they rely on membrane solubilization, which is likely to interfere with weak lateral protein associations. Chemical cross-linking is a tool that can maintain protein associations because the proteins become covalently connected. In conjunction with immunoprecipitation, cross-linking can be used to detect proteins that are associated in the cell membrane. We aimed to develop a strategy based on this method that allows *de novo* identification of proteins that can be cross-linked to the cell surface receptor CD4. Lateral associations of CD4 are especially important because of its function in the immune

system and as a receptor for HIV-1. There is still controversy about its function as a receptor for IL-16 as researchers have postulated that the real IL-16 receptor is a different molecule in the proximity of CD4 (*34*). Differences in susceptibility to different HIV-1 strains that cannot be explained by differences in tropism (*35*) could also be connected to different CD4 associating proteins in the membrane.

To cross-link adjoining proteins on the cell membrane, we used a protocol employing DSP that has previously been used to determine the oligomerization state of CD45 (*36*). DSP is a cross-linker with a short range of only 12 Å, which minimizes nonspecific cross-linking. To maximize solubilization of cross-linked complexes, we used a detergent combination containing SDS that had been shown to efficiently solubilize membrane proteins (*2*). We then investigated whether CD4 is still bound by the Q425 antibody after cross-linking, as this is a prerequisite for a purification of CD4-containing complexes generated by cross-linking. Using small-scale immunoprecipitations with Q425 followed by detection of CD4 by PolyT4-5, we found a significant reduction in the amount of CD4 that was precipitated with Q425. Such a reduction is likely to originate from the modification of lysine residues by the cross-linking reaction that leads to a poorer recognition of CD4 both by the Q425 antibody and by the PolyT4-5 antibody. Still, it can be seen that a large fraction of CD4 becomes part of high-molecular mass complexes that are present when surface molecules had been cross-linked. Reduction cleaves the cross-linker and yields monomeric CD4. Without cross-linking, the majority of CD4 remains in its monomeric form. The above result suggested that part of CD4 is indeed cross-linked into complexes with a sufficiently high molecular mass such that the complexes only just migrate into the very top section of the gel. Following purification of CD4-containing complexes out of a large number of cells, three transmembrane proteins CD45, CD71 (transferrin receptor), and CD98 (4F2 lymphocyte antigen) were identified along with CD4, lck, and in one of three experiments annexin II.

The detection of CD71 and CD45 together with CD4 is very unlikely to arise from nonspecific cross-linking with CD4 on the cell surface. If this were the case, the level of cell surface expression of those molecules should have correlated with the efficiency with which those proteins were cross-linked to CD4. Obviously, a highly abundant protein is more likely to be cross-linked to CD4 after nonspecific lateral diffusion than less abundant molecules. However, this is not the case here as CD98 was the most abundant molecule of those three on the surface of CemT4 cells in comparison with CD71 and CD45, but only one low-abundance peptide from CD98 was detected by MS/MS, showing that the method is not in some way artificially favoring the abundant molecules. CD71, however, is much less prominent on the cell surface but is cross-linked extremely efficiently to CD4 as shown by the detection of 17 CD71-derived peptides in the three experiments. Furthermore, given the large number of cell surface molecules, if nonspecific cross-linking were significant, one would then expect a broad band of many molecules in small amounts copurifying with CD4. Such a very high background level of tryptic peptides was derived from many nonspecifically cross-linked proteins and would make any MS/MS analysis extremely difficult. This was not the case, however, as peptides from CD45 and CD71 could easily be distinguished from the background, and MS/MS spectra were of a very good quality. Furthermore, cross-linking at 4 °C should result in a reduction in the lateral mobility of cell surface molecules and therefore should produce a decrease in the level of cross-linking of proteins that have no affinity for each other. Little reduction was observed for CD71 and CD45, supporting the notion that we are observing specific cross-linking. The possibility that CD71 or CD45 is nonspecifically binding to the resin can also be excluded. We have conducted extensive unrelated studies searching for molecules that are copurified with CD4 without cross-linking under similar conditions, but we were never able to identify CD45, CD71, or CD98 in any of those experiments (ref *19* and unpublished observations). Annexin II, however, was identified without cross-linking (unpublished results), and given its described ability to bind calcium ions, it is most likely to bind nonspecifically to the Q425 antibody via a calcium bridge. CD98, which is the most abundant molecule on the cell surface of the three, was only once detected in high abundance, and then only one peptide was detected so that nonspecific cross-linking for this protein cannot be excluded.

Our results suggest that there is an association of CD4 with both CD45 and CD71 on the cell surface. The interaction or association between CD4 and CD45 has been reported previously using techniques such as cocapping (*37*), co-immunoprecipitation (*38*), and FRET measurements (*18*). CD45 is thought to play a role in controlling the kinase activity of lck via its function as a tyrosine phosphatase. Whether the CD4−CD45 interaction involves direct or indirect binding has been controversial, and it has been suggested that the T cell receptor and an associated protein mediate the interaction between CD4 and CD45 (*39*). Were this the case, we would have expected the proteins to be cross-linked to CD4 and identified in this study. Our results therefore suggest that the CD4−CD45 interaction is direct or that the bridging molecule is transferrin receptor CD71 forming a cluster with CD4 and CD45.

The human transferrin receptor CD71 is a ubiquitous type II transmembrane glycoprotein. The number of CD71 molecules on the cell surface depends on the cell type and activation state (*40*). The receptor plays a role in iron metabolism and regulates the cellular uptake of iron, which is thought to control the proliferation of T cells (*41*). A direct CD71−CD4 association has not been reported, although noncovalent complexes of CD71 with CD4, CD3, CD45, and a range of other molecules on lymphocytes have been found (*40*). The transferrin receptor has been shown to form clusters on a number of cell types that incorporate various other molecules such as HLA-A, -B, and -C (*27*). The large number of peptides we identified from CD71 and the good quality of the MS/MS spectra suggest that a significant amount of CD71 was cross-linked to CD4. We have also shown that a part of CD4 can be cross-linked to CD71 and that CD71 forms clusters on the investigated cells.

Because of the differences in function between CD4 and CD71, the reason for their association remains elusive, like the described association between CD71 and HLA molecules (*27*). However, Matyus and co-workers, who reported the association between CD71 and the HLA molecules, suggested an explanation based on the endocytic properties of the molecules. Both HLA and CD71 molecules are internal-

ized via coated pits and coated vesicles on the cells where an association between them had been shown, indicating that the interaction results from clustering of the molecules during the endocytic process (*27*). On T lymphocytes and T cell lines, CD4 is being actively endocytosed because of a dileucine motif on its cytoplasmic tail. However, only a very small fraction of CD4 is endocytosed at any time because of the interaction with p56lck, which prevents targeting of CD4 to coated pits (*9*). CD71 carries a different endocytic signal on its cytoplasmic tail, but both signals target proteins to similar endocytic compartments (*42*). To investigate whether the observed association of a fraction of CD4 with clusters of CD71 is because of their endocytic properties, we investigated the association between CD4 and CD71 after treatment with PMA. PMA is a cell activator that strongly enhances endocytosis of CD4 in lymphoid cells (*32*). Thus, if the association of CD4 and CD71 were because of their combined endocytosis, a stronger association would be detectable after PMA stimulation and hence a fuller participation of CD4 in the endocytosis process. Using FRET measurements, we observed a significant increase in the level of energy transfer between CD4 and CD71 after PMA treatment, demonstrating that an association of those molecules can be induced via stimulating CD4 endocytosis. The observed energy transfer was not a result of a PMA-induced reduction in the level of CD4 expression because the energy transfer was dependent on the difference in the fluorescence signal between the donor and the FRET sample, both of which were treated with PMA. CD4 levels decreased only slightly in the PMA-treated and mock treated sample, with the decrease being slightly more intense in the PMA-treated sample where an increased level of CD4 endocytosis was expected. The similarity between the mock and PMA treated samples relates to the labeling of CD4 prior to PMA-induced endocytosis and/or CD4 still present in open endocytic pits. CD71 expression was monitored to determine whether the energy transfer efficiency was a result of a change in CD71 levels. However, despite a significantly decreased level of detection of CD71 after the (mock) activation, no difference was observed between the PMA-treated the mock treated samples. These results suggest that in unstimulated cells only CD71 is subject to bulk endocytosis and that a small fraction of CD4 is being endocytosed at the time, resulting in the detected cross-linking with CD71 clusters. These clusters are also targeted for endocytosis in the same coated pits. Treatment of cells with PMA then leads to a large portion of CD4 being targeted for endocytosis, and a significant association can be detected with FRET. Figure 9 shows a schematic diagram of the cell surface arrangement of the three molecules CD4, CD45, and CD71 without activation according to our proposed interpretation. Whereas the majority of CD4 is not associated with other cell surface molecules, a fraction of it is associated with the tyrosine phosphatase CD45. CD71 forms clusters on the cell surface and probably becomes associated with CD4 during endocytosis via coated pits. Future studies will examine colocalization in clathrin-coated pits and forming endocytic vacuoles by immunoelectron microscopy.

Several other cell surface molecules have been described as being associated with CD4, most importantly the T cell receptor (*39*, *43*) and HIV coreceptor CCR5 (*44*). However, we could not detect any peptides derived from these proteins probably because either such associations are with cytoplasmic domains or cross-linking of their external domains with CD4 is difficult. The association with AP-2 that mediates CD4 endocytosis (*9*) was not detected because it is characterized by a very weak affinity and the cross-linking occurred mainly extracellularly. The expression and associations of cell surface molecules may be altered on malignant cell lines (*45*) compared to those on resting native T cells. Further studies will therefore concentrate on primary or activated cells to address these questions.

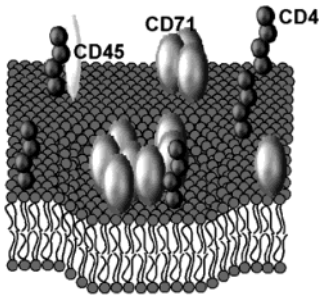

FIGURE 9: Hypothetical arrangement of cell surface molecules CD4, CD45, and CD71 according to our interpretation of the reported data. Whereas the majority of CD4 is not associated with other cell surface molecules, a fraction of it associates with the phosphatase CD45. When undergoing endocytosis, CD4 is targeted to coated pits (indicated by a developing pit at the front) where it becomes associated with clusters of CD71 molecules that are also targeted for endocytosis into the same pit. The sizes of the molecules are not to scale.

In summary, we have used a novel method to investigate association of CD4 with external domains of membrane proteins and have confirmed the previously reported association with CD45 and identified a new one, with CD71 within clathrin-coated pits on forming endocytic vacuoles. These results contribute to the task of defining the complex and changing quaternary associations of cell surface molecules and demonstrate the general utility of combining cross-linking with mass spectrometry.

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SUPPORTING INFORMATION AVAILABLE

MS/MS spectra of one peptide each from CD4, CD71, CD98, and lck, sequences of all peptides identified via MS/MS, and a list of excised gel slices and the proteins found therein. This material is available free of charge via the Internet at http://pubs.acs.org.

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