Proteomic and Biochemical Analyses of Human B Cell-derived Exosomes

POTENTIAL IMPLICATIONS FOR THEIR FUNCTION AND MULTIVESICULAR BODY FORMATION*

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Exosomes are 60–100-nm membrane vesicles that are secreted into the extracellular milieu as a consequence of multivesicular body fusion with the plasma membrane. Here we determined the protein and lipid compositions of highly purified human B cell-derived exosomes. Mass spectrometric analysis indicated the abundant presence of major histocompatibility complex (MHC) class I and class II, heat shock cognate 70, heat shock cognate 70 and 

α and β, actin, G0, and a multitude of other proteins. An α-integrin may direct B cell-derived exosomes to follicular dendritic cells, which were described previously as potential target cells. Clathrin, heat shock cognate 70, and heat shock protein 90 may be involved in protein sorting at multivesicular bodies. Exosomes were also enriched in cholesterol, sphingomyelin, and ganglioside GM3, lipids that are typically enriched in detergent-resistant membranes. Most exosome-associated proteins, including MHC class II and tetraspanins, were insoluble in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS)-containing buffers. Multivesicular body-linked MHC class II was also resistant to CHAPS whereas plasma membrane-associated MHC class II was solubilized readily. Together, these data suggest that recruitment of membrane proteins from the limiting membranes into the internal vesicles of multivesicular bodies may involve their incorporation into tetraspanin-containing detergent-resistant membrane domains.

Maturing endosomes accumulate vesicles in their lumen, resulting in their transformation into multivesicular bodies (MVB).

Endosomal limiting membrane and contain a selected cargo. Proteins that are sorted to the internal vesicles of MVB potentially may have three distinct fates. The first possibility is exemplified by ligand-activated epidermal growth factor receptor, which is ultimately transferred to lysosomes for degradation (2). A second possibility is that proteins may be stored temporarily in MVB, as observed for MHC class II in immature dendritic cells (3). MHC class II-carrying MVB in dendritic cells have also been termed MHC class II compartments (MIIC), in accordance with similar structures in B cells (4).

Exosomes are released by a great number of cell types, including reticulocytes (5), cytotoxic T cells (8), B lymphocytes (9, 10), dendritic cells (11–13), mast cells (14), platelets (15), and intestinal epithelial cells (16). The biological functions of exosomes are generally unclear. Increasing evidence, however, suggests that exosomes from hematopoietic cells may serve as intercellular communication vehicles that assist immune responses (6, 7). For example, B cell-derived exosomes that carry peptide-loaded MHC class II were demonstrated to stimulate CD4+ T cells (17) and to specifically bind follicular dendritic cells (18) in vitro. Furthermore, exosomes derived from cultured dendritic cells that were loaded in vitro with tumor-derived peptides on MHC class I stimulated cytotoxic T lymphocytes both in vitro and in vivo (11).

Functions of exosomes should be reflected by their protein composition. Given that exosomes are formed as the internal vesicles of MVB, exosomes can be expected to also contain factors required for MVB formation and protein sorting therein. Immunelectron microscopic studies, Western blot analyses, and peptide mass mapping of exosomes derived from dendritic cells (12, 13), B lymphocytes (9, 10), intestinal epithelial cells (16), and other cell types revealed the presence of common, as well as cell type-specific, proteins. For example,
MHC class II is especially enriched in exosomes derived from B lymphocytes, dendritic cells, mast cells, and intestinal epithelial cells. Ubiquitous proteins in exosomes include cytoplasmic proteins, such as tubulin, actin, and actin-binding proteins, the heat shock proteins hsc70 (also named hsc71 or hsp73) and hsp90, and trimeric G proteins, as well as membrane proteins, such as members of the tetraspanin family (CD9, CD63, CD81, CD92). Sorting of a number of membrane proteins into the MVB pathway involves ubiquitination of their cytoplasmic domain (19) and binding of these acquired ubiquitin moieties to Tsg101 (20). Indeed, Tsg101 (12), as well as c-Chi (21), a ubiquitin ligase required for ubiquitination of activated epidermal growth factor receptor (2), have been detected in isolated exosomes. Alternatively, membrane proteins may rely on ubiquitinated adaptors for their sorting into MVB (20, 22). Importantly, sorting of at least some proteins into the MVB pathway occurs independently of the ubiquitin system (23). The molecular factors and mechanism(s) behind such alternative sorting processes in MVB are unknown, and the analysis of exosomes may help their discovery.

In an approach to understand more about the formation and function of exosomes we developed a protocol that yielded highly purified exosomes from human B cells and studied their molecular content and biochemical properties. Based on exosome characteristics, we propose a model in which the incorporation of proteins into tetraspanin networks and detergent-resistant membranes (DRM) at the limiting membrane of MVB may be conditional for their sorting into the internal vesicles of MVB.

### MATERIALS AND METHODS

**Antibodies, SDS-PAGE, and Western Blotting—Rabbit anti-MHC class II (24)** was kindly provided by Dr. H. L. Ploegh (Harvard Medical School), rabbit anti-MHC class II α-chain was provided by Dr. N. Barois (INSERM-CNRS de Marseille-Luminy), and the mouse monoclonal antibodies anti-CD81 (clone M38) and anti-CD82 (clone C33) were provided by Dr. O. Yoshie (Kinki University School of Medicine, Osaka, Japan). Mouse monoclonal anti-MHC class II (CRI/43) was from DAKO (Glostrup, Denmark), mouse monoclonal anti-CD86 (BU36) was from Ancell (Lauflingen, Switzerland), mouse monoclonal anti-CD63 (435; CLB12) was from CLB (Amsterdam, The Netherlands), and polyclonal rabbit anti-biotin was from Sanver Tech (Beechout, Belgium). For the analysis of exosomes and cell fractions by SDS-PAGE and Western blotting, samples were incubated for 15 min at 65 °C in urea-containing sample buffer (5% SDS, 9 M urea, 10 mM EDTA, 5% β-mercaptoethanol, 120 mM Tris-HCl, pH 6.8). Proteins were separated on 12.5, 10, and 7.5% polyacrylamide gels (SDS-PAGE). For Western blotting, proteins were transferred from polyacrylamide gels to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were blocked and probed with antibodies in PBS containing 5% (v/v) non-fat dry milk (Protivar; Nutricia, Zoetermeer, The Netherlands) and 0.1% (w/v) Tween 20. Primary antibodies were probed with horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark) and detected with enhanced chemiluminescence (Roche Molecular Biochemicals). Cell Culture and Exosome Isolation—RN cells (HLA-DR15) were cultured as described (9). We observed that fetal calf serum contains exosomes (data not shown). To exclude bovine exosomes, the developed plate was cultured in medium supplemented with fetal calf serum that had been ultracentrifuged for 60 min at 141,000 × gmax. RN-derived exosomes were isolated routinely from 800 ml of culture medium containing ~10⁹ RN cells. As a first isolation step, exosomes were collected from the medium by differential centrifugation, as described (9). In short, cells were removed by centrifugation for 10 min at 2000 × g, Supernatants were collected and centrifuged sequentially twice for 10 min at 5000 × gmax once for 15 min at 20,000 × gmax once for 30 min at 100,000 × gmax and once for 60 min at 70,000 × gmax using a SW27 rotor (Beckman Instruments, Inc., Fullerton, CA). Exosomes were pelleted at the final centrifugation step and were resuspended in PBS and re-pelleted at 70,000 × gmax. The final pellet was resuspended in 5 ml of 2.6 M sucrose, 20 mM Tris-HCl, pH 7.2, and floated into an overlayer linear sucrose gradient (2.0–0.25 M sucrose, 20 mM Tris-HCl, pH 7.2) in a SW41 tube for 16 h at 270,000 × gmax. Gradient fractions of 1 ml were collected from the bottom of the tube and analyzed for the presence of MHC class II and α-HA by Western blotting. When indicated, gradient fractions were diluted with 3 ml of PBS each and centrifuged for 60 min at 350,000 × gmax and the pellets were analyzed by SDS-PAGE and Coomassie Blue staining. As a final purification step, 750-μl samples of pooled exosome-containing gradient fractions were added to 200 μl of Dynabeads M-450 (~8 × 10⁷ beads) coated with monoclonal mouse anti-human MHC class II (Dynal Biotech, Oslo, Norway). As a negative control, Dynabeads M-450 coated with goat anti-mouse IgG (Dynal) were used. The Dynabeads that were added to the exosomes suspensions were first extensively washed with and resuspended in PBS supplemented with 3 mg/ml bovine serum albumin. For adsorption, samples were rotated end-over-end for 16 h at 4 °C. The beads were washed extensively with PBS with the aid of a magnet (Dynal). Non-adsorbed membranes were diluted with PBS and collected by centrifugation for 30 min at 200,000 × gmax in a SW50 tube. Mass Spectrometric Protein Analyses—Proteins from Dynabead-associated exosomes were segregated by SDS-PAGE, stained with Coomassie Blue, excised from the gels, and analyzed by mass spectrometry by Protana (Denmark). In-gel tryptic digestion of proteins was performed as described (25). Approximately 2% of the tryptic digest was analyzed on a Bruker Reflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany), and the obtained peptide maps were queried against a non-redundant sequence data base. Search criteria was as follows: mass accuracy, 50 ppm; tryptic peptides, allowed missed cleavage one. Samples not identified in database 1. Samples not identifiable in database 2. Peptide sequences were analyzed using a QSTAR quadrupole-TOF mass spectrometer (Sciex) equipped with a nanoelectrospray source (Protea-Engineering). Prior to analysis, the mass spectrometer was calibrated to a mass accuracy of 20 ppm and a resolution of 9500. The data were processed with PPS2 (Protea’s Proteomics Software Suite), and the peptide sequence tags obtained were queried against a non-redundant sequence data base (26). Search criteria were as follows: MS mass accuracy, 1.1 Da; MS/MS accuracy, 0.1 Da; tryptic peptides, allowed missed cleavage sites, 1. For verification of a retrieved peptide sequence theoretical pattern was compared with the obtained collision-induced dissociation mass spectra. Lipid Analysis—PBS-washed RN cells, Dynabead-associated exosomes, and ultracentrifuged non-adsorbed membranes were suspended in a total volume of 3 ml of chloroform/methanol (1:1; v/v), and lipids were extracted overnight at 40 °C. The suspensions were then centrifuged for 10 min at 2000 × g, and the clear supernatants were dried in a stream of nitrogen. The residue was dissolved by first adding 60 μl of chloroform followed by 0.96 ml of methanol and 0.94 ml of water. Each resulting solution was freed of salts and sucrose by reverse phase chromatography. For this procedure small pieces of silanized glass fiber wadding were introduced into glass Pasteur pipettes, and a suspension of 3 mg Chloroprep RP1 (27) in 0.2 ml of chloroform followed by 0.96 ml of methanol and 0.94 ml of water. Each resulting solution was freed of salts and sucrose by reverse phase chromatography. For this procedure small pieces of silanized glass fiber wadding were introduced into glass Pasteur pipettes, and a suspension of 3 mg Chloroprep RP1 (27) in 0.2 ml of chloroform followed by 0.96 ml of methanol and 0.94 ml of water. Each resulting solution was freed of salts and sucrose by reverse phase chromatography.

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were eluted with chloroform/methanol/1 M ammonium acetate (3:1:1 by volume) and freed of salts, short chain fatty acids, and other less hydrophobic material by reverse phase chromatography on LiChroprep RP18 as described above.

For electrospray-MS, mass spectra were recorded in the negative or positive ion mode for acidic or uncharged lipids, respectively, on a Q-TOF 2 mass spectrometer (Micromass, Manchester, United Kingdom) operated with a nanospray source. Lipids were dissolved in chloroform/methanol (1:1) (v/v). Solutions were injected into the mass spectrometer by glass capillaries (long type; Protana, Odense, Denmark) using a capillary voltage of 1000 V and a cone voltage of 50 V at 70 °C. Instrument calibration was done with a mixture of sodium iodide and cesium iodide in 50% aqueous acetonitrile with 0.1% formic acid. For MS/MS experiments argon was used as collision gas, and fragmentation was observed at energy values from 20–50 eV.

For MALDI-TOF-MS, measurements were done on a TOFSpec E (Micromass, Manchester, United Kingdom) in positive or negative ion mode with an accelerating voltage of 20 kV. For lipid samples matrix solutions of 2,5-dihydroxybenzoic acid were used in a concentration of 10 mg/ml in methanol. Spectra were calibrated externally by using suitable reference substances.

Density Gradient Electrophoresis and Cell Surface Biotinylation—RN cells were collected and washed with PBS by centrifugation at 4 °C for 10 min at 200 × g. The cell surface was biotinylated for 15 min at 0 °C using 5 mg/ml sulfo-NHS-biotin (Pierce). Excess sulfo-NHS-biotin was quenched with 10 mM NH4Cl in PBS for 20 min at 0 °C after which the cells were collected and washed twice with homogenization buffer (1% Triton X-100, 1 mM EDTA, 0.25 M sucrose, pH 7.4) by centrifugation. Cells were homogenized using an EMBL-cell cracker (ball 8.011 applying 10 strokes), and nuclei were removed by centrifugation at 900 × g for 3 min. The post-nuclear supernatant was treated with trypsin (25 μg/ml protein) for 15 min at 37 °C after which trypsin inhibitor (100 μg/ml protein; Sigma) and protease inhibitor mix (Roche Molecular Biochemicals) were added. Density gradient electrophoresis was performed as described (27) for 30 min at a constant current of 10 mA. Fractions of 500 μl were collected from the anodic side and analyzed by Western blotting for MHC class II β-chain. Plasma membrane proteins were detected by probing Western blots with streptavidin-peroxidase (Sigma) and enhanced chemiluminescence (Amersham) for 1 h. Membrane protein bands that sedimented between 300,000 and 4,200,000 g were excised by using molecular weight markers.

RESULTS

Purification of Secreted Human Exosomes—Exosomes were purified from the human B cell line RN as described in three sequential steps. In the first step we performed differential centrifugation to collect membranes from the culture medium that sedimented between 300,000 and 4,200,000 g (10, 12). In the second purification step pelleted exosomes were washed and floated into sucrose density gradients to remove non-membranous serum protein (complexes). Exosomes, as identified by the presence of MHC class II, floated up to a density of 1.15 g/ml (Fig. 1A), consistent with previous observations (9, 10). The gradient fractions were diluted with PBS and ultracentrifuged, and the pellets were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 1A). The sample buffer used for SDS-PAGE was supplemented with urea (see “Materials and Methods”) as we found that this greatly enhanced the separation of exosome-associated proteins by SDS-PAGE. A number of proteins, possibly originating from fetal calf serum in the culture medium, remained in the bottom fractions (indicated by the arrows) whereas the majority of proteins co-distributed with MHC class II in the gradient. In addition to MHC class II, these proteins include CD86 and the tetraspanins CD37, CD53, CD63, CD81, and CD82 (data not shown) as identified previously (10) by Western blotting. As a third purification step, gradient fractions containing MHC class II (fraction 6–8) were pooled, and exosomes were immunoabsorbed onto anti-MHC class II-coupled magnetic beads. Membranes that did not associate with the beads were collected from the bead supernatant by ultracentrifugation. Bead-associated and non-associated proteins were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 2). The anti-MHC class II antibody-conjugated beads recovered nearly all proteins whereas beads coated with negative control antibodies did not collect any of these proteins. This indicates that all detected proteins were linked physically to MHC class II-carrying exosomes.

Protein Composition of Exosomes—To analyze the identity of
Lipid Composition of B Lymphocytes and Exosomes—Lipids extracted from [1-14C]sodium acetate-labeled lymphocytes were separated according to charge and relative mobility by TLC, stained, and quantified (see Fig. 3, lane 2 and Table II). The nature of these lipids was identified in parallel experiments (32). To investigate whether esosomes display DRM-like properties we determined their solubility in the presence of 1% Triton X-100 or 1% CHAPS (Fig. 5). Sucrose gradient fractions containing esosomes (as in Fig. 1) were pooled and washed with PBS. Aliquots were incubated as indicated for 30 min at 0 or 37 °C in the presence or absence of 1% CHAPS, 1% Triton X-100, 1 mM EDTA, 1 mM MgCl2, and/or 10 mM methyl-β-cycloextrin and then centrifuged. Pellets were analyzed for total protein content by SDS-PAGE and Coomassie Blue staining (Fig. 5). The solubility of some proteins slightly increased on secreted esosomes and much less abundant on the MIIC-limiting membrane and plasma membrane. We conclude, based on the morphological and biochemical characterization of esosomes, that esosomes are relatively enriched in cholesterol.

Electron Microscopic Detection of Cholesterol on Exosomes—To determine the morphological distribution of cholesterol, cryosections of RN cells were labeled for cholesterol with 10 nm of colloidal gold using Perfringolysin O and examined by electron microscopy (30). The sections were double-labeled for either MHC class II (15 nm of gold) or CD63 (15 nm of gold). MHC class II was detected on the plasma membrane, in MIIC, and on esosomes within MIIC-plasma membrane fusion profiles. Consistent with previous observations (10), CD63 was found predominantly on the internal membranes of MIIC and esosomes rather than on the plasma membrane. Fig. 4 shows fusion profiles of MIIC with the plasma membrane. Consistent with the lipid analyses, cholesterol was predominantly present on secreted esosomes and much less abundant on the MIIC-limiting membrane and plasma membrane.

Proteomic and Biochemical Analyses of B Cell-derived Exosomes

Exosomal proteins, discernable Coomassie Blue-stained bands in Fig. 2, lane 1 were excised from the gel and analyzed by mass spectrometry. Identified proteins are indicated in Fig. 2 and listed in Table I. Only proteins with a minimum of two matching peptides are shown. HLA-encoded proteins, including MHC class I heavy chain and several MHC class II subtypes, are dominantly present in esosomes. Other membrane proteins that were identified include Na+/K+-ATPase, the receptor tyrosine phosphate CD45, integrin α4, and the receptor-associated inhibitory signaling molecule Qa, which is linked to the cytoplasmic face of membranes by a palmitoyl anchor. Other identified proteins can be grouped in heat shock proteins (hsp90α and hac70), cytoskeletal proteins (α and β tubulin and actin), a member of the ERM (ezrin-radixin-moesin) family of cytoskeleton-associated proteins (moesin) and a set of enzymes involved in glycolysis (glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, α-enolase, and fructose-bisphosphate aldolase A). Clathrin heavy chain-1 and elongation factor 1A were detected, as well. Murine immunoglobulin heavy chain originated from the anti-MHC class II antibody that was conjugated to the Dynal beads. Tetraspanins were not detected by mass spectrometry, possibly because of low abundance and/or their poor resolution characteristics by SDS-PAGE (10).

Lipid Composition of B Lymphocytes and Exosomes—Lipids extracted from [1-14C]sodium acetate-labeled lymphocytes were separated according to charge and relative mobility by TLC, stained, and quantified (see Fig. 3, lane 2 and Table II). The nature of these lipids was identified in parallel experiments in which the position of 14C-labeled lipids was determined on film rather than by staining. These were then extracted from the TLC plates and analyzed by electrospray-time-of-flight-mass spectrometry and MALDI-TOF-MS (Tables III and IV). Among the acidic lipids were ganglioside GM3 with palmitoyl or nervonoyl residues. The majority of acidic lipids were, however, composed of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), bismonoacyl glycerophosphate (BMP), and cardiolipin (CL) with varying fatty acyl moieties. Their fatty acid composition was deduced from tandem mass spectra collected in the negative ion mode. The amount of BMP exceeded that of CL (see Fig. 3A). The uncharged lipids were comprised of sphingomyelin (SM), cholesterol (Chol), phosphatidyl choline, phosphatidylethanolamine (PE), and ether lipids with an ethanolamine phosphoryl head group. Like GM3, sphingomyelin contained either palmitoyl or nervonoyl residues, resulting in two distinct bands by TLC. SM containing C16:0 was not separated from ganglioside GM3 by TLC (lower band of the double band in Fig. 3A). Dynabead-bound esosomes and membranes from esosome-containing sucrose gradient fractions that did not bind to anti-MHC class II-coated Dynabeads were analyzed for their lipid content and compared with total cell membranes (see Fig. 3 and Table II). Esosomes were enriched in cholesterol (42 versus 20 mol % in total cell membranes) in sphingomyelin and ganglioside GM3 (23 versus 13 mol % in total cell membranes) on the expense of the presence of phosphatidyl ethanolamine and its respective ether lipids, as well as phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine, and phosphatidic acid. As expected, cardiolipin, a lipid that is predominantly found in mitochondria, was absent from esosomes. BMP, also referred to as lysobisphosphatidic acid (LBPA), could also not be detected in esosomes.

Electron Microscopic Detection of Cholesterol on Exosomes—To determine the morphological distribution of cholesterol, cryosections of RN cells were labeled for cholesterol with 10 nm of colloidal gold using Perfringolysin O and examined by electron microscopy (30). The sections were double-labeled for either MHC class II (15 nm of gold) or CD63 (15 nm of gold). MHC class II was detected on the plasma membrane, in MIIC, and on esosomes within MIIC-plasma membrane fusion profiles. Consistent with previous observations (10), CD63 was found predominantly on the internal membranes of MIIC and esosomes rather than on the plasma membrane. Fig. 4 shows fusion profiles of MIIC with the plasma membrane. Consistent with the lipid analyses, cholesterol was predominantly present on secreted esosomes and much less abundant on the MIIC-limiting membrane and plasma membrane. We conclude, based on the morphological and biochemical characterization of esosomes, that esosomes are relatively enriched in cholesterol.

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Exosomes Are Detergent-resistant—The enrichment in sphingomyelin, GM3, and cholesterol is a characteristic of so-called DRM or raft domains (31). Such domains are unusually resistant to solubilization by non-ionic detergents (32). To investigate whether esosomes display DRM-like properties we determined their solubility in the presence of 1% Triton X-100 or 1% CHAPS (Fig. 5). Sucrose gradient fractions containing esosomes (as in Fig. 1) were pooled and washed with PBS. Aliquots were incubated as indicated for 30 min at 0 or 37 °C in the presence or absence of 1% CHAPS, 1% Triton X-100, 1 mM EDTA, 1 mM MgCl2, and/or 10 mM methyl-β-cycloextrin and then centrifuged. Pellets were analyzed for total protein content by SDS-PAGE and Coomassie Blue staining (Fig. 5A) and for the presence of MHC class II by Western blotting (Fig. 5B). Many exosomal proteins, but not all, were resistant to solubilization by CHAPS, independently of divalent cations or temperature. The solubility of some proteins slightly increased when cholesterol was chelated with methyl-β-cycloextrin at 37 °C, suggesting that cholesterol is important for exosomal DRM. The solubility of esosomes was higher in Triton X-100

Fig. 2. Immunopurification of exosomes. Exosomes, isolated by differential centrifugation and floatation into sucrose gradients (as in Fig. 1), were immunoadsorbed to Dynal beads coated with anti-MHC class II or control IgG. The beads were collected and analyzed by 7.5% (lanes 1–4) or 12.5% (lane 5) SDS-PAGE and Coomassie Blue-stained. Essentially all proteins were immunoadsorbed to anti-MHC class II-coated beads (lanes 1 and 5), and little protein remained in the supernatant (lane 3). In contrast, essentially all protein remained in the supernatant (lane 4) of control IgG-coated beads (lane 2), indicating the specificity of the procedure. Coomassie Blue-stained proteins were excised from lanes 1 and 5 and analyzed by nanoelectrospray tandem mass spectrometry. Identified proteins are indicated in the figure, as well as in Table I. mIgHC is immunoglobulin-derived from the mouse anti-MHC class II antibody from the Dynal beads.
compared with CHAPS but remained incomplete. The same observations were made for exosome-associated MHC class II. MHC class II was solubilized to a significant degree only in the concomitant presence of CHAPS and methyl-β-cyclodextrin at 37 °C or in the presence of Triton X-100. DRM are characterized by the stable association of both proteins and lipids and, consequently, have a relative low buoyant density in sucrose density gradients. To test whether DRM are associated with exosomes, we performed flotation experiments for CHAPS-treated exosomes (Fig. 6). Pelleted exosomes were resuspended in 2.5 M sucrose containing 1% CHAPS and overlaid with a sucrose density gradient. After ultracentrifugation, gradient fractions were analyzed for the presence of MHC class II and the tetraspanins CD81 and CD63. About one-third of each of these markers floated up into the gradient, indicating their association with DRM.

The Solubility of MHC Class II Depends on Its Location—
Exosomes are formed as the internal vesicles of MVB/MIIC. Because the majority of MHC class II in MIIC of RN cells localizes to these internal vesicles, we hypothesized that MHC class II in MIIC, like exosomes, should be resistant to solubilization in CHAPS. To test this idea, we isolated MIIC from surface-biotinylated cells by density gradient electrophoresis (Fig. 7), a technique that segregates MIIC from other cellular membranes (33). Each fraction was tested for the presence of total protein, β-hexosaminidase (a marker for MVB and

**TABLE I**

Proteins from Fig. 2 as identified by mass spectrometry

Coomassie Blue-stained bands in Fig. 2; lane 1 were excised from the gel, trypsinized, and analysed by mass spectrometry for peptide mass fingerprints and, when indicated, peptide sequences.

| Name | Accession code | Mass | Matched peptides | Coverage (amino acids) |
|------|----------------|------|------------------|-----------------------|
| MHC class I heavy chain (MHC class I HC) | Q29638 | 40,419 | 8 | 146/362 (40%) |
| MHC class II α-chain (HLA DRA) | V00523 | 19,748 | 4 | 69–81/171 (40–47%) |
| MHC class II β-chain (HLA DR2) | Q29757 | 27,065 | 3 | 72/237 (30%) |
| MHC class II β-chain (HLA DR2.2) | P01911 | 22,964 | 4<sup>a</sup> | 97/198 (49%) |
| MHC class II β-chain (HLA DQB1*0602) | L34105 | 26,335 | 4 | 164/229 (72%) |
| T200 leukocyte common antigen (CD45) | Q16614 | 132,381 | 7<sup>b</sup> | 114/114 (100%) |
| Integrin α4 (VLA 4) | P13612 | 116,760 | 2<sup>b</sup> | 16/1038 (2%) |
| G<sub>α</sub>2z | P04898 | 40,890 | 8<sup>c</sup> | 16/354 (46%) |
| hsp90 or hsp86 | P07900 | 84,542 | 10 | 175/731 (24%) |
| Chitinase | P11142 | 70,888 | 11 | 242/446 (27%) |

<sup>a</sup> When mass fingerprints did not unambiguously match a specific protein, the peptides were sequenced by mass spectrometry.

**TABLE II**

Relative lipid compositions as determined from Fig. 3

Lipids from RN cells (Fig. 3, lane 2), immunosorbed exosomes (Fig. 3, lane 3), and membranes that did not bind to the immunobeads (Fig. 3, lane 4) were stained and quantified by comparison with known amounts of reference lipids. The data are representative of two independent experiments. Values shown are ±5%. The numbers represent relative amounts of lipid with respect to the total of identified lipids in the same membrane preparation. Because of the low amount of material, some lipids, especially those of the non-bound material derived from unlabeled cells, could not be determined (ND). Cholesterol, SM, and ganglioside GM3 are relatively enriched in adsorbed exosomes as compared with total cell membranes on the expense of the relative amount of PE and its respective ether lipids, as well as PC, PI, PS, and PA. Note that BMP (LBPA) and the mitochondrial lipid CL comigrate and were not detected in exosomes.

| Lipid | RN cells | Exosomes | Non-bound |
|-------|----------|----------|-----------|
| Cholesterol | 19.6 | 42.1 | 55.5 |
| PE + ether lipids | 27.5 | 14.6 | ND |
| BMP + CL | 6.3 | ND | ND |
| PC + (PI, PS, PA) | 34.0 | 20.3 | 8.9 |
| SM + GM3 | 12.6 | 23.9 | 35.6 |
| Total | 100 | 100 | 100 |

<sup>a</sup> When mass fingerprints did not unambiguously match a specific protein, the peptides were sequenced by mass spectrometry.

**FIG. 3.** Analysis of lipids from RN cells and exosomes. Lipids from total cellular membranes and Dynal bead-associated exosomes were extracted, subjected to thin-layer chromatography, and stained. Lane 1, reference lipids, their nature is indicated on the left side of the figure. Chol, cholesterol; DOPE, dioleoyl phosphatidyl ethanolamine; BHCL, cardiolipin from bovine heart; DOPC, dioleoyl phosphatidyl choline; SM, sphingomyelin from bovine brain containing stearic or nervonic acid, resulting in a double band by TLC. Lane 2, lipids from total RN membranes. Their nature is indicated at the right side of the figure and was determined in a parallel experiment in which metabolically labeled lipids were extracted from cells, separated by TLC, and analyzed by mass spectrometry. Spot X is a non-identified compound, spot Y is not identified but has the mobility of monohexosylceramides, and spot Z at the front could not be associated with any known lipid and is most likely related to compounds(s) from plastics or other paraphenyls that were extracted by the solvents. Lane 3, lipids from Dynal bead-associated exosomes (isolated as in Fig. 2, lane 1). Lane 4, lipids from non-bound material (as in Fig. 2, lane 3). The quantified relative amounts of lipids are depicted in Table II.
Cholesterol is predominantly present on exosomes. Note the presence of MHC class II on the plasma membrane and on exosomes. Cholesterol is predominantly present on exosomes. B indicates a similar structure with exosomes (E) relatively enriched in both lysosomes, and biotinylated proteins (positioning the plasma membranes) and Western blotted for MHC class II. As expected, most MHC class II was found in plasma membrane-containing fractions (fractions 16–21), but a significant amount was associated with MIIC, as indicated by its co-migration with β-hexosaminidase (fractions 5–10).

To compare exosomes with MIIC for the solubility of MHC class II, samples of pooled MIIC-containing fractions from density electrophoresis gradients and pooled exosome-containing sucrose gradient fractions were diluted with excess PBS, containing either CHAPS or Triton X-100 or lacking detergent. After 30 min at 0 or 37 °C, DRM were pelleted by ultracentrifugation and analyzed for the presence of MHC class II by Western blotting for MHC class II (B). Closed arrows indicate example proteins that were entirely solubilized by detergents, open arrows indicate partially solubilized proteins, and arrow heads indicate non-solubilized proteins. Molecular weight markers are on the right. The data are representative of three independent experiments.
Isolated exosomes were incubated in the absence or presence of CHAPS and layered at the bottom of a sucrose density gradient. After ultracentrifugation, gradient fractions were tested for the presence of MHC class II β-chain, CD81, and CD63 by Western blotting. Bottom fractions are at the left. About one-third of all three markers floated up into the gradient, indicating their association with DRM rafts. The data are representative of three independent experiments.

Western blotting (Fig. 8). Exosomes and MIIC were similar with respect to the solubility of MHC class II (Fig. 8A), consistent with the notion that the majority of MHC class II in MIIC localizes to internal vesicles and that these vesicles are released as exosomes upon exocytic fusion of MIIC with the plasma membrane.

To compare the detergent solubility of MHC class II at the plasma membrane with that of exosomes, intact cells and isolated exosomes were first biotinylated. This procedure allowed selective labeling of the exoplasmic domain of plasma membrane or exosome-associated MHC class II. After the addition of CHAPS, insoluble MHC class II was pelleted by centrifugation and solubilized in Triton X-100-containing PBS. Biotinylated MHC class II was collected using Neutravidin-conjugated Sepharose beads and analyzed by Western blotting. The solubilization of biotinylated MHC class II from exosomes in CHAPS was inefficient (Fig. 8B), possibly even more so than total MHC class II from non-biotinylated exosomes (Fig. 8A). In contrast to exosome-related and MIIC-derived MHC class II, however, plasma membrane-derived MHC class II was entirely solubilized by CHAPS (Fig. 8B). These data indicate that MHC class II is associated with DRM in exosomes and MIIC but not at the plasma membrane. Possibly, the incorporation of MHC class II in DRM at the MIIC-limiting membrane plays a role in its sorting into the MVB internal vesicles.

**DISCUSSION**

We developed a method to purify B cell derived exosomes to homogeneity, thus allowing determination of their protein and lipid composition. In a previous study we already demonstrated the presence of MHC class I and class II on B cell-derived exosomes (9, 10); here we show that they are among the most prominent proteins. MHC class I and class II have also been detected on exosomes from dendritic cells (11), intestinal epithelial cells (16), and T cells (21). MHC class I was also found to associate with tumor-derived exosomes (34), and MHC class II was found to associate with mast cell-derived exosomes (14). Previously, we also demonstrated by immunoelectron microscopy and Western blotting that the tetraspanins CD63, CD37, CD53, CD81, and CD82 are heavily enriched on B cell-derived exosomes and on the internal vesicles of MVB (10). One of these tetraspanins, CD63, is in fact also known as lysosome-associated membrane protein 3 (Lamp 3). Tetraspanins have also been demonstrated on exosomes derived from dendritic cells (11, 12), intestinal epithelial cells (16), T cells (21), and platelets (15). In the current study we failed to detect tetraspanins by the mass spectrometric analyses. Despite their relative enrichment, the only tetraspanin detected previously (12) in exosomes by mass spectrometric analyses is CD9, most likely because tetraspanins cannot be recovered as discrete bands from acrylamide gels (10, 13). Tetraspanins comprise a large group of ubiquitously expressed 25–50-kDa proteins that contain a number of conserved residues (35). Tetraspanins associate with each other, as well as with many Ig superfamily proteins, proteoglycans, integrins, growth factor receptors, and signaling enzymes, to form large transmembrane protein networks. Such networks are involved in a variety of processes at
Heijnen, H. F. G., Slot, J. W., and Geuze, H. J. (2003) at the MVB-limiting membrane. 

chored proteins are also enriched in reticulocytes exosomes (54), 
chored proteins have the tendency to be incorporated into 
have been implicated in HIV, type 1 assembly and release (53). 
macrophages, assemble HIV predominantly at the plasma 
formation of MVB internal vesicles. T cells, in contrast to 
class II and MHC class I localize to membrane microdomains, 
them with raft/DRM-like properties (37).

detergent solubility assays demonstrated the association of 
observed by immunoelectron microscopy that in RN cells BMP 
the internal membranes of MVB (48). Possibly, the amount and 
somes (18) or biochemically in a subcellular fraction containing 
we did not observe detectable amounts of BMP by TLC on 
the plasma membrane.

We found that exosomes are enriched in cholesterol, sphingomyelin, and GM3. These lipids are characteristically enriched in rafts/DRM. These features, together with the pres- 
ence of tetratraspanins and the stable association of lipids with 
chored-solubilized exosomal protein webs, indicate that exo- 
somes contain protein/lipid complexes that can be described as 
exes or DRM rafts. These rafts or DRM rafts may contribute to 
protein sorting in MVB but may also play a role in the gener- 
ation of membrane buds and even in membrane fission (31, 47).

We did not observe detectable amounts of BMP by TLC on 
exosomes. This is seemingly inconsistent with other studies in 
which BMP was detected either immunocytochemically on exo- 
somes (18) or biochemically in a subcellular fraction containing 
the internal membranes of MVB (48). Possibly, the amount and 
chord of BMP is cell type-dependent. Furthermore, we 
observed by immunoelectron microscopy that in RN cells BMP 
chored is enriched on multilaminar lysosomes rather than on MVB. 

Certain viruses, such as human cytomegalovirus (49) and HIV 
in macrophages (50), assemble at MVB in a process resembling 
the formation of MVB internal vesicles. T cells, in contrast to 
macrophages, assemble HIV predominantly at the plasma 
membrane in a process that requires elements of the same 


2 Möbius, W., Van Donselaar, E., Ohno-Iwashita, Y., Shimada, Y., Heijn, H. F. G., Slot, J. W., and Geuze, H. J. (2003) Traffic 4, in press.
shown to bind directly to hsc70, and this interaction may be important for the trafficking of MHC class II in MVB.

The transmembrane protein tyrosine phosphatase CD45 modulates the signal that is transduced via the B cell antigen receptor by regulating the phosphorylation state of Src family kinases and is required for normal B cell development, tolerance induction, and responsiveness to antigen (75). Interestingly, CD45 was found to be absent on T cell-derived exosomes (21). The relevance of the specific association of CD45 with B cell-derived exosomes is unclear. Na+/K+-ATPase is generally present at the plasma membrane. However, its cell surface expression can be regulated by endocytosis, and it plays a regulatory role in the acidification of endosomes and lysosomes (76).

The presence of a c4-integrin on B cell-derived exosomes is intriguing. Exosomes released by maturing reticulocyte also contain α4β1-integrin (77). B cell selection involves their homing to follicular dendritic cells in the germinal center in a process that is dependent on the interaction of α4β1 with VCAM-1 (78). B cell (RN)-derived exosomes bind specifically to VCAM-1 (79).

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