Selective Accumulation of the Heat Shock Protein hsc73

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Abstract. E xosomes are membrane vesicles secreted by hematopoietic cells upon fusion of late multivesicular endosomes with the plasma membrane. Dendritic cell (DC)-derived exosomes induce potent antitumor immune responses in mice, resulting in the regression of established tumors (Zitvogel, L., A. Regnault, A. Lozier, J. Wolters, C. Flament, D. Tenza, P. Ricciardi-Castagnoli, G. R. aposo, and S. A migorena. 1998. Nat. M ed. 4:594–600). To unravel the molecular basis of exosome-induced immune stimulation, we now analyze the regulation of their production during DC maturation and characterize extensively their protein composition by peptide mass mapping. E xosomes contain several cytosolic proteins (including annexin II, heat shock cognate protein hsc73, and heteromeric G protein GI2α), as well as different integral or peripherally associated membrane proteins (major histocompatibility complex class I, Mac-1 integrin, CD9, milk fat globule-EGF-factor VIII [M F G-E 8]). M F G-E 8, the major exosomal component, binds integrins expressed by DCs and macrophages, suggesting that it may be involved in exosome targeting to these professional antigen-presenting cells. A nother exosome component is hsc73, a cytosolic heat shock protein (hsp) also present in DC endocytic compartments. hsc73 was shown to induce antitumor immune responses in vivo, and therefore could be involved in the exosome’s potent antitumor effects. Finally, exosome production is downregulated upon DC maturation, indicating that in vivo, exosomes are produced by immature DCs in peripheral tissues. Thus, DC-derived exosomes accumulate a defined subset of cellular proteins reflecting their endosomal biogenesis and accounting for their biological function.

Key words: endosomes • antigen presentation • dendritic cells • exosomes • heat shock protein

The term "exosome" was originally used to describe small membrane vesicles (50–90 nm diameter) released by reticulocytes during their final stage of maturation into red blood cells (J ohnstone et al., 1987). E xosomes eliminate plasma membrane proteins that are not necessary for mature erythrocyte function, such as transferrin receptor (T FR)† (Pan and J ohnstone, 1983; H arding et al., 1984; J ohnstone et al., 1984) or acetylcholine esterase (J ohnstone et al., 1987). E M studies (Pan et al., 1985) have suggested that exosomes are not formed by budding from the plasma membrane, but that they originate from compartments of the endocytic pathway called multivesicular bodies (M VBs). The small vesicles present in the lumen of M VBs probably bud from the internal face of the M VB’s limiting external membrane (T rowbridge et al., 1993; v an D eurs et al., 1993), generating vesicles with the cytosolic face of the membrane facing the inside of the vesicle. U pon direct fusion of M VBs with the plasma membrane, these vesicles are released into the extracellular medium, and are then called exosomes (Pan et al., 1985).

However, exosomes are not uniquely produced by terminally differentiating reticulocytes, since E pstein-B arr virus-transformed B lymphocytes (B-E B V s) secrete similar vesicles (R aposo et al., 1996). In B lymphocytes, as well as in other antigen-presenting cells (A PCs), compartments of the late endocytic pathway, including M VBs, are sites...
of peptide loading on major histocompatibility complex (MHC) class II molecules (West et al., 1994; Morkowski et al., 1997; Pierre and Mellman, 1998). B-E BV–derived exosomes bear peptide–MHC class II complexes, and present them directly to CD4+ T lymphocytes (Raposo et al., 1996). M astrocytes also produce exosomes upon degranulation induced by a cytosolic Ca2+ rise (Raposo et al., 1997b), reenforcing the idea that exosomes originate from endocytic compartments and not from the plasma membrane.

More recently, we found that dendritic cells (DCs) also secrete exosomes (Zitvogel et al., 1998). DCs are the only A PC s to induce primary and secondary immune responses (H art, 1997; B anchereau and Steinman, 1998). To do so, they have developed a remarkable array of specific attributes. DCs exist under two maturation states: immature DCs are efficient for antigen endocytosis and phagocytosis, but inefficient for T cell activation. Cytokines, such as tumor necrosis factor α or interleukin 1, and bacterial compounds, such as lipopolysaccharide (LPS), induce maturation of DCs. Mature DCs downregulate their internalization capacities but express high levels of MHC and costimulation molecules, which makes them extremely efficient for T cell activation.

DC-derived exosomes accumulate MHC class II and also bear MHC class I molecules. Exosomes produced by DCs exposed to tumor-derived antigenic peptides induce potent immune responses, such as cytotoxic T lymphocytes–mediated responses, leading to the regression of established tumors in mice (Zitvogel et al., 1998). However, the mechanisms of this spectacular antitumor effect are still unknown.

In an attempt to understand the molecular bases of the effects of DC-derived exosomes on the immune system, we have determined the conditions of their production by DCs and analyzed their protein composition. We have identified by trypsin digestion and mass spectrometry (i.e., peptide mass mapping) eight major exosomal proteins. Besides MHC class II molecules, major exosomal proteins represent either cytosolic molecules potentially involved in exosome formation, or membrane proteins that are probably necessary to address exosomes to their cellular targets in vivo: a major exosomal compound is milk fat globule–EGF–factor VIII (MFG-E8), a soluble protein binding αvβ5 and αvβ3, two integrins expressed in DCS and macrophages. Importantly, hsc73, an efficient inducer of antitumor immune responses in vivo, also accumulates in exosomes.

Materials and Methods

Cells and Antibodies

The spleen-derived murine DC line D1 has been described previously (Winzler et al., 1997). It was cultured in Iscove’s modified Dulbecco’s medium (Sigma Chemical Co.) supplemented with 10% endotoxin-free FCS (Life Technologies, Inc.) and 30% R1 medium (granulocyte/macrophage colony-stimulating factor (GM-CSF)–transfected NIH–3T3 fibroblast conditioned medium). Cells were passaged twice a week in 145-mm nontissue culture–treated petri dishes (4–5 × 106 cells per dish). To control their nonactivated state, the level of cell surface expression of MHC class II and costimulatory molecules was checked regularly by FAC S® as described (Winzler et al., 1997). Fresh DCS were generated from bone marrow (BM) as described previously (Winzler et al., 1997). In brief, cells obtained from the femur BM of C57/B16 mice were cultured for 2–3 wk in granulocyte/macrophage colony-stimulating factor (GM-CSF)–containing medium, as described for DCS.

Tumor cell line TS/A (spontaneously arising undifferentiated mammary carcinoma, H-2d; kind gift from G. Forini, University of Turin, Turin, Italy) was cultured in RPMI 1640 supplemented with 10% endotoxin-free FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids, and sodium pyruvate.

Antibodies used here were: mouse anti–I-Aa mAb (Y3P) for FAC S® analysis and immunoprecipitation; rat mAb anti–mouse MHC II (M5114) for EM; rabbit antiserum to the cytosolic sequence of mouse MHC II α chain for Western blot. Rat anti-mouse invariant chain (II) (IN-1 mAb), rabbit anti–mouse calnexin (kindly provided by A. Helenius, Swiss Federal Institute of Technology, Zürich, Switzerland), mouse mAb anti–mouse annexin II (kindly provided by Dr. V. Gerke, Institut für Medizinische Biochemie, Münster, Germany), rabbit anti–hsp84 (Afinity BioReagents), and rat anti–glycoprotein (gp) 96 (SPA 850) for Western blot; rat mAb anti–mouse CD9 (PharMingen) and anti-hsc73 (SPA 851) (StressGen Biotechnologies Corp.) for Western blot, EM, and immunoprecipitation; rat mAb anti–mouse Mac-1 (M1/70) and rabbit anti–mouse annexin II antiserum (kindly provided by V. Gerke) for immunoprecipitation and EM; and FITC–conjugated rat anti–mouse CD40 (PharMingen) for FAC S® analysis.

Immunoelectron Microscopy

D1 cells, either immature or mature after treatment for 24 h with LPS, were fixed with 2% paraformaldehyde in phosphate buffer 0.2 M, pH 7.4 (PB), for 2 h at room temperature. Ultrathin sections of cells fixed and processed for ultracytomicroscopy (Raposo et al., 1997a), as well as whole mounts of exosomes (Raposo et al., 1996; Escola et al., 1998), were immunogold–labeled as described previously. Observations were made with a CM 120 Twin Phillips electron microscope.

Exosome Purification

Exosomes were prepared either from the supernatant of a 3-d-old DC culture, or from fresh culture medium incubated for 24 h with 3-d-old DCs. We could not evidence any difference in the overall composition of exosomes purified from 3-d or from 24-h supernatants. Exosomes were purified as described previously (Raposo et al., 1996), by three successive centrifugations at 300 g (5 min), 1,200 g (20 min), and 10,000 g (30 min) to eliminate cells and debris, followed by centrifugation for 1 h at 110,000 g. The exosome pellet was washed once in a large volume of PBS, centrifuged at 110,000 g for 1 h, and resuspended in 50–200 μl of PBS with 0.01% sodium azide. The amount of exosomal proteins recovered was measured by Bradford assay (Bio-Rad).

A different batches of FCS used for tissue culture contain variable amounts of endogenous bovine exosomes (W. Stoorvogel, personal communication), the batch used for DC culture was carefully characterized in terms of amount of bovine exosomes and markers expressed by these exosomes. Approximately 10% of the exosomal proteins recovered from a D1 or BM–DC supernatant come from FCS. Two antibodies used here or in a previous study (Zitvogel et al., 1998) recognize both murine and bovine proteins present in exosomes: anti-hsc73 and anti–TFR (H 68.4 hybridoma). Therefore, the actual presence of the murine, DC–derived protein in exosomes was demonstrated by immunoprecipitation from metabolically
cally labeled, DC-derived exosomes (see Fig. 7), and by Western blots performed on exosomes produced by DCs grown in medium depleted of bovine exosomes by overnight centrifugation at 110,000 g (data not shown). Under these conditions, TFR was detected but not enriched in exosomal preparations (data not shown).

Floatation of exosomes on a continuous sucrose gradient was performed as described (Rapooso et al., 1996), but in an SW41 rotor. Fractions of the gradient (1 ml each) were diluted in 2 ml of PBS, centrifuged for 1 h at 100,000 g, and the pellet was loaded on a 10% SDS gel for Western blot analysis.

**Protein Analysis by SDS-PAGE and Western Blots**

Total proteins were obtained from D1 cells lysed in 50 mM Tris, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.1% sodium azide, with a cocktail of antiproteases (chymostatin, leupeptin, aprotinin, pepstatin [CLAP], 100 μM each; Sigma Chemical Co.), and cleared from nuclei by centrifugation at 10,000 g. To separate cytosol and total membranes, D1 cells were homogenized in 10 mM triethanolamine, 1 mM EDTA, 10 mM acetic acid, 250 mM sucrose, pH 7.4 (TEA-sucrose), supplemented with CLAP, by 60 passages through a 25-G needle. The supernatant, cleared from nuclei and cell debris by centrifugation at 1,200 g, was centrifuged for 1 h at 100,000 g; total membranes were recovered in the pellet and cytosol in the supernatant.

Proteins (1–10 μg) were separated on 10 or 12% SDS-PAGE G, bloated on Immobilon (Millipore), and detected by Western blot using an enhanced chemiluminescence detection kit (Boehringer Mannheim).

**Protein Composition of Exosomes from Dendritic Cells**

Metabolic labeling of D1 cells or BM-DCs was performed: cells were starved for 1 h at 37°C in methionine/cysteine-free RPMI supplemented with 30% dialyzed R1 medium (labeling medium), and incubated overnight at 37°C in fresh labeling medium supplemented with 5% FCS and 10–20 μCi/ml [35S]methionine/cysteine (Promix 35S; ICN). Labeling medium was removed, then cells were washed and further incubated for 24 h in complete medium at 37°C. Exosomes were purified from the supernatant; total lysates, cytosol, and total membranes were prepared from the same cells. Proteins (20,000 cpm of each sample) were separated on 10 or 12% SDS-PAGE, blotted on Immobilon (Millipore), and detected by Western blot using an enhanced chemiluminescence detection kit (Boehringer Mannheim).

**Subcellular Fractionation**

Subcellular fractionation of D1 cells was performed as described previously for murine B lymphoma cells (A migrena et al., 1994). In brief, cells homogenized in TEA-sucrose were first fractionated on a discontinuous sucrose gradient, where the low density membrane (LDM) fraction was recovered at the junction between 1 and 0.25 M sucrose, and the high density membrane (HDM) fraction at the junction between 1.04 and 1 M sucrose. A 5 μg trypsin digestion (5 ng trypsin [Sigma Chemical Co.]) per μg of proteins, 10 min at 37°C, stopped by soybean trypsin inhibitor, 10 ng/ml trypsin). LDMs were loaded on a free-flow electrophoresis (FFE) chamber (Dr. Weber, GmbH, Ismaning, Germany). Fractions were collected, pooled pairwise, and analyzed for protein content (Bradford assay; Bio-Rad) and β-hexosaminidase activity (Green et al., 1987). 15 pools of fractions, within 10 fractions of the protein and β-hexosaminidase activity peaks, were kept for further analysis. They were centrifuged at 10,000 g for 1 h, and the pellets were resuspended in reducing SDS sample buffer and run on SDS-PAGE for Western blot analysis.

**Protein Analysis by Matrix-assisted Laser Desorption Ionization—Time of Flight Mass Spectrometry**

30 μg of proteins from exosomes or total lysates was run on an 8-15% SD S-PAGE and stained with Coomassie blue (Bio-Rad). The major bands in the exosomes preparation were excised from the gel, and in-gel digested with trypsin as described previously (Rabilloud et al., 1998). An aliquot of the digest solution was analyzed by matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF-MS), using a Bruker Biflex mass spectrometer (Bruker-Franzen A nalytik) equipped with a shoot multiprobe inlet and a gridless delayed extraction ion source. Ion acceleration voltage was 19.5 kV and the reflectron voltage was 20.0 kV. For delayed ion extraction, a 6.2-kV potential difference between the probe and the extraction lens was applied. Mass spectra of peptide mixtures were acquired as the sum of ion signals generated by irradiation of the target with 100 laser pulses. They were calibrated using ion signals from trypsin autodigestion peptides (MH + 842.50, MH + 1045.55, and MH + 2211.09). Monoisotopic peptide masses were assigned and used in database searches. Typical search parameters, using the Microsoft FIT program, were as follows: maximum allowed peptide mass error of 100 ppm; consideration of one incomplete cleavage per peptide; no restriction was placed on the isoelectric point of the protein, and a protein mass range of 0–200 kDa was allowed.

**Figure 1. In vivo effects of BM-DC-derived exosomes. Immunocompetent (A) or nude (B) mice bearing 4- to 5-day-old TS/A mammary tumors were injected intradermally with exosomes (5 μg/mouse) produced by BM-DCs pulsed with AEP (i.e., MHC-associated peptides) from either TS/A tumor cells (DC ex-TS/A AEP) or splenic cells (DC ex-Spleen A EP). Tumor size was monitored weekly. Tumor growth was significantly delayed in tumor-AEP exosome-injected mice, compared with splenic-AEP exosome- or saline-injected mice (A). In nude mice lacking T lymphocytes, TS/A tumor growth was not affected by the same treatments (B). Each experimental group included five mice. Experiments were reproduced twice with similar results. A sterisks represent significant results at 95% using Fisher’s exact method compared with injection of saline.
Results

DC-derived Exosomes Elicit Antitumor Immune Responses In Vivo

We have recently shown that murine BM-DCs secrete exosomes (Zitvogel et al., 1998). Exosomes were purified by ultracentrifugation from supernatants of BM-DCs exposed to peptides eluted from MHC class I molecules at the surface of a tumor cell line. Injection of tumor peptide-pulsed exosomes into mice bearing the tumor induced a strong delay in tumor growth, whereas injection of exosomes pulsed with normal spleen-eluted peptides had no

Figure 2. Immunelectron microscopy of D1 cells and exosomes. Ultrathin cryosections of immature (A) and mature (B) D1 cells were single immunogold-labeled with the rat anti-MHC class II mAb M5114 as described in Materials and Methods. (A) Immature D1 cells show numerous MHC class II-positive compartments with intraluminal vesicles. MHC class II are also detected at the plasma membrane (pm). (Insert) Whole-mounted exosomes immunogold-labeled with M5114. (B) In mature D1 cells (24 h in the presence of LPS), the plasma membrane (pm) shows numerous processes and is intensely labeled with the M5114 antibody. Very few intracellular compartments containing MHC class II molecules are observed. n, nucleus. Bars, 200 nm.
Théry et al. found in late endocytic compartments (Cella et al., 1997; In immature DCs, most MHC class II and Ii molecules are

Exosome Production during DC Maturation

To unravel the molecular mechanisms of exosome action, we used the well-characterized growth factor-dependent murine spleen DC line, D1 (Winzler et al., 1997). D1 cells show all the attributes of fresh immature DCs, including high endocytic capacity and abundant MHC class II–containing lysosomal compartments.

As shown by EM, immature D1 cells displayed numerous characteristic MVBs containing internal vesicles labeled by anti–MHC class II antibodies (Fig. 2 A). Such MVBs were also observed apposed or in close proximity to the cell surface, suggesting their possible fusion with the plasma membrane and the secretion of exosomes. Exosomes were indeed isolated from D1 culture supernatants by differential centrifugation as described previously (Raposo et al., 1996). After the last ultracentrifugation step, the pellet was washed once in PBS. Thus, we obtained routinely 15–20 µg of exosomal proteins (as measured by Bradford assay) from the supernatant of a 24-h culture of 2 × 10^6 immature D1 cells.

Whole-mount EM preparations of exosomes showed a population of vesicles (Fig. 2 A, insert), similar in size and morphology to BM–DC–derived exosomes (Zitvogel et al., 1998). As noted above for DC-derived exosomes, these vesicles are more heterogeneous in size and MHC class II labeling than B-EBV–derived exosomes (40–90 vs. 50–80 nm in diameter, respectively). Western blot analysis (Fig. 3 A) showed that exosomes secreted by D1 cells are, like exosomes produced by B-EBV or by BM–DCs (Raposo et al., 1996; Zitvogel et al., 1998), enriched in MHC class II, and devoid of Ii, which associates with newly synthesized MHC class II in the ER. D1-derived exosomes are also devoid of calnexin (an ER resident protein). Like exosomes from B-EBV cells (Raposo et al., 1996), D1-derived exosomes migrate on a continuous sucrose gradient at a density of 1.14–1.20 g/ml.

Therefore, exosomes produced by D1 cells resemble exosomes produced by BM–DCs or B-EBVs. We decided to use D1 cells to analyze the regulation of exosome production during DC maturation and to characterize the molecular composition of exosomes.

Exosome Production during DC Maturation

In immature DCs, most MHC class II and Ii molecules are found in late endocytic compartments (Cella et al., 1997; Pierre et al., 1997). As shown in Fig. 2 A, many of these compartments display intraluminal membrane vesicles. Upon maturation, these compartments disappear, and MHC class II molecules are redistributed to the plasma membrane (Cella et al., 1997; Pierre et al., 1997). Accordingly, we observed that after LPS treatment of D1 cells for 24 h, the number of MHC class II–positive compartments, as well as the amount of MHC class II in the remaining compartments, decreased (on average, the number of MVBs per cell profile decreased from five to eight in immature, to one to two in mature D1 cells) (Fig. 2 B). Concomitantly, MHC class II labeling at the cell surface is highly increased, and the plasma membrane displayed numerous processes (Fig. 2 B, pm). Although the precise pathway of MHC class II transport to the plasma membrane during this maturation process is still unknown, it is possible that direct fusion of endocytic compartments with the plasma membrane participates in this process. In this case, increased exosome production should occur upon DC maturation.

We tested this possibility by quantifying the amount of exosomal proteins produced by immature D1 cell in the presence or absence of LPS. Unexpectedly, we observed a lower production of exosomes in the presence of LPS (a 35% reduction) (Fig. 4 A, LPS 24h). Furthermore, if maturation was induced for 16 h, before the 24-h time period of exosome collection, the amount of produced exosomes was reduced by 67%, as compared with immature D1 cells (Fig. 4 A, LPS 40h). At the end of the total 40-h LPS treatment, D1 cells showed all the signs of maturation: increased surface expression of MHC class II and costimulatory molecules, and decreased internalization activity (Fig. 4 B). Similar results were obtained with murine BM–DCs and with human monocyte–derived DCs (data not shown).
Overall Protein Composition of Exosomes: A Few Proteins Selectively Accumulate in Exosomes

Therefore, we undertook an extensive characterization of the protein composition of exosomes. D1 cells were metabolically labeled overnight with [35S]methionine/cysteine, washed once, and fresh nonradioactive medium was added to the cells for 24 h. Metabolically labeled exosomes were purified from this supernatant. In four independent experiments, the amount of radioactivity recovered in exosomes was $0.17 \pm 0.1\%$ of the radioactivity incorporated into cells.

The protein composition of exosomes from metabolically labeled cells was analyzed by SDS-PAGE and autoradiography, and compared with that of whole D1 cells, cytosol, or total cellular membranes. As shown in Fig. 5 A, exosomes displayed a unique protein composition pattern. At least seven proteins (of ~180, 90, 70, 58, 43, 32, and 27 kD) (see dots in “exos” panel of Fig. 5 A) were strongly enriched in exosome preparations compared with whole cells, cytosol, or total membranes. At least eight other major proteins from whole cells, cytosol, or total membranes were either absent or less abundant in exosomes (see stars in “cells” panel of Fig. 5 A). Interestingly, two of the bands enriched in exosomes (70 and 58 kD) are not detected in the cytosol or in the cell lysates, suggesting a particularly strong mechanism of enrichment of this protein in exosomes. Similar analyses were performed on metabolically labeled primary BM-DCs, and the pattern of bands obtained was very similar to that of exosomes from D1 cells (data not shown). All the proteins present in exosomes comigrated on a continuous sucrose gradient at the expected density (1.15 g/ml), confirming that all the proteins in the exosome pellets are associated to vesicular structures of similar density. Therefore, DC-derived exosomes accumulate a unique subset of cellular proteins.

To identify these proteins, 30 µg of exosomes was loaded on an 8-15% gradient SDS gel; the pattern of bands stained with Coomassie brilliant blue was very similar to that of metabolically labeled exosomes (Fig. 6). All of these major bands (Fig. 6, bands 1-11) were excised, trypsin-digested, and the peptides generated were analyzed by MALDI-TOF-MS. The peptide profiles generated from the different bands were compared with the theoretical tryptic peptide profiles of known proteins from the databases (Jensen et al., 1996). The results are summarized in Table I. Among the identified proteins, three are transmembrane (Mac-1 α chain, MHC II β chain, and CD9), one is secreted and peripherally associated to membranes (MFG-E8), and four are cytosolic, often found in association with membranes (GÎ±2α, annexin II, gag from M RV provirus, and hsc73).

In two cases (MFG-E8 and gag), the theoretical molecular weight of the identified protein was not consistent with its migration on the SDS gel. Two forms of MFG-E8, due to various degrees of glycosylation, have been described in mice and cows (Stubbs et al., 1990; Aoki et al., 1995; Hvarregaard et al., 1996), probably accounting for the migration of MFG-E8 as three separate bands in our exosome preparation (Fig. 6 and Table I). Gag is first translated as a 60-kD precursor and subsequently cleaved into

Therefore, production of exosomes is developmentally regulated: it is effective in immature DCs and reduced upon maturation, when endocytic activity of DCs decreases. These results also suggest that exosome formation is an active process, requiring the selection of a limited set of proteins, whose identity defines exosomes as an independent cellular compartment and whose functions determine their biological roles.
three mature products: the gag peptides generated from band 10 all matched the mature 30-kD core shell protein.

The presence of hsc73, annexin II, MHC II, Mac-1, and CD9 in exosomes from D1 cells and primary BM-DCs was quantified by Western blot and immunoprecipitation from metabolically labeled cells (Fig. 7, A and B). Similar amounts of exosomes and cell lysates (2 and 6 μg) or the same number of counts (3 × 10^6 cpm) were analyzed by Western blot and immunoprecipitation, respectively. MHC II and CD9 were highly enriched (>10-fold) in exosomes compared with total cells. Mac-1 was enriched by 5–10-fold, annexin II and hsc 73 by 2–3-fold. These results confirm the MALDI-TOF-MS analysis and show that exosomes accumulate a defined set of cellular proteins.

Immunoelectron microscopy analysis (Fig. 7 C) of whole-mounted exosomes showed staining for MHC II, CD9, and Mac-1, but not for annexin II and hsc73, although the antibodies used stained D1 cells ultrathin cryosections (data not shown). These results suggest that in contrast to MHC class II, CD9, and Mac-1, hsc73 and annexin II are not exposed at the surface of exosomes but contained within their lumen.

**Selective Accumulation of hsc73 in Exosomes and Endocytic Compartments**

The MALDI-TOF-MS analysis of band 4 revealed a mixture of the MFG-E8 protein and another protein of potential interest in our study of exosome-mediated antitumor effects: the 70-kD hsp family member, hsc73. Several groups have reported that three hsp family members, hsc73, gp96, and hsp84, induce immune responses and rejection of established tumors (Ullrich et al., 1986; Arnold et al., 1995; Nicchitta, 1998; Srivastava et al., 1998). Since the presence of hsc73 in exosomes could be relevant for exosome’s antitumor effects, we analyzed in more detail the presence of different hsp family members in DC-derived exosomes.

As shown in Figs. 7 A and 8 A, when the same amount...
of proteins from total cells or from exosomes were analyzed by Western blot, approximately three times more hsc73 could be detected in the exosomal preparations than in D1 total cell lysates. The two other main hsps generating antitumor immune responses (Srivastava et al., 1998) were either absent (gp96), or present, but not enriched (hsp84) in exosomes, compared with cell lysates (Fig. 8 A).

In murine fibroblasts, hsc73, or a closely related protein, accumulates in lysosomes (A garraberes et al., 1997), raising the possibility that the presence of hsc73 in exosomes results from its selective accumulation in endocytic compartments. Therefore, we analyzed the subcellular distribution of the three main hsps involved in antitumor immunity, gp96, hsp84, and hsc73. Subcellular fractionation of D1 cells was performed first by discontinuous sucrose flotation to separate cytosol and H D M s ( E R , some plasma membrane, and G olgi apparatus) from L D M s (endosomes, lysosomes, plasma membrane, and G olgi apparatus). The L D M s were then further fractionated by FFE, to separate endosomes and lysosomes from the other cell membranes (M arsh et al., 1987).

As expected, gp96 was found in the H D M s and L D M s, but was absent from the cytosol (Fig. 8 B). In contrast, hsp84 was detected in the cytosol and the H D M s (which, because of the setting of the sucrose gradient, is often contaminated with cytosol), but totally absent from the L D M s. hsc73 was present in all three fractions, consistent with its proposed presence in the cytosol and endocytic pathway. Before the FFE, L D M s were mildly trypsin digested. This treatment generated a 60-kD tryptic fragment of hsc73, and a 80-kD fragment of gp96 (Fig. 8, B and C, right panel).

The L D M s were then further fractionated by FFE. Most cellular membranes are not negatively charged, and consequently, the bulk of cellular proteins are not shifted to the anode (Fig. 8 C, B Bradford curve). Negatively charged endosomal and lysosomal membranes, as detected by β-hexosaminidase enzymatic activity (a lysosomal resident enzyme), were found in the fractions deviated towards the anode (Fig. 8 C, β-H ex curve). Western blot analysis of the FFE fractions, using anti-M H C class II antibodies, showed that in D1 cells, as in human and mouse D Cs (Cella et al., 1997; Pierre et al., 1997), abundant M H C class II molecules are present in the endocytic pathway (Fig. 8 C).

To localize hsc73 and gp96 in the fractions, the same filters were hybridized with the corresponding specific antibodies. As expected, gp96 was only present in unshifted FFE fractions, containing plasma, E R, and G olgi membranes. In contrast, most hsc73 was found in shifted FFE fractions, together with endosomes and lysosomes (Fig. 8 C). Therefore, hsc73 accumulates in endocytic compartments in D1 cells, probably accounting for the selective presence of hsc73 in exosomes.

**Discussion**

D C-derived exosomes elicit potent T cell–dependent immune responses in mice (Zitvogel et al., 1998). This striking effect on the immune system in vivo prompted us to analyze the molecular structure of exosomes. Thus, here we identified eight major proteins from D C-derived exosomes. Fig. 9 shows a scheme of our current idea of the nature and topology of the major proteins present in exosomes. Exosomes contain a defined subset of cytosolic proteins, most likely involved in exosome function and biogenesis (Fig. 9, hsc73, annexin II, G i2a). Exosomes also accumulate membrane proteins potentially involved in their association to target cells (Fig. 9, M F G-E8, M ac-1, C D 9) or in T cell activation (Fig. 9, M H C class I and II, B7.2). The most abundant exosomal protein, M F G-E8, binds to integrins (αvβ3 and αvβ5) expressed in D Cs and macrophages, and could target D C-derived exosomes to other A P Cs. A nother exosomal protein, the hsp 70 family member hsc73, is a potent inducer of antitumor immune responses in vivo, suggesting a mechanism for exosome’s antitumor effects (Zitvogel et al., 1998).

The presence of hsc73 in exosomes is interesting in relation to two aspects of exosome biology: their biogenesis and their biological effects in vivo. We show that in D1
As a cytosolic protein, hsc73 should be inside the vesicles. Consistent with this possibility, exosome-associated hsc73 was partially protected from trypsin digestion (data not shown), and was not detected by immunoelectron microscopy on whole mounts of exosomes (Fig. 7), suggesting that it resides inside exosomes. However, hsc73 may also be taken up by a putative specific receptor (Arnold-Schild et al., 1999) and accumulate on MVB internal vesicles, and thereby on the surface of exosomes. D ice and co-workers have shown that hsc73 is involved in binding misfolded proteins and addressing them to lysosomes, suggesting that hsc73 may translocate across lysosomal membranes (Cuervo et al., 1995; Hayes and Dice, 1996). Moreover, in exosomes secreted by reticulocytes, the two major proteins (on Coomassie blue-stained gels) are TFR and hsc73 (Davis et al., 1986): hsc73 was postulated to associate with the TFR in order to help its segregation into exosomes. However, hsc73 in exosomes from DCs may play a totally different role.

The presence of hsc73 in exosomes of APCs is most interesting in the light of results showing that hsps induce antitumor immune responses (Srivastava et al., 1998). hsc73 is associated with endogenous peptides, and injection of hsc73 purified from the tumor in tumor-bearing mice stimulates potent cytotoxic T lymphocyte immune responses and tumor rejection (Udono and Srivastava, 1993). Furthermore, hsc73-associated peptides are efficiently transferred to APCs for MHC class I and II antigen presentation (Udono and Srivastava, 1994; Blachere et al., 1997). This may be due to the presence of a receptor for hsc73 at the surface of macrophages and DCs (Arnold-Schild et al., 1999). hsc73 can also induce macrophages to activate T cells independently of antigen (Breloer et al., 1999). The role of exosomes in peptide transfer between cells and their capacity to activate macrophages and DCs are under examination.

Indeed, it is still unclear whether exosomes interact directly with T lymphocytes or require the presence of an additional APC. Although direct stimulation of T cells by exosomes was observed in several antigenic systems in vitro (Raposo et al., 1996; Zitvogel et al., 1998), it was always inefficient, compared with stimulation induced by intact APCs. In contrast, our recent results show that T cell stimulation by exosomes is much more efficient in the presence of DCs (Regnault, A., A. Lozier, C. Théry, G. Ra- poso, S. A. migorena, and L. Zitvogel, manuscript in prepara- tion), suggesting an indirect mode of action for exosomes in vivo. In any case, to induce immune responses, exosomes must interact with target cells: T lymphocytes or APCs.

From this point of view, the strong enrichment of MFG-E8 in exosomes is very interesting. MFG-E8 is originally described at the surface of milk fat globules (Stubbs et al., 1990; Larocca et al., 1991; Harvaregaard et al., 1996). It has two EGF-like domains, with an integrin-binding, A qa-G ly- A sp motif and a phosphatidylserine-binding, factor Vll-like, domain. Our preliminary results show that exosomal membranes expose phosphatidylserine (Théry, C., B. Hug- gel, and J.-M. Freyssinet, unpublished data), suggesting that MFG-E8 may bind exosomes through phospholipids. The human and bovine homologues of MFG-E8 were described at the surface of milk fat globules (Stubbs et al., 1990; Larocca et al., 1991; Harvaregaard et al., 1996). It has two EGF-like domains, with an integrin-binding, A qa-G ly-A sp motif and a phosphatidylserine-binding, factor Vll-like, domain. Our preliminary results show that exosomal membranes expose phosphatidylserine (Théry, C., B. Hug- gel, and J.-M. 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pressed at the surface of macrophages and immature DCs, respectively, and mediate the phagocytosis of apoptotic bodies for clearance (Fadok et al., 1992) or for antigen presentation by MHC class I molecules (Albert et al., 1998). Therefore, MFG-E8 may target DC-derived exosomes to other APCs rather than to T lymphocytes.

Furthermore, several other major exosome components could be involved in exosome-T cell or exosome-APC interaction. The presence and abundance of several members of the tetraspan protein family (CD63, CD81, CD82) in exosomes of B-EBVs or DCs have been reported recently (Escola et al., 1998; Zitvogel et al., 1998). CD63 and CD82 interact with several membrane proteins, including integrins and MHC class I and II molecules, and probably potentiate cell-cell interactions (Hammond et al., 1998; Porter and Hogg, 1998). We show here that another tetraspan family member, CD9, is most likely the major tetraspan in DC-derived exosomes (see Table I). CD9 also associates to other transmembrane proteins, and is a coreceptor facilitating the interaction of an EGF-like growth factor and its receptor (Higashiyama et al., 1995; Nakamura et al., 1995; Indig et al., 1997; Rubinsteine et al., 1997). Therefore, CD9 could participate in the interaction of exosomes with either APCs or T cells in vivo.

A further component of exosomes potentially involved in their interaction with cells is the α chain of Mac-1 (a β2 integrin also known as the type 3 complement receptor, CR3) (Gahmberg et al., 1997). Since we have coprecipitated this chain with its β chain counterpart from exosomes (Fig. 7), it is likely that functional Mac-1 is present at the surface of exosomes. Observations made by EM using an anti-Mac-1 antibody on preparations of intact exosomes as well as the known orientation of MHC class II molecules at the surface of B-EBV- or DC-derived exosomes (Rappo et al., 1996, 1997c), suggest that Mac-1 is present at the surface of exosomes in a conformation suitable for binding to its ligands. Therefore, Mac-1-expressing exosomes could be addressed to ICAM-1 or ICAM-2-expressing cells (the known ligands for Mac-1) such as DCs, lymphocytes, or endothelial cells.

The presence in exosomes of plasma membrane-associated proteins, such as Mac-1 or CD9, could be explained by the extremely high constitutive internalization rate (including endocytosis and macropinocytosis) in immature DCs (Sallusto et al., 1995). As a result, most plasma membrane proteins are actively internalized and continuously recycled. This property may be important for the generation of exosomes, since mature DCs, with reduced internalization abilities, produce less exosomes than immature DCs (Fig. 4).

Concerning the cytosolic proteins found in exosomes, all of them are involved in vesicle budding or fusion of intracellular compartments. Annexin II plays a role in early endosome fusion, and in fusion of secretion granule with the plasma membrane (Chasserot-Golaz et al., 1996; Liu et al., 1996). The G12α subunit of heterotrimeric G proteins has been found in phagosomes (Garin, unpublished observations), but its role is still unclear. The eventual presence in exosomes of the β and γ subunits, which normally associate with Gα, will be tested.

Finally, detection of a murine retrovirus gag protein in exosomes may reflect the presence of endogenous retrovi-
Figure 9. A speculative model for the molecular structure of DC-derived exosomes. The proteins represented have been found in DC exosomes, here (MFG-E8, Mac-1, CD9, hsc73, annexin II, G12a, gag) or in our previous studies (MHC class I and II, B7.2) (Zitvogel et al., 1998). The topology of membrane proteins is based on previous studies on exosome membrane orientation (Pan et al., 1985; Raposo et al., 1997), and on EM observations of whole-mounted exosomes reported here (Fig. 7). MHC I and II and B7.2 may be involved in the induction of immune responses by exosomes (Fig. 1). MFG-E8, Mac-1, and CD9 could play a role in addressing exosomes to target cells expressing their respective ligands (integrins αvβ3 and αvβ5, ICAM-1, and -2). Annexin II, G12a, gag, and hsc73 may play a role in exosomes biogenesis and/or biological functions.

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