Purification of intracellular compartments involved in antigen processing: a new method based on magnetic sorting

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In the present study, we describe a method to specifically isolate intracellular compartments containing endocytosed antigen. We have demonstrated that isolated compartments represent a small proportion of the intracellular material, highly enriched in antigen. Antigen-containing vesicles are specifically sorted from other intracellular compartments, such as endoplasmic reticulum or Golgi apparatus, and from the plasma membrane. They remain functional *in vitro* since they can be acidified, and the antigen inside has been found to be partially proteolysed. In macrophages, kinetic analysis has revealed that the antigen is first found in compartments of endosomal density, carrying Rab 5 and Rab 7, then in late compartments of lysosomal density,

which are rich in proteases. The global protein content of the compartments was mapped by two-dimensional electrophoresis. In B lymphocytes, this method has allowed the isolation of endocytic compartments emerging from receptor-mediated endocytosis of the antigen. After 2 h of chase, the antigen reached vesicles containing large amounts of MHC-class II molecules, invariant chain and human leucocyte antigen-DM, where peptide loading can occur.

Key words: endocytosis, intracellular traffic, MHC-class II molecules, subcellular fractionation.

INTRODUCTION

Non-particulate antigens are internalized by fluid-phase or receptor-mediated endocytosis in B lymphocytes, or by macropinocytosis in macrophages. In antigen-presenting cells (APCs), antigens are proteolysed progressively along the endocytic pathway, and the peptides produced subsequently bind to neo-synthesized or recycling MHC-class II molecules co-localized in the endocytic compartments. Neosynthesized class II molecules are delivered to endocytic compartments, associated with the Invariant chain (Ii), which is degraded by acid proteases to a small fragment CLIP (class II associated-invariant chain peptide), remaining in the binding groove of the class II molecules. This peptide is then exchanged for an antigenic peptide, under histocompatibility locus antigen (HLA)-DM catalysis [1,2]. Another pool of class II molecules, corresponding to recycling molecules from the cell surface, may be found in early endocytic compartments where they can load an antigenic peptide [3]. In both cases, class II molecules are exported to the plasma membrane.

The compartment where loading of neosynthesized class II molecules takes place is poorly characterized. It might consist of specialized vesicles, rich in class II molecules [4,5], or conventional endocytic compartments [6]. Furthermore, intracellular localization of class II molecules varies among cell types. Human lymphocytes and macrophages accumulate these molecules in late-endocytic compartments called MIIC [7,8], which may be considered as special lysosomes [9,10], whereas in murine B cells, they are found in endosome-like vesicles named CIIV [11].

Several studies have shown that the receptor used for internalization of the antigen might determine its targeting in the APC [12,13]. Furthermore, B-cell-receptor-mediated internalization led to peptide loading on newly synthesized class II molecules [10,14], whereas fluid-phase internalization by B cells

led to presentation via both newly synthesized and recycling class II molecules [15,16]. This implies that there are different endocytic pathways with non-equivalent, although morphologically indistinguishable, types of endosomes or lysosomes.

In the present work, we describe a new method of purification of intracellular compartments involved in antigen processing. This entails isolation of these vesicles according to their antigen content, since they display no morphological differences from classical endocytic vesicles. Our method is on the basis of magnetic fractionation: after covalent binding of a 10-nm ferromagnetic particle (micro-bead) to the antigen and internalization of the complex by the cells, antigen-containing compartments are harvested using a magnet that retains the micro-beads. The method has been applied to human macrophage-like cells (activated U937) and B lymphocytes, using tetanus toxin (TeTx) as a model antigen.

MATERIALS AND METHODS

Materials

Cells used were U937 cells (purchased from the American Type Culture Collection, Rockville, MD, U.S.A.), TeTx-specific, Epstein-Barr-virus (EBV)-transformed B cell clone 4.2 (generously given by Dr. A. Lanzavecchia, Basle, Switzerland) and non-specific, EBV-transformed B cell line BL15 prepared as described previously [17]. Cells were grown in RPMI 1640 medium (Gibco BRL, Cergy Pontoise, France)/2 mM glutamine/10% (v/v) heat-inactivated fetal calf serum (Gibco BRL). Activation of U937 cells was induced by 20 ng of PMA/10⁶ cells per 1.25 ml for 24 h at 37 °C. Antibodies used were polyclonal anti-(human cathepsins B and D) (purchased from France Biochem, Meudon, France), polyclonal antibodies raised against Rab 5 and Rab 7

Abbreviations used: APC, antigen-presenting cell; Ii, Invariant chain; HLA, human leucocyte antigen; TeTx, tetanus toxin; EBV, Epstein-Barr virus; DMEM, Dulbecco's modified Eagle's medium; NMF, non-magnetic fraction; MF, magnetic fraction; PNS, post-nuclear supernatant; ER, endoplasmic reticulum; HRP, horseradish peroxidase; TGN, *trans*-Golgi network; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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(generously given by Dr. P. Chavrier, Marseille-Luminy, France) [18], polyclonal antibodies raised against Rab 2 and Rab 6 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), monoclonal antibodies raised against cytoplasmic tails of MHC-class II α and β chains (DA6-147 and DA6-231) [19] and Ii (Pin1) [20] (generously given by Dr. J. Salamero, Institut Curie, Paris, France), anti-HLA-DM α (generously given by Dr. C. Roucard, Institut A. Bonniot, Grenoble, France), horseradish-peroxidase (HRP)-conjugated polyclonal anti-(rabbit IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) and HRP-conjugated polyclonal anti-(mouse IgG) (Sigma Cocktail, St. Quentin-Fallavier, France). Tetanus toxin was kindly provided by Dr. J. R. Cartier, Institut Mérieux (Lyon, France). Ovalbumin (grade V) was purchased from Sigma.

Preparation of micro-beads

Micro-beads (10 nm), comprised of a magnetite core (Fe_3O_4) coated with T40-dextran (Pharmacia Biotech, Orsay, France), were synthesized mainly as described previously [21], except that half of the T40-dextran was substituted with amino-T40-dextran (Molecular Probes, Leiden, The Netherlands). The concentration of micro-beads was estimated from the concentration of elemental iron, as described previously [22].

Preparation of TeTx micro-beads

TeTx was iodinated using the Iodogen method described previously [23]. ^{125}I -TeTx (200 $\mu\text{g}/\text{ml}$) was covalently linked to micro-beads (with an iron concentration of 6 mg/ml) by addition of 0.025% (w/w) glutaraldehyde in 0.1 M phosphate buffer, pH 7.5. Excess glutaraldehyde was inactivated by ethanolamine (0.1 M, pH 7.5). The proportion of TeTx covalently linked to micro-beads (75–90%) was evaluated from the percentage of ^{125}I -TeTx remaining in the supernatant after ultracentrifugation [400 000 g for 30 min, using a TL100-1 rotor (Beckman Instruments, Gagny, France)].

Magnetic fractionation

Antigen internalization was achieved by the process of either pinocytosis or receptor-mediated endocytosis. For pinocytosis, activated U937 cells (250×10^6) were washed in cold Dulbecco's modified Eagle's medium (DMEM), resuspended to 10^8 cells/ml and incubated for the indicated times at 37 °C with TeTx micro-beads (final iron concentration 1 mg/ml). After three washes, cells were either lysed or incubated further at 37 °C in DMEM at 5×10^6 cells/ml for a chase period of up to 4 h.

For receptor-mediated endocytosis, TeTx-specific B lymphocytes (250×10^6) were washed in cold DMEM and resuspended at a concentration of 10^8 cells/ml, and incubated for 1 h on ice with TeTx micro-beads (final iron concentration of 1 mg/ml) to allow the fixation of TeTx on its receptor. Cells were washed three times with cold DMEM and resuspended at a concentration of 5×10^6 cells/ml. Internalization was performed for the indicated times at 37 °C.

Intracellular traffic was stopped by maintaining the cells at 0 °C. Cells were washed twice with 50 ml of PBS and resuspended in homogenization buffer (250 mM sucrose/1 mM Hepes/1 mM EDTA, pH 7.2).

For cell lysis, plasma membranes were disrupted by using a 'cell-disrupter' (1 shot-0.75 kW model, Constant systems Ltd., Warwick, U.K.) with a pressure of 350 bar (where 1 bar = 10^5 Pa). Nuclei and intact cells were removed by centrifugation (750 g , 10 min, 4 °C) and post-nuclear supernatant (PNS) was collected.

Separation of intracellular compartments was performed mainly as described previously [24] with certain modifications: a column filled with 1.5 g of slightly compacted steel wire (Spontex, Nanterre, France) was placed in a 0.6 Tesla U-shaped permanent magnet (MACS of Milteniy Biotec, Bergisch Gladbach, Germany) and equilibrated with homogenization buffer. PNS was pumped into the column at a flow rate of 1 ml/min. Non-retained material was eluted, which will be referred to subsequently as the non-magnetic fraction (NMF). After washing with 10 vol. of homogenization buffer, the retained material, or magnetic fraction (MF), was removed from the column (outside the magnetic field) in 4 ml of the same buffer. The MF was concentrated by ultracentrifugation (100 000 g for 1 h at 4 °C) in a SW60 rotor (Beckman). The pellet was resuspended in 100 μl of denaturing buffer [4 M urea/0.5% (v/v) Triton X-100/0.5% (v/v) Nonidet P40].

The amount of ^{125}I -TeTx was measured in all fractions. Total protein concentration was determined using a micro-bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.) with BSA as a standard.

Subcellular fractionation on a Percoll gradient

Centrifugation using a self-forming Percoll density gradient was performed in 13 ml of Quick-Seal tubes (Beckman) filled consecutively with 1 ml of a cushion of sucrose (60%, w/v), 11 ml of Percoll (17%, v/v) and 1 ml of the fraction to be analysed. All solutions were in homogenization buffer. Sealed tubes were centrifuged (20 000 g for 90 min at 4 °C) in a 70Ti rotor (Beckman). Gradients were collected from the bottom of the tube in 0.65-ml fractions.

Subcellular structures were localized on these gradients as described previously [25]; fractions 4–7, 12–15, 14–16 and 18–20 contained lysosomes, endosomes, plasma membrane and cytosol respectively. The endoplasmic reticulum (ER) and Golgi were found with the endosomes and the plasma membrane.

Acidification assay

The endocytic pathway was loaded with micro-beads and 10 mg/ml FITC-T70-dextran (Sigma) for 45 min at 37 °C. Cells were washed three times with homogenization buffer and broken in a cell-disrupter. Organelles were isolated by magnetic fractionation and diluted twice into isotonic buffer containing 125 mM KCl/5 mM MgCl_2 /2 mM EDTA/10 mM Hepes, pH 7.4. ATP-dependent acidification of compartments was recorded as changes in FITC-dextran fluorescence intensity, as a function of decreasing pH. Acidification was induced by the addition of 2.5 mM ATP (pH 7.4). The resulting proton gradient was dissipated with 7.8 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Sigma). Relative fluorescence intensities were measured at 37 °C using a spectrofluorimeter (Hitachi F2000), with excitation wavelengths set at 450 and 490 nm and emission wavelength set at 520 nm.

Immunoblotting

Samples were electrophoresed by using SDS/PAGE (either 10% or 12% gels, or 7.5–15% gradient gels). Proteins were electrotransferred (at 100 V for 1 h for Mini-gels or 30 V overnight for large gels) to PVDF membranes (Schleicher and Schuell, Elqueville, France) in 10 mM 3-(cyclohexylamino)propane-1-sulphonic acid/10% (v/v) methanol, pH 11. Blots were saturated with 5% (w/v) fat-free milk powder in PBS/0.1% (v/v) Tween 20. Washing and incubations with both primary and secondary antibodies were performed in PBS/0.1% (v/v) Tween 20. De-

Table 1 Antigen and protein content of the magnetically isolated compartments

U937 cells (250×10^6) were incubated for 30 min at 37 °C with ^{125}I -TeTx-micro-beads or ^{125}I -TeTx and micro-beads or ^{125}I -TeTx alone. 4.2 B cells (250×10^6) were either incubated for 1 h at 0 °C with ^{125}I -TeTx-micro-beads, washed and placed at 37 °C for 2 h, or incubated for 30 min at 37 °C with ^{125}I -TeTx and micro-beads or ^{125}I -ovalbumin and micro-beads. Iodinated protein and total protein content of the retained MB are expressed as a percentage of the equivalent quantities in the PNS, before magnetic fractionation. The means of retained TeTx were calculated when the experiments were repeated at least three times. n.d., not determined. MB = micro-beads.

Internalized material ...	Macrophages (U937)			B cells (4.2)		
	TeTx — MB	TeTx + MB	TeTx	TeTx — MB	TeTx + MB	Ova + MB
Retained TeTx	20	18	0.17	28	6	0.3
Retained total protein	0.5	0.83	0	0.25	n.d.	n.d.
Mean of retained TeTx	21 ± 3	20 ± 6	0.15 ± 0.05	22 ± 6	n.d.	n.d.

Figure 1 Electron microscopy of a magnetically isolated endocytic compartment

U937 cells were incubated for 30 min with micro-beads and magnetically fractionated. Isolated compartments were fixed and negatively stained. Panels A–F show characteristic views of the purified compartments. Arrows indicate some of the micro-beads inside the compartment. Bar = 0.1 μm .

tection was performed using HRP-conjugated secondary antibodies with the enhanced chemiluminescence kit (ECL, Amersham, Les Ulis, France). When quantification was needed, calculations were made using ImageMaster® software (Pharmacia), after scanning of the film. Biotinylated proteins were detected using streptavidin–HRP (Pierce) diluted to 1:200000 and 4-chloro-1-naphthol as the substrate.

Two-dimensional electrophoresis

Samples were solubilized in 9 M urea/0.5% (v/v) Triton X-100/2% (v/v) Pharmalytes (Pharmacia)/65 mM dithiothreitol, and loaded on to an immobilized pH gradient (pH 3–10) on a

pre-formed polyacrylamide gel (Immobiline® DryStrip), rehydrated as recommended by Pharmacia [26]. Isoelectric-focusing gels were run at 20 °C in a Multiphor II apparatus (Pharmacia) for 6.5 h at 300 V, 5 h up to 2000 V and 6 h at 2000 V, in sequence. After separation, the strips were stored at -80 °C.

Proteins were separated by vertical SDS/PAGE for the second dimension. The strips were equilibrated in 100 mM Tris/HCl, pH 6.8/1% (w/w) SDS/6 M urea/30% (v/v) glycerol with 5 mg/ml dithiothreitol for 10 min, then with 45 mg/ml iodoacetamide for 10 min, and applied on top of a 7.5–15% (w/w) polyacrylamide gel gradient. Gels were stained with silver nitrate, as described previously [27]. All gels were scanned and proteins were quantified using ImageMaster® software.

Cell-surface biotinylation

After washing with cold PBS, cells (1.5×10^7 cells/ml) were resuspended in 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce) in PBS, pH 7.4. After 30 min incubation at 0 °C with gentle stirring, cells were washed three times with PBS before further treatment.

Electron microscopy

Vesicles isolated by magnetic fractionation were adsorbed on to a Formvar-coated grid, washed with water, fixed for 1 min with 1% (v/v) *p*-formaldehyde and negatively stained for 2 min with 1% (w/v) uranyl acetate, before observation with a JEOL 1200 EX II transmission electron microscope.

RESULTS

MF is enriched in antigen

In order to isolate antigen-containing compartments, the antigen (TeTx) was linked to ferromagnetic micro-beads and internalized either by fluid-phase macropinocytosis in U937 macrophages or by receptor-mediated endocytosis in TeTx-specific 4.2 B lymphocytes. Micro-bead-containing compartments were selectively retained in a strong magnetic field.

When U937 macrophages were pulsed for 30 min with TeTx micro-beads, 0.5% of the PNS proteins were retained in the magnetic field, together with 20% of the internalized TeTx

Figure 2 Percoll gradient analysis of the density of compartments isolated by magnetic fractionation

U937 cells were pulsed for 15 min with TeTx micro-beads and chased for 0 h, 1 h or 2 h. Cells were broken down and PNS prepared. Magnetically isolated compartments and PNS were analysed on Percoll gradients. The amount of ^{125}I -TeTx has been plotted for each fraction of Percoll gradient. (A) Comparison of PNS and MF isolated after 15 min of pulse. (B) Comparison of magnetic fractions isolated after 15 min of pulse and 0 h chase (15/0), 1 h chase (15/1) and 2 h chase (15/2).

Figure 3 *In vitro* acidification of isolated compartments

U937 cells were incubated with micro-beads and FITC-dextran for 30 min. FITC-labelled endocytic compartments were magnetically isolated, treated in the presence or absence of FCCP and submitted to the acidification assay, as described in the Materials and methods section. (A) Untreated compartments; (B) compartments pretreated with FCCP.

(Table 1), whereas in control experiments in the absence of micro-beads, no cellular protein and only 0.17% of the ^{125}I -TeTx was trapped by the column (Table 1). When TeTx and micro-beads were not covalently linked, the amount of TeTx in the MF of U937 was unchanged, indicating that TeTx micro-beads are internalized, as found for any pinocytotic probe. Furthermore, micro-beads in solution were not retained by the magnet (results not shown).

To allow IgG-specific receptor-mediated endocytosis, TeTx-specific 4.2 B cells were first incubated on ice with TeTx micro-beads and washed before internalization at 37 °C. After a 2 h internalization of TeTx micro-beads by these cells, the MF contained only 0.25% of the PNS proteins with 28% of the internalized antigen (Table 1). Under the same conditions, a non-TeTx-specific B cell line, BL15, internalized 3–4 times less TeTx or TeTx micro-beads than specific 4.2 B cells (results not shown). When 4.2 B cells were incubated for 30 min at 37 °C with iodinated TeTx and micro-beads that were non-covalently linked, only 6% of the TeTx was retained by the magnet (Table 1), owing to a weak co-localization with micro-beads. In the same experiment, if TeTx was replaced by iodinated ovalbumin, which can only be internalized by pinocytosis, almost no ovalbumin was retained (Table 1), indicating that pinocytosis occurs less prevalently in these cells.

For both cell types, when using TeTx micro-beads an average

Figure 4 Partial proteolysis of TeTx in micro-bead-containing compartments

U937 cells were loaded with micro-beads and ^{125}I -TeTx for 30 min. Proteins of magnetically isolated compartments were analysed by SDS/PAGE in reducing conditions. Iodinated fragments were observed with a PhosphorImager[®]. The image of the gel is placed above the quantitative profile. Apparent molecular masses of the bands, indicated by arrows above the trace, were determined using molecular-mass markers.

of 21 % of the internalized iodinated TeTx was found in the MF (Table 1).

Characterization of isolated material

Purified intracellular compartments were observed by electron microscopy, after 30 min of internalization of micro-beads. Figure 1 shows six characteristic views of the purified compartments. The size of the vesicles ranges from 80 nm (Figure 1F) to 300–400 nm (Figure 1B). Micro-beads with a size of 10 nm are clearly visible inside all the vesicles (as shown by arrows in Figure 1A).

The MF was analysed on a 17 % self-forming Percoll gradient and compared with the PNS (Figure 2A). In the PNS of U937 cells, after a 15-min pulse TeTx was localized in endosomes and lysosomes, at the plasma membrane and also in the cytosol located on top of the gradient (see the Materials and methods section). This cytosolic TeTx could result from the breaking down of some endocytic compartments during treatment of the cells. Nevertheless, it was not retained in the MF, which was composed of compartments found in fractions 4–15, corresponding to lysosomes and endosomes. Approx. 50 % of the ^{125}I -TeTx-labelled compartments of the PNS (endosomes or lysosomes) were isolated in the MF. As a control, we found that the distribution of the endocytic marker ^{125}I -TeTx on a Percoll gradient was the same whether or not cells were loaded with micro-beads (results not shown), indicating that the compartment density was not modified by their micro-bead content.

TeTx-containing intracellular compartments were analysed by pulse–chase experiments, where U937 cells were pulsed for 15 min and chased for 0, 1 or 2 h. The density of isolated compartments labelled by iodinated TeTx changed with chase time (Figure 2B). Immediately after the pulse, 55 % of the TeTx micro-beads were recovered in vesicles of endosomal density, while the remaining 45 % were in lysosomes. After 1 h and 2 h of the chase, the amount of TeTx micro-beads in lysosomes increased to 64 and 83 % respectively.

In order to ensure that the MF was free from plasma

Figure 5 Evolution of compartment contents

U937 cells were pulsed for 20 min at 37 °C with TeTx micro-beads and chased for 0, 30, 60, 120 or 240 min. Compartments were magnetically isolated and analysed by Western blotting for the presence of Rab 5, Rab 7 and cathepsin D. (A) Detection of these proteins in the MF isolated after 20 min of pulse and no chase. (B) The amount of protein was quantified using ImageMaster[®] software, and plotted as a function of the chase time. (C) Detection of Rab 2 and Rab 6 in the NMF fraction and in the MF isolated after 2 h of chase.

membranes, cell-surface proteins were biotinylated after endocytosis of TeTx micro-beads for 30 min in U937 cells and for 2 h in 4.2 B cells. Micro-bead-containing compartments were isolated by magnetic fractionation, and biotinylated membrane proteins were quantified. Less than 1 % of the total amount of biotin present in the PNS was found to be associated with the MF (results not shown).

Isolated compartments are functional

The integrity of isolated compartments from U937 cells was checked by their ability to undergo ATP-dependent acidification *in vitro*. FITC-loaded compartments isolated by magnetic fractionation were incubated in the presence of ATP. ATPase-driven acidification was followed by the induced decrease of FITC fluorescence (Figure 3A). FCCP, which intercalates into the membranes, reversed the fluorescence change by destroying the pH gradient (Figure 3A). Endosomes and lysosomes isolated on a Percoll gradient gave quite similar fluorescence shifts (results not shown). Furthermore, FCCP-pretreated compartments showed no modification of fluorescence upon addition of ATP (Figure 3B). In the absence of compartments, FITC-dextran

Figure 6 Two-dimensional electrophoresis of isolated compartments

U937 cells were pulsed for 15 min at 37 °C with TeTx micro-beads and chased for 0 h (15/0), 1 h (15/1) or 2 h (15/2); then, compartments were magnetically isolated. Proteins (5 µg) were separated by isoelectric focusing on an immobilized pH gradient (pH 3–10) and by SDS/PAGE. Gels were silver-stained, scanned and analysed using ImageMaster® software. Representative images are of the gels of compartments isolated after a 2-h chase (A) and PNS (B). Arrows indicate either proteins that were highly enriched (2–6) or disappearing (1) in the MF. (C) Quantification of spot intensities for the six proteins annotated with arrows. Quantities are expressed in parts per million (p.p.m.) of the total protein in the gel.

fluorescence was not modified by addition of ATP or FCCP (results not shown).

Antigen processing in isolated compartments

Compartments magnetically isolated from U937 cells, loaded for 30 min with non-linked ^{125}I -TeTx and micro-beads, were analysed by SDS/PAGE under reducing conditions and the radioactivity profile was recorded by a PhosphorImager™ (Molecular Dynamics, Bondoufle, France) (Figure 4). The two bands at 100 and 50 kDa correspond to the heavy and light chains of TeTx respectively, whereas the bands at 80, 60, 40, 22 and < 10 kDa correspond to proteolytic fragments, indicating that TeTx has been processed in the APC.

Protein composition of antigen-processing compartments

To further characterize antigen-processing compartments, we analysed the modifications in the contents of the compartments along the endocytic pathway. In pulse–chase experiments, TeTx micro-beads were internalized by U937 cells for 20 min and then

chased for 0–240 min. Western blot analysis of MFs revealed three characteristic markers of endocytic compartments: Rab 5, Rab 7 and cathepsin D (Figure 5A). Rab 5 and Rab 7 were present at the highest concentration in the fractions isolated just after the pulse of micro-beads, and decreased rapidly during the first 30 min of the chase. They were, nevertheless, detected in all fractions, even after 4 h of the chase (Figure 5B). In contrast, cathepsin D was present at a maximum after 2 h, having increased 2-fold relative to its presence just after the 20-min pulse (Figure 5B). Similar variations were observed for cathepsin B (results not shown).

Rab 2 and Rab 6 have been defined as markers for the ER and Golgi apparatus respectively. By using Western blot analysis, small amounts of Rab 2 and Rab 6 were detected in the MFs from U937 cells (Figure 5C), representing less than 0.5% and 0.3% respectively of the total amount of these proteins in the cell.

Global protein analysis of isolated compartments was performed by two-dimensional electrophoresis. Analytical gels were made with 5 µg of total protein per gel. Figure 6 shows the

Figure 7 TeTx micro-beads are targeted to compartments containing MHC-class II molecules

TeTx micro-beads were internalized by receptor-mediated endocytosis in 4.2 B lymphocytes. After a 2-h chase, compartments were magnetically isolated. The NMF and MF were analysed by Western Blotting with antibodies raised against α or β chains of MHC-II (A), the cytoplasmic tail of Ii (B) or the α chain of the HLA-DM molecule (C).

contents of the MF isolated after 15 min of pulse and 2 h of chase (Figure 6A) compared with the proteins of the PNS (Figure 6B). Under these conditions, approx. 200 spots were detected in the MFs, whereas at least 1000 were detected in the PNS. Furthermore, some proteins appeared only in the MF. After quantification using the ImageMaster® software, at least 20 proteins appeared as being enriched in the isolated compartments. Of these, five are indicated by arrows in Figure 6(A) (nos. 2–6) and their equivalent location in the PNS is indicated in Figure 6(B). The protein numbered as one is an example of a PNS protein that disappears from the MF. Figure 6(C) represents the amount of each of these proteins in both the PNS and the MFs isolated after loading and after 1 or 2 h of chase.

Isolation of the peptide-loading compartment of B cells

Compartments of TeTx-specific 4.2 B cells were magnetically sorted after membrane IgG endocytosis of TeTx micro-beads. Their contents were analysed by Western blotting. The blots shown in Figure 7 are taken from one representative experiment of five, performed as described in the Materials and methods section.

In order to determine whether the antigen was in a compartment where peptide loading could occur, we looked for MHC-class II molecules. Compartments of the MF contained large amounts of these molecules detected with antibodies raised against α and β chains (Figure 7A). We also detected a lot of class II molecules in the NMF fraction (Figure 7A), comprising plasma membrane and exocytic compartments.

Using a monoclonal antibody raised against the cytoplasmic tail of Ii, a protein of 31 kDa was detected in the MF, corresponding to intact Ii (Figure 7B). The protein was highly enriched in the MF, as compared with the NMF. In the same compartments, large amounts of HLA-DM α -chain (33 kDa) were also detected (Figure 7C).

DISCUSSION

The method we have developed allows specific isolation of antigen-containing endocytic compartments. Micro-bead-loaded

compartments were selectively separated from the rest of the cell. The proportion of biotinylated membrane proteins and Rab 2 and Rab 6 found in these compartments was < 1% of that found in the PNS, therefore contamination of the isolated compartments by plasma membrane, ER or Golgi apparatus was at a low level. Micro-beads in solution are not retained by the magnet, i.e. TeTx micro-beads that would be released into the medium are not retained with the compartments. Approx. 50% of antigen-containing endosomes and lysosomes, as defined on a Percoll gradient, were retained specifically, owing to their micro-bead content. Furthermore, the antigen was found to be partially degraded in purified compartments, indicating that they contain proteases that are not inhibited by the presence of micro-beads. Magnetically isolated vesicles were able to be acidified *in vitro*, indicating that the membrane was not damaged and that the content was retained during the course of the purification. As a consequence, all the contents of the antigen-processing compartments could be analysed.

The density of antigen-containing compartments increased with the chase time of the TeTx micro-beads. The MF was enriched with light endosomes for short incubation times (< 15 min), which subsequently accumulated dense lysosomes. The traffic was rapid, since half of the antigen was already contained in the lysosomes after a 15-min pulse. Kinetic analysis of some markers was correlated to these results. The Rab proteins, which are small monomeric GTPases, bound to the cytoplasmic face of the intracellular compartments of both the endocytic and exocytic pathways, were used as specific markers of the compartments. Rab 5 has been localized to the plasma membrane and early endosomes, whereas Rab 7 resided preferentially on late endosomes [28]. Under the conditions of the present study, the compartments isolated after 20 min of pulse contained large amounts of both Rab 5 and Rab 7. These proteins were still detected after 4 h of chase, as has been observed previously on mouse macrophages (J774) [29,30]. We did not detect any difference in the pattern of accumulation of Rab 5 and Rab 7, which is unsurprising since after a 20-min pulse, micro-beads were already found in endosomes and lysosomes. Similar results were obtained by Desjardins et al. [29], using immunodetection of Rab 5 and Rab 7 in phagosomes.

Taken together, our data indicate that the early-antigen-processing compartments of U937 macrophages are endosomes carrying Rab 5 and Rab 7. Subsequently, the antigen reaches compartments of lysosomal density that are enriched with the mature proteases, cathepsin B and D. The fact that the antigen gains access to cathepsin D strengthens the hypothesis that cathepsin D plays a role in antigen processing, (either) directly in antigen proteolysis [31] and/or indirectly in Ii proteolysis [32].

Applying this method to B cells that express a TeTx-specific antibody, we have demonstrated that TeTx micro-beads are internalized by endocytosis via the membrane antibody. The pre-incubation of TeTx micro-beads at 0 °C allows their internalization to be synchronized. Covalent binding of the antigen to the micro-beads is essential for the specificity of the purification. Although micro-beads can be passively internalized in small quantities by coated pits during TeTx endocytosis (B cells incubated with TeTx and micro-beads), they have to be linked to the antigen in order to optimize the purification of antigen-containing compartments. This physical linkage allows a massive concentration of micro-beads in coated pits (during antigen-specific, receptor-mediated endocytosis), and ensures that micro-beads and the antigen follow the same intracellular pathway. With covalent binding of the ligand to micro-beads, the sorting is highly specific to compartments originating from the internalization of the ligand receptor. In the case of non-specific,

fluid-phase pinocytosis, the absence of covalent binding does not affect the yield of antigen retention, because the antigen and the micro-beads are co-internalized in a large volume of extracellular medium.

One advantage of this method is to separate the compartments of interest from the rest of the cell, so that their content can be analysed thoroughly by two-dimensional electrophoresis; we have detected some proteins that are specifically enriched in the compartment fraction. Starting from the same amount of total protein, the selectivity of the method is highlighted by the restricted number of spots in the MF, as compared with the PNS.

Late compartments, isolated from TeTx-specific B cells, are rich in MHC-class II molecules. Since we have shown that the plasma membrane does not contaminate the isolated compartments, these class II-molecules are actually of vesicular origin. P31 Ii has been described as the major isoform in B cells [33]. Ii associates with neosynthesized class II molecules in the ER, and is targeted to an endocytic compartment, where it is degraded by acid proteases. The presence of intact Ii in the antigen-containing vesicles indicates that at least some of them are the site of targeting of neosynthesized class II molecules. The simultaneous presence of MHC-II, Ii and HLA-DM molecules indicates that the antigen is localized in a compartment where peptide loading can occur. Interestingly, the *trans*-Golgi network (TGN) marker Rab 6 [34] was enriched in these isolated compartments (results not shown), whereas it was almost undetectable in compartments isolated from U937 cells, which do not synthesize class II molecules. This reinforces our hypothesis that compartments from 4.2 B cells, isolated after 2 h of chase, contain some MHC-class II-Ii complexes originating from the TGN. The purified fraction contains compartments localized at the point of intersection of the endocytic and exocytic pathways of class II molecules. This compartment might allow both the degradation of Ii and peptide loading on class II molecules, or these functions might take place in distinct compartments.

Our fractionation method can be used to isolate endocytic compartments in general, since a large cohort of proteins can be fixed to micro-beads, allowing a wide range of possible sites of entry to the endocytic pathways. Additionally, it allows the separation of vesicles emerging from the internalization of one type of receptor from other endocytic compartments. There is evidence, on the basis of antigen presentation by APC, that the fate of internalized antigens is not always the same, depending on the receptor that mediates entry [35–37]. This method of isolation of antigen-containing compartments will allow us to determine whether different receptors are targeting their respective ligands towards different compartments, or towards the same compartments with different kinetics.

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REFERENCES

- Weber, D. A., Evavold, B. D. and Jensen, P. E. (1996) *Science* **274**, 618–620
- Denzin, L. K., Hammond, C. and Cresswell, P. (1996) *J. Exp. Med.* **184**, 2153–2165
- Pinet, V. M. and Long, E. O. (1998) *Eur. J. Immunol.* **28**, 799–804
- Kleijmeer, M. J., Ossevoort, M. A., van Veen, C. J., van Hellemond, J. J., Neefjes, J. J., Kast, W. M., Melief, C. J. and Geuze, H. J. (1995) *J. Immunol.* **154**, 5715–5724
- Harding, C. V. and Geuze, H. J. (1993) *J. Immunol.* **151**, 3988–3998
- Kleijmeer, M. J., Morkowski, S., Griffith, J. M., Rudensky, A. Y. and Geuze, H. J. (1997) *J. Cell Biol.* **139**, 639–649
- Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. and Geuze, H. J. (1991) *Nature (London)* **349**, 669–676
- West, M. A., Lucocq, J. M. and Watts, C. (1994) *Nature (London)* **369**, 147–151
- Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H. and Pieters, J. (1994) *Nature (London)* **369**, 120–126
- Davidson, H. W., Reid, P. A., Lanzavecchia, A. and Watts, C. (1991) *Cell* **67**, 105–116
- Amigorena, S., Drake, J. R., Webster, P. and Mellman, I. (1994) *Nature (London)* **369**, 113–120
- Hewlett, L. J., Prescott, A. R. and Watts, C. (1994) *J. Cell Biol.* **124**, 689–703
- Bonnerot, C., Lankar, D., Hanau, D., Spehner, D., Davoust, J., Salamero, J. and Fridman, W. H. (1995) *Immunity* **3**, 335–347
- Kakiuchi, T., Takatsuki, A., Watanabe, M. and Nariuchi, H. (1991) *J. Immunol.* **147**, 3289–3295
- Soreng, K. M., Moore, J. C., Sherman, M. A. and Jensen, P. E. (1994) *Cell Immunol.* **157**, 277–290
- Kakiuchi, T., Okada, Y., Kokuho, T., Gytoku, Y., Mizuguchi, J. and Nariuchi, H. (1995) *Immunobiology* **193**, 84–97
- Tourne, S., Miyazaki, T., Wolf, P., Ploegh, H., Benoist, C. and Mathis, D. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9255–9260
- Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K. and Zerial, M. (1990) *Cell* **62**, 317–329
- Guy, K., van Heyningen, V., Cohen, B. B., Deane, D. L. and Steel, C. M. (1982) *Eur. J. Immunol.* **12**, 942–948
- Roche, P. A., Marks, M. S. and Cresswell, P. (1991) *Nature (London)* **354**, 392–394
- Rodriguez-Paris, J., Nolta, K. and Steck, T. (1993) *J. Biol. Chem.* **268**, 9110–9116
- Fisch, W. W. (1988) *Methods Enzymol.* **158**, 357–364
- Fraker, P. J. and Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Adessi, C., Chapel, A., Vincon, M., Rabilloud, T., Klein, G., Satre, M. and Garin, J. (1995) *J. Cell Sci.* **108**, 3331–3337
- Villiers, M. B., Villiers, C. L., Jacquier Sarlin, M. R., Gabert, F. M., Journet, A. M. and Colomb, M. G. (1996) *Immunology* **89**, 348–355
- Görg, A. (1991) *Nature (London)* **349**, 545–546
- Swain, M. and Ross, N. W. (1995) *Electrophoresis* **16**, 948–951
- Novick, P. and Zerial, M. (1997) *Curr. Opin. Cell Biol.* **9**, 496–504
- Desjardins, M., Celis, J. E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G. L. and Huber, L. A. (1994) *J. Biol. Chem.* **269**, 32194–32200
- Tjelle, T. E., Brech, A., Juvet, L. K., Griffiths, G. L. and Berg, T. (1996) *J. Cell Sci.* **109**, 2905–2914
- Rodriguez, G. M. and Diment, S. (1992) *J. Immunol.* **149**, 2894–2898
- Maric, M. A., Taylor, M. D. and Blum, J. S. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2171–2175
- Kampgen, E., Koch, N., Koch, F., Stoger, P., Heufler, C., Schuler, G. and Romani, N. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3014–3018
- Antony, C., Cibert, C., Géraud, G., Santa Maria, A., Maro, B., Mayau, V. and Goud, B. (1992) *J. Cell Sci.* **103**, 785–796
- Bonnerot, C., Briken, V. and Amigorena, S. (1997) *Immunol. Lett.* **57**, 1–4
- Amigorena, S., Lankar, D., Briken, V., Gapin, L., Viguier, M. and Bonnerot, C. (1998) *J. Exp. Med.* **187**, 505–515
- Mitchell, R. N., Barnes, K. A., Grupp, S. A., Sanchez, M., Misulovin, Z., Nussenzweig, M. C. and Abbas, A. K. (1995) *J. Exp. Med.* **181**, 1705–1714

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