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Accumulation of Major Histocompatibility Complex Class II Molecules in Mast Cell Secretory Granules and Their Release upon Degranulation

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To investigate the relationship between major histocompatibility complex (MHC) class II compartments, secretory granules, and secretory lysosomes, we analyzed the localization and fate of MHC class II molecules in mast cells. In bone marrow-derived mast cells, the bulk of MHC class II molecules is contained in two distinct compartments, with features of both lysosomal compartments and secretory granules defined by their protein content and their accessibility to endocytic tracers. Type I granules display internal membrane vesicles and are accessed by exogenous molecules after a time lag of 20 min; type II granules are reached by the endocytic tracer later and possess a serotonin-rich electron-dense core surrounded by a multivesicular domain. In these type I and type II granules, MHC class II molecules, mannose-6-phosphate receptors and lysosomal membrane proteins (lamp1 and lamp2) localize to small intralumenal vesicles. These 60–80-nm vesicles are released along with inflammatory mediators during mast cell degranulation triggered by IgE-antigen complexes. These observations emphasize the intimate connection between the endocytic and secretory pathways in cells of the hematopoietic lineage which allows regulated secretion of the contents of secretory lysosomes, including membrane proteins associated with small vesicles.

INTRODUCTION

Major histocompatibility complex (MHC) molecules present antigens to T cells. Class I molecules meet peptides derived from endogenous antigens in the biosynthetic pathway and class II molecules bind peptides arising from degradation of exogenous antigens internalized by the antigen-presenting cell (APC). The intracellular compartments representing the potential meeting points between antigenic peptides and MHC class II molecules have been characterized by morphological and biochemical criteria in human and murine APCs (Amigorena *et al.*, 1994; Castellino and Germain, 1995; Harding and Geuze, 1992, 1993; Kleijmeer *et al.*, 1995; Nijman *et al.*, 1995; Peters *et al.*, 1991b; Pieters *et al.*, 1991; Tulp *et al.*, 1994; West *et al.*, 1994). In the majority of cells analyzed so far, MHC class II compartments (MIICs) are heterogeneous and can be classified into at least four types according to their morphology, protein contents, and accessibility to endocytic tracers (Peters *et al.*, 1995; Glickman *et al.*, 1996). These types are MIICs with only a few internal vesicles and an irregular shape, multivesicular MIICs, multilaminar MIICs, and intermediate MIICs with both internal vesicles and concentrically arranged membrane sheets. All MIICs are mildly acidic and contain lysosomal components (β-hexosaminidase, cathepsin D, lamps, CD 63), although they show different accessibility to endocytic tracers indicating their different position in the endocytic pathway (reviewed in Kleijmeer *et al.*, 1996). Studies carried out with splenic cells and murine B cell lines revealed, however, that compartments positioned earlier in the en-

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docytic pathway and sharing features with early endosomes may be involved in peptide binding as well (Amigorena *et al.*, 1994; Castellino and Germain, 1995). The lysosomal environment of MIICs may be suitable for invariant chain degradation, antigen degradation, and peptide association with MHC class II, although it is conceivable that, depending on the APCs and on the type of antigen to be handled, different compartments may be involved. If so, different transport routes would be used by class II–peptide complexes to reach the plasma membrane.

Recent studies showed that prelysosomal multivesicular MIICs of B cells and melanoma cells fuse with the plasma membrane in an exocytic manner (Raposo *et al.*, 1996; Wubbolts *et al.*, 1997). This process is similar to the direct fusion of transferrin receptor-containing multivesicular bodies (MVBs) with the plasma membrane in reticulocytes (Harding *et al.*, 1984; Pan *et al.*, 1985), and to the exocytic process of cytolytic granules by cytotoxic T cells (Peters *et al.*, 1989, 1991a). Direct fusion of MIICs may account for the transfer of MHC class II molecules to the plasma membrane (Wubbolts *et al.*, 1997). However, a major consequence is the release of small membrane vesicles, called exosomes, into the extracellular environment (Raposo *et al.*, 1996).

To investigate the relationship between prelysosomal/lysosomal MIICs and well-defined secretory granules and to determine whether their fusion with the cell surface occurs during regulated exocytosis, we analyzed the localization of MHC class II molecules in mast cells using electron microscopy. Mast cells mature from bone marrow precursors into either mucosal mast cells found adjacent to respiratory and intestinal mucosal surfaces or connective tissue mast cells spread throughout the skin, peritoneal cavity, and musculature. Both types of mast cells have a relatively large number of secretory granules which are the site of accumulation of biogenic amines, proteoglycans, and numerous proteases (Razin *et al.*, 1981; Galli *et al.*, 1984; Dvorak, 1995). The two cell types differ from each other by their granule content. These cells play a prominent role in immediate hypersensitivity reactions because of the expression of high-affinity receptors for IgE. Cross-linking of IgE antibodies by multivalent antigens triggers mast cell degranulation, leading to the release of inflammatory mediators and proteases contained in secretory granules (Dvorak *et al.*, 1983, 1991). Additional roles for mast cells include host defense against intestinal helminth infection or dermal tick infestation (Matsuda *et al.*, 1990), bacterial phagocytosis (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996), and cytokine synthesis (Galli, 1993; Galli and Wershil, 1995). More recently, it has been suggested that mast cells are important in the APC family. Several groups have reported that MHC class II molecules are present on the surface of mast cells and mast cell lines. The function of MHC class II molecules in antigen presentation was observed in mouse bone marrow-derived mast cells (BMMCs) (Frandji *et al.*, 1993, 1996) and rat peritoneal mast cells (Fox *et al.*, 1994). It remains to be shown by biochemical and morphological approaches that mast cells synthesize MHC class II molecules and that they can correctly address these molecules to intracellular compartments that have a crucial role in peptide-binding to MHC class II molecules in several APCs.

In the present study, we show that MHC class II molecules synthesized by mast cells accumulate in secretory granules with lysosomal characteristics. These granules are classified on the basis of their protein content and accessibility to endocytic tracers. In these so-called secretory lysosomes, MHC class II molecules are associated with 60–80-nm vesicles which are exocytosed during degranulation triggered by IgE-antigen complexes.

MATERIALS AND METHODS

Animals

C3H/Heou, DBA/2, and C57BL/6 mice were purchased from Janvier (Laval France). Mice were used between 10 and 12 wk of age.

Generation of BMMCs

BMMCs were prepared as described by Razin (1981) and modified as described previously (Frandji *et al.*, 1993). Interferon-γ (IFN-γ) at 100 U/ml was added for the last 48 h of the 3-wk period of culture. The cells consisted of over 98% mast cells as assessed by toluidine blue staining and immunofluorescence staining for Mac-1, Nldc 145, and B220 cell surface antigens, indicating that the mast cell preparations were devoid of macrophages, dendritic cells, or B cells.

Preparation of B Cells

Cells were prepared by incubating spleen cells at 5×10^7/ml in the presence of anti-Thy.1 and anti-CD4 antibodies for 30 min at 0°C, and the cells were pelleted and resuspended in a 1:10 dilution of fresh low-tox rabbit serum complement (Cedarlane, Canada) and incubated for 45 min at 37°C. The complement lysis of T cells was repeated twice. The purity of B cells was consistently between 95 and 98%.

Reagents and Monoclonal Antibodies (mAbs)

RPMI 1640, fetal calf serum, phosphate-buffered saline (PBS), penicillin, streptomycin, sodium pyruvate, and L-glutamine were purchased from Life Technologies (Paisley, Scotland). RPMI 1640 depleted for methionine and cysteine and [^{35}S]methionine/cysteine labeling mix were from Amersham Corp. (Arlington Heights, IL). Protein A-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Recombinant interleukin 3, interleukin 4, and IFN-γ, were obtained from Immugenex (Los Angeles, CA).

The antibodies used for these experiments were anti-Thy-1 mAb from clone 9.37 (S. Kimura, Sloan-Kettering Memorial Institute, New York, NY) anti-CD4 mAb from clone GK1.5 (American Type Culture Collection, Rockville, MD). Fluorescein isothiocyanate-labeled anti-B220 (clone 6B2) and anti-MAC-1 (clone M1–70), anti-Nldc 145 mAbs from Dr. G. Powers (Hoffmann-La Roche, Nutley, NJ), mouse anti-mouse I-Ab mAb Y3P (Janeway *et al.*, 1984), rat

anti-mouse Ii chain mAb IN1 (directed against the Ii chain cytoplasmic tail) (Peterson and Miller, 1990), a rat mAb reactive against both I-Ab and I-Ad mouse MHC class II molecules (M5.114) (Bhattacharya *et al.*, 1981), and a polyclonal rabbit serum against the conserved cytoplasmic tail of the mouse MHC II-Ab,d b chain were obtained from Dr. R. Germain (National Institutes of Health, Bethesda, MD). Other antibodies used were rabbit sera anti-mannose-6-phosphate receptor (MPR; kindly provided by Dr. K. Von Figura and Dr. B. Hofflack), rat monoclonal anti-lamp1 and anti-lamp2 antibodies (Pharmigen, San Diego, CA), and rabbit serum against serotonin (Seralabo). Rat and rabbit antibodies were detected by Texas Red or fluorescein isothiocyanate-labeled $F(ab')_2$ fragments of donkey antiserum from Jackson Immunoresearch (West Grove, PA).

Immunofluorescence Staining and Confocal Microscopy

Cell labeling was performed as described previously. Cells, washed in PBS, were allowed to adhere on 0.2% poly-L-lysine-coated glass coverslips. After fixation in 3% paraformaldehyde for 10 min at room temperature, cells were permeabilized in PBS containing 0.05% saponin and 0.2% bovine serum albumin (BSA) and incubated with specific antibodies. After several washes, the cells were then stained with species-specific donkey $F(ab')_2$ fragments for 30 min. The coverslips were then mounted in Mowiol. Confocal laser scanning microscopy and double immunofluorescence analysis were performed using a Leica TCS microscope.

Pulse-Chase# Labeling and Immunoprecipitation

Experiments were performed as described previously (Amigorena *et al.*, 1994). Briefly, after a 2-h starving in methionine and cysteine-free medium, the cells were metabolically labeled for 16 h with [^{35}S]methionine/cysteine labeling mix (Amersham Corp., 100 μCi/ml). Cells were lysed in lysis buffer (0.5% Nonidet P40, 300 mM NaCl, 50 mM Tris, pH 7.4, plus 10 μg/ml mix of leupeptin, chemostatin, aprotinin, and pepstatin). Immunoprecipitations were performed on free nuclear cell lysate with Y3P or IN1-coated protein A-Sepharose beads (Pharmacia). After washing, the immunoprecipitates were eluted in 20 μl of sample buffer containing 0.5 M DDT for 30 min at room temperature to release SDS-stable complexes from the beads. Parallel samples were boiled for 3 min before analysis on 12% SDS-acrylamide gels.

Western Blot

Cells were lysed as described above. Cell lysates and exosomes were diluted in reducing sample buffer and boiled for 3 min before analysis on 12% SDS-acrylamide gels. Proteins were transferred on a polyvinylidene fluoride (PVDF) membrane (Millipore) at 2.5 mA/cm^2 after blocking solution treatment and incubation with antibodies followed by horseradish peroxidase-labeled species-specific antibody. Chemiluminescence was detected using a Boehringer kit.

Electron Microscopy and Immunogold Labeling

For conventional electron microscopy, BMMCs were fixed with a mixture of 2% paraformaldehyde and 1% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4), postfixed with 1% OsO_4 supplemented with 15% ferrocyanure, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were viewed with a TEM CM120 Philipps electron microscope after counterstaining with uranyl acetate and lead citrate. Mast cells processed for ultracryomicrotomy were fixed with 2% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4), embedded in 10% gelatin, and infused in 2.3 M sucrose as described (Kleijmeer *et al.*, 1996; Raposo *et al.*, 1997a). Gelatin blocks were frozen in liquid nitrogen and ultrathin sections were prepared with an ultracryomicrotome (UltracutS Leica, Wien, Austria) and a diamond knife (Drukker, Cuijk, the Netherlands). Ultrathin cryosections were collected with a mixture (vol/vol) of methylcellulose and 2.3 M sucrose (Liou *et al.*, 1996) and single- or double-immunogold labeled according to Slot *et al.* (1991; Raposo *et al.*, 1997b) with different antibodies and protein A coupled to 5, 10, or 15 nm of gold (PAG 5, PAG 10, and PAG 15) as indicated in the figure legends. Uptake of the endocytic tracer BSA coupled to 5-nm gold particles (BSAG) was performed on living cells before fixation and processing. As detailed in RESULTS, cells were pulsed for 10 min at 37°C with BSAG (OD = 5). After washing with ice-cold medium, the endocytic tracer was chased for 5, 20, or 80 min. Protein A-gold conjugates and BSAG were purchased from Dr. J.W. Slot (Department of Cell Biology, Utrecht University, the Netherlands).

RESULTS

Expression of MHC Class II Molecules in BMMCs Analyzed by Metabolic Labeling and Immunofluorescence

To perform biochemical and morphological analysis of MHC class II molecules, their synthesis by BMMCs was first increased by INF-γ treatment. This blocks APC function due to down-regulation of costimulatory molecules (Frandji *et al.*, 1996). To investigate the synthesis and conformation of MHC class II molecules in BMMCs, we examined their pattern of synthesis as compared with B cells. The two cell types, prepared from the same mice, were metabolically labeled with [^{35}S]methionine for 16 h. After cell lysis, class II molecules were immunoprecipitated with the anti-class II mAb Y3P and separated by SDS-PAGE. It is possible to distinguish between MHC class II-Ii complexes and MHC class II molecules with bound peptides on the basis of their SDS stability under reducing nonboiling

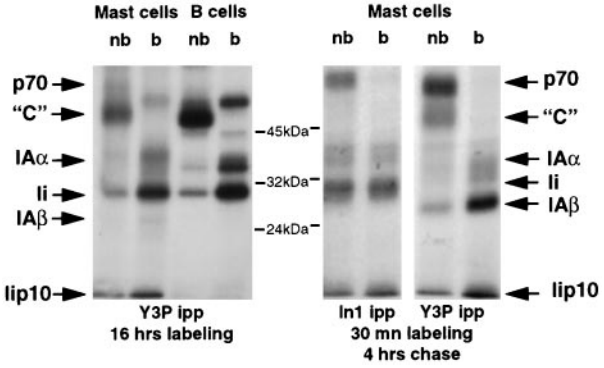


Figure 1. Metabolic labeling and immunoprecipitation of newly synthesized MHC class II molecules in BMMCs and B cells. BMMCs and B cells were metabolically labeled with [^{35}S]methionine for 16 h (left panel). Right panel, mast cells were pulsed for 30 min and then chased for 4 h. After cell lysis, MHC class II molecules were immunoprecipitated with the Y3P antibody recognizing mature α-β dimers or with the IN1 antibody directed against the cytoplasmic domain of the invariant chain. Before running on 12% SDS gels, samples were incubated in sample buffer at 20°C for 30 min (nonboiled conditions, nb) or at 95°C for 10 min (boiled conditions, b). The SDS-stable mature α-β dimer, named compact form, is indicated "C."

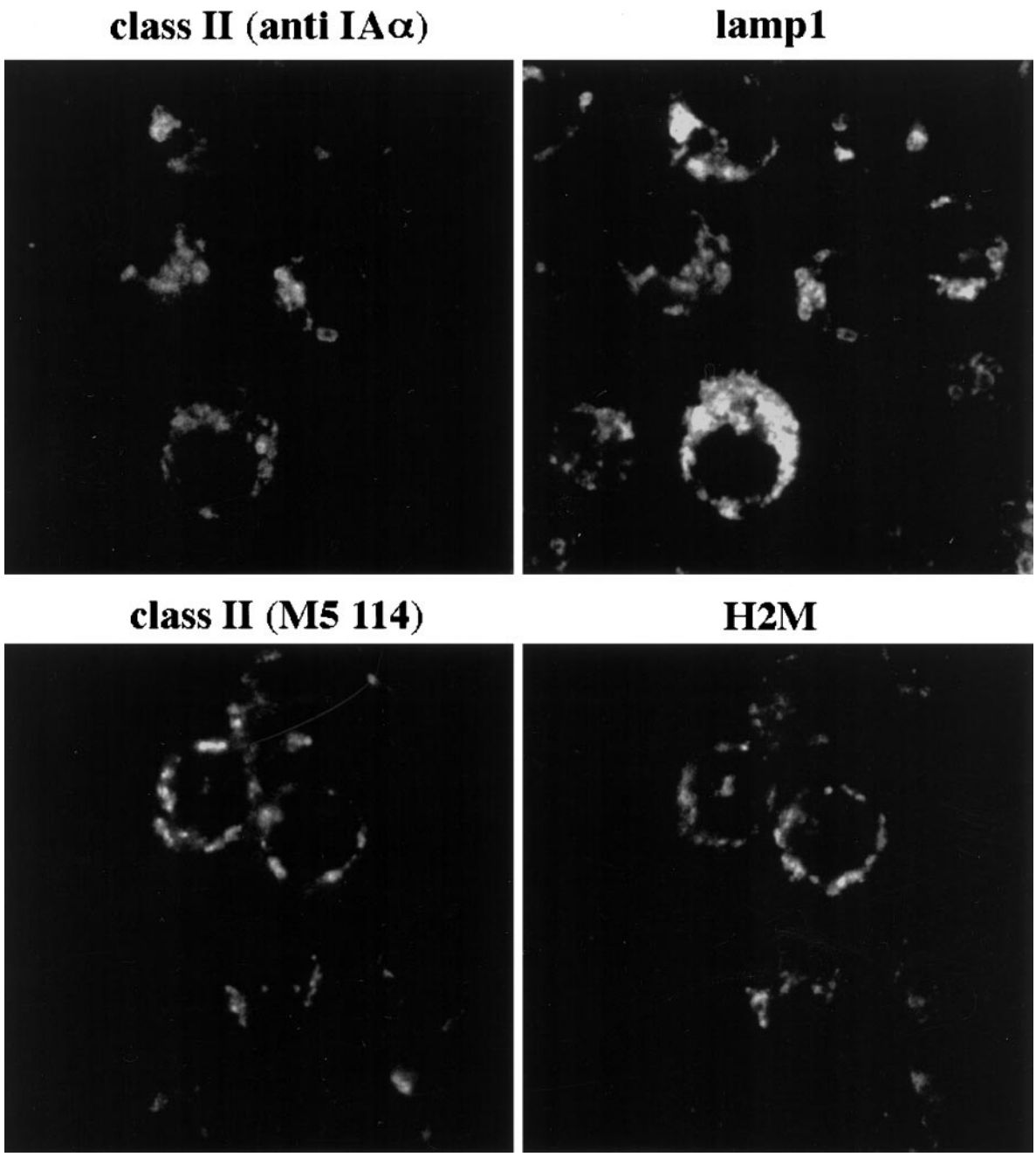


Figure 2. Immunofluorescence localization of MHC class II, H2-M, and lamp1. MHC class II molecules were detected after cell permeabilization with rabbit polyclonal antibodies specific for the cytoplasmic domain of the I-A molecule or with the rat monoclonal M5114 (anti-I-Ab,d). Upper left and lower right panels show that, despite some heterogeneity, MHC class II molecules are localized in H-2 M and lamp1 positive compartments.

conditions visualized by their different mobility by SDS-PAGE (Sadegh-Nasseri and Germain, 1991). As shown in Figure 1, the majority of MHC class II molecules of mast cells and B cells in the nonboiled samples migrate as SDS-stable α-β dimers of 60 kDa (compact form, "C"). Boiling allows dissociation of the complex in the two chains (α and β). The faint 70-kDa band observed in nonboiled BMMC sample could correspond to α-β dimers associated with the P10 kDa fragment of the invariant chain, mast cells were metabolically labeled with [^{35}S]methionine for 30 min and then chased in cold media for 4 h. Cell lysates were

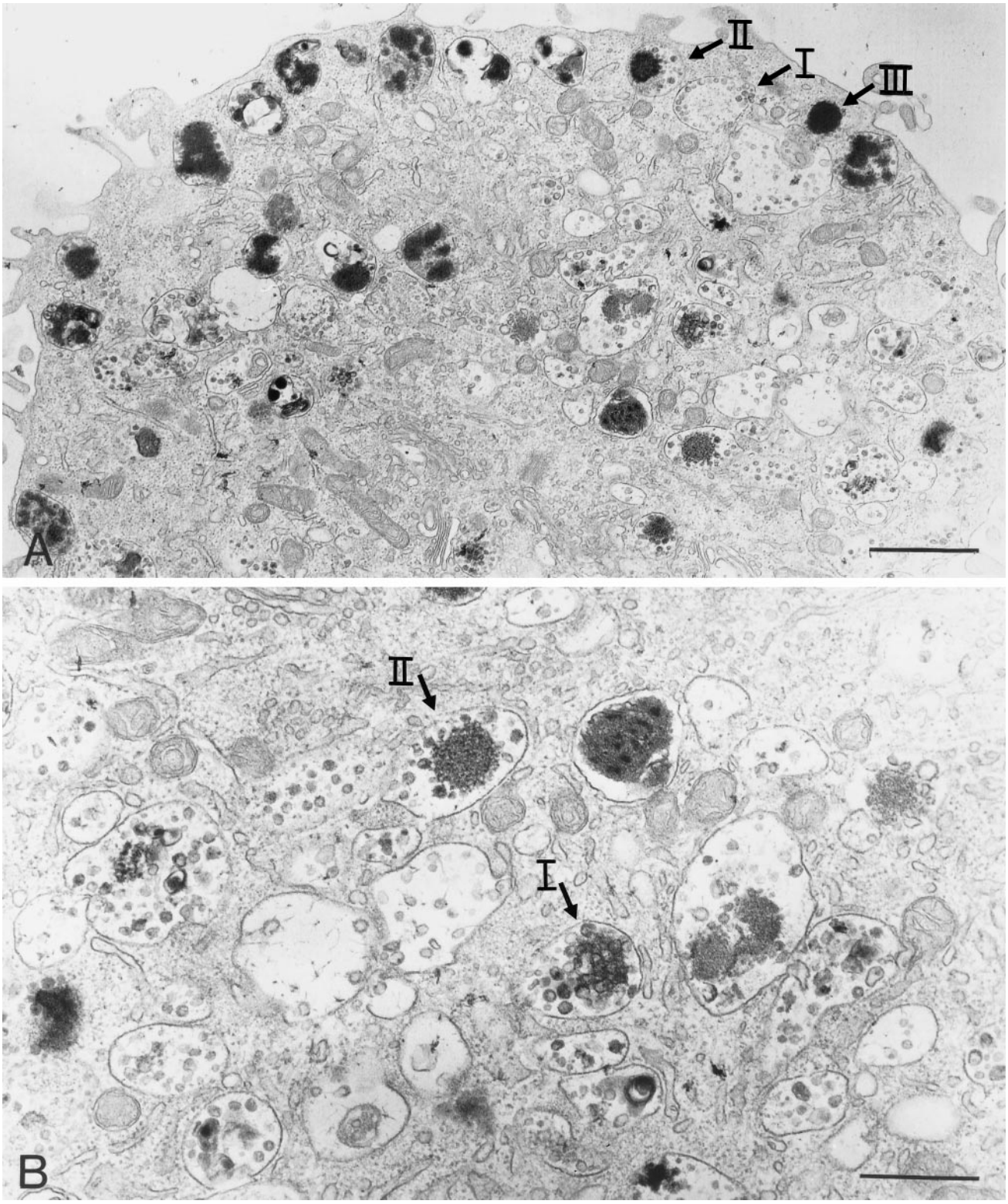


Figure 3. Ultrastructure of BMMCs. (A) Low magnification bar, 1 μm. (B) High magnification bar, 0.5 μm. The cytoplasm of BMMCs shows three types of morphologically distinct granules. Granules displaying internal vesicles (type I), granules with an electron-dense core surrounded by membrane vesicles (type II), and electron-dense granules (type III).

immunoprecipitated with either the Y3P antibody or the anti-invariant chain mAb, IN1. The two antibodies immunoprecipitated the same 70-kDa complex detected under nonboiled conditions and which dissociates after boiling into α-β chains and p10 fragment as shown by the increased intensity of these bands. The Y3P antibody, however, precipitated the mature SDS-stable form of the α-β dimers more efficiently than IN1 (Figure 1). In addition, in these short-pulse conditions the unidentified band was not detected. Thus, as compared with B cells, it appears that the degradation of the invariant chain is less efficient in mast cells but allows the formation of mature α-β dimers.

An overview of the distribution and localization of MHC class II molecules in BMMCs was obtained by immunofluorescence and confocal microscopy. After cell permeabilization, MHC class II molecules were visualized with a rabbit serum specific for the cytoplasmic domain of the I-A molecule or with the rat monoclonal M5114 specific for the I-A d, b haplotypes. MHC class II molecules accumulate in the periphery of large vesicular structures (Figure 2, A and B), whereas the cell surface is only faintly labeled, indicating that the bulk of MHC class II molecules expressed by BMMCs is localized intracellularly. To compare the location of MHC class II molecules in mast cells with that described in other APCs, comparisons were made to the class II molecule H-2 M (Figure 2C) and to a lysosomal membrane protein lamp1 (Figure 2D) which are present in MIICs (Kleijmeer *et al.*, 1996). As shown in Figure 2, MHC class II molecules were found to colocalize in mast cell compartments that contained H-2 M and lamp1, indicating that MHC class II molecules accumulate in compartments with characteristics of MIICs.

These observations suggest that in BMMCs, MHC class II molecules access intracellular compartments where they acquire their SDS-stable conformation.

Ultrastructure of BMMCs

Prior to a detailed study using immunoelectron microscopy of MHC class II localization in BMMCs, we performed conventional electron microscopy to examine the ultrastructural features of the secretory granules. In ultrathin sections of plastic-embedded BMMCs, we identified three types of morphologically distinct granules in the cytoplasm. By analogy to cytotoxic T cells and natural killer (NK) cells (Burkhardt *et al.*,, 1990; Peters *et al.*, 1991a), we classified the BMMC granules according to their contents (Figure 3A, low magnification; B, high magnification): multivesicular granules filled with small membrane vesicles ranging from 60 to 80 nm in diameter (type I, Figure 3); intermediate granules showing an electron-dense core surrounded by small vesicles (type II, Figure 3); and electron-dense granules devoid of membranous content (type III, Figure 3A).

Immunogold Localization of MHC Class II Molecules in Relation to Granule Contents, Prelysosomal and Lysosomal Components

The morphological features of the resident site of MHC class II molecules in BMMCs were then analyzed on ultrathin cryosections immunogold labeled with the rat monoclonal M5.114 antibody. The plasma membrane is only poorly labeled. The bulk of MHC class II molecules is detected in two morphological distinct compartments (Figure 4A). One compartment has densely packed membrane vesicles in its lumen, the other compartment possesses an electron-dense core surrounded by a multivesicular cortex. In both compartments, MHC class II molecules are restricted to the 60–80-nm internal vesicles. Few MHC class II molecules are detected in the limiting membrane of either type of compartment. This suggests that the distribution of MHC class II molecules in the periphery of the large vesicular structures revealed by immunofluorescence is because of their localization in the multivesicular cortex surrounding the core domain rather than in the limiting membrane itself. Quantitation of the labeling on ultrathin cryosections reveals that only 3% of the total number of gold particles detecting MHC class II molecules is on the plasma membrane, whereas 97% represents intracellular labeling. The bulk of MHC class II molecules (85% of the labeling) is present in the multivesicular type I granules and the intermediate type II granules. The remaining 12% is detected in the Golgi complex and small vesicles. The electron-dense type III granules do not show labeling for MHC class II molecules (see below Figure 6A).

To confirm that MHC class II molecules access the secretory granules of BMMCs, we performed double-labeling procedures to detect MHC class II molecules and the biogenic amine serotonin. As shown in Figure 4B, MHC class II molecules are localized in serotonin-

Figure 4 (facing page). Immunogold localization of MHC class II, serotonin, and MPR. (A) Ultrathin cryosections were single-immunogold labeled with anti-class II antibody M5.114 and PAG 10. MHC class II molecules were detected in compartments displaying intralumenal membrane vesicles (type I) and in the multivesicular domain surrounding the electron-dense core of a secretory granule (type II). (B) Ultrathin cryosections were double-immunogold labeled with anti-class II antibodies (PAG 10) and antiserotonin antibodies (PAG 5). Serotonin was present in the electron-dense core whereas MHC class II labeling was restricted to the surrounding vesicles. (C) Ultrathin cryosections were double-immunogold labeled with anti-class II (PAG 10) and anti-MPR antibodies (PAG 15). MHC class II and MPR were both detected in multivesicular compartments (type I granules) and in granules displaying an electron-dense core (type II). Bars, 200 nm.

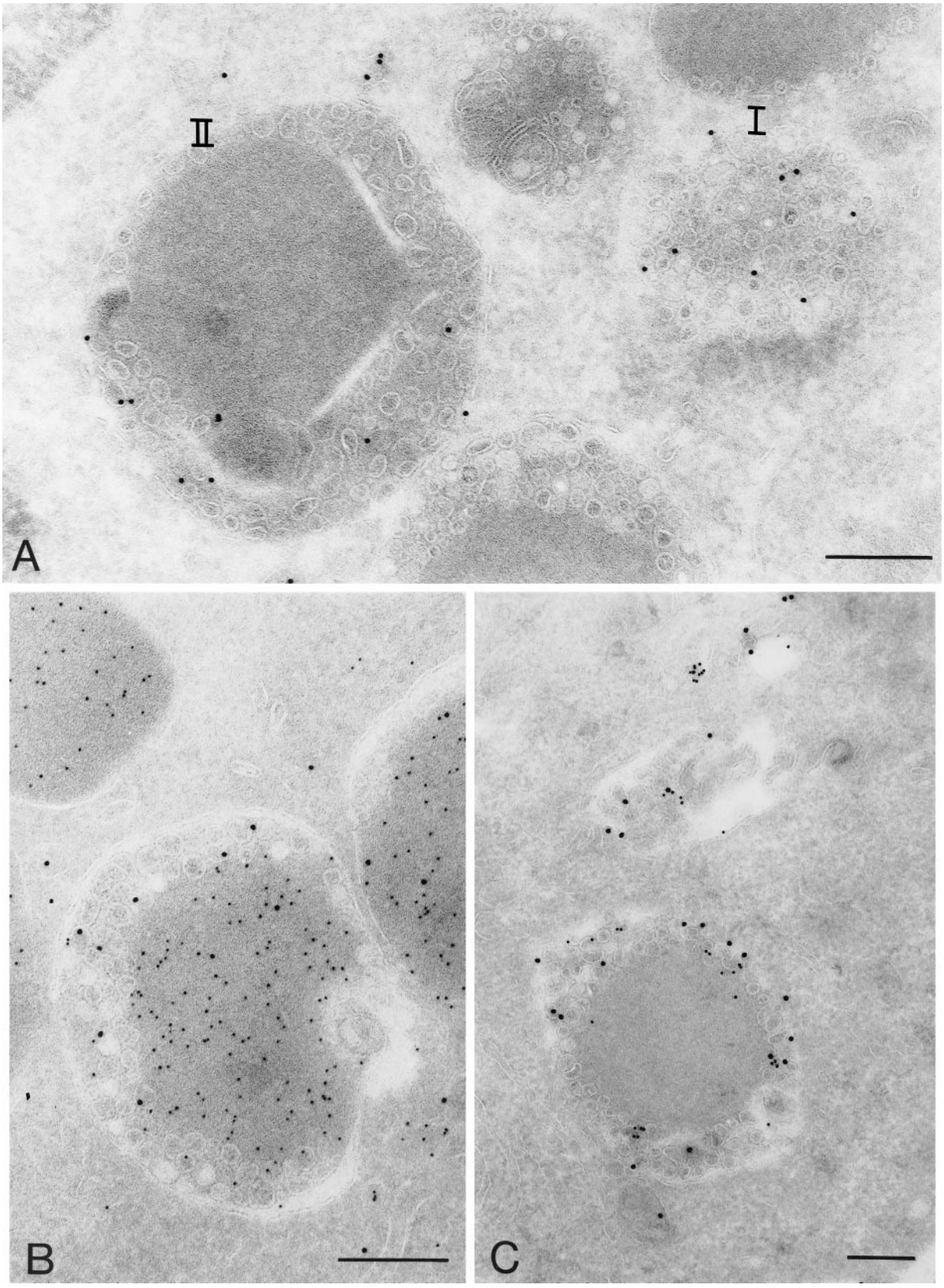


Figure 4.

containing type II granules. In these granules, the bulk of class II molecules localize to the multivesicular domain whereas serotonin preferentially accumulates in the electron-dense core. Occasionally, MHC class II labeling was observed in the electron-dense core of type II granules but this represented less than 2% of the total labeling. As shown below in Figure 5B, type I granules do not label with antiserotonin antibodies.

We compared the intracellular location of MHC class II molecules in BMMCs to that described for B lymphocytes and dendritic cells. We subsequently analyzed the relationship between type I and type II granules by double-immunogold labeling to detect I-A molecules with respect to membrane proteins associated with late endosomes and lysosomes. Unlike observations made in B cells, where the MPR is mainly detected in trans-Golgi network- (TGN) derived vesicles (Glickman *et al.*, 1996), a strict colocalization of MHC class II molecules and MPR are observed in type I granules (Figure 4C). This colocalization is also observed in the intermediate type II granules in which both class II and MPR are detected in the multivesicular domain (Figure 4B), indicating that the internal membrane vesicles are related to prelysosomes rather than lysosomes (Kornfeld and Mellman, 1989). Similar to MIICs, lysosomal membrane proteins (lamp1 and lamp2), which are mainly present in prelysosomes and lysosomes, are also detected in MHC class II-containing granules (Figure 5). Like MHC class II molecules, lamps are associated with the internal vesicles present in type I and type II granules (Figure 5) but are rarely detected in the limiting membrane of the electron-dense type III granules. In agreement with the slow degradation rate of the invariant chain observed in BMMCs, a small percentage of MHC class II-positive type I and type II granules are also labeled with the IN1 antibody specific for the cytoplasmic domain of the invariant chain (not shown).

These observations show that in BMMCs, MHC class II molecules accumulate in prelysosome/lysosome-related multivesicular granules (type I) and in intermediate granules (type II). The former contains MPRs and lamps. In addition to these markers, the latter contains serotonin.

Kinetics of Accessibility of MHC Class II-containing Granules to an Endocytic Tracer

In BMMCs, MHC class II molecules accumulate in granules with the characteristics of secretory lysosomes. To analyze the position in the endocytic pathway of these morphologically distinct compartments, we examined their kinetics of accessibility to an exogenously added tracer. BMMCs internalized by fluid phase BSA conjugated to 5-nm gold particles (BSAG). The cells were pulsed with BSAG for 10 min at 37°C and, after removing excess particles, the tracer was chased for 5, 20, and 80 min at 37°C. As shown in Figure 6, both type I and type II MHC class II-containing granules can be reached by BSAG after a 20-min chase. At this time point, 56% of class II-positive type I granules and 10% of type II granules contained the tracer, which was located in the multivesicular region. At an 80-min chase, only 5% of type I granules were positive whereas 60% of type II granules contained BSAG, mainly present in the dense core rather than associated with the multivesicular domain (Figure 6B). At all time points, type III granules did not contain BSAG, although it cannot be ruled out that they could be located later in the endocytic pathway.

These results show that type I and type II secretory granules are positioned in the endocytic pathway similarly to prelysosomal and lysosomal compartments, respectively.

Fate of MHC Class II Molecules during Degranulation Induced by IgE-Antigen Immune Complexes

In BMMCs, MHC class II molecules specifically accumulate in 60–80-nm membrane vesicles contained in lysosome-related granules called secretory lysosomes. A major question concerns the destiny of type I and type II compartments during regulated exocytosis or degranulation process. To elucidate this issue, BMMCs were sensitized with IgE anti-DNP and degranulation was triggered by incubation with DNP-BSA for 5 to 60 min. At the ultrastructural level, cells that have undergone degranulation showed an increase of cell surface processes and a reduced number of intracellular granules. Quantitation of the total number of intracellular granules revealed that after 30 min of degranulation mast cells show a reduction of 13% of the total number of granules. As shown in Figure 7, between the plasma membrane extensions reminiscent of an exocytic event, small vesicles similar to those present in intracellular granules and intensely labeled with anti-class II antibodies were observed. The presence of MHC class II-positive vesicles at the cell surface of BMMCs shows that during degranulation both soluble contents and membrane proteins associated with the intragranular vesicles are secreted.

Figure 5 (facing page). Immunogold localization of MHC class II, lamp1, and serotonin. (A) Ultrathin cryosections were double-immunogold labeled with anti-class II (PAG 10) and anti-lamp1 (PAG 15) antibodies. Both MHC class II molecules and lamp1 are associated with the internal membrane vesicles surrounding the electron-dense core of type II granules. (B) Ultrathin cryosections were double-immunogold labeled with anti-lamp1 (PAG 10) and antiserotonin antibodies (PAG 15). Serotonin was only detected in the electron-dense core of type II granules. lamp1 was present in the internal vesicles of multivesicular granules (type I) and intermediate granules (type II). Bars, 200 nm.

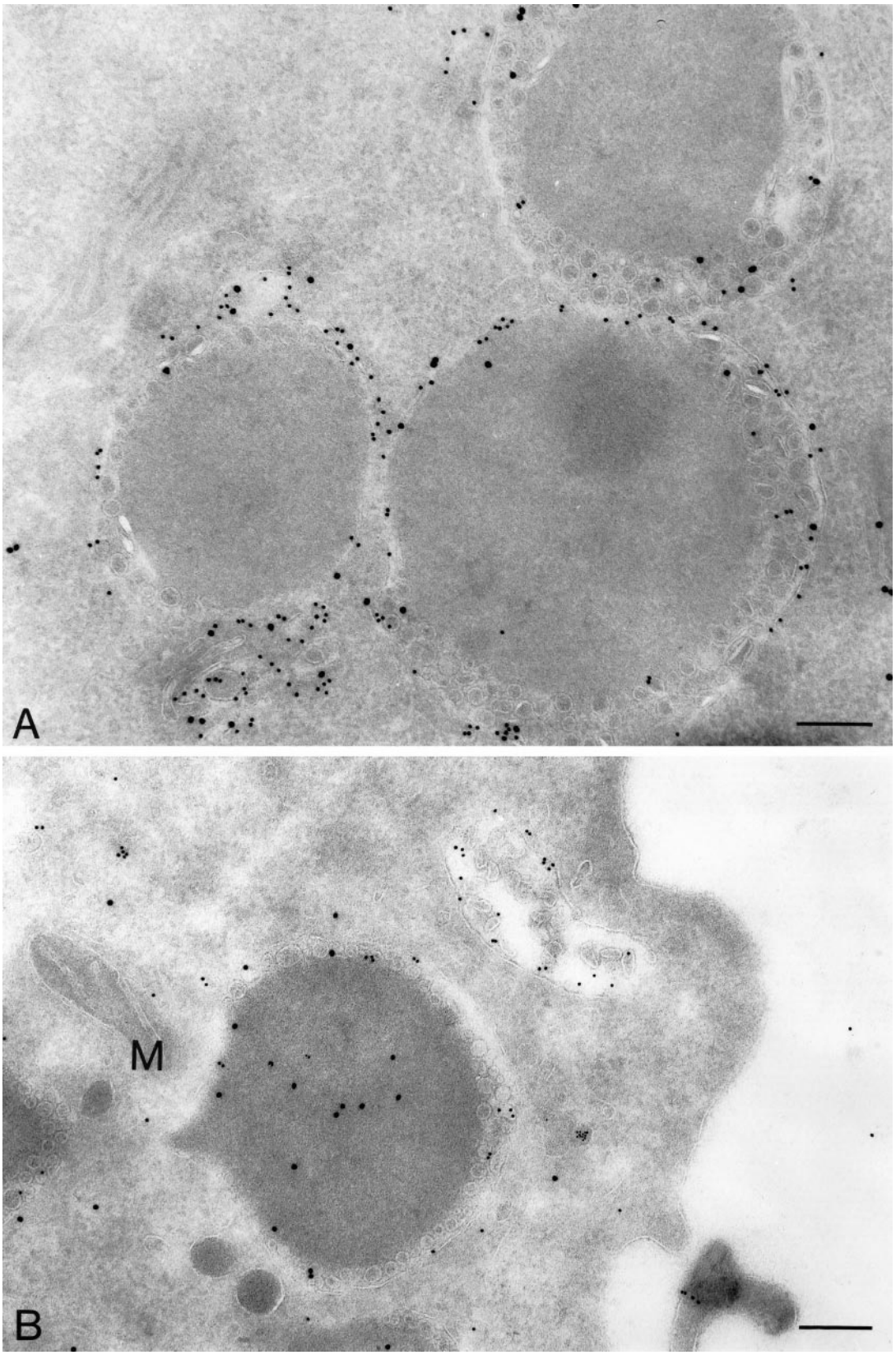


Figure 5.

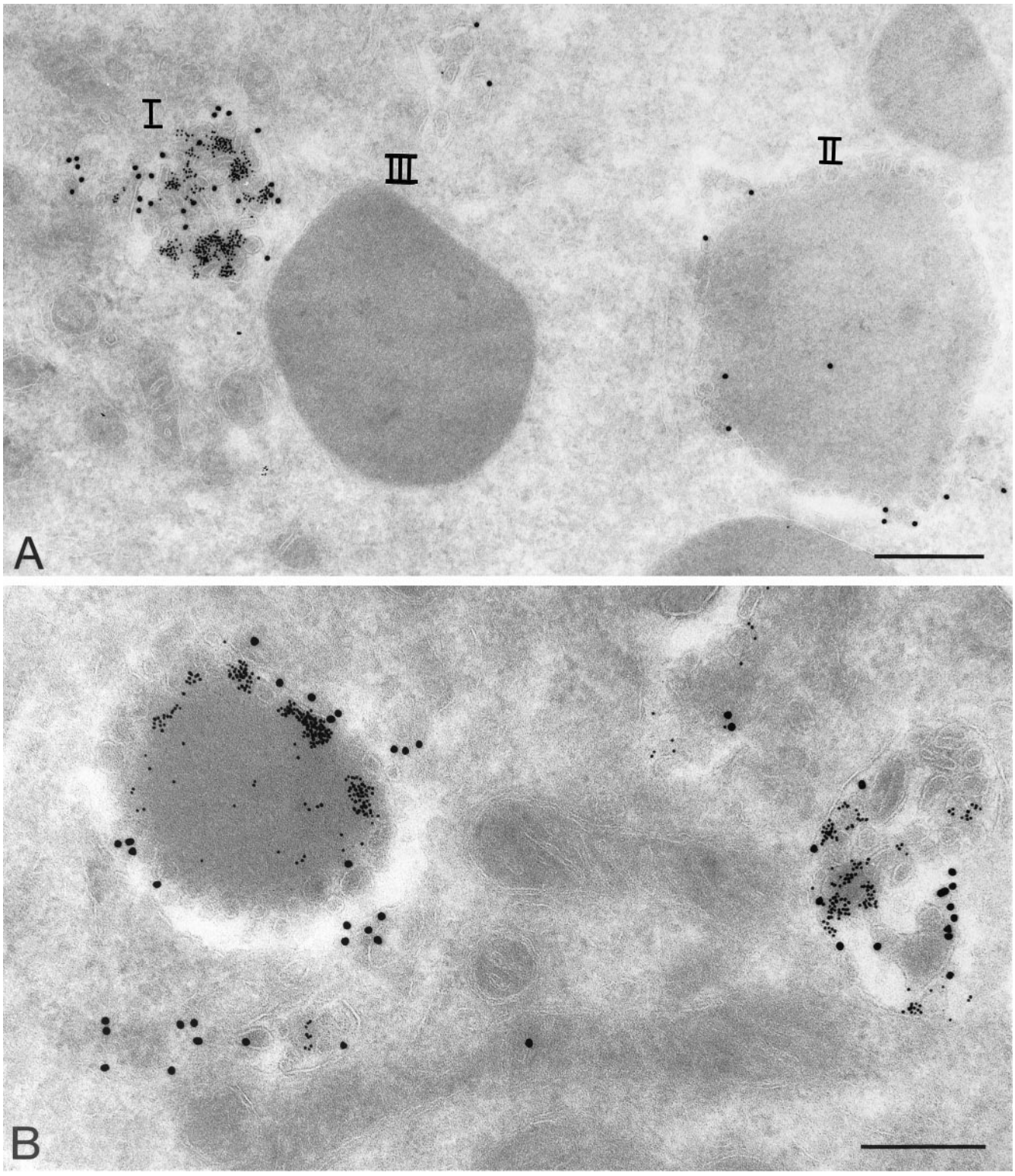


Figure 6. Kinetics of accessibility of mast cell granules to BSAG. BMMCs were pulsed for 10 min with BSA coupled to 5 nm of gold (BSAG) and then chased for 20 min (A) or 80 min (B). Ultrathin cryosections were labeled with the anti-class II antibody M5114 (PAG 10). After 20 min of chase, BSAG was detected in multivesicular compartments (type I granules) intensely labeled with anti-class II antibodies. The majority of type II granules as well as type III granules were not reached by the tracer. After 80 min of chase, the majority of class II-positive

To provide biochemical evidence for the release of MHC class II molecules during degranulation, membrane vesicles were isolated by differential centrifugation from the supernatant of mast cells that have been incubated with DNP-BSA at 37°C (degranulated cells) or at 4°C (nondegranulated cells). The 70,000-g pellets (S-P) were analyzed by Western blot with anti-I-A, anti-Ii, and anti-lamp1 antibodies. The secretion of both MHC class II molecules and lamp1 is calcium dependent and rapid, corresponding to the requirements for the release of inflammatory mediators during mast cell degranulation (Figure 8A). As shown in Figure 8B, in mast cells such as in B lymphoblastoid cell lines, the invariant chain is only detected in cell lysates, indicating that only MHC class II molecules free of invariant chain are recovered from the medium of degranulated cells.

We have shown that secretory lysosomes contain 60–80-nm vesicles in their lumen. By using MHC class II-specific antibody, we show that these vesicles are the major site of intracellular accumulation of MHC class II molecules in mast cells. During regulated exocytosis triggered by IgE-antigen complexes, these vesicles, identical to previously characterized exosomes (Raposo *et al.*, 1996), are released into the extracellular environment.

DISCUSSION

Mast cells synthesize MHC class II molecules which accumulate in secretory granules. This allows the investigation of the relation between MIICs and secretory granules, as well as the destiny of MHC class II-containing compartments during regulated exocytosis.

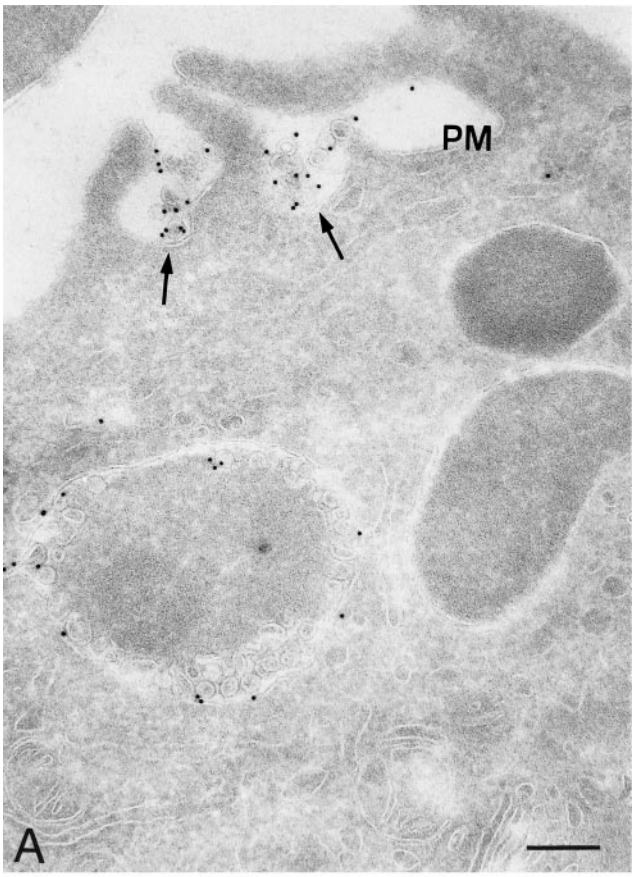
Two major features characterize the synthesis and the localization of MHC class II molecules in mast cells in comparison to other APCs. First, a slow degradation of the invariant chain whose P10 fragment remains associated with α-β dimers to give raise to a P70 complex observed by SDS-PAGE analysis under nonboiling conditions. Second, MHC class II molecules are present at very low levels on the cell surface and are largely retained intracellularly in compartments sharing features with both lysosomal compartments and secretory granules. Such high intracellular retention is probably a consequence of the slow degradation rate of li, which may control the transport of MHC class II molecules in and out of lysosomal compartments (Brachet *et al.*, 1997).

A major feature of the intracellular localization of MHC class II molecules in mast cells is their restricted location to the membrane of 60–80-nm internal vesicles similar to those present in the lumen of multivesicular MIICs (Peters *et al.*, 1995; Glickman *et al.*, 1996; Raposo *et al.*, 1996). Murine mast cells do not contain the characteristic multilaminar MIICs displayed by human B cells and dendritic cells (Nijman *et al.*, 1995; Peters *et al.*, 1995) or the electron-dense MIICs of murine B cells and dendritic cells (Kleijmeer *et al.*, 1995; Brachet *et al.*, 1997). Therefore, in mast cells, MHC class II molecules are specifically sequestered in internal membrane vesicles contained in the lumen of two morphologically distinct compartments: one displaying only vesicles and the other displaying both vesicles and an electron-dense core. Mast cells may be representative of a model to evaluate subcellular mechanisms implicated in the targeting and accumulation of MHC class II molecules in the internal membrane vesicles of multivesicular organelles. In addition, these cells may be used to examine involvement of such compartments in invariant chain degradation and peptide binding to MHC class II molecules.

The presence of MHC class II molecules in serotonin-containing secretory granules emphasizes the intimate connection between the lysosomal compartment and secretory granules previously described only in cytotoxic T cells and NK cells (Burkhardt *et al.*, 1990; Peters *et al.*, 1991a). Besides the presence of MHC class II molecules, which in several APCs accumulate largely in lysosomal compartments, we show that mast cell granules bear lysosomal membrane proteins (lamps). These observations extend previous reports showing that mast cell granules contain lysosomal enzymes such as β-hexosaminidase, β-glucuronidase, arylsulfatase, and carboxypeptidases (Bentfeld-Barker and Bainton, 1980; Schwartz and Austen, 1980, 1981). In addition, the convergence between the endocytic and secretory pathway in these cells is emphasized by the observation that mast cell granules are loaded with exogenously added tracers (BSAG) with time lags compatible with their location late in the endocytic pathway. Similar to prelysosomal MVBs, type I granules contain densely packed membrane vesicles bearing MPRs and lamps and are reached by BSA-gold conjugates after 30 min of internalization; type II granules display a classical secretory domain, i.e., the electron-dense core and they contain the same markers. The majority of these granules are reached by the tracer later, indicating that they are located in the endocytic pathway after the type I granules. However, the biogenesis of these organelles as well as how transport occurs to and in-between organelles is still obscure.

The biogenesis of the type I multivesicular granules previously defined, in particular, in cytotoxic T cells, NK cells, or platelets is not clear. Given their striking similarities with prelysosomal MVBs, one cannot rule out that they may represent the same compartment. In

Figure 6 (cont). type II granules contained BSAG. Only a small percentage of type I granules still contained BSAG. Bars, 200 nm.



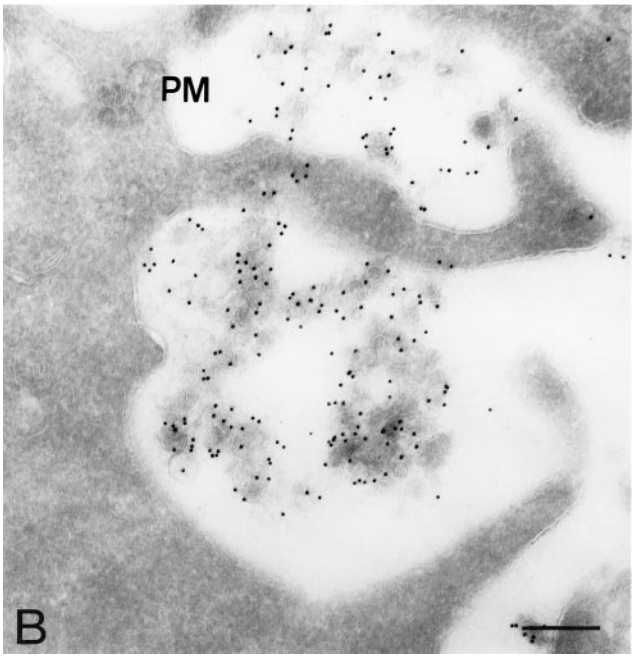


Figure 7. Release of membrane-associated MHC class II molecules by BMMCs during degranulation. BMMCs were incubated overnight with IgE anti-DNP antibodies, and degranulation was induced for 60 min with or without the addition of DNP-BSA. Ultrathin cryosections were immunogold labeled with anti-class II antibodies and PAG 10 (A and B). Upon degranulation, small vesicles (60–80 nm) highly labeled with anti-class II antibodies were detected at the cell surface between plasma membrane extensions (arrows). PM, plasma membrane. Bars, 200 nm.

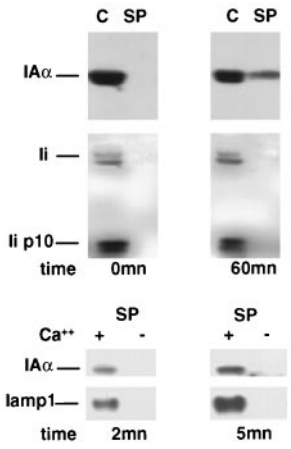


Figure 8. BMMCs were incubated overnight with IgE anti-DNP antibodies and then degranulation was induced by addition of DNP-BSA in the presence of 1 mM $CaCl_2$ or 1 mM EGTA for 2 min, 5 min, or for 60 min in calcium-containing medium (Lower panels). Western blot with anti-I-A and anti-lamp1 antibodies on the 70,000-g pellets of supernatants (S-P) of mast cells degranulated with or without calcium. (Upper and middle panels) Western blot with anti-I-A and anti-li antibodies of mast cell lysates and of the 70,000-g pellets of supernatants (S-P). MHC class II molecules were recovered in S-P only from degranulated cells whereas li was not detected.

this case, they find their origin in the endocytic pathway, namely, during the maturation process, allowing the formation of late endosomes and prelysosomes from early endosomes. In such a model, the internal membrane vesicles of the prelysosomal MVBs are thought to arise from budding of a portion of the limiting membrane of the early endosome into the endosomal lumen. During the invagination toward the intralumenal milieu, membrane proteins as well as soluble components undergoing transport to lysosomes are sequestered in the membrane and cytosol of the internal vesicles, whereas other proteins remain in the limiting membrane of the MVBs (Hopkins *et al.*, 1990; Trowbridge *et al.*, 1993; van Deurs *et al.*, 1993). However, as suggested for the biogenesis of MIICs in B cells, multivesicular organelles may also find their origin directly from the biosynthetic pathway. Indeed, the majority of MHC class II molecules are thought to enter the endocytic pathway directly from the TGN along with the invariant chain (Neefjes *et al.*, 1990; Benaroch *et al.*, 1995), likely at the level of an early MIIC displaying few internal vesicles (Glickman *et al.*, 1996). These early multivesicular MIICs may originate at the level of the TGN by a mechanism reminiscent of the biogenesis of secretory granules in endocrine cells (Dahan *et al.*, 1994; Glickman *et al.*, 1996). The expression and localization of MHC class II molecules in mast cell secretory granules may offer a unique opportunity to investigate the transport of MHC class II molecules to such compartments, namely, in relation to well-defined secretory proteins.

The origin of intermediate type II granules is also not completely clarified despite efforts to elucidate this issue, in particular, in cytotoxic T cells and NK cells. A number of reports have favored the hypothesis that such dual organelles represent a degradative compartment arising by fusion of lysosomes with secretory granules. This process, called crinophagy, may operate for disposal of unused granules (de Duve, 1989). However, our present study, along with data published by others, show that the multivesicular domain of such granules is of prelysosomal origin since it contains the MPR (Burkhardt *et al.*, 1990). Moreover, the content of these dual organelles is also secreted namely in the cleft between cytotoxic T cells and target cells (Peters *et al.*, 1989, 1991a). At present, two models can be proposed for the biogenesis of such granules. Peters *et al.* (1989, 1991a) from work on cytotoxic T cells have suggested that type I granules mature sequentially into type II and then type III granules by a condensation process in which internal vesicles fuse together to form an electron-dense core often surrounded by a membrane. The second model implies that type I granules fuse with type III granules similar to the mechanism proposed for fusion of multivesicular prelysosome with mature lysosomes (Futter *et al.*, 1996). Indeed, electron-dense type III granules may represent the lysosomal compartment of these cells. Despite that they are not considerably enriched in lysosomal membrane proteins, they do contain several proteolytic enzymes (Severson, 1969; Lagunoff *et al.*, 1970; Bentfeld-Barker and Bainton, 1980; Schwartz and Austen 1981). Our results, showing that the multivesicular granules are devoid of serotonin and that even after 2 h of internalization the type III granules do not seem to be accessed by exogenous molecules, are in favor of the second hypothesis implying a fusion event between the types I and III granules for the generation of the type II granules. The biogenesis of these organelles as well as the subcellular mechanisms and molecular machineries involved in such events will be fields for further investigations.

In the present work, we show that MHC class II molecules associated with vesicles identical to exosomes secreted constitutively by B lymphoblastoid cell lines (Raposo *et al.*, 1996) are exocytosed by mast cells only upon calcium-dependent triggering. This observation suggests, for the first time, that release of membrane-associated MHC class II molecules may be a regulated process. It is not known whether in mast cells MHC class II-enriched exosomes are released upon fusion with the cell surface of only type II granules or whether type I multivesicular granules are also targets for regulated secretion. Nevertheless, observations of fusions of multivesicular MIICs in B cells, multivesicular cytolytic granules in cytotoxic T cells, or transferrin receptor containing MVBs in reticulocytes emphasize that MVBs devoid of the so-called secretory domain have also the ability to fuse with the plasma membrane (Raposo *et al.*, 1997b). It seems likely that secretory lysosomes represent an heterogeneous population of organelles and that the molecular machinery needed for fusion is also present in MVBs. Thus, secretion of membrane and cytosolic proteins associated with exosomes may represent an important property of the endocytic and secretory pathway in hematopoietic cells. The physiological relevance of exosome secretion by APCs is still a matter of debate. In vitro studies indicate that MHC class II exosomes secreted by B cells are able to stimulate T cell proliferation likely through their expression of adhesion and costimulatory molecules (Raposo *et al.*, 1996). Their role in intercellular communication as well as their possible implication in the maintenance of T cell memory or T cell tolerance has been proposed. Mast cells, due to their widespread localization and to their ability to secrete membrane-bound class II molecules as well as cytokines, are good candidates to play a key role in the recruitment of circulating T cells at the site of the inflammatory reaction.

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