INTRODUCTION

Presentation of antigenic peptides to T cells in the context of MHC class II molecules by antigen presenting cells, such as macrophages, B lymphocytes, and dendritic cells, requires a complex sequence of intracellular events. Antigens are internalized, then degraded into peptides which associate with MHC class II molecules before reaching the cell surface to stimulate T cells (Watts, 1997). MHC class II α and β subunits assemble in the endoplasmic reticulum with the invariant chain (Ii), then reach endocytic compartments (Cresswell, 1994). In these compartments, Ii is degraded, leaving MHC class II molecules free to bind peptides derived from endocytosed antigens. Depending on the cell type analyzed, compartments either related to endosomes (Class II vesicles; CIIVs in murine B cells) or to lysosomes (MHC class II compartments; MIICs in human B cells, dendritic cells, macrophages) have been identified. CIIVs and MIICs have been proposed as the meeting point between class II molecules and processed antigenic peptides (Amigorena et al., 1994; Tulp et al., 1994; West et al., 1994).

In bone marrow-derived mast cells (BMMCs) MHC class II molecules accumulate in secretory granules with lysosomal characteristics, called secretory lysosomes (Raposo et al., 1997). Three types of secretory granules were identified on the basis of their ultrastructural morphology, their protein content and their accessibility to endocytic tracers. The majority of MHC class II molecules accumulate in Type I granules displaying intralumenal vesicles and in Type II granules containing an electron dense core, rich in serotonin, surrounded by a multivesicular cortex (Raposo et al., 1997). Two questions arise from these observations: what are the molecular mechanisms responsible for the retention of MHC class II molecules in secretory granules and what are the consequences of this peculiar localization of MHC class II molecules on antigen presentation in mast cells?

To approach these issues, BMMCs are not the best experimental model. First, these cells need to be cultured in a cocktail of lymphokines (IL3, IL4 and GM-CSF) which may have pleiotropic effects on MHC class II transport and functions. Second, BMMCs are an heterogeneous cell population since only 30% of cells express MHC class II molecules. Therefore, we designed a new experimental model to study MHC class II transport and functions in mast cells, by expressing IA α and β chains, as well as murine Ii, in the MHC class II negative rat basophilic cell line, RBL-2H3 (Barsumian et al., 1981). In the present study, we show that most MHC class II molecules were retained in intracellular compartments displaying markers of lysosomes and secretory granules. In these compartments, MHC class II molecules were found in fragments. This defect, resulting in a slower rate of MHC class II maturation, was ascribed to a low cathepsin S activity. Second, although a small fraction of class II dimers matured (i.e. became free of invariant chain), allowing their association with antigenic peptides, they were retained in secretory granules. As a consequence of this intracellular localization, cell surface expression of class II molecules was strongly increased by cell activation stimuli which induced the release of the contents of secretory granules. Our results suggest that antigen presentation, and thereby antigen specific T cell stimulation, are regulated in mast cells by stimuli which induce mast cell activation.

Key words: MHC class II, Mast cell, Invariant chain, Secretary granule, Antigen presentation

SUMMARY

Bone marrow-derived mast cells as well as dendritic cells, macrophages and B lymphocytes express major histocompatibility complex (MHC) class II molecules. In mast cells, the majority of MHC class II molecules reside in intracellular cell type-specific compartments, secretory granules.

To understand the molecular basis for the localisation of MHC class II molecules in secretory granules, MHC class II molecules were expressed, together with the invariant chain, in the mast cell line, RBL-2H3. Using electron and confocal microscopy, we observed that in RBL-2H3 cells, mature and immature class II molecules accumulate in secretory granules. Two particular features of class II transport accounted for this intracellular localization: first, a large fraction of newly synthesized MHC class II molecules remained associated with invariant chain fragments. This defect, resulting in a slower rate of MHC class II maturation, was ascribed to a low cathepsin S activity. Second, although a small fraction of class II dimers matured (i.e. became free of invariant chain), allowing their association with antigenic peptides, they were retained in secretory granules. As a consequence of this intracellular localization, cell surface expression of class II molecules was strongly increased by cell activation stimuli which induced the release of the contents of secretory granules. Our results suggest that antigen presentation, and thereby antigen specific T cell stimulation, are regulated in mast cells by stimuli which induce mast cell activation.
two different forms: the majority of class II molecules were immature, i.e. associated to a partially digested form of Ii, the p10 fragment, which was due to a low cathespin S activity in mast cells. However, a fraction of class II molecules retained in the secretory granules matured into â² dimers able to bind antigenic peptides. Consequently, we show that stimuli which induce mast cell activation, and therefore degranulation, also allow exposure of functional MHC class II-peptide complexes at the cell surface and thus T cell stimulation.

MATERIALS AND METHODS

Chemical reagents and antibodies

Chemical reagents used in this study were all from Sigma (St Louis, MO, USA). RPMI 1640, fetal calf serum (FCS), phosphate buffered saline (PBS), penicillin – streptomycin, sodium pyruvate and L-glutamine were purchased from Gibco (Paisley, Scotland). RPMI depleted for methionine and cysteine, and [35S]methionine/cysteine labelling mix were obtained from ICN (Orsay, France). Protein G-Sepharose was obtained from Pharmacia (Uppsala, Sweden). The antibodies used were the mouse anti-mouse IAb mAb MKD6 (Kappler et al., 1981), the mouse anti-mouse IAb mAb Y3P (Janeway et al., 1984), the mouse mAb YaE anti-mouse IAb associated with peptide 52-68 of IEd â (Murphy et al., 1992), the rat anti-IAb and IAd mAb M5/114 (Bhattacharya et al., 1981), a polyclonal rabbit serum against the conserved cytoplasmic tail of the â chain of the mouse MHC class II molecules IAb, d (anti-IAb6), the rat anti-mouse Ii chain mAb IN1 (directed against Ii’s cytoplasmic tail) (Peterson and Germain, 1992), the rat anti-serotonin anti-serum (Seralabo), a rabbit anti-mouse IAb associated with Ii chain or anti-Ii-NH2 chain or anti-Ii-NH2 (Kappler et al., 1981), the mouse anti-mouse IAb associated with peptide 52-68 of IEd â (Murphy et al., 1992), the rat anti-IAb and IAd mAb M5/114 (Bhattacharya et al., 1981), a polyclonal rabbit serum against the conserved cytoplasmic tail of the â chain of the mouse MHC class II molecules IAb, d (anti-IAb6), the rat anti-mouse Ii chain mAb IN1 (directed against Ii’s cytoplasmic tail) (Peterson and Miller, 1990) and a rabbit anti-serum specific for the cytoplasmic tail of Ii chain (IiNH2) (a gift from J. Davoust, CIML, Marseille, France). Other antibodies were used the mouse anti-rat CD63 mAb AD1 (a gift from Dr R. P. Siragian, NIH, Bethesda, MD) (Kitani et al., 1991), the mouse anti-rat lamp1 mAb LY1C6 (a gift from Dr W. Hunziker, Epalinges, Switzerland) (Lewis et al., 1985), the mouse anti-rat p80 (protein contained in the secretory granules of RBL-2H3; Bonifacino et al., 1989) 5G10 (a gift from J. Bonifacino, NIH, Bethesda, MD), a rabbit anti-serotonin anti-serum (Seralabo), a rabbit anti-mouse cathespin S anti-serum (a gift from Dr H. Chapman, Harvard University, Boston, MA). To specifically detect mouse, rat or rabbit antibodies, horseradish peroxidase (HRP-), FITC- or Texas Red-coupled F(ab')2 fragments of donkey antiserum were obtained from Jackson ImmunoResearch (Jackson laboratory, West Grove, PE, USA).

Cells

RBL-2H3 (Barsumian et al., 1981), B414, IIA1.6 and T cell hybridoma were grown in RPMI 1640, 10% FCS, 1% penicillin-streptomycin, 0.1% ß-mercaptoethanol, 2% sodium pyruvate. The B lymphoma IIA1.6 is a FcγR-defective variant of A20 B cells (Bonnerot et al., 1992). The B lymphoma B414 is a A20 B lymphoma cell line transfected with cDNAs encoding IAb ß and Ii chains (kindly provided by A. Barlow and C. Janeway, Yale University, New Haven, CT). TH30 is a T cell hybridoma which recognizes ßτ 52-68 peptide associated with IAb (Barlow et al., 1998). Peptides have been synthesised by Syntec (Nice, France).

cDNAs were cloned downstream of an ßτ promoter (Takebe et al., 1988) in expression vectors carrying resistance genes for hygromycin B (NTHygro), neomycin (NTNeo) or zeocin (NTZeo). The IAd and IAb cDNAs (respectively provided by Dr R. Germain, National Institutes of Health, and Drs A. Barlow and C. Janeway, Yale University, New Haven, CT) were subcloned in the NTHygro vector for ß chains and NTNeo vector for Ii chains. The cDNA encoding murine Ii chain (provided by Dr R. Germain) was subcloned in the NTZeo vector. Expression vectors were co-transfected into the RBL-2H3 cell line as described (Bonnerot et al., 1992). Briefly, cells were electroporated at 260 V, 975 μF with 50 μg of linearized plasmids. Two days after transfection, cells were switched into selection medium (containing 1 mg/ml of hygromycin, neomycin-G418- and zeocin, respectively). Growing cells were subcloned by limiting dilution in 96-well plates. Clones were then analyzed for surface MHC class II molecules expression by cytofluorimetry with a FACScan® flow cytometer (Becton Dickinson) after staining with MS/114 and FITC-coupled anti-rabbit antibodies.

Western blot

Cells were lysed in lysis buffer: 0.5% Triton X-100, 300 mM NaCl, 50 mM Tris, pH 7.4, 10 μg/ml of leupeptin, chemostatin, aprotinin, pepstatin, 20 mM N-ethylmaleimide, 1 mM PMSF and centrifuged at 20,000 g for 15 minutes. Cell lysates were diluted in Laemmli buffer and boiled at 95°C for 5 minutes before analysis on 12% SDS-polyacrylamide gels. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). After incubation with blocking solution, membranes were incubated with specific antibodies followed by HRP-conjugated anti-rabbit IgG antibodies. Chemiluminescence was detected using a Boehringer kit.

Cell fractionation and immunoblotting

Percoll gradients were performed as described (Bonnerot et al., 1998). Briefly, 3×107 RBL IAbIi cells were washed twice in PBS and resuspended in 1 ml of 10 mM trithionalamine, 10 mM acetic acid, 1 mM EDTA, 250 mM sucrose, pH 7.4 (TEA). They were homogenized in a ball-bearing homogenizer (Balch and Rothman, 1985) and centrifuged for 10 minutes at 300 g. Three hundred microliters of the resulting postnuclear supernatant (PNS) were diluted with TEA 250 mM sucrose and 90% Percoll to obtain a final concentration of 22% Percoll, loaded on top of a 1.5 M sucrose cushion and centrifuged for 30 minutes at 33,000 rpm in a TLA100.4 rotor (Beckman Instruments, Inc., Palo Alto, CA). One milliliter fractions were collected. ß-Hexosaminidase activity in 1/100 of each fraction was revealed with 6 mM 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminide (Sigma) in NaCitrate-PO4 buffer, pH 4.5 (Green et al., 1987; Harms et al., 1980; Pool et al., 1983). Alkaline phosphodiesterase activity was revealed on 1/10 of each fractions using thymidine-5'-monophosphate-p-nitrophenyl ester (Sigma) (Green et al., 1987; Pool et al., 1983). Before being analysed by SDS-PAGE, fractions were lysed for 30 minutes at 4°C in 1/10th volume of 10× lysis buffer (3 M NaCl, 5% Tx-100, 0.5 M Tris-HCl, pH 7.4) with 1 mM PMSF at 4°C for 30 minutes, adjusted to pH 12 with 2 M NaOH and centrifuged at 20,000 g for 30 minutes at 4°C in order to pellet Percoll (Marsh et al., 1987). Proteins were analysed by western blotting with the rabbit anti-IA â chain or anti-li-NH2 antibodies.

Metabolic labelling and immunoprecipitation

Immunoprecipitations were performed as described (Amigorena et al., 1994). After 45 minutes starving in methionine- and cysteine-free medium, the cells were metabolically labelled with [35S]methionine/cysteine labelling mix for 30 minutes and chased for various times. Cells were lysed in 0.5% Nonidet P40, 300 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 1 mM PMSF. Immunoprecipitations were performed on nucleus-free cell lysates with Y3P-, IN1- or M5/114-coated Protein G-Sepharose beads. After washing, 30% of immunoprecipitates were eluted in 20 μl of Laemmli buffer containing 50 mM DTT for 30 minutes at room temperature to release SDS-stable complexes from the beads. The remaining 70% were eluted in 50 mM DTT Laemmli buffer, heated at 95°C for 5 minutes. Samples were analysed on 12% SDS-acrylamide gels.

Assays for antigen presentation

For antigen presentation assay by degranulated cells, RBL IAbIi cells were incubated overnight with various concentrations of IEd â 52-68 peptide. They were then incubated at 37°C for 30 minutes with or without 1 μM ionomycin in DMEM (Gibco) then fixed at 4°C for 30
seconds with 0.05% glutaraldehyde. TH30 cells were added at 10^6 cells/ml. After 24 hours, IL-2 release by the T-cell hybridoma into the culture supernatants was determined as described before (Bonnerot et al., 1995), using a CTL.L2 proliferation assay: 50 µl of supernatants were removed, frozen at -80°C for 1 hour, thawed and added to IL-2-dependent CTL.L2 cells for 16 hours. [3H]Tdr ([H]-labelled deoxythymidine) incorporation was measured after an additional 6 hours incubation in the presence of 0.25 µCi [3H]Tdr/well. Each point represents the average of duplicate samples which varied by less than 5%.

Immunofluorescence staining and confocal microscopy

Cells were washed in complete medium and allowed to adhere on glass coverslips for 2 hours. All solutions were completed with Ca^{2+} and Mg^{2+} in order to allow the adhesion of RBL-2H3 cells on coverslips. Cells were fixed in 3% paraformaldehyde for 10 minutes at room temperature and then incubated with 100 mM glycine in PBS. For staining with rabbit anti-serotonin antibody, cells were fixed for 10 minutes at room temperature with 2% glutaraldehyde and then incubated with sodium borohydride (Sigma) at 1 mg/ml for 15 minutes at room temperature. After permeabilization with 0.05% saponin in PBS 0.2% BSA, cells were incubated with specific antibodies. The mouse mAbs Y3P, MKD6, YAc, AD1, the rat mAb M5/114, the rabbit sera anti-serotonin, anti-IiNH2, anti-IAβ chain were all used at 7-10 µg/ml. After three washes the mouse, rat or rabbit antibodies were, respectively, revealed by (F(ab')2 donkey anti-mouse, anti-rat or anti-rabbit IgG coupled to FITC or TR. Coverslips were mounted in Mowiol. Confocal laser scanning microscopy was performed using a Leica TCS microscope based on a DM microscope interfaced with a mixed gas Argon/Krypton laser (Leica Laser Technik, Germany) as previously described (Bonnerot et al., 1995).

Immunoelectron microscopy

RBL-IAbIi cells were fixed with a mixture of 2% paraformaldehyde in phosphate buffer 0.2 M, pH 7.4 (PB) and 0.125% glutaraldehyde for 2 hours at room temperature. Fixed cells were processed for ultrathin sectioning and immunolabelling as described previously (Raposo et al., 1997). After washing with PB and PB-50 mM glycine, cells were embedded in 7.5% gelatin. Small blocks were infiltrated with 2.3 M sucrose at 4°C for 2 hours and then frozen in liquid nitrogen. Ultrathin cryosections prepared with a Leica ultracut FCS (Wien, Austria) were retrieved with a mixture of 2% methylcellulose and 2.3 M sucrose (vol/vol) and indirectly immunogold labelled with M5/114 followed by a rabbit anti-rat antibody (Dako, Danemark) and a rabbit polyclonal anti-serotonin antiserum. Rabbit antibodies were detected with Protein A coupled to 10 and 15 nM gold particules (Raposo et al., 1997). After washing with PB and PB-50 mM glycine, cell surface expression of IAb and IAβ chain was measured using a CM120 Twin Phillips electron microscope (Eindhoven, The Netherlands).

Fluorometric test for cathepsins activities

PNS of RBL-IAbIi and B414 cells were prepared as described above. Membranes were obtained by centrifugation of PNS at 70,000 g for 1 hour at 4°C. The same amounts of proteins (measured by Bradford assay) diluted in 1 M NaAc, 0.2M EDTA, 0.5 M DTT, 5% CHAPS (pH 5.5 or pH 7.0) were incubated for 1 hour at 37°C in a 96-well plate with the substrates of cathepsins: 200 µM Z-Val-Val-Arg-NHMec (a gift from P. Morton, Chesterfield, MO, USA) for cathepsin S in the presence or not of 200 nM of the inhibitor N-morpholinureaurea-NHMec (a gift from P. Morton, Chesterfield, MO, USA) for cathepsins B/L. Reactions were quantified at λexc.=355 nm and λem.=460 nm with a fluorometer (1420 VICTOR® multilabel counter EG&G WALLAC) (Manoury et al., 1998). The same samples were tested for β-hexosaminidase activity as described before. LHVS was kindly provided by Dr H. Ploegh (Harvard University, Boston, MA).

Labelling of active site of cysteine proteases

RBL-IAbIi and B414 were incubated in complete RPMI with or without 50 nM cathepsin S inhibitor LHVS. Detection of active cysteine proteases with Mu-125I-Tyr-Ala-CH2F was performed as described elsewhere (Riese et al., 1996). The cysteine protease inhibitor Mu-Tyr-Ala-CH2F was iodinated as previously reported (Mason et al., 1989). RBL-IAbIi and B414 cells (10^7 cells/sample) were incubated with LHVS (50 nM) at 37°C for 1 hour prior to labelling. Cells were then incubated with 50 nM Mu-125I-Tyr-Ala-CH2F for 2 hours at 37°C, washed twice with cold PBS 1% FCS. Cell lysates were boiled for 5 minutes before analysis in a 12% SDS-acrylamide gel.

RESULTS

RBL-2H3 cells accumulate MHC class II molecules in secretory lysosomes

To study MHC class II transport in mast cells, we have expressed recombinant murine class II molecules in the rat basophilic cell line RBL-2H3. This mast cell line has a functional phenotype of mucosal mast cell: i.e. expresses high affinity receptors for IgE (FceRI) and contains secretory granules that accumulate biogenic amines, such as histamine and serotonin (Barsumian et al., 1981). These amines are released by the cells upon stimulation through IgE receptor or a calcium ionophore (Dvorak et al., 1983).
RBL-2H3 cells have been transfected with cDNAs encoding the α and the β chains of the b or the d haplotypes of IA MHC class II molecules as well as murine Ii. Numerous clones were isolated and characterized by immunofluorescence using monoclonal antibodies specific for the αβ dimers of IAd (MKD6), of IAb (Y3P) (data not shown) or of IAb/d (M5/114).

Fig. 2. MHC class II complexes are mainly retained in lysosomes and secretory granules. RBL-2H3 cells expressing class II molecules were fixed with 3% paraformaldehyde (A,B) or 1% glutaraldehyde (C) before permeabilization with 0.05% saponin and incubation with various antibodies. Staining was detected with FITC-coupled or Texas red-coupled secondary antibodies, and was analyzed by confocal microscopy. (A) MHC class II molecules and Ii co-accumulate in the same vesicles. RBL IAbIi and IAdIi cells have been stained with rat mAb anti-β chain, M5/114 (green) and a rabbit antiserum specific for the cytoplasmic tail of murine Ii (red). (B) MHC class II molecules accumulate in lysosomal compartments. RBL IAbIi were stained with the rabbit anti-IAα chain cytoplasmic tail and two antibodies directed against lysosomal proteins: AD1 specific for CD63 and LY1C6 specific for Lamp1 (red). (C) MHC class II molecules accumulate in secretory granules of RBL-2H3 cells. RBL IAbIi cells were labelled with the anti-CD63 (AD1) or the anti-class II (M5/114) antibodies (green) and a rabbit anti-serotonin antiserum (red). (D) MHC class II-peptide complexes accumulate intracellularly. RBL IAbIi cells incubated overnight with IEα 52-68 peptide were fixed and permeabilized with 0.05% saponin. IAb/IEα 52-68 peptide complexes were detected with the mouse mAb YAc (red) and MHC class II molecules with the rat anti-IAb/d mAb, M5/114 (green). Intracellular staining was analyzed by confocal microscopy. (B,C,D) Arrows indicate colocalisation of the two labellings.
class II molecules. The RBL IAbIi cells as well as the RBL IAdIi cells, shown in Fig. 1, have been chosen because they expressed a high and homogenous level of MHC class II molecules at their surface as analyzed by cytofluorimetry (Fig. 1) and similar levels of Ii as detected by western blotting (see Fig. 4D).

The intracellular localization of MHC class II was determined by immunofluorescence. IAb and IAd molecules were detected with rabbit antiserum directed against the cytoplasmic domain of the IA α chain or with monoclonal antibodies specific for mature IAb (Y3P) or mature IAd (MKD6) αβ dimers, while the murine Ii was detected with a rabbit antiserum specific for the cytosolic NH2 domain. The labelling was analyzed by confocal microscopy in both RBL IAbIi and RBL IAdIi cells. Class II molecules and Ii were mostly detected in intracellular vesicular compartments (Fig. 2A). Only a small fraction of class II molecules was detectable at the cell surface.

The intracellular site of MHC class II accumulation was characterized by colocalization with different markers of endo/lysosomal compartments. The results obtained by immunofluorescence and confocal analysis indicated that class II molecules, CD63 (Kitani et al., 1991; Nishikawa et al., 1992) and Lamp 1 (Kleijmeer et al., 1996) were mostly detected in the same compartments (Fig. 2B). In contrast, class II molecules never colocalized with the transferrin receptor, a marker of early endosomes (Marsh et al., 1995) (data not shown). We then analyzed the intracellular localization of class II molecules into secretory granules containing serotonin. The cells were fixed with 2% glutaraldehyde to preserve the composition of the secretory granules, then class II molecules, CD63 and serotonin were labelled with specific antibodies. The results shown in Fig. 2C illustrate a clear accumulation of class II molecules or CD63 with serotonin in vesicular compartments sharing molecular components of typical and secretory lysosomes.

The next question was: are intracellular MHC class II molecules able to bind antigenic peptides? To answer this question, we used the monoclonal antibody, Y Ae, specific for the cytoplasmic domain of the IA α chain with the murine IAb class II molecules (Farr et al., 1996; Murphy et al., 1992). RBL IAbIi cells were incubated overnight with the Y Ae antibody specific for the cytosolic NH2 domain. The labelling was analyzed by confocal microscopy in both RBL IAbIi and RBL IAdIi cells. Class II molecules and Ii were mostly detected in intracellular vesicular compartments (Fig. 2A). Only a small fraction of class II molecules was detectable at the cell surface.

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To define the ultrastructure of the compartments where MHC class II molecules accumulate, ultrathin cryosections of RBL IAbIi were immunogold-labelled with the rat monoclonal anti-class II M5/114, and the anti-serotonin rabbit antiserum. Fig. 3 shows that in these cells, MHC class II molecules mainly localized in compartments displaying internal membrane vesicles. Class II molecules’ labelling was present on the small internal vesicles of 60 to 80 nM diameter and on the limiting membrane of the granule (Fig. 3). Serotonin could also be detected in the same compartments although it is difficult to state if the labelling is present in the lumen or associated with the cytosolic side of the internal vesicles. The MHC class II rich compartments in RBL-2H3 cells are similar to the lysosomal Type I granules observed in BMMCs (Rapooso et al., 1997).

These morphological approaches allow to conclude that in RBL-2H3, most MHC class II molecules are found in secretory compartments related to lysosomes where they can be loaded with antigenic peptide.

The maturation of MHC class II-invariant chain trimers is slowed down in RBL-2H3 cells

To analyze further the trafficking of mature (αβ-peptide) and immature (αβ-Ii) class II molecules in mast cells, we followed their biosynthesis. RBL IAbIi cells and B cells were metabolically labelled for 30 minutes with 35S-labelled cysteine and methionine, then chased for different times in non radioactive medium. IAb class II molecules were immunoprecipitated with Y3P specific for IAb αβ dimers free or associated with Ii fragments. The samples were heated or not at 95°C and analyzed by 12% polyacrylamide SDS-PAGE. When Y3P-immunoprecipitates were boiled with SDS, we observed that similar amounts of class II molecules matured with the same kinetics in RBL-2H3 cells and murine B cells. When the same samples were not heated, αβ SDS-stable dimers, which account for the full maturation of class II molecules, were detected early (2 hours) in murine B cells whereas, in RBL-2H3 cells, only a small fraction of class II molecules were detected in SDS-stable conformation after 20 hours of chase. Surprisingly, an additional SDS-stable 70 kDa complex was observed in RBL-2H3 cells after 6 hours of chase (Fig. 4A). The molecular mass of this complex could correspond to immature class II dimers (60 kDa) associated to the p10 fragment of Ii (10 kDa).

To define the composition of this 70 kDa complex in RBL-2H3 cells, immunoprecipitations were performed with antibodies recognizing non conformational epitopes on the β chain of class II molecules (i.e. M5/114) or the cytoplasmic domain of Ii (i.e. IN1). In boiling conditions, we observed (Fig. 4B) that the rate of Ii biosynthesis was similar in B cells and RBL-2H3 cells. In contrast, its half-life was clearly different since most of the p31 was degraded in B cells after 6 hours of chase whereas 50% of p31 Ii remained detectable in RBL-2H3 cells after 20 hours of chase in complete medium (Fig. 4B). In addition, a p10 fragment accumulated in RBL-2H3 cells from 2 hours to 21 hours of chase. When the same samples were not boiled to preserve SDS-stable conformation, IN1 antibody immunoprecipitated a p90 complex at time 0 which remained detectable after 2 hours of chase whereas a p70 complex appeared at 2 hours to peak after 6 hours of chase and remained detectable after 21 hours. In control B cells, IN1 did not immunoprecipitate the p70 complex. To further identify the proteins contained in p90 and p70 complexes, class II molecules were immunoprecipitated with the anti-class II β chain, M5/114 (Fig. 4C). The same bands were obtained than with the anti-Ii antibody, IN1 (Fig. 4B). Indeed, M5/114 and IN1 immunoprecipitated the same p90 and p70 complexes with the same kinetics when the samples were run in unboiled conditions to dissociate, in
Fig. 3. Immuno-electron microscopy of RBL-2H3 transfected cells.
(A) Ultrathin cryosections of RBL IAbIi cells were immunogold labelled with the rat monoclonal anti-IA Ab M5/114, a rabbit anti-rat bridging antibody and Protein A coupled to 10 nM gold particles (PAG 10). MHC class II molecules are detected in the numerous compartments filling the cytoplasm of RBL-2H3 cells. (B) Higher magnification showing the multivesicular appearance of the compartments accumulating MHC class II molecules. (B, inset) Ultrathin cryosections were also double immunogold labelled with the rat monoclonal Ab M5/114 (PAG 15) and a rabbit polyclonal anti-serotonin (PAG 10). Serotonin was detected to some extent in the MHC class II positive compartments. Bars, 200 nM.
Fig. 4. Defect of MHC class II maturation in RBL-2H3 cells is related to a slow degradation of Ii. RBL IAblIi and the B lymphoma cells B414, both expressing IAbl molecules, were metabolically labelled with [35S]methionine/cysteine then chased for the indicated times. At each time, immunoprecipitations of newly synthesized MHC class II molecules were performed with different monoclonal antibodies. Before analysis by 12% SDS-PAGE, the samples were incubated in reducing Laemmli buffer at RT for 30 minutes (non-boiled conditions, NB, left panel) or at 95°C for 5 minutes (boiled conditions, B, right panel).

(A) αβ dimers did not mature into a compact SDS-stable conformation in RBL-2H3 cells but remained as p70 isoforms. Immunoprecipitations of MHC class II molecules were carried out with the Y3P antibody recognizing αβ IAbl dimers free or associated with Ii fragment. (B,C) Invariant chain degradation was slowed down and it remained associated with class II dimers in RBL-2H3 cells. Ii was immunoprecipitated with IN1, an antibody directed against the cytoplasmic tail of murine Ii. Whereas class II molecules were immunoprecipitated with the M5/114 antibody recognizing the IA β chain in mature or immature αβ complexes (C).

(D) Accumulation of p10 Ii fragment in RBL-2H3 cells expressing class II molecules. Lysates of RBL IAblI and IAdIi cells as well as lysates from IIA1.6 and untransfected RBL-2H3 cells were analyzed by western blotting with a rabbit monoclonal antibody directed against the cytoplasmic tail of murine Ii. Arrowheads indicate the bands corresponding to α chain (α) or β chain (β) of MHC class II molecules, Ii and its degradation fragments (p10 and p20), SDS-stable compact form of class II molecules (αβ) or SDS-stable class II molecules associated with the entire Invariant chain (p90) or its p10 degradation fragment (p70).

the same ratio, into α, β chains of class II molecules and the entire or the p10 fragment of Ii. These data indicate that, in RBL-2H3 cells, MHC class II molecules slowly mature into a compact αβ mature dimer because Ii is poorly degraded and remains associated with class II molecules in its entire form or p10 and p20 fragments.

Lysates of RBL IAblI and RBL IAdIi cells were analyzed by western blotting with rabbit antiserum specific for the cytoplasmic tail of Ii. In RBL-2H3 cells, Ii mostly accumulated as different forms corresponding to p20 and p10 fragments of the p31 Invariant chain (Fig. 4D) in agreement with the previous data. In murine B cells only a small proportion of Ii corresponded to the p10 fragment.

In order to quantify the accumulation of MHC class II molecules in secretory granules, RBL IAbl cells were fractionated and lysosomes were purified by ultracentrifugation on Percoll gradient (Bonnerot et al., 1998). The different fractions were characterized by their β hexosaminidase activity (found in secretory granules and lysosomal compartments) and their alkaline phosphodiesterase (APDE) activity (found at the plasma membrane) (Fig. 5A). Each fraction was analyzed by western blotting with the rabbit antiserum against MHC class II molecules.
II molecules. A minor fraction of MHC class II molecules was found in light fractions corresponding to the cell surface whereas 80% accumulated in dense fractions, corresponding to lysosomes-related compartments, together with p10 Ii degradation fragment (Fig. 5B).

These results were very similar to those obtained after leupeptin (a protease inhibitor) treatment of B cells (Brachet et al., 1997) or in immature dendritic cells (Pierre and Mellman, 1998) where $\alpha$-p10 complexes accumulated in lysosomal compartments due to a decreased activity of cathepsin S. We therefore measured Cathepsin S activity in RBL-2H3 cells by three independent assays (Fig. 6). Using a fluorometric assay, low cathepsin S activity can be detected at pH 5.5 or pH 7 in membrane preparations of RBL-2H3 cells, whereas normal cathepsin L, cathepsin B and $\beta$-hexosaminidase activities were measured in the same samples (Fig. 6A). Significant cathepsin S activity was detected in membrane preparations of B414 cells in the same experiment. Since this fluorometric assay was reported as not perfectly specific for cathepsin S activity, we then labelled RBL-2H3 and B414 cells with a iodinated-peptide which specifically bound the active site of cathepsin S. A 26 kDa protein, corresponding to cathepsin S, was specifically labelled in these conditions in B414 cells whereas no corresponding protein was detected in RBL-2H3 cells (Fig. 6B). This protein was suspected to be cathepsin S because it has the right size and its labelling by the iodinated peptide was inhibited in the presence of a cathepsin S inhibitor, the LHVS peptide (Xing and Mason, 1998). This result was...
Antigen presentation in mast cells

supported by the lack of cathepsin S signal in RBL-2H3 lysates by western blot using a rabbit anti-cathepsin S antiserum (Fig. 6C). Therefore, the absence of cathepsin S in mast cells such as RBL-2H3 cells is likely to contribute to the low rate of II degradation and the slow MHC class II maturation.

Therefore, mature MHC class II molecules loaded with peptides or immature class II molecules associated with the p10 fragment accumulate in secretory granules. Since the content of secretory granules is known to be released upon a cytosolic calcium increase (Stump et al., 1987), mast cells activation should induce cell surface arrival of MHC class II molecules.

A cytoplasmic Ca²⁺ increase regulates cell surface MHC class II molecules peptide complexes expression in RBL-2H3 cells. We therefore checked if MHC class II molecules, loaded or not with antigenic peptides, reach the cell surface after a short intracellular calcium increase induced by a calcium ionophore, ionomycin. RBL IAb Ii cells were loaded for 2 hours with the IEd α 52-68 peptide (10 µM) then cell surface expression of total class II molecules (Y3P) or peptide-class II complexes (YAe) was measured using specific mAbs (LY1C6, AD1, and 5G10) before and after addition of 1 µM ionomycin. This calcium signaling, which induced cell degranulation as measured by serotonin release (data not shown), uniformly increased the labelling detected by the Y3P mAb of 15% of the cells (Fig. 7A, right panel). To test if redistributed peptide-class II complexes were recognized by specific T cells, RBL IAbIi cells were incubated with different concentrations of IEd α 52–68 peptide and antigen presentation was detected, before or after ionomycin treatment (1 µM in DMEM), by the T hybridoma TH30, specific for this peptide presented by IAb MHC class II molecules. The results shown in Fig. 7B illustrate that a calcium signaling increased by a factor 15 the ability of mast cells to stimulate antigen specific T cells. These data confirmed that class II molecules loaded with antigenic peptides in secretory granules were delivered to the cell surface upon mast cell activation.

In conclusion, mast cells are able to process antigens into peptides that bind to MHC class II molecules in secretory lysosomes. This feature of MHC class II transport in mast cells allows therefore to coordinate, upon similar stimuli, the exocytosis of inflammatory mediators and the cell surface expression of class II-peptide complexes.

DISCUSSION

We recently reported that BMMCs accumulate MHC class II molecules in secretory granules (Raposo et al., 1997). These cells are currently used as a model for studying mast cell
function. However, the level of MHC class II molecules is too low to allow biochemical approaches and MHC class II expression is under the control of various cytokines, such as GM-CSF and IL4, which could interfere with MHC class II transport in BMMCs (Frandji et al., 1995). Expressing IA molecules in RBL-2H3 cells allowed us to analyze the intracellular transport of MHC class II in an homogenous mast cells population expressing physiological levels of class II molecules without the addition of any lymphokines in the culture media. We mostly found that secretory granules or secretory lysosomes of RBL-2H3 cells, like in BMMCs (Raposo et al., 1997), are the intracellular site of accumulation of mature class II molecules, i.e. αβ dimers loaded with antigenic peptides, and of immature class II molecules, i.e. αβ dimers associated with p10 fragment of Ii.

How do mast cells prevent MHC class II transport to the cell surface? Our results suggest that several mechanisms may be used by these cells. First, a delay in the maturation of class II molecules, which remained associated with a fragment of Ii, likely due to a low cathepsin S activity, blocking the access of the class II pocket to the peptides; second, a defect in the transport of class II molecules-peptide complexes from lysosomal compartments to the cell surface. Altogether these mechanisms contribute to the intracellular accumulation of class II molecules.

One important observation is that the intracellular maturation of MHC class II molecules in the rat mast cell line RBL-2H3, as well as in BMMCs, is altered. Indeed, although a small fraction of αβ dimers became SDS stable, the majority of class II molecules remained associated with Ii as well as its degradation intermediates fragments, p10 and p22. Similar results have been obtained in B cells treated with leupeptin, an inhibitor of cysteine-proteases (Amigorena et al., 1995; Brachet et al., 1997). In the presence of leupeptin, the degradation of Ii was inhibited, which resulted in its accumulation as a p10 form. As a consequence, MHC class II/II complexes were retained in lysosomal compartments (Brachet et al., 1997). The hypothesis that Ii may indeed regulate the localization and transport of MHC class II molecules along organelles of the endocytic pathway has been recently extended by Pierre and coll (Pierre and Mellman, 1998). Immature dendritic cells express high levels of cystatin C, an inhibitor of cathepsin S, inducing the accumulation of αβp10 complexes in lysosome-related compartments (MIICs). In LPS-induced mature dendritic cells, the down regulation of cystatin C expression induced cathepsin S activity and the degradation of p10 Ii form, thus allowing the redistribution of mature αβ dimers at the cell surface of dendritic cells (Pierre and Mellman, 1998). The low cathepsin S activity we observed in RBL-2H3 cells therefore accounts for the accumulation of p10 Ii fragment with class II dimers in secretory lysosomes of mast cells.

It remains that, even in the absence of cathepsin S, a small fraction of class II molecules became SDS stable and could be loaded by antigenic peptide in RBL-2H3 cells. This suggest that cathepsin S is not absolutely necessary for MHC class II maturation and antigen presentation. Indeed, mature or immature dendritic cells of cathepsin S knock-out mice efficiently present antigens and express similar level of cell surface class II molecules than wild-type mice (Villadangos et al., 1999). In addition, it has been reported that cathepsin L can replace cathepsin S in some cell types such as thymic epithelial cells or macrophages (Nakagawa and Rudensky, 1999; Villadangos et al., 1999); mast cells or RBL-2H3 cells could be other examples where other enzymes such as cathepsin L degrade lip10 or where lip10 is slowly displaced from the MHC class II dimer. Thus, it seems that class II molecules in RBL-2H3 cells and in immature dendritic cells have very similar behaviors since αβlip10 complexes and αβ-peptide complexes mainly accumulate in compartments with lysosomal features (Pierre and Mellman, 1998) and they may, both, express high levels of class II molecules at the cell surface and present antigen after cell activation.

What are the intracellular compartments of MHC class II accumulation in mast cells? Cell fractionation of RBL-2H3 cells indicated that 80% of the total class II molecules were found in dense fractions of Percoll gradient, enriched in conventional lysosomes and secretory lysosomes (Shiver et al., 1992). In addition, MHC class II and Ii were strictly colocalized by immunofluorescence with CD63, a common marker of these two types of lysosomal compartments. When the intracellular localization of class II molecules was compared with that of a specific marker of secretory granules, a significant fraction of class II molecules was found in serotonin-positive compartments. In BMMCs, we previously observed that the bulk of class II molecules is contained in two distinct compartments (type I and type II) with features of both lysosomal compartments and secretory granules as defined by their morphology, their protein content and their accessibility to endocytic tracers. Type I granules are multivesicular compartments where MHC class II molecules co-localize with mannose-6-phosphate receptors and lysosomal membrane proteins such as lamp1 and lamp2. Type II granules display in addition to small membrane vesicles, an electron dense secretory domain rich in serotonin (Raposo et al., 1997). The morphological features of the intracellular compartments of RBL-2H3 cells expressing murine MHC class II molecules are somehow distinct from those of BMMCs. In RBL-2H3 cells we observed compartments morphologically similar to Type I granules of BMMCs since they display intralumenal membrane vesicles. Typical Type II granules with their electron dense core were not detected, instead compartments with internal membrane sheets morphologically similar to lysosomes were observed. Interestingly, serotonin could be detected together with MHC class II molecules in the multivesicular compartments. Thus, it seems that in mast cells, the bulk of class II molecules and lysosomal proteins are localized in particular MIICs which are secretory granules.

What are the consequences of the accumulation of MHC class II molecules in secretory granules? Antigen presentation is the result of numerous intracellular steps which require accessory molecules to obtain MHC class II molecules loaded with antigenic peptides (Kropshofer et al., 1997). In mast cells, although the degradation of Ii is largely inhibited, a fraction of αβ dimer matured and was able to bind antigenic peptides in secretory granules. This unique intracellular localization of MHC class II molecules in mast cells has several consequences on the transport of class II molecules towards the plasma membrane. First, since the content of secretory granules remains inside the cells in the absence of signals which determine exocytosis, immature and mature MHC class II molecules are mostly retained intracellularly in mast cells.
Second, cell surface expression of class II molecules, loaded or not with antigenic peptides occurs upon cytosolic calcium increase, a process which induces mast cell degranulation. Thus the nature of the intracellular compartment where MHC class II molecules accumulate in mast cells must have important consequences on mast cell functions during antigen processing and antigen presentation. An hypothesis could be that, during inflammatory or allergic reaction, mast cells might be able to determine a two-steps process: mast cells capture antigens in a primary body site, then process antigens into peptides that are loaded on MHC class II molecules in secretory granules, the content of which could be released, together with inflammatory mediators, after cell migration into a second body site upon IgE-mediated stimulation. Therefore, mast cells may have developed an original mechanism to deliver, into mucosal tissue, antigen specific signals to modify T cell response.

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