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The lysosomal protease cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the rat basophilic/mast cell line RBL

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SUMMARY

Basophils and mast cells contain a peculiar class of inflammatory granules that discharge their content upon antigen-mediated crosslinking of IgE-membrane receptors. The pathways for granule biogenesis and exocytosis in these cells are still largely obscure. In this study we employed the rat basophilic leukemia (RBL)/mast cell line to verify the hypothesis that inflammatory granules share common bioactive molecules and functional properties with lysosomes. We demonstrate that inflammatory granules, as identified by the monoclonal 5G10 antibody (which recognises an integral membrane protein) or by Toluidine Blue staining, have an intralumenal acidic pH, possess lysosomal enzymes and are accessible by fluid-phase and membrane endocytosis markers. In addition, we studied the targeting, subcellular localisation and regulated secretion of the lysosomal aspartic protease cathepsin D (CD) as affected by IgE receptor stimulation in order to obtain information on the pathways for granule biogenesis and exocytosis. Stimulation with DNP-BSA of specific IgE-primed RBL cells led to a prompt release of processed forms of CD, along with other mature lysosomal hydrolases. This release could be prevented by addition of EGTA, indicating that it was dependent on extracellular calcium influx. Antigen stimulation also induced exocytosis of immature CD forms accumulated by ammonium chloride, suggesting the existence of an intermediate station in the pathway for granule biogenesis still sensitive to regulated exocytosis. The targeting of molecules to secretory granules may occur via either a mannose-6-phosphate-dependent or mannose-6-phosphate-independent pathway. We conclude that endosomes and lysosomes in basophils/mast cells can act as regulated secretory granules or actually identify with them.

Key words: Lysosomal protease, Secretory lysosome, Mast cell

INTRODUCTION

Lytic granules of cytotoxic T lymphocytes and of natural killer cells have been shown to share several biochemical properties with lysosomes such as the content of some lysosomal proteins and the acidic pH (Burkhardt et al., 1990; Peters et al., 1991; Griffiths and Issaz, 1993). Together with the finding that the targeting of granzymes A and B to the lytic granules is accomplished by the mannose-6-phosphate (M6P)-receptors (MPRs), i.e. the same system used for the segregation of soluble lysosomal proteins, these observations have led to the hypothesis that cells of the myeloid lineage possess a specialised class of lysosomes, the so called 'secretory lysosomes' (Griffiths, 1996; Stinchombe and Griffiths, 1999). However, differences in the biogenesis and intracellular accumulation of lysosomes and secretory granules also exist between cell types of the haemopoietic lineage. For instance, in mature T lymphocytes the majority of lysosomes identifies with secretory granules (Stinchombe and Griffiths, 1999), whereas neutrophils seem to possess both true lysosomes and 'secretory lysosomes' (Brumell et al., 1995). The presence of some soluble lysosomal enzymes (Schwartz and Austen, 1980) and lysosomal membrane proteins (Raposo et al., 1997) in secretory granules of mastocytes has been reported. Yet, it remains to be shown by morphological and biochemical approaches whether lysosomes and secretory granules in mast cells fully or partially overlap and how in these cells newly synthesised lysosomal enzymes are segregated into secretory granules. Sorting of the molecules destined for either the endosomal-lysosomal apparatus or the regulated secretory granules occurs at the trans-Golgi network (TGN) site. Both mannose-6-phosphate (M6P)-dependent or 'alternative' pathways may be involved in directing secretory granular molecules and lysosomal resident proteins to their final compartments. Whether these routes converge to the same organelle in basophils and in mast cells is so far not known.

In this study we adopted the RBL cells, a rat basophilic leukaemia/mast cell line that exhibits a calcium-regulated degranulation upon antigen stimulation of the high-affinity IgE receptor (Jouvin et al., 1995), as a cell model to verify whether inflammatory secretory granules have the features of true endosomes and lysosomes and whether these post-Golgi compartments share common biosynthetic pathways. In a previous work we have already shown that activation of the IgE receptor in RBL cells causes the missorting of molecules destined to secretory granules at TGN or post-TGN/pre-endosomal level (Baldassarre et al., 2000). Here we demonstrate by a range of morphological and biochemical criteria that the bulk of secretory granules of RBL cells biochemically and functionally overlap with endosomes and lysosomes. Furthermore, to gain an insight into the pathways for granule biogenesis and exocytosis we also studied the biogenesis, trafficking and compartmentalisation of the lysosomal protease cathepsin D (CD) as affected by antigen stimulation of the IgE receptor.

MATERIALS AND METHODS

Cells, cell culture and treatments

Rat basophilic/mast leukaemia (RBL-2H3 type) cells were grown as a monolayer in DMEM supplemented with 16% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics as previously described (Buccione et al., 1994). For the experiments, growing cells were plated at a density of 10^6 cells/P_{35}mm dish and allowed to adhere for at least 24 hours before use. Unless otherwise specified, all incubations were performed in 1 ml of fresh serum-free medium. Treatments included 100 ng/ml DNP-BSA (2,4 dinitrophenyl-bovine serum albumin), 5 mM EGTA, 20 mM ammonium chloride. At the end of the incubations, medium and cells were collected and processed for enzyme assays and protein analysis as detailed below. For studies involving degranulation, RBL cells were incubated overnight with DNP-BSA specific IgE (1 μg/10^6 cells) and the excess unbound antibody washed out prior to antigenic stimulation.

Morphological studies

Cells were adhered to sterile glass coverslips, cultured for 24 hours, presensitised with DNP-specific IgEs and further incubated for the times indicated in serum-free fresh medium in the absence or the presence of substances as indicated. For Toluidine Blue staining or immunofluorescence the cells were then fixed with 3.7% formaldehyde in phosphate buffered saline, pH 7.4, for 20 minutes and permeabilised with 0.1% Triton X-100 for 7 minutes. Alternatively, for identification of acidic compartments living cells adherent on coverslips were incubated for 15 minutes with 1 μg/ml Acridine Orange (AO), washed three times with PBS and immediately examined under a fluorescent microscope. CD immunolocalisation was performed by incubation with specific antiserum (1:50 in phosphate buffered saline, pH 7.4, containing 0.1% Triton X-100 and 1:25 foetal calf serum) followed by incubation with secondary antibodies (see below). Inflammatory granules were stained with the 5G10 monoclonal antibody (a kind gift from Dr. Bonifacino) which recognises an integral membrane protein of RBL granules (Bonifacino et al., 1986). The 5G10 antibody was diluted and used as above. Depending on the experiment and as specified in the figure legends, secondary antibodies used were Texas Red-conjugated goat anti-mouse IgG, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma Chem. Co), Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes Europe, The Netherlands) and Cy5-conjugated goat anti-mouse IgG (Amersham). As a negative control cells were incubated with the secondary antibodies alone or with pre-immune antiserum. Fluoresceinated Concanavalin A (Sigma Chemical Co.) or Dextran (Molecular Probes Europe) was employed as membrane-bound or fluid phase endocytosis tracers, respectively, to label endocytic compartments. In this case living cells were allowed to uptake the fluorescent tracer and then fixed and processed as above for granule staining.

Enzymatic assays

CD assays were performed at pH 3.65 using ^{3}H-labelled haemoglobin as substrate (Isidoro et al., 1995). About 96% of the acid proteolytic activity was inhibitable by 1 μM pepstatin (Sigma Chem. Co.), a CD inhibitor. Cathepsins B and L were assayed by using fluorimetric substrates as previously described (Isidoro et al., 1995). β-Hexosaminidase was assayed in sodium-citrate buffer at pH 4.5 using *p*-nitrophenyl-N-acetyl β-D glucosaminide as a substrate. Enzyme activity was referred to cell protein, determined according to the method of Lowry et al. (1951).

Analysis of CD expression, maturation and secretion

Cell-associated and secreted CD molecular forms were revealed by standard western blotting techniques using specific polyclonal rabbit anti-rat CD antiserum (Démoz et al., 1999). Secreted CD was analysed in RBL cells cultured in serum-free medium. In this case, with respect to cell homogenate a 10× volume of medium was subjected to TCA-precipitation and total secreted proteins were then washed with acetone and denatured in electrophoresis loading buffer. Proteins were separated by SDS-polyacrylamide (12.5%) gel electrophoresis and electroblotted onto nitrocellulose sheets. CD-related bands were revealed by incubation with an anti-CD antiserum followed by a peroxidase-conjugated goat-anti-rabbit antibody and subsequent peroxidase-induced chemiluminescence (ECL) as recommended by the manufacturer (Amersham). Alternatively, CD was immunoprecipitated from medium and cell extracts of RBL cultures metabolically labelled with a ^{35}S-cysteine/methionine mix and visualised by fluorography or from non labelled cultures and visualised by western blotting. In some cases immunoprecipitates or total cell and secreted proteins were treated with Endoglycosidase H for 2 hours at 37°C as recommended by the manufacturer. Transport and maturation of CD in each experimental condition were analysed at least four times. Representative gels are shown.

RESULTS

In RBL cells acidic compartments are sensitive to antigen-stimulated calcium-dependent exocytosis

Endosomes and lysosomes are acidic organelles usually confined in a perinuclear region of the cell (Matteoni and Kreis, 1987). These organelles can be easily recognised by fluorescent labelling with acridine orange (AO) that permeates the membrane of living cells and accumulates in acidic compartments, where, upon protonation, it emits orange or red fluorescence under excitation. AO staining is also informative of lysosomal membrane integrity, since disruption of the proton gradient in this organelle impairs the emission of orange or red fluorescence. In this study we employed AO to monitor the effect of IgE receptor stimulation on the integrity of the endosomal-lysosomal compartment in RBL mast cells. In parallel, we monitored the effects of antigen stimulation on RBL cells in which the cytoplasmic granules were conventionally stained with Toluidine Blue (TB). As shown in Fig. 1, in control unstimulated cells AO-stained acidic compartments are distributed throughout the cell, though the majority is located in a perinuclear region as expected for

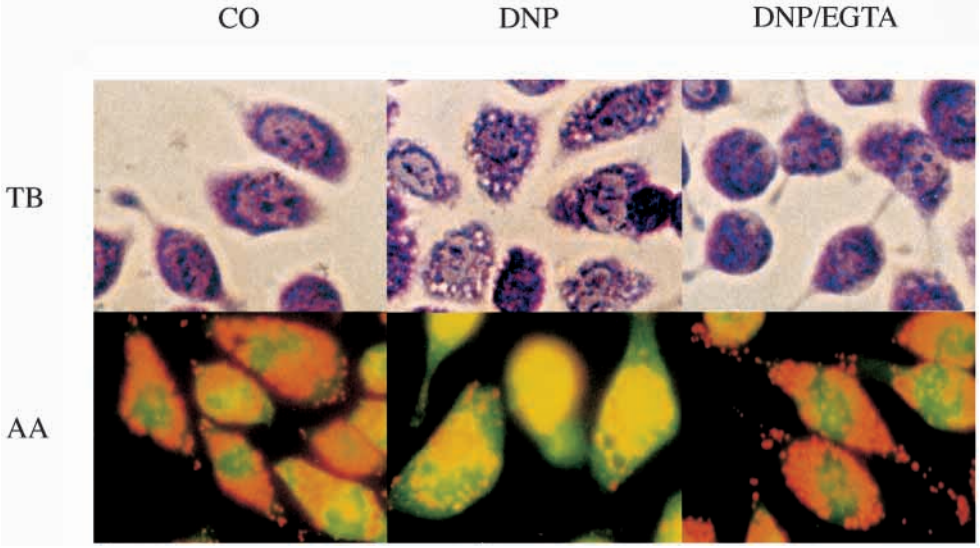

Fig. 1. Acidic compartments of mastocytes behave as regulated secretory organelles. Two sets of adherent RBL cells grown on glass coverslips were treated as indicated (Co, control; DNP, incubated for 30 minutes with DNP-BSA; DNP/EGTA, incubated for 30 minutes with DNP-BSA in the presence of EGTA). Samples were then processed for conventional staining with Toluidine Blue (TB), which identifies inflammatory granules, or with Acridine Orange (AO), which fluorogenically labels acidic compartments.

endosomes and lysosomes. This also corresponds to the distribution of TB-stained granules. In myeloid granular cells activation of cell surface receptors causes the translocation of secretory granules to the periphery of the cell and their eventual fusion with the plasma membrane. When IgE-primed RBL cells were exposed to DNP-BSA, only cytoplasmic vacuoles not stainable with TB were apparent, consistent with efficient degranulation. In parallel, living cells that have undergone degranulation stained with AO showed a (diffuse) yellow-green, rather than (granular) orange-red, fluorescence as expected for organelles that lost the ability to acidify their lumen. The third panel of Fig. 1 clearly demonstrates that antigen stimulation in the presence of the calcium-chelator EGTA is ineffective. In fact, organelles stained either with TB or AO accumulate within the cell and concentrate at the periphery, in proximity of the plasma membrane.

In RBL mast cells cathepsin D is sorted to inflammatory granules and is exocytosed upon antigenic stimulation

The data in Fig. 1 indicate that in RBL cells acidic vacuolar organelles behave as regulated secretory compartments. The formal proof that in basophils and mast cells lysosomes and secretory granules coincide is still lacking. To clarify this point we performed a double-labelling immunofluorescence confocal analysis using polyclonal antibodies against Cathepsin D (CD), a soluble protease resident within endosomes and lysosomes (Hasilik, 1992), and the monoclonal 5G10 antibody, which recognises an integral membrane protein of RBL secretory granules (Bonifacino et al., 1986). As shown in Fig. 2, most if not all organelles stained with both the anti-CD and 5G10 monoclonal antibodies. Yet, a number of organelles stained only for either of the antibodies. In this respect, organelles labelled with anti-CD were more abundant and located in the perinuclear area. These organelles might be regarded as true lysosomes.

We performed immunofluorescence studies to investigate further the effects of antigenic stimulation on the localisation of CD-containing organelles in RBL cells. As shown in Fig. 3, in control unstimulated RBL cells, CD-positive organelles were distributed throughout the cytoplasm, although a major site of accumulation was in the perinuclear area. This is well in agreement with the usual localisation of late endosomes and lysosomes (Matteoni and Kreis, 1987). After 30 minutes of antigenic stimulation with DNP–BSA, the number of CD-positive organelles was severely reduced, the very few remaining being essentially perinuclear (not clearly visible in the picture). However, when EGTA was present during the incubation with the antigen, this did not occur. Of interest, in this case CD-positive organelles translocated to the periphery

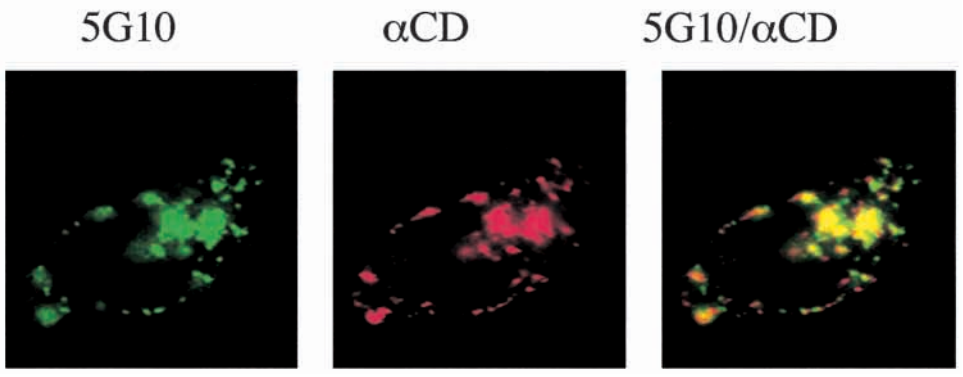

Fig. 2. Inflammatory secretory granules contain CD. RBL cell monolayers were processed for confocal immunofluorescence microscopy with 5G10 monoclonal and anti-CD polyclonal antibodies to identify inflammatory granules and lysosomes, respectively. FITC-conjugated and Texas Red-conjugated secondary antibodies were used for 5G10 and anti-CD, respectively. Computer superimposed images demonstrates nearly complete overlap between the two antigens recognised by the antibodies. Yet, a subpopulation of true lysosomes is clearly visible. Representative images of several experiments are shown.

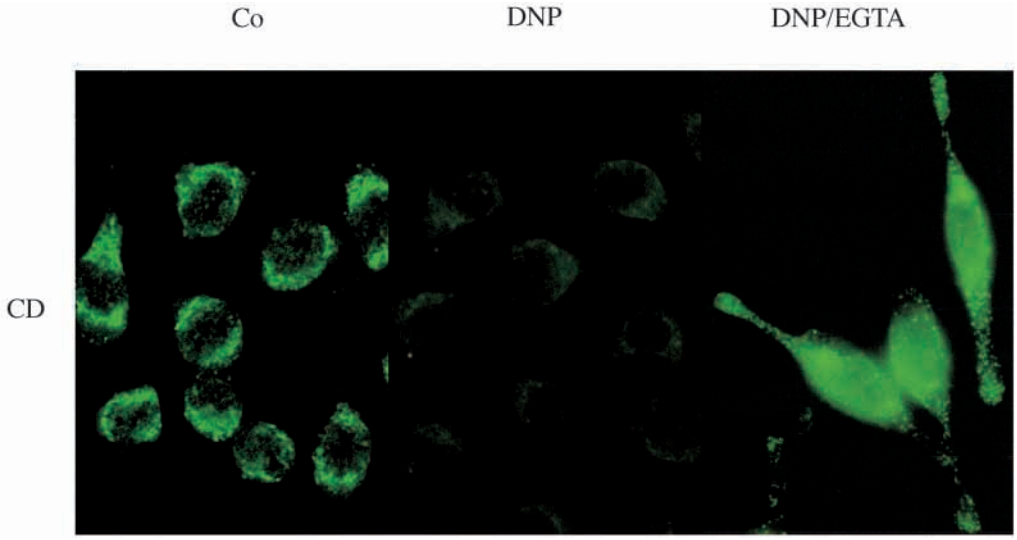

Fig. 3. Immunolocalisation of CD-containing organelles in RBL cells: effects of antigen stimulation. IgE-primed RBL cells were treated with DNP-BSA in the absence (DNP) or presence (DNP/EGTA) of EGTA for 30 minutes. Control cells were incubated for the same time (Co). Monolayers were then processed for immunofluorescence microscopy with anti-CD antibodies. Antigen stimulated cells show a marked reduction of CD-positive organelles. However, if EGTA is present in the incubation medium degranulation is abrogated and most of CD-containing organelles accumulates close to the plasma membrane. Experiments were performed several times with similar results.

of the cells and mostly accumulated close to the plasma membrane along the edges of long cellular processes (see also Fig. 1). Changes in the morphology of RBL cells upon antigenic stimulation have been reported to precede degranulation and seem to reflect cytoskeletal rearrangements proposed to facilitate fusion of secretory granules with the plasma membrane (Edgar and Bennett, 1997).

Inflammatory granules are connected to the endocytosis pathway

Finally, we investigated whether inflammatory granules were interconnected with the endocytic pathway as would be expected for authentic lysosomes. For this purpose, RBL cells were incubated with dextran-FITC, a tracer of fluid-phase endocytosis, at 37°C for 2 hours to allow the tracer to reach and accumulate in late endosomal/lysosomal compartments. The same cells were then fixed, permeabilised and prepared for triple labelling with the monoclonal antibody 5G10 specific for a granule-membrane antigen and anti-CD to label secretory granules and endosomes/lysosomes, respectively. As shown in Fig. 4, the vast majority of endosomal tracer, 5G10 antigen and CD colocalise. Similar results were obtained with fluorescinated concanavalin A (not shown). These observations emphasise the intimate connection between the endocytic pathway and the regulated secretory pathway in basophilic/mast cells.

Antigen stimulation of IgE-primed RBL triggers the exocytosis of mature lysosomal proteases

We further investigated the possibility that lysosomes indeed share common biochemical markers and functional properties with mast cell secretory granules. In a previous study we have already shown that in RBL cells the activation of IgE receptor affects the targeting of CD and proteoglycans, the latter being a soluble component of inflammatory granules (Baldassarre et al., 2000). In the following experiments we asked whether antigen stimulation of IgE-presensitised RBL cells would

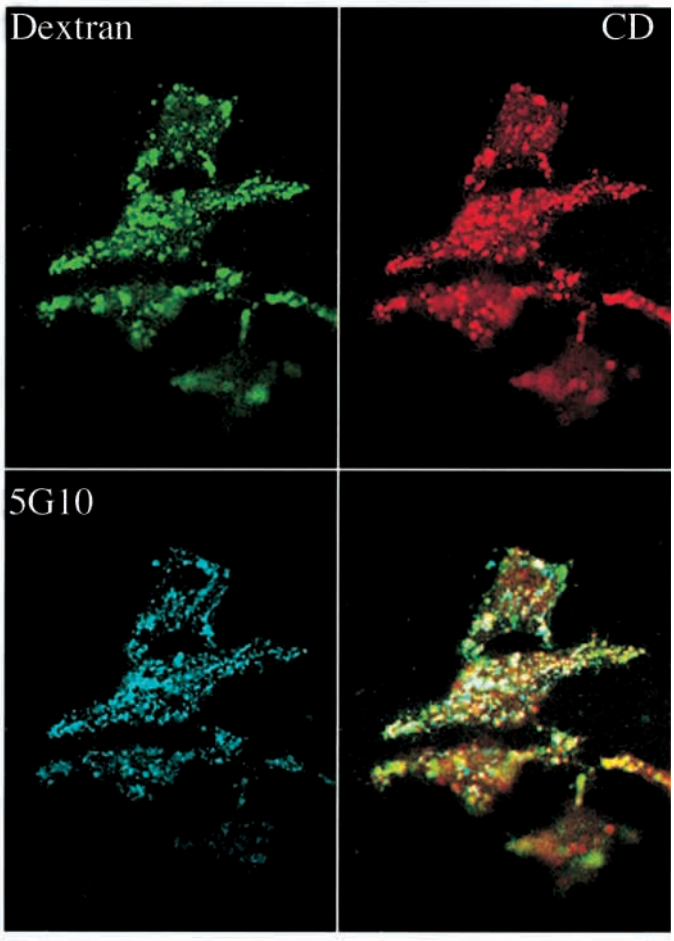

Fig. 4. Secretory granules in RBL are accessible via endocytosis. RBL cells were incubated with the the fluid phase endocytosis marker dextran-FITC for 2 hours at 37°C to label late endocytic/lysosomal compartments. The same cells were then processed for triple label immunofluorescence microscopy with anti-5G10 monoclonal antibodies using a Cy5-conjugated secondary antibody and anti-CD polyclonal antibodies antibodies using an Alexa 546-conjugated secondary antibody to label secretory granules and endosomal-lysosomal organelles, respectively. Experiments were performed at least 3 times with similar results. A representative image is shown.

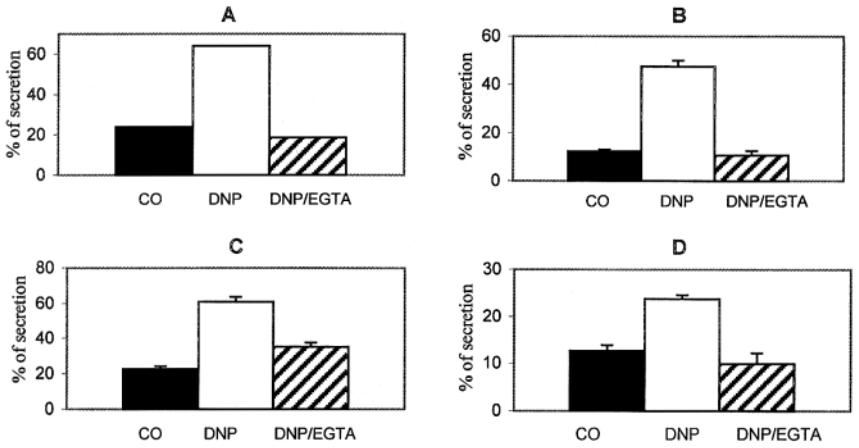

Fig. 5. Effects of antigen stimulation on the exocytosis of lysosomal hydrolases. IgE-primed RBL cells were treated or not with 100 ng/ml DNP-BSA to induce degranulation in the presence or absence of EGTA for 180 minutes and the activity of CD (A), β-hexosaminidase (B), cathepsin B (C) and cathepsin L (D) was measured in cell homogenates and culture media. Data are given as percentage (± s.d.) of activity found in medium. In the medium of stimulated cultures the levels of CD, β-hexosaminidase and cathepsin B activity were about 3-fold and the levels of cathepsin L activity were about 2-fold higher than those in controls, respectively. The experiment also demonstrates that antigen stimulation-induced release of the lysosomal enzymes was Ca^{2+} dependent.

affect the intracellular retention of mature enzymatically active lysosomal hydrolases. IgE-primed RBL cells were subjected to an adequate antigenic stimulation and the proportion of CD within the cell and secreted in the medium was estimated by assaying pepstatin-inhibitable proteolytic activity. In this condition, extracellular release of CD activity accounted for almost 70% of total and appeared to be maximal within 30 minutes of stimulation (not shown). Next we investigated the role of extracellular calcium in antigen-dependent exocytosis of this lysosomal enzyme. As shown in Fig. 5, even after a prolonged (3 hour) stimulation of the IgE receptor in the presence of a calcium-chelating agent (EGTA) the release of CD activity did not exceed that of non-stimulated cultures. In parallel, we also evaluated the changes in intracellular retention of three other lysosomal acid hydrolases resident in endosomes and lysosomes, namely β-hexosaminidase (known to be released by RBL cells upon antigenic stimulation) and cathepsins B and L. Relatively to the activity levels found in unstimulated cultures, antigenic stimulation raised extracellular release up to 3-fold for β-hexosaminidase and cathepsin B and up to 2-fold for cathepsin L (Fig. 5). Again, this effect was abolished when EGTA was added concomitant with DNP (Fig. 5).

Biogenesis of CD in RBL cells: role of the mannose-6-phosphate system

We wished to exploit CD as protein reporter to better understand the pathways of granules biogenesis and exocytosis. To this end we first characterised the synthesis, transport and processing of this molecule in RBL cells. The complex pattern of CD molecular forms generated in the rat basophilic leukaemia cell line, as revealed by western blotting of immunoprecipitates, is shown in Fig. 6A. The antibody recognised several molecular species which likely correspond to: (i) the proenzyme (P) having an approximate molecular mass of 53 kDa, (ii) an intermediate form (I) of approximately 47 kDa; (iii) a mature single-chain form (Msc) of approximately 43 kDa; (iv) two large chains (LC) of approximately 34 and 30 kDa and two small chains (SC) of 14 kDa and 9 kDa arising from the double-chain forms of CD. The sizes of these molecular species correspond to the predicted forms of rat CD, based on the predicted sequence and the proteolytic cleavage sites (Yonezawa et al., 1988; Fujita et al., 1991) and with the molecular forms of CD isolated from other rat cell types (Ludwig et al., 1991; Démoz et al., 1999; Chiarpotto et al., 1999). More in detail, it appears that, at steady-state, the 43 kDa species is by far the most prominent molecular form and that the 34 kDa molecule is the most represented large-chain within the cell. A similar pattern of CD expression was observed in bone marrow derived mastocytes (not shown). In a separate study we analysed the biogenesis of the various molecular forms of CD in RBL cells by pulse-chase and

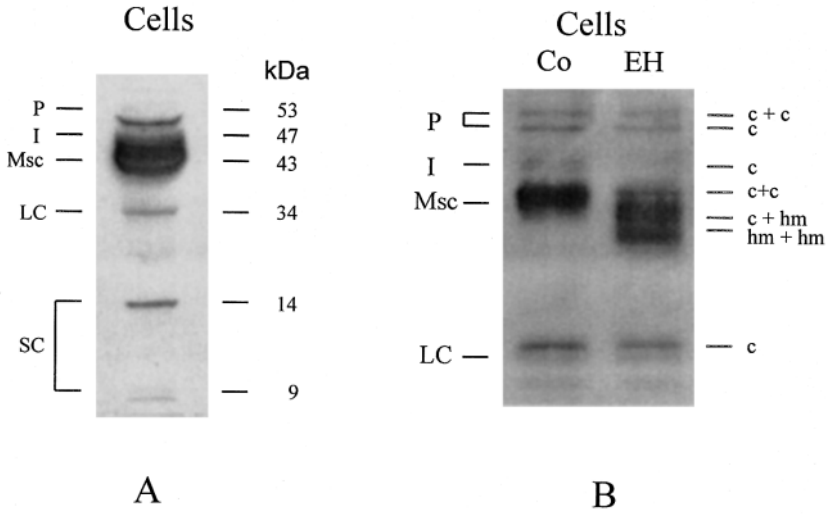

Fig. 6. Synthesis, transport and processing of CD in RBL. (A) CD molecular forms expressed by RBL. RBL cells were lysed and processed for SDS-PAGE and western blotting. CD molecular forms were revealed by enhanced chemiluminescence of immunocomplexes. 'P' indicates the 53 kDa precursor, 'I' the 47 kDa immature intermediate, 'Msc' the 43 kDa mature single chain, 'LC' the 34 and 30 kDa mature large chains and 'SC' the 14 and 9 kDa mature small chains. (B) EndoH analysis of CD. CD immunoprecipitates from RBL cell extracts were treated or not with Endoglycosidase H (EndoH) for 2 hrs at 37°C, and then processed for SDS-PAGE and western blotting to reveal the various CD molecular forms. 'P', 'I', 'Msc' and 'LC' indicate CD molecular forms as in A; 'c' and 'hm' indicate the presence of complex and high mannose-type saccharides, respectively.

immunoprecipitation techniques and under conditions known to interfere with transport along the exocytic pathway (such as 20°C temperature block, brefeldin A, ammonium chloride). These experiments revealed that CD was synthesised as a 53 kDa precursor rapidly converted into a smaller molecule of 47 kDa during a short (30 minute) pulse period; the 43 kDa form was generated within 2 hours, while the double-chain processed forms became apparent only after a 4-6 hour chase (not shown; see also Baldassarre et al., 2000).

Within the Golgi complex the oligosaccharides on proCD can be modified into the complex type or acquire the M6P group, the latter serving as a recognition marker for its receptor-mediated segregation into lysosomes (von Figura and Hasilik, 1986). We further investigated to what extent high mannose type carbohydrates on CD were processed into complex type by treating the immunoprecipitates with endoglycosidase H (EndoH), which removes only high mannose type carbohydrates. The results shown in Fig. 6B suggest that in steady-state conditions: (i) proCD and the 47 kDa intermediate, i.e. the Golgi-associated forms, bear complex-type carbohydrates; (ii) the 43 kDa Msc bears either high mannose- or complex-type carbohydrates; and (iii) the 34 kDa LC bears one complex type carbohydrate. It should be emphasised that the prominent form of mature CD (i.e. the 43 kDa species) bears at least one oligosaccharide of the high-mannose type, while the intermediate form (i.e. the 47 kDa species) and the precursor bear complex-type oligosaccharide(s). By analogy with other studies on CD biogenesis it can be assumed that high mannoses were further phosphorylated (von Figura and Hasilik, 1986). The above results may therefore be interpreted as meaning that only the 47 kDa intermediate molecules that acquired the M6P signal could be further transported and processed to generate the 43 kDa species. On the other hand, the presence of a small amount of mature CD bearing only complex-type oligosaccharides suggests that a portion of its precursor could be segregated into endosomal-lysosomal compartments via an MPR-independent pathway.

The involvement of a receptor system in the targeting of CD in RBL cells was assessed by studying the effects of a 24 hour incubation in the presence of 20 mM ammonium chloride. This drug is known to alkalinise the endosomal pH, thus impairing the receptor-ligand dissociation and re-utilisation of free receptors (Maxfield, 1982), and to inhibit the formation of the M6P signal on lysosomal enzymes (Isidoro et al., 1990). Under this experimental condition we observed an accumulation of the 47 kDa form and an increase of immature CD molecules (53 and 47 kDa forms) secreted in the medium (not shown). It should be pointed out that in untreated cultures the proportion of secreted vs intracellularly retained CD accounted for by less than 10-15%. These data are consistent with: (i) efficient sorting and targeting to post-Golgi compartments of CD precursor; (ii) exocytosis of both the 53 kDa and 47 kDa molecules from pre-endosomal compartments; and (iii) a role for the M6P system in the transport of CD to post-Golgi compartments. Thus, both M6P-dependent and M6P-independent pathways are operative for the transport of CD in RBL cells.

Antigen stimulation triggers the exocytosis of CD from different compartments of the biosynthetic pathway in RBL cells

It should be noted that the assays for β-hexosaminidase and cathepsins B and L reveal only the mature enzymes released from post-Golgi compartments, whereas in the assay conditions used to determine CD activity the immature form of this enzyme autoactivates and therefore, if present, contributes to the substrate hydrolysis (data in Fig. 5). We therefore analysed the CD molecular forms released by RBL cells stimulated as for the experiments described above by western blotting. Within 30 minutes of incubation only a 43 kDa species is released under effective antigen stimulation (not shown), while after a 3 hour incubation stimulated cells also released a small amount of a 53 kDa molecular form (Fig. 7). In this condition, in the medium of control or antigen stimulated plus EGTA cultures only the 53 kDa CD molecular form was faintly detectable. Thus, under effective antigen stimulation, secretion of the 53 kDa form was doubled, while that of the 43 kDa form increased by nearly 40-fold (see densitometric analysis, Fig. 7C). This fact strongly suggests that these molecular forms reside in different compartments which are differentially sensitive to the exocytosis triggered by

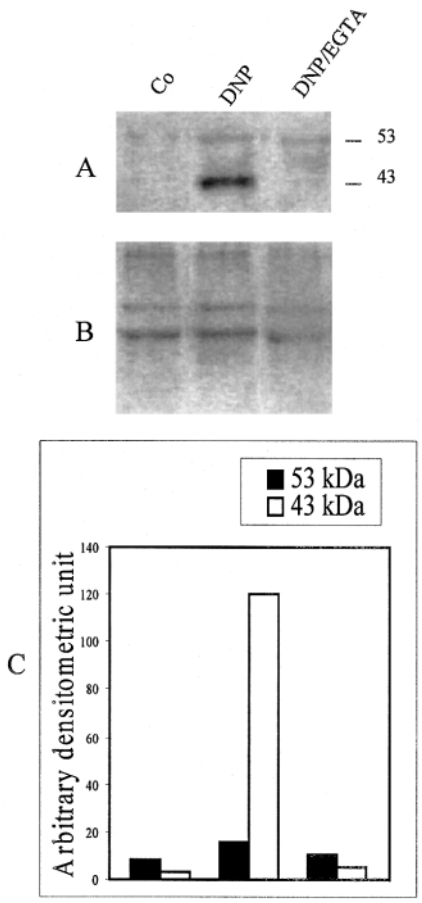

Fig. 7. CD molecular forms secreted by antigen stimulation. RBL cells were incubated with DNP-BSA (DNP) or with DNP-BSA and EGTA (DNP/EGTA) for 180 minutes. Control cultures (Co) were also included. Incubation were made in serum free medium. (A) Media were TCA-precipitated and processed for SDS-PAGE and western blotting as described in Materials and Methods. For CD molecular forms refer to the legend to Fig. 6. (B) Coomassie blue-stained gel showing equal loading of samples (remnant after blotting). (C) densitometric evaluation of gel A.

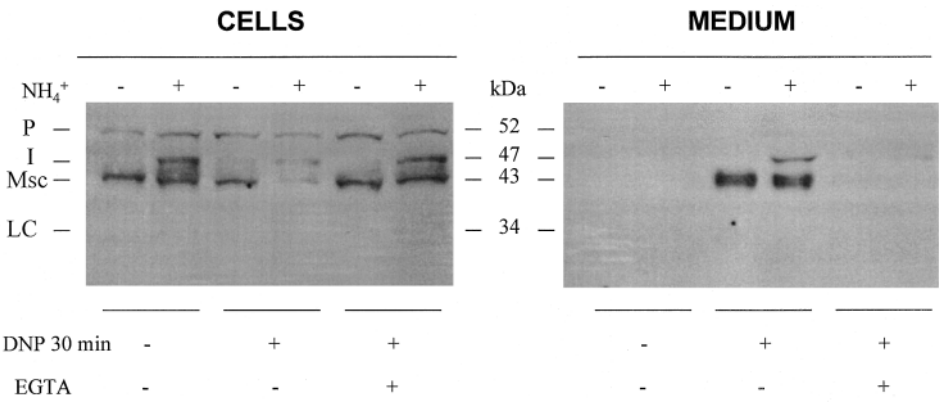

Fig. 8. Antigen stimulation induces secretion of mature and immature CD. IgE-primed RBL cells, preincubated or not with 20 mM ammonium chloride for 24 hours, were treated or not for 30 minutes with 100 ng/ml DNP-BSA in the presence or absence of EGTA. CD molecular forms (see Fig. 6) in cells and media were revealed by western blotting.

IgE receptor stimulation. In this respect, the compartment containing the 43 kDa CD form seems to be the most sensitive (see also below).

Since the processed molecular forms of CD result from proteolytic cleavage of proCD occurring while it travels through the compartments of the secretory and endocytic pathways (Hasilik, 1992) we exploited the powerful features of CD as protein reporter to investigate the intracellular compartments along the biosynthetic pathway sensitive to antigen-induced exocytosis. In the following experiments we asked whether interfering with the receptor-dependent segregation of the intermediate CD would cause its accumulation into a compartment sensitive to antigen-dependent exocytosis. For this purpose RBL cells were incubated for 24 hours in the presence of 20 mM ammonium chloride. Control and ammonium chloride-treated RBL cells were then stimulated with DNP-BSA in the presence or the absence of EGTA and the secretion accumulated during 30 minutes of incubation in serum-free medium was analysed by western blotting. The prolonged incubation with ammonium chloride caused, as expected, the intracellular accumulation of the 47 kDa intermediate species (Fig. 8). Based on the mechanism of action of this drug it is reasonable to assume that the intermediate CD was accumulating in a compartment upstream of the acidic endosomal compartment (Maxfield, 1982), although its transport to post-Golgi compartments via a receptor-independent (ammonium chloride insensitive) pathway cannot be excluded. The results shown in Fig. 8 demonstrate that both the intermediate and the mature forms of CD are exocytosed under effective antigenic stimulation, most likely from two distinct compartments. It is of note that secretion of the 47 kDa induced by antigen stimulation also was abrogated in the presence of EGTA.

DISCUSSION

Basophils and mast cells play a central role in immediate allergic and inflammatory responses. When activated through their high-affinity IgE-receptors by an allergen these cells immediately release various inflammatory mediators, such as histamine, cytokines, chemotactic factors and proteases, previously stored in the secretory granules (Gordon et al., 1990; Galli et al., 1993; Dvorak et al., 1995). In addition, under certain circumstances (such as cytokine stimulation) mast cells also feature antigen presentation ability to T lymphocytes (Fox et al., 1994; Frandji et al., 1996), a function that relies on the presence in these cells of the so-called 'major histocompatibility complex class II compartment' (Raposo et al., 1997). It is known that this compartment belongs to the endosomal-lysosomal type of cellular organelles (Amigorena et al., 1994; Glickman et al., 1996). Therefore, understanding the relationship between lysosomes and secretory granules in basophils and mastocytes is of primary importance to clarify whether lysosomal hydrolases actively participate in the allergic/inflammatory reaction, as well as in the processing of exogenous antigens by these cells.

Here we provide evidence that the inflammatory granules of RBL cells, having the phenotype of mucosal mastocytes, contain lysosomal acid proteases which are released upon allergen stimulation. This observation, together with the acid pH and the accessibility to endocytosed molecules, emphasises the intimate connection between the endosomal-lysosomal apparatus and secretory granules previously described only in natural killer and T cytotoxic lymphocytes (Burkhardt et al., 1990; Peters et al., 1991). Lysosomes are considered to be the terminal station for hydrolysis of extra- and intracellular substrates therein transported via endocytosis, (auto)phagocytosis or chaperons. Generally speaking, these organelles do not release their content in the extracellular space. Recently, Rodriguez and co-workers (1997) have shown that in epithelial cells lysosomes can be induced to undergo exocytosis by increasing intracellular calcium levels; this involved, however, only 10% of total cellular lysosomes. Lysosomes of basophils/mast cells seem to behave in a different way. A 30 minute stimulation with DNP-BSA of specific IgE-coated basophils led to a prompt and massive release of mature CD from post-Golgi compartments. Parallel experiments showed that antigenic stimulation also raised up to 2- or 3-fold the secretion of three other lysosomal enzymes (β-hexosaminidase and cathepsins B and L). This could be prevented by addition of EGTA, indicating that secretion was dependent on the influx of calcium. These features correspond to the requirements for the release of inflammatory mediators from activated mastocytes. It is of note that under conditions that prevented degranulation (i.e. in the presence of EGTA), allergen stimulation induced the translocation of CD-containing organelles toward the periphery of the cell and their accumulation close to the plasma membrane (Figs 1 and 3).

We have also shown that secretory granules of RBL cells are acidic and that degranulation is associated with the rupture of the lysosomal membrane integrity and consequent inability to

maintain intralumenal acid pH. We further investigated the relationship between secretory compartments and the endocytosis pathway by examining the fate of exogenously added fluorescent tracers. Data are consistent with the convergence in RBL cells of the secretory and endocytosis pathways at the level of inflammatory granules. The presence of lysosomal cathepsins, the acidic pH and the accessibility to exogenous molecules via endocytosis are all features that might enable the secretory compartment of mastocytes to perform antigen processing functions (Raposo et al., 1997).

An open question is the mechanism of granule biogenesis in regulated secretory cells. Proteins destined to the granules may be recruited at TGN through direct or indirect binding to receptors or enter the immature granule (IG) in a soluble state and be retained therein by progressive condensation during granule maturation. In the first model ('sorting for entry'), nongranule secretory proteins are shunted to the constitutive secretory pathway by default at TGN level, while in the second model ('sorting by retention') nongranule secretory proteins are removed from maturing granules by constitutive-like vesicle budding (reviewed by Arvan and Castle, 1998). Since lysosomal cathepsins are found in regulated secretory granules of epithelial (Kulliawat et al., 1987) or myeloid (Burkhardt et al., 1990; Peters et al., 1991; the present study) cells, they might provide a model of soluble proteins suitable for studying the biogenesis of these organelles. To our knowledge no previous study has addressed the question of the targeting, maturation and regulated secretion of a lysosomal enzyme in basophilic/mast cells.

CD can be considered the paradigm of soluble lysosomal hydrolases and, due to its well known transport-linked post-translational processing, it also represents a good protein reporter for studying the traffic within the endocytic/exocytic pathways (Isidoro et al., 1996). Based on the kinetics of maturation it appears that in RBL cells proCD is initially converted into a 47 kDa form within the Golgi, and later into the 43 kDa molecular species within post-Golgi compartments. EndoH treatment demonstrated the presence of high mannose-type oligosaccharides on mature CD, which is compatible with the formation of the M6P recognition marker for the receptor-mediated lysosomal delivery. Accordingly, the experiments with ammonium chloride indicated that most of the 47 kDa intermediate CD is further transported from TGN by a receptor-mediated mechanism. Yet, the presence in RBL cells of an alternative, MPR-independent mechanism for segregation of lysosomal enzymes similar to that described in other myeloid cells (cf. Glickman and Kornfeld, 1993) seems also possible, as ammonium chloride- or chloroquine-treatment did not preclude completely CD maturation (not shown). We exploited these features of CD maturation to discriminate between intracellular compartments sensitive to antigen-dependent exocytosis in RBL cells. The experiments revealed that the compartment most sensitive to allergen-induced degranulation appears to be the 43 kDa mature CD containing-one. Moreover, the data shown in Figs 7 and 8 are consistent with the view that beside secretory organelles, pre-endosomal compartments of basophils also are able to respond to antigenic stimulation by releasing their content. This rises the question whether in basophils the pathway for lysosomal proenzymes segregation includes an intermediate compartment with the features of immature granules which might fuse with the plasma membrane upon adequate stimulation. Such an itinerary has been shown to take place in pancreatic β-cells (Kulliawat et al., 1997). In these cells, both cathepsins L and B were found in IG, while only the former persisted in mature β-granules. This fact has been attributed to the different affinity that the two enzymes exhibit for the MPRs responsible of their sorting at TGN and IG levels (Kulliawat et al., 1997). ProCD and proCathepsin B bind very efficiently to the MPRs, yet in this study both these enzymes are largely found in secretory granules in a mature, enzymatically active molecular form. This might be explained admitting a missorting of the two cathepsins at IG level. Acidification of maturing granules is one plausible cause, as it compromises binding to MPRs and sorting of lysosomal enzymes. In RBL cells inflammatory granules are in fact acid, as assessed by labelling with acidotropic fluorogenic probes. On the other hand, if one considers that myeloid secretory granules coincide with lysosomes, then the presence of lysosomal enzymes in secretory granules would not arise from a missorting, rather it would well be explained by the 'sorting for entry' model (Arvan and Castle, 1998). Our data seem to favour this latter hypothesis. The observation that the antigen-induced extracellular release of CD and cathepsin B was enhanced by 3-folds, while that of cathepsin L was enhanced only by 2-folds, lend support to this view.

In conclusion, the present work demonstrates that in basophils/mastocytes inflammatory secretory granules are lysosomal-like organelles, based on the following: (i) intralumenal acidic pH; (ii) connection with the endocytosis pathway; (iii) content of mature lysosomal hydrolases. The morphological (Fig. 2: co-localisation with 5G10 and anti-CD) and biochemical (Figs 7 and 8: secretion of single-chain forms of CD, not of the double-chain form) data provided in this paper lend support to the view that in basophils/mastocytes prelysosomal and lysosomal compartments share with secretory granules the ability to fuse with the plasma membrane in response to IgE-receptor stimulation and that in these cells there might exist a population of lysosomes that have lost this feature. This issue, however, needs to be investigated further, for instance employing immuno-electron microscopy. We also demonstrate that targeting of lysosomal cathepsins to mature inflammatory granules is mostly sensitive to vacuolar alkalinisation by ammonium chloride (implying that it is mainly receptor-mediated) and includes an intermediate station (IG?) sensitive to antigen-stimulated calcium-dependent degranulation. Most interestingly from a pathophysiological point of view, we show that antigen-mediated cross-linking of mast cell IgE receptor results in a prompt and extensive release of mature, enzymatically active, lysosomal proteases. Granules of immunocompetent cells of the myeloid lineage are designed to effect the elimination of the antigen. The meaning of the presence of lysosomal enzymes in such granules is still unknown. One can speculate as to whether the concurrent secretion of lysosomal acidic hydrolases in degranulating myeloid cells is instrumental to the elimination of the antigen or if extracellular lysosomal proteases exert a regulatory role in the inflammatory response. In this regard it is worth noting that lysosomal cathepsins participate in the maturation of procollagen (Helseth and Veis, 1984), in the degradation of fibrinogen and fibrin (Simon et al., 1994), fibronectin (Humphries and Ayad, 1983), and of several other components

of the extracellular matrix (Briozzo et al., 1988) and in the activation of procaspase 3 (Heinrich et al., 1999) and procaspase 11 (Schotte et al., 1998), which are involved in the onset of apoptotic cell death and inflammation, respectively.

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REFERENCES

Amigorena, S., Drake, J. R., Webster, P. and Mellman, I. (1994). Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature* **369**, 113-120.

Arvan, P. and Castle, D. (1998). Sorting and storage during secretory granule biogenesis: looking backward and looking forward. *Biochem. J.* **332**, 593-610.

Baldassarre, M., Dragonetti, A., Marra, P., Luini, A., Isidoro, C. and Buccione, R. (2000). Regulation of protein sorting at the TGN by plasma membrane receptor activation. *J. Cell Sci.* **113**, 741-748.

Bonifacino, J. S., Perez, P., Klausner, R. D. and Sandoval, I. V. (1986). Study of the transit of an integral membrane protein from secretory granules through the plasma membrane of secreting rat basophilic leukemia cells using a specific monoclonal antibody. *J. Cell Biol.* **102**, 516-522.

Briozzo, P., Morisset, M., Capony, F., Rougeot, C. and Rochefort, H. (1988). In vitro degradation of extracellular matrix with Mr 52,000 cathepsin D secreted by breast cancer cells. *Cancer Res.* **48**, 3688-3692.

Brumell, J. H., Volchuck, A., Sengelov, H., Borregaard, N., Cieutat, A. M., Bainton, D. F., Grinstein, S. and Klip, A. (1995). Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. *J. Immunol.* **155**, 5750-5759.

Buccione, R., Di Tullio, G., Caretta, M., Marinetti, M. R., Bizzarri, C., Francavilla S., Luini, A. and De Matteis, **M. A.** (1994). Analysis of protein kinase C requirement for exocytosis in permeabilised rat basophilic leukaemia RBL-2H3 cells: a GTP-binding protein(s) as a potential target for protein kinase C. *Biochem. J.* **298**, 149-156.

Burkhardt, J. K., Hester, S., Lapham, C. K. and Argon Y. (1990). The lytic granules of natural killer cells are dual-function organelles combining secretory and pre-lysosomal compartments. *J. Cell Biol*. **111**, 2327-2340.

Chiarpotto, E., Domenicotti, C., Paola, D., Vitali, A., Nitti, M., Pronzato, M. A., Biasi, F., Cottalasso, D., Marinari, U. M., Dragonetti, A., Cesaro, P., Isidoro, C. and Poli, G. (1999). Regulation of rat hepatocyte protein kinase C β isoenzyme by the lipid peroxidation product 4-hydroxy-2, 3-nonenal: a signaling pathway to modulate vesicular transport of glycoproteins. *Hepatology* **29**, 1565-1572.

Démoz M., Castino, R., Dragonetti, A., Raiteri, E., Baccino, F. M. and Isidoro, C. (1999). Transformation by oncogenic Ras-p21 alters the processing and subcellular localization of the lysosomal protease cathepsin D. *J. Cell. Biochem.* **73**, 370-378.

Dvorak, A. M., MacGlashan, Jr., D. W., Morgan, E. S. and Lichtenstein L. M. (1995). Histamine distribution in human basophil secretory granules undergoing FMLP-stimulated secretion and recovery. *Blood* **86**, 3560-3566.

Edgar, A. J. and Bennett, J. P. (1997). Circular ruffle formation in rat basophilic leukemia cells in response to antigen stimulation. *Eur. J. Cell Biol.* **73**, 132-140.

Fox, C. C., Jewell, S. D. and Whitacre, C. C. (1994). Rat peritoneal mast cells present antigene to a PPD-specific T cell line. *Cell Immunol.* **158**, 253-264.

Frandji, P., Tkaczyc, C., Oskeritzian, C., David, B., Desaymard, C. and Mécheri, S. (1996). Exogenous and endogenous antigen are differentially presented by mast cells to CD4+T lymphocytes. *Eur. J. Immunol.* **26**, 2517-2528.

Fujita, H., Tanaka, Y., Noguchi, Y., Kono, A., Himeno, M. and Kato, K. (1991). Isolation and sequencing of a cDNA clone encoding rat liver lysosomal cathepsin D and the structure of three forms of mature enzymes. *Biochem. Biophys. Res. Commun.* **179**, 190-196.

Galli, S. J., Gordon, J. R. and Wershil, B. K. (1993). Mast cell cytokines in allergy and inflammation. *Agents Actions* Suppl. **43**, 209-220.

Glickman, J. N. and Kornfeld, S. (1993). Mannose 6-phosphate-independent targeting of lysosomal enzymes in cell disease B lymphoblasts. *J. Cell Biol.* **123**, 99-108.

Glickman, J. N., Morton, P. A., Slot, J. W., Kornfeld, S. and Geuze, H. J. (1996). The biogenesis of the MHC class II compartment in human I-cell disease B lymphoblasts. *J. Cell Biol.* **132**, 769-785.

Gordon, J. R., Burd, P. R. and Galli, S. J. (1990). Mast cells as a source of multifunctional cytokines. *Immunol. Today* **11**, 458-464.

Griffiths, G. M. and Isaaz, S. (1993). Granzymes A and B are targeted to the lytic granules of lymphocytes by the mannose-6-phosphate receptor. *J. Cell Biol.* **120**, 885-896.

Griffiths, G. M. (1996). Secretory lysosomes – a special mechanism of regulated secretion in haemopoietic cells. *Trends Cell Biol.* **6**, 329-332.

Helseth, D. L. Jr and Veis, A. (1984). Cathepsin D-mediated processing of procollagen: lysosomal enzyme involvement in secretory processing of procollagen. *Proc. Nat. Acad. Sci. USA* **81**, 3302-3306.

Hasilik, A. (1992). The early and late processing of lysosomal enzymes: proteolysis and compartmentation. *Experientia* **48**, 130-151.

Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Weber, T., Brunner, J., Krönke, M. and Schütze, S. (1999). Cathepsin D targeted by acid sphingomyelidae-derived ceramide. *EMBO J.* **18**, 5252-5263.

Humphries, M. J. and Ayad, S. R. (1983). Stimulation of DNA synthesis by cathepsin D digests of fibronectin. *Nature* **305**, 811-813.

Isidoro, C., Radons, J., Baccino, F. M. and Hasilik, A. (1990). Suppression of the 'uncovering' of mannose-6-phosphate residues in lysosomal enzymes in the presence of NH_4Cl. *Eur. J. Biochem.* **191**, 591-597.

Isidoro, C., Demoz, M., De Stefanis, D., Baccino, F. M. and Bonelli G. (1995). High levels of proteolytic enzymes in the ascitic fluid and plasma of rats bearing the Yoshida AH-130 hepatoma. *Invasion Metast.* **15**, 116-124.

Isidoro, C., Maggioni, C., Démoz, M., Pizzagalli, A., Fra, A. M. and Sitia, R. (1996). Exposed thiols confer localization in the endoplasmic reticulum by retention rather than retrieval. *J. Biol. Chem.* **271**, 26138-26142.

Jouvin, M. H., Numerof, R. P. and Kinet, J. P. (1995). Signal transduction through the conserved motifs of the high affinity IgE receptor Fc epsilon RI. *Semin. Immunol.* **7**, 29-35.

Kulliawat R., Klumperman, J., Ludwig, T. and Arvan, P. (1997). Differential sorting of lysosomal enzymes of the regulated secretory pathway in pancreatic β-cells. *J. Cell Biol.* **137**, 595-608.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Ludwig, T., Griffiths, G. and Hoflack, B. (1991). Distribution of newly synthesized lysosomal enzymes in the endocytic pathway of normal rat kidney cells. *J. Cell Biol.* **115**, 1561-1672.

Matteoni, R. and Kreis, T. E. (1987). Translocation and clustering of endosomes and lysosomes depends on microtubules. *J. Cell Biol.* **105**, 1253-1265.

Maxfield, F. R. (1982). Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol*. **95**, 676-681.

Paul, W. E., Seder, R. A. and Plaut, M. (1993). Lymphokine and cytokine production by Fc∈RI+ cells. *Adv. Immunol.* **53**, 1-29.

Peters, P. J., Borst, J., Oorshot, V., Fukuda, M., Krähenbühl, O., Tschopp, J., Slot, J. W. and Geuze, **H. J.** (1991). Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **173**, 1099-1109.

Raposo, G., Tenza, D., Salahedine, M., Peronet, R. Bonnerot, C. and Desaymard, C. (1997). Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol. Biol. Cell* **8**, 2631-2645.

Rodriguez, A., Webster, P., Ortego, J. and Andrews, N. W. (1997). Lysosomes behave as Ca^{2+}-regulated exocytic vesicles in fibroblasts and epithelial cells. *J. Cell Biol.* **137**, 93-104.

Schotte, P., Van Criekinge, W., Van de Craen, M., Van Loo, G., Desmedt, M., Grooten, J., Cornelissen, M., De Ridder, L., Vandekerckhove, J.,

Fiers, W. et al. (1998). Cathepsin B-mediated activation of the proinflammatory caspase-11. *Biochem. Biophys. Res. Commun.* **251**, 379-387.

Schwartz, L. B. and Austen, K. F. (1980). Enzymes of the mast cell granule. *J. Invest. Dermatol.* **74**, 349-353.

Simon, D. I., Ezratty, A. M. and Loscalzo, J. (1994). The fibrin(ogen)olytic properties of cathepsin D. *Biochemistry* **33**, 6555-6563.

Stinchcombe, J. C. and Griffiths, G. M. (1999). Regulated secretion of hemopoietic cells. *J. Cell Biol.* **147**, 1-5.

von Figura, K. and Hasilik, A. (1986). Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* **55**, 167-193.

Yonezawa, S., Takahashi, T., Wang, X. J., Wong, R. N., Hartsuck, J. A. and Tang, J. (1988). Structures at the proteolytic processing region of cathepsin D. *J. Biol. Chem.* **263**, 16504-16511.