Actin microfilaments control the MHC class II antigen presentation pathway in B cells

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SUMMARY

Newly synthesised major histocompatibility complex class II molecules associate with invariant chains (Ii) to form nonameric complexes. These complexes are transported to endosomes, where proteolytic enzymes generate αβ class II dimers associated with nested Ii-derived peptides. These peptides are then exchanged with antigen peptide, and mature class II molecules reach the cell surface. The role of the actin cytoskeleton in the transport and maturation of class II molecules has not been studied. We show here that upon treatment with cytochalasin D (cyto D), the rate of Ii degradation is drastically reduced in B cells. Cyto D treatment also leads to a delayed appearance of stable forms of class II molecules, and a reduced presentation efficiency of antigen determinants requiring newly synthesised class II molecules. Under such conditions, we found that invariant chain fragments and class II molecules are accumulated in early and late endosomal compartments, whereas the leupeptin protease inhibitor induces their accumulation in lysosomal compartments. The addition of cyto D to leupeptin blocks the delivery of class II/invariant chain complexes to lysosomes, and further inhibits degradation of Ii. The dynamics of the actin cytoskeleton can therefore control the meeting point between newly synthesised class II molecules and lysosomal proteases, involved in Ii degradation and antigen peptide loading.

Key words: Antigen processing, Cytoskeleton, H-2-M molecule, Invariant chain, Lysosome

INTRODUCTION

Major histocompatibility complex (MHC) class II molecules present peptide determinants, generally derived from the uptake of exogenous antigens in antigen-presenting cells (APC), to CD4+ T lymphocytes (Germain, 1994). MHC class II αβ heterodimers associate with invariant chains (Ii) in the endoplasmic reticulum to form (αβ)3Ii3 nonameric complexes (Roche et al., 1991). Ii has multiple roles during the maturation of class II molecules (Bertolino and Rabourdin-Combe, 1996). The Ii amino acid sequence 84-104, called CLIP, prevents the association of endogenous peptides present in the endoplasmic reticulum with newly synthesised MHC class II dimers (Germain and Hendrix, 1991; Long et al., 1994). CLIP occupies the peptide-binding groove of αβ dimers (Romagnoli and Germain, 1994; Ghosh et al., 1995) and MHC class II molecules have to be free from CLIP to bind antigen peptides (Roche and Cresswell, 1991). The cytoplasmic domain of Ii contains two dileucine motifs, which promote the transport of newly synthesised (αβ)3Ii3 nonamers to endosomal compartments (Pieters et al., 1993; Odorizzi et al., 1994; Arneson and Miller, 1995). During the transport of class II/Ii complexes to endosomal compartments, Ii is sequentially degraded by proteolytic enzymes into several intermediate fragments, which can remain associated with αβ dimers through CLIP (Nguyen et al., 1989; Xu et al., 1994; Marie et al., 1994).

Several groups have shown that the last transmembrane Ii degradation product to be associated with αβ dimers was a p12 Ii fragment containing CLIP (Nguyen et al., 1989; Xu et al., 1994; Morkowski et al., 1995; Barois et al., 1997; Brachet et al., 1997). Another p10 transmembrane Ii fragment lacking CLIP, but not associated with class II molecules, was identified in murine B cells (Barois et al., 1997). The production of p12 Ii fragments precedes the formation of the free p10 Ii molecules, suggesting that class II/CLIP complexes are formed during the conversion of p12 into p10 fragments (Ghosh et al., 1995; Morkowski et al., 1995). Riese et al. (1996) have shown that this last step of invariant chain degradation is performed by cathepsin S, which is a cysteine protease (Riese et al., 1996). The non-polymorphic H-2M molecules, in mouse, and HLA-DM, in human, can associate with class II/CLIP complexes (Sanderson et al., 1994; Fernandez-Borja et al., 1996; Sanderson et al., 1996); they catalyse the removal of CLIP from the class II binding groove and facilitate antigen peptide binding (Denzin and Cresswell, 1995). Class II/peptide complexes were also shown to gain access to the cell surface from the specialised pre-lysosomal compartment (Wubbolts et al., 1996; Pond and Watts, 1997). The recycling of αβ dimers from the cell surface to the endosomal compartment, has also been described (Harding et al., 1989; Salamero et al., 1990; Pinet et al., 1995) and allows the binding and the presentation of antigen, independently of invariant chain, protein synthesis and HLA-
DM molecules (Harding et al., 1989; Nadimi et al., 1991; Griffin et al., 1997).

Actin microfilaments are known to be important for the intracellular organisation of the cell cytoplasm and for cell motility. Several recent studies performed in MDCK cells or in hepatoma cells have shown that actin microfilaments are also involved during receptor-mediated endocytosis (Gottlieb et al., 1993; Durrbach et al., 1996). Cytochalasin D (cyto D), which inhibits actin polymerisation, blocks the uptake of exogenous proteins and the internalisation of cell surface receptors (MacLean-Fletcher and Pollard, 1980; Gottlieb et al., 1993; Durrbach et al., 1996). Furthermore, Durrbach et al. (1996) have shown that the dynamics of the actin cytoskeleton control the transport between endosomal compartments at different stages; the transport of α2-macroglobulin from late endosomes to lysosomes was clearly sensitive to the depolymerisation of actin microfilaments. The participation of actin cytoskeleton in the processing of soluble antigen has also been reported (Soreng et al., 1995); however, no precise localisation of the step(s) controlled by actin microfilaments was defined. Furthermore, actin was shown to be partially associated with purified murine class II molecules (Newell et al., 1988) and class II molecules lacking the cytoplasmic domains of the α chain have an increased surface mobility (Wade et al., 1989b), suggesting that the actin cytoskeleton could interact directly with MHC class II molecules.

To assess whether the cytoskeleton participates in the maturation of class II molecules in murine B cells, we studied the effect of actin microfilament disruption by cyto D on the MHC class II pathway and on the internalisation of B cell receptor (BCR) ligands. Our results show that actin microfilament depolymerisation induces the accumulation of newly formed MHC class II/ı complexes in non lysosomal compartments. Cyto D was also shown to reduce the internalisation of cross-linked surface immunoglobulins, indicating that the dynamics of the actin cytoskeleton control the MHC class II presentation pathway in B cells.

MATERIALS AND METHODS

Antibodies and reagents

The rabbit polyclonal anti-H-2Mβ (αCyt.Mβ) antibody was obtained after immunisation with the peptide sequence of the β cytoplasmic domain corresponding to RKSFIGSSTPLGSTEPGRH243, coupled with glutaraldehyde to keyhole limpet hemocyanin as protein carrier. The rabbit polyclonal anti-ı (αCyt.ı) and anti-I-ıp (αCyt.ıAıp) antibodies were raised against synthetic peptides as previously described (Barois et al., 1997). The αCyt.ı Antibody was conjugated to the NHS-LC-biotin (Pierce, Rockford, IL) for double-staining with Texas Red (suitable for double-labeling experiments) and biotinylated or FITC-coupled donkey anti-mouse immunoglobulins (to cross-link the BCR) were purchased from Jackson ImmunoResearch (West Grove, PA).

Leupeptin and cyto D were from ICN and Calbiochem, respectively. The antigens, hen egg lysozyme (HEL) and ribonuclease A (RNase A), used in antigen presentation assays, were obtained from Sigma.

Cells

The F6 B cell lymphoma was derived from the M12C3 B cell line, I- A negative, transfected for expression of I-AK molecules (Wade et al., 1989a). The 3A9 T cell hybridoma (Allen and Unanue, 1984) and the TS12 T cell hybridoma (Lorenz et al., 1989a) are both I-AK restricted and specific for HEL 46-61 and RNase A 43-56 peptides, respectively. IL-2 dependent CTLL-2 cells were purchased from ATCC (Rockville, MD). Cell lines were cultured in DME medium (Gibco BRL) supplemented with 10% FCS, 20 μM β-mercaptoethanol, 1 mM sodium pyruvate and 1 mM glutamine.

35S metabolic labelling

F6 cells were washed twice and incubated for 45 minutes at 37°C in cysteine/methionine-free RPMI medium (Gibco BRL). In 3 ml of this medium containing 5% dialysed FCS, cells were incubated for 30 minutes at 37°C with 0.6 μCi [35S]cysteine/methionine. When indicated, pulse-labelled cells were chased for different periods of times at 37°C in RPMI medium containing cold cysteine and methionine with or without 10 μg/ml cyto D. Cells were washed with ice-cold PBS and solubilised in lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing a cocktail of protease inhibitors (0.25 mM PMSF, 0.5 mM iodoacetamide, and 1 μg/ml leupeptin, pepstatin and aprotilin). Lysates were preclarified with protein A-Sepharose beads and supernatants were added to αCyt.ı antibody or 10.2 μM mouse anti-I-ı antibody for 4 hours. Immunoprecipitates were extensively washed and resuspended in reducing sample buffer (10 mM Tris-HCl, 2 mM EDTA, 33% glycerol, 2% SDS, 5% β-mercaptoethanol). To detect sodium dodecyl sulfate (SDS)-stable αβ dimers, immunoprecipitates were left at room temperature for 1 hour before being resolved by 15% SDS-PAGE. Labelled proteins were intensiﬁed and revealed by autoradiography.

Antigen presentation

F6 antigen-presenting cells were pulsed with various doses of HEL and RNase A for 2 hours and treated or not with 10 μg/ml cyto D for 3 hours. After washing, 5.10⁴ APCs were co-cultured for 24 hours at 37°C with 10⁴ 3A9 or 10⁵ TS12 T cell hybridomas. IL-2 production in culture supernatants was measured using thiazolyl blue MTT (Sigma) to evaluate the growth of the IL-2 dependent CTLL-2 cell line as previously described.

Immunofluorescence staining and confocal microscopy

F6 cells cultured to 75% conﬂuence on glass coverslips were treated or not with 10 μg/ml cyto D or with 500 μM leupeptin or with both for 4 hours. Using a concentration of 10 μg/ml of cyto D for 4 hours, the cells were not signiﬁcantly detached from the coverslip support. Cells were ﬁxed for 15 minutes at room temperature with 4% paraformaldehyde in PBS. After washing, cells were permeabilised with PBS containing 0.05% saponin and 1% BSA. Unbound antibodies were removed by washing in the same medium and cells were incubated for 30 minutes with secondary labelled antibody, washed, incubated with rabbit preimmune serum, washed again, post-fixed with 2% paraformaldehyde, incubated for 30 minutes with the second primary antibody coupled to biotin, rinsed and labelled with Texas Red-conjugated streptavidin. After washing in PBS and distilled water, the coverslips were mounted onto glass slides with mowiol plus DABCO (Sigma). In the case of anti-MPR antiserum, cells were permeabilised with PBS containing 0.5% saponin. To label early endosomes, FITC-coupled transferrin was internalised for 20 minutes just before fixing and labelling with αCyt.ı antibody as previously described (Barois et al., 1997). For studying BCR internalisation, cells were incubated for 1 hour at 37°C with or without 10 μg/ml cyto D, then incubated for 30 minutes at 37°C with labelled anti-mouse immunoglobulins, just before fixation and mounting. Confocal microscopy was performed using the Confocal Laser Scanning Microscopy TCS 4D.
(Leica Lasertechnik GmbH, Heidelberg Germany) interfaced with an argon/krypton ion laser and with fluorescence filters and detectors allowing simultaneous recording of FITC and Texas Red markers as previously described (Barois et al., 1997).

**Western blotting**

F6 cells were treated for 1 hour or longer as indicated with 10 µg/ml cyto D or 500 µM leupeptin or both. Cells were washed in ice-cold PBS and solubilised in 1 ml of lysis buffer as previously described. Lysates were either suspended in reducing sample buffer and directly analysed by 15% SDS-PAGE or incubated for 2 hours with 10.2.16 mAb bound to protein A-Sepharose beads (Pharmacia). After extensive washing, pellets were suspended in reducing sample buffer, boiled in SDS and analysed by 15% SDS-PAGE. Separated proteins were transferred onto Immobilon-P membranes (Millipore), and proteins were blotted with anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Labelled proteins were incubated with anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Labelled proteins were detected using the ECL immunodetection kit (Amersham).

**Flow cytometry**

For flow cytometry analysis of BCR internalisation, 1.5 Flow cytometry detected using the ECL immunodetection kit (Amersham). peroxidase (Jackson Immunoresearch). Labelled proteins were incubated with anti-rabbit antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Labelled proteins were detected using the ECL immunodetection kit (Amersham).

**RESULTS**

**Cytochalasin D treatment delays the maturation of newly synthesised class II molecules**

To analyse the kinetics of class II peptide loading in the presence or absence of cyto D, we monitored the resistance of metabolically labelled IA<sup>β</sup>/peptide complexes to denaturation by SDS at 20°C (Fig. 1A), reflecting the presence of peptide loaded class II αβ heterodimers as previously reported (Germain and Hendrix, 1991). For these studies we used a dose of 10 µg/ml of cyto D, which was sufficient to induce a rapid disruption of actin microfilaments as revealed by fluorescent phalloidin labelling (data not shown). In untreated cells, newly synthesised class II molecules acquired resistance to SDS dissociation at 20°C, 1 hour after their biosynthesis. The mature form of class II α chains (αm), which is terminally glycosylated, appeared after 30 minutes of chase, while immature α chains (αi) disappeared after 1 hour. Immature β chains (βi) disappeared after 30 minutes of chase but since mature β chains (βm) migrate at the same position as the p31 Ii forms, we cannot detect the initial rate of β chain maturation on these SDS gels. Cyto D treatment did not affect the rate of class II α chain maturation but delayed the formation of SDS-resistant αβ dimers (CF) for 2 hours following biosynthesis. The integrity of actin microfilaments was therefore required for the peptide loading of IA<sup>β</sup> class II molecules in B cells. Furthermore, we observed a higher level of association of p12 and p21 Ii fragments with MHC class II molecules after 4 hours of chase in the presence of cyto D (Fig. 1B). In untreated cells, the p21 fragment was degraded within 1 hour of chase while it was still associated with MHC class II molecules after 4 hours of chase with cyto D. The degradation rate of the p12 fragment was also slower in cyto D-treated cells compared to untreated cells, and the overall Ii degradation pattern was affected (Fig. 1B). In untreated cells, the quantity of the p31 Ii form slowly decreased at 1 hour of chase and we observed a transient appearance of the intermediate p21, p12 and p10 Ii fragments. In cells treated with cyto D, the p31, p21 and p12 Ii forms were prominently accumulated at 1 hour, 2 hours and 4 hours of chase. Moreover, the short cytoplasmic p10 Ii fragment appeared only at 4 hours of chase. These results show that actin microfilaments play a role in invariant chain degradation and in class II peptide loading.

**Differential sensitivity of antigen presentation pathways to cyto D treatment**

To assess the importance of actin microfilaments in the MHC class II antigen presentation pathway, we studied the response

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![Fig. 1. Cyto D treatment delays the formation of SDS-stable MHC class II heterodimers (A) and reduces degradation of the invariant chain (B). The cells were pulsed with [35S]Met/Cys and chased for 0-4 hours in the absence or presence of cyto D. The cells were solubilised and immunoprecipitated with mouse anti-I-A<sup>k</sup> mAb or rabbit αCyt.Ii antibody. Non-boiled samples were resolved by SDS PAGE. αi, αm, βi and βm indicate immature and mature α and β chains; p31, 21, 12 and 10, Ii peptide fragments; CF, compact forms of IA<sup>k</sup> stable in SDS at 20°C. MHC class II peptides were blotted with mAb or rabbit anti-I-A<sup>k</sup> mAb and labelled with a biotinylated mAb bound to protein A-Sepharose beads and detected with Streptavidin-alkaline phosphatase.](image-url)
of two T cell hybridomas requiring different pathways of antigen processing. The I-A\(^k\) restricted presentation of HEL to the HEL 46-61 specific 3A9 T cell hybridoma (Allen and Unanue, 1984) is sensitive to protein synthesis inhibitors, membrane transport inhibitors (Adorini et al., 1990; St-Pierre and Watts, 1990; Soreng et al., 1994), and requires Ii expression (Nadimi et al., 1991). The presentation of RNase A to the RNase A 43-56 specific TS12 T cell hybridoma requires neither protein synthesis nor Ii chain expression for an optimal response (Lorenz et al., 1988; Nadimi et al., 1991). These two antigenic determinants are thought to use different pathways of processing for their presentation to helper T cells. The RNase A 43-56 peptide is mainly presented by resident MHC class II molecules, which are thought to recycle (Harding et al., 1989; Salamero et al., 1990; Pinet et al., 1995), whereas the HEL 46-61 peptide is presented by newly synthesised class II molecules.

To determine whether these two pathways of antigen presentation are sensitive to actin microfilament disruption, we incubated F6 cells with the antigens for 2 hours prior to cyto D treatment for 3 hours (Fig. 2). This protocol was chosen to eliminate side effects of cyto D treatment on the internalisation of antigens from the fluid phase during the first 2 hours. At low doses of antigen, the presentation of HEL to the I-A\(^k\)-restricted 3A9 T cell hybridoma was reduced by about tenfold when the APCs were treated with cyto D (Fig. 2A). The presentation of HEL became independent of actin depolymerisation at high concentration of antigen. In contrast to HEL, the efficiency of the presentation of RNase A to the I-A\(^k\)-restricted TS12-specific T cell hybridoma was not affected by treatment with cyto D (Fig. 2B). Cyto D treatment selectively altered the presentation pathway of the HEL-derived 46-61 peptides, which requires newly synthesised MHC class II molecules after processing of the HEL protein.

**Cytochalasin D treatment induces the accumulation of class II/Ii complexes in non-lysosomal compartments**

To determine whether cyto D treatment leads to the accumulation of Ii and class II molecules into particular intracellular compartments, we used confocal microscopy and immunofluorescence labelling of I-A\(^k\) and Ii molecules. In untreated cells, MHC class II molecules labelled with \(\alpha\text{Cyt.IAk}\) antibody were mainly localised at the cell surface. Ii chain visualised with \(\alpha\text{Cyt.Ii}\) antibody gave mostly a reticulated intracellular staining, consistent with an endoplasmic reticulum expression pattern (Fig. 3A). After 4 hours of cyto D treatment, MHC class II molecules appeared in intracellular vesicles in conjunction with the invariant chain (Fig. 3B). Since the F6 B cells rounded up upon cyto D treatment, we had to collect several sections through elongated cells. The confocal section shown in Fig. 3B is representative of focal series in many fields. This intracellular accumulation of MHC class II/Ii complexes induced in cyto D-treated B cells was sensitive to the cycloheximide protein synthesis inhibitor, indicating that these compartments contain newly synthesised complexes (data not shown). These vesicles containing Ii are partially positive for internalised transferrin (Fig. 3C). In F6 cells, the lysosomal compartments labelled with anti-lgp110-B mAb (Granger et al., 1990), contain H-2M molecules (Fig. 3D) (Sanderson et al., 1994; Fernandez-Borja et al., 1996), and we found the same colocalisation between lgp110-B and H-2M in cyto D-treated cells (data not shown). H-2M molecule was therefore used as a lysosomal marker equivalent to lgp110-B in F6 cells. To visualise the intracellular distribution of the class II/Ii complexes in relation to the lysosomal compartments, we double-labelled the invariant chain with either \(\alpha\text{Cyt.IM}\) or anti-lgp110-B antibodies (Fig. 3E,F, respectively). After cyto D treatment, we found no superimposition of Ii with these two lysosomal markers, showing that class II molecules did not accumulate in lysosomal compartments.

To further analyse the kinetics of invariant chain degradation in presence of cyto D, we performed western blotting with \(\alpha\text{Cyt.Ii}\) either after class II molecule immunoprecipitation, or on total cell lysates (Fig. 4). We observed a prominent accumulation of different Ii fragments commmunoprecipitated with class II \(\alpha\beta\) dimers (Fig. 4A). The quantity of associated p12 and p21 Ii fragments increased after 1 hour of cyto D treatment. On total cell lysates (Fig. 4B), the effect of cyto D was also dependent on the duration of exposure to the drug. The quantity of p12 fragments already increased after 1 hour,


Fig. 3. Cyto D treatment accumulates MHC class II molecules and invariant chain in an early compartment. (A) In untreated cells, MHC class II molecules are expressed at the cell surface (in green) and invariant chain is localised in the endoplasmic reticulum (in red). (B) After cyto D treatment, MHC class II molecules (in green) and invariant chain (in red) are accumulated in intracellular vesicles (in yellow). (C) After cyto D treatment, invariant chain (in red) are accumulated in transferrin-positive vesicles (in green). (D) In control cells, H-2M molecules (in red) are completely localised in lysosomal compartments with lgp110-B (in green). (E and F) After cyto D treatment, the accumulated invariant chain (in red) was not localised in the lysosomal compartments (in green), labelled with H-2M molecules (E) or lgp110-B (F). Cells treated or not with 10 μg/ml cyto D for 4 hours were fixed, permeabilised and double-labelled for the invariant chain with the biotinylated αCyt.Ii antibody (in red in A, B and E) and for I-A^k molecules with αCyt.IAβ antibody (in green in A and B) or for H-2M molecules with αCyt.Mβ antibody (in green in E). For D, untreated cells were labelled with anti-lgp110-B mAb (in green) and with αCyt.Mβ antibody (in red). For C and F, cyto D-treated cells were labelled with αCyt.Ii antibody (in red) and internalised transferrin (C, in green) or lgp110-B mAb (F, in green). Arrowheads show the colocalisation. Bar, 10 μm.

suggesting that p12 fragments are rapidly induced. This result was consistent with the accumulation of Ii fragments in intracellular compartments observed by immunofluorescence (Fig. 3B). Furthermore, the quantity of the p10 fragment lacking CLIP decreased in total cell lysates after cyto D treatment, indicating that cyto D treatment blocks partially the degradation of p12 fragments into p10 fragments. Our results show that a late step of MHC class II molecule maturation, i.e. the release of class II molecules from p12 Ii fragment and consequently the generation of class II/CLIP complexes is inhibited by the cyto D-induced disruption of actin cytoskeleton.

Disruption of actin microfilaments alters the transport of the MHC class II molecules and the internalisation of the BCR

Durrbach et al. (1996) showed that the actin cytoskeleton plays a role in the transport of α2-macroglobulin from endosomal compartments to compartments of degradation (Durrbach et al., 1996). To analyse whether cyto D treatment can block the transport of MHC class II molecules to the endosomal compartments where class II/CLIP complexes were generated, we compared the treatment of leupeptin and leupeptin plus cyto D on invariant chain degradation and on accumulation of class II/Ii complexes (Nguyen et al., 1989; Neefjes and Ploegh, 1992; Amigorena et al., 1995). High concentrations of leupeptin can induce the accumulation of Ii fragments in vesicular compartments largely coincident with lysosomal proteins in B cells (Brachet et al., 1997). Since cyto D alone induced the accumulation of MHC class II/Ii complexes in a non-lysosomal compartment, it was tempting to combine the two drugs to dissect the pathway of MHC class II transport.

We first performed western blotting with αCyt.Ii on total cell lysates from cells treated with 500 μM leupeptin (Fig. 5A) or with 500 μM leupeptin plus 10 μg/ml cyto D (Fig. 5D) for various periods of times. After leupeptin incubation alone, p12 and p10 Ii fragments were rapidly accumulated (Fig. 5A). When cells were treated with leupeptin plus cyto D, we observed a strong accumulation of p12 Ii fragments as in cells treated with cyto D alone (Fig. 4B). The quantity of p10 Ii fragments decreased in the presence of cyto D during 1 hour and increased thereafter but remained always smaller than the level of p12 Ii fragments (Fig. 5D), indicating that the degradation of p12 fragment into p10 fragment was dependent on the integrity of the actin cytoskeleton. We also observed an increase of p31 fragment that was probably due to the cumulative effect of the two drugs. To assess whether cyto D treatment also blocked the transport of class II/Ii complexes to lysosomes exemplified in the presence of leupeptin, we monitored the intracellular distribution of Ii, in comparison with H-2M molecules and internalised transferrin. In cells treated with leupeptin alone for 4 hours, the invariant chain fragments were accumulated in lysosomes labelled with H-2M molecules (Fig. 5B) and were absent from early endosomes containing internalised transferrin (Fig. 5C). In cells treated with leupeptin and cyto D, Ii fragments were absent in H-2M-positive lysosomal compartments (Fig. 5E) and present in transferrin-positive compartments (Fig. 5F). This result indicates that disruption of the actin cytoskeleton reverses the accumulation of class II/Ii complexes from lysosomal compartments in leupeptin-treated cells to endosomal compartments. We have further analysed the distribution of Ii fragments in late endosomes labelled with anti-MPR antibody
When cells are treated with cyto D alone, invariant chain is scarcely accumulated in late MPR-positive endosomes (Fig. 6A,D). In cells treated with leupeptin alone, the distribution of Ii was also partially coincident in MPR-containing compartments (Fig. 6B,E). However in cells treated with both leupeptin and cyto D, numerous Ii-positive vesicles contain MPR, indicating that the invariant chain was accumulated in late as well as in early endosomal compartments under these circumstances. These experiments show that effects of the two drugs are cumulative: leupeptin inhibits proteases involved in Ii degradation at different stages in the endocytic pathway and cyto D blocks the access of the invariant chain to lysosomal proteases, such as cathepsin S involved in the generation of class II/CLIP complexes (Riese et al., 1996).

Surface immunoglobulins can internalise and target their ligand to endosomal and lysosomal compartments (Liu et al., 1994; Mitchell et al., 1995). Since actin microfilaments are also involved in endocytosis of ligands (Gottlieb et al., 1993; Durrbach et al., 1996), we studied the effect of actin disruption on internalisation of immunoglobulins by immunofluorescence and FACS analysis. The cells were pre-incubated or not for 1 hour with cyto D before BCR cross-linking for 30 minutes. In untreated cells, 50% of cross-linked surface immunoglobulins were internalised and localised in large intracellular vesicles (Fig. 7A,C). In cyto D-treated cells, the surface labelled immunoglobulins were not internalised but patched at the cell surface (Fig. 7B,D). This result shows that actin microfilaments also participate in the internalisation of cross-linked BCR and consequently in the uptake of B cell-specific antigens. The actin cytoskeleton plays multiple roles in the transport of MHC class II molecules, and in the uptake of antigens recognised by the B cell receptor.

**Fig. 4.** Cyto D treatment accumulates in cells p12 Ii fragments but not p10 Ii fragments. (A) Cyto D increases the association of p12 fragments with αβ dimers. (B) Degradation of p12 fragments into p10 fragments is blocked by cyto D. Cells were treated for different periods of times with 10 μg/ml cyto D. Then, 50 μg of proteins from lysates were boiled before being resolved by SDS-PAGE or class II molecules were immunoprecipitated before being charged on the gel. Proteins were transferred on immobilon-P membrane and blotted with αCyt.Ii antibody. Revelation was performed with the ECL detection kit.

**Fig. 5.** Cyto D treatment blocks the degradation and the transport of MHC class II-associated invariant chains in the endosomal pathway. (A) Cells treated with leupeptin accumulate p10 and p12 Ii fragments. (D) Cells treated with leupeptin and cyto D only accumulate p12 fragments. Cyto D treatment also increases p21 and p31 Ii forms. Cells were treated for different periods of times with 500 μM leupeptin or with both 500 μM leupeptin and 10 μg/ml cyto D. Then, 50 μg of proteins from lysates were boiled before being resolved by SDS-PAGE. Proteins were transferred on immobilon-P membrane and blotted with αCyt.Ii antibody. Revelation was performed with the ECL detection kit. (B,C) Leupeptin treatment induces the accumulation of Ii (in red) in compartments mostly coincident with H-2M positive compartments (B, in green) producing double-labelled vesicular structures (yellow, arrowheads) but not in the transferrin-positive compartment (C, in green). (E,F) Cells treated with cyto D and leupeptin, invariant chain (in red) has a distinct localisation with H-2M positive compartment (E, in green) but is localised with internalised transferrin (F, in green). Cells treated with 500 μM leupeptin or with 500 μM leupeptin plus 10 μg/ml cyto D for 4 hours were fixed, permeabilised and double-labelled for the invariant chain with the biotinylated αCyt.Ii antibody (in red) and for either H-2M molecules with αCyt.Mβ antibody (B and E, in green) or internalised transferrin (C and F, in green). Arrowheads show the colocalisation. Bars, 10 μm.

**DISCUSSION**

We showed recently that B cell activation controls the antigen presentation function by acting on the maturation pathway of newly synthesised MHC class II/IIi complexes (Barois et al., 1997). In the present report, we have analysed the role of the actin cytoskeleton on the MHC class II antigen presentation pathway. The treatment of B cells with cyto D, which blocks actin polymerisation, reduced here the degradation of the
**invariant chain**, as in activated B cells. The p12 and p21 Ii fragments were associated for a longer period of time with newly synthesised MHC class II \(ab\) dimers in cyto D-treated cells than in untreated cells. The accumulation of invariant chain fragments associated with class II molecules in presence of this drug also explains the 2-hour delay found in the appearance of class II SDS-stable forms. The effect of cyto D was reversible since the drug had to be present during the whole experiment and the SDS-stable forms of class II molecules reappeared rapidly after a chase in control medium (data not shown). \(^{35}\)S metabolic labelling also showed that p12 and p21 Ii fragments remained associated with \(\alpha\beta\) dimers for a longer time in cyto D-treated cells than in untreated cells. Actin depolymerisation and B cell activation lead to a similar delay in the formation of mature MHC class II molecules and we have further analysed the function and the intracellular distribution of class II molecules in cyto D-treated B cells.

**Disorganisation of actin microfilaments modifies the transport of newly synthesised class II molecules**

The internalisation of endocytic markers was recently shown to be affected by the disruption of the actin cytoskeleton (Gottlieb et al., 1993; Durrbach et al., 1996). In B cells, surface immunoglobulins internalise antigens into the endosomal and lysosomal compartments for class II presentation (Liu et al., 1994; Mitchell et al., 1995). We show here that disruption of actin microfilaments blocks the internalisation of surface immunoglobulins cross-linked with divalent donkey anti-mouse antibody uniquely bound on the BCR (Fig. 7). The actin cytoskeleton is therefore involved in antigen uptake and the effect of cyto D treatment on antigen internalisation should not be confused with its effect on the transport and the maturation of MHC class II molecules. To avoid an effect of cyto D treatment on the uptake of antigens via fluid phase in antigen presentation assays, we incubated the cells with antigens for 2 hours before drug treatment. We observed a selective inhibitory effect of cyto
D treatment on the conventional presentation pathway of HEL to the 3A9 hybridoma without alteration of the alternative presentation pathway of RNase A to the TS12 hybridoma. These results support the idea that actin microfilaments influence the antigen presentation pathway in compartments that are only accessible to newly synthesised class II molecules, and not to recycled αβ dimers. At high concentrations of antigen, cyto D treatment did not alter the presentation of the HEL 46-61 determinant. The classical pathway uses newly synthesised MHC class II molecules and is dependent on invariant chain expression, protein synthesis and H-2M molecules (Adorini et al., 1990; St-Pierre and Watts, 1990; Nadimi et al., 1991; Soreng et al., 1994; Griffin et al., 1997). The alternative pathway uses αβ dimers, which are expressed at the cell surface and recycled in endosomes (Harding et al., 1989; Salamero et al., 1990; Pinet et al., 1995). This pathway is independent of invariant chain and H-2M molecules (Griffin et al., 1997). Recycled class II molecules are thought to bind peptides generated in early endosomes while newly synthesised class II molecules can bind peptides generated in late endosomes after disruption of disulfide bonds (Griffin et al., 1997). Cyto D treatment delayed invariant chain degradation and the appearance of αβ SDS-stable dimers. Altogether these results indicate that the dynamics of actin microfilaments are important for optimal peptide loading of newly synthesised MHC class II molecules.

As recently shown, actin microfilaments are involved in the transport from late endosomes to lysosomes in hepatoma cells (Durrbach et al., 1996). In agreement with this, cyto D treatment induced here the accumulation of class II/ii fragment complexes in early and late intracellular compartments, identified by internalised transferrin and endogenous MPR, respectively (Figs 4 and 5). This accumulation was not observed in the presence of protein synthesis inhibitors (data not shown). The fact that II degradation was partially inhibited could be due to a reduced access of newly formed MHC class II/ii complexes to lysosomal proteases in absence of intact actin microfilaments.

**Cytochalasin D blocks the transport of the class II/p12 complexes to lysosomes**

Leupeptin, which inhibits cysteine proteases (Nguyen et al., 1989), has been extensively used to inhibit the degradation of invariant chain, and to study the intracellular transport of MHC class II molecules (Neefjes and Ploegh, 1992; Brachet et al., 1997). In cells treated with leupeptin alone, p10 and p12 II fragments are accumulated in lysosomal compartments containing H-2M molecules (Brachet et al., 1997; Fig. 5B). In cells treated with leupeptin plus cyto D, II fragments were accumulated in compartments distinct from the lysosomes but coincident with internalised transferrin and endogenous MPR (Figs 5 and 6). Furthermore, the degradation of the CLIP containing p12 II fragment into the CLIP minus p10 II fragment was slowed down, suggesting that this maturation step of class II/ii complexes depends on the integrity of the actin cytoskeleton. Our results indicate that cyto D blocks the meeting point between formed MHC class II/ii complexes and lysosomal proteases involved in the formation of class II/CLIP complexes and H-2M molecules, which normally catalyse the exchange of CLIP with antigen peptides. Cyto D could affect either the transport of the lysosomal proteins to MHC class II-rich compartments or the transport of the class II/p12 complexes to lysosomal compartments. The schematic diagram in Fig. 8 summarises the results from the colocalisation experiments between II and transferrin, MPR or lysosomal markers performed in B cells treated with cyto D and/or leupeptin. The most probable hypothesis is that actin microfilaments allow the transport of the MHC class II molecules to a subset of lysosomal compartments. This is compatible with numerous reports suggesting that the peptide loading process of αβ dimers can occur in a pre-lysosomal compartment containing HLA-DM molecules (Qi et al., 1994; West et al., 1994; Tulp et al., 1994; Sanderson et al., 1994; Fernandez-Borja et al., 1996). The accumulation of class II/p12 and class II/p21 complexes results from an inhibition of invariant chain degradation and this can be correlated with an inhibition of transport between late endosomes and lysosomes as previously shown in hepatoma cells (Gottlieb et al., 1993; Durrbach et al., 1996).

Actin has a high dynamics of assembly and disassembly (MacLean-Fletcher and Pollard, 1980). Here, we report that an alteration of this dynamic equilibrium by cyto D treatment modifies the internalisation of BCR, the transport, and the maturation of newly synthesised MHC class II molecules and their access to lysosomal proteases. In a previous report, we showed that cellular activation through the BCR controls the transport of MHC class II molecules in a similar way (Barois et al., 1997). Moreover, engagement of the BCR modifies the dynamics of actin microfilaments by increasing the state of actin polymerisation (Melamed et al., 1991). We think that upon activation, antigen-presenting cells could modulate their antigen presentation function by acting on MHC class II transport and BCR-mediated antigen uptake, through a reorganisation of the actin cytoskeleton. The regulation of antigen presentation pathways by the dynamics of actin microfilaments could also be of importance in another APC type such as dendritic cells, in which inflammatory cytokines induce a drastic reorganisation of the actin cytoskeleton (Winzler et al., 1997), in parallel with a redistribution of MHC class II molecules to the cell surface, a loss of antigen uptake ability and the acquisition of migratory phenotype (Winzler et al., 1997; Cella et al., 1997; Pierre et al., 1997).
REFERENCES


