Selective Presentation of the Major Histocompatibility Complex Class II Antigen Presentation Pathway following B Cell Receptor Ligation and Protein Kinase C Activation*

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We noticed that B cell receptor ligation or phorbol 12-myristate 13-acetate treatment induced intracellular vesicles containing major histocompatibility complex (MHC) class II and invariant chain (Ii), and increased the amount of transmembrane p12 Ii fragments coinm noprecipitated with class II molecules. To determine the influence of protein kinase C activation on the MHC class II presentation pathway, we analyzed the subcellular distribution of Ii, the induction of SDS-stable forms of class II molecules, and their ability to present different antigens. Ii chains visualized with luminal and cytoplasmic directed antibodies appeared in early endosomal compartments accessible to transferrin in response to phorbol 12-myristate 13-acetate treatment, whereas transmembrane Ii degradation products equivalent to the p12 Ii fragments were colocalized with the B cell receptors internalized after cross-linking. Protein kinase C activation delayed in parallel the formation of SDS-stable forms of class II molecules and reduced the presentation of antigenic determinants requiring newly synthesized class II αβ-II complexes. These data indicate that B cell activation affects Ii processing and MHC class II peptide loading in endosomal compartments intersecting the biosynthetic pathway.

MHC* class II molecules bind in their groove antigenic peptides generally derived from endocytosed proteins for their presentation to specific CD4+ T lymphocytes (reviewed in Refs. 1–3). Shortly after their biosynthesis, the MHC class II α and β chains assemble with Ii through a sequence from amino acid 81 to 104 of Ii called CLIP for class II associated Ii peptide (4–6), and form (αβ)2 Ii3 nonameric structures (7, 8). Ii prevents the association of antigenic peptides with class II αβ heterodimers (9–11) through CLIP peptide (5), which occupies the peptide-binding groove in crystallized MHC class II molecules (12). The trimerization of Ii cytoplasmic domains, containing two dileucine motives each, drives the targeting of newly synthesized class II molecules to specialized endosomal compartments (13–15), which were further characterized as compartments of antigen processing and of peptide loading (16–19). The amount of class II molecules located in these intracellular compartments can differ to a considerable extent, depending on the cell type (20–23), and proteolytic cleavage of Ii occurs at this stage as indicated by ultrastructural observations (22). Upon removal of CLIP from class II molecules catalyzed by HLA-DM or its murine equivalent H2-M class II molecules (24–26), peptide-loaded MHC class II complexes are exported to the cell surface by a poorly defined pathway. Recycling of class II molecules, from the plasma membrane through endosomes, has also been observed in B cells (27–29), allowing binding and presentation of different antigens to T cells (27, 30). MHC class II molecules can therefore gain access to the antigen-processing compartments by at least two different routes, a direct targeting of newly synthesized αβ-II complexes and an indirect targeting of resident αβ heterodimers. These two pathways of antigen presentation coexist in B cells. One is sensitive to protein synthesis and membrane transport inhibitors (31–33), and requires the expression of Ii (34). The other one requires preexisting αβ class II heterodimers with intact cytoplasmic domains (30).

In B lymphocytes, the most efficient pathway of antigen uptake is mediated by the BCR (reviewed in Ref. 35). Upon BCR ligation, antigens are internalized independently of a phosphorylation of the immunoreceptor tyrosine-based activation motives present on the cytoplasmic tail of the coreceptors Igα and Igβ molecules (36). Ligation of the BCR transduces activation signals, through the Igα and Igβ coreceptors, leading to a cascade of protein tyrosine kinase activation (reviewed in Refs. 37 and 38) and to the production of second messengers, such as inositol triphosphate, and PKC activators, such as diacylglycerol (39). Several routes dependent on protein tyrosine kinase and PKC are then converging at the level of the activation of the mitogen-activated protein kinase pathway (40).

Since the BCR delivers signals leading to PKC activation (37, 41), it was of interest to analyze the effects of BCR cross-linking and of direct PKC activation, through phorbol ester, on the biosynthetic transport and the function of MHC class II molecules. Our results show that different mechanisms of PKC activation decreased the endosomal degradation of MHC class II associated Ii chains and induced an intracellular accumulation of p12 Ii protein fragments previously identified in MHC class II-Ii transfectants (20) and equivalent to the p10 Ii protein fragment SLIP (23, 42). This is correlated with a selective modulation of antigen presentation, and a regulation of vesicular traffic as shown for the uptake of transferrin (43).
FIG. 1. Subcellular distribution of class II molecules (A, C, and E) and II (B, D, and F). The F6 B lymphoma cells were double labeled with aCyt.IAβ Ab and biotinylated aCyt.II Ab, in control condition (A and B), following BCR cross-linking (C and D) or following PMA treatment (E and F). Arrows indicate the sites of colocalization. Field of 50 × 35 μm.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-II rabbit polyclonal Abs were raised against synthetic peptides of the cytoplasmic and CLIP domain of the mouse II (aCyt.II and aCLIP). Anti-MHC class II rabbit polyclonal Ab was raised against cytoplasmic domains of the mouse I-β molecule (aCyt.IAβ). The corresponding amino acid sequences of the peptides used for immunization were: M^3DQDQLISNHEQLPILGNRPREPES- RCKSY and YR^3MKLPSKAKPV SQMRMATFLLMRFPSMDMMLG^229 and YH^222RSQKPGRPAPPAGILG^229 for the aCyt.II, aCLIP, and aCyt. IAβ respectively. Peptides were coupled to keyhole limpet hemocyanin as a protein carrier with the cross-linker bisdiazobenzidine. The biochemical characterization of aCLIP and aCyt.IAβ Abs was previously performed (44). The rabbit antiserum aCyt.II was conjugated to the NHS-LC-biotin (Pierce) for double staining with other rabbit anti-serum. The mouse hybridoma 10.2.16 producing an I-Ak-specific mAb was performed (44). The rabbit antiserum used for immunization were: M1DDQRDLISNHEQLPILGNRPREPES- RCKSY31,YR77MKLPKSAKPVSQMRMATPLLMRPMSMDNMG109, and F7H222RSQKPGRPAPPAGILG229 for the Ii, CLIP, and IIpeptides, respectively. Peptides were coupled to keyhole limpet hemocyanin as a protein carrier with the cross-linker bisdiazobenzidine. The biochemical characterization of aCLIP and aCyt.IAβ Abs was previously performed (44). The rabbit antiserum aCyt.II was conjugated to the NHS-LC-biotin (Pierce) for double staining with other rabbit anti-serum. The mouse hybridoma 10.2.16 producing an I-Ak-specific mAb was obtained from the American Type Culture Collection (Rockville, MD). The rabbit antiserum against luminal domain of human II (aLum.II), the mouse anti-IgG110-B mAb GL2A7 (45), and the mouse anti-Golgi apparatus mAb CTR433 (46) were kindly provided by Drs. Salamero, Amigorena, and Bornens (Curie Institute, Paris) respectively. The secondary reagents (donkey anti-mouse IgGs, anti-rabbit IgGs, and streptavidin) coupled to FITC, Texas Red, or Cyanine 5 suitable for multiple labeling experiments and unlabeled donkey anti-mouse IgGs to cross-link the BCR were purchased from Jackson Immunoresearch (West Grove, PA). Human transferrin (Sigma) was conjugated to FITC and purified on G25 column. PMA and leupeptin were from Sigma and ICN respectively.

Cells—The F6 B cell lymphoma was derived from the M12C3 B cell line, I-A negative, transfected for expression of I-Ak molecules (47) and were solubilized in 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 33% glycerol, 2% SDS, 5% iodoacetamide, and 1 μg/ml leupeptin, pepstatin, and aprotinin). Half of the lysate was directly charged on 15% SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred onto Immobilon-P membrane (Millipore). II and Ii fragments containing intact cytoplasmic tail were detected with the aCyt.II Ab. The second half of the lysate was incubated for 2 h with 10.2.16 mAb bound to the protein A-Sepharose beads (Pharmacia Biotech Inc.). Then immunoprecipitates were washed, and pellets were suspended in reducing sample buffer (10 mM Tris-HCl, 2 mM EDTA, 33% glycerol, 2% SDS, 5% β-mercaptoethanol). Immunoprecipitates were boiled in SDS and run on 15% SDS-PAGE. After transferring onto membrane, proteins were blotted with aLum.II, aCyt.II, or aCLIP Abs. After washing, membranes were incubated with anti-rabbit Ab conjugated to horseradish peroxidase (Jack- son Immunoresearch). Labeled proteins were detected using the ECL immunodetection kit (Amersham Corp.).

35S Metabolic Labeling—F6 cells were either untreated or treated with 100 μg/ml leupeptin, with 50 ng/ml PMA, or with 10 μg/ml donkey anti-Ig Ab to cross-link the BCR for 1 h at 37 °C. Cells were washed in ice-cold PBS and solubilized in 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 33% glycerol, 2% SDS, 5% iodoacetamide, and 1 μg/ml leupeptin, pepstatin, and aprotinin). Half of the lysate was directly charged on 15% SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred onto Immobilon-P membrane (Millipore). II and Ii fragments containing intact cytoplasmic tail were detected with the aCyt.II Ab. The second half of the lysate was incubated for 2 h with 10.2.16 mAb bound to the protein A-Sepharose beads (Pharmacia Biotech Inc.). Then immunoprecipitates were washed, and pellets were suspended in reducing sample buffer (10 mM Tris-HCl, 2 mM EDTA, 33% glycerol, 2% SDS, 5% β-mercaptoethanol). Immunoprecipitates were boiled in SDS and run on 15% SDS-PAGE. After transferring onto membrane, proteins were blotted with aLum.II, aCyt.II, or aCLIP Abs. After washing, membranes were incubated with anti-rabbit Ab conjugated to horseradish peroxidase (Jackson Immunoresearch). Labeled proteins were detected using the ECL immunodetection kit (Amersham Corp.).

35S Metabolic Labeling—F6 cells were washed twice and incubated for 45 min at 37 °C in cysteine/methionine-free RPMI medium (Life Technologies, Inc.). In 3 ml of this medium containing 5% dialyzed FCS, cells were incubated for 30 min at 37 °C with 0.6 μCi of [35S]cysteine/methionine in the presence or in the absence of 50 ng/ml PMA. When indicated, pulse-labeled cells were chased for different periods at 37 °C.
in RPMI medium containing cold cysteine and methionine with or without PMA. Cells were washed with ice-cold PBS and solubilized in lysis buffer previously described. Lysates were precleared with protein A-Sepharose beads, and the supernatants were mixed with a Cyt.Ii Ab or 10.2.16 mAb previously bound to protein A-Sepharose beads. Immunoprecipitates were extensively washed and pellets were resuspended in reducing sample buffer. To detect SDS-stable dimers, immunoprecipitates were left at room temperature 1 h before being resolved on 15% acrylamide SDS-PAGE. Radiolabeled proteins were intensified and revealed by autoradiography.

Antigen Presentation—F6 and CH27 antigen-presenting cells (APC) were untreated or treated with 50 ng/ml PMA for 1 h then washed. Cells were pulsed with various doses of HEL and RNase A for 2 h. After extensive washing, 5-10^4 APCs were cocultured for 24 h at 37 °C with 10^5 3A9 or 10^5 TS12 T cell hybridomas. Interleukin-2 production in culture supernatants was measured using thiazolyl blue 3-(4,5-dimethyl thiazol-2-xy)-2,5-diphenyl tetrazolium bromide (Sigma) to evaluate the growth of the interleukin-2-dependent CTLL-2 cell line.

RESULTS

Intracellular Distribution of MHC Class II and Ii Molecules—To determine whether B cell activation influenced the accumulation of Ii and of class II molecules into specialized intracellular compartments, we used confocal microscopy and immunofluorescence labeling of I-A^b and Ii molecules in F6 B lymphoma cells. In untreated cells, MHC class II molecules labeled with a Cyt.IA^b polyclonal Ab were localized mainly at the cell surface (Fig. 1A). Ii chain visualized with a Cyt.Ii polyclonal Ab gave mostly a reticulated intracellular staining consistent with the endoplasmic reticulum (Fig. 1B). After cross-linking of the surface Igs, MHC class II molecules appeared in intracellular vesicles (Fig. 1C, arrows) colocalized with Ii (Fig. 1D, arrows). A similar colocalization of MHC class II and Ii was found in PMA-treated cells (Fig. 1, E and F, arrows).

The presence in class II-rich compartments of Ii proteins and Ii degradation fragments was reported in human B cell lines (22) and in murine B lymphoma cells treated with the leupeptin protease inhibitor (23). To visualize the subcellular distribution and the processing of Ii molecules in PMA (Fig. 2B) and in leupeptin (Fig. 2A)-treated cells, we double labeled the cells with rabbit aCyt.Ii and aLum.Ii Abs. A set of vesicles was found to contain colocalized luminal and cytoplasmic Ii epitopes, likely from full length Ii proteins (Fig. 2, A and B, yellow vesicles), while many peripheral vesicles were labeled with the aCyt.Ii Ab only (Fig. 2, A and B, red vesicles). These
vesicles contain only short Ii fragments lacking their luminal domains. Since distinct vesicular compartments contained different forms of Ii, it was of importance to localize Ii fragments with respect to Golgi, lysosomal, and endosomal markers in PMA-treated cells. The mouse Golgi CTR433 mAb which recognizes a marker present in the Golgi complex (46), was partially colocalized with the αLum.Ii (Fig. 2C, yellow vesicles) and weakly colocalized with αCyt.Ii labeling (Fig. 2D). Some vesicles positive for the αLum.Ii Ab and many peripheral vesicles, containing Ii degradation products recognized by the αCyt.Ii Ab, were not coincident with this Golgi marker (Fig. 2D). Next, to identify the lysosomal compartments, we used a mAb directed against the lgp110-B marker (45) and red vesicles. Some vesicles positive for the αLum.Ii Ab and many peripheral vesicles, containing Ii degradation products recognized by the αCyt.Ii Ab, were not coincident with this Golgi marker (Fig. 2D). Since antigens bound to the BCR are transported to peptide-loading compartments for processing and presentation by the MHC class II molecules (35), it was of importance to determine whether the BCR could reach vesicles containing the different forms of Ii. Internalization of the BCR was triggered by cross-linking with anti-Ig Ab in PMA-treated cells. The anti-Ig Ab internalized for 0.5 h were highly colocalized with peripheral vesicles containing cytoplasmic Ii fragments (Fig. 2F).

To further define the intracellular localization of Ii degradation products with respect to the early endosomes in activated cells, FITC-coupled transferrin was internalized through its receptor during PMA treatment. The cells were then fixed and processed for double immunofluorescence staining with the αLum.Ii and αCyt.Ii Abs. We found a limited level of colocalization between the early endosomes loaded with FITC-transferrin and the Ii positive vesicles labeled with the αCyt.Ii Ab (Fig. 2G, yellow vesicles). To determine whether the Ii-positive early endosomes contained also the luminal Ii epitope, we used an image processing program to surround FITC-transferrin-positive structures above a fluorescence value of 10% with black contours. These contours were superimposed on the double fluorescence image obtained with αLum.Ii and αCyt.Ii Abs (Fig. 2H). This representation shows triple positive vesicles as double labeled objects surrounded with black contours. Some early endosomes contain the two Ii epitopes (Fig. 2H, yellow vesicles with black contours), while only a few contain short Ii cytoplasmic fragments (Fig. 2H, red vesicles with black contours). We think that newly synthesized MHC class II-Ii complexes bearing unprocessed Ii chains gain access to early endosomal compartments. PKC activation also generates Ii fragments that are colocalized with internalized surface Igs in later elements of the endosomal pathway (Fig. 2F).

Generation of Ii Fragments and Induction of Class II Compact Forms—The cytoplasmic Ii fragments identified in endosomal compartments after PKC activation are probably equivalent to cytoplasmic derived peptide-loading compartment Ii fragments associated with intracellular class II molecules (23, 42) and to the p12 Ii fragments identified in αβ-II-transfected fibroblasts (20). To test this hypothesis, we performed Western blotting with αCyt.Ii, αLum.Ii, and αCLIP antisera in total cell lysates from leupeptin (100 μg/ml) and PMA (50 ng/ml)-treated cells (Fig. 3A, lanes L and P, respectively). The αCyt.Ii polyclonal Ab recognized the p10, p12, p31, and to a lesser extent the p41 forms of Ii in the total cell lysate. After leupeptin or PMA treatment, there was no change in the amount of p10 and p31 Ii forms; however, a slight increase in the p12 Ii form was detected in PMA treated cells (Fig. 3A). Since Ii is produced in excess compared to class II molecules, we identified also I-Aβ-associated Ii proteins on Western blots after immunoprecipitation with the nonconformational 10.2.16 anti-I-Aβ mAb (Fig. 3B). Compared to the controls, no change occurred for p31 and p41 Ii forms associated with class II molecules; however, PMA and leupeptin treatments increased the class II-associated p12 Ii fragments, revealed with rabbit αCyt.Ii and αCLIP Abs (Fig. 3B, lanes L and P). The cytoplasmic derived p10 Ii fragments identified in the total cell lysates do not bind to class II molecules (Fig. 3, A and B). In another set of experiments, we compared the effect of PMA treatment and BCR ligation (Fig. 4, lanes P and B) on the pattern of MHC class II-associated Ii fragments revealed with the αCyt.Ii Ab (Fig. 4A) and the αCLIP Ab (Fig. 4B). Both PKC activation pathways increased the level of association of p12 Ii fragments with class II molecules (Fig. 4, A and B).

To test whether PKC activation modified Ii turnover, we performed a 35S pulse-chase labeling followed by SDS-PAGE analysis of Ii products immunoprecipitated with the αCyt.Ii Ab. In untreated cells, Ii was rapidly degraded and a p12 Ii fragment appeared after 30 min of 35S pulse (Fig. 5A) analog to SLIP. Faint p10 Ii fragments were detected after 2 h of chase, and most of the p31 and p41 Ii forms were degraded within 3 h. In cells treated with PMA, degradation of Ii was consistently reduced with more p12 fragment at 2 h and more p31 form at 3 h of chase compared with the untreated cells (Fig. 5A). Rather than increasing the biosynthesis of Ii, PMA reduced the rate of Ii turnover. This could affect peptide loading on newly synthesized αβ class II dimers as in leupeptin-treated cells (52). To analyze the kinetics of antigen binding we monitored the resistance of class II complexes to denaturation by SDS detergent at 20 °C, reflecting the presence of peptide loaded class II αβ.
The I-Ak-restricted presentation of HEL to the HEL 46–61 hybridoma requires different pathways of antigen processing. Minimizing the effects of PKC activation on the MHC class II antigen processing for their presentation to helper T cells. Using antigenic determinants are thought to use different pathways of processing for their presentation to helper T cells. Using anti-peptide Abs raised against the cytoplasmic N-terminal and the luminal C-terminal portion of Ii, we found by immunofluorescence microscopy a reticulated cytoplasmic staining in untreated cells and a vesicular pattern in cells activated by BCR ligation and PMA treatment. Most of the Ii-positive vesicles were labeled with anti-MHC class II Ab and probably contain newly synthesized MHC-Ii complexes since

**FIG. 5.** Time course of Ii degradation and of MHC class II SDS stable forms production. The F6 cells were pulsed with [35S]Met and Cys and chased for 0–4 h in the absence or in presence of PMA. The cells were lysed and immunoprecipitated with rabbit anti-I-Ak mAb (A) or with mouse anti-I-Ak mAb (B). Immunoprecipitates were resolved on SDS-PAGE. ai, am, βi, βm, indicate the position of the immature and mature α and β chains; CF, compact forms stable in SDS at 20 °C.

In order to assess the function of newly synthesized and of resident class II molecules depending on PKC activation, we delivered the antigen by uptake from the fluid phase for 2 h in F6 B lymphoma cells (Fig. 6, A and B), and in the nonadherent CH27 B lymphoma cells for the sake of comparison (Fig. 6, C and D). At low doses of antigen, the presentation of HEL to the I-Ak-restricted 3A9 T cell hybridoma was reduced by about 10-fold when the APCs were pretreated for 1 h with 50 ng/ml PMA (Fig. 6, A and C). The presentation of HEL became insensitive to PMA at high concentration of antigen. In contrast to HEL, the efficiency of the presentation of RNase A to the I-Ak-restricted TS12 specific T cell hybridoma was not affected by pretreatment with PMA for 1 h in F6 and in CH27 B cell lines (Fig. 6, B and D). PKC activation altered selectively the presentation pathway of the HEL-derived 46–61 peptides, which are thought to meet newly synthesized MHC class II molecules after processing of the protein.

**FIG. 6.** Effect of PMA on antigenic presentation. F6 (A and B) and CH27 (C and D) B lymphoma cells untreated (open circles) or treated (closed circles) with PMA were used as APCs to present HEL to the 3A9 T cell hybridoma (A and C) and RNase A to the TS12 T cell hybridoma (B and D). Interleukin-2 production was determined using the growth of the interleukin-2-dependent cell line CTLL with an 3-(4,5-dimethyl thiazol-2-xy)-2,5-diphenyl tetrazolium bromide assay.

**DISCUSSION**

The ligation of activation receptors such as surface Igs and the direct activation of PKC generate a cascade of phosphorylation events in B cells (37–40) which influences protein-protein interactions in the cytoplasm, activates gene transcription and modifies the cell morphology. Our goal was to investigate whether B cell activation influences the MHC class II presentation pathway. Initial experiments showed that surface Ig cross-linking and phorbol ester treatment can induce the appearance of MHC class II/iii-containing intracellular vesicles in murine B lymphoma cells. These observations led us to evaluate the effects of PKC activation on Ii processing and transport, MHC class II peptide loading, and antigen presentation.

**Redistribution of Ii Protein Fragments in PMA-treated B Cells—**Using anti-peptide Abs raised against the cytoplasmic N-terminal and the luminal C-terminal portion of Ii, we found by immunofluorescence microscopy a reticulated cytoplasmic staining in untreated cells and a vesicular pattern in cells activated by BCR ligation and PMA treatment. Most of the Ii-positive vesicles were labeled with anti-MHC class II Ab and probably contain newly synthesized MHC-Ii complexes since
they appeared after 30 min and were susceptible to protein synthesis inhibitors.\(^2\) The Ii-positive vesicles were further divided into two subsets according to the stage of Ii processing. One subset contained colocalized luminal and cytoplasmic Ii epitopes and the other contained only the cytoplasmic Ii epitope. Short-term treatment with the leupeptin serine protease inhibitor induced a similar redistribution of Ii proteins and Ii fragments (Fig. 2, A and B).

Most of the vesicular compartments containing the luminal Ii epitope were also labeled with the CTR433 mAb, which is characterized at the ultrastructural level as a marker of the mid-Golgi compartment (46). However, many vesicles containing only transmembrane Ii fragments were not labeled with this Golgi marker and all of them were distinct from the lysosomes identified with the lgp110-B marker (45). Ii-positive vesicles were also distinct from the H2-M molecules present in the lysosomes (54), and from the late endosomes\(^2\) defined by the anti-Rab7 mAb and by the presence of the cation-independent mannose 6 phosphate receptor (55). The absence of colocalization with lysosomal and late endosomal markers indicates that Ii cytoplasmic fragments are present in vesicles equivalent to endosomal compartments defined by subcellular fractionation in murine B cells (16, 17). Using multiple immunofluorescence and confocal microscopy, we found that PKC activation induces the redistribution of intact Ii from the endoplasmic reticulum to the Golgi complex and to other elements of the endosomal pathway. After cross-linking and internalization of the BCR, numerous endocytic vesicles defined by their Ig content were labeled with the oCyt.Ii Ab.

To determine whether the vesicles in which MHC class II-Ii complexes are delivered correspond to early endosomes, we performed a double immunofluorescence staining with a Lumin.Ii and oCyt.Ii Abs following internalization of FITC-transferrin (Fig. 2, G and H). Some early endosomes labeled with transferrin contain no Ii molecules, whereas other ones are labeled with both luminal and cytoplasmic Ii-directed Abs. Intact Ii chains are probably reaching the early endosomal compartments first, whereas later elements of endosomal pathway are loaded with Ii fragments partially colocalized with internalized Igs. This scheme is compatible with the steady state distribution of Ii degradation products found in distinct compartments in human B cell lines at the ultrastructural level (22). Our results are also in agreement with recent analyses of subcellular fractions derived from leupeptin-treated cells (23) and with subcellular fractionation experiments performed in murine B lymphoma cells (56), since class II molecules were colocalized with Ii proteins and Ii fragments after BCR engagement or after PMA treatment. In conclusion, PKC activation triggers the accumulation of intact Ii presumably associated with newly synthesized MHC class II molecules in vesicular structures accessible to transferrin uptake. Later elements of the endosomal pathway, in which surface Igs are internalized, contain apparently many Ii fragments, suggesting an initial targeting of \(\alpha\beta\)-Ii complexes to transferrin positive compartments as reported in leupeptin treated cells (23, 56).

**Peptide Loading of MHC Class II Molecules**—Looking at the biosynthetic pathway of MHC class II transport, we report here that PKC activation reduces Ii turnover and delays the induction of class II SDS stable forms. PMA treatment also triggers the accumulation of CLIP containing p12 Ii fragments previously identified in fibroblast transfectants (20) and analogously to p10 cytoplasmic derived fragments obtained in leupeptin-treated B cells (23, 42). The p12 Ii fragments contain in their cytoplasmic portion two dileucine based targeting motives able to direct Ii chain and Ii chimeric constructs to endosomal compartments (13, 14). The fact that Ii proteins and p12 Ii fragments remain associated with \(\alpha\beta\) heterodimers for a longer time in PMA treated cells provides an explanation for the intracellular retention of \(\alpha\beta\)-Ii complexes and for the delay in the induction of SDS stable forms. Moreover, intracellular Ii and MHC class II molecules remained highly colocalized in PKC activated cells, indicating that the CLIP portion of p12 Ii fragments presumably lies in the groove of newly synthesized MHC class II molecules and impairs peptide loading in the endosomes.

To identify whether PKC activation influences the antigen presentation capacity of B cells in relation to the biochemical perturbation of Ii processing, we have analyzed the presentation efficiency of Ii-dependent and Ii-independent epitopes. In the B cell populations selected here, newly synthesized class II molecules are required to present HEL to the HEL 46–61 specific I-A\(^b\) restricted, 3A9 T cell hybridoma (31, 32), and this antigen presentation pathway is critically dependent on Ii expression in B cells (34). We showed here that this presentation event is partially impaired by PMA treatment. However, we found no correlation with a reduction in class II surface expression analyzed by flow cytometry and no correlation with a reduction in the presentation of the HEL 46–61 peptide.\(^3\) The MHC class II recycling pathway of antigen presentation was resistant to protein synthesis inhibitors here. This pathway does not require Ii (34) but requires the integrity of MHC class II cytoplasmic domains (30). As an example of this class II recycling pathway, we analyzed the presentation of RNase A to the RNase A 43–56 specific I-A\(^b\) restricted, TS12 T cell hybridoma (31, 32) and observed no influence of PKC activation, indicating in addition that reorganization of the plasma membrane is not responsible for the inhibition of HEL presentation to the 3A9 T cell hybridoma. From our results, we think that the biosynthetic pathway of class II presentation requires a rapid degradation of Ii fragments to be fully functional. In splenic B cells derived from mice lacking H2-M molecules, a similar defect occurs for the presentation of antigenic determinants requiring both Ii expression and MHC class II synthesis (44). In the absence of H2-M which catalyze the exchange of CLIP peptides, Ii processing is normal but MHC class II molecules present at the cell surface are massively loaded with CLIP and do not reach their final SDS stable forms (44). In PKC activated cells in which Ii processing is partially inhibited, we found a higher degree of intracellular retention of MHC class II and Ii molecules. This is probably due to the fact that dileucine motives present in multiple copies in the \((\alpha\beta)_2\)-Ii and the \((\alpha\beta)_2\)-I(p12Ii), nonamers can retain the complexes in endosomal compartments (13–15).

The molecular mechanism leading to the reduction of Ii degradation and Ii-dependent antigen presentation after PMA treatment and BCR ligation remains, however, unclear. We looked for a PMA-induced phosphorylation of MHC class II and Ii, but we had no evidence in favor of this hypothesis. As shown previously in human B cells (57), Ii chains can be phosphorylated on serine residues lying in the cytoplasmic domain, and this event may regulate Ii-dependent antigen presentation. We have detected a basal level of \(^{32}\)P incorporation in p31 and p12 forms of Ii immunoprecipitated with oCyt.Ii Ab, but found no modification of Ii and MHC class II phosphorylation after PMA treatment.\(^5\) We cannot exclude that PMA induced another type of post-translational modification of Ii, or reduced the activity and the expression of proteases involved in Ii degradation (58, 59). Many endosomal proteases are synthesized as precursors and need a proteolytic step occurring in the trans-Golgi network and in subsequent compartments to become fully active (60). PMA could also influence the maturation of proteases resulting in a slower degradation and the accumulation of Ii.
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fragments partially associated with class II molecules. It should be noted that inhibitors of protein phosphatases such as okadaic acid can regulate endocytic vesicle formation in vitro (61) and inhibit endocytosis in Hela cells (62) consistent with other reports showing that PMA can modulate the uptake of transferrin and of fluid phase markers (43). Additional events such as a reorganization of the cytoskeleton perturbing the exocytic traffic of MHC class II complexes remain also possible.

Using BCR ligation and PKC activation, we observed for the first time a negative regulation of the MHC class II biosynthetic pathway of antigen presentation. Phorbol esters reduce peptide loading and conversion of class II molecules into SDS-resistant heterodimers and impaired presentation of Ii-dependent exoge-

Peptides are generated in endosomal compartments involved in peptide loading of αβ heterodimers, delayed maturation of SDS-resistant ab dimers, and impaired presentation of Ii-dependent exoge-

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REFERENCES

1. Germain, R. N. (1994) Cell 76, 287–299
2. Cresswell, P. (1994) Annu. Rev. Immunol. 12, 259–293
3. Busch, R., and Mellins, E. D. (1996) Curr. Biol. 8, 51–58
4. Freiweninkel, I. M., Schenck, K., and Koch, N. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9703–9706
5. Romagnoli, P., and Germain, R. N. (1994) J. Exp. Med. 180, 1107–1113
6. Bijl-Vonken, M. E., Bornens, V., and Ploegh, H. L. (1994) J. Exp. Med. 180, 623–629
7. Roche, P. A., Marks, M. S., and Cresswell, P. (1991) Nature 354, 392–394
8. Lamb, C. A., and Cresswell, P. (1992) J. Immunol. 148, 3478–3482
9. Teytown, L., O’Sullivan, D., Dickson, P. W., Lotteau, V., Sette, A., Fink, P., and Peterson, P. A. (1990) Nature 348, 39–44
10. Roche, P. A., and Cresswell, P. (1990) Nature 345, 615–618
11. Roche, P. A., Teletski, C. L., Krap, D. R., Fren, V., Bakke, O., and Long, E. O. (1992) EMBO J. 11, 2841–2847
12. Ghosh, P., Amaya, M., Mellins, E., and Wiley, D. C. (1995) Nature 375, 457–462
13. Pieters, J., Bakke, O., and Dobberstein, B. (1993) J. Cell. Sci. 106, 831–846
14. Odorizzi, C. G., Towbridge, I. S., Xue, L., Hopkins, C. R., Davis, C. D., and Collawn, J. F. (1994) J. Cell Biol. 126, 317–330
15. Arneson, L. S., and Miller, J. (1995) J. Cell Biol. 129, 1217–1228
16. Amigorena, S., Drake, J. R., Webster, P., and Mellman, I. (1994) Nature 369, 113–120
17. Qiu, Y., Xu, X., Wadlinger-Ness, A., Dalke, D. P., and Pierce, S. K. (1994) J. Cell Biol. 125, 595–605
18. Tulp, A., Voorwouw, D., Dobberstein, B., Ploegh, H. L., and Pieters, J. (1994) Nature 369, 120–126
19. West, M. A., Luooco, J. M., and Watts, C. (1994) Nature 369, 147–151
20. Humbert, M., Raposo, G., Cosson, P., Reggio, H., Davoust, J., and Salamero, J. (1995) Eur. J. Immunol. 25, 3158–3166
21. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995) J. Exp. Med. 182, 389–400
22. Peters, P. J., Raposo, G., Neefjes, J. J., Oorschot, V., Leijendekker, R. L., Geuze, H. J., and Ploegh, H. L. (1995) J. Exp. Med. 182, 325–334
23. Amigorena, S., Webster, P., Drake, J., Newcomb, J., Cresswell, P., and Mellman, I. (1995) J. Exp. Med. 181, 1729–1741
24. Denizot, L. K., and Cresswell, P. (1995) Cell 89, 155–165
25. Sherman, M. A., Weber, D. A., and Jensen, P. E. (1995) Immunity 3, 197–205
26. Sloan, V. S., Cameron, P., Porter, G., Gammon, M., Amaya, M., Mellins, E., Chen, R., and Zaller, D. M. (1995) Nature 375, 802–806
27. Harding, C. V., Roof, R. W., and Unanue, E. R. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 4230–4234
28. Salamero, J., Humbert, M., Cosson, P., and Davoust, J. (1990) EMBO J. 9, 3499–3496
29. Reid, A. P., and Watts, C. (1990) Nature 346, 655–657
30. Pinet, V., Vergelii, M., Martin, R., Bakke, O., and Long, E. O. (1995) Nature 375, 601–606
31. Adorini, L., Ulrich, S. J., Appella, E., and Fuchs, S. (1990) Nature 346, 63–66
32. St-Pierre, Y., and Watts, T. H. (1990) J. Immunol. 145, 812–818
33. Sorensen, M. M., Moore, J. C., Sherman, M. A., and Jensen, P. E. (1994) Cell. Immunol. 157, 277–290
34. O’Donnell, D. F., and Robinson, J. H. (1992) Cell 74, 923–940
35. Teyton, L., O'Sullivan, D., Dickson, P. W., Sette, A., Fink, P., and Peterson, P. A. (1990) Nature 348, 39–44
36. Patel, K. J., and Neuberger, M. S. (1992) EMBO J. 11, 411–416
37. Spyrou, D., and Watts, T. H. (1993) J. Immunol. 150, 2223–2232