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Assembly and intracellular transport of the human B cell antigen receptor complex

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Abstract

The B cell antigen receptor (BCR) complex consists of transmembrane (m) Ig, in non-covalent association with a disulphide-linked heterodimer of *mb-1* and B29 gene products. The MB-1–B29 heterodimer is required for deposition of the BCR at the plasma membrane, as well as for coupling of the antigen receptor to intracellular signal transduction cascades. We have performed biosynthetic labelling studies using the mature B cell line Ramos to investigate the process of assembly of the BCR components. We conclude that association of the four components, Ig-heavy chain (HC) and -light chain (LC), MB-1 and B29, is required and sufficient to permit exit of the BCR complex out of the endoplasmic reticulum (ER). With the short pulse labelling procedures used, no evidence was found for transient participation of other molecules in complex formation. A 32 kDa glycoprotein was identified, which is serologically related to MB-1, but has a more acidic isoelectric point (pI) and a protein backbone of 21 kDa, as compared with 25 kDa for MB-1. This protein did not appear to participate in BCR complex formation and is most likely degraded prior to reaching the *cis*-Golgi. The MB-1 component was found to be the rate-limiting step in BCR complex formation, while Ig-HC, -LC and B29 are synthesized in excess. Ig-HC and -LC form disulphide-linked tetrameric complexes within 3 min after biosynthesis, with which B29 and MB-1 components associate independently, followed by disulphide bond formation between these heterodimeric partners. While partial BCR complexes containing B29 and mIg-H_2L_2 tetramers are rapidly formed and have a half-life of a few hours in the ER, entry of MB-1 into these complexes controls exit out of this compartment.

Introduction

The B cell antigen receptor (BCR) consists of two membrane (m) Ig heavy chains in covalent association with two light chains. In addition, the BCR complex contains a disulphide-linked heterodimer formed by the *mb-1* and B29 gene products (which have been designated CD79a and CD79b by the 5th International Workshop on Human Leukocyte Differentiation Antigens) (1–3). As evidenced particularly by illuminating experiments in transgenic mice, the transmembrane μ-heavy chain (HC) directs B cell survival, proliferation and differentiation throughout development, and is involved in the regulation of Ig gene rearrangement (4–6), functional silencing or deletion of autoreactive B cells (7,8) and activation of the mature B cell population (9,10).

While the transmembrane μ-HC itself has not conclusively been shown to interact with cytoplasmic effector molecules, the MB-1–B29 heterodimer clearly endows the BCR complex with signal transduction properties (9,10). Both proteins contain an extracellular Ig-like domain, a transmembrane segment and a cytoplasmic tail of ~50 amino acids (11–14), which harbours a motif sequence including two tyrosine residues (15). This motif is subject to phosphorylation upon antigen receptor stimulation and couples the BCR to intracellular molecules initiating signal transduction cascades (1–3,9,10). Since the MB-1–B29 heterodimer is essential for the signalling capacity of the receptor, it is important that only fully assembled BCR complexes appear at the cell surface. Therefore, expression levels of the individual components, assembly of oligomers and transport through intracellular compartments are expected to be subject to various intracellular control mechanisms.

Association with MB-1 and B29 is essential for intracellular transport and plasma membrane deposition of newly synthe-

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sized mIg molecules (16,17). The product of the MB-1 gene (11) was in fact recognized as a component of the BCR complex by virtue of its requirement for cell surface expression of mIgM in a transfected plasmacytoma cell line (18,19). Transfection studies have also demonstrated that the four subunits of the BCR complex are required and sufficient to achieve plasma membrane expression of the BCR in non-B cells (20,21). Reconstitution of the BCR complex in T cells does not seem to require MB-1, but it cannot be excluded that a TCR complex component can substitute for MB-1 in this case (22).

Intracellular retention of mIgM in absence of the heterodimer was found to be dictated by the transmembrane segment of the μ chain, since replacement by the homologous region derived from MHC class I rescued plasma membrane expression (19,23). Mutational analysis identified a polar patch within the transmembrane spanning region of the μ chain, the TTAST sequence, as essential for retention (23). It can be envisioned that interaction between the transmembrane regions of the μ, MB-1 and B29 chains alleviates this specific retention signal. Proteins involved in interaction with the μ chain, which mediate this retention, have thus far not been identified.

A second retention signal is known to reside in the first constant (C_H1) domain of Ig-HC (24), which is important for association with the endoplasmic reticulum (ER)-localized HC binding protein BiP (25). In absence of LC synthesis, secretory HC remains associated with BiP and is not exported (26). Strikingly, the transmembrane μ-HC can come to the cell surface independent of association with LC and the MB-1–B29 heterodimer if its C_H1 domain is deleted (27). This result suggests that BiP-mediated ER retention of mIgM is essential to allow its association with the MB-1–B29 heterodimer.

In this work, we have investigated biosynthesis of the human BCR complex, using the Burkitt lymphoma line Ramos as a model system. Specifically, we have studied the order of assembly of the BCR components using biosynthetic pulse–chase labelling procedures and immunoprecipitation with antibodies directed at the four different subunits of the complex. Intracellular transport was monitored by susceptibility of newly synthesized complexes to digestion with Endoglycosidase H (Endo H). These procedures have also allowed us to address the question whether additional proteins interact either constitutively or transiently with the human BCR complex.

Methods

Cells and antibodies

The human Burkitt lymphoma cell line Ramos was maintained in 5% CO_2 at 37°C in RPMI 1640 medium, supplemented with 5% FCS and antibiotics. Anti-human μ-HC mAb CLB-MH15 was purchased from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) and goat anti-human λ-LC polyclonal serum from Tago Immunologicals (Burlingame, CA). Polyclonal rabbit anti-B29 serum was raised in our laboratory against a recombinant glutathione *S*-transferase fusion protein containing the cytoplasmic tail of B29. The HM57 mAb directed against a C-terminal MB-1 peptide (28) and the JCB112 mAb, directed against a recombinant fusion protein of rat CD4 and the extracellular domain of human MB-1 (29), were used to detect human MB-1.

Radiolabelling

For pulse–chase metabolic labelling, 30×10^6 cells per chase-point were washed in PBS, resuspended in Met-/Cys-free RPMI 1640 (Selectamine kit; Gibco, Grand Island, NY), supplemented with 10% FCS and pre-incubated at 10×10^6 cells/ml at 37°C, 5% CO_2 for 1 h. Subsequently, cells were suspended at 30×10^6 cells/ml in Met-/Cys-free medium, 125 μCi/ml of an L-[^{35}S]Met and L-[^{35}S]Cys mixture (Amersham, Amersham, UK) was added and cells were cultured for the indicated pulse time. Next, 10 volumes of RPMI 1640 medium containing a 10-fold molar excess of unlabelled L-Met and L-Cys were added and cells were cultured for the indicated chase times, after which cell aliquots were harvested and lysed.

Immunoprecipitation and gel electrophoresis

After labelling, cells were lysed in immunoprecipitation buffer (IPB) consisting of 10 mM tri-ethanolamine–HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml ovomucoid trypsin inhibitor, 1 mM Nα-*p*-tosyl-L-lysine chloromethyl ketone and 0.02 mg/ml leupeptin, containing 1% digitonin or 1% NP-40 as detergent. Lysates were precleared and immunoprecipitation was carried out as described (30), using CLB-MH15 or HM57 mAb covalently linked to Protein A–Sepharose CL4B (Prot A) beads, or other antibodies that were added to the lysates and recovered by incubation with Prot A beads. For immunodepletion, lysates were incubated with the same antibody for 10 subsequent times, at least 2 h per immunoprecipitation. For re-immunoprecipitation, Prot A beads containing the protein of interest were boiled in 50 μl IPB containing 5% SDS for 5 min. After dilution with 1 ml IPB containing 1.5% NP-40 and addition of 50 μg myoglobin as carrier protein, the supernatant was precleared with Prot A beads coated with normal mouse Ig, followed by immunoprecipitation with specific antibodies. For SDS–PAGE, 7.5–15% polyacrylamide gradient gels were used, according to a modification of the Laemmli procedure. Samples were analysed either under reducing (5% β-mercaptoethanol in SDS sample buffer) or non-reducing (1 mM iodoacetamide in SDS sample buffer) conditions. Two-dimensional gel electrophoresis was performed according to O'Farrell (31), using ampholytes (LKB, Bromma, Sweden) of isoelectric points (pI) 3.5–10, 4–6, 5–8 and 9–11 at a ratio of 10:1:1:1. Isoelectric focusing (IEF) was followed by SDS–PAGE in the second dimension on 7.5–15% SDS–polyacrylamide gradient gels. Gels were treated with 1 M sodium salicylate, pH 5.4, before autoradiography, which took place at −70°C, using Kodak XAR-5 films in combination with intensifier screens.

Endo H digestion

Washed immunoprecipitates were resuspended in 20 μl 50 mM sodium citrate, pH 5.5, 0.2% SDS, heated for 5 min at 95°C and incubated for 20 h at 37°C with 2 mU Endo H (Boehringer Mannheim, Mannheim, Germany) per sample in the presence of protease inhibitors.

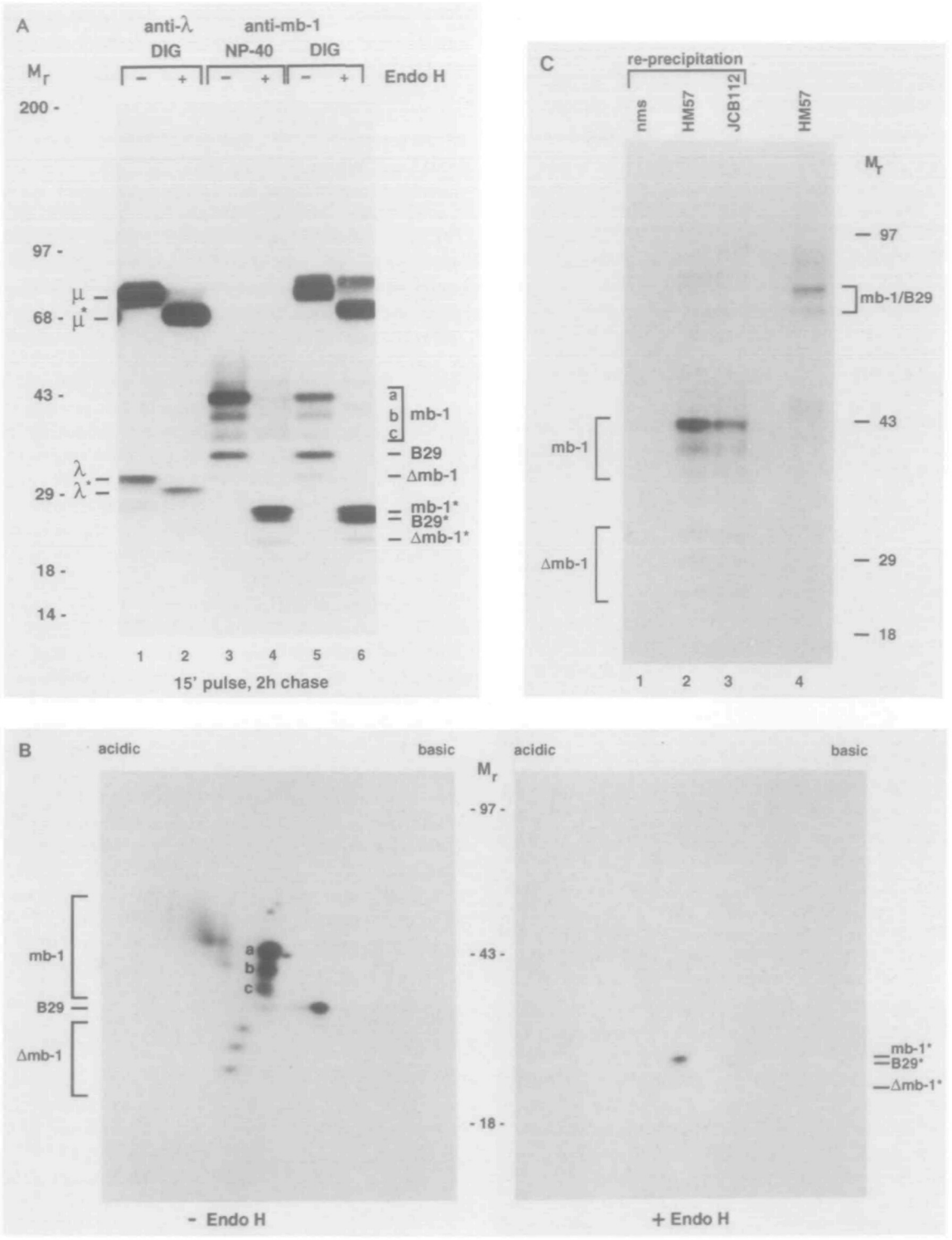

Fig. 1. Introduction to the BCR complex components. (A) Ramos cells were pulse labelled with ^{35}S-labelled L-Met and L-Cys for 15 min, chased for 2 h, lysed with digitonin or NP-40 and used for immunoprecipitation with anti-λ or -MB-1 reagents. Isolated proteins were mock-treated or subjected to Endo H digestion and analysed by SDS–PAGE under reducing conditions. (B) Two-dimensional gel electrophoresis according to O'Farrell (31) of the same precipitates analysed in lanes 3 and 4 of (A). (C) Identification of ΔMB-1. Cells were labelled as in (A), lysed with NP-40 and subjected to precipitation with HM57 anti-MB-1 mAb. The precipitated material was eluted from the mAb, reduced and alkylated, re-precipitated with normal mouse serum (lane 1), followed by HM57 mAb directed at a C-terminal MB-1 epitope (lane 2) or JCB112 directed at an epitope on the Ig domain of MB-1 (lane 3). Lane 4 shows the HM57 precipitate prior to re-precipitation. Samples were analysed by SDS–PAGE under non-reducing conditions.

Results

Description of the biosynthetically labelled BCR components

To study assembly and intracellular transport of the BCR complex, we have used the human Burkitt lymphoma line Ramos as a model system. This cell line represents mature, IgM bearing B cells (32). The known subunits of the BCR complex, Ig-HC, -LC, MB-1 and B29 chains, are glycoprotein products of distinct genes, which are synthesized on the rough ER membrane and transported into the ER lumen. The ER-localized glycoproteins bear immature *N*-linked glycans that are sensitive to Endo H digestion, while resistance to enzymatic digestion is acquired after transport of the glycoproteins to the medial–Golgi compartment. In the mature, cell surface exposed BCR complex of Ramos cells, the disulphide-linked MB-1–B29 heterodimer is non-covalently associated with the $\mu_2\lambda_2$ disulphide-linked tetramer (30,33).

Figure 1(A) introduces the subunits of the BCR complex as they appear after pulse labelling for 15 min and 2 h of chase. Immunoprecipitation from a digitonin lysate with anti-λ serum allows isolation of μ-HC and λ-LC (lane 1). At this chase time point, the majority of both μ and λ chains is sensitive to Endo H digestion (lane 2). Although MB-1 and B29 remain associated with μ/λ complexes in digitonin lysates, these components are not visible in lanes 1 and 2, since μ and λ chains are synthesized in great excess over MB-1 and B29 chains (see below). Immunoprecipitation from a digitonin lysate with anti-MB-1 mAb allows isolation of a predominant MB-1 form of 43 kDa, as well as the associated 34 kDa B29 chain (lane 5). Both MB-1 and B29 contain multiple *N*-linked carbohydrate groups and have protein backbones of 25 and 24 kDa respectively (lane 6). Under these lysis conditions, μ-HC remains associated with the heterodimer (lane 5 and 6). Part of the co-immunoprecipitated μ protein has aquired Endo H resistance, indicative for intracellular transport of completely assembled complexes. In an NP-40 lysate, the interaction between μ-HC and the heterodimer is disrupted, and anti-MB-1 mAb recovers the major 43 kDa MB-1 form (band a) and the B29 chain (lane 3). In addition, proteins of 40 and 37 kDa (bands b and c) are visible in lane 3, which appear to be differentially glycosylated MB-1 forms (Fig. 1B). Moreover, anti-MB-1 immunoprecipitates contain an undefined glycoprotein with a backbone of 21 kDa (ΔMB-1 in lanes 4 and 6).

To conclusively determine the identity of the proteins present in the anti-MB-1 precipitates, the same preparations used for SDS–PAGE in lanes 3 and 4 of Fig. 1(A) were separated by two-dimensional IEF/SDS–PAGE (Fig. 1B). The major 43, 40 and 37 kDa proteins (bands a, b and c in Fig. 1A) have very similar pI before Endo H treatment (left panel) and shift to the same 25 kDa protein species with homogeneous charge after deglycosylation (right panel). This clearly indicates that all three are differentially glycosylated, immature MB-1 forms. In addition, the left panel shows a small amount of mature MB-1 forms at 41–50 kDa, which are heterogeneous in size and charge due to their content of sialylated, complex *N*-linked carbohydrate groups (30). These cannot be observed in the right panel due to the lower labelling intensity of this sample. The 34 kDa immature B29 protein can be discriminated from the MB-1 forms by virtue of its more basic pI, both before and after deglycosylation and has a protein backbone of 24 kDa.

Identification of a truncated form of the MB-1 protein.

The left panel of Fig. 1(B) shows in addition to the MB-1 and B29 protein forms, three molecules of 32, 28 and 25 kDa with similar pI. The result of Endo H treatment depicted in the right panel indicates that these are differentially glycosylated forms of the same 21 kDa protein, which has a more acidic p*I* than both MB-1 and B29.

Since this additional protein was only observed in anti-MB-1 precipitates, both in Ramos (see Fig. 3A and B) and other cell lines representing different developmental stages (data not shown), we investigated its relationship to MB-1 by a re-immunoprecipitation experiment (Fig. 1C). Cells were labelled as described for Fig. 1(A), lysed in NP-40 and MB-1 was recovered with the HM57 mAb as before. Subsequently the precipitated material was eluted from the antibody and reduced and alkylated to irreversibly disrupt disulphide bridges. The eluate was subjected to immunoprecipitation with the HM57 mAb, which is directed at a C-terminal MB-1 epitope and the JCB112 mAb, which is directed at the N-terminal extracellular domain of MB-1. Samples were analysed by SDS–PAGE under non-reducing conditions to check the efficiency of the reductive alkylation procedure. The starting material, analysed under non-reducing conditions, is shown for comparison (lane 4).

Comparison of Fig. 1(C, lanes 2 and 3) with Fig. 1(A, lane 3) indicates that both anti-MB-1 mAb recognize the 43, 40 and 37 kDa differentially glycosylated MB-1 forms, while the B29 protein is absent from the re-precipitates, as expected after disruption of the interchain disulphide bond. In addition, the 32, 28 and 25 kDa proteins, which are weakly visible in Fig. 1(A) and have been described in Fig. 1(B), can be recovered with both anti-MB-1 mAb. We therefore conclude that an alternative MB-1 form exists, which has a protein backbone of 21 kDa and a more acidic pI than the conventional MB-1 protein. Apparently, this MB-1 form contains a deletion of ~4 kDa, which leaves the C-terminus and at least part of the extracellular domain intact and preserves a number of *N*-linked glycosylation sites. The relationship of this protein to MB-1 was confirmed by Western blotting with JCB112 mAb on a HM57 immunoprecipitate. The signal was very weak, indicating that this deleted MB-1 form (ΔMB-1) occurs at very low steady state levels (data not shown).

Order of assembly of the BCR components

Next, we aimed to gain insight into the order of assembly of the components of the BCR complex. To look at these early biosynthetic events, cells were pulse labelled for 3 min and chased for up to 1 h. Lysis was performed in digitonin to maintain interactions between all subunits. Lysates were split in three portions, depleted for either μ, MB-1 or B29 and associated components by exhaustive immunoprecipitation and remaining partial complexes were isolated by subsequent precipitation with mAb directed against these three subunits (Fig. 2).

μ-HC is synthesized in excess, since it can be recovered from both MB-1- and B29-depleted lysates (Fig. 2A, lanes 10-17). Association of μ-HC with labelled λ-LC occurs independently of interaction with MB-1 or B29, since complexes are detected in both MB-1- and B29-depleted lysates. This can more clearly be observed after analysis of the anti-μ

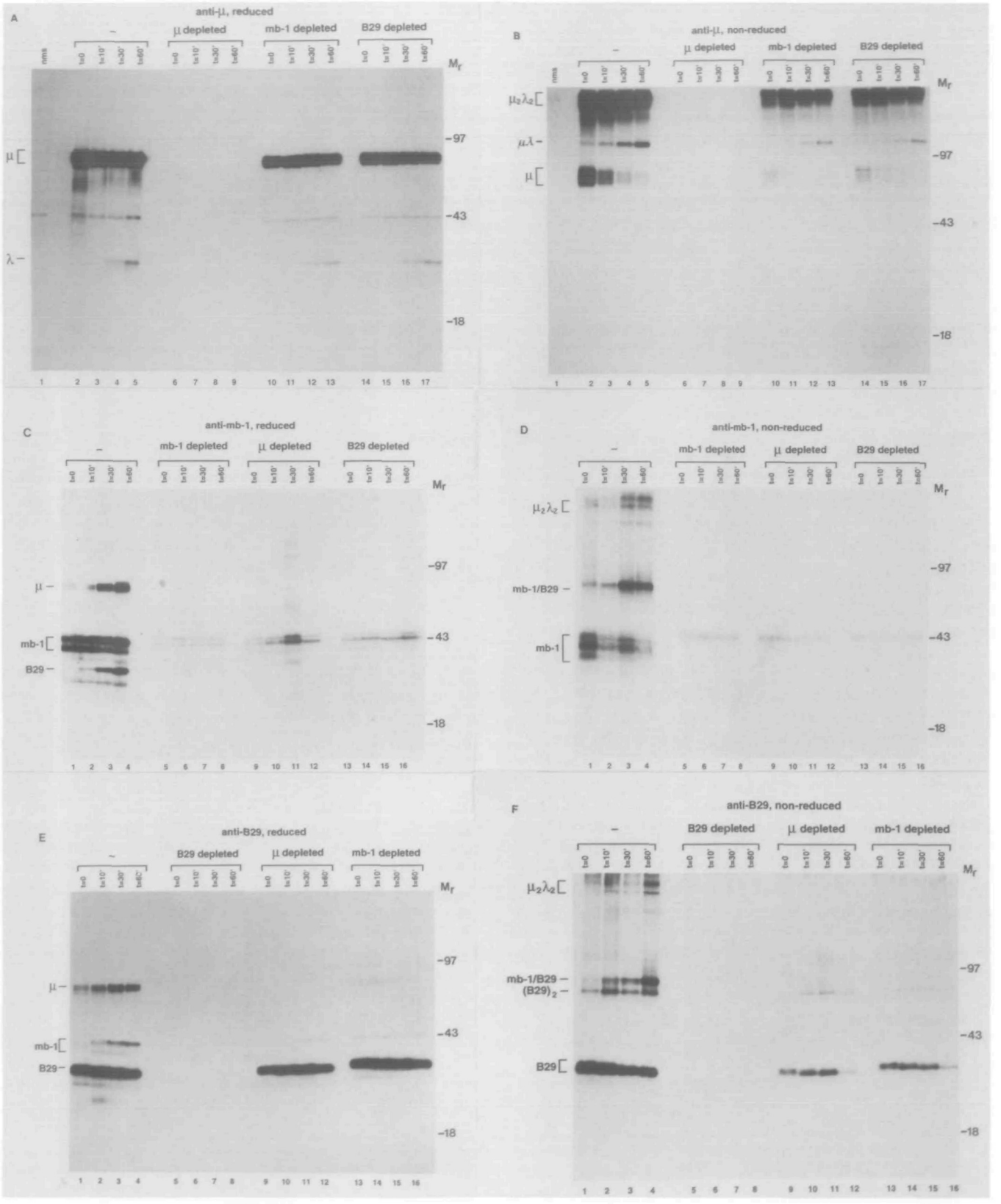

Fig. 2. Order of assembly of the BCR components. Ramos cells were labelled for 3 min and chased for up to 1 h as indicated. Cells were lysed with digitonin and immunoprecipitations were performed in parallel with anti-μ-HC (A and B), -MB-1 (C and D) and -B29 (panels E and F) reagents. One quarter of each lysate was directly used for the relevant immunoprecipitation (lanes 1–4 of each panel). Three quarters were depleted for either μ-HC, MB-1 or B29, after which the relevant precipitation was performed. All samples were analysed by SDS–PAGE under reducing (A, C and E) or non-reducing (B, D and F) conditions.

precipitates under non-reducing conditions (Fig. 2B). Formation of $\mu_2\lambda_2$ tetramers is visible directly after the 3 min pulse, also in MB-1- and B29-depleted lysates (lanes 2, 10 and 14). While free μ chains are initially present (lanes 2 and 3), virtually all have become incorporated into dimers or tetramers after 60 min chase (lane 5). Labelled λ-LC is detected only at later chase points (Fig. 2A), indicating that newly synthesized μ chains initially associate with an available pool of unlabelled λ chains. The 110 kDa protein that appears concomitantly with labelled λ chain under non-reducing conditions represents μλ dimers (Fig. 2B).

MB-1 associates efficiently with labelled B29 and μ-HC throughout the 60 min chase period (Fig. 2C, lanes 2–4). Concomitant with association between MB-1 and labelled B29, a 70 kDa disulphide-linked MB-1–B29 heterodimer increases in intensity throughout the chase (Fig. 2D, lanes 1–4). However, association between MB-1 and predominantly unlabelled BCR components already occurs immediately after the 3 min pulse, as can be concluded from the presence of a disulphide linked MB-1–B29 heterodimer (Fig. 2D, lane 1), in absence of labelled B29 (Fig. 2C, lane 1). Moreover, no MB-1 can be recovered from lysates depleted for μ-HC (Fig. 2C, lanes 9–12) or B29 (Fig. 2C, lanes 13–16) protein, even after short chase periods. This indicates that the seemingly monomeric MB-1 observed under non-reducing conditions (Fig. 2D) is already incorporated into partial BCR complexes, but not yet disulphide linked to B29. In conclusion, there is no pool of free MB-1 subunits. The two bands seen at 44 and 45 kDa in lanes 5–16 do not correspond to MB-1, since they migrate at different positions under reducing conditions and remain at 45 kDa under non-reducing conditions (Fig. 2D, lanes 5–16), while MB-1 engages in dimer formation. No multimeric mIg complexes can be recovered with anti-MB-1 mAb after removal of B29 (Fig. 2D, lanes 13–16). Apparently, all newly synthesized MB-1 chains are rapidly incorporated into already existing complexes of the other three BCR subunits.

There is a large steady state pool of free B29 molecules, since removal of μ-HC (Fig. 2E, lanes 9–12) or MB-1 (lanes 13–16) does not deplete B29. After the 3 min pulse, part of the labelled B29 chains are already associated with μ-HC (Fig. 2E, lane 1), while labelled MB-1 is seen to enter this complex in increasing amounts during the 60 min chase period (lanes 2–4). Precipitation from MB-1-depleted lysates shows that B29 can associate with μ-HC independent of MB-1 (lanes 13–16). Analysis under non-reducing conditions indicates that these B29-associated μ-HC are part of $\mu_2\lambda_2$ tetrameric complexes (Fig. 2F, lanes 13–16). Under non-reducing conditions, a 70 kDa dimer is seen to form concomitant with entry of MB-1 into the complex (Fig. 2F, lanes 1–4). Apparently, disulphide bond formation between B29 and MB-1 occurs immediately upon entry of the newly synthesized MB-1 chains into the $\mu_2\lambda_2$/B29 complexes. Formation of these 70 kDa MB-1–B29 heterodimers does not take place efficiently in absence of μ-HC, since MB-1 could not be identified in anti-B29 precipitates after depletion of μ-HC (Fig. 2E, lanes 9–12). This is confirmed by analysis of the same precipitates under non-reducing conditions (Fig. 2F) where the 70 kDa MB-1–B29 dimer is not observed in lysates depleted for either μ-HC (lanes 9–12) or MB-1 (lanes 13–16). Whereas the 70 kDa dimer is also detected in anti-MB-1 precipitates (Fig. 2D, lanes 1–4), the anti-B29 precipitates contain an additional disulphide-linked dimer of 64 kDa (Fig. 2F, lanes 1–4), which does not involve MB-1, since it can also be isolated from MB-1-depleted lysates (lanes 13–16). Most likely, the excess B29 chains engage in homodimer formation.

We conclude that single B29 and MB-1 chains associate independently with preformed tetrameric $\mu_2\lambda_2$ complexes, whereby B29 probably precedes MB-1, and subsequently become disulphide-linked.

Intracellular transport

Intracellular transport of partially or fully assembled BCR complexes was monitored by determining their susceptibility to Endo H digestion. Cells were pulse labelled for 15 min and chased for up to 8 h, followed by lysis with digitonin. Immunoprecipitations were performed with mAb directed against μ, MB-1 or B29. As demonstrated above, interaction between μ-HC and the MB-1–B29 heterodimer is not readily observed in anti-μ precipitates since μ and λ chains are synthesized in excess over MB-1 and B29 chains. The anti-μ precipitations demonstrated that although $\mu_2\lambda_2$ tetramer formation occurs within the first minutes after biosynthesis and is completed after 1–2 h of chase, the tetramer becomes Endo H resistant only after ~4 h of chase (data not shown). This indicates that tetramerization precedes exit out of the ER, as expected, but is not sufficient to permit ER exit.

Since MB-1 appears to be the rate-limiting step in BCR complex formation, while the other components are synthesized in excess, transport of the BCR complex was studied using anti-MB-1 immunoprecipitates. Consistent with the results depicted in Fig. 2(C), MB-1 is seen to associate throughout the chase with increasing amounts of labelled μ-HC, λ-LC and B29 (Fig. 3A, lanes 2–5). Concomitant with this association, the subunits acquire Endo H resistance. The majority of the labelled subunits are Endo H resistant after 4 h of chase (lane 12). This is most easily observed for the μ-HC, whereas mature MB-1 and B29 have a typical heterogeneous appearance after complete glycosylation and are not easily detectable in Fig. 3(A and B). The 32 kDa ΔMB-1 protein, which co-migrates with λ before Endo H digestion but can clearly be seen at 21 kDa after deglycosylation (lanes 8–11), is probably degraded prior to reaching the medial-Golgi, since we have no indication that an Endo H resistant form of this protein is generated.

Aliquots of the same lysates were used for parallel immunoprecipitation with anti-B29 reagent (Fig. 3B). In agreement with the results depicted in Fig. 2(E), B29 is seen to associate with labelled μ-HC within the 15 min pulse (lane 2). Labelled MB-1 enters the complex after 0.5 h of chase. This can most easily be observed after Endo H digestion where a 24–25 kDa doublet appears (lane 9), representing deglycosylated B29 and MB-1 respectively. ΔMB-1 is not observed in anti-B29 precipitates, indicating that this truncated form does not participate in BCR complex formation. After prolonged chase periods, a significantly smaller proportion of B29-associated μ chains has acquired Endo H resistance, as compared to MB-1-associated μ chains.

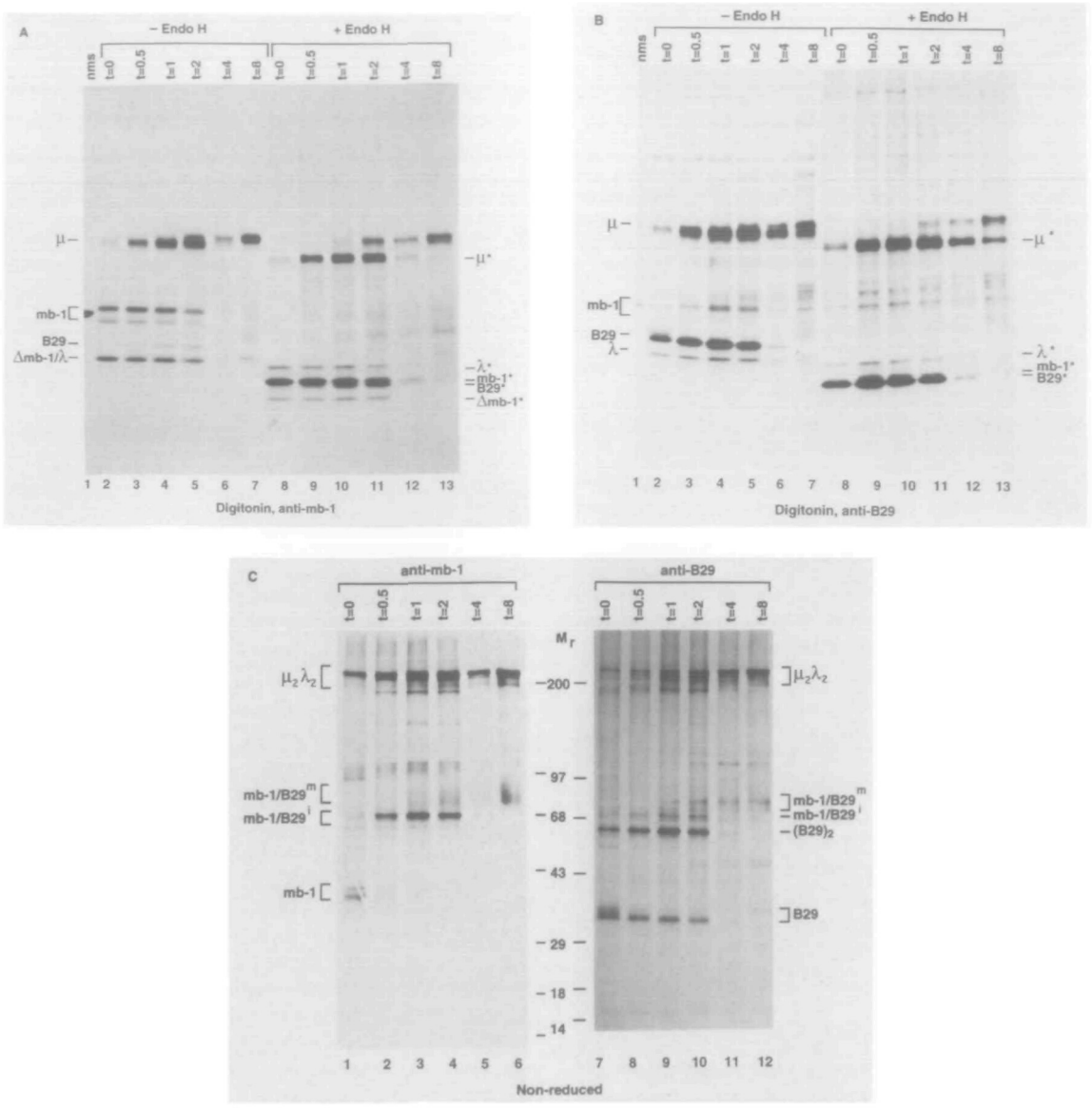

Fig. 3. Intracellular transport of the BCR complex. Ramos cells were pulse labelled for 15 min, chased for several periods up to 8 h as indicated and lysed with digitonin. Immunoprecipitations with anti-MB-1 (A) or -B29 (B) mAb were performed, samples were mock treated or subjected to Endo H digestion and analysed by SDS–PAGE under reducing (A and B) or non-reducing conditions (C).

This indicates that MB-1-associated μ-HC is more efficiently transported out of the ER.

Figure 3(C) shows analysis of the same immunoprecipitates as analysed in panels (A) and (B) under non-reducing conditions. Both MB-1 (lane 1) and B29 (lane 7) are associated with disulphide-linked $\mu_2\lambda_2$ tetramers after the 15 min pulse. Newly synthesized MB-1 is rapidly incorporated into disulphide-linked MB-1–B29 heterodimers, since monomeric MB-1 chains are detectable after the 15 min pulse (lane 1), but not at later chase points. In contrast, a significant pool of newly synthesized B29 chains remains monomeric even after 2 h of chase (lanes 7–10). Two types of disulphide-linked dimers are observed in anti-MB-1 precipitates (lanes 1–4), one predominating as a band at 70 kDa, the other appearing as a minor band at 66 kDa. Resolution of the proteins extracted from these bands by SDS–PAGE under reducing conditions confirmed that both represent MB-1–B29 heterodimers (results not shown). The heterodimers appear until the 2 h chase point as discrete bands, indicating that they contain immature *N*-linked carbohydrate groups. Consistent with the acquisition of Endo H resistance seen in panels (A) and (B), the heterogeneously glycosylated, mature MB-1-B29 heterodimer predominates from the 4 h chase timepoint on (lanes 4–6). In anti-B29 precipitates, initially the 64 kDa presumed B29 homodimer is observed (lanes 7–10). When MB-1 comes into the complex, a heterodimer of 70 kDa is formed that is subject

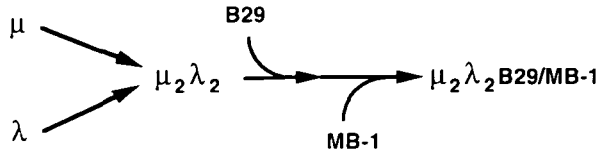

Fig. 4. Schematic representation of the of assembly of the BCR components. MB-1 and B29 associate independently and most likely sequentially, in the order indicated, with a $\mu_2\lambda_2$ tetramer, and consecutively form disulphide linked dimers. Our results do not allow a conclusion regarding the stoichiometry of the complex.

to complex glycosylation (lanes 9–12). Excess B29 monomers and dimers are subject to degradation, as judged from the decrease in labelling intensity between the 2 and 4 h chase points.

The collective data from these experiments indicate that MB-1 and B29 associate with preformed $\mu_2\lambda_2$ complexes sequentially, most likely first B29, followed by MB-1, and heterodimerize after association (Fig. 4). The amount of MB-1 synthesized appears to be the rate-limiting step in BCR complex formation, since all newly synthesized MB-1 protein is incorporated in complexes that are subject to intracellular transport, whereas the other subunits are synthesized in excess. Complete complex assembly is first achieved about 2 h after biosynthesis of the individual chains and the entire newly synthesized pool of MB-1 is fully assembled and transported out of the ER ~4 h after biosynthesis. Degradation of excess B29 chains occurs between 2 and 4 h after biosynthesis and takes place in or near the ER, since these molecules do not acquire Endo H resistance. In contrast, most newly synthesized MB-1 proteins participate in BCR complexes that are transported to the cell surface.

Discussion

Although biosynthesis and intracellular transport of mIg have been studied previously, this has never included the participation of the MB-1 and B29 components. Since the identification of MB-1 and B29 proteins, the striking parallels in structural organization and function of antigen receptor complexes of T and B lymphocytes have become increasingly apparent (reviewed in 2). Given the early identification of the TCR-associated CD3 components, detailed analysis of TCR complex biosynthesis and intracellular transport has preceded that of the BCR complex (reviewed in 34). A functional TCR complex consists of the TCR α and β (or γ and δ) chains, CD3 γ, δ and ε proteins, and a disulphide-linked dimer of a ζ chain, paired with either a ζ, η or γ partner. All components assemble in the ER, with the ζ-containing dimer as the rate-limiting step for intracellular transport (35). MB-1 and B29 can be considered the structural homologues of CD3 γ/δ and ε components. No ζ homologue has been identified in the BCR complex with biochemical approaches, including the work presented here. In addition, association of Ig-HC and -LC with MB-1 and B29 is sufficient to allow cell surface expression of the BCR complex in non-T cells (20,21), indicating that these four subunits form the complete, mature complex. In this study, we have established that assembly with the MB-1 subunit is the rate-limiting step in BCR complex formation and intracellular transport.

During assembly in the ER, partial TCR complexes are associated with the TRAP or ω protein (34,40), which may play a role in complex formation. In our experiments, we have not observed a similar biosynthetic intermediate in the BCR complex. Obviously, it cannot be excluded that such a protein would have a low turnover and did not incorporate sufficient radioactivity in the short pulse labelling procedures. However, TRAP is not absolutely required for TCR complex formation, since correct assembly occurred upon introduction of the individual TCR–CD3 components into non-lymphoid cells in the absence of TRAP (41).

Upon biosynthetic labelling, several MB-1 species were detected. After deglycosylation, MB-1 and B29 can be discriminated by virtue of their distinct pI and were found to have polypeptide backbones of 25 and 24 kDa respectively. This is in agreement with the size predicted from cDNA cloning, as well as with our previous analysis of cell surface iodinated components (30). Human MB-1 contains six potential *N*-glycosylation sites, two of which are not used efficiently, since we find a significant amount of intermediately glycosylated species. Indeed, two glycosylation sites in the MB-1 protein are in proximity to proline residues, which may interfere with the addition of *N*-linked glycans (13).

In these deglycosylation experiments, glycoprotein species of 32, 28 and 25 kDa were identified, which have a common backbone of 21 kDa with a more acidic pI than that of either MB-1 or B29. This molecule was designated ΔMB-1, since it appeared to share with MB-1 a well defined epitope at the very C-terminus, as well as an epitope on the extracellular domain. These data suggest that ΔMB-1 may be encoded by the *mb-1* gene, but contains a deletion of ~35 amino acids (4 kDa). ΔMB-1 could result from alternative splicing of the *mb-1* mRNA or from proteolytic processing. If we assume that the human *mb-1* gene has a similar organization as the murine gene (36), deletion of exon 3, which includes the transmembrane segment but no *N*-glycosylation sites, would be compatible with the observed molecular mass of ΔMB-1. There are precedents for truncated antigen receptor associated molecules that participate in antigen receptor complexes at the plasma membrane: an alternative B29 form in the murine system (37) and the η chain in the TCR complex (38). Also, Ishihara *et al.* (39) have reported a cell surface expressed 23 kDa protein that is associated with murine B29 and shares an extracellular epitope with MB-1. However, we have no evidence that ΔMB-1 engages in complex formation and is transported to the cell surface.

We have identified two MB-1-containing disulphide-linked heterodimers: a major species migrating at 70 kDa and a minor species of 66 kDa. Both contain MB-1 and B29 subunits as established by recovery of the proteins and analysis by SDS–PAGE under reducing conditions (results not shown). The 66 kDa form may involve an incompletely glycosylated MB-1 subunit or represent a folding intermediate. Curiously, a 64 kDa disulphide-linked dimer was found, which contains B29, but not MB-1. We favour the interpretation that this

concerns a B29 homodimer, since no additional chain was observed upon reduction. Formally, however, it cannot be excluded that B29 pairs with a partner chain that has not incorporated radioactivity in our experiments. Whether such dimer formation occurs *in vivo*, or is a post-lysis event, has not been determined.

It has clearly been established in this study, that tetramerization of transmembrane μ-HC and λ-LC occurs rapidly after biosynthesis, but is not sufficient to allow ER exit. It would indeed be expected that control mechanisms exist, that mediate ER retention of the tetramer until correct assembly with MB-1 and B29 subunits has occurred. Three proteins have been described which are involved in ER retention of Ig chains. BiP was identified as the secretory HC binding protein (25), which most likely also binds transmembrane HC. Both secretory μ-HC (25) and λ-LC (42) can interact with BiP until they are correctly folded and form $\mu_2\lambda_2$ complexes. This assembly alleviates the retention signal that involves the N-terminal portions of μ-HC and λ-LC. However, transmembrane $\mu_2\lambda_2$ complexes are subject to an additional retention mechanism that involves the transmembrane segment of the HC. The ER-resident protein calnexin would be a good candidate for this function. Unlike BiP, which is a luminal protein, calnexin has a transmembrane orientation and has indeed been shown to associate with the BCR complex (43). It will be of interest to investigate which BCR subunits interact with calnexin and whether this involves the transmembrane segments of the participating components. Recently, it has become clear that apart from BiP, another luminal heat shock protein, the HSP90 family member GRP94 (44), can interact with free Ig-HC and -LC. GRP94 was shown to act at a later step of biosynthesis than BiP (45). However, its possible association with transmembrane μ, MB-1 and/or B29 chains has thusfar not been investigated.

The findings reported here would predict that association of MB-1 with the partially assembled BCR complex is the final step in the release of ER retention. Consequently, the level of MB-1 protein expression would determine the efficiency of intracellular transport and cell surface deposition of the functional BCR complex.

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Abbreviations

BCR	B cell antigen receptor
Endo H	endoglycosidase H
ER	endoplasmic reticulum
HC	heavy chain
IEF	isoelectric focusing
IPB	immunoprecipitation buffer
LC	light chain
mIg	membrane Ig
pI	isoelectric point
Prot A	Protein A–Sepharose CL-4B

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