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Two types of μ chain complexes are expressed during differentiation from pre-B to mature B cells

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Immunoglobulin μ chains synthesized in murine pre-B cells are known to be associated with surrogate light chains designated as omega (ω), iota (ι) and B34. In addition to these molecules, we identified the complexes of polypeptides (50, 40, 27 and 15.5 kd) associated with surface or intracellular μ chains of pre-B cell lines. Most of these polypeptides were continuously synthesized and associated with μ chains in virgin B cells lines, although some of them scarcely bound to the μκ dimer or μ2κ2 tetramer concomitantly present in the same clone or population. However, in mature B cells they were no longer detectable except B34. Cross-linking of μm chains on the surface of pre-B cells resulted in an increase in intracellular free Ca^{2+}, indicating that the μm chain complex on the surface of pre-B cell lines acted as a signal transduction molecule. However, the receptor cross-linkage of pre-B cell lines did not induce the increased inositol phospholipid metabolism usually observed in virgin and mature B cell lines. These results suggest that, during the differentiation from pre-B to mature B cells, the cells express two types of μ chain complexes which exhibit different structures as a whole and possess different signal transducing capacities.

Key words: B cell differentiation/Ig expression/μ chain complexes/signal transduction/ts mutant of A-MuLV

Introduction

Immunoglobulin μ chains synthesized in pre-B cells are in general believed not to be expressed on the cell surface unless they are associated with κ or λ light chains following gene rearrangement (Burrows *et al.*, 1979; Siden *et al.*, 1979, 1989; Levitt and Cooper, 1980). However, there is some evidence that murine or human pre-B lymphomas express μm on the surface in the absence of conventional light chain expression (Gordon *et al.*, 1981; Paige *et al.*, 1981; Findley *et al.*, 1982; Hendershot and Levitt, 1984; Hardy *et al.*, 1986; Pillai and Baltimore, 1987; Kerr *et al.*, 1989). Recent reports have revealed that murine pre-B cell lines synthesize polypeptides of relative M_r of 18 kd and 14 kd, designated as ω and ι chains, which associate with μ chains to form μ2ω2 and μ2ι2 tetramers (Pillai and Baltimore, 1987, 1988). In addition, another μ chain associated protein of M_r 34 kd has recently been identified in a pre-B cell line as well as in mature B cells (Hombach *et al.*, 1988), and this molecule is assumed to be encoded by a B cell specific gene mb-1 (Hombach *et al.*, 1988; Sakaguchi *et al.*, 1988). These molecules are believed to help the μ chain mediated stage specific function (Nussenzweig *et al.*, 1987; Reth *et al.*, 1987) and to support μ chain transport to the cell surface as surrogate light chains (Pillai and Baltimore, 1987; Hombach *et al.*, 1988). In the present study, in addition to these molecules, we identified new polypeptides associated with membrane-formed μ chains (μm) on the surface or intracellular μ chains of pre-B cell lines. We characterized the function as well as the fate of these molecules in association with surface or intracellular μ chains as B cell differentiation progressed.

Results

Immunoprecipitation of the complex associated with μm chains of pre-B cell and virgin B cell clones transformed with A-MuLV

An immature B cell clone 46-6 was obtained by transforming BALB/c bone marrow cells with a ts mutant of Abelson murine leukemia virus (A-MuLV, Takemori *et al.*, 1987). When the temperature was raised from 35°C to 37.5°C, most of the cells of this clone expressed μ chains on the surface as a result of IgH gene rearrangement, and then later co-expressed κ chains following κ light chain gene rearrangement (data not shown). Stable clones expressing μ (Ig6.3) and μ and κ (Ig6.11) were established by subcloning 46-6 that had been cultured at a non-permissive temperature. Southern blot analysis using a Jκ or Jλ probe indicated that κ and λ genes were at germline gene configuration in Ig6.3, but that the former was rearranged on both alleles in Ig6.11 (data not shown). Immunofluorescence analysis showed that most of the Ig6.3 cells expressed μ chains on the cell surface and that >95% of Ig6.11 cells co-expressed μ and κ chains (data not shown).

When Ig6.3 was radioiodinated and the lysates were immunoprecipitated with anti-μ antiserum, it was observed that polypeptides of M_r of 50, 15.5 and 14 kd were specifically detected on SDS–polyacrylamide gels under reducing or non-reducing conditions as well as 40 (with an arrow) and 27 kd molecules under reducing conditions only (Figure 1A and B). None of these molecules nor the μ chains were detected in the cell lysates immunoprecipitated with anti-κ antibodies (Figure 1A and B, lane b). These polypeptides were precipitated from cell lysates prepared by extraction with the mild detergent digitonin (Figure 1C) as well as with PBS lysis buffer (Figure 1A and B). The association of the 14 kd molecule with μ chains of pre-B cell lines

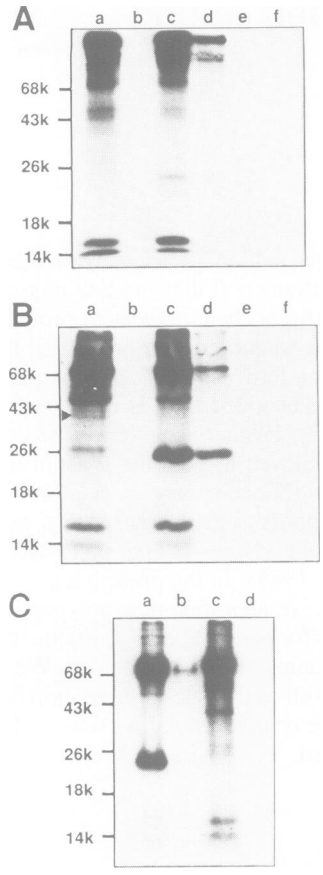

Fig. 1. The expression of small polypeptides associated with μm chains on the surface of A-MuLV transformed pre-B (Ig6.3), virgin B cell (Ig6.11) line or splenic B cells. More than 95% of the cells of Ig6.11 co-express μ and κ chains on the surface, as defined by flow cytometry analysis for the cells double stained with purified anti-μ and anti-κ antibodies. Ig6.3 (lanes a, b and e in **A** and **B**, lanes c and d in **C**), Ig6.11 (lanes c, d, and f in **A** and **B**), and splenic B cells (lanes a and b in **C**) were labeled with ^{125}I and lysed in PBS lysis buffer (A and B) or in digitonin lysis buffer (C). They were pre-cleaned with normal goat Ig (nGIg) and immunoprecipitated with goat anti-μ antiserum (lanes a and c in A, B and C), monoclonal anti-κ antibody (lanes b and d in A and B), or nGIg (lanes e and f in A and B, lanes b and d in C). Immunoprecipitates were resolved on 12% SDS–polyacrylamide gels under non-reducing (A) or reducing (B and C) conditions.

has already been observed, and this chain designated as the iota (ι) chain (Pillai and Baltimore, 1988). The 50, 15.5 and 14 kd molecules as well as κ light chains (M_r 24 kd) were also co-precipitated with anti-μ antiserum from the lysate of Ig6.11 which differentiated further from the stage of Ig6.3; however, these molecules were not detectable in splenic B cells which expressed polypeptides of M_r 16–17 and 13–14 kd, although at very low levels (Figure 1C, lane a). The 50, 15.5 and 14 kd polypeptides in Ig6.11 were not precipitated with anti-κ antibody, indicating that they were hardly associated with μκ dimer or μ2κ2 tetramer in this clone (Figure 1B, lane d).

As observed in Figure 2, the resolution of the immunoprecipitates of Ig6.3 on two-dimensional diagonal electrophoresis showed the 27 kd molecule running at off-diagonal position, the 50 kd molecule migrating either at diagonal or off-diagonal position, and the 15.5 kd at on-diagonal position. In the case of Ig6.11 (Figure 2B), off-diagonal spots

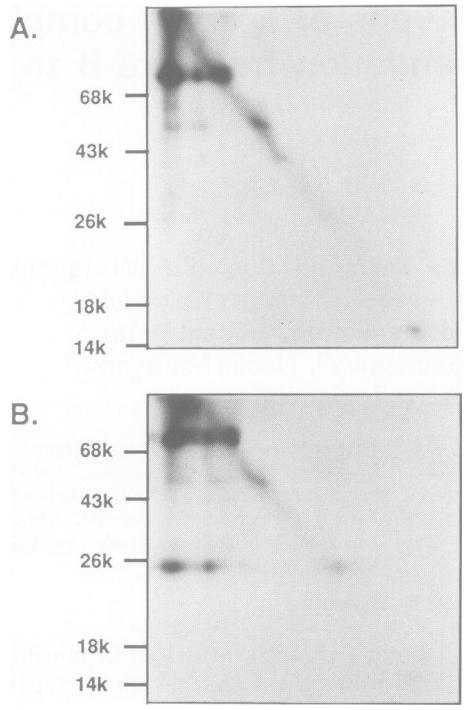

Fig. 2. The analysis of small polypeptides associated with μm chains on diagonal gels. The Ig6.3 (A) and Ig6.11 (B) were iodinated, lysed and immunoprecipitated with anti-μ antiserum as described in Figure 1. The precipitates were run on 12% SDS–polyacrylamide gel. The gel was sliced, reduced and the precipitates were further resolved on 13% (A) or 14% (B) SDS–polyacrylamide gels. The same precipitates were concomitantly resolved on a 7% SDS–polyacrylamide gel to analyze the size of M_r of μ chains disulfide linked to other polypeptides (data not shown).

contained molecules of 24 kd (κ light chain) and 50 kd, and on-diagonal spots contained those of 50, 24 and 15.5 kd. The spot corresponding to the size of 40 or 14 kd was barely observed under this condition. The detection of ι chain by immunoprecipitation of total cell lysates with anti-μ antibodies sometimes accompanied weak resolution on the gel, the reason for which is unknown. When the same immunoprecipitates of Ig6.3 or Ig6.11 were resolved on 7% polyacrylamide–SDS gels under non-reducing conditions, molecules migrated at ~ 220, 190, 110–105, 90–86, and 72 kd (μ chain) in Ig6.3, and at 220, 180, 115, 90 and 72 kd in Ig6.11 (data not shown). These results suggest that μm chains on the surface of the pre-B cell line Ig6.3 form disulfide or non-disulfide linkage to the 50, 40, 27, 15.5 and 14 kd molecules by different combinations, and also that these molecules except the 27 kd continuously link to μm chains on Ig6.11 which concomitantly expresses μκ disulfide linkage.

The complex of polypeptides with μ chains expressed in virgin B cell lines

The expression of μm chains under the association of complexes of polypeptides is not peculiar to A-MuLV-transformed cell lines. As shown in Figure 3, the chemically induced 70Z/3 also expressed μm chains on the surface under association with the 50, 40 or 15.5 kd molecule, although the 50 and 40 kd molecules were not well dissolved on the gel under non-reducing conditions. The 27 and 14 kd molecules were hardly detectable in this cell line (Figure

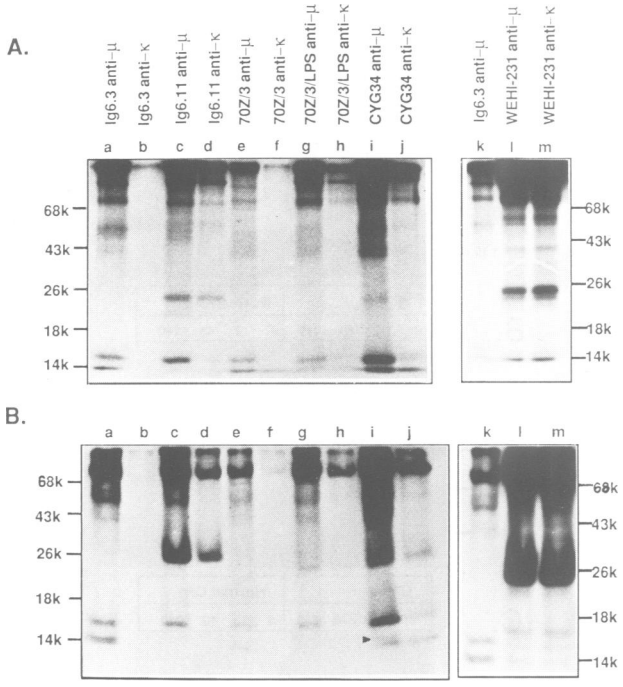

Fig. 3. The association of small polypeptides with μm chains on the surface of pre-B or virgin B cell lines. Ig6.3, Ig6.11, 70Z/3, 70Z/3 stimulated with LPS (20 μg/ml) for 18 h and CYG34 were labeled with [^{125}I]NaI, lysed and immunoprecipitated with purified anti-μ (lanes a, c, e, g and i) or anti-κ (b, d, f, h and j) antibodies as described in Figure 1. Their precipitates were analyzed by SDS−PAGE under non-reducing (**A**) or reducing (**B**) conditions. Double staining experiments indicate that 80% of 70Z/3 cells express μ chains on the surface and 70% of total cells co-express μ and κ chains within 18 h of stimulation with LPS and that >95% of CYG34 cells co-express μ and κ chains on the cell surface (data not shown).

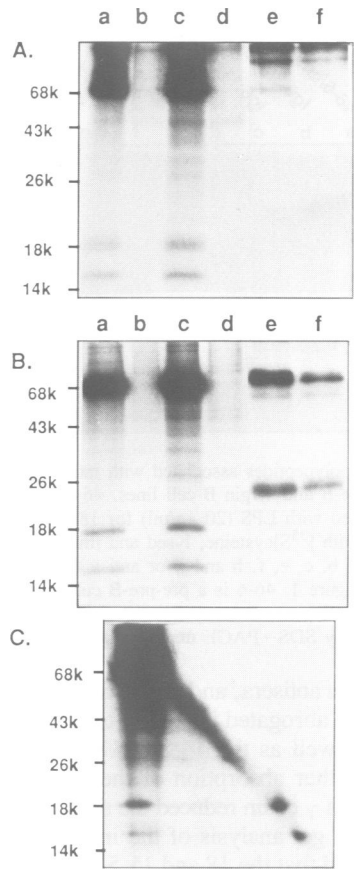

Fig. 4. The association of small polypeptides with intracellular μ chains in pre-B cell lines. 70Z/3 (lanes a and b), Ig6.3 (lanes c and d), and spleen cells (lanes e and f) were metabolically labeled with [^{35}S]cysteine, lysed, and immunoprecipitated with anti-μ (lanes a, c, e) or anti-κ (lanes b, d, f) antiserum as described in Figure 1. They were analyzed by SDS−PAGE under non-reducing (**A**) or reducing (**B**) conditions. The immunoprecipitates from Ig6.3 were resolved on diagonal gels as described in Figure 2. These small molecules were not detected on the gels when immunoprecipitation was done in the presence of excess of cold MOPC104E (μ, λl) (data not shown).

3, lane e). The cell lines matured upon stimulation with LPS, and 70% of the cells co-expressed μ and κ chains on the surface as determined by flow cytometry analysis (data not shown). The 50, 40 and 15.5 kd molecules were continuously expressed in this cell line after differentiation from the stage of late pre-B cells to that of virgin B cells which expressed μ and κ chains on the surface (Figure 3, lane g). The 50 and 40 kd molecules were not well dissolved on the gel under non-reducing conditions. These molecules were also expressed in a permanent virgin B cell clone, CYG34, of which >95% of the cells co-expressed μ and κ chains on the surface. The 14 kd molecule was also expressed in this clone, although at a low level (Figure 3B, lane i with an arrow). The 50 and 40 kd molecules were barely detectable in the precipitates of 70Z/3 and CYG34 when precipitated with anti-κ antibody, indicating that κ and these molecules hardly co-associated with the same μm molecule on the surface. A faint band on the gel corresponding to the κ chain in CYG34 and 70Z/3 stimulated with LPS could be attributable to inefficient labeling on this chain, since the same anti-κ antibody used in this assay precipitated a significant amount of intracellular κ light chains in both cells (see Figure 5). On the other hand, mature B cell lymphoma WEHI 231 did not express μm chains under the association with the 27, 15.5 and 14 kd molecules (Figure 3, f and g). Instead, a minor fraction of μm chains of WEHI 231 was found to be associated with small polypeptides of relative M_r 16−17, 15 and 13−14 kd. The association of these small polypeptides was also detectable at similar intensity in the fraction of μ chains linked to κ light chains (Figure 3, l and m). The analysis of immunoprecipitates by diagonal gel electrophoresis revealed that the μm chains of WEHI 231 were in addition associated with 53, 45 and 33−35 kd (probably B34) molecules (data not shown).

The polypeptide complex associated with intracellular μ chains in pre-B or virgin B cell clones

Ig6.3, 70Z/3, or splenic B cells were metabolically labeled and their lysates were immunoprecipitated with anti-μ or anti-κ antiserum. As depicted in Figure 4, the polypeptides of the M_r 18 and 15.5 kd were co-precipitated under reducing or non-reducing conditions with intracellular μ chains in Ig6.3 and 70Z/3, but not with those in splenic B cells (Figure 4A and B). The association of the 18 kd molecule with intracellular μ chains in pre-B cell lines has already been reported, and this molecule was designated as the ω chain (Pillai and Baltimore, 1987). The 14 kd molecule was also precipitated from Ig6.3 lysates, but at low level. The co-precipitation of these molecules was observed with different batches of

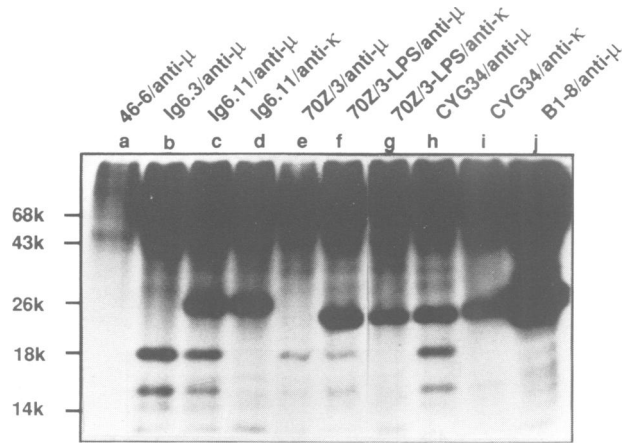

Fig. 5. Small polypeptides associated with intracellular μ chains were observed in pre-B and virgin B cell lines. 46-6, Ig6.3, Ig6.11, 70Z/3, 70Z/3 stimulated with LPS (20 μg/ml) for 18 h, CYG34 and B1-8 were labeled with [^{35}S]cysteine, lysed and immunoprecipitated with anti-μ (lanes a, b, c, e, f, h and j) or anti-κ (d, g and i) antisera as described in Figure 1. 46-6 is a pre-pre-B cell line, and B1-8 is a hybridoma secreting monoclonal anti-NP antibody. Their precipitates were analyzed by SDS−PAGE under reducing conditions.

purified anti-μ antisera, and absorption of one of the antisera with μ chains abrogated its ability to precipitate the 15.5 kd molecule as well as the 14 and 18 kd molecules. On the contrary, neither absorption of the antiserum with κ or λ chain nor with γ chain reduced the activity (data not shown). The diagonal gel analysis of the immunoprecipitates from Ig6.3 revealed that the 18 and 15.5 kd molecules were both covalently and non-covalently associated with intracellular μ chains (Figure 4C). Mu chain disulfide linked to these proteins migrated at sizes of 190, 150 and 120 kd under non-reducing conditions on 7% SDS−polyacrylamide gels (data not shown).

As shown in Figure 5, these intracellular μ chain complexes were also detected in B cell lines at the stage equivalent to virgin B cell, such as Ig6.11, 70Z/3 stimulated with LPS, or CYG34, but not in a hybridoma B1-8 and a mature B cell line WEHI 231 (data not shown). These polypeptides were not associated with the majority of $\mu\kappa$ dimers and/or $\mu 2\kappa 2$ tetramers co-expressed in these virgin B cell lines (lanes d, g, and i).

Assembly of μ chains with small polypeptides

The assembly of μ chains and polypeptide complex in Ig6.3, Ig6.11 and splenic B cells was followed, after a 10 min pulse and 10−240 min chase, by immunoprecipitating the cell lysates with anti-μ antiserum and analyzing the precipitates under reducing or non-reducing conditions on SDS gels. The result of immunoprecipitations under reducing conditions is illustrated in Figure 6. A significant amount of μ chain of Ig6.3 formed the complex with the 18 kd and/or 15.5 kd molecules within short periods of the chase, whereas a fraction of μ chain linked to the 14 kd molecule within the pulse period and reduced the level of association during the chase periods (Figure 6A). In Ig6.11, μ chains associated with κ chains became dominant soon after heavy chain synthesis, in contrast to their late assembly to the 18 and 15.5 kd molecules formed during long chase periods (Figure 6B). In addition, a minor fraction of μ chains in both cells linked to 30, 34 (probably B34), and 40 kd molecules

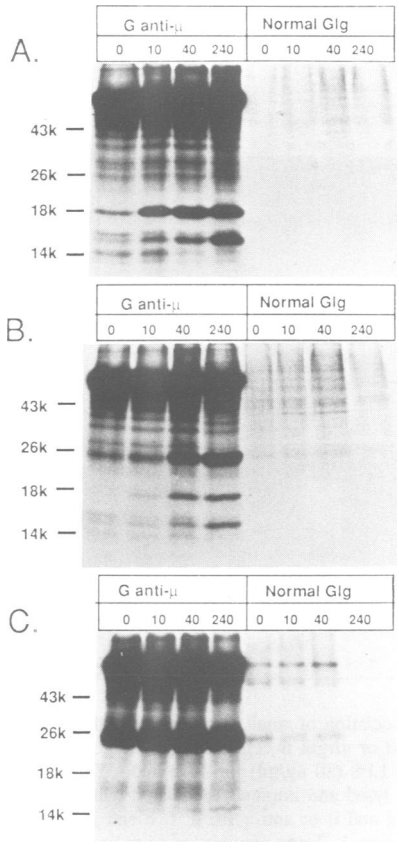

Fig. 6. Assembly of small polypeptides and intracellular μ chains of pre-B and virgin B cell lines. 1×10^8 Ig6.3, Ig6.11, or splenic B cells were metabolically labeled with 1 mCi [^{35}S]cysteine for 10 min, and each was chased in the presence of excess L-cysteine (300 μg/ml). The cell lysates were prepared at the end of pulse (0) and at 10 min (10), 40 min (40) and 240 min (240) chase period. The lysate corresponding to 5×10^7 cells was immunoprecipitated with either 10 μg goat anti-μ antiserum or 10 μg normal goat Ig (nGIg) after pre-cleaning as described in Figure 1. The immunoprecipitates were analyzed by SDS−PAGE under reducing conditions.

immediately after the heavy chain synthesis. The association of a 26 kd molecule was also observed in Ig6.3. The significant assembly of μ chains with these polypeptides was not evident in splenic B cells, however, a minor fraction of μ chains appeared to associate with molecules migrating at the size of 17−16 kd on SDS gel under reducing conditions (Figure 6C).

μ chains on the pre-B cell clones act as a signal transduction molecule

To learn whether μ chains on the pre-B cell lines act as a signal transduction molecule as well as those on mature B cells (Klaus *et al.*, 1987), Ig6.3, 70Z/3 or Ig6.11 cells were loaded with Fura 2/AM and their receptors were cross-linked with anti-μ antiserum. As shown in Figure 7, a cross-linking of surface μ chains on 70Z/3 (A) or Ig6.3 (B) resulted in a significant increase in intracellular free calcium concentrations ($[Ca^{2+}]_i$), as observed in a virgin B cell line Ig6.11 (Figure 7C) and mature B cell line WEHI 231 (data not shown). The results indicate that μ chains on the surface of pre-B cell lines act as signal transduction molecules eliciting an increase in $[Ca^{2+}]_i$.

It has generally been believed that increases in inositol

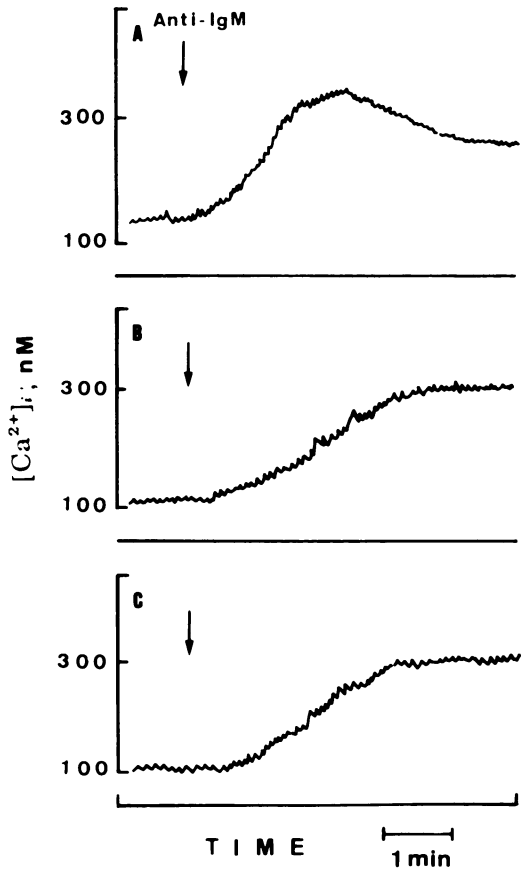

Fig. 7. Increase in $[Ca^{2+}]_i$ in various B cell lines after receptor cross-linkage. 70Z/3 (**A**), Ig6.3 (**B**), or Ig6.11 (**C**) was loaded with Fura 2 and stimulated with 30 μg/ml anti-IgM antibodies. The fluorescence was monitored on a fluorometer. In a control experiment, irrelevant antibodies were shown not to cause these increases in pre-B cell clones (data not shown).

phospholipid metabolism are tightly coupled with the increase in $[Ca^{2+}]_i$ in mature B cells (Klaus *et al.*, 1987) as well as in various other types of cells in response to ligands (Berridge, 1987). Therefore, inositol phospholipid metabolism was tested in pre-B cell lines upon stimulation with purified anti-μ antiserum. As expected, a significant increase in inositol phospholipid metabolism was observed in Ig6.11 when their receptors cross-linked with anti-μ or anti-κ antibodies (Table I). However, it was undetectable in Ig6.3 or 70Z/3 through μ chains cross-linking with anti-μ antibodies, whereas NaF plus $AlCl_3$, an activator of G proteins, led to a significant increase in inositol phospholipid metabolism in these cells lines. These results indicate that μ chains of pre-B cell lines possess the functional capacity to transduce the signal for $[Ca^{2+}]_i$ metabolism, but not for inositol phospholipid metabolism, quite distinct from Ig receptors in virgin and mature B cells upon the stimulation.

Discussion

It is known that pre-B cell lines synthesize several polypeptides to link covalently or non-covalently to μ chains as surrogate light chains. Up to now, three proteins, designated as ω, ι and B34, have been identified; the ω chain is an 18 kd protein which forms disulfide linked tetramers with μm chains (Pillai and Baltimore, 1987). This protein is suggested

Table I. Accumulation of inositol triphosphate (IP3) in pre-B and virgin B cell clones in response to anti-μ or anti-κ antibody.

	Accumulation of IP3[b] (c.p.m./sample)		
stimulant[a]	Ig6.3	70Z/3	Ig6.11
Experiment 1			
buffer	164 ± 13	189 ± 7	173 ± 10
anti-IgM	175 ± 5	198 ± 5	280 ± 25
anti-κ	ND[3]	184 ± 16	246 ± 18
Experiment 2			
buffer	525 ± 62	ND	79 ± 6
anti-IgM	574 ± 97	ND	126 ± 4
anti-κ	557 ± 49	ND	107 ± 3
NaF + $AlCl_3$	966 ± 116	ND	ND

[a]Cells were loaded with myo[^{3}H]inositol and stimulated with anti-μ (30 μg/ml), anti-κ (30 μg/ml) or NaF (30 mM) and $AlCl_3$ (10 μM) for 5 min.
[b]Results are presented as mean ± SD in triplicate assays.
[c]ND: not determined.

to be encoded by the λ-5 gene (Sakaguchi and Melchers, 1986; Pillai and Baltimore, 1988). The ι chain is a 14 kd pre-B cell specific protein associated non-covalently with μm chains. On the other hand, B34 is synthesized in pre-B cell lines as well as mature B cell lines, and appears to play an important role in transporting μ chains on the surface (Hombach *et al.*, 1988). This protein is assumed to be encoded by B cell specific gene mb-1 (Sakaguchi *et al.*, 1988). In addition, it has also been observed that a fraction of μ chains in human pre-B cell lines can reach the surface in association with 22, 18 and 16 kd molecules (Kerr *et al.*, 1989).

In addition to these molecules, we have identified new polypeptides of relative M_rs of 50, 40 and 15.5 kd in association with membrane-formed μ chains (μm) on the surface of virally or chemically induced pre-B cell lines, Ig6.3 and 70Z/3. The 50 kd molecule linked either covalently or non-covalently to μm chains and the 15.5 kd molecule associated non-covalently with μm chains. According to the molecular size, the 15.5 kd molecule is considered to be the product of the Vpre B-1 gene (Kudo and Melchers, 1987; Kerr *et al.*, 1989), although the expression of the gene at the stage of virgin B cells is not clear. In addition, the 27 kd protein was detected in covalent association with μ chains of Ig6.3, but was undetectable in late-stage pre-B cell line 70Z/3 and in virgin or mature B cell lines.

We observed that a significant amount of intracellular μ chain in the same pre-B cell lines also linked to the 15.5 kd molecule, with or without the ω and ι chains and that a minor fraction of intracellular μ chains was also associated with the 40, 30 and 26 kd molecules as well as B34. However, the association of the 50 kd molecule was obscure in the populations observed under our experimental conditions, probably reflecting the size in the total fraction of μ chains, or by a rapid turnover of the 50 kd and μ chain complex in the cytoplasm. The association of these molecules with intracellular μ chains followed different time courses; the ι chain assembled in a small fraction of μ chains within a short time after the heavy chain synthesis, whereas the linkage of the ω chain and the 15.5 kd molecule was induced in a relatively larger fraction of the μ chains during a lengthy

period after the synthesis. At this stage, μ chains associated with the ι chain reduced their level in the fraction. From this result we speculated that only a small fraction of μ chains that had been linked to ι chains was only transported to the cell surface. The linkage of the 15.5 kd molecule and/or the ω chain appears to stabilize the intracellular μ chains synthesized, and we speculate that μ chains associated with the 15.5 kd molecule are efficiently transported to the cell surface.

The expression of these polypeptides appears to be regulated in a stage specific manner. As observed in virally and chemically induced pre-B cell lines, the 50, 40 and 15.5 kd proteins as well as the ι and ω chains were continuously expressed as associated forms with intracellular or cell surface μ chains, or both, during differentiation from the pre-B stage to that of virgin B cells. They were also detected significantly in a stable virgin B cell clone established by a long-term bone marrow culture. However, the 50, 40 and 15.5 kd molecules as well as the ω and ι chains were no longer detectable in mature B cells. On the other hand, mature B cells appear to express other μ chain associated molecules which were not detectable in pre-B and virgin B cell lines.

The fact that these cell lines equivalent to virgin B cells co-expressed either μ chains associated with these polypeptides or those with conventional light chains (κ chains in our cases) at a single cellular level indicates that the expression of μ chains on the surface under the association with these polypeptides does not cause an inhibition of κ-locus rearrangement. The cells hardly expressed μ chain molecules associated with both the conventional light chains and these polypeptides (50, 40 and 15.5 kd molecules and ω chain), implying that the binding of one of each protein to the μ chain molecule affects the conventional light chain binding, probably by steric hindrance or by local changes in the μ chain structure. We do not know at the moment how μm chains were selectively linked to either small polypeptides or conventional light chains synthesized in virgin B cell lines. These results led to the possibility that some fractions of virgin B cells, prior to further maturation and emigration to the peripheral organs from bone marrow, may express two types of μm chain molecules on the surface. The lack of mature B cells in the periphery expressing these μm chain complexes may imply that the synthesis of these additional polypeptides is switched off as B cell maturation proceeds, or alternatively, that these cells are eliminated at the periphery.

We observed that the μm chain complex on the surface of pre-B cell lines acts as a signal transduction molecule; a cross-linking of μm chains of pre-B cell lines by anti-μ antiserum led to a significant increase in $[Ca^{2+}]_i$. As observed in other systems, the increase of $[Ca^{2+}]_i$ in lymphocytes is considered to result from the release of Ca^{2+} stored in the endoplasmic reticulum (Ransom and Cambier, 1986), from the entry of extracellular Ca^{2+} across the plasma membrane (MacDougall *et al.*, 1988), or both (Pozzan *et al.*, 1982). In these cases, it is assumed that the phosphatidylinositol (PI) metabolic cycle is activated by surface Ig ligation, and that inositol 1,4,5-triphosphate (IP_3), the product from the break-down of phosphatidylinositol, mediates Ca^{2+} mobilization (Ransom *et al.*, 1985; Kuno and Gardner, 1987; Mizuguchi *et al.*, 1987) as a consistent view in other systems (Streb *et al.*, 1983; Berridge, 1987).

It was observed under our experimental conditions that the stimulation of virgin B cells with anti-μ or anti-κ antibody induced a reproducible increase in PI metabolism. However, cross-linking of surface μ or pre-B cell lines did not bring about the same effect, indicating that the increase in $[Ca^{2+}]_i$ via μ chain ligation was not mediated by IP_3 in pre-B cell lines and that the stimulation associated with surface μm chains of pre-B cells lacked the capacity to stimulate PI turnover. Since the amount of μ chain on the surface was indistinguishable in pre-B and virgin B cell lines, as indicated from immunoprecipitation analysis and cell surface staining, and since the direct activation of G protein by AlF_4 led to an increase in IP_3 in pre-B cell lines, it is suggested that the defect in activation of PI turnover in pre-B cell lines lies in μ chain associated molecules; these molecules in pre-B cells act as signal transmitters for stimulating Ca^{2+} metabolism without linkage to PI turnover. The increase of $[Ca^{2+}]_i$ in the absence of PI hydrolysis was also recently observed in a variant of mature B cell line WEHI 231; SIg-coupled response led to activation of PI turnover, c-*fos* induction, and down-regulation of proliferation in WEHI 231, but not in the variant (Monroe *et al.*, 1989). The lack of PI turnover with the response is assumed to be related either to an intermediary coupling molecule between μ chains and G proteins or to the signaling domain of μ chains.

It is known that anti-μ manipulation at nanogram concentrations on immature B cells in bone marrow results in the arrest of their differentiation and growth, while the same treatment on mature B cells at the periphery does not have such an effect (Nossal, 1983). Our results suggest that, during differentiation from pre-B to mature B cells, the cells express two types of μ chain complexes exhibiting different structures as a whole and possessing different signal transducing capacities. Although it is not known to what extent these μ chain complexes are expressed on immature B cells, the different sensitivity to anti-μ manipulation between immature and mature B cells can be attributed to the different activities of μ chain expression on those B cells. Further investigations should shed some new light on B cell tolerance and selection of B cell repertoire.

Materials and methods

Cell lines

Immature B cell clone 46-6 was obtained by transforming BALB/c bone marrow cells with a ts mutant of A-MuLV at 35°C (Takemori *et al.*, 1987). 46-6 lacks the expression of B cell marker B220 and intracellular μ chains but carries rearranged IgH genes composed of non-productive VDJ and DJ segments on each allele. Kappa and λ genes were at germline gene configurations in this cell line (data not shown). When cultured at 37.5°C, >95% of the cells of this clone express μ chains on the surface ($s\mu^+$), followed by further rearrangement of the IgH gene by V_H to $V_HD_HJ_H$ joining and later expression of μ and κ chains on the surface as a result of κ gene rearrangement (T.Shirasawa and T.Takemori, unpublished). Stable clones Ig6.3 expressing μ chains and Ig6.11 expressing μ and κ chains on the surface were established by limiting dilutions (0.1 cell/well) from 46-6 cultured at the non-permissive temperature. 70Z/3 is a chemically induced pre-B cell lymphoma (Kincade *et al.*, 1981), a kind gift from Dr Paige. The cell line was subcloned once on feeder cells of bone marrow and maintained under standard culture conditions. The cells possess rearranged lgH and κ light chain genes and 80% of them expressed μ chains on the surface. They express both μ and κ chains when stimulated with lipopoly-

saccharide (LPS) (Perry and Kelley, 1979), indicating that this cell line is in the late stage of pre-B cells. A virgin B cell clone, CYG34, was a generous gift of Dr Nishikawa (Nishikawa *et al.*, 1988). It was established from bone marrow cells by culturing on stroma cells of bone marrow. CYG34 was initially at the pre-B cell stage, >95% of which afterwards became μ and κ chain positive during cultivation in the presence of feeder cells. WEHI 231 is a mature B cell line (Boyd and Schrader, 1981) and B1-8 is a hybridoma cell line which produces antibody (μ/λ) with specificity for the hapten NP (Reth *et al.*, 1978). They were cultured at 37–37.5°C except 46-6 which was cultured at 35.5°C. In some experiments, 70Z/3 was cultured in the presence of LPS (*Escherichia coli*, 055:B5, Difco Laboratories, Detroit, USA) at a final concentration of 10–20 μg/ml for 18–24 h.

Antibodies

Goat anti-μ antiserum was prepared by repeated immunization of the animal with purified MOPC104E (μ/λ) antibody. The antiserum was extensively absorbed with NP specific monoclonal antibody P8-86-9 ($\gamma 1/\lambda$; Takemori and Rajewsky, 1984) and eluted from sepharose 4B coupled with purified NP specific monoclonal antibody B1-8 (μ/λ; Reth *et al.*, 1978). The specificity of purified antiserum was analyzed by radioimmunoassay using a panel of myeloma and hybridoma antibodies as described previously (Takemori and Rajewsky, 1981). In some experiments, 20 μg of purified antiserum was absorbed with either 1 mg MOPC104E, HOPC-1 ($\gamma 2a/\lambda$), or J606 ($\gamma 3/\kappa$) and the residual activity was analyzed by immunoprecipitation. Purified monoclonal anti-κ antibody 187.6 was a kind gift of Dr Tsubata. Properties of monoclonal anti-λ antibody (4/1-101) and preparation and purification of anti-$\lambda 2$ antiserum were described previously (Takemori and Rajewsky, 1981).

Cell staining

The cells were stained with anti-μ, anti-κ or anti-λ antibodies as described previously (Takemori and Rajewsky 1981; Kimoto *et al.*, 1989). Briefly, when stained with biotin-conjugated antibodies, avidin–FITC or avidin–RITC was used as the developing reagent at the second step. FITC-conjugated rabbit anti-goat antiserum was used as the developing reagent for staining with purified goat anti-$\lambda 2$ antibodies. In the case of double staining for μ and κ chains, the cells were incubated with FITC-conjugated goat anti-μ antiserum and biotin-conjugated anti-κ antibody followed by incubation with avidin–RITC. The frequency of double positive cells was analyzed by fluorescence activated cell sorter IV (Becton Dickinson, Sunnyvale, CA) equipped with an argon ion laser.

Cell labeling and immunoprecipitation

Surface iodination was carried out with 1 mCi of ^{125}I per 1×10^8 viable cells by chemical reaction catalyzed by lactoperoxidase or 2, 3, 4, 6-tetrachloroglycoluril (Iodogen, Pierce Chemicals Co., Rockford, IL) (Hanstein, 1975; Kubo and Pelanne, 1983). Metabolic labeling was performed by culturing $2-3 \times 10^7$ viable cells with 300 μCi of [^{35}S]cysteine or [^{35}S]methionine for 2–3 h. Before labeling, the cells were pre-incubated in cysteine or methionine-free RPMI 1640 medium containing 15% dialyzed fetal calf serum for 2–3 h. In the pulse–chase experiments, 1×10^8 cells were labeled with 1 mCi of [^{35}S]cysteine and an excess of cold cysteine (300 μg/ml) was added to the culture at the time of chase (8–10 min). The cells were washed in PBS and lysed in 8–10 ml PBS lysis buffer [1% Triton-X; 0.5% sodium deoxycholate; 0.1% SDS, 10 mM phosphate buffer pH 7.4; 100 mM NaCl; 5 mM EDTA (Kunopka *et al.*, 1984)] containing 15% glycerol, supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF), 50 μg/ml leupeptin, 100 μg/ml pepstatin and 20 μg/ml aprotinin before use. In some cases, the cells were also lysed in 4 ml digitonin lysis buffer [1% digitonin, 10 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, adjusted to pH 7.5 (Oettgen *et al.*, 1986)]. In pulse–chase experiments with a short chase period, 2× lysis buffer was added directly to the culture. Cell lysates were pre-cleaned by incubation with 200–350 μg purified normal goat Ig or normal rat Ig overnight at 4°C and further incubated with 300–400 μl of Staph A (50% vol/vol) for 6–8 h at 4°C. After that, they were centrifuged at 35 000 r.p.m. (RP-55T-348, Hitachi) for 60 min followed by incubation with 300–400 μl of 50% vol/vol protein A sepharose (Pharmacia, Uppsala, Sweden). Specific immunoabsorption was carried out by incubating 4–5 ml of the pre-cleaned lysates with 5–10 μg purified goat anti-μ antiserum or 10–20 μg rat monoclonal anti-κ antibody 187.6 for 12 h at 4°C, followed by a second incubation with 60 μl of 50% w/v protein A sepharose for 2–3 h at 4°C. Pellets were washed twice with PBS lysis buffer before being resuspended in electophoresis sample buffer containing 8 M urea.

Binding inhibition assay

Metabolically labeled cell lysates were pre-cleaned with normal goat serum and incubated with 5 μg purified goat anti-μ antiserum for 2 h in the presence or absence of 150 μg MOPC-104E, HOPC-1, J606 or Ls136 ($\gamma 1/\kappa$). The immunoprecipitates were collected on protein A sepharose and the samples were analyzed on SDS–polyacrylamide gels as described.

Gel electrophoresis

One-dimensional SDS–PAGE was carried out as described previously (Takemori *et al.*, 1987). Two-dimensional diagonal electrophoresis was performed according to Goding and Harris (1981). Cell lysates immunoprecipitated with anti-μ antiserum from iodinated or metabolically labeled cells were electrophoresed under non-reducing conditions on a 12% SDS–acrylamide gel. The materials in a sliced gel were reduced in the presence of 10% 2-ME, 0.1% SDS and 100 mM Tris, pH 6.8, for 2–3 h. The sliced gel was then placed on top of a 13 or 14% polyacrylamide–SDS gel and run in the second dimension. We used pre-stained protein mol. wt standards (6041LA, BRL, Bethesda) to standardize the size of immunoprecipitates on SDS gel. BRL standards contained hen egg lysozyme (14.4 kd), β-lactoglobulin (18.4 kd) and α-chymotrypsinogen (25.7 kd) for low mol. wt markers.

Measurement of accumulation of inositol phosphate

Accumulation of inositol phosphatase was measured by the modified method as previously described (Mizuguchi *et al.*, 1987). Briefly, cells (1×10^7/ml) were loaded with 20 μCi of myo[^{3}H]inositol and incubated overnight. They were washed and resuspended in RPMI 1640 supplemented with 5% FCS and 10 mM LiCl. The cells were stimulated with 30 μg/ml $F(ab')_2$ fragment of goat anti-mouse IgM antibodies. The reactions were stopped with the addition of chloroform/methanol buffer. The upper aqueous phase was applied to a Dowex-1 column and inositol triphosphate was eluted. The ^{3}H content was determined by liquid scintillation counting.

Determination of intracellular free calcium concentration ($[Ca^{2+}]_i$)

The cells were loaded with 5 mM Fura 2/AM (Dojin, Kumamoto, Japan) for 30 min at 37°C as previously described (Mizuguchi *et al.*, 1988). The fluorescence of the Fura 2 loaded cells was monitored with a spectrofluorometer (Shimadzu RF-5000).

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