Biochemical Nature of an Fcμ Receptor on Human B-Lineage Cells

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Summary

An IgM-binding protein of ~60 kD has been identified on activated B cells, but not on resting and activated T cells, monocytes, or granulocytes. Here, we characterize this IgM-binding protein as a receptor for the Fc portion (CH3 and/or CH4 domains) of IgM molecules (FcμR). The FcμR can be expressed as a cell surface activation antigen throughout the pre-B and B cell stages in differentiation. Receptor expression is not directly linked with IgM production, as both μ pre-B cells and isotype-switched B cells may express the FcμR. The receptor molecules produced by both pre-B and B cells are identical in size and are characterized as an acidic sialoglycoprotein with O-linked, but no N-linked, oligosaccharide. The FcμR is anchored to the surface of B-lineage cells via a glycosyl phosphatidylinositol linkage. The FcμR is thus the third member of a family of Fc receptors expressed on B-lineage cells, and its preferential expression on activated B cells suggests a potential role in the response to antigens.

Receptors for the Fc portions of all Ig isotypes (FcR)1 have been identified on cells of the immune system (for review see references 1–3). The binding of Ig molecules to cell surface FcR can trigger immunologic functions that vary according to their Ig isotype specificity and the cell type. IgG antibodies mediate cellular cytotoxicity by macrophages and NK cells (3, 4) and phagocytosis of opsonized particles by macrophages and granulocytes (3, 5). IgE antibodies induce the release of chemical mediators by mast cells and basophils (6, 7). Because the FcR are essential for multiple immune system functions, understanding their structure and function has become an important focus in immunology. Considerable progress has been made in elucidating the structural diversity of FcγR and FcεR, especially through the use of mAbs and gene cloning (for review see references 8–13), but FcR for the other Ig isotypes are still largely operationally defined entities.

Functional FcμR have been reported on subpopulations of T and B cells in both humans and mice (14–22), and indirect evidence suggests that IgM binding may enhance lymphocyte responses to antigens and polyclonal mitogens (22–24). In an earlier study, we identified an IgM-binding protein of ~60 kD on activated B cells, but could not detect this molecule on resting and activated T cells, monocytes, or granulocytes (25). In the present study, we characterize the IgM-binding protein further, show that it is truly an FcμR, and demonstrate its expression as an activation antigen throughout the pre-B and B cell stages in differentiation.

Materials and Methods

Antibodies. The following mouse mAbs were used in this study: (a) MH-59-10 (mouse μK isotype), SA-DA4-4 (γκ), LP-13B2-3 (γκ), and LP-13A3-6 (γκ) are specific for human μ chains (25, 26); (b) BH-EB2-1 (μκ), NC-BB2-6 (γκ), and GH-EA5-1 (γκ) are specific for human γ chains (25, 27); (c) CH-EB6-8 (γκ) is an anti-human α chain antibody (27); (d) 57.1 (μκ) and 35.3 (γκ) have anti-DNP specificity (25); (e) Cla (μκ) is specific for a chicken la determinant (26); (f) 1A10 (γκ,κ, a kind gift of Dr. V. Nussenzweig, New York University School of Medicine, New York) is specific for decay-accelerating factor (DAF; reference 29); and (g) Leu-16 (γκ) is an anti-CD20 mAb (Becton Dickinson & Co., Mountain View, CA). All mAbs with specificity for human Ig determinants were purified from ascites fluid by affinity chromatography on Sepharose 4B columns coupled with the appropriate human Ig. Other mAbs, except for 1A10 and Leu-16, were isolated by DEAE ion-exchange cellulose- and Sepharose-6B gel filtration chromatography as described (26, 27). FITC-labeled or biotinylated, affinity-purified goat antibodies specific for mouse Ig, μ, or κ determinants and free of crossreactivity with human Ig (Southern Biotechnology

References

1. Abbreviations used in this paper: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; DAF, decay-accelerating factor; FcR, Fc receptor; GPI-PLC, glycosyl phosphatidylinositol-specific phospholipase C; rLT, r-Lymphotoxin; SAC, Staphylococcus aureus Cowan I.
Preparation of Myeloma Proteins, IgM Fragments, and IgM Deletion Mutants. Human IgM, IgA, and IgG myeloma proteins and normal IgG were purified from serum samples by euglobulin or salt fractionation, DEAE ion-exchange cellulose, and Ultrogel AcA 22 gel filtration column chromatography as described (26, 27, 31). The Fc and Fab fragments were prepared from a human IgMx preparation as described by Plaut and Tomasi (32). The purity of each fragment was confirmed by SDS-PAGE analysis under both reducing and nonreducing conditions and by ELISA using anti-μ and anti-κ mAbs. For studies using IgM domain deletion mutants, culture supernatants were obtained from the mouse hybridoma clones secreting IgM with well-characterized heavy chain (CH) domain deletions: clone 43 (CH1 deletion, H2L2), clone 427 (CH1-2 deletion, H2L3), and their parental clone Sp6.18 (intact IgM), and clone 128 (CH1-3 deletion, HL), clone 208 (CH1-3 deletion, HL), and 482 (CH4 deletion, H2L3) (33-35) (generous gifts from Dr. M. J. Shulman, University of Toronto, and Dr. G. M. Köhler, Max Planck Institute for Immunology, Freiburg). The concentration of IgM, estimated by ELISA using rat anti-μ/mouse κ mAb for coating plates and alkaline phosphatase-labeled goat anti-mouse κ antibody as a developing reagent was adjusted to 0.05 mg/ml.

Cell Preparation and Activation. The preparation of blood mononuclear cells from normal donors and the enrichment of B cells by depletion of T cells with SRBC rosette formation and of monocytes with adherence to plastic dishes have been described elsewhere (25, 26). Acute and chronic lymphocytic leukemia (ALL and CLL) populations (>95% purity estimated by immunophenotypic profiles) were obtained from patients’ blood samples. The hemopoietic cell lines used in this study are listed in Table I. All were free of Mycoplasma when tested by Bisbenzimide H33258 Fluorochrome staining (Hoeschst dye 33258; Calbiochem-Behring Corp., San Diego, CA). For cell activation, cells (0.7-1.0 x 10^6) resuspended in 1 ml of RPMI 1640 containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5 x 10^-5 M 2-ME were cultured at 37°C for 16 h with or without the following stimuli: 0.1-100 nM PMA, a mouse IgM mAb (C1a, 3-1,000 μg/ml), 0.0001-0.1% heat-killed Staphylococcus aureus Cowan I (SAC), a mitogenic anti-human μ mAb (SA-DA4-4; 0.1-100 μg/ml), rIFN-α (Roche-Takeda Co., Tokyo, Japan), rIFN-β (Kyowa Hakko, Tokyo, Japan), rIFN-γ (Amgen Biologicals, Thousand Oaks, CA), H2-L2 (BIOgene Co. Ltd., Geneva, Switzerland), rIL-4 (Inmunex, Seattle, WA), rTNF (Genetech, San Francisco, CA), or r-Lymphotoxin (rLT Genetech) at concentrations of 10-1,000 U/ml. In other experiments, cells were incubated with PMA (10 nM) or mouse IgM (100 μg/ml) for various intervals (0.5-72 h).

Immunofluorescence Analysis of Cell Surface Molecules. For studies of IgM binding, viable cells (0.5-1.0 x 10^6) were incubated for 20 min at 4°C with or without 10 μl of highly purified mouse IgM mAbs with irrelevant specificities (C1a or 57.1; 0.1 mg/ml), washed, and stained with 10 μl of biotinylated goat anti-mouse μ antibody (0.05 mg/ml) or rat anti-mouse κ mAb (0.1 mg/ml) as described (25, 31). PE-labeled streptavidin (10 μl; 0.01 mg/ml) was used as a developing reagent. Cells were analyzed by flow immunocytometry using a FACSScan (Becton Dickinson & Co.) equipped with a Consort 30 data managing program. In an inhibition assay for IgM binding, cells were incubated with PMA (10 nM) or mouse IgM (100 μg/ml) for various intervals (0.5-72 h).

Table 1. Expression of the IgM Receptor

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell line</th>
<th>PMA (-)</th>
<th>PMA (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-B cell</td>
<td>BLIN-1</td>
<td>1.5</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>697</td>
<td>1.3</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>SMS-SB</td>
<td>1.2</td>
<td>15.2</td>
</tr>
<tr>
<td>B cell</td>
<td>Ramos</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Daudi</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>CESS</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Dakiki</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Plasma cell</td>
<td>RPMI 8226</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>T cell</td>
<td>Jurkat</td>
<td>1.2</td>
<td>1.3</td>
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<tr>
<td></td>
<td>CEM6</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Hut78</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Myeloid</td>
<td>U937</td>
<td>1.1</td>
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</tr>
<tr>
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<td>PLB985</td>
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<td>1.2</td>
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<tr>
<td>Erythroid</td>
<td>K562</td>
<td>1.4</td>
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* The ratio of mean fluorescence intensity between IgM binding and background.

Activated cells (3 x 10^6) were surface labeled with 1 mCl Na^22I (Amersham Corp., Arlington Heights, IL) by the lactoperoxidase method (36) and lysed in 1 ml of 1% NP-40 in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, leupeptin (1 μg/ml), antipain (2 μg/ml), trypsin (10 U/ml), benzamidine (10 μg/ml), and 1 mM PMSF (Mabey Chemical Co., New York). After centrifugation (15,600 g for 20 min at 4°C), IgM-binding molecules were isolated from the cleared lysates by either the previously described solid phase immunoaffinity technique (25-27) or the following immunoadsorbent bead-mediated immunoprecipitation. Briefly, 1 ml of the cell lysate was sequentially incubated with 100 μl of Sepharose 4B coupled with human albumin (5 mg/ml of beads) and with normal human IgG (5 mg/ml of beads) for 6-15 h at 4°C with rotation. This precleared lysate was incubated with 20 μl of Sepharose 4B coupled with a human IgMx myeloma or a C1a (mouse IgMx) mAb (2.5 mg/ml of beads) for 15 h at 4°C. After extensive washing with lysis buffer, bound molecules were dissociated by addition of Laemmli's sample buffer (37), and resolved by SDS-PAGE analysis using 10% acrylamide. For glycosidase digestion, IgM-bound molecules were incubated at 37°C with: (a) N-glycanase (Genzyme, Boston, MA) at 2-50 U/ml for 16 h; (b) neuraminidase (Sigma Chemical Co.) at 0.05 U/ml for 12 h; or (c) neuraminidase at 0.05 U/ml for 12 h and O-glycanase (Genzyme) at 4 U/ml for 4-12 h according to the manufacturer's recommendation. The digested materials were then analyzed by SDS-PAGE. For two-dimensional gel analysis, IgM-bound molecules were resuspended in 9.5 M urea/2% NP-40/2% Ampholines (pH range 3.5-9.5)/5% 2-ME, and separated first in tube gels according to isoelectric points, overlaid onto slabs of SDS-PAGE, and then run in the second dimen-
sion according to molecular weight as described by O'Farrell et al. (38).

**Biosynthetic Analysis of IgM-R.** Cells (3 × 10⁶) pretreated with PMA (10 nM for 12 h) were metabolically labeled with ³⁵S-Met and ³⁵S-Cys (100 µCi each) for 6 h in the presence of PMA. For inhibition of N-linked glycosylation, cells were incubated with tunicamycin (2.5 µg/ml; 1.5 h at 37°C), washed, and then labeled in the presence of PMA and tunicamycin. Radiolabeled IgM-R molecules were isolated from both the culture supernatant and cell lysate (1% NP-40 lysis buffer), and resolved by SDS-PAGE analysis.

**Enzyme Treatment of Cells.** PMA-activated cells (5 × 10⁶/ml) were incubated for 30 min at 37°C in 10 mM Hepes-HBSS, pH 7.4, containing various amounts of glycosyl phosphatidylinositol-specific phospholipase C (GPI-PLC; from Bacillus thuringiensis; enzyme activity: 0.35 µM/min/ml), a generous gift of Dr. M. G. Low (Columbia University, New York) (39). After treatment, cells were washed with PBS containing 5% FCS, centrifuged on a Ficoll-Hypaque gradient to eliminate dead cells, and assayed for IgM-R and cell surface antigens by flow immunocytofluorometry. In some experiments, surface-iodinated cells were similarly treated with GPI-PLC, and the treated cell pellet and the supernatant were subjected to immunoprecipitation of IgM-R molecule and SDS-PAGE analysis.

**Results**

**Cellular Distribution and Regulation of IgM-R Expression.** A panel of hemopoietic cell lines representative of different lineages and of different stages in B cell differentiation were examined in order to confirm the B-lineage restriction of IgM-R expression (25) and to identify a prime cell source for isolation of the IgM-R molecules. The pre-B cell lines (697, BLIN-1, and Nalm6) exhibited a slightly shifted immunofluorescence profile for IgM binding (Fig. 1), but none of the other cell lines constitutively expressed IgM-R in detectable levels. However, IgM binding was easily demonstrated on PMA-activated pre-B cell lines (697, BLIN-1, SMS-SB > Nalm6) and to a lesser extent on PMA-activated B cell lines (Raji > Ramos) (Fig. 1 and Table I). Maximal IgM-R expression was obtained at a PMA concentration between 1 and 100 nM (Fig. 2 A). The induction of IgM-R expression by the cultured pre-B cells occurred as early as 16 h after PMA activation and persisted beyond 72 h without substantial cell death (Fig. 2 B). In agreement with previous findings (40-43), these pre-B cell lines were found to express µ chains both in the cytoplasm and on the cell surface in small amounts, the surface level of which was downregulated by PMA stimulation (data not shown). None of the cell lines of other lineages (three T cell, three myeloid, and one erythroid) were capable of binding IgM even after PMA activation (Table I).

Unlike normal B cells (25), the 697 pre-B cells did not express IgM-R after treatment with a mitogenic anti-µ mAb (DA4.4; 0.1-100 µg/ml) or SAC (0.0001-0.1%), or with a variety of other stimuli, including rIFN-α, -β, or -γ, rIL-2 or -4, rTNF, and rLT (10¹-10³ U/ml). In contrast, both mouse and human IgM induced the expression of IgM-R on 697 pre-B cells in a dose-dependent manner (Fig. 2 A). IgM-R expression was maximal within 30 min after incubation with IgM, whereas longer exposure to PMA was required for maximal stimulation (Fig. 2 B). Note also the higher level of IgM-R expression induced by its natural ligand. This upregulation by IgM was dependent on the continuous presence of the ligand, as removal of IgM from the culture resulted in a time-dependent decline of IgM-R expression by 697 pre-B cells.

Fresh leukemic samples of B-lineage cells (four ALL, eight CLL) were examined for their IgM-binding capabilities. In most cases, the leukemic cells were capable of binding IgM at significant levels, and all of them exhibited enhanced binding of IgM after overnight culture with PMA (see Fig. 1). This result contrasts with the low or undetectable levels of IgM-R expression by unstimulated pre-B and B cell lines, but parallels the IgM-R expression noted previously on subpopulations of fresh tonsillar or splenic B cells (17, 25). The results of this survey also indicate that IgM-R expression is not directly associated with the intracytoplasmic or surface expression of IgM molecules, since one of the ALL samples had neither cytoplasmic nor surface µ, and two of the eight CLL samples had undergone isotype switching: one expressed surface IgGk and the other surface IgAll.

**Characterization of IgM-R Molecules.** To determine whether IgM-R on pre-B cells are the same as the polypeptide chain of ~60 kD previously identified on activated B cells (25),
iodinated IgM-R molecules from the four pre-B cell lines were examined by SDS-PAGE analysis. An IgM-binding molecule with an apparent Mr of 58,000 ± 3,000 (mean ± 1 SD from 30 experiments) was identified on all four pre-B cell lines by using either mouse or human IgM as the ligand (see Fig. 3). The same molecular mass estimate was obtained under both reducing and nonreducing conditions. The molecular size identity of IgM-R from pre-B and B cells was confirmed on a gel in which the IgM-R isolated from different cell sources (i.e., PMA-activated, pre-B cell lines, IgMK/IgDK ALL, and normal B cells) were subjected to electrophoresis in adjacent lanes (data not shown). In agreement with the immunofluorescence results, PMA-activated pre-B cells yielded a more intense 58-kD band than did unstimulated pre-B cells (Fig. 3, lanes 6 and 7 vs. 2 and 3). The inverse relation was observed for the ~78-kD μ H chain expressed on their cell surface; PMA-activated pre-B cells gave a less intense μ chain band than did the unstimulated pre-B cells (Fig. 3, lane 5 vs. 1). When an IgG1κ anti-μ mAb was used to immunoprecipitate the μ H chains on the surface of pre-B cells, a protein with the same molecular mass as the IgM-R was coprecipitated, suggesting (15) that the IgM-R may associate with its crosslinked IgM neighbors (Fig. 3, lane 1). Additional discrete proteins of 45 and 18-22 kD were also coprecipitated with μ chains on the pre-B cell line. The former is most likely actin, while the latter represents the surrogate L chain complex, thought to be encoded by Vpre-B and A5 genes and to be human homologues of mouse ω and L proteins (44-48; Ohno et al., manuscript in preparation).

When isotype-switched B CLL samples (IgG1κ and IgA1λ) were examined for IgM-R molecules, the same 58-kD band

![Figure 2](image.png)

**Figure 2.** Effect of PMA and IgM stimulation on the expression of IgM-R by the 697 pre-B cell line. (A) Dose-dependent induction of IgM-R expression. Cells were incubated for 16 h with various doses of either PMA or IgM, washed, then assessed for IgM binding by immunofluorescence as described in Materials and Methods. Results were expressed as an IgM-binding index, which was determined by: (x of staining of PMA- or IgM-stimulated cells - x of background control staining of PMA- or IgM-treated cells)/(x of staining of nonstimulated cells - x of background control staining of nonstimulated cells); where x indicates the mean fluorescence intensity value. (B) Time-course analysis of PMA- and IgM-induced IgM-R expression. Cells were incubated for varying time intervals with PMA (10 nM) and mouse IgM (100 μg/ml), washed, then examined for IgM-R expression as described above.

![Figure 3](image.png)

**Figure 3.** PMA-induced upregulation of IgM-R expression on the 697 pre-B cell line. The 697 pre-B cells (3 × 10⁶) incubated with or without PMA (10 nM, 16 h) were labeled with 1 mCi Na²¹⁵I, and their NP-40-solubilized membrane lysates were added to wells precoated with the following mouse mAbs: lanes 1 and 5, SA-DA4-4 anti-human μ (γκε); lanes 2 and 6, Cla anti-chicken Ia (μκ); lanes 3 and 7, 57.1 anti-DNP (μκ); lanes 4 and 8, 33.5 anti-DNP (γκε). Bound materials were analyzed by SDS-10% PAGE under reducing conditions. An IgM-binding molecule of 58 kD was also identified on the BLIN-1, SMS-SB, and Nalm 6 pre-B cell lines after PMA activation by using either mouse or human IgM as the ligand.
was identified on both types of PMA-activated CLL cells using IgM mAbs regardless of their specificity (data not shown). mAbs of other isotypes but with the same specificity as the IgM mAb did not precipitate this molecule.

To determine their glycosylation status, IgM-R were isolated from either PMA-activated B-CLL or 697 pre-B cells, digested with various glycolytic enzymes, and then analyzed by SDS-PAGE. The molecular mass of the IgM-R from both cell sources was not altered by treatment with N-glycanase (2–50 U/ml), while the size of the surface μ H chain as a control was reduced to ~68 kD (data not shown). This suggests that N-linked oligosaccharides are not present on IgM-R molecules, since N-glycanase-resistant N-linked carbohydrates have not been reported (49). Removal of sialic acid residues with neuraminidase from the 58-kD IgM-R molecule resulted in a decrease of molecular mass to 51 kD (Fig. 4 A, lanes 5 and 3). Sequential digestions of the IgM-R with neuraminidase and O-glycanase did not result in further reduction in size of the desialylated IgM-R molecule (Fig. 4 A, lane 1). In two-dimensional IEF gel analysis, the IgM-R isolated from PMA-activated B CLL cells was resolved into a spot with isoelectric points (pI) of 5.6–6.0 (Fig. 4 B). The desialylated IgM-R molecule was shifted to a more basic spot with pI > 7.2 (not shown).

These findings indicate that the IgM-R on both pre-B and B cells is a single sialoglycoprotein, with M₉ of ~58,000 and pI of 5.6–6.0, that has O-linked, but no N-linked, oligosaccharides.

IgM C Regions Involved in Binding to the IgM-R. A quantitative immunofluorescence inhibition assay using various Ig isotypes and IgM fragments as inhibitors was used to examine the binding specificity of the IgM-R. Human IgMk inhibited the binding of a mouse IgMk to PMA-activated 697 pre-B cells in a dose-dependent manner, while human IgG and IgA did not (Fig. 5 A). The IgM binding was inhibited by Fc₃μ fragments, but not by Fab₃μ fragments (Fig. 5 B). Fig. 6 A shows a representative inhibition profile for IgM binding with a 64-fold excess of inhibitor proteins. To confirm that the Fc portion of IgM molecules is the specific ligand for the 58-kD cell surface receptor, the same panel of inhibitors was used during immunosolagation of the IgM-R molecule from activated pre-B cells. As shown in Fig. 6 B, addition of a 100-fold excess of the intact IgM or its Fc₃μ fragments inhibited completely the binding of the 58-kD surface protein to IgM-coated beads (lanes 4 and 2), while Fab₃μ fragments had no effect on ligand-mediated isolation of the IgM-R molecule (lane 3).

To determine which C region domain(s) of the μ H chain (C₉) is involved in IgM-R binding, culture supernatants from mouse hybridoma clones secreting mutant IgM molecules with C₉ domain deletions were used as ligands in both immunofluorescence and immunosolagation assays. The C₉₁ deletion mutant 43 bound to activated pre-B cells as well as did the parental, intact IgM from Sp6 (Fig. 7 A). A lesser, but significant binding was also observed using two different C₉₁-2 deletion mutants, 128 and 427. In contrast, the C₉₁-3 deletion mutant 208 and the C₉₄ deletion mutant 482 did not exhibit binding activity. The same conclusion was drawn from the immunosolagation assay in which the IgM mutants with either C₉₁ or C₉₁-2 deletion precipitated the 58-kD IgM-R molecule from activated pre-B cells, whereas those with either C₉₁-3 or C₉₄ deletion did not (Fig. 7 B).

Phosphatidylinositol Linkage of the Cell Surface IgM-R. When IgM-binding activity was examined by immunofluorescence after treatment of PMA-activated pre-B cells with GPI-PLC, the level of IgM binding was reduced by 89%. By compar-

![Figure 4. Characterization of the IgM-R as an acidic sialoglycoprotein. (A) Glycosidase digestion of IgM-R molecule. The membrane lysates from radiolabeled PMA-activated CLL cells were added to the wells precoated with a mouse IgMk mAb (57.1 anti-DNP; lanes 1, 3, and 5) or a mouse IgG1a mAb (35.3 anti-DNP; lanes 2, 4, and 6). Bound materials were digested without (lanes 5 and 6) or with neuraminidase (lanes 3 and 4) or neuraminidase plus O-glycanase (lanes 1 and 2), and analyzed by SDS-10% PAGE. (B) Two-dimensional gel electrophoretic analysis of IgM-R molecule. IgM- or IgG-bound membra materials isolated from radiolabeled PMA-activated CLL cells were separated first according to isoelectric points in tube gels, and then run according to molecular size in slabs of SDS-PAGE.]
Figure 5. Evaluation of the Ig isotype specificity of the IgM-R. (A) PMA-activated 697 pre-B cells (5 x 10^5) were incubated with the inhibitor human paraproteins: IgA1X (●), IgG4X (▲), or IgMA (O) before incubation with the indicator mouse IgM mAb (anti-chicken λ; 0.1 mg/ml). Cell-bound IgM was detected by biotinylated rat anti-mouse κ mAb and PE-labeled streptavidin. Immunofluorescence was analyzed by flow cytometry. Results were expressed as percent mean fluorescence estimated by: 100 x [(X of IgM binding with inhibitors - X of background control)/(X of IgM binding without inhibitors - X of background control)]; where X indicates the mean fluorescence intensity value. (B) PMA-activated 697 pre-B cells (5 x 10^5) were incubated with a human IgMparaprotein (O) and its Fc5μ (●) and Fabp (▲) fragments for 30 min at 4°C. The cells were then stained and analyzed by flow cytometry as described above.

Figure 6. Fcμ-dependent inhibition of IgM-R binding by IgM. (A) Representative inhibitory activity of IgM and its fragments. PMA-activated 697 cells (5 x 10^5) were first incubated with a 64-fold excess of inhibitor proteins, IgMx, Fc5μ, and Fab fragments for 30 min at 4°C. The cells were then examined by immunofluorescence for their capacity to bind mouse IgM as described in Fig. 5. Dashed lines indicate immunofluorescence profile for background controls; dotted lines for IgM binding without inhibitors; solid lines for IgM binding with the test inhibitors. (B) The membrane lysate from radiolabeled PMA-activated 697 cells (3 x 10^7) was incubated with mouse IgM (Cla)-coated Sepharose beads in the absence (lane 1) or the presence of 100-fold excess of the following inhibitors: Fc5μ (lane 2), Fabp (lane 3), and IgMx (lane 4). Bound material was analyzed by SDS-10% PAGE.

Comparison, GPI-PLC treatment reduced the expression of the GPI-anchored DAF protein (50) by 70%, while the expression of the transmembrane CD20 glycoprotein (see reference 51) was unaffected (Fig. 8, lower panel). GPI anchoring of the IgM-R molecule was also indicated by SDS-PAGE analysis. When radiolabeled pre-B cells were treated with GPI-PLC, large amounts of the 58-kD cell surface IgM-R were released into the supernatant (Fig. 9, lanes 1 and 4). In agreement with the immunofluorescence results (Fig. 8, upper panel), a significant amount of the IgM-R was also released into the medium in which radiolabeled cells were incubated at 37°C without GPI-PLC (Fig. 9, lane 2), suggesting the possibility of endogenous PLC or pre-B cell protease activity.

Biosynthesis of IgM-R Molecule. To determine whether the IgM-R molecule is actively secreted, PMA-stimulated pre-B cells were metabolically labeled and the cell supernatant and the cell lysate assessed for the presence of IgM-R molecules. As shown in Fig. 10, none could be detected on IgM-coated beads incubated with the culture supernatant of either unstimulated pre-B cells (not shown) or those activated with PMA (lane 5), indicating no active secretion of IgM-R molecule. In contrast, an IgM-binding protein with essentially the same molecular mass (57 ± 2 kD from 11 experiments) as the surface IgM-R was identified in the cell lysate of four pre-B cell lines and a pre-B ALL under both reducing and non-reducing conditions (Fig. 10, lane 2). The binding of this molecule to the IgM-coated beads was inhibited by addition of soluble IgM (Fig. 10, lane 3). Unlike cell surface IgM-R, the cytoplasmic form of the IgM-R was not obviously upregulated after PMA stimulation (data not shown). When pre-B cells were metabolically labeled in the presence of tunicamycin, the molecular mass of the newly synthesized
IgM-R molecule was unaffected (Fig. 10, lane 10 vs. 11). In contrast, the tunicamycin treatment reduced the size of \( \mu \) H chains from 79 to 68 kD, leaving the 78-kD BiP retention molecule (52) behind (Fig. 10, lane 8 vs. 9).

**Discussion**

These experiments define the IgM-binding molecule of \( \sim 60 \) kDa as an FcR on B lineage cells, its GPI linkage to the cell surface, and the stages in B cell differentiation when it can be expressed. The restricted expression of Fc\( \mu \)R on B-lineage cells indicated in our assay system is somewhat surprising in view of the numerous reports describing IgM binding by T cells. Early studies indicate that subpopulations of human T and B cells can bind IgM-coated ox erythrocytes in an isotype-specific manner (14, 17, 18, 22, 23), and IgM-binding T cells have also been identified in chickens and mice (15, 20, 24, 53). Detection of IgM-R on human T cells usu-

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**Figure 8.** Susceptibility of IgM-R to PI-PLC assessed by flow cytometry. PMA-activated 697 pre-B cells were incubated with or without PI-PLC, and then examined for the expression of IgM-R, DAF, and CD20. (Top) Solid lines indicate profiles of cells incubated at 37°C without PI-PLC. Dotted lines indicate profiles of cells at 4°C without PI-PLC. Dashed lines indicate background profiles for developing antibody controls, which were the same for the two different cell treatments. Note modest reduction (49%) in mean fluorescence intensity of IgM-R, but not of DAF or CD2, profile after cell incubation alone. (Bottom) Solid lines indicate profiles of cells incubated at 37°C with PI-PLC. Dotted lines indicate profiles of cells incubated at 37°C without PI-PLC. Dashed lines indicate background profiles for developing antibody. Note the striking reductions in mean fluorescence intensity of IgM-R (89%) and DAF (70%) profiles after PI-PLC treatment.
assay method. Given the variety of FcyR and FceR molecules that can be expressed by different cell types (8, 51), it seems possible that the 58-kD FcγR identified on B-lineage cells in the present study could be expressed on other types of cells after appropriate stimulation or may be a member of a fundamental difference in expression of FcγR by human B lineage cells via their Fc portion has been well documented (17, 25), both of which contain activated B cells. In many cases, leukemic cells of B-lineage constitutively expressed FcγR on their surface in significant levels (16-19, 24, 54). FcγR thus appears to belong to the family of cell surface molecules that have been characterized as activation antigens on B-lineage cells (51). Since some of these have been identified by IgM mAb (see references 55 + 56), the assignation to this category will require confirmation using assays assuring that antibody binding to the activated B cells is mediated by the Fab portion.

One objective of the present study was to obtain a suitable cell source for isolation of the FcγR molecule for further biochemical characterization. In this regard, several pre-B cell lines were found to be excellent sources since they expressed relatively large amounts of the FcγR after stimulation with PMA. The FcγR was characterized as a 58-kDialoglycoprotein with O-linked oligosaccharides. The lack of N-linked glycosylation, an unusual feature for a cell surface glycoprotein, was suggested by two observations. First, the molecular mass of iodinated surface FcγR was unaltered by treatment with N-glycanase in various concentrations, and N-linked oligosaccharides resistant to this enzyme have not been reported (49). Second, FcγR isolated from metabolically labeled cells were identical in size both in the presence and absence of tunicamycin. Removal of sialic acid residues with neuraminidase treatment instead resulted in a reduction of the apparent molecular mass from 58 to 51 kD and a change in pl from 5.6-6.0 to >7.2.

The FcγR appears to recognize configurations of the IgM molecule that are conferred by the Cγ3 and/or the Cγ4 domain(s). First, FcγR fragments inhibit IgM binding to FcγR, whereas the Fab fragments do not. FcγR fragments generated by the hot trypsin digestion of IgM are mainly composed of the Cγ4 domain(s), since the major cleavage site for this digestion is located within the Cγ4 domain (57). Second, IgM mutants with either Cγ1, Cγ3 or Cγ4 domain deletions could not bind the 58-kD FcγR.

It is interesting to contrast these results on FcγR expression by B-lineage cells in humans with those that have been obtained in mice. While a murine FcγR molecule has not yet been identified, the specific binding of IgM molecules to B-lineage cells via their Fc portion has been well documented using an immunofluorescence assay (21). Studies with domain deletion mutants indicated an essential role for the Cγ4 domain in the IgM binding to murine B cells (21). Only a minor difference has been observed in the pattern of IgM-binding capacity in the mouse in that pre-B cells and IgM+/IgD+ B cells could bind IgM molecules, but isotype-switched B cells and plasmacytomas or hybridomas apparently could not (21). In view of these findings, it seems likely that the FcγR, that we have characterized on human B-lineage cells will be found to be conserved in mice.

The FcγR in humans appears to be anchored via phos-
phatidyl-inositol to the surface of pre-B cells, as indicated by susceptibility to GPI-PLC enzymatic cleavage. GPI-PLC treatment of the PMA-activated pre-B cells resulted in almost complete elimination of FcµR expression and efficient release of the 58-kD surface FcµR into the medium. However, we also noted that significant amounts of soluble FcµR with similar molecular mass were released from PMA-activated pre-B cells incubated without GPI-PLC. Similar spontaneous release has been described for other GPI-anchored proteins: Qa-2 antigen, acetylcholinesterase (AChE), Thy-1 antigen, FcγR III. A soluble, but not actively secreted, form of the Qa-2 antigen can be identified in the supernatant of Con A-stimulated T cells (58, 59). AChE is released from certain areas of the brain and adrenal chromaffin cells in response to various stimuli (60). Similarly, the Thy-1 antigen on T cells (61) and FcγR III on granulocytes (62) can be spontaneously released from the cell surface. One possible mechanism for this spontaneous release of GPI-anchored proteins is that it is mediated by endogenous phospholipases, and the existence of GPI-specific phospholipase D in plasma (39) would suggest that this might occur in vivo. However, it is also noteworthy that DAF, another GPI-anchored protein present on pre-B cells, was not found to be released spontaneously in our experiments. The selective nature of the release might therefore favor the alternative possibility that the release could be the consequence of proteolytic cleavage near the position where the FcµR molecule might be processed by exchanging its COOH-terminal hydrophobic residues with the PI linkage elements. This mechanism has been invoked to explain the spontaneous release of a transmembrane form of FcγR III on NK cells (62). Whatever the true mechanism, the data suggest that the FcµR molecule may function not only as a versatile cell surface recognition unit but also as a soluble factor in immunoregulatory networks.

The IgM-R that we have characterized is thus the third member of a family of FcRs that are expressed by B-lineage cells. Members of this family exhibit binding specificity for IgG, IgE, or IgM. IgA may be the only major class of Ig for which an FcR cannot be found on human B cells (31, 63). Low affinity FcγR II in both humans and mice have been identified on B cells and phagocytic cells (64-66). However, they differ from each other in that alternate splicing of its nuclear transcript in B cells lends an additional intracytoplasmic segment that may prevent the phagocytic signal (64-66). Instead, occupation of the receptor by IgG antibodies that crosslink with neighboring membrane-bound Ig serves to inhibit B cell activation (67-69). The low affinity FcγR II (CD23) present on activated B cells instead serves to enhance responsiveness (70). While more information is needed to predict the precise role of the FcµR, its preferential expression on activated B cells and ability to bind both secreted IgM molecules and neighboring membrane-bound IgM molecules after their crosslinkage suggest a potential role in the B cell response. The enhancement of specific antibody responses by infusion of IgM antibodies, in contrast to their inhibition by IgG antibodies (71, 72), may imply the transmission of a positive signal by the FcµR molecule.
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