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 (C_{H3} and/or C_{H4} 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 μκ isotype), SA-DA4-4 ($γ_{1}κ$), LP-13B2-3 ($γ_{2a}κ$), and LP-13A3-6 ($γ_{2b}κ$) are specific for human μ chains (25, 26); (b) BH-EB2-1 (μκ), NC-BB2-6 ($γ_{1}κ$), and GH-EA5-1 ($γ_{2a}κ$) are specific for human γ chains (25, 27); (c) CH-EB6-8 ($γ_{1}κ$) is an anti-human α chain antibody (27); (d) 57.1 (μκ) and 35.3 ($γ_{1}κ$) have anti-DNP specificity (25); (e) CIa (μκ) is specific for a chicken Ia determinant (28); (f) IA10 ($γ_{2a}κ$, 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 ($γ_{1}κ$) 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 IA10 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

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[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.

Associates, Birmingham, AL) were used as developing reagents. In some experiments, biotinylated, affinity-purified 187.1 rat anti-mouse κ mAb (30) was used as the second antibody.

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 Fc5μ and Fabμ fragments were prepared from a human IgMκ 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 H chain (C_H) domain deletions: clone 43 (C_{H1} deletion, $H_{10}L_{10}$), clone 427 (C_{H1-2} deletion, H_2L_2), and their parental clone Sp6.18 (intact IgM), and clone 128 (C_{H1-2} deletion, HL), clone 208 (C_{H1-3} deletion, HL), and 482 (C_{H4} deletion, H_2L_2) (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–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 H 33258 Fluorochrome staining (Hoeschst dye 33258; Calbiochem-Behring Corp., San Diego, CA). For cell activation, cells (0.7–1.0 × 10^6) resuspended in 1 ml of RPMI 1640 containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5 × 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 (CIa; 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), rIL-2 (BIOgene Co. Ltd., Geneva, Switzerland), rIL-4 (Immunex, 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 × 10^6) were incubated for 20 min at 4°C with or without 10 μl of highly purified mouse IgM mAbs with irrelevant specificities (CIa 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 immunocytofluorometry using a FACScan (Becton Dickinson & Co.) equipped with a Consort 30 data managing program. In an inhibition assay for IgM binding, cells were incubated (30 min at 4°C) with various inhibitor proteins before assay for IgM binding. For the expression of CD20 and DAF antigens, Leu-16 and IA10 mAbs were used as well as isotype-matched control mAbs.

Immunoprecipitation of Iodinated Membrane Proteins. Activated cells

Table 1. *Expression of the IgM Receptor*

Cell type	Cell line	MFI* PMA (−)	MFI* PMA (+)
Pre-B cell	BLIN-1	1.5	19.8
	697	1.3	17.3
	SMS-SB	1.2	15.2
	Nalm6	1.4	3.5
	207	1.1	1.3
B cell	Raji	1.3	5.9
	Ramos	1.4	2.5
	Daudi	1.2	1.8
	CESS	1.4	1.4
	Dakiki	1.3	1.2
Plasma cell	RPMI 8226	1.0	1.2
T cell	Jurkat	1.2	1.3
	CEM6	1.1	1.4
	Hut78	1.4	1.5
Myeloid	U937	1.1	1.5
	PLB985	1.3	1.2
Erythroid	K562	1.4	1.2

* The ratio of mean fluorescence intensity between IgM binding and background.

(3 × 10^7) were surface labeled with 1 mCi Na^{125}I (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), trasylol (10 U/ml), benzamidine (10 μg/ml), and 1 mM PMSF (Mobay 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 immunoisolation 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 IgMκ myeloma or a CIa (mouse IgMκ) 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 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 × 10⁷) preactivated 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 > Nalm 6) 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 IgG1κ and the other surface IgA1λ.

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),
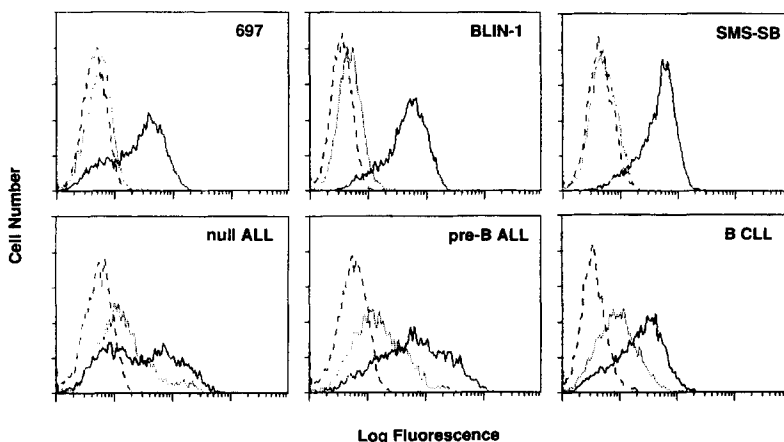

Figure 1. Immunofluorescence analysis of IgM binding by various types of B-lineage cells. Cells incubated with or without PMA (10 nM for 16 h) were stained as described in Materials and Methods. Solid lines represent the immunofluorescence profiles of cells cultured with PMA, and dotted lines represent the cells cultured without PMA. Dashed lines represent control profiles, developed with the secondary antibody, which were virtually identical for cells cultured with or without PMA. Note that PMA consistently induces an increase in IgM-binding capacity for pre-B and B cells.

A

B
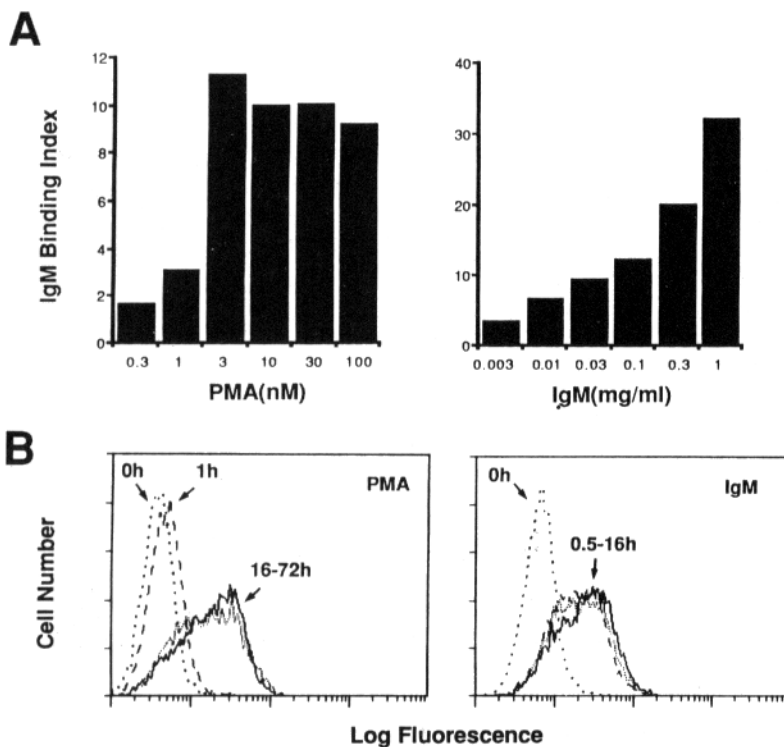
Figure 2. Effect of PMA and IgM stimulation on the expression of IgM-R by the 697 pre-B cell line. (*A*) Dose-dependent induction of IgM-R expression. Cells were incubated for 16 h with various doses of either PMA or IgM, washed, then assessed for IgM binding by immunofluorescence as described in Materials and Methods. In the case of incubation with mouse IgMκ, biotinylated rat anti–mouse κ mAb was added immediately after washing the cultured cells. Results were expressed as an IgM-binding index, which was estimated by: (*x* of staining of PMA- or IgM-treated cells − *x* of background control staining of PMA- or IgM-treated cells)/(*x* of staining of nontreated cells − *x* of background control staining of nontreated 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.

iodinated IgM-R molecules from the four pre-B cell lines were examined by SDS-PAGE analysis. An IgM-binding molecule with an apparent M_r 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, IgMκ/IgDκ 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 λ5 genes and to be human homologues of mouse ω and ι 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
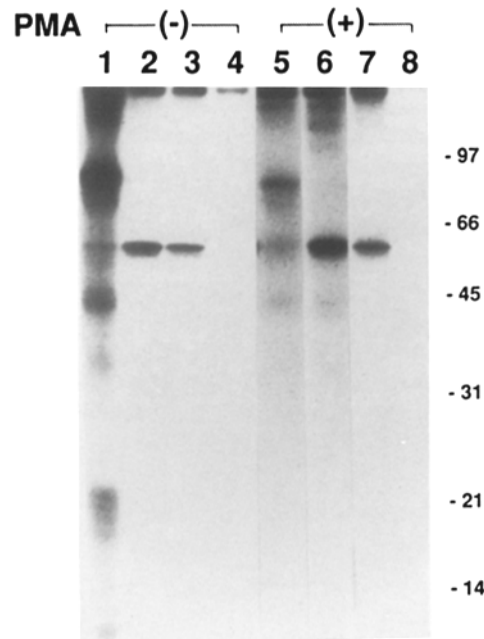
Figure 3. PMA-induced upregulation of IgM-R expression on the 697 pre-B cell line. The 697 pre-B cells (3 × 10⁷) incubated with or without PMA (10 nM, 16 h) were labeled with 1 mCi Na¹²⁵I, and their NP-40-solubilized membrane lysates were added to wells precoated with the following mouse mAbs: lanes *1* and *5*, SA-DA4-4 anti–human μ (γ₁κ); lanes *2* and *6*, CIa anti–chicken Ia (μκ); lanes *3* and *7*, 57.1 anti-DNP (μκ); lanes *4* and *8*, 35.3 anti-DNP (γ₁κ). 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 M_r 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 IgMκ inhibited the binding of a mouse IgMκ 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 Fc5μ fragments, but not by Fabμ 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 immunoisolation 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 Fc5μ fragments inhibited completely the binding of the 58-kD surface protein to IgM-coated beads (lanes 4 and 2), while Fabμ 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 (C$_H$) is involved in IgM-R binding, culture supernatants from mouse hybridoma clones secreting mutant IgM molecules with C$_H$ domain deletions were used as ligands in both immunofluorescence and immunoisolation assays. The C$_{H1}$ 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 C$_{H1-2}$ deletion mutants, 128 and 427. In contrast, the C$_{H1-3}$ deletion mutant 208 and the C$_{H4}$ deletion mutant 482 did not exhibit binding activity. The same conclusion was drawn from the immunoisolation assay in which the IgM mutants with either C$_{H1}$ or C$_{H1-2}$ deletion precipitated the 58-kD IgM-R molecule from activated pre-B cells, whereas those with either C$_{H1-3}$ or C$_{H4}$ 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-
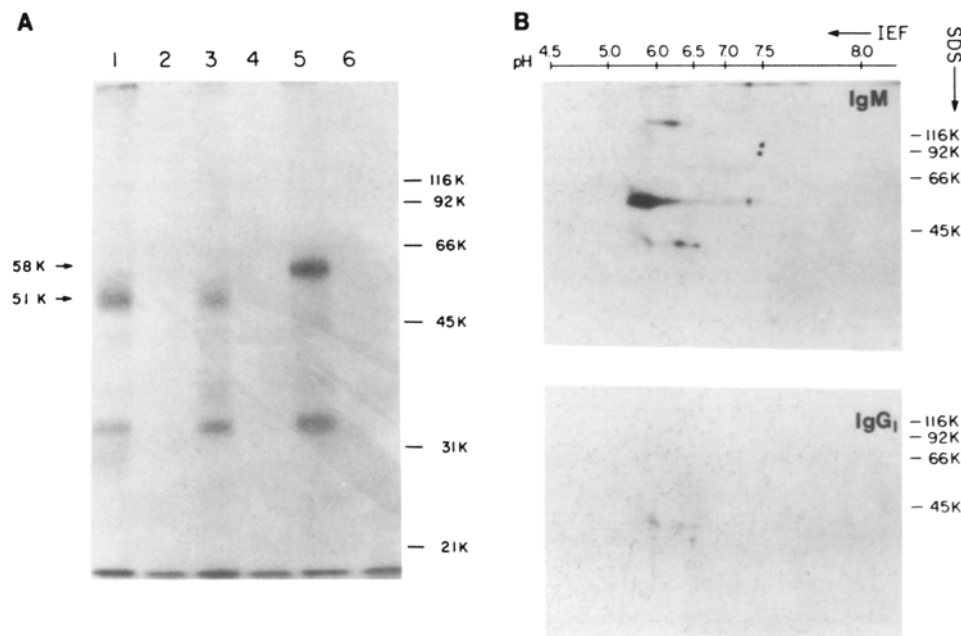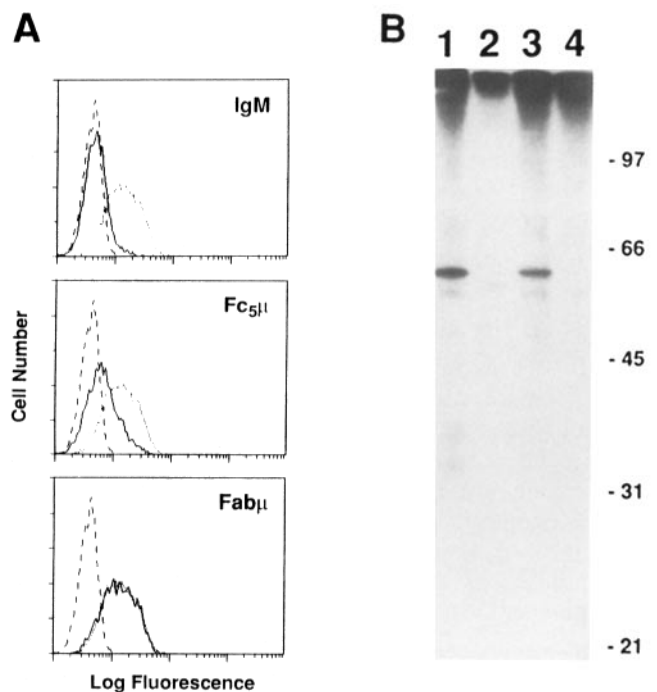

Figure 4. Characterization of the IgM-R as an acidic sialoglycoprotein. (A) Glycosidase digestion of IgM-R molecule. The membrane lysates from radiolabeled PMA-activated CLL cells were added to the wells precoated with a mouse IgMκ mAb (57.1 anti-DNP; lanes 1, 3, and 5) or a mouse IgG1κ mAb (35.3 anti-DNP; 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 membrane 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.
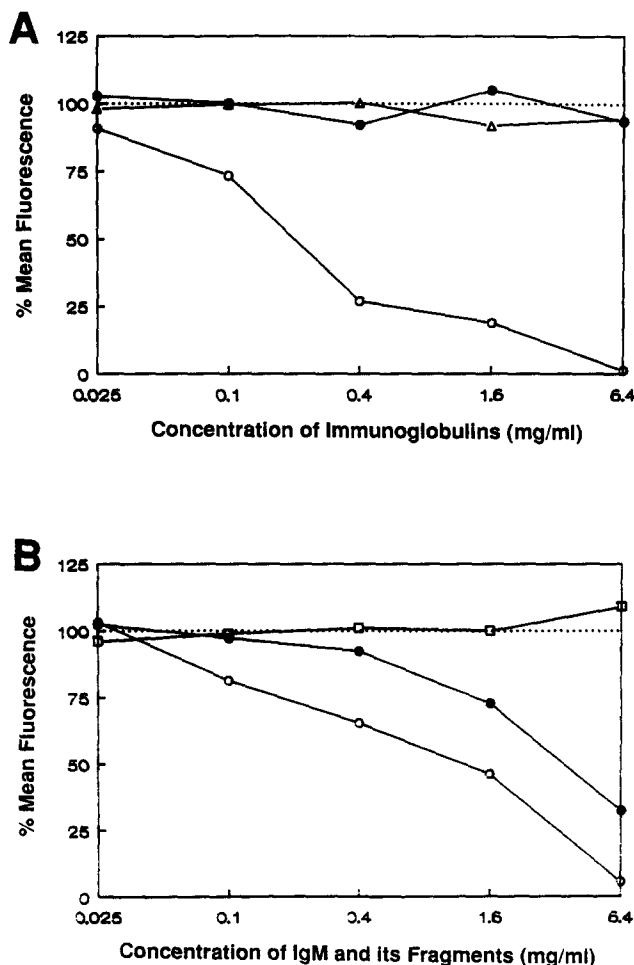
Figure 5. Evaluation of the Ig isotype specificity of the IgM-R. (A) PMA-activated 697 pre-B cells (5 × 10⁵) were incubated with the inhibitor human paraproteins: IgA1λ (●), IgG4λ (△), or IgMλ (○) before incubation with the indicator mouse IgM mAb (anti–chicken Ia; 0.1 mg/ml). Cell-bound IgM was detected 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 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 × 10⁵) were incubated with a human IgMκ paraprotein (○) and its Fc5μ (●) and Fabμ (□) 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 × 10⁵) were first incubated with a 64-fold excess of inhibitor proteins, IgMκ, 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 × 10⁷) was incubated with mouse IgM (CIa)-coated Sepharose beads in the absence (lane 1) or the presence of 100-fold excess of the following inhibitors: Fc5μ (lane 2), Fabμ (lane 3), and IgMκ (lane 4). Bound material was analyzed by SDS-10% PAGE.

ison, 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 culture 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

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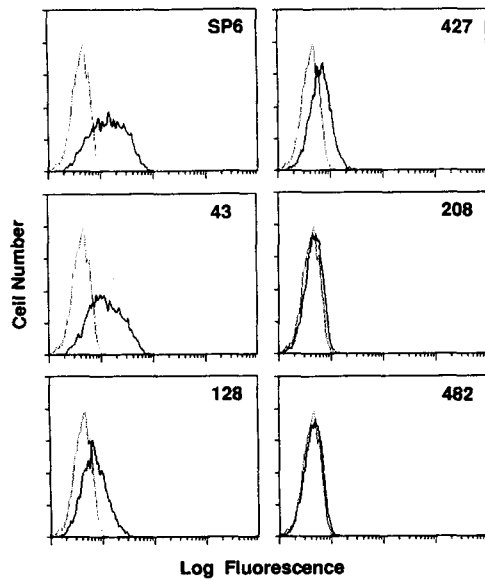
B
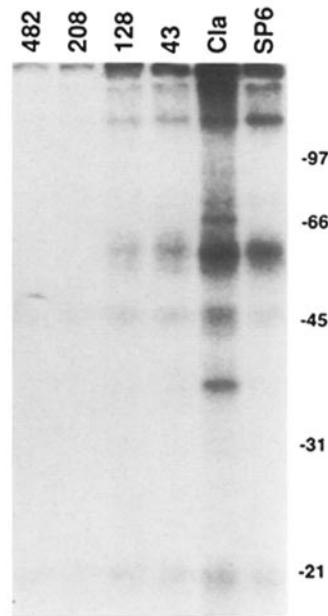
Figure 7. IgM C_H domain(s) involved in binding to IgM-R. (*A*) PMA-activated 697 cells were incubated with C_H deletional mutants or their parental wild-type SP6 IgM (0.5 μg/5 × 10^5 cells). IgM binding (*solid lines*) was assessed by immunofluorescence using biotinylated rat anti–mouse κ mAb and PE-labeled streptavidin. C_H deletional mutants used here are 43 (C_{H1}^-, $H_{10}L_{10}$), 128 ($C_{H1,2}^-$, HL), 427 ($C_{H1,2}^-$, H_2L_2), 208 (C_{H1-3}, HL), and 482 (C_{H4}^-, H_2L_2). (*B*) The membrane lysate from radiolabeled PMA-activated 697 cells was added to the wells precoated with the indicated C_H deletional mutants or intact mouse IgM (SP6 and CIa). Bound membrane proteins were analyzed by SDS-10% PAGE.

IgM-R molecule was unaffected (Fig. 10, lane *10* vs. *11*). In contrast, the tunicamycin treatment reduced the size of μ 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 ~60 kD 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 FcμR 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-
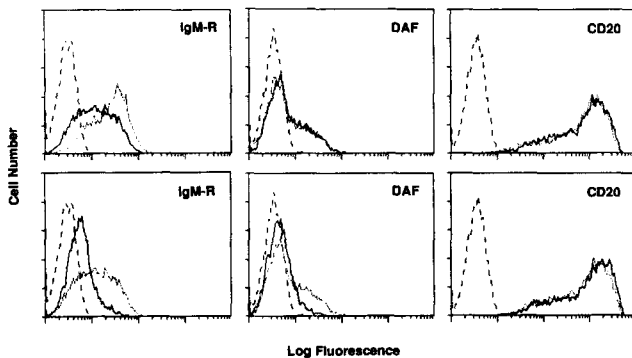
Figure 8. Susceptibility of IgM-R to PI-PLC assessed by flow cytometry. PMA-activated 697 pre-B cells were incubated with or without PI-PLC, and then examined for the expression of IgM-R, DAF, and CD20. (*Top*) Solid lines indicate profiles of cells incubated at 37°C without PI-PLC. Dotted lines indicate 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.
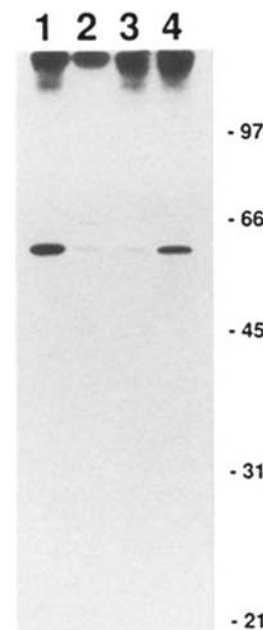
Figure 9. Release of membrane-bound IgM-R into the supernatant after PI-PLC treatment. Radiolabeled 697 pre-B cells (PMA activated) were incubated with (lanes *3* and *4*) or without PI-PLC (lanes *1* and *2*), centrifuged, and then lysed with NP-40 lysis buffer. Cell supernatants (lanes *2* and *4*) and cell lysates (lanes *1* and *3*) were examined for the presence of IgM-R using IgMκ-coated Sepharose beads and SDS-10% PAGE.
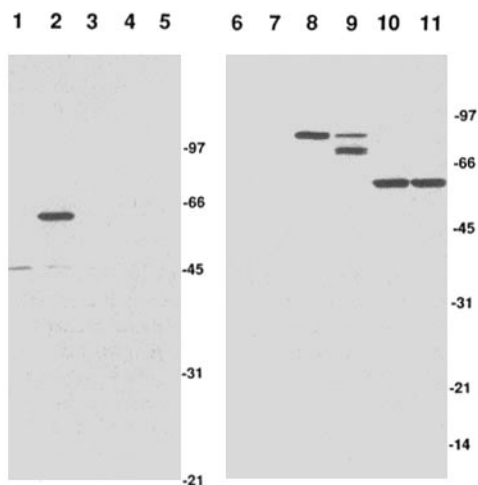
Figure 10. Biosynthetic analysis of IgM-R molecule. PMA-activated 697 pre-B cells (3 × 10^7) were metabolically labeled with ^{35}S-Met and ^{35}S-Cys (100 μCi each) for 6 h in the absence (lanes 1–6, 8, and 10) or presence (lanes 7, 9, and 11) of tunicamycin (2.5 μg/ml). The 1% NP-40-solubilized cell lysate (lanes 1–3 and 6–11) and the culture supernatant (lanes 4 and 5) were subjected to immunoprecipitation with the following Ig-coated Sepharose beads: lanes 1 and 4, normal human IgG; lanes 2, 5, 10, and 11, a human IgMκ paraprotein; lane 3, a human IgMκ paraprotein in the presence of a 100-fold excess of soluble mouse IgMκ (CIa); lanes 6 and 7, 35.3 anti-DNP mAb (γ₁κ); lanes 8 and 9, SA-DA4-4 anti–human μ mAb (γ₁κ). Bound materials were analyzed by SDS-10% PAGE under both nonreducing (not shown) and reducing conditions. The same results were also obtained from three other pre-B cell lines (BLIN-1, SMS-SB, Nalm 6) and a pre-B ALL (not shown).

ally requires overnight incubation in appropriate media to generate free binding sites, previously occupied by serum IgM (14, 17, 22, 23). More IgM antibodies are required to coat ox erythrocytes in order to detect IgM-R on B cells (17, 22), and the rosettes formed around B cells are more fragile than the T cell–mediated rosettes (16, 17). In contrast, subsequent studies using an immunofluorescence assay reveal that heat-aggregated IgM binds exclusively to B cells from normal lymphoid tissues as well as to neoplastic B cells from patients with CLL, hairy cell leukemia, or lymphoma (19, 54). While the differences in these earlier results may depend in part on the methods used, it is noteworthy that IgM-R expression by B cells is consistently demonstrable regardless of the assay method. Given the variety of FcγR and FcεR molecules that can be expressed by different cell types (8, 51), it seems possible that the 58-kD FcμR 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 an IgM-R family. This issue will require further study to reach final resolution, but the present data clearly indicate a fundamental difference in expression of FcμR by human B and T cells.

The same FcμR was identified on cytoplasmic μ⁻ ALL cells, μ⁺ pre-B ALL or cell lines, IgM⁺ B cells or cell lines, and isotype-switched CLL cells, indicating that this IgM-R can be expressed throughout pre-B and B cell differentiation and is not directly linked with IgM expression. The surface

expression of FcμR was more closely associated with the activation status of cells. The level of its expression was increased after exposure of B-lineage cells to the IgM ligand or the phorbol ester PMA. The FcμR could also be detected on subpopulations of B cells obtained from tonsils and spleen (17, 25), both of which contain activated B cells. In many cases, leukemic cells of B-lineage constitutively expressed FcμR on their surface in significant levels (16–19, 24, 54). FcμR 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 FcμR 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 FcμR after stimulation with PMA. The FcμR was characterized as a 58-kD sialoglycoprotein 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 FcμR was unaltered by treatment with N-glycanase in various concentrations, and N-linked oligosaccharides resistant to this enzyme have not been reported (49). Second, FcμR 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 pI from 5.6–6.0 to >7.2.

The FcμR appears to recognize configurations of the IgM molecule that are conferred by the C$_{H3}$ and/or the C$_{H4}$ domain(s). First, Fc5μ fragments inhibit IgM binding to FcμR, whereas the Fabμ fragments do not. Fc5μ fragments generated by the hot trypsin digestion of IgM are mainly composed of the C$_{H3-4}$ domains, since the major cleavage site for this digestion is located within the C$_{H2}$ domain (57). Second, IgM mutants with either C$_{H1-3}$ or C$_{H4}$ domain deletions could not bind the 58-kD FcμR.

It is interesting to contrast these results on FcμR expression by B-lineage cells in humans with those that have been obtained in mice. While a murine FcμR 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 C$_{H3}$ 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 FcμR that we have characterized on human B-lineage cells will be found to be conserved in mice.

The FcμR 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 phospolipases, 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 FcεR 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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