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Immunology

IgM antigen receptor complex contains phosphoprotein products of B29 and *mb-1* genes

(membrane immunoglobulin/B lymphocyte/signal transduction)

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ABSTRACT **Membrane immunoglobulin M (mIgM) and mIgD are major B-lymphocyte antigen receptors, which function by internalizing antigens for processing and presentation to T cells and by transducing essential signals for proliferation and differentiation. Although ligation of mIgM or mIgD results in rapid activation of a phospholipase C and a tyrosine kinase(s), these receptors have cytoplasmic tails of only three amino acid residues (Lys-Val-Lys), which seem ill suited for direct physical coupling with cytoplasmic signal transduction structures. In this report, we identify the α, β, and γ components of the mIgM-associated phosphoprotein complex, which may play a role in signal transduction. Proteolytic peptide mapping demonstrated that the IgM-α chain differs from Ig-β and Ig-γ. The chains were purified, and amino-terminal sequencing revealed identity with two previously cloned B-cell-specific genes. One component, IgM-α, is a product of the *mb-1* gene, and the two additional components, Ig-β and Ig-γ, are products of the B29 gene. Immunoblotting analysis using rabbit antibodies prepared against predicted peptide sequences of each gene product confirmed the identification of these mIgM-associated proteins. The deduced sequence indicates that these receptor subunits lack inherent protein kinase domains but include common tyrosine-containing sequence motifs, which are likely sites of induced tyrosine phosphorylation.**

Membrane-bound immunoglobulin M (mIgM) and mIgD are major B-lymphocyte surface structures that specifically bind antigen and subsequently transduce growth-modulating signals. Ligation of either mIgM or mIgD with polyvalent antigen or anti-immunoglobulin antibodies results in the rapid activation of a polyphosphoinositide-specific phospholipase C, which generates calcium-mobilizing inositol polyphosphates and protein kinase C-activating diacylglycerol (1, 2). Recent evidence from several laboratories has established that a tyrosine kinase is also activated upon B-cell antigen receptor ligation (3, 4) and that ligand-induced phospholipase C activation is kinase dependent (5). Candidates for the tyrosine kinase include Lyn, which has been shown to associate with mIgM (6), and Blk, which is B-cell-specific (7). Interestingly, mIgM and mIgD have minimal cytoplasmic structure [a Lys-Val-Lys (KVK) sequence] on each of two membrane-spanning heavy chains (8–10), which is probably incapable of direct physical coupling to such cytoplasmic signaling structures.

Recently B-cell antigen receptor-associated structures have been identified that may play an important role in physical coupling to signal transduction structures, analogous to CD3 components in association with the T-cell antigen receptor. Multiple components of the mIgM-associated glycoprotein complex have been defined biochemically by several laboratories (11–16) and designated IgM-α (32 kDa), Ig-β (37 kDa), and Ig-γ (34 kDa) (13, 14). All of these components are inducibly phosphorylated on tyrosine residues upon receptor ligation or treatment of cells with aluminum fluoride (ref. 13 and K.S.C. and J.C.C., unpublished observations). IgM-α is found disulfide-linked as a heterodimer with either Ig-β or Ig-γ (13, 14).

Studies by Reth and colleagues (11, 12) have provided evidence that expression of the *mb-1* gene product, MB-1, is crucial for mIgM surface expression. Concurrently, they have correlated expression of an mIgM-associated protein, designated IgM-α, with surface expression of the receptor (11, 12). These results indicate that MB-1 is required for mIgM transport to the cell surface and suggest that it may be a component of the B-cell antigen receptor complex.

In this report we have identified the primary structure of three components of the mIgM antigen receptor complex in normal B cells by using proteolytic peptide mapping, amino-terminal sequencing, and immunoblotting with anti-peptide antibodies. IgM-α (pp32) was identified as a product of the *mb-1* gene. Ig-β (pp37) and Ig-γ (pp34) were characterized as products of the B29 gene.

MATERIALS AND METHODS

Cell Preparation. B lymphocytes were isolated from spleens of BDF_1 mice (6–12 weeks old, The Jackson Laboratory) as previously described (17). High and intermediate-density B cells (1.066–1.092 g/cm^3) were isolated by centrifugal sedimentation through discontinuous Percoll (Pharmacia) gradients as described by Ratcliffe and Julius (18).

Radiolabeling and Immunoprecipitation of the Complex. B cells were permeabilized with α-lysophosphatidylcholine, equilibrated with [γ-^{32}P]ATP, stimulated with AlF_4^- for 30 min at 37°C, and lysed in 1% digitonin buffer (containing phosphatase and protease inhibitors) before mIgM was immunoprecipitated from the detergent-soluble fraction with Sepharose-derivatized monoclonal anti-μ antibody b-7-6 (19), as previously described (13). The anti-major histocompatibility complex (MHC) class I (H2K) monoclonal antibody M1/42.398 (20) was also used in control immunoprecipitations.

V8 Protease Peptide Mapping. Immunoprecipitates (40 million cell equivalents per lane) were analyzed by SDS/PAGE under reducing conditions (21), and the gel was dried and autoradiographed. Individual mIgM-associated protein bands were excised and proteolytically digested and analyzed in a second gel according to the method of Cleveland *et al.* (22), using 10 μg of V8 protease (*Staphylococcus aureus*; ICN) per lane. The second separating gel contained 20% polyacrylamide and was prepared according to the method of Giulian and Graham (23).

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Abbreviations: mIgM, membrane IgM; mIgD, membrane IgD; MHC, major histocompatibility complex.

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Amino-Terminal Sequencing. Mononuclear cells were prepared from spleens of 200 mice and lysed in 1% digitonin lysis buffer as above. Particulate material was removed by centrifugation (12,000 × *g* for 20 min) and mIgM was affinity purified on a Sepharose column derivatized with monoclonal anti-μ antibody (b-7-6). The Sepharose was washed extensively with 0.2% digitonin buffer, and the mIgM-associated protein complex was selectively eluted with 1% octyl glucoside [which is dialyzable, yet dissociates the complex from membrane immunoglobulin as effectively as 0.5% Triton X-100 (13); data not shown]. Eluate was concentrated by vacuum dialysis, dialyzed against 0.2% SDS, and lyophilized. The samples were trace labeled with a ^{32}P-labeled anti-IgM immunoprecipitate (prepared as above) and separated on SDS/PAGE with an agarose stacker under nonreducing conditions. Radiolabeled protein (about 70 kDa) was localized in the wet gel by using an automated β emission scanner (AMBIS Systems, San Diego) and again electrophoresed on SDS/PAGE under reducing conditions. Separating gels were aged 2 days and the reducing gel was run with 0.1 mM thioglycolate in the cathode running buffer to avoid artificial blockage of amino termini. Proteins in the second-dimension gel were transferred electrophoretically to Immobilon-P membrane (Millipore) and autoradiographed. ^{32}P-labeled protein bands were excised, washed extensively, and subjected to amino-terminal sequence analysis by the Edman degradation method (24–26) on an Applied Biosystems 470A gas-phase sequencer using on-line phenylthiohydantoin (PTH) determination with an Applied Biosystems 120A analyzer. The sequencing data lacked a significant background of contaminating amino acids, indicating a high degree of purity in the preparations.

Anti-Peptide Antibody Production. Macvector (IBI Pustell) computer analysis of MB-1 and B29 sequences, predicted that peptide sequences PPVPLGPGQGTTQ and DMPDDYEDENL from MB-1 (peptide 254, extracellular residues 72–85 and peptide 298, cytoplasmic residues 171–181, respectively) and sequences DKDDGKAGMEED and FRKRGSQQPQ from B29 (peptide 296, cytoplasmic residues 181–192 and peptide 300, extracellular residues 76–85, respectively) would have high antigenicity. These peptides were synthesized by using *t*-butoxycarbonyl (*t*-Boc) chemistry (27–28) on a PAM resin with an Applied Biosystems 430A peptide synthesizer and purified by using reversed-phase HPLC (Beckman) on a C_{18} column (Vydac, Hesperia, CA). Identity was confirmed by sequence analysis, and individual peptides were coupled to keyhole limpet hemocyanin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC; Bio-Rad). Individual rabbits (New Zealand White; Hazleton Research Products, Philadelphia) were immunized subcutaneously with 1000 μg of peptide-hemocyanin conjugate in complete Freund's adjuvant (GIBCO) and boosted at 2- to 3-week intervals with 100 μg of antigen in incomplete Freund's adjuvant. Antigen-specific antibodies were affinity purified from rabbit serum by using peptide-bovine serum albumin-derivatized Sepharose (Pharmacia) and elution with 3.5 M $MgCl_2$.

Immunoblotting. Digitonin (1%) lysates were prepared from splenic B cells (10^8 cells per sample), immunoprecipitated with Sepharose derivatized with anti-μ monoclonal antibody (b-7-6) or anti-H2K monoclonal antibody (M1/42.398) and subjected to reducing SDS/PAGE, and proteins were transferred electrophoretically to nitrocellulose. After blocking with 2% bovine serum albumin, nitrocellulose was incubated with affinity-purified anti-MB-1 peptide antibody or affinity-purified anti-B29 peptide antibody (10 μg/ml) for 2–4 hr at room temperature. Some blots were incubated with antibodies (10 μg/ml) that had been preincubated with their corresponding peptide immunogen (20 μg/ml) for 15 min at room temperature. Blots were washed with 0.01 M Tris-buffered saline, pH 7.3 (with or without 0.05% Triton X-100), prior to incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad) for 1 hr at room temperature. After washing, blots were developed with a substrate kit purchased from Vector Laboratories.

RESULTS

Peptide Mapping. To compare primary structure of components of the IgM antigen receptor complex, IgM-α, Ig-β, and Ig-γ were subjected to peptide mapping analysis. As can be seen in Fig. 1, the patterns of ^{32}P-labeled fragments of Ig-β (pp37) and Ig-γ (pp34) are very similar (5.5- and 18.2-kDa fragments), although 21.1- to 22.7-kDa fragments appear to be unique to pp34. Alternatively, most fragments from IgM-α (pp32) (4.8, 5.2, 5.6, and 20.3 kDa) are distinct from those of Ig-β and Ig-γ. These results indicate that the primary sequence of IgM-α differs from the sequences of Ig-β and Ig-γ, and that Ig-β and Ig-γ are very similar to one another. These peptide patterns are highly reproducible and are essentially identical to patterns of mIgD-associated components (data not shown).

Amino-Terminal Sequencing. To determine partial amino acid sequences of IgM-α, Ig-β, and Ig-γ, these mIgM-associated proteins were purified from normal B cells and subjected to amino-terminal sequencing using Edman degradation. As shown in Fig. 2, the sequence obtained for IgM-α is identical to the predicted amino-terminal sequence of MB-1, based on nucleotide sequence of the *mb-1* gene (29). Sequences of Ig-β and Ig-γ match exactly the predicted amino-terminal sequence of B29, deduced from B29 gene nucleotide sequence (30). These 7- to 9-residue amino-terminal sequences lack significant homology with any other relevant mammalian protein sequence reported in GenBank (version 63, March 1990). MB-1 and B29, the predicted protein products of previously cloned B-cell-specific genes (29–32), have predicted molecular masses of 20 and 25 kDa, respectively, and N-linked carbohydrate attachment sites. The protein products of B29 and *mb-1* genes have not been isolated or characterized to date. As shown in Fig. 2, signal

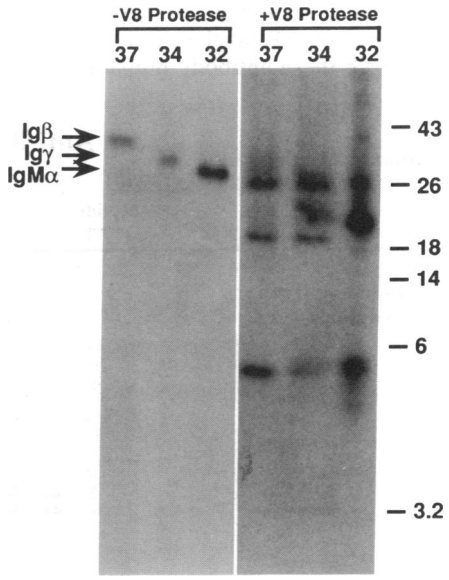

FIG. 1. Comparative V8 protease peptide mapping of the mIgM-associated ^{32}P-labeled phosphoproteins IgM-α (pp32), Ig-β (pp37), and Ig-γ (pp34). Shown is an autoradiograph demonstrating the electrophoretic mobility of purified ^{32}P-labeled IgM-associated proteins (−V8 protease) and proteolytic fragments generated by treatment of these proteins with V8 protease. Positions of markers (kDa) are given on the right.

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IgMα (pp32)          ?-R-V-E-G-G-P-P-S-L
MB-1          P-G-C-Q-A-L-R-V-E-G-G-P-P-S-L-T-V
                ⇧       ⬆

Igγ (pp34)           ?-?-?-L-P-L-N-F-Q-G
Igβ (pp37)           ?-?-D-L-P-L-N-F-Q-G
B29          P-V-P-A-M-T-S-S-D-L-P-L-N-F-Q-G-S-P
               ⇧       ⬆
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FIG. 2. Amino-terminal sequence analysis of purified mIgM-associated IgM-α (pp32), Ig-β (pp37), and Ig-γ (pp34). The experimentally determined sequences are compared with the deduced sequences of the amino termini of MB-1 and B29 (29, 30). Resulting sequences are presented in one-letter code of the amino acids that were present in each Edman degradation cycle. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Question marks signify amino acids that were unidentifiable. Solid arrows mark the actual signal peptidase cleavage sites, while outlined arrows mark originally predicted cleavage sites (29, 30).

peptidase cleavage sites which generate the actual amino termini (solid arrows) differ from those predicted in original reports (outlined arrows) that described these genes (29, 30).

Immunoblotting. To confirm identity of the mIgM-associated proteins with MB-1 and B29, polyclonal rabbit antibodies were produced by immunization with peptide sequences deduced from the *mb-1* and B29 genes and used for immunoblotting. As can be seen in Fig. 3, two affinity-purified anti-MB-1 peptide antibody preparations (peptide 254, which is extracellular residues P^{72}–Q^{85}, and peptide 298, which is cytoplasmic residues D^{171}–L^{181}) reacted with a band that migrates identically with ^{32}P-labeled IgM-α (pp32) in mIgM immunoprecipitates but not with Ig-β or Ig-γ bands. Conversely, two anti-B29 peptide antibody preparations (peptide 296, which is cytoplasmic residues D^{181}–D^{192}, and peptide 300, which is extracellular residues F^{76}–Q^{85}) reacted with bands corresponding exactly to Ig-β (pp37) and Ig-γ (pp34) in immunoprecipitates of mIgM but not with the IgM-α band. The reactivity of the anti-B29 antibodies suggests that Ig-β is more prevalent than Ig-γ, and this correlates with the ability to reproducibly ^{32}P label Ig-β more than Ig-γ. The activities of both of these antibody preparations were completely eliminated by coincubation with the respective peptide immunogen (Fig. 3).

DISCUSSION

These data identify homology of IgM-α with multiple regions of MB-1 and the homologies of Ig-β and Ig-γ with multiple regions of B29. The structural basis for different molecular weight forms of B29 (Ig-β and Ig-γ) is unknown, but it may be differential RNA splicing, the existence of a second B29 gene, or differential post-translational modification, such as glycosylation or phosphorylation. It is unlikely that Ig-γ is a proteolytic fragment of Ig-β because Ig-γ is reproducibly coisolated with Ig-β at identical stoichiometry. Furthermore, four protease inhibitors are added during isolation, and proteolysis has not been observed in a number of other proteins that have been isolated by using this procedure.

These experiments confirm and extend the recent findings of Reth and colleagues (33), which demonstrated that the amino-terminal amino acids of IgM-α and Ig-β are identical to those predicted for MB-1 and B29, respectively. The use of anti-peptide antibodies provides an additional confirmation of three points of identity on each protein with that predicted from nucleic acid sequence of the *mb-1* and B29 genes. In addition this report identifies Ig-γ as an additional product of the B29 gene(s).

Comparison of deduced sequences of MB-1 and B29 (29, 30) reveals some interesting structural motifs (see Fig. 4 *Left*). Both are predicted to be transmembrane proteins, to be members of the immunoglobulin gene super family (extracellular domains) (29–32), and to share minor sequence homology with components of the T-cell CD3 complex (intracellular domains) (36). Both have transmembrane domains 22 amino acids in length and substantial intracellular domains, which may provide the antigen receptor with adequate structure for interaction with secondary transducers, such as the tyrosine kinase Lyn, which has been shown to associate with the mIgM receptor complex (6). Both MB-1 and B29 contain two cysteines and one tryptophan, which are appropriately configured to form an immunoglobulin domain (34, 35) (see Fig. 4 *Left*). When aligned at the transmembrane domains, the apposition of multiple extracellular cysteines on the two chains provides for several potential interchain disulfide linkages. These sequence characteristics are con-

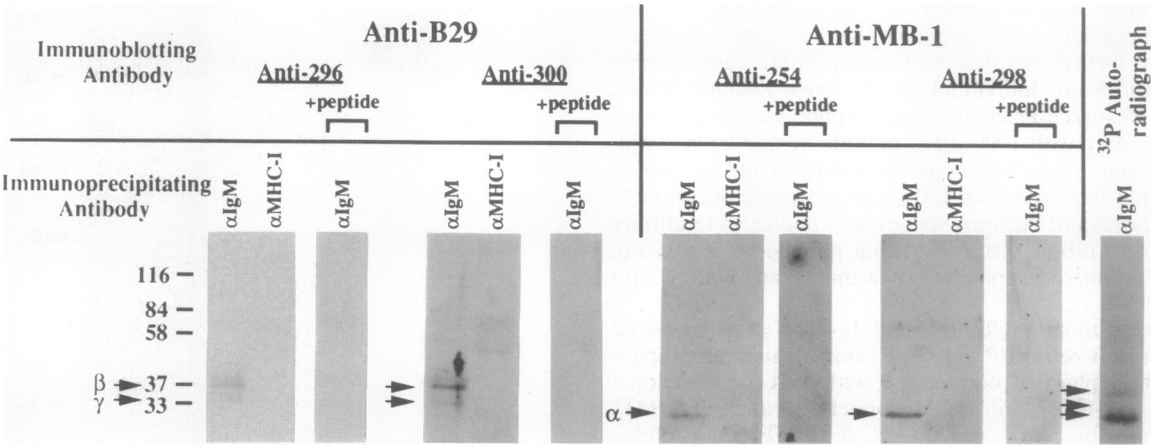

FIG. 3. Affinity-purified rabbit anti-B29 peptide antibody reacts on immunoblots with the mIgM-associated proteins Ig-β and Ig-γ, and anti-MB-1 peptide antibody reacts with IgM-α. mIgM and MHC class I immunoprecipitates were prepared from mouse splenocytes by using antibodies (α-) b-7-6 and M1/42.398, respectively, and immunoblotted with anti-B29 (peptides 296 and 300) or anti-MB-1 (peptides 254 and 298) antibody preparations as indicated. Relevant reactive bands that correspond to IgM-α, Ig-β, and Ig-γ are labeled with arrows. The anti-MB-1 antibody reacted with a species corresponding to ^{32}P-labeled IgM-α. The anti-B29 reacted with bands corresponding with Ig-β and Ig-γ. The corresponding peptide for each antibody preparation eliminated reactivity. Some cross-reactivity of the secondary antibody (goat anti-rabbit immunoglobulin) with the heavy chains of the immunoprecipitating antibodies was also noted. ^{32}P-labeled mIgM immunoprecipitates were run in an adjacent lane and visualized by autoradiography (far right). Positions of markers (kDa) are given on the left.

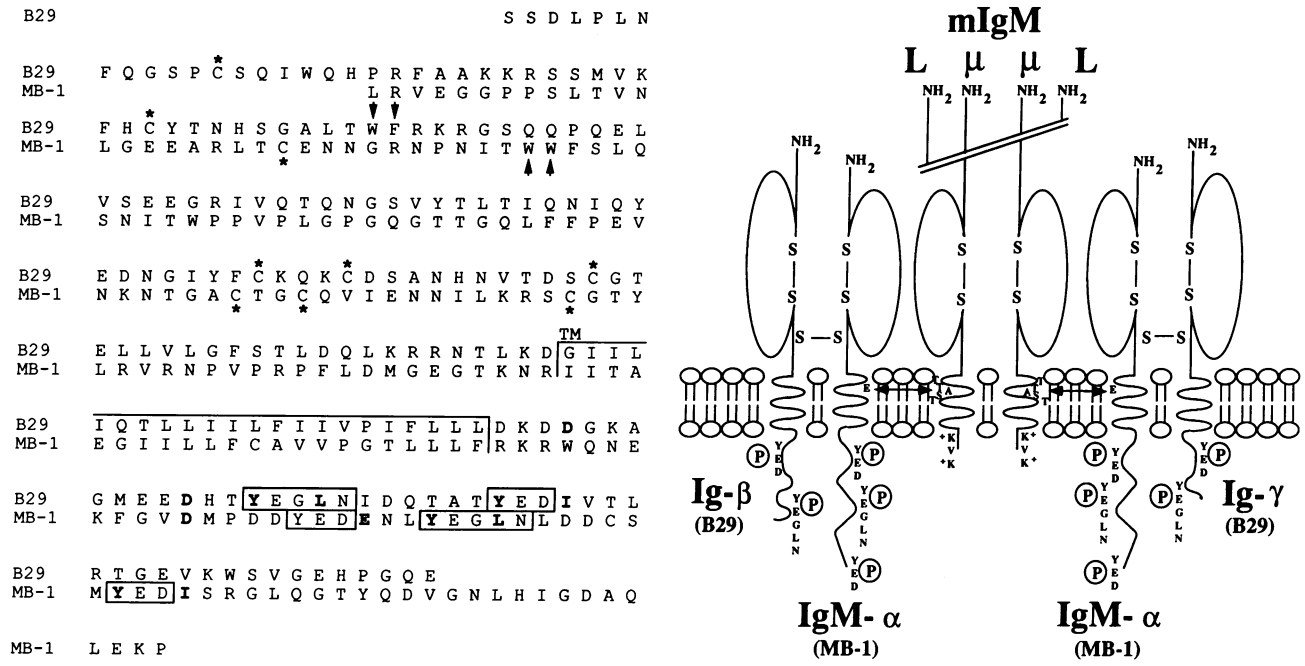

FIG. 4. Comparison of the predicted amino acid sequences of the *mb-1* and B29 gene products, and a model of MB-1/B29 interactions with mIgM. (*Left*) Predicted amino acid sequences of MB-1 and B29 (29, 30) aligned at their 22-amino acid transmembrane segments. The amino-terminal sequences begin with residues as determined by sequence analysis. The common cytoplasmic tyrosine motifs are indicated in boxes as potential phosphorylation sites and the predicted transmembrane domains (TM) are bracketed. Extracellular cysteines are marked with stars. Arrows mark the extracellular tryptophan residues followed by aromatic residues, which are characteristic of immunoglobulin variable domains and contribute to interchain dimer formation (V_H to V_L) (34, 35). As is characteristic of immunoglobulin-superfamily members, individual cysteines (site 1) are located 10–11 residues on the amino-terminal side of the tryptophan residues and a cluster of three cysteines (site 2) is located toward the carboxyl terminus; one of the latter presumably provides for the intrachain disulfide linkage (site 1 to one of the site 2 cysteines) that defines the immunoglobulin-like domain (34, 35). Boldface characters mark a cytoplasmic sequence motif found in both MB-1 and B29 that is also found in CD3 components and ζ chain (36). (*Right*) Model of IgM-α/Ig-β and IgM-α/Ig-γ heterodimer interactions with the two membrane-spanning heavy chains of mIgM (μ). L, light chain. The stoichiometry of actual numbers of heterodimer interacting with each mIgM has not been confirmed experimentally. Extracellular loops designate immunoglobulin-like domains, and common intracellular tyrosine-containing motifs are designated as potential phosphorylation sites.

sistent with the observed occurrence of MB-1 and B29 as a disulfide-linked heterodimer (13, 14), the structure of which is modeled in Fig. 4 *Right*. The transmembrane segment of MB-1 contains an acidic glutamic residue that might hydrogen bond with hydroxyl groups of several threonine and serine residues (TTAST) found in the same region on μ heavy chains. Interestingly, Williams *et al.* (37) have found that mutation of these threonine and serine residues to valine and alanine, respectively, appears to overcome the MB-1 requirement for transport of the receptor to the cell surface.

Though the role of these molecules in signal transduction is not defined, their tyrosine phosphorylation is induced by receptor ligation (ref. 13 and K.S.C. and J.C.C., unpublished observations). Importantly, B29 and MB-1 both contain cytoplasmic YED and YEGLN sequences, which may be substrate motifs for specific tyrosine kinases and tyrosine phosphatases. The YEGLN sequence is unique to MB-1 and B29 as determined by a search of GenBank (version 63, March 1990). It is intriguing to note that two of the tyrosines within YED and YEGLN sequences on each protein are part of a common sequence motif (Fig. 4 *Left*) that is also found on CD3 components and ζ chain, which associate with the T-cell antigen receptor (36). Further, phosphorylation of MB-1 and B29 appears to be dynamically regulated by interplay of an undefined tyrosine kinase and the phosphotyrosine phosphatase CD45 (38). In fact, cellular expression of CD45 and tyrosine kinase activation are both required for activation of phospholipase C and calcium mobilization by mIgM ligation (5, 38).

These findings provide evidence that MB-1 and B29 are components of the IgM antigen receptor complex, existing as disulfide-linked heterodimers in noncovalent association with mIgM. In addition to our data, findings that these genes are exclusively expressed in B cells (29, 30), that expression correlates with surface expression of mIgM [in fact, MB-1 is required for receptor expression (11, 12) and B29 overexpression increases receptor expression (33)], and that the proteins are inducibly phosphorylated strongly imply that these gene products are important for antigen receptor function.

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