Developmentally Regulated Association of a 56-kD Member of the Surface Immunoglobulin M Receptor Complex

By Amy J. Yellen-Shaw and John G. Monroe

From the Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Summary

Immature and mature B cells differ in the signals generated and transduced through their antigen receptor, surface immunoglobulin M (sIgM). Whereas signals generated through sIgM on mature B cells initiate a program leading to the positive activation of these cells, signaling through this receptor at the immature stage of development leads to a state of induced unresponsiveness or tolerance. Our previous studies have described developmental differences in sIgM transmembrane signaling that are independent of ligand-receptor affinity. In an attempt to understand the molecular basis for signaling differences between immature and mature B cells, we have analyzed the sIgM receptor complex in neonatal and adult mouse splenic B cells. While previously described components of this complex do not exhibit marked developmentally regulated differences in their association with sIgM, we have identified a 56-kD protein that associates with sIgM in mature (antigen-responsive), but not immature (tolerance-sensitive) B cells. This protein (p56) associates with sIgM as a homodimer, is constitutively phosphorylated on tyrosine, and is coimmunoprecipitated with IgM but not IgD. The observed inability to iodinate p56 suggests it is an intracellular component of the receptor complex. Based upon its migration in one- and two-dimensional gel electrophoresis we show, however, that p56 is distinct from the blk, lyn, or fyn src family kinases that have been shown to be associated with sIgM in mature B cells. The developmentally regulated participation of p56 in the B cell antigen receptor complex suggests a role in the differential signaling mediated via sIgM on immature and mature B cells.

B lymphocytes at different stages of maturation undergo different responses to antigen receptor engagement (1, 2). In contrast to the response of mature B cells to receptor crosslinking, immature B cells are not activated to proliferate or secrete antibody in response to such stimulation, but rather are rendered nonresponsive to future antigenic stimulation (2–5). We have, through previous work, established that regulation of responsiveness in immature (surface [s]^{1}IgM$^+$, sIgD$^-$) B cells occurs at two levels: (*a*) silencing of a critical activation-associated gene (*egr-1*) (6); and (*b*) uncoupling of sIgM from phosphatidylinositol (PI) hydrolysis (5). Our studies have suggested that silencing of *egr-1* induction characterizes only early stage immature B cells (7), while all populations of immature B cells manifest the transmembrane signaling defect. Indeed, this membrane-associated signaling difference appears to be the primary mechanism accounting for the unresponsiveness of neonatal splenic B cells to anti-μ antibody stimulation (5).

The molecular basis for the membrane-associated signaling defect is as yet undefined. Our previous studies (5) are consistent with the defect residing upstream of G protein-coupled phospholipase C, implicating sIgM-associated signaling molecules analogous to the TCR–CD3 complex. To date, several proteins have been shown to be associated with the B cell antigen receptor. These include a 28–30 kD protein (8, 9), and more recently, three disulfide bonded proteins, migrating in reducing SDS-PAGE gels with molecular masses corresponding to 32, 34, and 38 kD (10–14). Currently named Igα, Igβ, and Igγ, respectively, these proteins maintain weak noncovalent associations with the antigen receptor, as evidenced by the fact that the associations are stable in digitonin, but not in NP-40 detergent. Although it appears likely that these molecules may play a role in coupling sIgM to signal transduction machinery, it is possible that they might not themselves possess intrinsic signaling capabilities. Indeed, it has been shown that Igα and IgM coexpression alone does not constitute a receptor capable of transducing activation signals (10).

Given the differences in sIgM transmembrane signaling in

[1] *Abbreviations used in this paper:* IEF, isoelectric focusing; NRS, normal rabbit sera; PI, phosphatidylinositol; sIg, surface Ig.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/07/0129/09 $2.00
Volume 176 July 1992 129–137

immature and mature B cells, we have undertaken studies to identify molecule(s) that manifest differential association with sIgM at these critical stages of B cell development. In the studies reported here, we fail to observe differences in the sIgM receptor complex between immature and mature B cells with respect to the proteins described above. Rather, we have observed a 56 kD IgM-associated protein whose participation in the receptor complex is developmentally regulated and correlates with developmentally associated signaling differences. The potential role of this protein in determining the signaling characteristics of immature B cells and its relationship to other sIgM-associated proteins is discussed.

Materials and Methods

Mice. Mice used in all experiments were BALB/c, obtained originally from The Jackson Laboratory (Bar Harbor, ME), then bred and maintained in our colony.

Reagents. Rabbit anti-mouse μ chain was produced in rabbits by injection of the purified product of the H013.4.9 hybridoma (11) in CFA, followed by subsequent immunizations using the 8792.6 hybridoma (12) product in saline. Antisera were adsorbed using normal mouse IgG-Sepharose to remove L chain activity, and affinity purified on IgM-coupled Sepharose. Rabbit anti-mouse Fab fragment antibody was kindly provided by Dr. M. Cancro (University of Pennsylvania, School of Medicine, Department of Pathology, Philadelphia, PA). Goat anti-mouse μ chain affinity-purified antibody was obtained from Sigma Chemical Corp. (St. Louis, MO). Goat anti-mouse μ chain $F(ab')_2$ fragments were obtained from Chemicon (Temecula, CA). Rabbit anti-δ affinity-purified polyclonal antibody was kindly provided by R. Woodland (University of Massachusetts, Worcester, MA). Anti-class I $H\text{-}2D^dK^d$ antibody was ammonium sulfate cut of 34-1-2S hybridoma supernatant (American Type Culture Collection, Rockville, MD). Rabbit antiphosphotyrosine antibody was kindly provided by Dr. J. Casnellie (University of Rochester, Rochester, NY), and was prepared as previously described (13). Protein A-Sepharose was obtained from Sigma Chemical Co. Rabbit antibodies to blk, lyn, and fyn were kindly provided by Dr. J. Bolen (Bristol-Myers Squibb Research Institute, Princeton, NJ) (14).

Preparation of Splenic B Cells. Adult mice were killed by cervical dislocation and neonatal mice (pooled litters $\leq$4 d of age) were killed by decapitation. Mature splenic B lymphocytes were then isolated from adult mice ($\geq$6 wk of age), and purified as previously described (15). The neonatal splenic B cell preparations were depleted of any mature B lymphocytes by treatment with anti-δ antibodies and complement.

Panning for $sIgM^+$ Cells. Enrichment for $sIgM^+$ neonatal splenic B cells was performed exactly as previously described (5).

Metabolic Labeling with [^{35}S]Methionine-Cysteine. Purified splenic B cells were resuspended in methionine/75% cysteine-free RPMI (Selectamine; Gibco, Gaithersburg, MD) at 1–2 $\times$ 10^7 cells/ml with 100 μCi/ml (Express ^{35}S Labeling kit; New England Nuclear, Boston, MA) for 4 h at 37°C. Cells were then washed and resuspended at 10^7 cells/100 μl and lysed for 10 min on ice in 10 mM Tris pH 8, 1% NP-40 detergent with freshly added 2 mM PMSF and 8 mM iodoacetamide, plus a cocktail of protease inhibitors. Lysate was spun at 14,000 rpm for 10 min, and supernatant was harvested for immunoprecipitation.

Surface Labeling with Na-^{125}I. Purified splenic B cells (>10^7) were washed twice with ice-cold PBS, then resuspended in 100 μl PBS, with the addition of 30 μl lactoperoxidase, 10 μl Na-^{125}I (1 μCi, Amersham, Arlington, Heights, IL), and 10 μl 0.5% H_2O_2. The cells were incubated at room temperature and mixed every min for 5 min. 15 μl of H_2O_2 was added, and the cells were mixed every minute for 10 min, then 20 μl of H_2O_2 was added and the cells were mixed every minute for 15 min. The cells were then washed three times with cold PBS and lysed as with metabolic labeling.

Immunoprecipitation of Labeled Proteins. Lysates were precleared with protein A-Sepharose overnight at 4°C. 5–50 μl of either affinity-purified or ammonium sulfate-cut antibody was then added to 50 μl labeled cell lysate, and samples were incubated on ice for 90 min at which time 10 μl protein A-Sepharose was added to each tube. Samples were rotated for 30 min at 4°C, then washed four times with lysis buffer. Pellets were resuspended in 2$\times$ reducing or nonreducing sample buffer and analyzed by SDS-PAGE. Gels were fixed and soaked in Amplify (Amersham) before analysis by autoradiography.

Two-dimensional Nonreducing-reducing SDS-PAGE Analysis. After immunoprecipitation, samples were resuspended in 2$\times$ nonreducing SDS-PAGE sample buffer, with the addition of 10 μl of prestained protein standards (Bethesda Research Laboratories, Gaithersburg, MD), then analyzed on a 7.5–15% gradient gel. Lanes were then excised, incubated for 30 min in 2$\times$ reducing sample buffer, then laid across the stack of a 10% gel and analyzed as above.

Two-dimensional Isoelectric Focusing (IEF) Gel Analysis of Labeled Proteins. After immunoprecipitation, pellets were resuspended in IEF sample concentrate (9 M urea, 0.1% β-ME, 0.8% Bio-Lyte 5/7, and 0.2% Bio-Lyte 3/10; Bio-Rad Laboratories, Richmond, CA), then analysis on tube gels by IEF was carried out at 400-V constant voltage for 12–15 h, followed by 2 h at 800 V-constant voltage. After the run, gels were expelled, at which time they were incubated for 30 min in 2$\times$ reducing SDS-PAGE sample buffer. Tubes were laid across the stack of a slab gel and electrophoresed as in the one-dimensional SDS-PAGE analyses. Position of ^{14}C-labeled markers in the second dimension was as indicated.

Immune Complex Kinase Assay. Cells were stimulated as indicated, then lysed for 10 min on ice in 10 mM Tris, pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, and 1% NP-40 detergent with freshly added 2 mM PMSF, 100 μM sodium orthovanadate, and 8 mM iodoacetamide, plus a cocktail of protease inhibitors. Lysates were incubated at 4°C for 1 h with 2.5 μl of the appropriate antisera, and then rotated for an additional 30 min with protein A-Sepharose. Samples were washed twice with lysis buffer, then once with 10 mM Tris, pH 7.0, 100 mM NaCl, and sodium orthovanadate. Pellets were resuspended in kinase reaction buffer (20 mM Hepes, pH 7.0, 10 mM $MnCl_2$), mixed with 10 μCi/sample of γ[^{32}P]ATP (New England Nuclear) and incubated for 15 min at room temperature. Samples were then washed with ice-cold lysis buffer, resuspended in nonreducing SDS-PAGE sample buffer, and analyzed by two-dimensional nonreducing-reducing SDS-PAGE. Gels were treated with 1 M KOH for 1 h at 56°C to cleave all serine/threonine-bound phosphate groups before analysis by autoradiography.

Results

The 28-, 32-, 34-, and 38-kD Components of the Mature B Cell sIgM Receptor Complex Are also IgM-associated in Immature B Cells. Potential differences in the sIgM receptor complex in immature and mature B cells were evaluated with regard to previously identified IgM-associated proteins. Im-

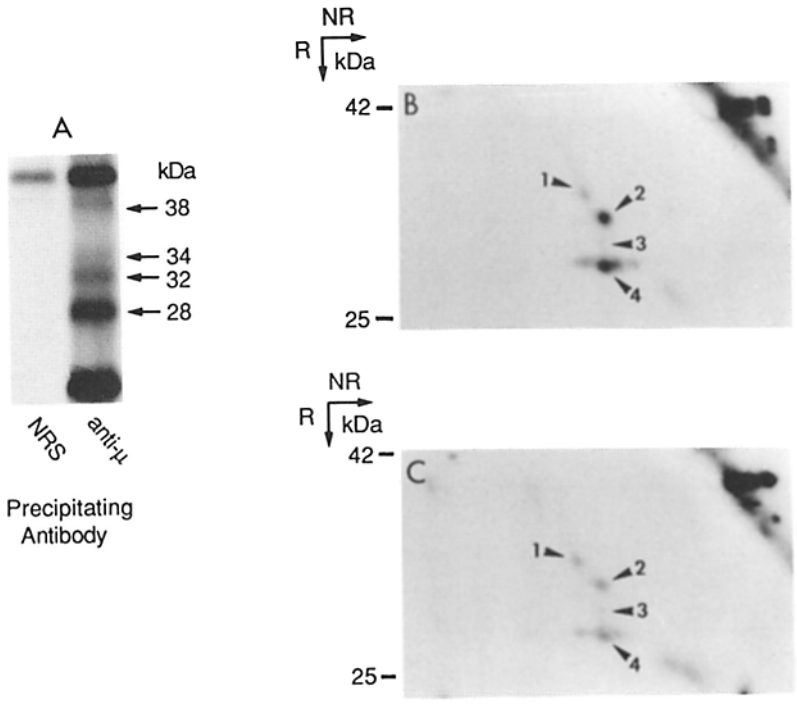

Figure 1. Coprecipitation of sIgM with four protein species in both immature and mature splenic B cells. (*A*) ^{35}S-labeled proteins from 1% digitonin-lysed immature splenic B cells were immunoprecipitated using either rabbit anti-μ antibodies or normal rabbit sera (NRS) followed by the addition of Protein A-Sepharose. Samples were analyzed by reducing SDS-PAGE using a 10% gel. (*B* and *C*) ^{35}S-labeled proteins from 1% digitonin-lysed immature (*B*) or mature (*C*) splenic B cells were immunoprecipitated using rabbit anti-μ chain antibodies, then analyzed by nonreducing SDS-PAGE. *Arrows* point to protein species corresponding to those designated in *A*.

mature (day 4 neonatal) or mature (6 wk) splenic B cells were labeled with [^{35}S]methionine-cysteine, and then solubilized in lysis buffer containing 1% digitonin detergent. Although prior studies have primarily used ^{32}P (16) or cell surface labeling (9, 17–19) to visualize sIg-associated proteins in nontransformed B cells, we reasoned that the use of metabolic labeling would enable visualization of a broader range of receptor-associated components. The labeled lysates were immunoprecipitated using polyclonal anti-μ antibodies, and the resulting complexes analyzed using SDS-PAGE. Shown in Fig. 1 are one- and two-dimensional SDS-PAGE analyses of IgM-associated proteins from immature and mature B cells. We observed four specific proteins coprecipitating with IgM from immature B cells (Fig. 1 *A*) that migrated with molecular weights consistent with four previously described receptor-associated proteins in mature B cells (8, 16, 19–21). The undesignated band at the bottom of the gel represents the sIgM-associated L chains.

A comparison of immature and mature B cells with respect to these proteins was accomplished by nonreducing/reducing SDS-PAGE. Each of the proteins visualized in Fig. 1 *A* was identified (1–4; 38, 34, 32, and 28 kD, respectively). It is important that each protein species was associated with IgM in both immature (Fig. 1 *B*) and mature (Fig. 1 *C*) B cells. In addition, these proteins migrate to positions below the diagonal, indicating their participation in reducible covalent associations, as previously reported (16, 21). Finally, the labeling intensities of the 34- (no. 2) and the 28-kD (no. 4) proteins were relatively increased in immature versus mature B cells. Although this is a reproducible finding, we do not know whether this reflects true stoichiometric differences or differences in the turnover rates of these proteins in immature and mature B cells.

Differential Association between IgM and a 56-kD Protein Distinguishes the Mature and Immature B Cell Populations. Although no qualitative differences were observed between immature and mature B cells with respect to the association of IgM with the 28-, 32-, 34-, and 38-kD components of the receptor complex, these analyses revealed a differential association between IgM and a protein migrating with a relative molecular mass of 56 kD (Fig. 2). ^{35}S-labeled mature and immature B cells were solubilized in lysis buffer containing 1% NP-40. Precleared lysates were immunoprecipitated with rabbit anti-μ or normal rabbit serum and protein A-Sepharose, and analyzed by SDS-PAGE (Fig. 2). A protein migrating with an apparent molecular mass of 56 kD (p56) coprecipitated with IgM in the mature, but not the immature B cells. These gels were intentionally overexposed to maximize our ability to detect a p56 association in the immature B cells. However, p56 was not found in IgM precipitates from immature cells.

Coprecipitation of p56 and IgM in 1% NP-40 suggests a relatively strong association between these molecules in mature cells. The lack of an association in the immature B cells was not due to the use of this relatively strong detergent, as identical results were obtained using 1% digitonin (not shown). We have, therefore, relied on NP-40 lysis conditions (unless stated otherwise) for our subsequent studies of p56 because immunoprecipitations were much cleaner under these conditions.

Studies shown in Fig. 3 demonstrate that coprecipitation of p56 is specific to Ig, as no association was detected in im-

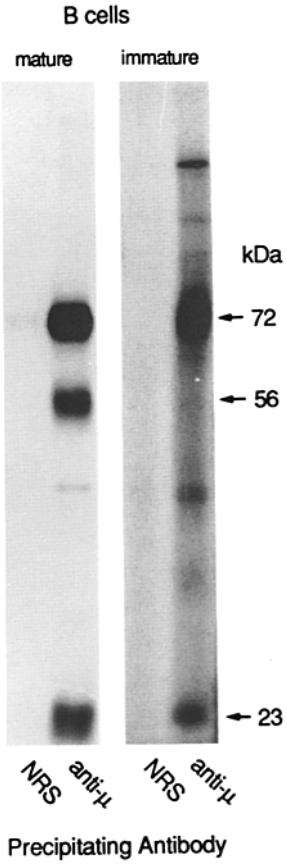

Figure 2. Association between sIgM and a 56-kD protein distinguishes the mature and immature B cell populations. Mature or immature splenic B cells were labeled with [^{35}S]methionine-cysteine and lysed in 1% NP-40 detergent. Labeled proteins were immunoprecipitated using NRS or rabbit anti-μ antibodies, and were then analyzed by SDS-PAGE under reducing conditions as in Fig. 1. Molecular weights were then calculated relative to ^{14}C-labeled protein standards (New England Nuclear, Boston, MA).

munoprecipitates from normal rabbit serum, normal rabbit Ig, anti-class I antibodies, or with anti-FcRγ or anti-class II antibodies (not shown). Coprecipitation of IgM and p56 is mouse strain-independent (Fig. 3 *a*) and was observed using two other affinity-purified polyclonal antibody preparations, goat anti-mouse μ and rabbit anti-mouse Fab (Fig. 3 *b*).

p56 Is Specifically Associated with IgM but Not IgD. As shown in Fig. 4, both rabbit anti-δ antibodies and anti-Fab antibodies immunoprecipitate IgD, with the ^{125}I- and ^{35}S-labeled δ protein migrating at 67 kD under reducing conditions. However, ^{35}S-labeled p56 was only observed in precipitations using the anti-μ and anti-Fab reagents. These results indicate that p56 specifically associates with IgM. In addition to the observed differences in p56 association with IgM and IgD, the results in Fig. 4 also demonstrate our repeated inability to iodinate p56, despite efficient ^{125}I-labeling of μ or δ chain. These results suggest limited or no expression of this protein on the cell surface, or a lack of exposed tyrosine residues.

p56 Migrates as a Dimer under Nonreducing Conditions. Analysis of anti-μ-precipitated proteins under nonreducing conditions failed to resolve a 56-kD protein (Fig. 5 *A*), but instead yielded two major bands migrating at ~114 and 180–220 kD. The larger band represents assembled Ig molecules (H_2L_2). The loss of the 56-kD band, coinciding with the appearance of a 114-kD band, suggests that p56 may exist and associate with IgM as a disulfide-linked homodimer. This interpretation was confirmed by analyzing these complexes

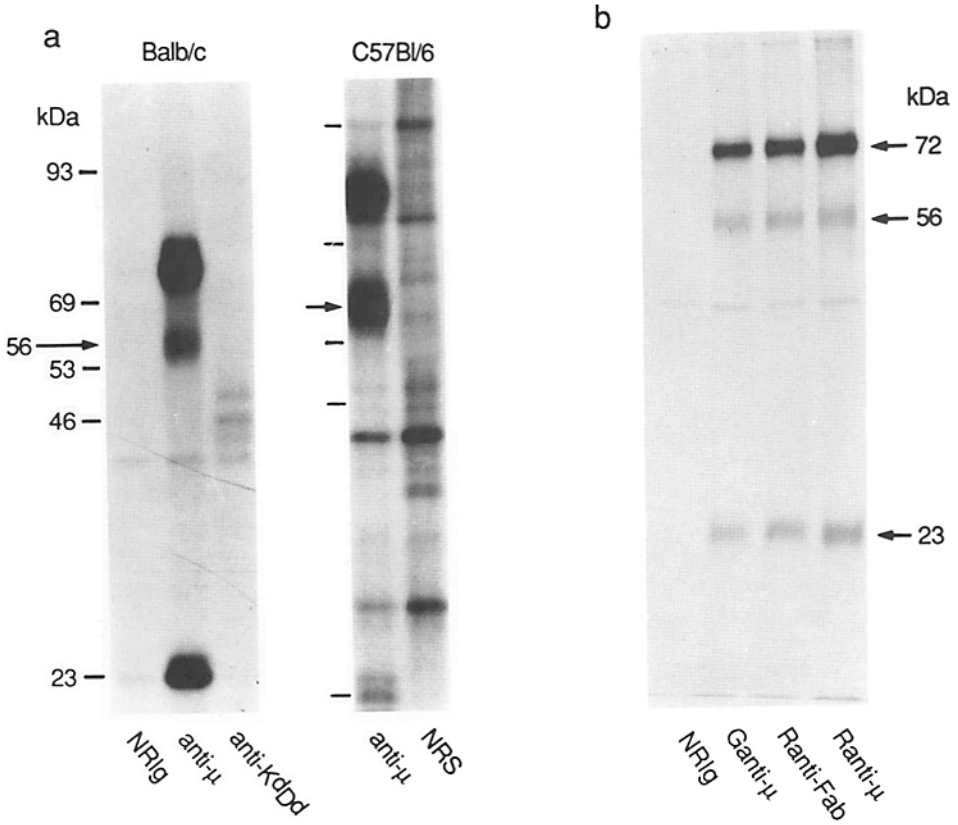

Figure 3. Specificity of the sIgM-p56 association. (*a*) ^{35}S-labeled proteins from 1% NP-40 lysed mature splenic B cells from either BALB/c or C57Bl/6 (B6) mice were immunoprecipitated using either NRS, rabbit anti-μ antibody (anti-μ) or anti-class I antibody (saturated ammonium sulfate cut of 34-1-2S, H-2D^{d}K^d-specific; American Type Culture Collection) followed by addition of Protein A-Sepharose. Samples were analyzed by reducing SDS-PAGE using a 10% gel. (*b*) ^{35}S-labeled proteins from mature splenic B cells from BALB/c mice were immunoprecipitated using normal rabbit immunoglobulin (NRIg) (Protein A-Sepharose purified), goat anti-μ, rabbit anti-Fab, or rabbit anti-μ antibody preparations, then analyzed as above. The positions of ^{14}C-labeled protein standards were as indicated.

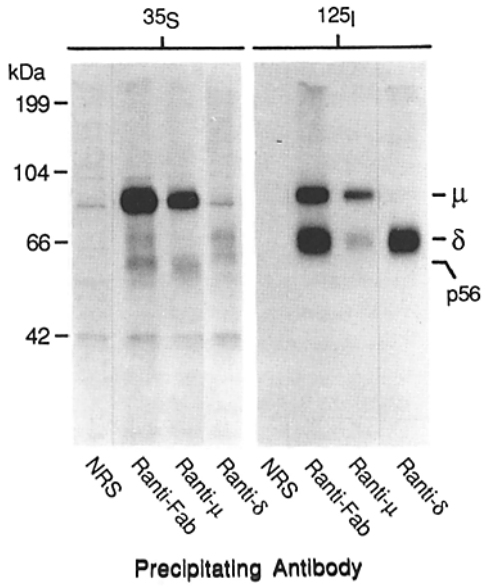

Figure 4. p56 associates specifically with sIgM and not sIgD. Mature splenic B cells were either metabolically labeled with ^{35}S or surface labeled with ^{125}I, lysed in 1% NP-40, and then subjected to immunoprecipitation using NRS, rabbit anti-Fab, rabbit anti-μ or rabbit anti-δ antibodies. Samples were then analyzed by reducing SDS-PAGE on a 10% gel.

on two-dimensional nonreducing-reducing SDS-PAGE gels (Fig. 5 *B*). Under these conditions, p56 migrates to a position below the diagonal with a nonreduced apparent molecular mass of ~110–120 kD and a reduced molecular mass of 56 kD. Immature B cells are also shown in Fig. 5 *C* for comparison to reconfirm the absence of the p56–IgM association.

Coprecipitation of IgM and p56 Using Phosphotyrosine-specific Antibodies. Phosphorylation at tyrosine characterizes several members of the sIgM receptor complex (22, 23). Additionally, we have previously observed a 56-kD plasma-membrane–associated protein in B cells that is phosphorylated in unstimulated B cells (24). The sIgM-associated p56 molecule was evaluated for tyrosine phosphorylation by immunoprecipitation of ^{35}S-labeled B cells using antiphosphotyrosine antibody (anti-tyrP). Shown in Fig. 6, both anti-μ and anti-tyrP precipitated a ^{35}S-labeled 56-kD protein. Because no evidence exists to support tyrosine phosphorylation of IgM under these conditions, the immunoprecipitation of p56 by anti-tyrP suggests that this protein is phosphorylated in unstimulated B cells. More importantly, the observation that anti-tyrP also precipitated the 72-kD μ chain confirms an association between p56 and IgM.

To ensure that we were immunoprecipitating the same protein using either anti-μ or antiphosphotyrosine antibody, we analyzed the immunoprecipitates by two-dimensional IEF and SDS-PAGE. Shown in Fig. 7, p56 migrates as a series of proteins (*highlighted* region) that differ in their isoelectric points. This heterogeneity could be due to differences in levels of phosphorylation or glycosylation. It is important that the protein patterns were identical in both the antiphosphotyrosine and anti-μ immunoprecipitates. Furthermore, the 72-kD proteins precipitated by anti-tyrP correspond to the heterogeneous μ chains detected after anti-μ immunoprecipitation. In addition, the anti-tyrP antibody immunoprecipitated

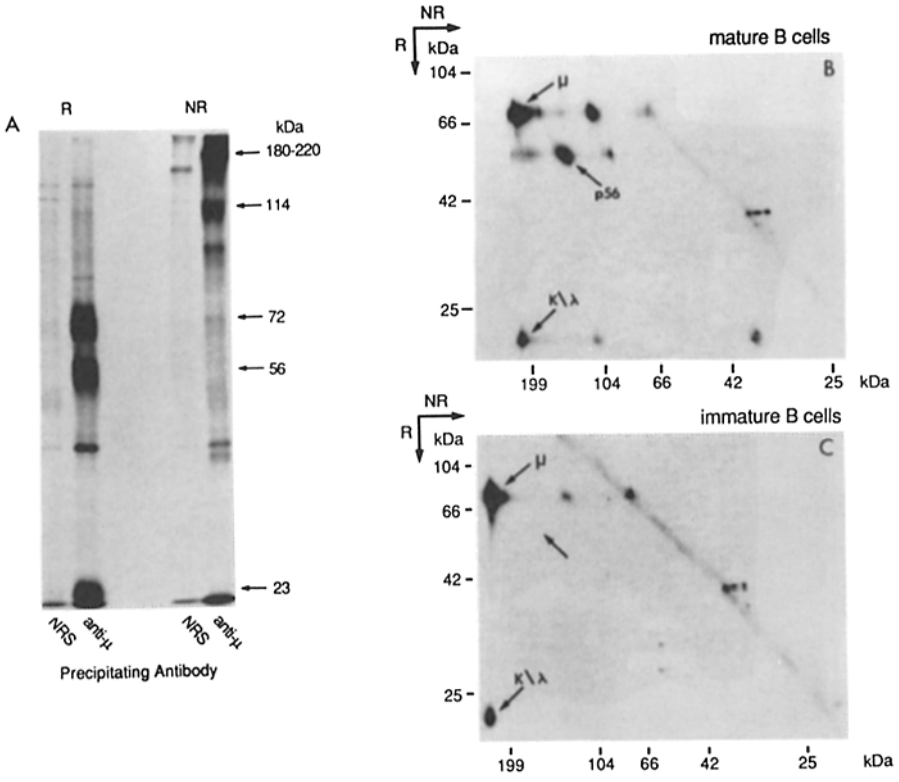

Figure 5. Two-dimensional, nonreducing-reducing analysis of IgM-coprecipitated p56. (*A*) Mature splenic B cell proteins were metabolically labeled, lysed using 1% digitonin, immunoprecipitated using either NRS or anti-μ antibody, and then analyzed under reducing (*R*) or nonreducing (*NR*) SDS-PAGE conditions. (*B*) Mature or (*C*) immature splenic B cell anti-μ antibody immunoprecipitated material was analyzed by two-dimensional nonreducing-reducing gels. The first dimension was performed under nonreducing conditions using a 7.5–15% gradient gel. The second dimension was performed on a 10% gel. The positions of protein molecular weight standards under each condition were as indicated along each axis. Approximately 5×10^7 cell equivalents were used per gel for this analysis.

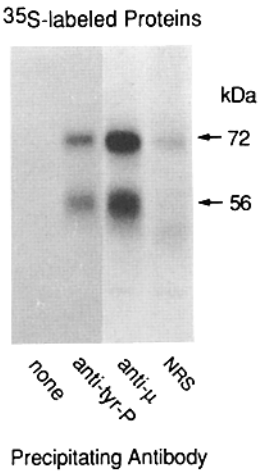

Figure 6. Coprecipitation of IgM and p56 using antiphosphotyrosine antibodies. ^{35}S-labeled proteins from NP-40-lysed mature splenic B cells were immunoprecipitated using NRS, rabbit anti-μ chain, or rabbit anti-phosphotyrosine (anti-Tyr-P) antibodies, then analyzed on a 10% SDS-PAGE gel under reducing conditions. The associated Ig L chain, migrating at ~23 kD, has been cut off in this figure.

a number of proteins not observed in the anti-μ precipitates, indicating, not surprisingly, a number of phosphoproteins not associated with IgM in unstimulated B cells. The prominent protein migrating at 46 kD in these gels is a common contaminant in our system.

These results are important primarily because they confirm an association between p56 and μ chain, and secondarily, because they suggest tyrosine kinase-mediated modification of the p56 protein in unstimulated cells. However, in the latter case we cannot unequivocally state that the precipitation of p56 by antiphosphotyrosine antibody is not indirectly mediated (i.e., via an associated tyrosine phosphoprotein intermediate). Studies are currently underway to analyze the effects of sIgM crosslinking on the phosphorylation state of this protein.

p56 Is Distinct from the blk, fyn, lck, and lyn src-family Kinases. Several characteristics of p56 are consistent with a relationship to one of a number of src-related tyrosine kinases known to be preferentially expressed in lymphoid cells (25, 26). Its apparent molecular weight is similar to the blk, lyn, and fyn kinases, all of which are expressed in B cells (25, 26), and which have been suggested to be associated with sIgM (14, 27). Furthermore, the inability to iodinate p56 suggests an intracellular localization. To address the relationship between p56 and these tyrosine kinases, we compared their behavior in SDS-PAGE under reducing and nonreducing conditions, conditions which we have used here to characterize p56. To evaluate differences between p56 and p56-blk, immature, and mature B cells were labeled with ^{35}S-methionine, solubilized with 1% NP-40, and immunoprecipitated with anti-μ or anti-blk antibodies (Fig. 8 *A*). As we have already established, p56 was observed only in the mature B cells, primarily in the anti-μ immunoprecipitates. However, the possibility of faint expression in the anti-blk immunoprecipitates is suggested in this experiment. In contrast, blk expression was observed in both the immature and mature B cells. Furthermore, its migration was slower than that of p56, consistent with a relative molecular mass of 58 kD in our gel system. This band corresponding to blk was not observed in the anti-μ immunoprecipitations in which p56 was detected. Thus, based upon relative migration by one-dimensional SDS-PAGE, p56 and blk are distinct proteins.

p56 and blk were further distinguished by two-dimensional SDS-PAGE. p56 runs below the diagonal on two-dimensional nonreducing-reducing gels (shown in Fig. 5 *B*). In contrast, blk runs on the diagonal under these conditions (Fig. 8 *B*). In this study, blk was immunoprecipitated by anti-blk and then incubated with γ[^{32}P]ATP to allow labeling by autophosphorylation (14). This approach allows greater specific activity in the labeling and, therefore, greater sensitivity in the detection of these kinases. Two prominent proteins were observed in the molecular weight range of blk. Both were on the diagonal, the higher molecular weight form we believe to be blk based upon its position in the one-dimensional

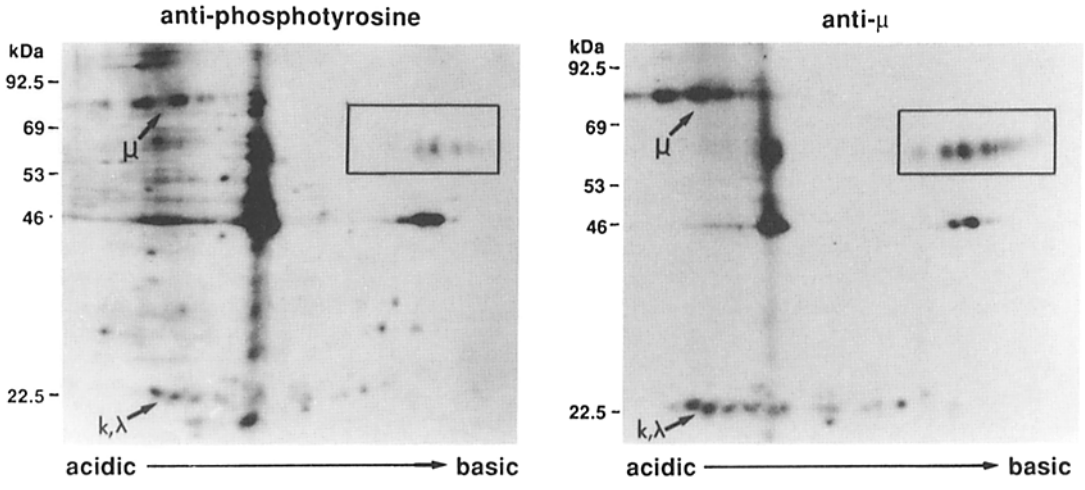

Figure 7. Two-dimensional IEF analysis of p56 precipitated by either anti-μ or antiphosphotyrosine antibodies. ^{35}S-labeled proteins from NP-40–lysed mature splenic B cells were immunoprecipitated using either rabbit anti-μ or rabbit anti-TyrP antibodies, then analyzed first by IEF using tube gels, followed by reducing SDS-PAGE on a 10% gel.

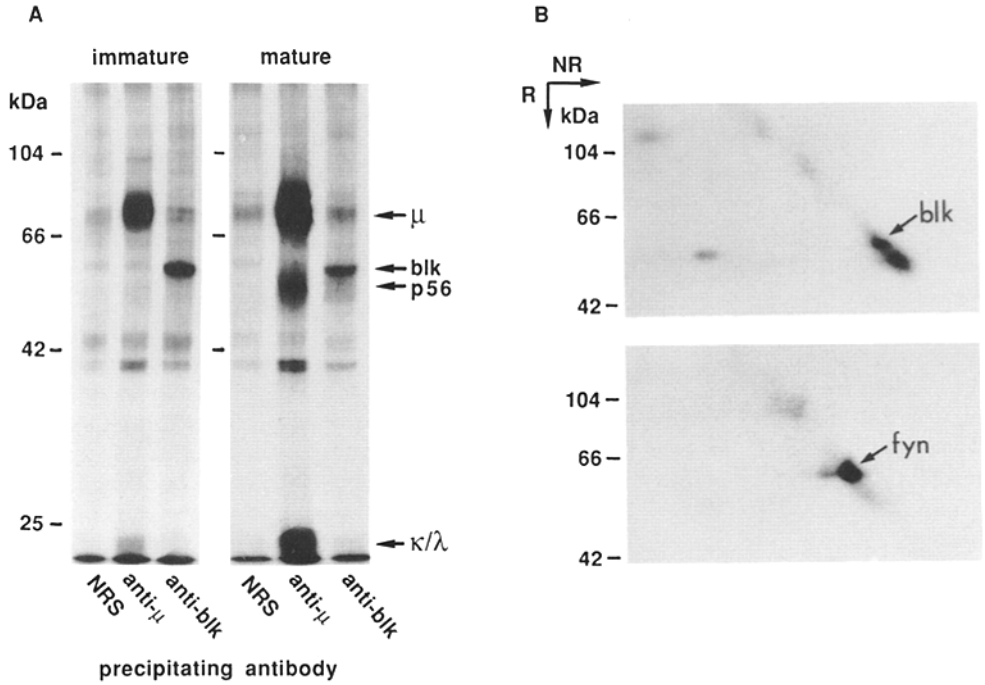

Figure 8. One- and two-dimensional gel analysis of p56, blk, and fyn. (*A*) ^{35}S-labeled proteins from 1% NP-40–lysed immature or mature splenic B cells were immunoprecipitated using either NRS, rabbit anti-μ or rabbit anti-blk antibody, then analyzed by SDS-PAGE on a 10% gel. (*B*) Mature splenic B cells were stimulated with 10 μg/ml goat anti-μ F(ab')$_2$ antibody for 2 min. Lysates were immunoprecipitated using either rabbit anti-blk or rabbit anti-fyn antisera. Immune complex kinase reactions were carried out, and samples were then analyzed by two-dimensional nonreducing-reducing SDS-PAGE.

gel system (Fig. 8 *A*) and published reports where two bands were also observed using this antibody (14). It is interesting that a faint spot with an apparent reduced molecular weight intermediate between these proteins was detected below the diagonal, in the region where p56 is usually observed. The possibility exists that this protein may be p56 in its phosphorylated form, suggesting that p56, although distinct from blk, may be able to noncovalently associate with this kinase. This possibility is consistent with our analysis of Fig. 8 *A*, in which we observe a small amount of a protein comigrating with p56 in anti-blk from mature but not immature B cells.

Differences in electrophoretic mobility also argue against the possibility that p56 may be either the fyn or lyn src-family kinases. For these studies, the in vitro kinase assay was used exclusively because neither fyn nor lyn labels efficiently with [^{35}S]methionine in the primary B cells (A. Yellen-Shaw, unpublished observations; this characteristic also distinguishes these proteins from p56). Immunoprecipitation using anti-fyn antibody showed that like blk, fyn also migrates on the diagonal (Fig. 8 *B*), and is therefore, quite distinct from p56. In contrast to blk, the off diagonal spot in the p56 region was not observed in the fyn or lyn (not shown) immunoprecipitation. Previous studies (27) have shown that lyn from murine B cells migrates at ~56 kD under both reducing and nonreducing conditions, which is again inconsistent with the pattern of p56 migration. Consistent with this published report, we also observe lyn to migrate on the diagonal in our gel system and have, therefore, not shown these data here.

Finally, recent studies of Campbell and Sefton (34) have shown p56^{lck} to be associated with I_gM in murine splenic B cells. In our hands, lck is either not expressed in B cells or does not label well with [^{35}S] methionine. Furthermore, p56 and p56^{lck} migrate differently under nonreducing conditions; lck migrating at 56 kD (Campbell, M.-A. and B. M. Sefton, personal communication). As such, we believe p56 and these src-family kinases to be distinct members of the receptor IgM complex.

Discussion

Our previous studies comparing sIgM-linked signaling in mature and immature B cells suggested that developmentally regulated expression and/or function of receptor-associated components may play a key role in determining responsiveness to antigenic stimulation at different stages of development (5). Our studies reported here have established that four previously described members of the sIgM receptor complex are not developmentally regulated with respect to their IgM association, although qualitative differences may exist. These results suggest that these proteins are not involved in the differential signaling associated with sIgM on immature and mature B cells, although we cannot formally rule out developmentally regulated functional differences in these proteins. We have, however, identified a novel μ-associated protein, p56, which manifests the developmentally regulated expression, or association with μ, that correlates with antigenic responsiveness.

As yet, we have no direct evidence that p56 plays a role

in IgM signal transduction. However, the developmental regulation of the p56-IgM association is provocative, as it may provide a molecular explanation for differential signaling in mature and immature B cells. If p56 is directly involved in the transduction of sIgM-generated signals, it may act to couple the receptor to transducing molecules, such as G proteins (28–30), or tyrosine kinases (14, 27). In this respect, the potential of an association between p56 and blk is intriguing and remains an area of investigation. Alternatively, p56 could itself manifest the enzymatic activity associated with one of these transducers, by, for example, having its own intrinsic tyrosine kinase activity. We have explored the possibility that p56 represents one of the src-family tyrosine kinases that have been previously shown associated with the B cell antigen receptor (blk, fyn, lck, and lyn). However, SDS-PAGE analysis strongly argues against this possibility but rather, allows us to conclude that p56 is distinct from these other members of the sIgM receptor complex. Therefore, p56 appears to represent a novel member of the B cell antigen receptor complex. The interplay between p56, blk, fyn, lck, lyn, GTP-binding proteins, as well as the other proteins associated with this increasing complex receptor remains to be established.

We have also considered the possibility that p56 may be related to the 56-kD protein that was previously reported by Koch and Haustein to be associated with IgM but not IgD (17). However, this protein was visualized after iodination, using conditions very similar to those that failed to label p56 in our studies. Likewise, ^{125}I has been used to detect an association between sIg and the vitamin D-binding Gc protein and a 55-kD myosin-binding protein found to associate with sIgM in chicken B lymphocytes (31–33). Based upon our inability to iodinate p56, and its existence as a homodimer, it seems unlikely that p56 is related to these proteins. We consider it more likely that we may be visualizing the same protein observed by Gold et al. (23), who performed an antiphosphotyrosine Western blot analysis of anti-mb-1 (IgMα) immunoprecipitated material from the WEHI 231 B lymphoma, and observed an inducibly tyrosine phosphorylated substrate that migrated as a 54-kD homodimer. However, these investigators did not directly analyze anti-IgM-precipitated material, nor did they evaluate the developmental characteristics of the 54-kD protein in nontransformed B cells.

Developmentally based differences in sIgM signaling remain largely unexplored at the molecular level. The differential association of sIgM and p56 distinguishes the mature and immature B cell populations. The correlation between this difference and the observed differences in their sIgM-linked transmembrane signaling raises the possibility that these phenomena may be related. Cloning of p56 followed by expression studies of the wild type and mutated protein are needed to establish this connection.

The authors would like to thank Drs. Julie Carman and Helen Quill for critical review of this manuscript.

This work was supported by American Cancer Society grant IM-497, National Institutes of Health grant AI-23568, the CTR, and the Lucille Markey Foundation. J. C. Monroe is a Scholar of the Leukemia Society of America.

Address correspondence to Dr. John J. Monroe, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Rm. 538A Clinical Research Building, 422 Curie Boulevard, Philadelphia PA 19104–6142.

Received for publication 15 November 1991 and in revised form 23 March 1992.

References

1. Monroe, J.M., and V.L. Seyfert. 1988. Studies of surface immunoglobulin-dependent B cell activation. *Immunol. Res.* 7:136.
2. Nossal, G.J.V. 1983. Cellular mechanisms of immunologic tolerance. *Annu. Rev. Immunol.* 1:33.
3. Cooper, M.D., J.F. Kearney, W.E. Gathings, and A.R. Lawton. 1980. Effects of anti-Ig antibodies on the development and differentiation of B cells. *Immunol. Rev.* 52:29.
4. Sieckmann, D.G., I. Scher, R. Asofsky, D.E. Mosier, and W.E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus-independent response by a mature subset of B lymphocytes. *J. Exp. Med.* 148:1628.
5. Yellen, A.J., W. Glenn, V.P. Sukhatme, X. Cao, and J.G. Monroe. 1991. Signaling through surface IgM in tolerance-susceptible immature murine B lymphocytes. *J. Immunol.* 146:1446.
6. Seyfert, V.L., S.B. McMahon, W.D. Glenn, A.J. Yellen, V.P. Sukhatme, X. Cao, and J.G. Monroe. 1990. Methylation of an immediate-early inducible gene as a mechanism for B cell tolerance induction. *Science (Wash. DC).* 250:797.
7. Monroe, J.G., A.J. Yellen-Shaw, and V.L. Seyfert. 1992. Molecular basis for unresponsiveness and tolerance induction in immature stage B lymphocytes. *In* Advances in Molecular and Cellular Immunology. B. Singh, editor. JAI Press, Greenwich, CT. In press.
8. Sidman, C.L., T. Bercovici, and C. Gitler. 1980. Membrane insertion of lymphocyte surface molecules. *Mol. Immunol.* 17:1575.
9. Haustein, D., and D. Von der Ahe. 1986. A 30-kDa protein is disulfide linked to IgM on normal and neoplastic murine B lymphocytes. *Eur. J. Immunol.* 16:113.
10. Justement, L.B., J. Wienands, J. Hombach, M. Reth, and J.C. Cambier. 1990. Membrane IgM and IgD molecules fail to trans-

duce Ca^{2+} mobilizing signals when expressed on differentiated B lineage cells. *J. Immunol.* 144:3272.
11. Marshak-Rothstein, A., P. Fink, T. Gridley, D.H. Raulet, M.J. Bevan, and M.L. Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* 122:2491.
12. Noseworthy, J.H., B.N. Fields, M.A. Dichter, C. Sobotka, E. Pizer, L.L. Perry, J.T. Nepom, and M.I. Greene. 1983. Cell receptors for the mammalian reovirus. I. Syngeneic monoclonal anti-idiotypic antibody identifies a cell surface receptor for reovirus. *J. Immunol.* 131:2533.
13. Casnellie, J.E., and R.J. Lamberts. 1986. Tumor promoters cause changes in the state of phosphorylation and apparent molecular weight of a tyrosine protein kinase in T lymphocytes. *J. Biol. Chem.* 261:4921.
14. Burkhardt, A.L., M. Brunswick, J.B. Bolen, and J.J. Mond. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 88:7410.
15. Monroe, J.G. 1988. Up-regulation of c-fos expression is a component of the mIg signal transduction mechanism but is not indicative of competence for proliferation. *J. Immunol.* 140:1454.
16. Campbell, K.S., and J.C. Cambier. 1990. B lymphocyte antigen receptors (mIg) are noncovalently associated with a disulphide-linked, inducibly phosphorylated glycoprotein complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:441.
17. Koch, N., and D. Haustein. 1983. Association of surface IgM with two membrane proteins on murine B lymphocytes detected by chemical crosslinking. *Mol. Immunol.* 20:33.
18. Parkhouse, R.M.E. 1990. Three B-cell surface molecules associating with membrane immunoglobulin. *Immunology.* 69: 298.
19. Chen, J., A.M. Stall, L.A. Herzenberg, and L.A. Herzenberg. 1990. Differences in glycoprotein complexes associated with IgM and IgD on normal murine B cells potentially enbable transuction of different signals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2117.
20. Hombach, J., L. Leclerq, A. Radbruch, K. Rajewsky, and M. Reth. 1988. A novel 34-kd protein co-isolated with the IgM molecule in surface IgM-expressing cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3451.
21. Hombach, J., T. Tsubata, L. Leclerq, H. Stappert, and M. Reth. 1990. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature (Lond.).* 343:760.
22. Campbell, M.-A., and B.M. Sefton. 1990. Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2125.
23. Gold, M.R., L. Matsuuchi, R.B. Kelly, and A.L. DeFranco. 1991. Tyrosine phosphorylation of components of the B cell antigen receptors following crosslinking. *Proc. Natl. Acad. Sci. USA.* 88:3436.
24. Monroe, J.G., and G.N. Gaulton. 1985. Comparison of protein phosphorylation induced by mitogen and phorbol diester stimulation of murine T and B lymphocytes. *Surv. Immunol. Res.* 4:192.
25. Dymecki, S.M., J.E. Niederhuber, and S.V. Desiderio. 1990. Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. *Science (Wash. DC).* 247:332.
26. Perlmutter, R.M., J.D. Marth, S.F. Ziegler, A.M. Garvin, S. Pawar, M.P. Cooke, and K.M. Abraham. 1988. Specialized protein tyrosine kinase proto-oncogenes in hematopoietic cells. *Biochim. Biophys. Acta.* 948:245.
27. Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima. 1991. Association of B cell antigen receptor with protein tyrosine kinase lyn. *Science (Wash. DC).* 251:192.
28. Gold, M.R., J.P. Jakway, and A.L. DeFranco. 1987. Involvement of a guanine nucleotide-binding component in membrane IgM stimulated phosphoinositide breakdown. *J. Immunol.* 139:3604.
29. Harnett, M.M., and G.G.B. Klaus. 1988. G protein coupling of antigen receptor-stimulated polyphosphoinositide hydrolysis in B cells. *J. Immunol.* 140:3135.
30. Monroe, J.G., and S. Haldar. 1989. Involvement of a specific guanine nucleotide binding protein in receptor immunoglobulin stimulated inositol phospholipid hydrolysis. *Biochim. Biophys. Acta.* 1013:273.
31. Petrini, M., D.L. Emerson, and R.M. Galbraith. 1983. Linkage between surface immunoglobulin and cytoskeleton of B lymphocytes may involve Gc protein. *Nature (Lond.).* 306:73.
32. Petrini, M., P.A.M. Galbraith, D.L. Werner, and P. Arnaud. 1984. Gc (vitamin D binding protein) binds to cytoplasm of all human lymphocytes and is expressed on B cell membranes. *Clin. Immunol. Immunopathol.* 31:282.
33. Rosenspire, A.J., and Y.S. Choi. 1982. Relation between actin-associated proteins and membrane immunoglobulins in B cells. *Mol. Immunol.* 19:1515.
34. Campbell, M.-A, and B.M. Sefton. 1992. Association between B-lymphocyte membrane immunoglobulin and multiple members of the src family of protein tyrosine kinases. *Mol. Cell. Biol.* 12:2315.