Ligand-independent Signaling Functions for the B Lymphocyte Antigen Receptor and Their Role in Positive Selection during B Lymphopoiesis

Gregory Bannish,[1] Ezequiel M. Fuentes-Pananá,[1] John C. Cambier,[2] Warren S. Pear,[1] and John G. Monroe[1]

[1]University of Pennsylvania School of Medicine, Department of Pathology and Laboratory Medicine, Philadelphia, PA 19104

[2]Department of Immunology, University of Colorado Health Science Center and National Jewish Medical and Research Center, Denver, CO 80206

Abstract

Signal transduction through the B cell antigen receptor (BCR) is determined by a balance of positive and negative regulators. This balance is shifted by aggregation that results from binding to extracellular ligand. Aggregation of the BCR is necessary for eliciting negative selection or activation by BCR-expressing B cells. However, ligand-independent signaling through intermediate and mature forms of the BCR has been postulated to regulate B cell development and peripheral homeostasis. To address the importance of ligand-independent BCR signaling functions and their regulation during B cell development, we have designed a model that allows us to isolate the basal signaling functions of immunoglobulin (Ig)α/Igβ-containing BCR complexes from those that are dependent upon ligand-mediated aggregation. In vivo, we find that basal signaling is sufficient to facilitate pro-B → pre-B cell transition and to generate immature/mature peripheral B cells. The ability to generate basal signals and to drive developmental progression were both dependent on plasma membrane association of Igα/Igβ complexes and intact immunoregulatory tyrosine activation motifs (ITAM), thereby establishing a correlation between these processes. We believe that these studies are the first to directly demonstrate biologically relevant basal signaling through the BCR where the ability to interact with both conventional as well as nonconventional extracellular ligands is eliminated.

Key words: B lymphocytes • pre-B cell receptor • B cell development • B cell antigen receptor • signal transduction

Introduction

All cells respond to environmental stimuli by processing signals generated as a consequence of plasma membrane receptors interacting with extracellular ligands. The traditional model imposes a requirement for extracellular ligand binding in order to initiate biologically relevant changes in cellular physiology. Ligand binding results in either conformational changes in the receptor which unmask intrinsic enzymatic activity or facilitation of interactions between nonenzymatic motifs in the transmembrane or cytoplasmic domains and intracellular signal transducing proteins. Another mechanism by which ligands facilitate signal initiation is through their ability to aggregate receptor complexes, leading to the sequestration and focusing of positive regulators of signal transduction and excluding negative regulators. The antigen receptors on B and T lymphocytes are classic examples of these latter types of receptors (1–5). Because of the polymorphic nature of the ligand binding components which allow these receptors the potential to interact with a wide diversity of ligands (antigens), signals are induced through aggregation of nonpolymorphic signaling proteins constitutively associated with the receptor complex.

Both immature and mature stage B cells recognize and generate signals for B cell responses to polymorphic antigens through these multi-protein complexes termed the B cell antigen receptor (BCR)*. The BCR is composed of the ligand-recognizing Ig heavy and light chains in noncovalent association with Igα and Igβ transmembrane proteins. Igα

*Abbreviations used in this paper: aa, amino acid; BCR, B cell receptor; HA, hemagglutinin; ITAM, immunoregulatory tyrosine activation motif.

and Igβ are associated as disulfide-linked heterodimers and mediate the signal transduction function of the BCR. Antigen-mediated aggregation of BCR complexes is required to trigger and sustain the signaling processes necessary for generating a signal of sufficient strength and duration to elicit responses by immature and mature B cells. Aggregation triggers an intracellular signaling cascade that requires immunoregulatory tyrosine activation motifs (ITAMs) present on the cytoplasmic domains of Igα and Igβ (6, 7). Tyrosine residues on these motifs become phosphorylated, probably by Src family tyrosine kinases, and function as platforms for the recruitment of additional kinases and adaptor proteins, and subsequent activation of multiple signaling cascades (8). Recent models have predicted that aggregation of the BCR results in complexes which are more accessible to receptor proximal signal transduction (9).

B cell development is an ordered process that allows for the sequential expression and assembly of the BCR. This process is associated with the expression of intermediate forms of the BCR that reflect progress in this ordered process and whose expression correlate with distinct checkpoints in B cell development (10–17). The earliest stages, called pro-B cells (18), express pro-BCR complexes that contain Igβ in the absence of Ig heavy or light chain expression. Mice that do not express Igβ are blocked in development at the early pro-B cell stage indicating a potential role for basal signaling through the pro-BCR (16, 19). The Ig heavy chain is recombined at the late pro-B cell stage to allow surface expression of the pre-BCR, which consists of the heavy chain, λ5, VpreB, Igα, and Igβ (12, 13). Deleting components of the pre-BCR, such as the heavy chain, λ5, or VpreB1 and VpreB2, leads to a pro-B cell block in development (20–22). This developmental arrest implies that signaling occurs through the pre-BCR and is further supported by a block at the pro-B cell stage in knockout mice that lack expression of the BCR-associated tyrosine kinase Syk (23) or the signaling adaptor Blnk (SLP65) (24, 25), and in humans lacking Igα, λ5, Blnk, or the protein tyrosine kinase Btk (26).

These genetic studies demonstrate a requirement for intermediate forms of the BCR and their signaling in order for proper B cell development to proceed. However, the mechanism by which these signals are generated through these complexes, in particular the role played by extracellular ligands in the initiation and maintenance of these signals, is not established. In one study, the extracellular ligand-binding domain of IgM was fused to the intracellular domain of either Igα or Igβ, and the chimera was able to drive B cell development in B cell–deficient mice (27). The ability of both the pro-BCR and a pre-BCR, in which the conventional sites for ligand interaction has been removed, to promote development (28) argues against a requirement for conventional ligand for these Igα/Igβ containing complexes to function. Alternatives to conventional ligand binding, however, include interactions with nonpolymorphic regions of these complexes such as the extracellular regions of the Igα and Igβ proteins themselves. On the other hand, a role for ligand-independent or basal sig-

naling functions of these complexes has been proposed (29–31).

Previous studies have suggested a role for basal signals in the homeostatic survival of peripheral B cells (32). In addition, basal ligand-independent signaling has been proposed to play a role in developmental progression and the function of pre-BCR (33). However, while indirect evidence exists to imply the independence of these Igα/Igβ containing complexes on extracellular ligand for B cell development and survival, there currently exists no direct biochemical evidence for this type of signaling in developing and mature B cells. For example, while 20–30% of the surface pre-BCR localizes to glycolipid enriched membrane domains called rafts (for a review, see reference 34), this localization could be the result of in vivo ligand encounter before isolation from the bone marrow. Although the Src family kinase Fyn has been found to associate with the resting BCR through a noncovalent association with Igα, its activity in this context has not been explored (35, 36). At the functional level, while previous studies that documented developmental progression despite an inability of the pre-BCR to interact with conventional ligands through its polymorphic μ-heavy chain, they have not eliminated possible interactions through nonpolymorphic regions of the complex (28). Similar studies for the pre-TCR (37) also have not established a role for similar regions of the CD3 complex to mediate interaction with nonpolymorphic ligands.

Perhaps the best direct biochemical evidence for basal signaling through the BCR comes from the studies of Wienands et al. (38). These studies demonstrated BCR-dependent signaling in the presence of the protein tyrosine phosphatase inhibitor pervanadate. Presumably the effect of pervanadate in these studies is to block the activity of protein tyrosine phosphatases that normally counterbalance signals generated through the resting BCR (6, 39), thereby stabilizing signals that are either weak, transient, or both. Although these studies cannot formally exclude the possibility of homotypic ligands expressed on the B cells themselves or of artifactual stimulation, they nevertheless support the idea that assembled BCR complexes at the plasma membrane are generating signals in the absence of aggregation by conventional antigen. However, these studies did not evaluate the relevance of these signals to B cell function or physiology.

The current study was designed to test the existence of ligand-independent signaling functions of BCR complexes on developing and mature B cells and to evaluate their role in developmental progression. To do so, we have designed an experimental model that allows us to isolate ligand-independent functions of the BCR and its intermediate forms. Our studies were specifically designed in order to test the hypothesis that basal signals are generated as a consequence of plasma membrane localization of Igα and Igβ. The described studies establish the ability of plasma membrane localized Igα/Igβ complexes to drive early and late B cell development despite the absence of transmembrane or extracellular regions with which to interact with extracellular ligands. Furthermore, this model allows us to correlate

the ability to generate basal signals with the ability to promote developmental progression.

Materials and Methods

Construction of MAHB and Derivatives. Each component of MAHB was subcloned independently into Bluescript SK (Stratagene). Overlapping oligonucleotides for the myristoylation domain of murine Lck contained a 5′ EcoRI site with a consensus Kozak sequence and 3′ BamHI site: MYR-S (sense) 5′-AAT-TCCACCATGGGCTGTGTCTGCAGCTCAAACCCTGAA-GATG-3′ and MYR-A (antisense) 5′-GATCCATCTTCAG-GGTTTGAGCTGCAGACACAGCCCATGGTG-3′. Annealed oligonucleotides were subcloned into Bluescript SK cut with EcoRI and BamHI to form SK-Myr. Igα (*mb-1*) and Igβ (B29) were cloned by reverse transcription (RT)-PCR using cDNA prepared from the B cell line Bal-17 using the following oligonucleotides: Igα (sense) 5′-CGCATGGGGATCCAGGAAACGGTGG-CAAAATGAGAAG-3′, Igα (antisense) 5′-TGCTCTAGATG-GCTTTTCCAGCTGGGCATCTCC, Igβ (sense) 5′-CGGGG-TACCGACAAGGATGACGGCAAGGCTGGGATG-3′, Igβ (antisense) 5′-CCCAAGCTTCATTCCTGGCCTGGATGCT-CTCCTAC-3′. Overlapping oligonucleotides for the hemagglutinin (HA) epitope tag contained a 5′ XbaI site and a 3′ KpnI site: (sense) 5′-GATCCCAGTCTAGATACCCATACGACGTCC-CAGACTACGCTGGTACCTGA-3′, and (antisense) 5′-TAG-CTTCAGGTACCAGCGTAGTCTGGGACGTCGTATGGG-TATCTAGACTGG-3′. Mutations of the ITAM tyrosine residues were created with overlapping oligonucleotides as follows: Igα-ITAMmt (sense) 5′-CTGAACCTTGATGACTGTTCTATGT-TCGAGGACATCTC-3′, Igα-ITAMmt (antisense) 5′-ACAG-TCATCAAGGTTCAGGCCCTCGAAGAGATTTTCA-3′, Igβ-ITAMmt (sense) 5′-TTGAACATTGACCAGACAGCCA-CCTTTGAAGACATAG-3′, Igβ-ITAMmt (antisense) 5′-TGT-CTGGTCAATGTTCAAGCCCTCAAAGGTGTGAT-3′.

Immunofluorescence Analysis of MAHB Expression. HeLa cells were grown on coverslips and fixed in 2% formaldehyde, 0.1% glutaraldehyde, in PBS for 20 min, followed by washing three times in PBS for 5 min. The cells were incubated with anti-HA-biotin (Boehringer) diluted to 0.2 μg/μl in incubation buffer (PBS containing 0.2% saponin, 0.1% BSA, and 0.02% sodium azide) for 1 h in a humid chamber. The coverslips were washed three times and then incubated with streptavidin-FITC (BD PharMingen; 1:100) in incubation buffer for 1 h. After washing three times, the coverslips were mounted onto slides using Fluoromount-G (Southern Biotechnology Associates, Inc.) and visualized by immunofluorescence microscopy.

Electron Microscopy. Electron microscopy was performed by the biomedical imaging core facility at the University of Pennsylvania. Cultured J558L-MAHB19A4 cells were washed in PBS and fixed in 4% paraformaldehyde plus 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h. After the dehydration in graded alcohol they were infiltrated and embedded in LR White resin and cured at 58°C for 18 h. 90-nm thick sections were cut using a Diatome Diamond Knife and a Leica ultracut S microtome. Sections were picked up on 200 mesh nickel grids and nonspecific binding was blocked with 1% ovalbumin plus 0.2% cold water fish skin gelatin in PBS at room temperature (RT) for 60 min and then transferred to anti-HA (Boehringer 12CA5) overnight at 4°C. The next day the grids were washed in Tris buffer and incubated with 10 nm labeled anti–mouse Ig for 1 h at RT. After washing and staining with 2% Uranyl acetate for 3 min

the sample was observed in JEOL JEM 1010 transmission electron microscope and images captured using the Hamamatsu CCD camera and AMT 12-HR imaging software.

J558L Infection and Derivation of Cell Lines. The murine myeloma J558L and J558L-μM3 cell lines were previously modified to express CD19 and CD45 (40, 41), and all J558L cell lines used in this study were derived from these variants. The J558L cell lines and clones were grown in Iscove's Modified Dulbecco's Medium with 25 mM HEPES, 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, 50 μM β-mercaptoethanol, and 100 U/ml Penicillin-streptomycin, at 37°C and 5% CO_2. For retroviral infection of J558L cells, constructs subcloned into the MIGR1 vector were transfected via CaPO4 into the Bosc 23 packaging cell line as described (42, 43). 2×10^6 J558L cells were spin infected by resuspending in 2 ml of 1:1 growth medium to retroviral supernatant containing polybrene at a final concentration of 4 μg/ml and centrifuged at 1,000 *g* for 1.5 h at 25°C. After infection, the cells were resuspended in medium. Retrovirally infected J558L cell lines were analyzed for CD45 expression using biotinylated anti-CD45 (I3/2.5) followed by streptavidin-PE (BD PharMingen). Cells (10^6) were washed twice and resuspended in 200 μl of culture medium containing 0.5% FBS instead of 5% FBS. Cells were sorted based upon GFP and CD45 expression directly into 96-well plates using a Becton Dickinson FACStar[PLUS™].

Sucrose Density Gradient Centrifugation. The J558LμM3 cell line was infected with MAHB to create the cell line μM3-MAHB3. These cells were lysed on ice in a 1% Triton X-100 lysis buffer and subjected to sucrose gradient separation as described (44).

Analysis of Protein Phosphotyrosine Substrates in Pervanadate Treated J558L-derived Cell Lines. Pervanadate/ H_2O_2 was made by mixing 1 ml 20 mM orthovanadate with 330 μl 30% H_2O_2 at 25°C for 5 min, yielding a solution of 6 mM pervanadate plus remaining H_2O_2. This stock was diluted to 100 μM pervanadate in RPMI to make a 2× solution. Cells (5×10^6 cells) from J558L subclones were washed with RPMI and resuspended in 0.5 ml RPMI. Prewarmed cells (0.5 ml) and pervanadate (0.5 ml) were mixed together and incubated for 2 min at 37°C. Cells were pelleted by spinning 5 s in a microfuge, and then resuspended in 100 μl RIPA lysis buffer (PBS containing 1% N-P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM orthovanadate, and 1.5 μg/ml each of pepstatin A, leupeptin, chymostatin, and antipain) for 15 min at 4°C. Lysates were clarified by centrifugation at 12,000 rpm in a microfuge for 10 min. Protein concentration was determined and 100 μg of each sample were loaded onto an SDS-PAGE 6–12% polyacrylamide gradient gel, followed by transfer to nitrocellulose membranes. After blocking with 10% FBS in TBST (10 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20), Western blot analysis was performed using anti–phosphotyrosine antibody (4G10, UBI, diluted 1:5,000). Phosphoproteins were visualized using peroxidase-labeled horse anti–mouse IgG (Vector Laboratories) as a secondary antibody followed by enhanced chemiluminescence (ECL).

HA and Fgr protein levels on stripped and reprobed membranes were accomplished in a similar manner except that 3% milk in TBST was used for blocking. Mouse anti-HA (16B12, diluted 1:2,000) or rabbit anti-Fgr antibodies were used as primary reagents with peroxidase-conjugated horse anti–mouse IgG (Vector Laboratories) or donkey anti–rabbit IgG (Amersham Pharmacia Biotech), respectively, used as secondary antibodies.

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Retroviral Infection of Progenitor-enriched Cultures and Bone Marrow Transfer. Infection of bone marrow cells from female *Rag2^{−/−}* BALB/c mice (The Jackson Laboratory) with green fluorescent protein (GFP)-normalized retroviral supernatants and transplantation of these cells into lethally irradiated (950 rad) 6–8-wk-old female syngeneic recipients was performed as described (42). Spinoculations were performed in medium containing IL-3 (6 ng/ml; R&D Systems), IL-6 (10 ng/ml; R&D Systems), stem cell factor (100 ng/ml; R&D Systems), and 5% WEHI-conditioned supernatant as described. On day 3 after bone marrow harvest, 5×10^5 cells were injected into syngeneic 6–8-wk-old female mice that had been lethally irradiated (950 rad).

Flow Cytometry Analysis of Bone Marrow and Splenic B Cells. Mouse bone marrow or splenocytes were erythrocyte-depleted using Gey's solution. Cells were analyzed for expression of B cell developmental markers using CD19-biotin(1D3) and CD43-PE (S7) (BD PharMingen) followed with streptavidin-allophycocyanin (APC; BD PharMingen), or B220-APC (RA3–6B2), IA/IE-PE (2G9), and CD23-biotin (B3B4) (BD PharMingen) followed with streptavidin-Red670 (GIBCO BRL). Samples stained with CD19 and CD43 were treated with 10 μg/ml propidium iodide immediately before analysis to allow for dead cell exclusion.

Results

MAHB Is a Model for Aggregation-independent, Basal Signaling Functions of BCR Complexes. The chimeric protein MAHB (Fig. 1 A) was designed to model potential ligand-independent functions of the BCR complex and its intermediate forms that are expressed during B cell development (13, 45–47). MAHB consists of the entire cytoplas-
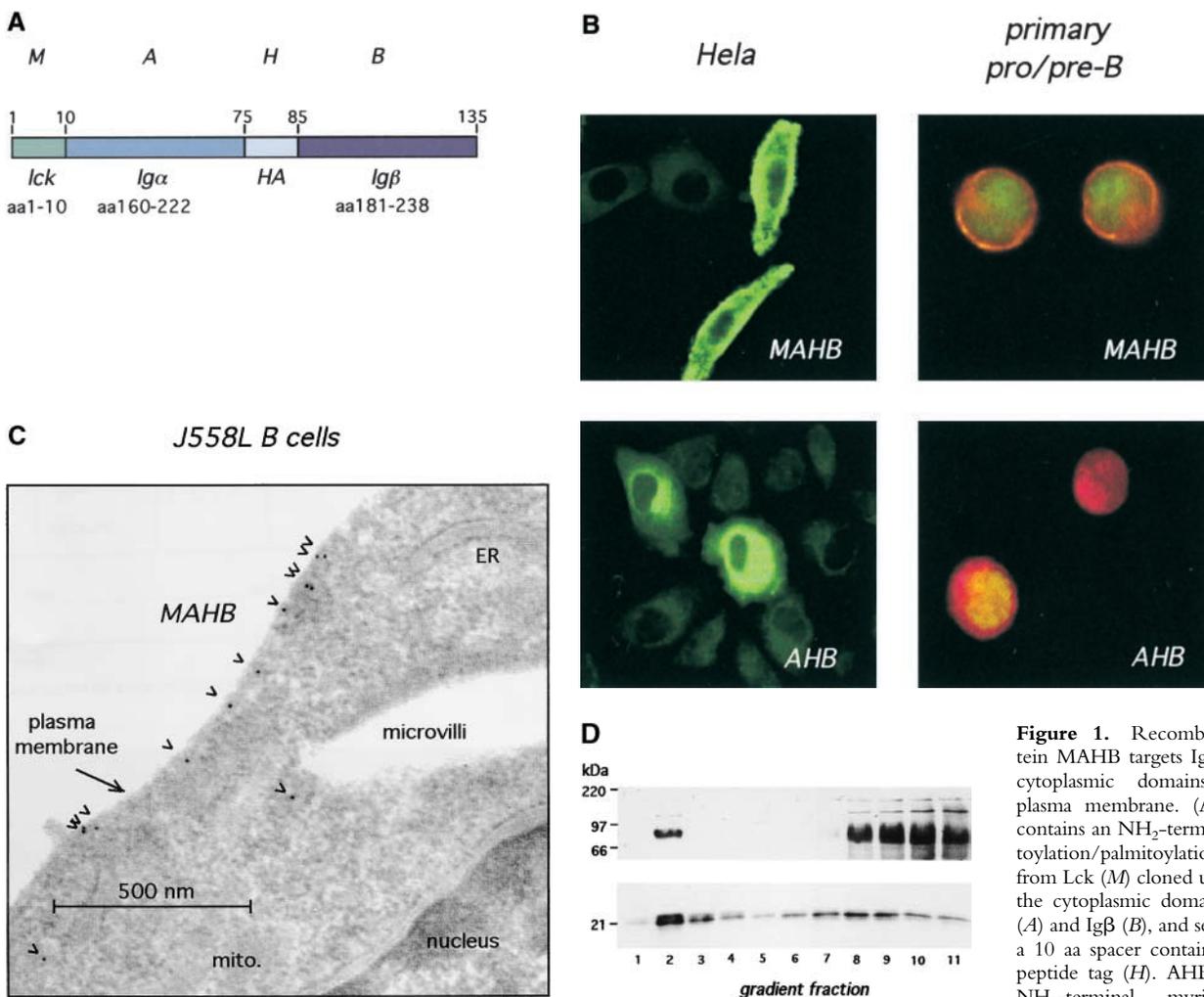

Figure 1. Recombinant protein MAHB targets Igα and Igβ cytoplasmic domains to the plasma membrane. (A) MAHB contains an NH2-terminal myristoylation/palmitoylation domain from Lck (*M*) cloned upstream of the cytoplasmic domains of Igα (*A*) and Igβ (*B*), and separated by a 10 aa spacer containing a HA peptide tag (*H*). AHB lacks the NH2-terminal myristoylation/palmitoylation site. (B) Expression and subcellular distribution of MAHB. (Left) HeLa cells were transiently transfected with the expression vector pSV2Zeo containing MAHB (top) or AHB (bottom). Cells were fixed and stained with biotinylated 12CA5 (anti-HA) followed with SA-FITC, which detects MAHB as green. (Right) Pro-B cells from RAG2^{−/−} mice were grown in vitro with IL-7 and were infected with the retrovirus MIGR1.str, which coexpresses GFP (shown in green) and either MAHB (top) or AHB (bottom). Cells were fixed and stained with 12CA5-rhodamine, which detects MAHB by red fluorescence. (C) Electron microscopy of the MAHB myeloma clone J558L-MAHB19A4. Cells were fixed in resin, and sections were stained with a primary antibody reacting against HA and a secondary antibody containing gold particles (18 nm). MAHB is detected as black spots. (D) Sucrose gradient fractionation. The BCR-positive J558L-μM3 cell line was infected with MAHB and the resting cell line was lysed on ice and subjected to sucrose gradient fractionation as described in Materials and Methods and reference 44). Fractions were removed, equal volumes were loaded onto a polyacrylamide gel, and blots were subjected to Western blot analysis using antibodies to IgM (top) or MAHB (bottom). Fractions 1–3 contain buoyant raft fractions, and fractions 8–11 contain soluble fractions.

mic regions of the murine Igα (CD79a; amino acid [aa] 160–222) and murine Igβ (CD79b; aa181–238) signaling components of the BCR. Each of these regions contain not only the ITAM motif for which there is a known requirement in BCR signal transduction and B cell development (48, 49), but also additional regions that are less well defined (50, 51). These portions of MAHB are separated by a 10 aa spacer containing an epitope tag from the influenza virus protein HA.

To isolate ligand and aggregation-independent processes from those mediated by extracellular ligand-mediated aggregation, we used the Lck myristoylation/palmitoylation sequence (murine Lck aa1–10) as a targeting motif. By so doing, MAHB could be targeted to the inner leaflet of the plasma membrane without transmembrane and extracellular sequences that could mediate potential ligand-induced effects. In so doing, this design allows the modeling of α/β plasma membrane complexes in a manner that obviates interactions with extracellular conventional as well as nonconventional ligands. The Lck targeting sequence was specifically chosen for this model because previous studies had demonstrated noncovalent interactions of murine Lck with the BCR in "resting" primary murine B cells and B cell lines (52, 53). Therefore, we reasoned that this targeting strategy would direct MAHB to regions of the plasma membrane that are normally occupied by the BCR when in its unligated and nonaggregated state.

Fig. 1 B demonstrates the ability to target MAHB to the plasma membrane in Hela cells (Fig. 1 B, left panel) and primary murine pro-B cells (Fig. 1 B, right panel). The latter were derived from IL-7 maintained Hardy Fraction C and C' (late pro-B) (18) murine B cells that had been infected with the MIGR1 retrovirus expressing MAHB. In both cases, localization was dependent upon the targeting motif since its removal in the AHB truncation results in cytoplasmic localization of the chimeric protein.

The relatively uniform distribution of MAHB in the plasma membrane of the HeLa and pro-B cells suggests that MAHB does not exist in a constitutively aggregated state nor is it selectively targeted to specialized regions of the plasma membrane. The former conclusion was further substantiated by transmission election microscopic analysis (Fig. 1 C). A representative section of J558L B cells expressing MAHB shows that the protein is dispersed at the plasma membrane. Furthermore, sucrose density gradients of Triton X-100 solubilized membranes from J558L B cells that coexpressed MAHB and conventional BCR (IgM form) determined that MAHB and IgM reside within the same fractions (Fig. 1 D). In our hands, conventional BCR (and also MAHB) is resident in both the buoyant raft fractions (1–4) and nonbuoyant fractions even in the absence of ligand-induced aggregation. Notably, aggregation results in a redistribution and increase in the number of receptor complexes in the buoyant fractions as has been shown by others (44). These latter observations suggest that MAHB and the conventional BCR localize to similar plasma membrane regions in resting B cells.

Although our localization data in Fig. 1 suggested that MAHB did not self aggregate, we attempted to rule this out using a more functional readout. Fig. 2, A and B, are phosphotyrosine immunoblots comparing conventional BCR-expressing (J558L-μm3) and MAHB-expressing (MAHB19A4) variants of the J558L B cell line. The parental J558L B cells lack surface expression of the BCR as well as CD45 and CD19. The variants used in our studies have been engineered to express the BCR in the case of the μm3 variant, and all lines have been transfected so that they express both B220 and CD19 (41, 54). MAHB was introduced into the BCR nonexpressing variant through retroviral infection, followed by sorting and limiting dilution cloning, so as to generate MAHB-expressing lines.

In Fig. 2 A one can observe that a significant signal is only detectable when the conventional BCR is aggregated with anti-IgM antibodies. The blots were stripped and reprobed with an antibody to the Src family kinase Fgr for use as a protein loading control. If MAHB was generating signals as a consequence of forming spontaneous stable aggregates or localizing to regions of the plasma membrane that bypasses a need for ligand, one would predict that it should resemble anti-IgM stimulated J558L-μm3 in the absence of pervanadate. The lower band present in the MAHB-expressing B cells and absent in the J558L-μm3 is the phosphorylated MAHB protein (asterisk). A similar but faint band migrating slightly higher in the anti-IgM stimulated μM3 lane (Fig. 2 A) is Igα as determined by Western blotting (unpublished data). In contrast to these results, both MAHB and the conventional BCR expressing B cells demonstrate a detectable signal after incubation with the protein tyrosine phosphatase pervanadate (Fig. 2 B). Importantly, in the absence of MAHB or the conventional BCR, J558L B cells and B cells infected with an empty MIGR retrovirus failed to demonstrate the extensive pattern of inducible protein phosphotyrosine substrates. The dependence on MAHB or the conventional BCR for generating pervanadate-induced signals supports the notion that these structures are generating basal signals which are stabilized by tyrosine phosphatase inhibition (38). Furthermore, the similar pattern of phosphotyrosine substrates in the pervanadate-treated J558L-μm3 and MAHB19A4 argues that the chimeric protein accurately models these stabilized signals, both qualitatively as well as quantitatively.

This conclusion is further supported by the dose–response data shown in Fig. 2 C. Here we have employed lower doses of pervanadate in order to detect potential differences in the quantitative levels of signal between MAHB and the conventional BCR that could be obscured by the higher doses. Our ability to first discern a pervanadate-associated signal occurs at 5 μM pervanadate. At this point, the BCR signal is equal to or greater than that dependent on MAHB expression. Although these assays are variable from one experiment to another, the experiment shown in Fig. 2 C is typical in failing to demonstrate a marked, reproducible, quantitative difference in the level of pervanadate-stabilized signal when comparing conventional BCR with MAHB.
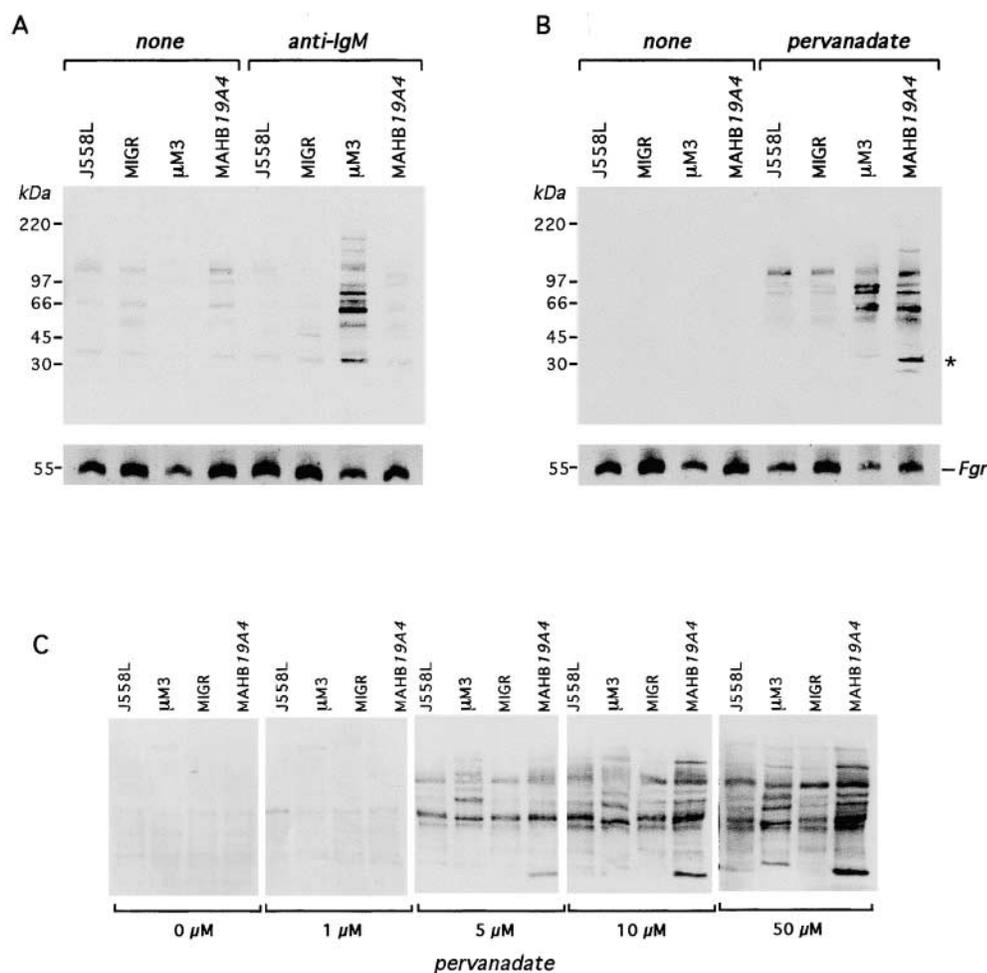
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Figure 2. Comparison of ligand-induced and basal signaling through MAHB and the conventional BCR. (A) The indicated derivatives of the J558L B cell line were either untreated (left) or treated with anti-IgM (10 μg/ml) for 5 min (right), and proteins from whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by Western blotting using the antiphosphotyrosine antibody 4G10. The blot was stripped and reprobed with an antibody to Fgr for use as a protein loading control. (B) The same conditions as in panel A except that the cell lines were treated with pervanadate (50 μM) instead of anti-IgM. The (*) indicates the position of the MAHB protein. (C) Titration of pervanadate. The indicated concentrations of pervanadate were included in the media of J558L, μM3, MIGR12B3, and MAHB19A4 cell lines. Then, 166,000 cell equivalents per lane were subjected to Western analysis using the antiphosphotyrosine antibody 4G10 as in panel A. At 50 μM pervanadate, the intensities of bands in the BCR^pos μM3 and the MAHB19A4 cell lines were more intense than the negative control cell lines J558L and MIGR12B3.

The apparent lack of evidence for self-aggregation or membrane compartmentalization argues that MAHB is an appropriate model of the BCR in its unligated state. The similarity of the pervanadate stabilized signals when comparing MAHB to the conventional BCR supports the conclusion that MAHB accurately models what little published biochemistry exists for basal signaling through the conventional BCR (38). Using this model, we wished to now address the hypothesis that targeting of Igα and Igβ to the plasma membrane is sufficient to generate basal signals and that these signals are biologically relevant to developing B cells.

MAHB Can Mediate Positive Selection of B Cells through the Pro-B → Pre-B Checkpoint. The above studies provide biochemical evidence to support the conclusion that MAHB provides an appropriate experimental model to isolate and test the biological relevance of aggregation–independent functions of the BCR. To test whether these signals are sufficient to mediate the positive selection normally generated through the pre-BCR, we retrovirally infected MAHB into hematopoietic progenitors from μMT mice. B cell development in these mice is arrested at the pro-B → pre-B transition due to the inability to assemble a pre-BCR at the plasma membrane (20). This arrest can be assessed by the inability of these mice to generate CD22^posCD43^neg B cells in the bone marrow and a complete absence of IgM^pos B cells in the periphery (20, 21).

Stem cell progenitors from μMT mice were infected with empty (MIGR) and MAHB-expressing (MAHB) retrovirus and transferred into lethally irradiated μMT recipient mice. The GFP marker afforded us the ability to distinguish infected from uninfected cells. In so doing, direct comparisons between wild-type μMT and MAHB-expressing μMT cells (or MIGR controls) could be accomplished in the same recipient mouse. The top panels are analyses of unmanipulated C57Bl/6 and μMT bone marrow and spleen (Fig. 3, A and B, respectively). As expected, the μMT mice lack the B220^pos, CD22^pos population in the bone marrow that is comprised of pre-B, immature, and recirculating mature B cell subsets present in the normal C57Bl/6 mice. μMT mice exhibit a complete lack of splenic CD19^pos B cells (top panel, Fig. 3 B). Compare this to C57Bl/6 spleen which has a significant population of CD19^pos splenocytes, the vast majority of which are CD43^neg indicating progression past the proB cell stage (18). Fig. 3, C and D, compares CD22 and CD43 expression on B cells generated from μMT progenitors expressing MAHB with those expressing empty virus
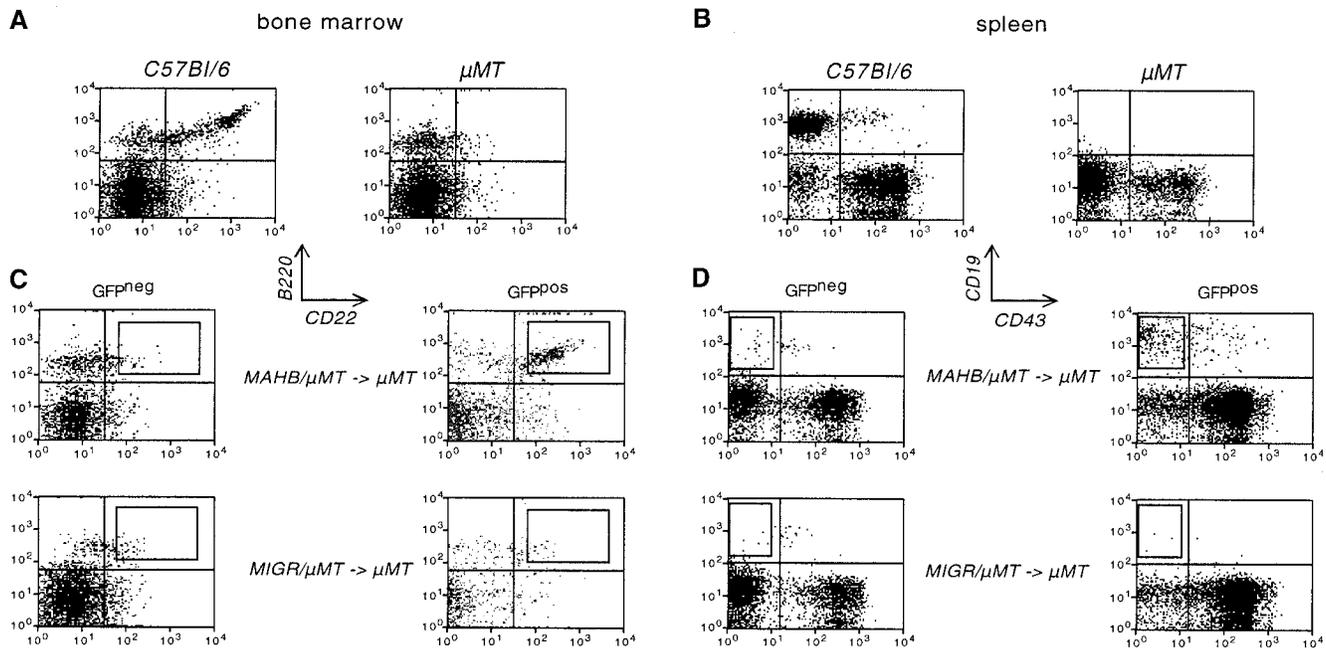
Figure 3. Bone marrow B cell development in MAHB-expressing µMT progenitors. Bone marrow from wild-type and µMT mice were analyzed by flow cytometry for surface expression of CD22 and CD43, along with the pan B cell markers B220 or CD19, in order to identify proB stage cells (CD22^neg, CD43^pos) from those B cells which have traversed the pro-B → pre-B checkpoint (CD22^pos, CD43^neg). Dead cells were excluded by propidium iodide exclusion in order to focus analysis on all live cells in the population. (A) B cell expression in the bone marrow. Wild-type mice (left panel) contain B cells (B220^pos) at the pro-B stage (CD22^neg) and at later stages (CD22^neg). In contrast, B cells in µMT mice (right panel) are all at the pro-B stage. (B) B cell expression in the spleen. Wild-type mice (left panel) contain B cells (CD19^pos) in the spleen, and they have progressed past the pro-B stage because they lack CD43. The µMT mice (right panel) completely lack B cells in the spleen (no CD19^pos cells). (C) Bone marrow of lethally irradiated µMT mice adoptively transferred with retrovirus infected µMT bone marrow hematopoietic progenitors expressing either MAHB (top) or the empty virus vector MIGR (bottom panels). Analyses were performed 4 wk after adoptive transfer. GFP^pos cells (right) are those derived from progenitors successfully infected with the indicated retrovirus; GFP^neg cells (left) represent noninfected cells for comparison. The boxed region represents B cells that have progressed past the pro-B stage. Only cells from GFP^pos, µMT mice contain a significant fraction of these developed B cells (boxed region of top right panel). (D) B cell development in splenic B cells from MAHB-expressing µMT mice. Spleens from adoptively transferred µMT mice were analyzed for expression of the B cell marker CD19 and the pro-B marker CD43, and results are shown as in C. Only GFP^pos cells from MAHB mice contain a significant fraction of B cells (CD19^pos), and they have developed past the pro-B stage (boxed region of top right panel).

(MIGR) or uninfected B cells (GFP^neg). Uninfected GFP^neg B cells present in both experimental groups fail to down-regulate CD43 or up-regulate CD22 surface expression in the bone marrow and spleen, respectively (see left-most panels of Fig. 3, C and D). This indicates that in the absence of MAHB, B cell progenitors maintain the developmental arrest characteristic of the µMT mouse. In contrast, GFP^pos B cells from the MAHB mouse express MAHB and resemble the wild-type mouse in that they express CD22 in the bone marrow, are present in the spleen, and lack CD43 expression (see top right panels of Fig. 3, C and D). The small fraction of GFP^negCD19^posCD43^pos B cells in the spleens of MAHB mice could be the result of a low proportion of stem cells homing to the spleen during the adoptive transfer (55) and subsequent B cell development within the spleen. This notion is further supported by similar findings in MIGR mice (Fig. 3, C and D, bottom right panels) and in mock–infected adoptive transfer experiments (unpublished data).

These results clearly demonstrate that MAHB can overcome the arrest in B cell development that is associated with the µMT mutation. More importantly, they argue for biological relevance for the aggregation-independent sig-

nals modeled by MAHB. These results imply that basal signals generated as a consequence of plasma membrane targeting of Igα/Igβ complexes have a biological function that is linked to the process of positive selection during B lymphopoiesis.

MAHB-mediated Signals Drive Progression to the Peripheral Transitional Immature/Mature B Compartments of B Cell Development. To determine whether the MAHB-expressing cells continue through development or alternatively arrest at the pre-B stage, we evaluated expression of two markers present in later stages of B cell development, MHC class II and CD23. MHC class II is not expressed on pro-B cells and is only expressed weakly or not at all at the pre-B stage. However, it is expressed strongly on a subset of the more advanced immature B cells in the bone marrow (56) and on mature B cells. CD23 is expressed only on peripheral transitional immature and mature B cells (57, 58). Splenic cells from MAHB or MIGR mice were stained with B220, CD23, and MHC class II antibodies, gated on a forward/side scatter lymphocyte gate, and divided into GFP^neg (Fig. 4, left) or GFP^pos (Fig. 4, right) fractions. Similar to the previous analyses, the GFP^neg fraction of cells that do not express MAHB likewise contain few splenic B
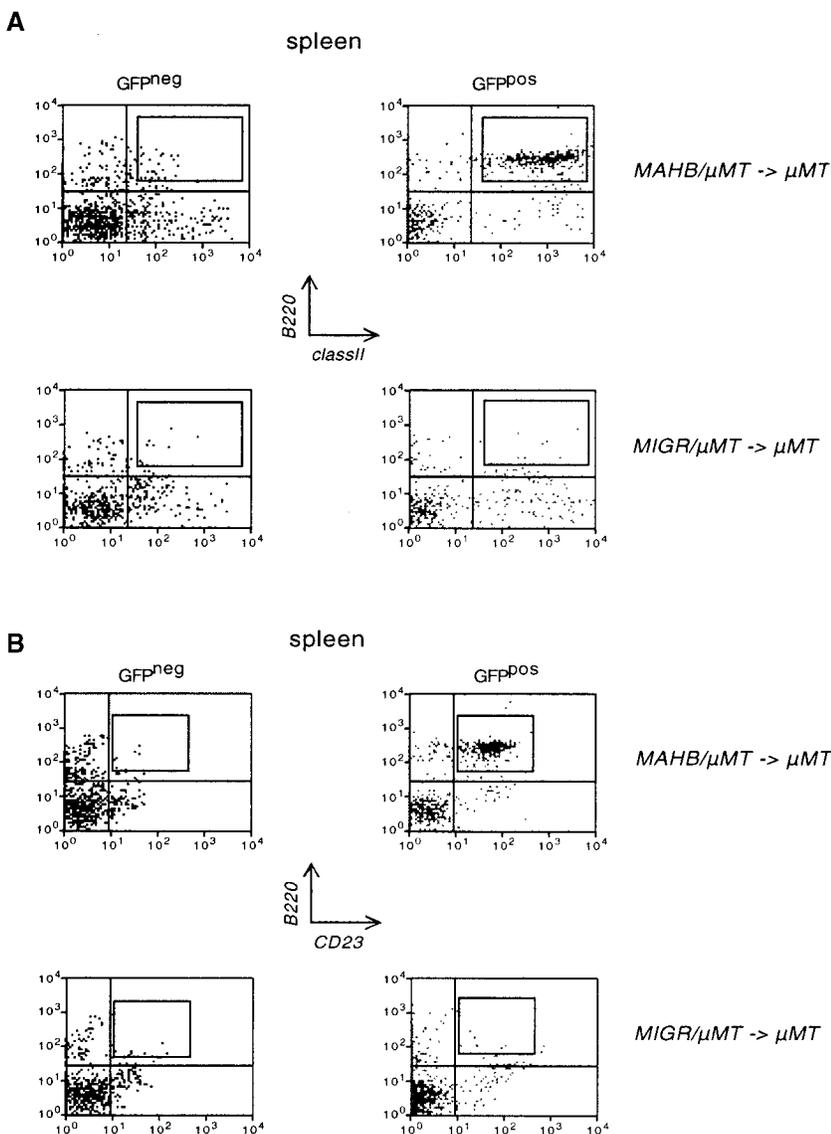
A

B

Figure 4. Peripheral B cell development after adoptive transfer of MAHB-expressing μMT progenitors. Splenocytes were obtained from lethally irradiated μMT mice that had been adoptively transferred with μMT hematopoietic progenitors infected with MAHB or the empty virus vector MIGR. All panels depict flow cytometric analysis of surface B220 on splenocytes gated with forward and side scatter to eliminate dead cells. (A) MHC class II expression on splenocytes from MAHB (top) or MIGR (bottom) expressing μMT mice. Retrovirus-expressing cells were selected by gating on GFP (right panels), whereas uninfected GFP^{neg} cells represented an internal negative control (left panels). Only GFP^{pos} cells from MAHB μMT mice contain B cells that express MHC class II (boxed region of top right panel). (B) Same as in panel A except analyzed for CD23 expression. The MHC class II positive B cells from panel A coexpress the CD23 marker shown in B (unpublished data).

cells and lack CD23 and MHC class II on their surface. The MIGR mouse also shared this phenotype, as well as a control μMT spleen, which lacks B cells (see Fig. 3). However, most splenic B cells from the GFP[pos] MAHB mouse expressed both MHC class II and CD23 (Fig. 4, A and B, top right quadrants of top right panels) and these cells coexpressed both markers (unpublished data). This was consistent with B cells seen in a wild-type mouse spleen. Therefore, we conclude that signals generated by MAHB are sufficient for transition through both early and late checkpoints of B cell development.

Low Levels of MAHB Expression Mediate Positive Selection. The pre-BCR is expressed at low levels on the surface of late pro-B and pre-B cells and is almost undetectable by surface staining and flow analysis. One potential caveat to our studies is that retroviral infection leads to overexpression of MAHB relative to physiological levels of the surface pre-BCR. If the difference in signaling between an activated versus resting pre-BCR or BCR is purely quantitative, then high levels of MAHB may imitate an aggregated receptor and aggregation of the pre-BCR might be required for developmental progression. One argument against this caveat is our observation that basal signals mediated through MAHB in J558L B cells are not stronger than those observed for the conventional resting BCR (Fig. 2 B, and see Fig. 7 A to follow). In addition, preliminary data comparing the intracellular calcium levels between MAHB versus BCR expressing J558L cells indicates that individual cells from both lines contain baseline levels of calcium far below levels of an aggregated BCR (unpublished data). Additionally, if high MAHB levels were mimicking an activated BCR, then immature B cells would undergo negative selection via apoptosis and would not be present in the periphery. Any B cells that could survive this negative selection and develop into mature B cells would express activation markers typical for a BCR cross-linked B cell, such

as CD69, CD80, and CD86. However, a significant population of peripheral immature/mature B cells is present in the spleens of MAHB mice (Fig. 4), and they do not exhibit upregulated expression of these activation markers (unpublished data).

To directly test whether only high MAHB levels could be responsible for B cell developmental progression, we compared the levels of GFP on B cells in the MAHB mouse. B cells in the bone marrow had lower levels of GFP than a large population of non-B, CD43^{pos} cells, but were similar to levels of GFP in thymocytes (Fig. 5 A, and unpublished data). Notably, the mean GFP level of B cells in the bone marrow was similar to that of B cells in the spleen, indicating that high levels of MAHB did not confer a selective advantage (Fig. 5 B, and unpublished data). Multiple gates were defined in order to isolate bone marrow cells with various levels of GFP, and the percentage of B cells that had transited the pro-B → pre-B block (CD43^{neg}CD22^{pos}) was determined. As can be seen, even low levels of GFP, as seen in gate 4 and 5, corresponded to a significant fraction of B cells that were CD43^{neg}CD22^{pos} (34 and 54%, respectively; Fig. 5 C) compared with GFP^{neg} (gates 2 and 3 are 12 and 10%, respectively) and GFP^{high} (gate 8, 82%). Similar results were obtained when we analyzed the additional developmental markers MHC class II and CD23 from splenic B cells (unpublished data). These results suggest that signals derived from the pre-BCR may be may be similar to the basal signals derived from MAHB.

Basal Signaling Functions of Igα/Igβ Complexes Require Plasma Membrane Localization. To begin to define the minimal requirements for generating biologically relevant ligand–independent BCR signals, we have initiated structure/function analysis of MAHB in the context of pervanadate–dependent signal initiation and proB to preB transition.

To determine whether surface localization was a requirement for the generation of pervanadate–induced signals, we generated stable J558L B cell clones expressing MAHB with amino acid substitutions at the myristoylation and palmitoylation sites in the targeting motifs (MPP). These mutations eliminate the fatty acid modifications that allow targeting to the plasma membrane. MPP rather than AHB was used as the model for surface dependence because for reasons that remain unknown, AHB protein was degraded more rapidly in the J558L B cell lines and could not be detected. However, like AHB, MPP expression was found to be undetectable at the cell surface by immunofluorescence and found entirely localized to the cytoplasm (unpublished data). We observed that signaling by the conventional BCR and MAHB was nearly identical in the presence of pervanadate (Fig. 6 A). The band at ∼21 kD in the MAHB-expressing cells but absent in the BCR–expressing cells represents the MAHB protein. Interestingly, signals from the cytoplasmic MPP protein were indistinguishable from J558L or the empty retrovirus clone MIGR 12B3, indicating that surface expression of Igα and Igβ is necessary for generating these signals. Moreover, the MPP mutation eliminated the ability of MAHB to drive developmental
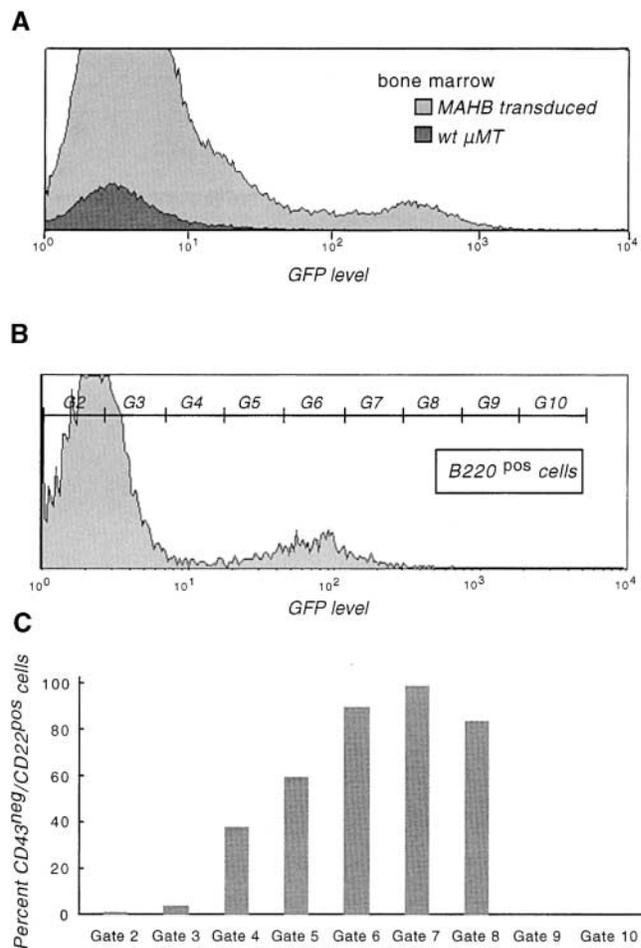

Figure 5. Expression level dependence of MAHB driven B cell development from μMT progenitors. Erythrocyte-depleted cells from the bone marrow of an MAHB reconstituted μMT mouse were analyzed for expression of B220, CD22, and CD43. (A) Live cells were selected using a forward and side scatter gate, and analyzed for levels of GFP expression. The reconstituted MAHB is lightly shaded, and bone marrow from a normal mouse was used as a negative control (dark shaded). (B) B cells from the bone marrow were selected by gating on B220 and analyzed for GFP expression. A series of gates were drawn to distinguish GFP negative (gates 2 and 3), low (gates 4 and 5), and high (gates 6–10). (C) Cells were gated for B220 and the levels of GFP expression in B, and CD43 versus CD22 plots were obtained. For each gate, the percentage of CD22^{pos} CD43^{neg} cells is shown, which represents B cell progression past the pro-B stage.

progression of either RAG2$^{-/-}$ or μMT progenitors (unpublished data), indicating that the ability to generate basal signals correlates with the ability to mediate positive selection (see the following section).

Fig. 6 B is a comparison of the relative levels of MAHB protein expression for each of the infected lines depicted in Fig. 6 A. Each are detected by Western blotting with anti-HA antibody and shown as compared with protein levels of the Fgr tyrosine kinase, also detected by Western blotting; in this case with anti-Fgr antibodies. As expected, no MAHB is observed in the parental J558L or J558L-μm3 or in the empty virus infected cells, whereas detectable signal is observed in the MAHB and MPP lanes.
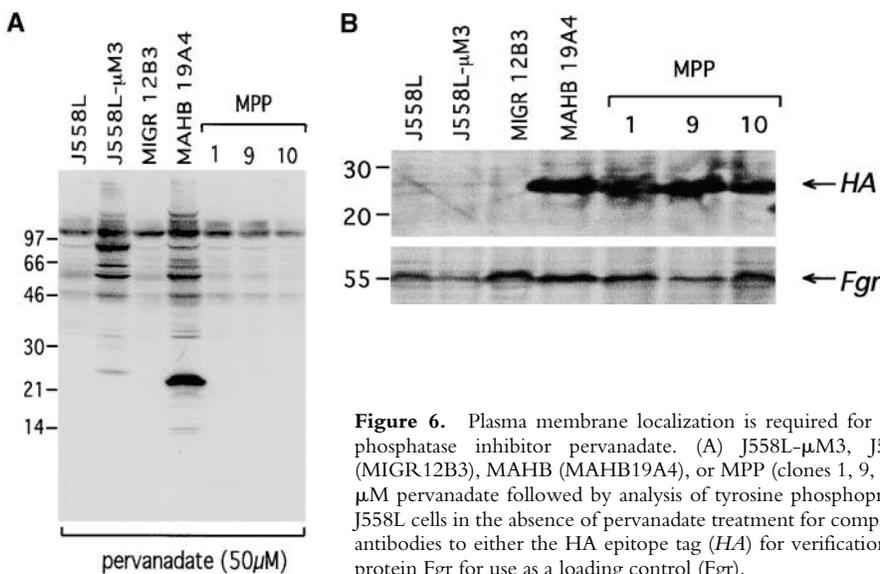
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Figure 6. Plasma membrane localization is required for basal signaling in the presence of protein tyrosine phosphatase inhibitor pervanadate. (A) J558L-μM3, J558L expressing the empty MIGR retrovirus (MIGR12B3), MAHB (MAHB19A4), or MPP (clones 1, 9, 10) were cultured for 2 min in media containing 50 μM pervanadate followed by analysis of tyrosine phosphoproteins as in Fig. 2 B. The far leftmost lane depicts J558L cells in the absence of pervanadate treatment for comparison. (B) The blot was stripped and reprobed with antibodies to either the HA epitope tag (*HA*) for verification of MAHB expression, or to the B cell expressing protein Fgr for use as a loading control (Fgr).

Aggregation-independent Signaling and Positive Selection are Both ITAM Dependent. Ligand-induced BCR signaling depends upon the tyrosine residues associated with the ITAMs of Igα and Igβ (48, 59). To assess whether aggregation-independent signaling also required the Igα/Igβ ITAMs, we generated J558L cell lines expressing MAHB with Y→F substitutions in $Y_{182, 193}$ and $Y_{195, 206}$ of Igα and Igβ, respectively. These mutations functionally destroy the ability of Igα/Igβ ITAMs to transduce aggregation-dependent signals (60). A panel of clones expressing either the wild-type MAHB or the ITAM mutant was generated. For the studies reported here, we selected four clones of each that were matched for GFP and B220 expression levels. As illustrated in Fig. 7 A, each of the 4 MAHB expressing J558L B cells generated signals that were detectable in the presence of pervanadate. In contrast, the four ITAM mutant expressing cell lines (mITAM) failed to signal in the presence of pervanadate. Clone 3–12 contained levels of phosphotyrosine proteins that were higher than the negative controls J558L and MIGR 12B3, but were lower than all four of the MAHB clones. Therefore, like conventional ligand-dependent BCR signaling, basal or ligand-independent signaling is also ITAM dependent.

Also shown in Fig. 7 are the functional effects of mutating the Igα/Igβ ITAMs associated with MAHB. Correlating with the ablation of the signaling in the J558L B cells, the ITAM mutated MAHB (mITAM) was unable to generate signals sufficient to drive pro-B → pre-B transition by μMT progenitors (Fig. 7 B). These studies not only confirm previous reports for ITAM dependence for pre-BCR–mediated positive selection, they extend these reports by establishing the role of the ITAMs under conditions where ligand-dependent signaling is eliminated. Furthermore, the pervanadate studies in Fig. 7 A extend those reported for conventional BCR signaling in the J558L system (38) by the ITAM-dependence for basal signaling under these conditions.

Discussion

We have designed a model system that allows us to isolate ligand-independent functions of the BCR and its developmental-associated intermediates from those functions that are dependent on ligand-induced aggregation. By doing so, we are able to focus on basal signals that are generated through these receptor complexes and to analyze their regulation and biological relevance to B cell physiology. In this report, we have studied the ability of basal signals to mediate positive selection at the pro-B → pre-B checkpoint. In establishing that signals modeled by MAHB are able to mediate positive selection of B cell progenitors that lack the ability to express pre-BCR or BCR complexes at their plasma membrane, we extend and integrate previous studies to evaluate ligand binding requirements for the pre-BCR and biochemical studies of putative basal signaling functions of the mature BCR. We believe that our studies represent the first demonstration for biologically relevant signaling through Igα/Igβ containing complexes in which the ability to interact with extracellular ligand has been definitively eliminated.

The implications for these studies with respect to basal signals for survival of developing and peripheral T and B lymphocytes is clear. In both cases, the activity of their respective antigen receptors is determined by the balance of transiently and constitutively active positive and negative regulators such as CD45, Src family protein tyrosine kinases (SPTK), CD22, CD5, and SHP-family protein tyrosine phosphatases, among others (61). The transient signals that are generated as a consequence of balancing the constitutive activity of CD45 and its ability to activate receptor-associated SPTK (62) with the inducible phosphorylation of ITIM-containing negative regulators such as CD22 and its ability to recruit SHP-1 to the receptor complex (61) are postulated to constitute the basal signals that mediate the biological effects reported in these studies. Of more general relevance is the possibility that other receptors that initiate
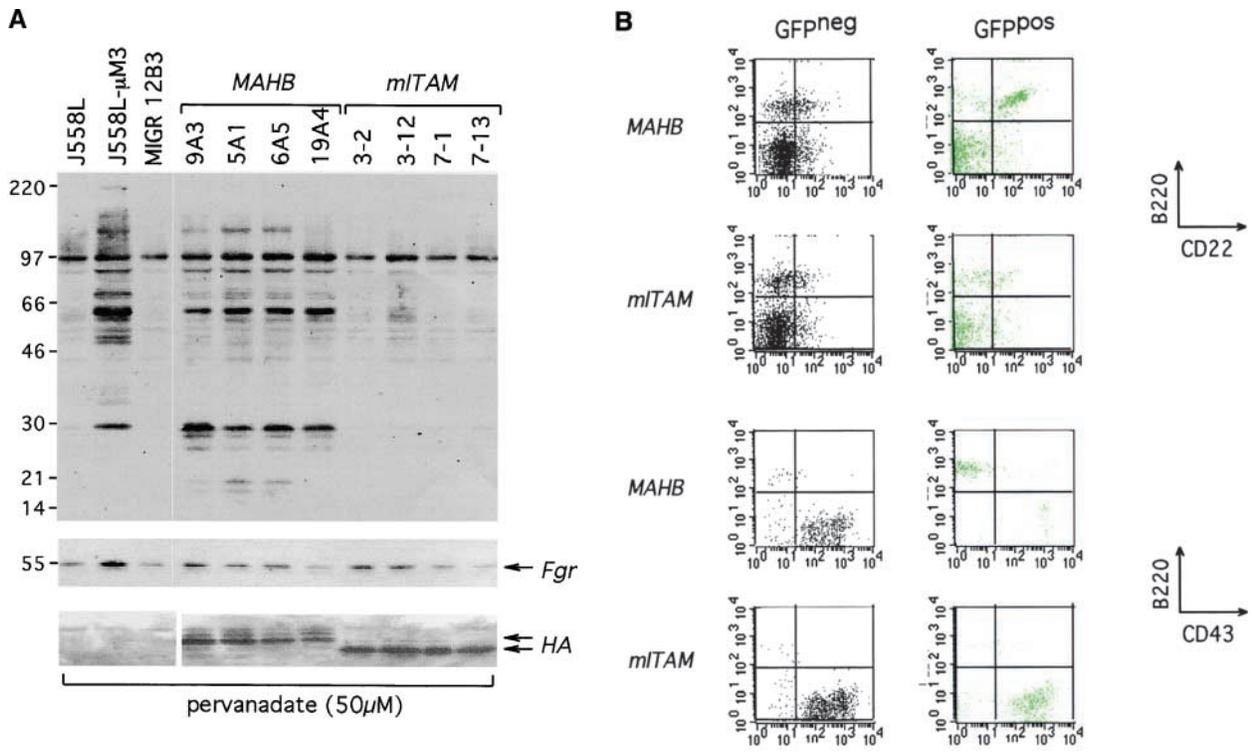
Figure 7. ITAM dependence for aggregation–independent signaling and basal signaling. (A) Pervanadate stimulation and comparison of wild-type MAHB (clones 9A3, 5A1, 6A5, 19A4) and MAHB with mutated ITAM motifs (mITAM clones 3–2, 3–12, 7–1, 7–13) expressing J558L B cells. The indicated cell lines were stimulated with pervanadate, and Western blots were probed with antiphosphotyrosine (top), anti-Fgr (middle), and anti-HA (bottom; same as Fig. 6). (B) Splenic cells from μMT mice containing MAHB or mITAM were analyzed for expression of B220 and CD22 (top), or for B220 and CD43 (bottom; same as for Fig. 3, C and D).

their signals via protein tyrosine kinase activation may also have basal signaling components for their function. In all cases, ligand induced conformational changes or aggregation may alter the signal qualitatively, or provide a mechanism to sustain the signal. Given that different cellular responses are known to be triggered at different signaling thresholds (63), the ability to elicit appropriate cellular responses to different environmental stimuli by evoking both ligand independent as well as ligand dependent functions for receptors provides a mechanism for enhanced flexibility in regulating cellular physiology.

The ability of MAHB expression to drive B cell development from μMT progenitors to the peripheral immature/mature stage definitively demonstrates the lack of a requirement for overt ligand-mediated aggregation for transition through the pro-B → pre-B checkpoint. Identical results have been obtained using progenitors derived from bone marrow of $Rag2^{-/-}$ mice (unpublished data). We interpret these findings to indicate that a low level "basal" signal generated through this receptor provides the signals necessary for positive selection at the pro-B → pre-B cell stage. We further suggest that plasma membrane localization of the Igα/Igβ cytoplasmic domains is sufficient for generating this signal. These results imply that a primary function of the calnexin pro-B, surrogate light chain pre-B, and fully assembled immature/mature BCR is to bring Igα

and Igβ to the plasma membrane. By doing so, these receptor complexes herald the successful expression of *mb-1* and *B29* genes (pro-BCR), followed by the successful completion of Ig heavy chain recombination (pre-BCR), and finally the complete assembly and function of the mature BCR. The aggregation-independent signal generated by this surface expression may then function to positively select those B cells in which the ordered and sequential assembly of the BCR is proceeding properly.

These findings argue against a requirement for either conventional (i.e., CDR binding) or nonconventional ligands for generating signals for B cell–positive selection for all but the most terminal stages of B cell development. The lack of a requirement for conventional ligand could be inferred from previous studies in which the antigen recognition elements of the Ig heavy chain were deleted. For example, Shaffer and Schlissel have shown that expression of a truncated Ig heavy chain–light chain heterodimer lacking all CDRs and therefore, the ability to bind conventional antigen is able to generate signals sufficient to transit through the pro-B → pre-B cell checkpoints (28). Similarly, Rosado and Freitas have shown that a human μ-heavy chain lacking its variable region sequences and unable to pair with surrogate or conventional light chains is nonetheless able to support B cell development through the CD23pos stage (64). Again, these results argue against

conventional BCR ligand interactions for generating signals for B cell positive selection through the pro-B → pre-B cell transition. In the latter studies the CD23^{pos} B cells were unable to enter the long-lived pool of peripheral mature B cells suggesting that maintenance of this pool may be dependent upon conventional antigen engagement (65). While these studies argue against a requirement for conventional CDR-dependent ligand engagement in the generation of signals required for developmental progression, they have not addressed the possibility of ligand interactions mediated via other regions of the Ig heavy chain for transition through the pro-B → pre-B cell checkpoint. Similarly, they have not addressed the possibility that Igα or Igβ through their extracellular domains are involved in interactions with nonpolymorphic ligands. Our studies extend these previous reports by ruling out involvement of nonconventional extracellular ligands during positive selection. In addition, they establish the concept that targeting Igα and Igβ proteins to the plasma membrane is by itself sufficient to generate low level and/or transient signals. These signals, we believe regulate the survival and/or differentiation pathways associated with positive selection and developmental progression.

Recently, the role of plasma membrane lipid rafts in BCR signaling has been examined. It has been reported that in resting B cells, very few BCRs are present in rafts as demonstrated by fluorescence confocal imaging (66) or sucrose gradient density fractionation (44, 67). However, antigen cross-linking of BCRs markedly increases the detectable colocalization of the receptor complex with these plasma membrane domains (44, 66, 67). This shift allows for colocalization of the BCR with proximal intracellular signaling components such as doubly acylated Src family tyrosine kinases (44, 68, 69), and possibly the exclusion of inhibitory coreceptors such as CD45 and CD22 (70). Although the published data for the BCR suggests raft localization only after receptor aggregation, it remains undetermined whether the BCR exists in small rafts such as those present for other raft–associated receptors (71). These small (26 nm) entities are undetectable by fluorescence microscopy and are not buoyant in sucrose density gradients. BCR aggregation, like these other systems, would function to coalesce "micro-rafts" and the aggregated structure could be envisioned to function mainly to facilitate exclusion of negative regulators of BCR signal transduction. Important in this regard, we find that MAHB and the conventional BCR localize to both raft and nonraft compartments in resting B cells as determined by sucrose density gradients. Therefore, we do not believe that MAHB is inappropriately targeted to regions of the membrane not normally occupied by the conventional BCR in its nonaggregated state. It remains an issue for further study as to whether basal signaling occurs for Igα/Igβ complexes when they are localized outside of rafts, or alternatively, is mediated by complexes transiently associated with these structures as has been postulated for the pre-BCR (72).

The finding that B cell development proceeds to the classII^{pos}CD23^{pos} stage under the direction of MAHB raises interesting possibilities. If these peripheral B cells have reached the mature stage one could argue that B cell development is regulated solely by antigen-independent mechanisms. However, while these MAHB-expressing B cells have the phenotype of more mature B cells, we have not yet determined whether they exhibit the homing and survival characteristics of long-lived peripheral mature B cells. Recent studies have indicated decreased survival of mature peripheral B cells that are induced to lose surface expression of the BCR (32) reflecting a role for either BCR surface expression or antigen interactions for the maintenance of this pool. The decreased survival of peripheral B cells with surface BCR that lack the ability to bind antigen (64), as well as the finding that peripheral naive T cells may require interaction with some form of ligand for their continued survival (73), suggests that development and maintenance may depend upon different signals. While our studies argue that developmental progression is ligand independent, it remains to be determined whether MAHB-directed targeting of Igα/Igβ is sufficient for maintenance of these peripheral B cells. In future studies it will be interesting to test the relative lifespan of the CD23^{pos} MAHB-expressing cells as well as to determine their ability to compete with normal BCR-expressing cells for follicular entry and survival.

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