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Engagement of the Human Pre-B Cell Receptor Generates a Lipid Raft–Dependent Calcium Signaling Complex

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Summary

Pre-B cell receptor (pre-BCR) expression is critical for B lineage development. The signaling events initiated by the pre-BCR, however, remain poorly defined. We demonstrate that lipid rafts are the major functional compartment for human pre-B cell activation. A fraction of pre-BCR was constitutively raft associated, and receptor engagement enhanced this association. These events promoted Lyn activation and Igβ phosphorylation and led to the generation of a raft-associated signaling module composed of tyrosine phosphorylated Lyn, Syk, BLNK, PI3K, Btk, VAV, and PLCγ2. Formation of this module was essential for pre-BCR calcium signaling. Together, these observations directly link the previously identified genetic requirement for the components of this module in B lineage development with their functional role(s) in human pre-BCR signaling.

Introduction

The pre-B cell receptor (pre-BCR) complex is comprised of μ heavy chain, surrogate light chain (ψLC; consisting of the λ5 and VpreB proteins), and associated Igα (Mb-1, CD79a)/Igβ (B29, CD79b) heterodimers. Expression of the pre-BCR is essential for the transition from the pro/pre-B to the immature B cell stage and for generation of the normal B cell repertoire (reviewed in Karasuyama et al., 1996). Genetic studies in mice and studies of B cell–immunodeficient humans clearly demonstrate the requirement for the signaling function of the pre-BCR (reviewed in Karasuyama et al., 1996; Conley and Cooper, 1998). In mice, disruption of pre-BCR components including μ, *Igβ*, or *λ5* (Kitamura et al., 1991, 1992; Gong and Nussenzweig, 1996) or loss of signaling molecules including *Syk*, the *p85* subunit of phosphoinositide 3-kinase (PI3K), and *BLNK* each leads to a significant inhibition of early B lineage development at the pro- to pre-B cell stage (Cheng et al., 1995; Fruman et al., 1999; Jumaa et al., 1999; Pappu et al., 1999). Similarly, in humans, mutations in μ, *Igα*, *λ5*, *Btk*, or *BLNK* result in nearly complete developmental arrest at the pro- to pre-B transition (Tsukada et al., 1993; Yel et al., 1996; Minegishi et al., 1998, 1999a, 1999b).

In contrast to the mature BCR, however, the signaling events mediated by the pre-BCR remain poorly defined. Genetic studies indicate the immune tyrosine activation motifs (ITAMs) of either the Igα or Igβ chains are required for pre-BCR function (Papavasiliou et al., 1995). Biochemical studies of human and murine pre-BCR signaling have yielded limited and inconsistent results. Various studies have identified a low-amplitude, or absent, pre-BCR-dependent calcium signal (Bossy et al., 1993; Matsuo et al., 1993; Kuwahara et al., 1996; Nakamura et al., 1996). This was associated with only minimal changes in protein tyrosine phosphorylation, and specific substrates have not been consistently identified.

Recent studies have demonstrated a key role for membrane "lipid rafts" in signal transduction by cell surface receptors in a broad range of cell types. These sphingolipid- and cholesterol-rich structures exist as phase-separated rafts within the liquid crystalline plasma membrane bilayer (reviewed in Brown and London, 1998). Lipid rafts are enriched for a variety of signaling molecules, including Src kinases and transmembrane proteins, glycosylphosphatidylinositol (GPI)-linked proteins, growth factor receptors, heterotrimeric G proteins, inositol triphosphate receptors, and phosphatidylinositol 4,5-bisphosphate (Brown and London, 1998). Signal transduction by the mature T cell antigen receptor (TCR) and the high affinity receptor for IgE (FcεRI) are both dependent upon the integrity of lipid rafts (Field et al., 1995; Stauffer and Meyer, 1997; Montixi et al., 1998; Moran and Miceli, 1998; Xavier et al., 1998; Janes et al., 1999). These observations have led to a general model for lipid rafts as preformed platforms essential for sustaining immunoreceptor signaling and/or cytoskeletal reorganization.

The very low level of pre-BCR surface expression suggests that in order to signal efficiently this receptor system requires precise compartmentalization of protein–protein and protein–lipid interactions. To begin to test this hypothesis, we have examined the potential role for lipid rafts in these events. We characterized the structural composition of lipid rafts in both primary human pre-B cells and pre-B cell lines and determined the biochemical events following pre-BCR engagement. Our results suggest that lipid rafts are the major compartment responsible for initiation and nucleation of higher order signaling complexes critical for pre-BCR signaling.

Results

Cross-Linking of the Human Pre-BCR Induces Calcium Signaling and Weak Tyrosine Phosphorylation

Sustained calcium flux is an indicator of cell activation and is linked to critical downstream transcriptional events in both mature B and T cells (reviewed in Berridge, 1993; Dolmetsch et al., 1997). Previous studies have reported conflicting data on the capacity of murine and human pre-B cells to generate a calcium signal in

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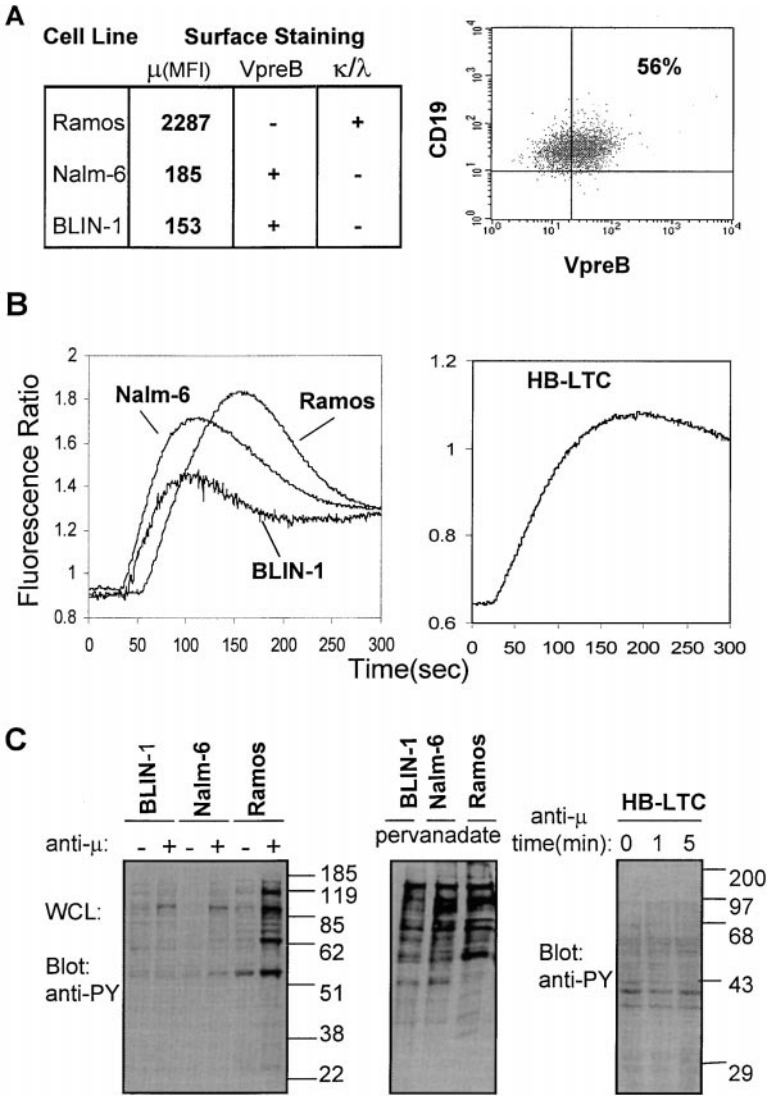

Figure 1. Pre-BCR Engagement Induces a Sustained Calcium Signal but Limited Tyrosine Phosphorylation

(A) (Left) Summary of flow cytometry analysis of pre-B and mature B cell lines for surface μ (MFI, mean fluorescence intensity), κ or λ LC, and the VpreB component of ψLC. (Right) Representative FACS plot of purified HB-LTC B progenitors stained with anti-CD19 and anti-VpreB antibodies.

(B) (Left) Ca^{2+} mobilization was monitored by spectrofluorimetry in human pre-B cell lines (Nalm-6 and BLIN-1) and IgM^+ immature B cells (Ramos) loaded with Indo-1. Cells were cross-linked with anti-μ [10 μg/ml $F(ab)_2$] after a 30 s baseline measurement. (Right) Primary human B progenitors were isolated from HB-LTC and Ca^{2+} mobilization in response to anti-μ cross-linking.

(C) Total cell lysate from anti-μ activated pre-B and B cells (left), cells treated with pervanadate (middle), and resting and activated HB-LTC B progenitors (right) were subjected to anti-phosphotyrosine (anti-PY) immunoblotting. Representative results of more than three independent experiments are shown for this and subsequent figures.

response to pre-BCR cross-linking (Bossy et al., 1993; Matsuo et al., 1993; Karasuyama et al., 1996; Kuwahara et al., 1996). To evaluate the consequences of pre-BCR engagement, we utilized the both pre-BCR^+ (surface IgM^-) human pre-B lines (Nalm-6 and BLIN-1) and primary human B lineage progenitors maintained in long-term, stroma-supported cultures (HB-LTC; Rawlings et al., 1995; Fluckiger et al., 1998b). Flow cytometry and molecular studies were performed confirming the low-level surface expression of the pre-BCR on these pre-B cell populations. Pre-B cells comprised 50%–75% of the primary B progenitor population with the remaining cells consisting of $c\mu^-$ pro-B cells and $<5\%$ $sIgM^+$ cells (Figure 1A and data not shown). The calcium signal following pre-BCR cross-linking was evaluated. As shown in Figure 1B, cross-linking of pre-BCR led to a sustained increase in intracellular calcium concentration in pre-B cell lines. In multiple independent experiments, primary pre-B cells also exhibited a sustained calcium signal. Control antibodies failed to induce calcium influx, and, as expected, ionomycin-induced strong calcium mobilization (data not shown). These findings clearly demonstrate that pre-BCR engagement leads to a sustained calcium signal in human pre-B cells.

Calcium mobilization in response to antigen receptor cross-linking in mature B and T cells is initiated by the coordinate activation of three families of nonreceptor tyrosine kinases (reviewed in Kurosaki, 1999; Rawlings, 1999). A hallmark of these events is a rapid increase in tyrosine phosphorylation of protein substrates in response to receptor engagement. Analysis of total cellular proteins from pre-B cell lines and primary pre-B cells stimulated by anti-μ cross-linking revealed minimal changes in tyrosine phosphorylation (Figure 1C). Earlier studies of pre-B cells have reported similar findings (Bossy et al., 1993; Matsuo et al., 1993; Kuwahara et al., 1996). In contrast, Ramos B cells and primary B cells exhibited robust increases in phosphorylation after anti-μ stimulation (Figure 1C and data not shown). The weak protein phosphorylation in pre-B cell lines was unlikely the result of deficient expression of nonreceptor tyrosine kinases because a strong tyrosine phosphorylation signal was observed in cells treated with the protein tyrosine phosphatase inhibitor, pervanadate (Figure 1C). In addition, Western blotting demonstrated equivalent expression of Lyn, Syk, and Btk in pre-B and mature B cell lines (data not shown), indicating that the dosage of these proteins is not limiting in pre-B cells.

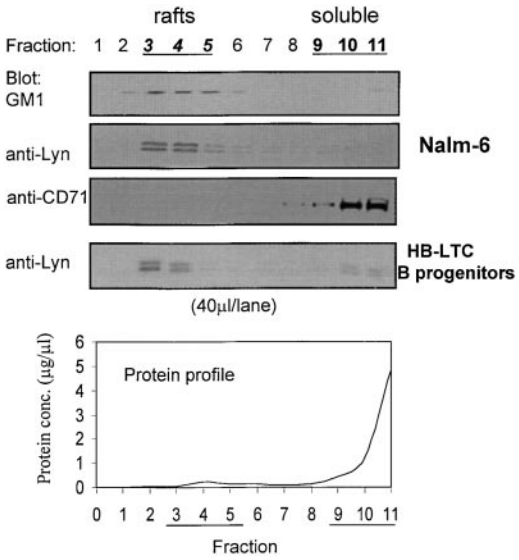

Figure 2. Characterization of Human Pre-B Cells Lipid Rafts

Nalm-6 pre-B cells or HB-LTC B progenitors were lysed in 0.5% Triton X-100 and subjected to sucrose gradient ultracentrifugation. Equal volumes of gradient fractions separated by SDS-PAGE and blotted with cholera toxin to detect the ganglioside, GM1, or with anti-Lyn, or anti-CD71 antibodies. The protein profile for each fraction (bottom) and fractions containing predominantly lipid "rafts" versus the "soluble" membrane are noted.

The generation of a sustained calcium signal in response to pre-BCR engagement was consistent with the abundant genetic evidence demonstrating an important functional role for the pre-BCR in human B lineage development. The low pre-BCR surface density and weak tyrosine phosphorylation signal, however, raised the question of how the pre-BCR is capable of generating this or other signals. These observations suggested that receptor compartmentalization might be required for nucleation of pre-BCR signaling complexes and that this might permit efficient signaling despite limited surface expression.

Structural Characterization of Lipid Rafts in Human Pre-B Cells

To begin to test this hypothesis, we isolated and characterized detergent-resistant membrane fractions in pre-B cell lines and primary pre-B cells. Lipid rafts were isolated using sucrose gradient ultracentrifugation following cell lysis in cold 0.5% Triton X-100. Similar approaches have been used previously to isolate cholesterol- and sphingolipid-enriched microdomains from mature T and B cells (Montixi et al., 1998; Xavier et al., 1998; Cheng et al., 1999). We collected 11 gradient fractions. Fractions 3–5 at the 5%–30% interface contained the low-density lipid raft fractions. Fractions 9–11 contained the high-density material and comprised the majority of Triton-soluble proteins and soluble membrane. The protein profile of these fractions confirmed that most of the cellular protein was present in the soluble fractions 9–11 (particularly in fraction 11), while fractions 3–5 accounted for 5% or less of total cellular proteins (Figure 2).

Detergent-insoluble membranes are characterized by the enrichment of glycosphingolipids and lipid-modified membrane proteins. The ganglioside, GM1, represents a well-characterized marker of lipid rafts in multiple cell types (Fra et al., 1995; Stauffer and Meyer, 1997; Montixi et al., 1998; Xavier et al., 1998). Figure 2 shows that GM1 was highly enriched in fractions 3–5 and difficult to detect in the remaining gradient fractions. Detergent-insoluble membranes are also enriched for dual-acylated Src kinases. Lyn is the predominant Src family kinase member expressed in mature B cells. We therefore evaluated the distribution of Lyn in the pre-B cell gradient fractions. Similar to GM1, Lyn was highly enriched in fractions 3–5 in both pre-B cell lines and primary pre-B cells (Figure 2). In contrast, the predominantly membrane associated protein, CD71 (transferrin receptor), was excluded from the detergent-insoluble raft fractions (Figure 2; Montixi et al., 1998; Xavier et al., 1998). Together, these findings support the conclusion that the buoyant fractions (fractions 3–5) isolated from human pre-B cells exhibited properties consistent with detergent-insoluble membrane microdomains or lipid rafts and that the extraction methods utilized resulted in limited cross-contamination between these fractions.

A Fraction of Pre-B Cell Receptors Localize to Lipid Rafts prior to Receptor Cross-Linking

To evaluate the membrane distribution of the pre-BCR, sucrose gradient fractions were subjected to Western blot analysis using anti-μ heavy chain and anti-Igβ antibodies. While the majority of the pre-BCR was present in the soluble fractions, a subset of receptors consistently localized to membrane rafts in unstimulated pre-B cells (Figure 3A). We compared the relative distribution of the pre-BCR and the mature BCR in sucrose fractions generated from resting pre-B cells and Ramos B cells (Figure 3B and data not shown). Consistent with the significantly higher surface expression of μHC, the soluble fractions from Ramos cells contained significantly greater amounts of μ than in pre-B cells. A fraction of mature receptors was also detected in lipid rafts. Interestingly, in both the pre-B cell lines and primary pre-B cells, the relative fraction of pre-BCR associated with lipid rafts was significantly higher than the respective fraction in mature B cells (Figures 3B and 3C). In unactivated pre-B cells, approximately 20%–30% of total μ chain localized within the raft fraction, while in IgM^+ B cells approximately 1% of μ chain was associated with lipid rafts. We also utilized subcellular fractionation as an independent approach to isolate and characterize the cytosolic, membrane, and detergent-insoluble membrane (DIM) fractions. The DIM fraction is enriched in lipid raft and/or cytoskeleton-associated cell membranes and has been used in previous studies of membrane microdomains (Hartley and Corvers, 1996; reviewed in Caplan and Baniyash, 2000). As shown in Figure 3D, pre-B cells express very low levels of μ chain, but the relative amount of μ chain in DIM fractions is significantly higher than that in mature B cells. Together, these findings suggest that a fraction of surface expressed pre-BCR may be constitutively associated with lipid rafts prior to receptor engagement.

Pre-BCR Engagement Enhances Its Association with Lipid Rafts and Leads to Recruitment of Multiple Signaling Molecules to the Raft Fraction

Antigen receptor engagement in mature B and T cells and mast cells leads to the translocation of signaling molecules to activated receptor subunits. We therefore

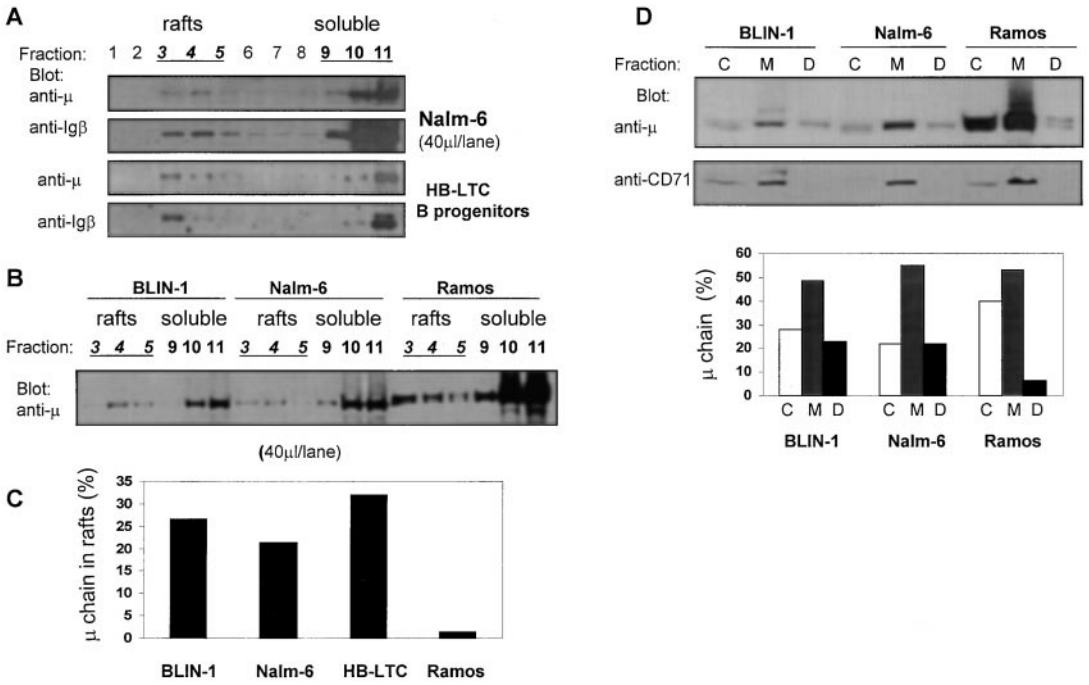

Figure 3. A Fraction of Pre-BCR Is Constitutively Associated with Lipid Rafts

(A) Equal volumes of gradient fractions from unactivated Nalm-6 or HB-LTC B progenitors were blotted with anti-μ or anti-Igβ.

(B) Comparison of anti-μ blotting of equivalent volume gradient fractions from BLIN-1, Nalm-6, and Ramos B cells.

(C) Quantitation by densitiometry of the relative fractions of pre-BCR versus BCR associated with lipid rafts.

(D) Subcellular distribution of μ chain pre-B and mature B cells. Equal volumes of cytoplasmic (C), membrane (M), and DIM (D) subcellular fractions were loaded in each lane and blotted with anti-μ or anti-CD71.

evaluated whether pre-BCR cross-linking led to additional receptor recruitment and/or recruitment of signaling molecules into lipid microdomains. We examined protein redistribution by Western blot analysis using both equivalent amounts of total protein and equal volumes of material from each gradient fraction. Overall, both approaches lead to similar and consistent findings as discussed below.

We initially evaluated the distribution of the pre-BCR following receptor activation. As showed in Figure 4A, the amount of both μ chain and Igβ in the raft fractions increased following anti-μ stimulation. The nearly equivalent increase in both μ and Igβ suggested that this represented a change in distribution of the intact pre-BCR. Interestingly, the consistently greater amount of Igβ in fraction 3 also suggested the potential for an independent pool of Igα/β (Vilen et al., 1999) and/or that Igβ was more tightly raft associated than μ. Overall, these results indicated that pre-BCR cross-linking resulted in recruitment of the pre-BCR complex to lipid rafts and/or stabilized the association of the pre-BCR with these microdomains.

The amount of Lyn localized in the raft fractions, and the total amount of Lyn protein present in pre-B cells showed little or no change after anti-μ stimulation (data not shown; Figures 4C and 5B). In contrast, other signaling molecules redistributed to the lipid raft fractions upon pre-BCR cross-linking. Figure 4B shows results of anti-PLCγ2 Western blotting before and after pre-BCR cross-linking in Nalm-6 and BLIN-1 pre-B cell lines. Blots using either equivalent protein loading or equivalent volumes of cell lysate each clearly demonstrated redistribution of PLCγ2 in activated cells in both cell lines. Figure 4C demonstrates redistribution of Syk, Btk, and BLNK. Similar results were also obtained for p85 (PI3K), VAV, cbl, and SHC (data not shown). We also evaluated recruitment to lipid rafts in primary pre-B cells. Because of the 100-fold lower number of cells utilized in these experiments, PLCγ2 Western blot analysis did not provide sufficient sensitivity to evaluate protein redistribution. For this reason, we immunoprecipitated PLCγ2 from pooled lipid raft or soluble fractions of unactivated and activated primary cell populations. Consistent with the results in pre-B cell lines, pre-BCR engagement led to a significant enrichment in the amount of PLCγ2, Btk and Syk in the raft fraction in primary pre-B cells (Figure 4D). Together, these results clearly demonstrate that pre-BCR engagement promotes the redistribution of signaling molecules from the soluble fraction into lipid rafts.

Pre-BCR Engagement Leads to Lyn Activation and a Marked Enrichment of Tyrosine-Phosphorylated Proteins in Lipid Rafts

The constitutive association of Lyn and the recruitment of additional tyrosine kinases, including Syk and Btk, to lipid rafts in response to pre-BCR activation suggested that these events could lead to kinase activation. This would be predicted to result in subsequent tyrosine phosphorylation of receptor subunits and additional cellular substrates. To initially examine this possibility, we compared the phosphotyrosine content of the soluble and raft fractions from pre-B cells following receptor

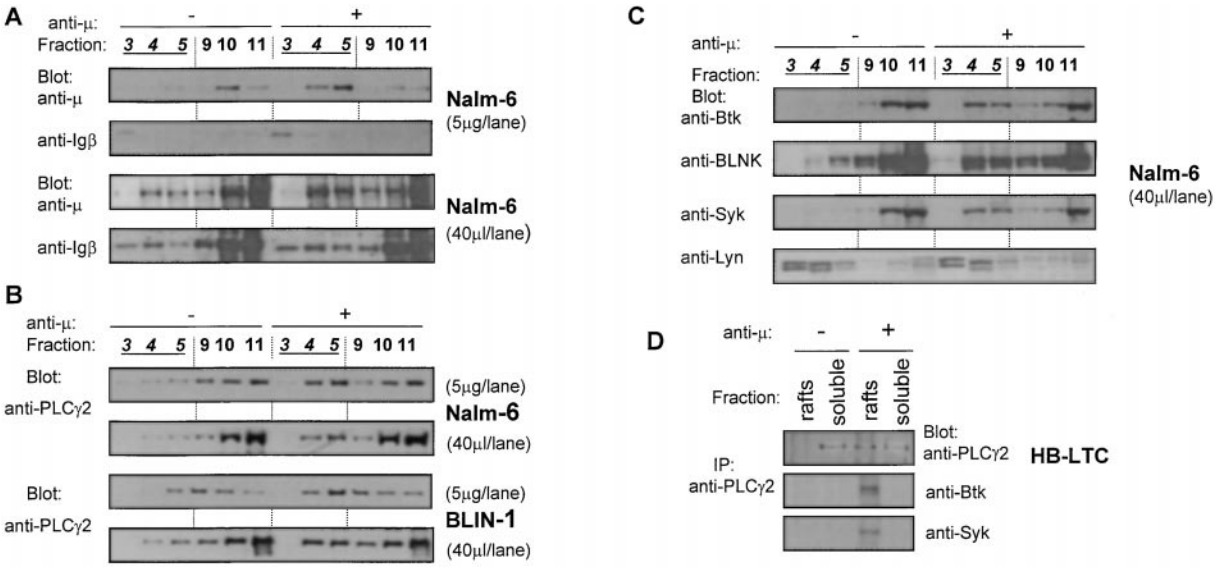

Figure 4. Pre-BCR Engagement Recruits the Pre-BCR and Signaling Molecules to Rafts

(A–C) Equal protein amounts (5 μg) or equal volumes (40 μl) of gradient fractions from resting and activated Nalm-6 or BLIN-1 cells were separated by SDS-PAGE and blotted with: (A) anti-μ or anti-Igβ antibodies; (B) anti-PLCγ2 antibody; or (C) antibodies specific for Btk, BLNK, Syk, or Lyn. Protein identities were confirmed using a combination of blotting of overexpressed protein constructs, immunoprecipitation, protein molecular weight, and analysis of pre-B and mature B cell lysates (data not shown).

(D) HB-LTC B progenitors were activated with anti-μ for 2 min, and pooled raft or soluble fractions were immunoprecipitated with anti-PLCγ2 antibody. Proteins were separated by SDS-PAGE and blotted with anti-PLCγ2, anti-Btk, and anti-Syk antibodies.

engagement. As shown in Figure 5A, pre-BCR cross-linking was associated with a significant phosphotyrosine signal within the raft fraction. Little change was observed within the soluble fraction despite the presence of up to 50-fold more protein in that fraction. The phosphorylated substrates within the raft fraction included a protein doublet migrating at approximately 53–55 kDa. This was consistent with the expected size of Lyn, and Western blotting confirmed comigration with Lyn (Figure 5B and data not shown). As shown in Figure 5B, the phosphotyrosine content of Lyn increased and peaked within 2 min and declined by 30 min after receptor cross-linking. To confirm that pre-BCR engagement led to Lyn activation, Lyn was immunoprecipitated from raft fractions at 0 min and 2 min after pre-BCR cross-linking, and in vitro kinase assays were performed. Receptor engagement resulted in a significant increase in Lyn kinase activity (Figure 5C). Thus, pre-BCR engagement leads to an increase in the activity of tyrosine kinases including Lyn, and the recruitment of tyrosine phosphorylated proteins to the lipid raft fraction.

The Activated Pre-BCR Generates Higher Order Signaling Complexes within the Lipid Raft Fraction

The essential role for PLCγ2 in BCR-dependent calcium signaling, and the prominent redistribution of PLCγ2 to the lipid raft fraction, suggested that PLCγ2 might be recruited into a raft-associated signaling complex. To evaluate this possibility, we immunoprecipitated PLCγ2 from pooled lipid raft versus soluble fractions in resting and activated pre-B cells and performed anti-phosphotyrosine Western blotting. Strikingly, a series of phosphorylated proteins coimmunoprecipitated with PLCγ2 only in raft fractions from activated cells (Figure 6A). These proteins were not detectable in soluble fractions despite the presence of significantly greater amounts of PLCγ2. The same blots were serially reprobed with specific antibodies in order to identify coimmunoprecipitating proteins. This approach clearly demonstrated coimmunoprecipitation of Lyn, Syk, Btk, BLNK, p85, and VAV (Figure 6A). Of these proteins, Lyn, Syk, Btk, BLNK, and VAV each comigrated with a phosphoprotein identified by anti-phosphotyrosine Western blotting. Notably, the PLCγ2 immunoprecipitates included several 30–35 kDa phosphoproteins (Figure 6A and data not shown). Western blotting with anti-Igβ indicated that at least one of these proteins was tyrosine phosphorylated Igβ. Together, these finding suggest that receptor activation leads to phosphorylation of residues within the Igβ ITAMs and recruitment and activation of additional nonreceptor tyrosine kinases, adaptor molecules, and effectors.

The tyrosine kinase Btk is required for human pre-B cell expansion and for generation of the sustained calcium signal in response to BCR cross-linking (reviewed in Rawlings, 1999). We therefore evaluated whether Btk was coimmunoprecipitated with additional signaling proteins within to the lipid raft compartment. Several prominent phosphoproteins of 60–80 kDa coimmunoprecipitated with Btk only in raft fraction from activated pre-B cells (Figure 6B). This pattern was distinct from that observed using anti-PLCγ2 antibodies, but included several species of similar molecular size and phosphotyrosine content. Western blotting confirmed the presence of both BLNK and Syk among these proteins. In addition, anti-PLCγ2 blotting demonstrated association of PLCγ2 with this complex, although the phosphotyrosine content of PLCγ2 was significantly less prominent than the other substrates at this time point (2 min post

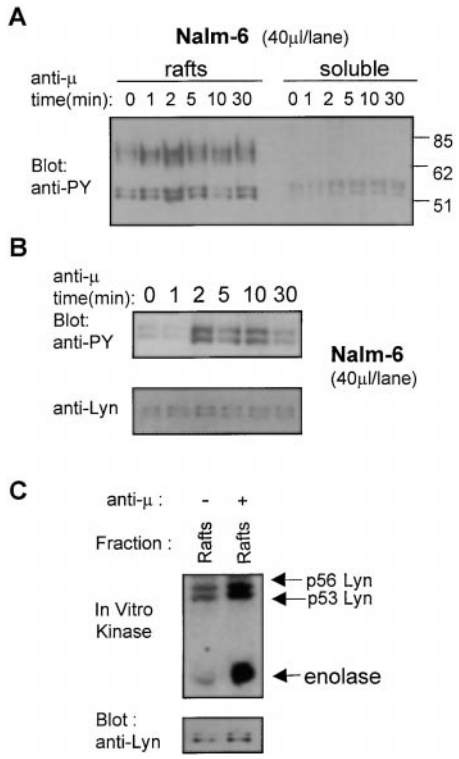

Figure 5. Pre-BCR Engagement Activates Lyn and Results in Phosphorylation of Raft-Associated Proteins

(A) Nalm-6 cells were activated with anti-μ for the time period indicated, and equal volumes of pooled raft (3–5) and soluble (9–11) fractions were blotted with anti-PY antibody.

(B) In a separate experiment, Nalm-6 cells were activated and equal volumes of pooled raft fractions were blotted with an anti-phosphotyrosine. The blot was stripped and reprobed with anti-Lyn.

(C) (Top) Lyn was immunoprecipitated from pooled raft fractions from resting and activated (2 min) Nalm-6 cells, and in vitro kinase assays were performed. Positions of Lyn (p53 and p56) and the exogenous substrate, enolase, are noted. (Bottom) Blot was probed with anti-Lyn antibody.

activation). Consistent with these findings, immunoprecipitation with anti-BLNK antibodies led to recovery of an overlapping subset of phosphorylated proteins including Btk, Syk, and PLCγ2 (data not shown). None of these respective proteins were present in immunoprecipitates of soluble fractions using either the anti-Btk or anti-BLNK antibodies.

The increase in the phosphotyrosine content of raft-associated Btk suggested that raft recruitment led to Btk activation. Btk is activated in mature B cells by Lyn (or another Src family kinase) via transphosphorylation of a tyrosine (Y551) within the Btk kinase domain (reviewed in Rawlings, 1999). This leads to Btk autophosphorylation at Y223 within its SH3 domain. Phosphorylation of these residues occurs rapidly in response to BCR engagement and is nearly undetectable in resting mature B and mast cells (Wahl et al., 1997; Nisitani et al., 1999). Anti-Btk phosphotyrosine site–specific antibodies were used to evaluate the phosphorylation status of raft-associated Btk (Figure 6C). Btk was immunoprecipited from pooled raft or soluble fractions of resting and activated pre-B cells, and Western blotting was performed. Despite the presence of significantly greater amounts of Btk in the soluble fraction, phosphorylation of Btk Y223 and Y551 was detectable only in raft-associated Btk from activated pre-B cells (Figure 6C and data not shown). These findings indicate that recruitment of Btk into the raft fraction leads to an increase in its enzymatic activity.

Disruption of Lipid Rafts Inhibits Recruitment of PLCγ2, IP3 Production, and Pre-B Cell Calcium Signaling

To determine whether lipid rafts function as an obligate compartment for generation of pre-BCR-dependent signals, we evaluated the consequences of disrupting lipid rafts. Cholesterol is an essential component of lipid rafts, and reduction of cholesterol content significantly alters lipid microdomain structure (Brown and London, 1998). We utilized two independently acting chemical agents to evaluate the consequences of altering the cholesterol content of pre-B cell lipid rafts. These agents included methyl β cyclodextrin (MCD), which preferentially extracts plasma membrane cholesterol and disrupts lipid rafts microdomains, and the antifungal agent, nystatin, which leads to a dispersion of cholesterol-rich membrane domains (Moran and Miceli, 1998; Xavier et al., 1998). Treatment with MCD (2.5–10 mM; Figure 7A) or with nystatin (25–100 μg/ml; data not shown) each led to a dose-dependent reduction in the pre-BCR-dependent calcium signal. Notably, while cell viability was reduced at higher drug dosages for both drugs, viability (as assessed by trypan blue exclusion and cell expansion following recovery from drug treatment) was not affected at the doses utilized for these experiments. Consistent with these observations, recovery in serum containing media following drug treatment led to a partial or complete (with longer recovery periods) restoration of pre-BCR-dependent calcium signaling (Figure 7A).

We next evaluated the effects of disruption of lipid rafts on the recruitment of signaling proteins. Consistent with its effect on the pre-BCR calcium signal, MCD treatment interfered with recruitment of PLCγ2, Btk and Syk to rafts (Figure 7B). The inhibitory effect of MCD was most specific for recruitment of signaling proteins, as 10 mM MCD had little or no effect on the distribution of Lyn but completely abrogated the association of PLCγ2 (Figure 7B and data not shown). Calcium mobilization is dependent upon the second messenger inositol-1,4,5-trisphosphate (IP3), which is converted from PtdIns-4,5-P2 by activated PLCγ. To further characterize the effect of MCD treatment on PLCγ2 activation, we measured the generation of IP3 in response to pre-BCR engagement in untreated and in drug-treated Nalm-6 cells. In untreated cells, IP3 levels increased 2.5- to 4-fold in response to activation and peaked within 1 min of pre-BCR cross-linking. MCD treatment led to a dose-dependent reduction in IP3 production that was also partially restored following cell recovery (Figure 7C and data not shown). Together, these results clearly demonstrate that formation of the PLCγ2 signaling complex and generation of the sustained calcium signal in pre-B cells requires an intact lipid raft compartment.

Discussion

Lipid Rafts Comprise the Major Signaling Compartment in Human Pre-B Cells

Our biochemical studies strongly support the model that lipid rafts are required to initiate (via colocalization with

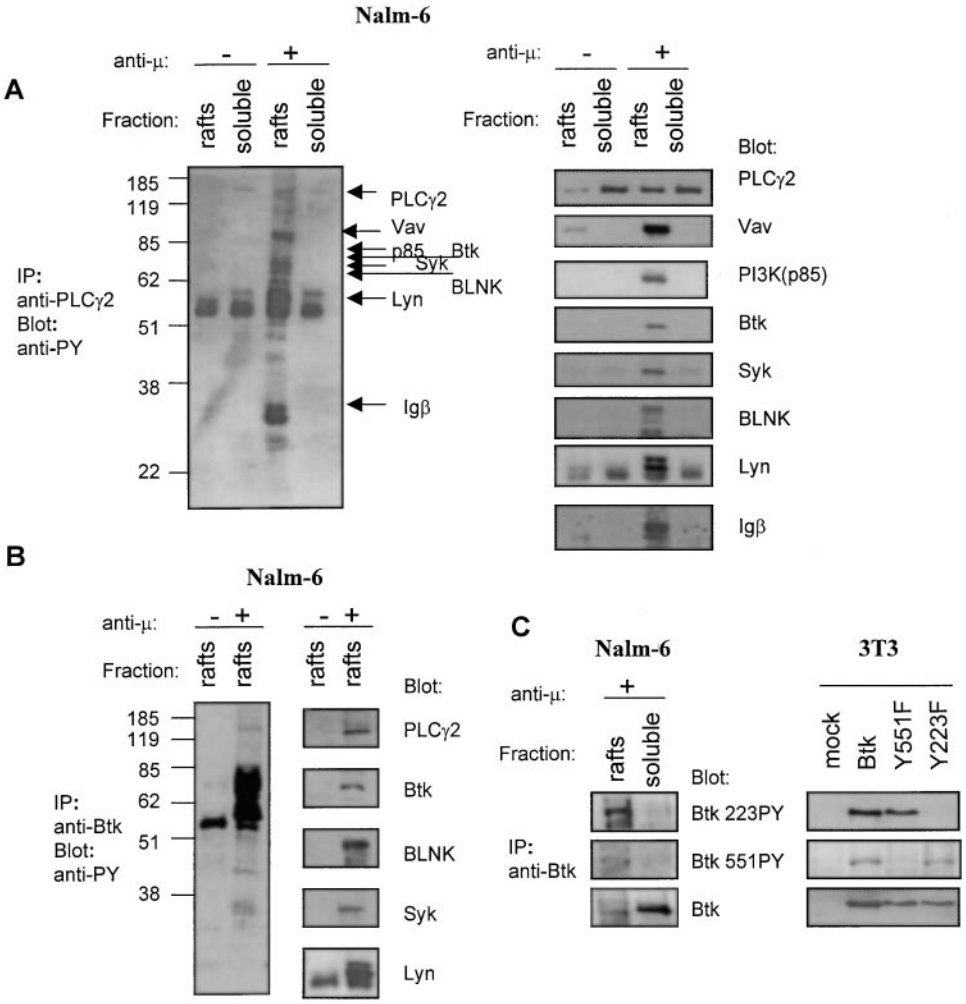

Figure 6. Pre-BCR Engagement Generates Raft-Associated PLCγ2 and Btk Signaling Complexes

(A) (Left) Nalm-6 cells were activated with anti-μ for 2 min, and pooled raft or soluble fractions were immunoprecipitated with anti-PLCγ2 antibody. Proteins were separated by SDS-PAGE and blotted with anti-PY antibody. (Right) The blot was stripped and serially reprobed with antibodies to the proteins indicated.

(B) Nalm-6 cells were activated as in (A) and immunoprecipitated with anti-Btk antibody, then blotted with anti-PY antibody (left) and serially reprobed with antibodies to the proteins indicated (right). Rabbit preimmune serum failed to immunprecipitate these protein complexes (data not shown). Alignment of blots identified comigrating tyrosine phosphorylated bands for each of the proteins noted at left.

(C) (Left) Pooled lipid rafts or soluble fractions from Nalm-6 cells were immunoprecipitated with anti-Btk antibody and blotted sequentially with anti-Btk-223 PY, anti-Btk-551PY, and anti-Btk antibodies. (Right) Concurrent blotting of control lysates from NIH 3T3 cells that were mock infected or coinfected with vaccinia viruses expressing Lyn and wild-type Btk, or Y223F, or Y551F Btk mutant proteins.

activated Lyn) and to propagate (via formation of a higher order signaling complex) human pre-BCR signaling. The lipid rafts fractions isolated from pre-B cells exhibited biochemical features consistent with the properties of lipid microdomains previously characterized in mast cells (Field et al., 1995), T cells (Montixi et al., 1998; Xavier et al., 1998), and mature B cells (Cheng et al., 1999). These data are consistent with the essential role for lipid rafts in generation of signals by other multichain immunoreceptors, including the TCR and FcεRI. Consistent with the predicted dual-acylation and with studies of mature B and mast cells (Field et al., 1995; Cheng et al., 1999; B. G. and D. J. R., unpublished data), Lyn was highly enriched in pre-B cells lipid rafts. Pre-BCR engagement led to a rapid increase in the kinase activity of Lyn, the accumulation of tyrosine phosphorylated Igβ, and recruitment of Syk, BLNK, Btk, p85, PLCγ2, and VAV to rafts. While only a relatively minor fraction of the total cellular content of each of these proteins was associated with lipid rafts, this fraction was markedly enriched for activated substrates as judged by their phosphotyrosine content. Most notably, pre-BCR engagement led to the formation of a higher order pre-BCR signaling complex(es) as demonstrated by the marked enrichment of phosphorylated substrates by immunoprecipitation of PLCγ2 (the major PLC isoform expressed in B lineage cells). The prominent phosphotyrosine signal observed within the lipid rafts of activated pre-B cells stands in distinct contrast to the minimal signal in total cell lysates described in this and previous studies (Figure 1C; Bossy et al., 1993; Kuwahara et al., 1996; Nakamura et al., 1996). Finally, partial disruption

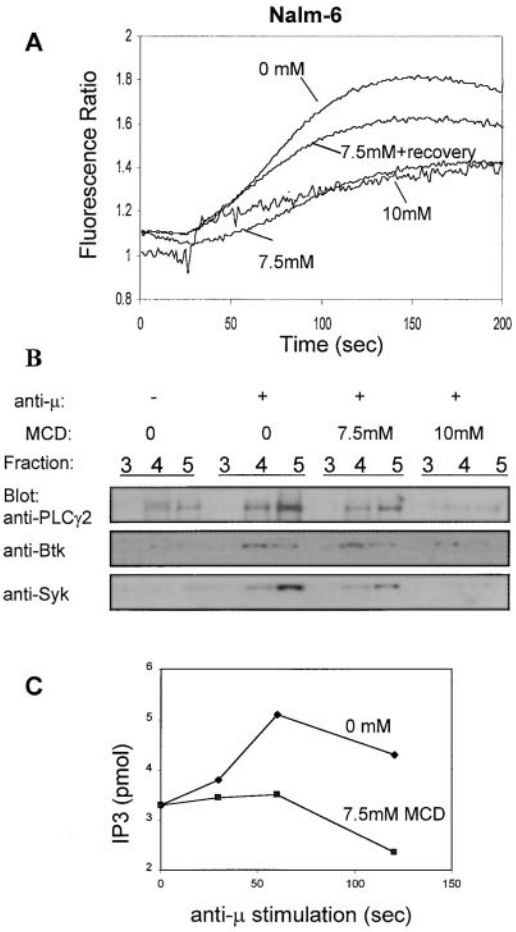

Figure 7. MCD Treatment Inhibits Pre-BCR Calcium Signaling

Nalm-6 cells were untreated, treated with MCD at dosages indicated, or treated and allowed to recover for 6 hr prior to activation.

(A) Ca^{2+} mobilization of was monitored as in Figure 1.

(B) Sucrose gradient fractions were prepared and blotted with anti-PLCγ2, anti-Btk, and anti-Syk antibodies.

(C) Kinetic analysis of IP3 production was performed by radioreceptor assay and displayed as total IP3 versus time post anti-human μ cross-linking.

Similar results were obtained using BLIN-1 pre-B cells.

of lipid raft structure using two alternative cholesterol-depleting agents blocked both the recruitment of PLCγ2 into rafts and the pre-BCR calcium signal. These events were restored following drug removal and cell recovery, supporting a specific effect of these drugs on raft structure. While cell numbers precluded extensive biochemical analysis, engagement of the pre-BCR on primary pre-B cells also led to the recruitment of PLCγ2, Btk, and Syk into rafts, and a sustained calcium signal that was similarly sensitive to cholesterol depletion. Together, these observations underscore the critical role for membrane compartmentalization in human pre-BCR-dependent signaling.

Pre-BCR Engagement Leads to Formation of a Raft-Associated Calcium Signaling Module

A striking feature of this work is identification and characterization of a lipid raft–associated module essential for generation of the pre-BCR calcium signal. The mature BCR calcium signal results from the sustained activation of PLCγ through the coordinate activation of Src, Syk, and Btk family kinases (reviewed in Rawlings, 1999; Kurosaki, 1999). Strikingly, each of the key signaling molecules implicated in BCR-dependent calcium signaling coimmunoprecipitated with PLCγ2 in activated pre-B cells. These associated proteins included Igβ, Lyn, Syk, Btk, BLNK, PI3K, and VAV. These proteins exhibited enhanced tyrosine phosphorylation only in activated pre-B cells, and disruption of raft structure specifically inhibited recruitment and IP3 production and led to a dose-dependent reduction pre-BCR calcium signal.

Our data provide a direct demonstration of recruitment and activation of Btk/Tec family kinases in response to pre-BCR engagement. Activation of Btk required association with rafts. These findings are consistent with previous confocal microscopy data demonstrating colocalization of the mature BCR and Btk phospho-Y223 and Y551 in activated B cells (Nisitani et al., 1999). Notably, activated Btk was predominantly associated with a subset of the proteins within the PLCγ2 signaling module, including Syk, BLNK, and PLCγ2. This clearly demonstrates the association of endogenous Btk with these proteins in B lineage cells. The specific molecular interactions between these proteins and/or additional unidentified molecules cannot be determined from our current data. Nonetheless, these observations are consistent with work suggesting that Btk/Tec kinases may interact via their SH2 domains with tyrosine phosphorylated adapter proteins including BLNK and SLP-76 (reviewed in Kurosaki and Tsukada, 2000). This interaction (BLNK/Syk/Btk) may further modulate Btk activation via increased accessibility to Lyn, as has been proposed for an analogous interaction (LAT/Zap-70/Itk) in Itk activation (Shan and Wange, 1999). These observations are also consistent with a model whereby the BLNK mediates access of Btk to PLCγ2. The association of activated Btk with PLCγ2 in pre-B cells suggests that Btk may regulate the sustained pre-BCR calcium signal via mechanisms analogous to those operating in mature B cells (Fluckiger et al., 1998a).

Constitutive Association of the Pre-BCR with Lipid Rafts May Promote Ligand-Independent Signaling

Interestingly, a significant fraction of the pre-BCR in both pre-B cell lines and primary pre-B cells was associated with lipid rafts in the absence of receptor engagement. While the quantities of surface receptors associated with lipid rafts were similar in pre-B and mature B cells, the relative fraction of raft-associated receptors was significantly higher in pre-B cells. The mechanism for this constitutive association is currently unclear. One possibility is that export of the pre-BCR to the cell surface requires (or is more efficient in) association with lipid-rich structures. In support of this idea, Lyn copurifies with the post-ER pre-BCR pool targeted to the cell membrane (Brouns et al., 1996). Perhaps, as receptor transport rates increase (as in mature B cells), this pathway becomes saturated, and the fraction of receptors that are raft-associated declines.

Our data indicate that surface expression, albeit at low levels, is likely to be required for pre-BCR function. It remains unclear, however, how raft-dependent pre-BCR signaling is initiated. Transgenic studies suggest

that the extracellular domains of the pre-BCR are dispensible for initiation of signals driving clonal expansion and differentiation (Corcos et al., 1995). This, together with the uniform specificity and low surface expression level of these receptors, has suggested that they may signal in the absence of an extracellular ligand. Our data suggest at least two possible mechanisms for ligand-independent, pre-B cell expansion. First, a constitutive raft-associated pre-BCR complex might be capable of generating a low-level tonic signal analogous to that proposed for the naive BCR (Lam et al., 1997). In such a model, oligomerization of constitutively associated receptors might initiate signaling through an increase in the local concentration of Src kinases and/or by excluding inhibitory molecules. This low-level signal might be sufficient to initiate transcriptional events promoting survival and expansion of pre-B cells with a productive μ chain rearrangement. Alternatively, engagement of GPI-linked or other raft-associated coreceptors could promote aggregation of the pre-BCR and subsequent signaling. Studies of T cell activation support such a potential model. Engagement of GPI-linked raft-associated accessory molecules alone or coengagement of these proteins and the TCR leads to raft aggregation, enhancement of receptor activation, and cytoskeletal reorganization (Brown, 1993; Moran and Miceli, 1998). Engagement of non-GPI-linked, raft-associated coreceptors (Yahsiro-Ohtani et al., 2000) or the non-raft-associated, CD28 coreceptor also enhance lipid raft redistribution and sustained TCR signaling (Viola et al., 1999). Similarly, the pre-BCR calcium signal in μ transgenic mice was detected only in response to co-cross-linking of μ and CD19 (Krop et al., 1996). Together, the biochemical approaches described in this work and recently established human B lineage culture systems (Rawlings et al., 1995; Fluckiger et al., 1998b) will facilitate identification of raft-associated, pre-B cell coreceptors and will permit direct evaluation of these models.

Lipid Raft–Dependent Pre-BCR Signals Provide a Biochemical Framework for Understanding Primary B Cell Immunodeficiencies

This work helps to directly link the previously identified genetic requirement for several B lineage signaling molecules, to their functional role(s) in human pre-BCR signaling. Disruption of pre-BCR components (including μ, Igα, Igβ, or λ5) or of the signaling molecules (including Syk, Btk, p85, and BLNK) results in partial or complete pre-B cell developmental arrest. The more severe phenotype associated with the human immunodeficiencies highlights the critical nature of this transition in human B lineage development and the importance of studying these events in human cells. Our work provides biochemical data demonstrating recruitment and activation of each of these molecules in response to human pre-BCR engagement. It strongly supports a direct role for these proteins in pre-BCR signaling and the genetic prediction that deficient function leads to developmental arrest via altered pre-BCR function. Our results also suggest that mutations in other components of the PLCγ2 signaling module may account for additional cases of non-*X*-linked agammaglobulinemia.

Finally, by analogy with the BCR, pre-BCR engagement is predicted to initiate multiple downstream signals in addition to the calcium signal. Notably, constitutive activation of the Ras/Raf/MAP kinase pathway in *RAG*-deficient mice leads to expansion and maturation of pre-B cells and rescue of B cell development (Iritani et al., 1999). These events, however, fail to induce allelic exclusion, suggesting that this signal is propagated independently of Ras activation. Application of the approaches described in this work should facilitate the biochemical evaluation of signaling complexes essential for pre-B development. The shared features of the pre-B and pre-T antigen receptors suggest that analogous studies may aid in understanding pre-TCR signaling.

Experimental Procedures

Cells and Reagents

The human pre-B cell lines, Nalm-6 and BLIN-1 (provided by Dr. Tucker LeBien, Univ. Minnesota), and the B cell line, Ramos, were cultured in RPMI-1640 media containing 10% fetal calf serum. Human B progenitor long-term cultures (HB-LTC) were generated and $CD19^+$ progenitors were purified as described (Rawlings et al., 1995; Fluckiger et al., 1998b). Antibody reagents included: polyclonal goat anti-human μ heavy chain antibody (Southern Biotechnology); anti-human μ heavy chain mAb (Dr. Andrew Saxon, UCLA); anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology); anti-Igβ (Dr. Randolph Wall, UCLA); anti-Lyn (Dr. Andrew Scharenberg, Harvard); anti-PI3K (p85) (Dr. David Fruman, UCLA); affinity-purified anti-Btk (Wahl et al., 1997); anti-Btk phosphotyrosine site–specific antibodies (Wahl et al., 1997; Nisitani et al., 1999); anti-human BLNK mAb (Dr. Andrew Chan, Washington University); anti-PLCγ2, anti-VAV, and anti-Syk polyclonal antibodies (Santa Cruz Biotechnology); horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories); and anti-VpreB mAb (Dr. Eva Sanz; Centro de Investigaciones Biologicas, Spain). Reagents included: Triton X-100 (Fisher Scientific); MES, methylcyclodextrin (MβCD), Nystatin, and HRP-conjugated cholera toxin β subunit (Sigma-Aldrich).

Sucrose Gradient Centrifugation and Subcellular Fractionation

Pre-B cells (1×10^8/ml) were stimulated with goat $F(ab)_2$ anti-human μ heavy chain (10 μg/ml) at 37°C for 2 min or as indicated at 37°C and lysed in 1 ml of ice-cold MBS (25 mM MES [pH 6.5], 150 mM NaCl [pH 6.5]), 0.5% Triton X-100, 1 mM Na_3VO_4, and protease inhibitors). Lysates were mixed with an equal volume of 85% sucrose (w/v) in MBS and transferred to a SW41 centrifuge tube. The sample was then overlaid with 6 ml of 35% sucrose and 3 ml of 5% sucrose in MBS and centrifuged at 200,000 × g for 16 hr at 4°C (Xavier et al., 1998). Following centrifugation, 11 fractions, of 1 ml each, were collected starting at the top of the gradient. Hypotonic lysis and subcellular fractionation were performed as described (Hartley and Corvera, 1996).

Immunoblotting, Immunoprecipitation, and In Vitro Kinase Assays

Samples were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Western blot analyses were performed using standard procedures. For GM1 staining, immunoblots were directly incubated with HRP-conjugated cholera toxin. For immunoprecipitations, the gradient fractions 3–5 containing the lipid rafts were pooled, mixed with three volumes of MBS buffer, and centrifuged at 200,000 × g for 2–4 hr. Raft pellets were resuspended, immunoprecipitated with the indicated antibody, and incubated with protein A–Sepharose overnight at 4°C. Lyn was immunoprecipitated using polyclonal rabbit anti-human Lyn antibody with protein A–Sepharose for 1 hr at 4°C. In vitro kinase assays were performed as described (Tsukada et al., 1993). Reaction samples were separated with 10% SDS-PAGE, blotted onto nitrocellulose, and visualized by Phosphorimaging (Molecular Dynamics).

Calcium Mobilization Assay and IP_3 Analysis

Pre-B and B cells at 10^7/ml loaded with 1 mM indo-1 acetoxymethylester (Molecules Probes) at 37°C for 30 min. To test drug effects on lipid microdomains, loaded cells were treated with 0–100 μg/ml nystatin for 60 min or 0–20 mM $M_\beta CD$ for 30 min at 37°C. Intracellular

calcium was measured by a bulk spectrofluorimeter (Photon Technology International). For IP3 analysis, cells were activated with anti-μ and IP_3 content was measured by ^3H radioreceptor assay as recommended by the manufacturer (DuPont NEN).

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