Involvement of LAT, Gads, and Grb2 in Compartmentation of SLP-76 to the Plasma Membrane

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Abstract

B cell linker protein (BLNK) and Src homology 2 domain–containing leukocyte protein of 76 kD (SLP-76) are adaptor proteins required for B cell receptor (BCR) and T cell receptor function, respectively. Here, we show that expression of SLP-76 cannot reconstitute BCR function in Zap-70^+BLNK^- B cells. This could be attributable to inability of SLP-76 to be recruited into glycolipid-enriched microdomains (GEMs) after antigen receptor cross-linking. Supporting this idea, the BCR function was restored when a membrane-associated SLP-76 chimera was enforcedly localized to GEMs. Moreover, we demonstrate that addition of both linker for activation of T cells (LAT) and Grb2-related adaptor downstream of Shc (Gads) to SLP-76 allow SLP-76 to be recruited into GEMs, whereby the BCR function is reconstituted. The Gads function was able to be replaced by overexpression of Grb2. In contrast to SLP-76, BLNK did not require Grb2 families for its recruitment to GEMs. Hence, these data suggest a functional overlap between BLNK and SLP-76, while emphasizing the difference in requirement for additional adaptor molecules in their targeting to GEMs.

Key words: glycolipid-enriched microdomain • antigen receptor signaling • adaptor molecule • translocation • lymphocyte

Introduction

Recent advances in membrane biology have led to the identification of subdomains of the plasma membrane known as glycolipid-enriched microdomains (GEMs)[1] or detergent-insoluble rafts. This membrane heterogeneity is caused by the self-association of sphingolipids, cholesterol, and protein components. GEMs have been proposed to be involved in membrane trafficking, cell morphogenesis, and signal transduction mechanisms. Indeed, a variety of signaling molecules are concentrated in GEMs, including Src family tyrosine kinases and monomeric and heterotrimeric G proteins, as well as molecules involved in Ca^{2+} influx (for reviews, see references 1–3).

Although immune receptors such as B cell receptor (BCR), TCR, and FcR are not significantly enriched in the GEMs before stimulation, aggregation of these receptors results in the accumulation of tyrosine-phosphorylated receptors and signaling molecules in the GEMs (4–10). For example, after TCR stimulation, Zap-70 translocates from the cytoplasm to the GEMs, and the tyrosine phosphorylated phospholipase C (PLC)-γ1 becomes enriched within the GEMs (6, 7, 11). In the case of FcεRI, biochemical analysis or direct monitoring in living cells has documented that after aggregation of FcεRI, Syk and PLC-γ1, in addition to FcεRI by itself, are rapidly recruited to the punctate plasma membrane microdomains, presumably reflecting GEMs (9, 10). The functional importance of membrane compartmentation in immune receptor signaling has been recently suggested by the findings that disruption of GEMs by the depletion or the sequestration of membrane cholesterol resulted in attenuation of antigen receptor–mediated calcium mobilization (5, 7).

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[1]*Abbreviations used in this paper:* BCR, B cell receptor; BLNK, B cell linker protein; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Gads, Grb2-related adaptor downstream of Shc; GEM, glycolipid-enriched microdomain; HA, hemagglutinin; IP_3, inositol 1,4,5-trisphosphate; JNK, c-Jun NH_2-terminal kinase; LAT, linker for activation of T cells; PLC, phospholipase C; PTK, protein tyrosine kinase; SH, Src homology; SLP-76, Src homology 2 domain–containing leukocyte protein of 76 kD.

Linker for activation of T cells (LAT) could provide the molecular basis by which signaling molecules are accumulated into GEMs upon engagement of TCR (12, 13). LAT is palmitoylated and is localized in GEMs before TCR ligation (11, 14). Once LAT is tyrosine phosphorylated by Zap-70, critical downstream signaling molecules are subsequently bound to the phosphorylated LAT, either directly or indirectly (12, 13, 15, 16). One of the associated molecules is Src homology (SH)2 domain–containing leukocyte protein of 76 kD (SLP-76), tyrosine phosphorylation of which requires LAT (15–17). Because Grb2 is able to bind to the tyrosine-phosphorylated LAT and the proline-rich region of SLP-76 by using its SH2 and SH3 domains, respectively, it was initially thought that Grb2 might act as a bridge between LAT and SLP-76. However, as a recently cloned Grb2-like adaptor protein, Grb2-related adaptor downstream of Shc (Gads; alternatively named as Grf40 or GrpL) was found to bind to SLP-76 more tightly than does Grb2, it has been proposed that Gads rather than Grb2 might connect phosphorylated LAT to SLP-76 in TCR signaling (18–20).

In contrast to expression of SLP-76 in T cells, NK cells, mast cells, macrophages, and platelets, B cell linker protein (BLNK; alternatively named as SLP-65, B cell adaptor containing SH2 domain [BASH], or activation-related B cell gene [BCA]) is predominantly expressed in B cells (17, 21–24). In addition, bone marrow macrophages are shown to express both adaptor molecules (25). The importance of SLP-76 and BLNK for T and B cell development and signaling has been underscored by gene targeting experiments in mice and cell lines (26–33). SLP-76 and BLNK are essential for PLC-γ and the subsequent calcium pathway in T and B cells (28–31).

Although BLNK and SLP-76 share some structural and functional similarities, differences between BLNK and SLP-76 have been also documented. For instance, SLP-76 binds to Grb2, Nck, and Vav, whereas BLNK interacts with PLC-γ in addition to these three molecules. Instead, in the case of T cells, LAT was shown to bind to PLC-γ (for reviews, see references 34, 35). Based on these differences between SLP-76 and BLNK, together with the evidence that neither LAT nor Gads is expressed in B cells (12, 13, 18–20), we hypothesized previously that the functions of BLNK are split between SLP-76, Gads, and LAT (21, 36). To test this hypothesis, we have examined the BCR functions in $Syk^{-}BLNK^{-}$ DT40 B cells that carry various combinations of Zap-70, SLP-76, Gads, and LAT. Here, we demonstrate that LAT is essential but not sufficient for targeting of SLP-76 to GEMs and its subsequent functions. In addition to LAT, exogenous expression of Gads or overexpression of Grb2 was required. Together, our findings suggest that LAT and a redundant role of Gads and Grb2 are required for exerting SLP-76 functions.

Materials and Methods

Expression Constructs, Cells, and Antibodies. Various mutant chicken DT40 B cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 50 μM 2-ME, 4 mM L-glutamine, penicillin, and streptomycin.

Chicken SLP-76 cDNA was isolated by screening chicken thymus cDNA library using human SLP-76 cDNA fragment (17) as a probe under a low stringent condition. The complete sequence of the chicken SLP-76 cDNA has been deposited into EMBL/GenBank/DDBJ under accession no. AF226988. FLAG-tagged chicken SLP-76 cDNA and membrane SLP-76 chimera cDNA (mSLP-76; see Fig. 3 A) were constructed by using PCR method and cloned into expression vector pAzeo. The mSLP-76 is composed of the extracellular domain of human CD16 (amino acids 1–206; reference 37), the transmembrane domain of the mouse CD45 (amino acids 424–454; reference 38), and the complete chicken SLP-76 with a FLAG tag at its COOH terminus. FLAG-tagged chicken BLNK (39), human LAT (13), and human Gads (18) cDNAs were cloned into pAzeo, pAhygro, and pApuro (40), respectively. The cDNAs of myc-tagged human Gads and myc-tagged human Grb2 in pcDNA3.1 were described previously (18). These cDNAs were transfected by electroporation at 550 V, 25 μF. Expression levels of transfected cDNAs were determined by Western blot analysis. Cell surface expression of BCR or CD16 was analyzed by FACScan™ (Becton Dickinson) using FITC-conjugated anti–chicken IgM Ab (Bethyl) or FITC-conjugated anti–human CD16 mAb (BD PharMingen), respectively.

For generation of $Syk^{-}BLNK^{-}$ DT40 cells (E37-5; see Table I), the targeting vector pSyk-bsr (40) was transfected into $BLNK^{-}$ cells (29). Clones were selected in the presence of 50 μg/ml blasticidin S. For isolation of E40-4 and -6 clones, hemagglutinin (HA)-tagged human Zap-70 cDNA in pApuro (41) was transfected into E37-5 cells (see Table I). FLAG-BLNK, FLAG–SLP-76, or mSLP-76 cDNA was transfected into E40-4 cells, generating E42-10 and -11, E48-1 and -3, or E77-8 and -9 clones, respectively. LAT, Gads, or both of them were transfected into E48-1 cells, generating E57-3 and -11, E88-8 and -22, or E67-1 and -3 clones, respectively. myc-Gads or myc-Grb2 cDNA was transfected into E57-11 cells, generating E61-9 and -18, or E87-17 and -23 clones, respectively.

Anti–chicken IgM mAb (M4), anti–chicken BLNK Ab, anti–chicken PLC-γ2 Ab, anti–chicken Lyn Ab, anti–human Gads Ab, anti–human c-Jun NH_2-terminal kinase (JNK)1 mAb, antiphosphotyrosine mAb (4G10), anti-myc mAb (9E10), and anti-FLAG mAb were described previously (18, 29, 39, 42). Anti–human LAT Ab, anti-HA mAb, anti–human CD16 mAb (3G8), and anti–α-tubulin mAb were purchased from Upstate Biotechnology, Roche, BD PharMingen, and Santa Cruz Biotechnology, Inc., respectively.

Preparation of GEM Fractions. Cells (4×10^8) were stimulated with M4 (4 μg/ml) or with anti-CD16 mAb (15 μg/ml) for 3 min in 5 ml RPMI 1640 at 37°C for BCR or mSLP-76 cross-linking alone, respectively. For coligation of mSLP-76 and BCR (see Fig. 3 D), E77-9 cells were treated with rabbit anti–mouse IgM Ab (Zymed Laboratories; 15 μg/ml) for 5 min at 37°C before stimulation by M4 (4 μg/ml). Cells were washed with PBS containing 4 mM EDTA, and were resuspended in 1 ml of TNEV buffer (25 mM Tris-HCl, pH 7.5, 0.5% Triton-X 100, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate) supplemented with protease and phosphatase inhibitors as described previously (40), and were homogenized with 20 strokes in a loose-fitting dounce homogenizer (Wheaton). The lysates were gently mixed with 1 ml of 80% (wt/vol) sucrose in TNEV buffer and placed in the bottom of a centrifuge tube. The samples were then overlaid with 6.5 ml of 30% sucrose and 3.5 ml of 5% sucrose in TNEV buffer and supercentrifuged for 16 h at 200,000 *g*

in a RPS40T rotor (Hitachi Instruments) at 4°C. Among 12 fractions collected from the top of the gradient, fraction 4, at the interface between 5% and 30% sucrose, was confirmed as GEMs by detecting both ganglioside GM1 and Lyn by Western blot analysis.

Calcium Analysis. Cells (5×10^6) were suspended in PBS containing 20 mM Hepes (pH 7.2), 5 mM glucose, 0.025% BSA, and 1 mM $CaCl_2$, and were loaded with 3 μM Fura-2/AM at 37°C for 45 min. Cells were washed twice, adjusted to 10^6 cells/ml, and stimulated by M4 (2 μg/ml). Cross-linking of mSLP-76 alone was performed by anti-CD16 mAb (10 μg/ml), while coligation of BCR and the chimera was carried out by adding rabbit anti–mouse IgM (10 μg/ml) followed by anti–chicken IgM mAb, M4 (2 μg/ml). Continuous monitoring of fluorescence from the cell suspension was performed using Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instruments) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described (40).

Immunoprecipitation, Western Blot Analysis, and In Vitro Kinase Assay. For immunoprecipitation, cells were solubilized in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors as described previously (40), and precleared lysates were sequentially incubated with proper Abs and protein G sepharose. Lysates or immunoprecipitates were separated by SDS-PAGE gel, transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membrane, and detected by appropriate Abs and enhanced chemiluminescence system (Amersham Pharmacia Biotech). For in vitro kinase assay for JNK, the assay conditions were described previously (42). Immunoprecipitated JNK1 was suspended in kinase assay buffer in the presence of [γ-^{32}P]ATP and glutathione *S*-transferase (GST)–c-Jun fusion protein as a substrate. The reaction mixtures were separated by 12.5% SDS-PAGE gel, dried, and subjected to autoradiography.

Results

SLP-76 Cannot Replace the Function of BLNK in B Cells. Among the Syk family protein tyrosine kinases (PTKs; Syk and Zap-70), Syk is expressed in B cells, thymocytes, mast cells, platelets, and neutrophils, whereas Zap-70 expression is limited to natural killer cells and T cells, including thymocytes (43). When Zap-70 was expressed in Syk$^-$ DT40 B cells, the early biochemical events, including BCR-induced tyrosine phosphorylation of cellular proteins, phosphorylation of Zap-70, and mobilization of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), were reconstituted (41). Because BLNK is essential for BCR-mediated calcium mobilization (29), these results suggest that BLNK is able to couple Zap-70 as well as Syk to PLC-γ2 activation in B cells. To formally demonstrate that BLNK is required for Zap-70–mediated PLC-γ2 activation, Syk$^-$BLNK$^-$ DT40 B cells expressing Zap-70 were generated (E40-4, E40-6). As expected, these cells failed to evoke BCR-induced PLC-γ2 tyrosine phosphorylation and the subsequent $[Ca^{2+}]_i$ increase (Fig. 1, A–C). For a summary of the cell lines and a schematic representation of the construct used in this study, see Table I and Fig. 3 A. The levels of cell surface expression of BCR in various clones used in this study were essentially the same as that of parental DT40 cells.

To examine whether SLP-76 can replace the function of BLNK in B cells, E40-4 cells, which express Zap-70 in Syk$^-$BLNK$^-$ DT40 B cells, were transfected with chicken BLNK or chicken SLP-76 cDNA and selected in zeocin-containing media. For comparison of the expression extent between BLNK and SLP-76, these cDNAs were tagged with a FLAG epitope at their NH_2 termini. Endogenous chicken SLP-76 could not be detected by the reverse transcriptase PCR method (data not shown). Among various stable clones, two independent clones expressing BLNK (E42-10, E42-11) or SLP-76 (E48-1, E48-3) were estab-

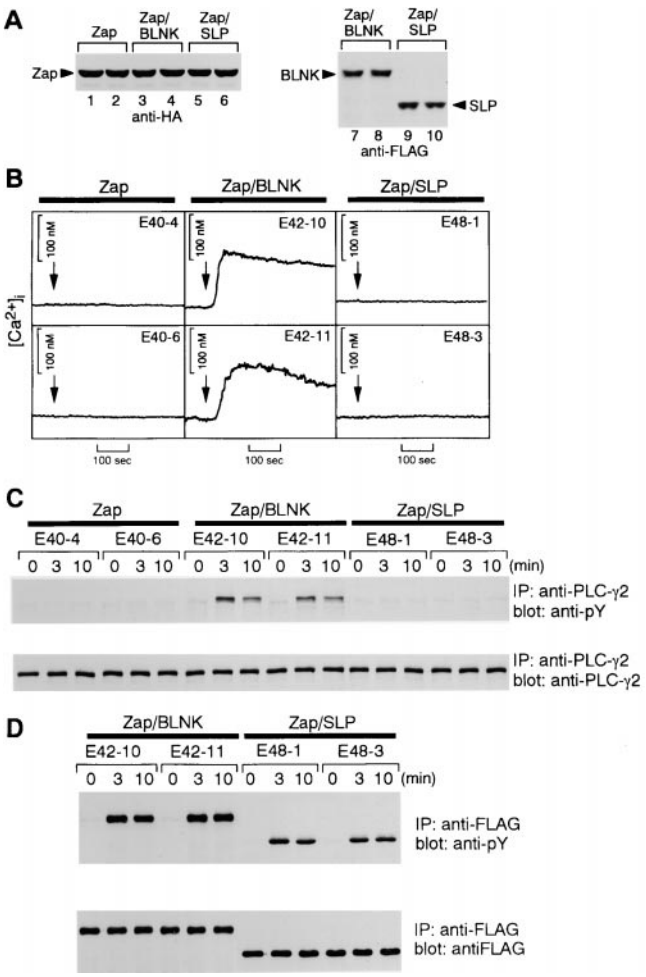

Figure 1. Functional differences between BLNK and SLP-76 in Zap-70$^+$BLNK$^-$ DT40 cells. (A) Expression levels of HA–Zap-70, FLAG-BLNK, and FLAG–SLP-76 proteins. Whole-cell lysates prepared from 2×10^6 cells were analyzed by Western blotting using anti-HA mAb (left) or anti-FLAG mAb (right). Lanes: 1, E40-4; 2, E40-6; 3 and 7, E42-10; 4 and 8, E42-11; 5 and 9, E48-1; 6 and 10, E48-3. (B) Calcium mobilization analysis. Arrows indicate the time point for adding M4 mAb. (C) Tyrosine phosphorylation of PLC-γ2. At the indicated time points after M4 stimulation (4 μg/ml), immunoprecipitates with anti–PLC-γ2 Ab (10^7 cells/lane) were divided in half, separated on a 7% SDS-PAGE gel, and analyzed by Western blotting with antiphosphotyrosine mAb (top) or anti–PLC-γ2 Ab (bottom). IP, immunoprecipitate. (D) BCR-induced tyrosine phosphorylation of BLNK and SLP-76. Cells (2×10^6) were similarly examined as in C using anti-FLAG mAb. Western blotting with antiphosphotyrosine mAb (top) or anti-FLAG mAb (bottom) is shown. IP, immunoprecipitate.

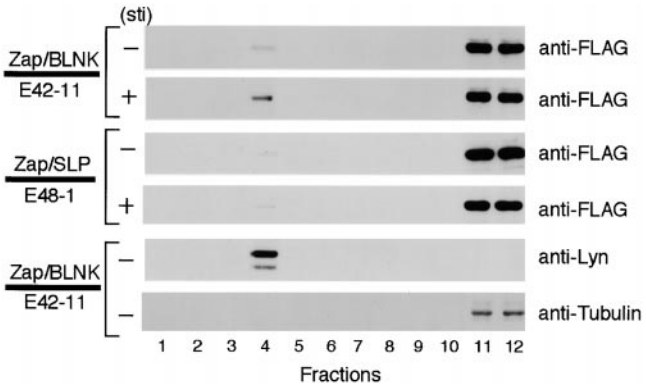

Figure 2. Western blot analysis of GEM fractions. After stimulation by M4 for 3 min, GEMs were prepared from E42-11 or E48-1 cells (4×10^8) by sucrose density gradient centrifugation. 40 μl of the individual fractions, except for fractions 11 and 12 (2 μl), were analyzed by Western blotting using anti-FLAG mAb, anti-Lyn Ab, or antitubulin mAb.

lished that expressed the comparable levels of BLNK and SLP-76 (Fig. 1 A), judged by Western blot analysis with anti-FLAG mAb. In E42-10 and E42-11 clones, the level of exogenous BLNK expression, detected by using Ab against chicken BLNK, was almost comparable to that present in parental DT40 cells (data not shown). In contrast to E42-10 and E42-11 clones, BCR-mediated PLC-γ2 tyrosine phosphorylation and calcium mobilization were not reconstituted in E48-1 and E48-3 clones (Fig. 1, B and C), despite the comparable expression level of SLP-76 to BLNK. As $BLNK^-$ DT40 cells expressing endogenous Syk and exogenous SLP-76 also failed to evoke the BCR-mediated calcium mobilization (data not shown), we conclude that BLNK, but not SLP-76, is able to couple Syk/Zap-70 PTKs to PLC-γ2 activation upon BCR cross-linking.

Coligation of Membrane SLP-76 and BCR Restores the PLC-γ2 Activation. To explore the mechanism underlying the functional difference between BLNK and SLP-76, we first examined tyrosine phosphorylation status of BLNK and SLP-76 after BCR ligation. As shown in Fig. 1 D, the BCR-induced tyrosine phosphorylation of SLP-76 was significant, but approximately fourfold lower than that of BLNK, determined by the ratio of the antiphosphotyrosine signal to the anti-FLAG signal.

It has been recently shown that BCR and the associated Igα rapidly translocate to GEMs upon receptor cross-linking, which is required for efficient signal transduction by the BCR (4, 5, 8). We therefore compared translocation of BLNK and SLP-76 into GEMs upon BCR cross-linking. After receptor stimulation, DT40 B cells were solubilized with 0.5% Triton X-100, and lysates were subjected to supercentrifiguration over a sucrose density gradient. As reported previously (4, 5), Lyn was enriched in the fraction 4, and tubulin was found to be completely excluded from the GEMs (Fig. 2). Although the stoichiometry of GEM-associated BLNK was substantially enhanced after BCR ligation, the significant translocation of SLP-76 to the GEMs could not be detected (Fig. 2). Taken together, these two lines of findings suggest that the failure of SLP-76 to evoke PLC-γ2 activation in B cells is due to its lower phosphorylation and/or its inability to be recruited into GEMs.

If the latter is a major cause, this predicts that the enforced localization of SLP-76 in GEMs should rescue the defect of PLC-γ2 activation. To test this prediction, we have made a membrane chimera of SLP-76 possessing SLP-76 and CD45 in place of the cytoplasmic and the trans-

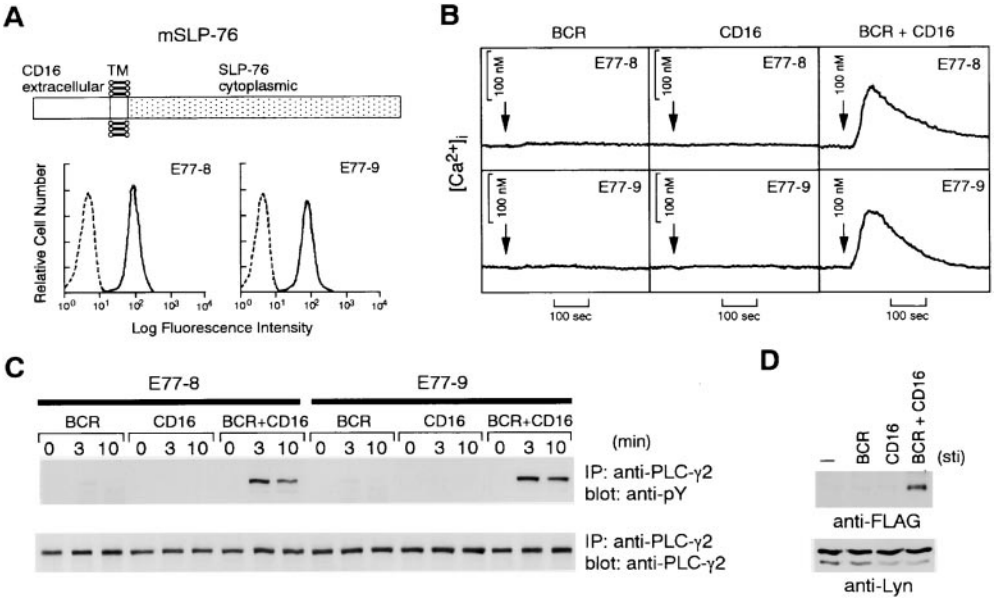

Figure 3. GEM-localized SLP-76 restores PLC-γ2 activation. (A) Schematic diagram of membrane SLP-76 chimera (mSLP-76 [top]). Cell surface expression of CD16 on transfected cells is shown in lower panels. Unstained cells were used as negative controls (dot lines). (B) Calcium mobilization after BCR stimulation, CD16 cross-linking, or coligation of BCR to mSLP-76. (C) Tyrosine phosphorylation of PLC-γ2. After BCR stimulation, CD16 cross-linking, or coligation of BCR to mSLP-76, immunoprecipitates (10^7 cells/lane) with anti–PLC-γ2 Ab were divided into half and analyzed by Western blotting with 4G10 (top) or anti-PLC-γ2 Ab (bottom). IP, immunoprecipitate. (D) Western blot analysis of GEM fractions. GEMs were prepared from E77-9 cells (4×10^8) and analyzed by Western blotting with anti-FLAG mAb (top) or anti-Lyn Ab (bottom). Among 12 fractions (see Fig. 2), fraction 4 is shown.

membrane domains of CD16, respectively (mSLP-76; shown in Fig. 3 A). It has been reported that CD45 is excluded from the GEMs (4, 7, 8, 44). Based upon the assumption that the transmembrane domain of CD45 contributes to the exclusion of CD45 from GEMs, this chimera was designed. The mSLP-76 was introduced into Syk$^-$BLNK$^-$ DT40 cells expressing Zap-70 (E40-4; Table I). As expected, significant localization of mSLP-76 into GEMs was observed only after coligation of BCR and mSLP-76 (Fig. 3 D). Correlated with this translocation, only coligation of the BCR to the chimera resulted in a $[Ca^{2+}]_i$ increase as well as PLC-γ2 tyrosine phosphorylation (Fig. 3, B and C). Thus, the failure of recruitment of native SLP-76 into GEMs, wherein the BCR translocates, likely causes its inability to couple Syk/Zap-70 PTKs to downstream signaling events such as PLC-γ2 activation. However, we cannot completely exclude the possibility that the decreased phosphorylation extent of native SLP-76 compared with that of BLNK (Fig. 1 D) could contribute, at least to some extent, to its inability to mediate downstream signaling.

LAT, Gads, and Grb2 Are Involved in Targeting SLP-76 to GEMs. The above results suggest the involvement of T cell–specific factor(s) in targeting SLP-76 to GEMs, whereby SLP-76 couples Syk/Zap-70 PTKs to downstream effectors. Likely candidates are LAT and/or Gads, both of which are expressed predominantly in T cells (12, 13, 18–20). Consistent with previous reports, DT40 B cells express neither LAT nor Gads as detected by Northern blot analysis or reverse transcriptase PCR method (data not shown). To examine the requirement for LAT and Gads in BCR function, E48-1 cells, which express Zap-70 and SLP-76 in Syk$^-$BLNK$^-$ DT40 cells, were transfected with various combinations of human LAT and Gads cDNAs and selected in hygromycin-containing media. Among various stable transformants, two independent clones were selected (Table I and Fig. 4). In all clones examined, the expression levels of LAT and Gads were not significantly greater than those in Jurkat T cells (data not shown). As observed in human T cells, two forms of LAT and Gads were recognized by anti-LAT and anti-Gads Abs, respectively, in transfected DT40 B cells (12, 13, 19). Fig. 5 A demonstrates that either LAT or Gads alone was not sufficient to restore BCR-mediated $[Ca^{2+}]_i$ increase, whereas this calcium defect was restored when both LAT and Gads, in addition to SLP-76, were introduced.

The calcium data suggest the requirement for both LAT and Gads, in addition to SLP-76, in BCR-dependent phosphorylation of PLC-γ2. Expression of LAT was essential for BCR-mediated tyrosine phosphorylation of PLC-γ2, because DT40 cells lacking LAT (E88-8, E88-22, E48-1, and E48-3) failed to induce PLC-γ2 tyrosine phosphorylation (Fig. 5 B). In contrast to a complete loss of PLC-γ2 tyrosine phosphorylation in DT40 cells lacking LAT, reconstituted cells by Zap-70, LAT, and SLP-76 (E57-3 and E57-11) showed the BCR-induced tyrosine phosphorylation of PLC-γ2, although the phosphorylation extent was approximately threefold lower than that in the completely reconstituted clones (E67-1 and E67-3). Despite the PLC-γ2 tyrosine phosphorylation, E57-3 and E57-11 clones failed to induce inositol 1,4,5-trisphosphate (IP_3) generation after BCR stimulation (data not shown), consistent with the calcium mobilization defect.

As DT40 B cells express Grb2 (42), the above findings suggest that Gads, but not Grb2, is essential for PLC-γ2 activation. However, it might be argued that expression level of endogenous Grb2 is not sufficient for evoking PLC-γ2 activation. To examine this possibility, E57-11 cells (expressing Zap-70, SLP-76, and LAT) were transfected with human Grb2 or Gads cDNA, both of which were tagged with a myc-epitope at their NH_2 termini. As shown in Fig. 6, the BCR-mediated PLC-γ2 activation, judged by its phosphorylation and calcium mobilization, was reconstituted in DT40 B cells expressing relatively high levels of myc-Gads (E61-9) and myc-Grb2 (E87-17). In contrast, DT40 cells expressing relatively low levels of these tagged proteins failed to restore the PLC-γ2 defect (E61-18 and E87-23). Based on these data, we conclude that LAT, in addition to SLP-76, is essential but not sufficient for reconstituting the BCR function, and that a redundant function of Gads and Grb2 is additionally required.

Table I. *Expression of BCR and Proteins in DT40 Clones*

Clone name	BCR	Syk or Zap-70	BLNK or SLP-76	LAT	Gads or Grb2
DT40 (wt)	+	Syk	BLNK	–	–
E37-5	+	–	–	–	–
E40-4, 6	+	Zap-70	–	–	–
E42-10, 11	+	Zap-70	BLNK	–	–
E48-1, 3	+	Zap-70	SLP-76	–	–
E77-8, 9	+	Zap-70	mSLP-76	–	–
E57-3, 11	+	Zap-70	SLP-76	LAT	–
E88-8, 22	+	Zap-70	SLP-76	–	Gads
E67-1, 3	+	Zap-70	SLP-76	LAT	Gads
E61-9, 18	+	Zap-70	SLP-76	LAT	myc-Gads
E87-17, 23	+	Zap-70	SLP-76	LAT	myc-Grb2

wt, wild-type.

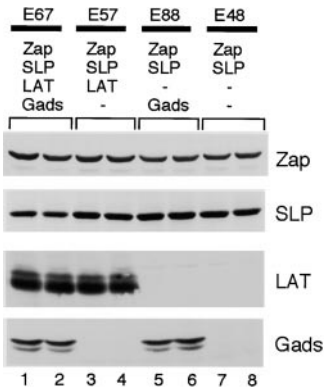

Figure 4. Expression levels of HA–Zap-70, FLAG–SLP-76, LAT, and Gads proteins. Total cell lysates (2.5×10^6 cells) were prepared and analyzed by Western blotting using anti-HA mAb (top), anti-FLAG mAb (second panel), anti-LAT Ab (third panel), or anti-Gads Ab (bottom), respectively. Lanes: 1, E67-1; 2, E67-3; 3, E57-3; 4, E57-11; 5, E88-8; 6, E88-22; 7, E48-1; 8, E48-3.

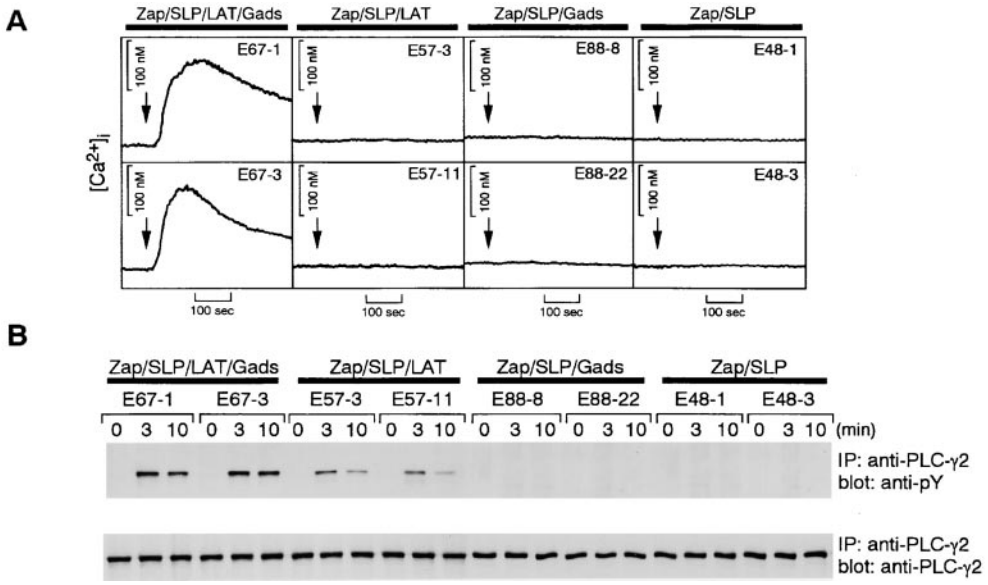

Figure 5. Expression of LAT and Gads in addition to SLP-76 restores PLC-γ2 activation in Zap-70^{+}BLNK^{-} DT40 cells. (A) Calcium mobilization analysis. (B) BCR-induced tyrosine phosphorylation of PLC-γ2. IP, immunoprecipitate.

As a functional readout of SLP-76, we also examined whether BCR-mediated JNK activation requires LAT and Gads, because we had shown previously that not only PLC-γ2 but also JNK activation is dependent on BLNK in BCR signaling (29). As shown in Fig. 7, SLP-76 was capable of inducing the BCR-mediated JNK activation in the presence of both LAT and Gads. As expected, E87-17 cells expressing LAT and a high level of Grb2 was also capable of reconstituting the JNK activation (data not shown).

Given the evidence that LAT is palmitoylated and is localized in GEMs before TCR stimulation (11, 14), the functional data described above suggest that Gads and Grb2 act as a bridge between LAT and SLP-76, thereby recruiting SLP-76 to GEMs. To test this idea, we first investigated whether Gads or Grb2 is required for an interaction between LAT and SLP-76 and the subsequent targeting of SLP-76 to GEMs. As demonstrated in Fig. 8 A, BCR-induced association of SLP-76 with LAT was observed even in the absence of Gads, albeit at a very low level (E57-11), whereas this association was greatly enhanced by addition of Gads (E67-3). Similarly, when Grb2 was overexpressed (E87-17), the BCR-mediated association of SLP-76 with LAT was significantly enhanced. In contrast to this inducible association, SLP-76 was constitutively associated with Gads (E67-3 and E88-8) or Grb2 (E87-17).

As reported, even in the absence of Gads, LAT was constitutively localized in GEMs (Fig. 9 B) and was tyrosine phosphorylated by BCR ligation (Fig. 8 B). SLP-76 was slightly translocated into GEMs even in the absence of Gads. However, 4.8-fold and 3.1-fold increases in translocation of SLP-76 to GEMs (averages among three independent experiments) were observed in E67-3 and E87-17 clones, respectively (Fig. 9, A and B). BCR-mediated tyrosine phosphorylation of SLP-76 appeared to be correlated with the translocation extent of SLP-76 to GEMs. The SLP-76 phosphorylation was enhanced ~1.5-fold by addition of LAT, and this phosphorylation was further increased about twofold by addition of

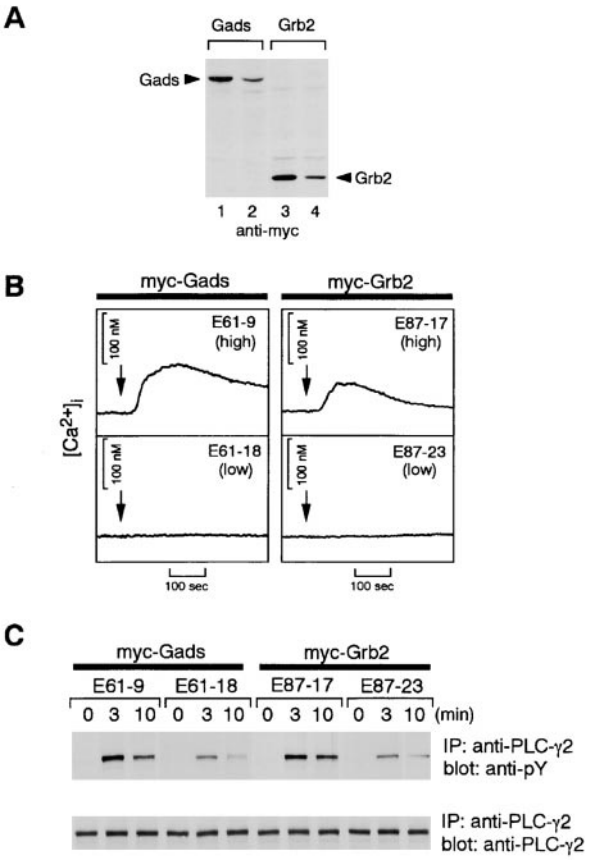

Figure 6. Overexpression of Grb2 replaces the Gads function. (A) Expression levels of myc-Gads and myc-Grb2 proteins. Total cell lysates (2.5×10^6 cells) were prepared and analyzed by Western blotting using anti-myc mAb. When anti-Gads Ab was used instead of using anti-myc mAb, two forms like those described in the legend to Fig. 4 were detected, suggesting that posttranslational modifications, such as cleavage of Gads, might occur. Lanes: 1, E61-9; 2, E61-18; 3, E87-17; 4, E87-23. (B) BCR-induced calcium mobilization. (C) Tyrosine phosphorylation of PLC-γ2 after BCR engagement. IP, immunoprecipitate.

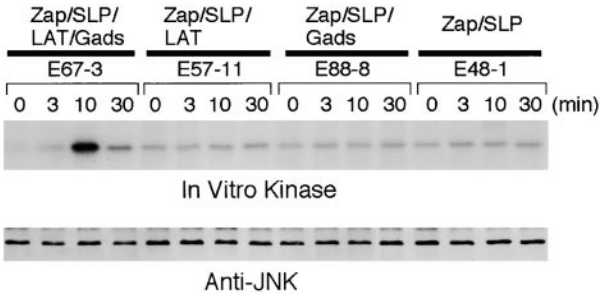

Figure 7. BCR-induced JNK activation. After M4 stimulation, JNK was immunoprecipitated with anti-JNK1 mAb. Half of immunoprecipitates was used for the in vitro kinase assay (top). The remaining half was used for Western blotting with anti-JNK1 mAb (bottom).

Gads or Grb2 (Fig. 8 B). Collectively, these results demonstrate that Gads or Grb2 is involved in the formation of a complex containing SLP-76 and LAT, and thereby participating in efficient translocation of SLP-76 to GEMs.

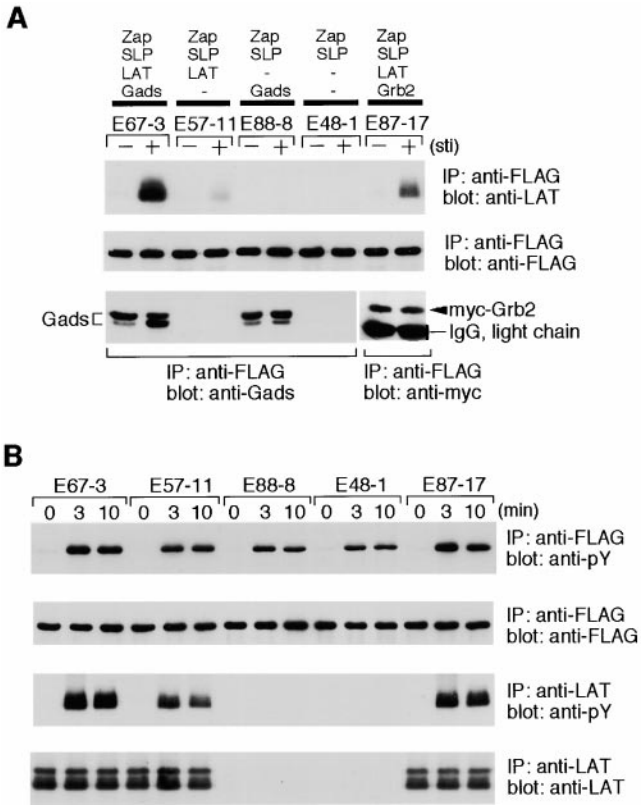

Figure 8. Association of SLP-76 with LAT, Gads, or Grb2 in DT40 cells. (A) After stimulation by M4 (4 μg/ml) for 3 min, SLP-76 proteins were immunoprecipitated with anti-FLAG mAb (5×10^7 cells/lane). Samples were divided, separated on an 8.5% SDS-PAGE gel, and analyzed by Western blotting with anti-LAT Ab (top), anti-FLAG mAb (middle), anti-Gads Ab (bottom), or anti-myc mAb (bottom). IP, immunoprecipitate. (B) Tyrosine phosphorylation of SLP-76 and LAT. At the indicated time points after M4 stimulation (4 μg/ml), immunoprecipitates (2×10^6 cells/lane) with anti-FLAG mAb or anti-LAT Ab were divided in half, separated on a 9.6% SDS-PAGE gel, and analyzed by Western blotting with antiphosphotyrosine mAb (top and third panels), anti-FLAG mAb (second panel), or anti-LAT Ab (bottom). IP, immunoprecipitate.

BLNK Couples Syk to Downstream Effectors Even in the Absence of Grb2 and Grap. The above results prompted us to examine the requirement for Grb2 families, Grb2 and Grap, in exerting BLNK function. To address this issue, we monitored calcium mobilization in $Grb2^-Grap^-$ DT40 cells (45). As shown in Fig. 10 A, these mutant cells still evoked calcium mobilization, demonstrating that in contrast to SLP-76, BLNK still activates PLC-γ2 pathway even in the absence of Grb2 families. Consistent with these functional data, BLNK was translocated to GEMs even in the absence of Grb2 and Grap (Fig. 10 B).

Discussion

Functional similarities between the Zap-70 and Syk, though not identical, have been demonstrated by studies in many systems. Defects of BCR function in Syk-deficient DT40 B cells can be restored by the expression of Zap-70 (41). Conversely, expression of Syk in $Zap\text{-}70^{-/-}$ mice and Zap-70–deficient Jurkat T cells results in restoration of thymocyte development and TCR function, respectively (46, 47). In contrast, we report here the inability of SLP-76 to compensate for the absence of BLNK in the DT40 B cell line. Expression of SLP-76 in the $Zap\text{-}70^+BLNK^-$ B cells cannot reconstitute BCR signaling properties as measured

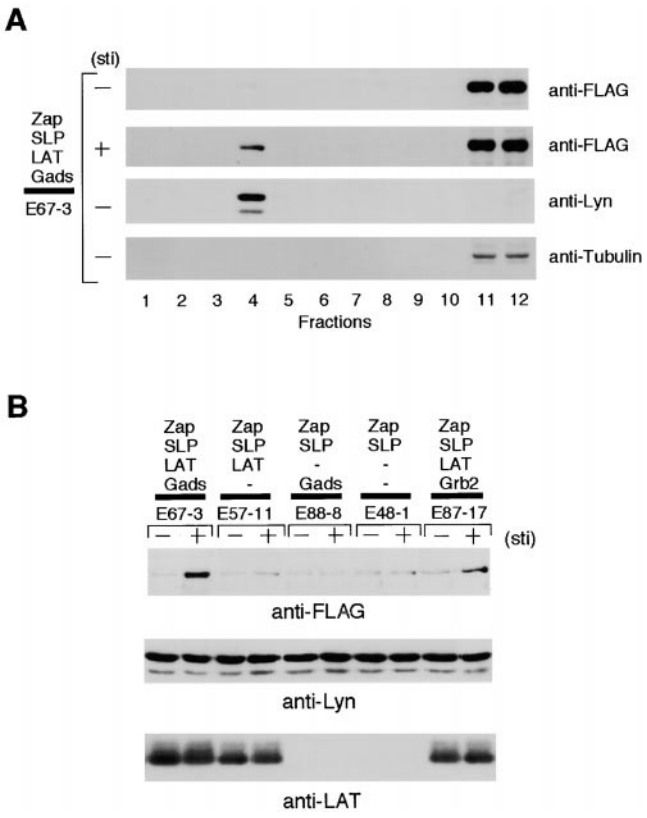

Figure 9. LAT, Gads, and Grb2 are involved in targeting SLP-76 into GEM fractions. (A) GEMs were prepared from E67-3 cells (4×10^8) and analyzed by Western blotting as described in the legend to Fig. 2. (B) Western blot analyses of GEM fractions (fraction 4) with anti-FLAG mAb (top), anti-Lyn Ab (middle), and anti-LAT Ab (bottom) are shown.

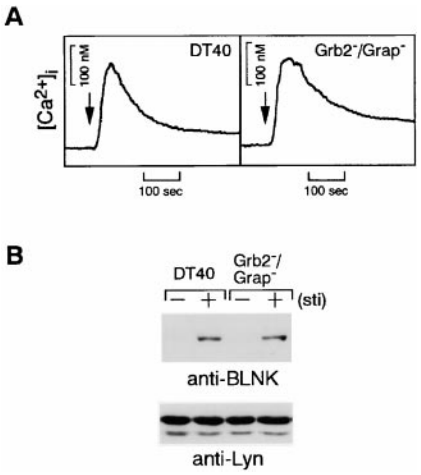

Figure 10. BLNK is translocated to GEMs in the absence of Grb2 and Grap in B cells. (A) Calcium mobilization in wild-type and Grb2^{-}Grap^{-} DT40 cells. (B) Western blot analyses of GEM fractions (fraction 4) are shown. BLNK and Lyn were determined by Western blotting with anti-BLNK Ab (top) or anti-Lyn Ab (bottom).

by mobilization of cytoplasmic free calcium and JNK activation (Fig. 1 and 7).

CD45 is excluded from the GEMs, and this exclusion has been proposed to be required for efficient antigen receptor signaling (4, 7, 8, 44). As expected, mSLP-76 harboring the transmembrane domain of CD45 is not localized in GEMs, whereas upon coligation of mSLP-76 and BCR, a substantial fraction of the mSLP-76 is enforcedly translocated into GEMs (Fig. 3 D). In this condition, BLNK-dependent downstream signaling events such as calcium mobilization can be reconstituted by SLP-76. Thus, our observations suggest that SLP-76 possesses a potentiality to couple Zap-70 to downstream effectors even in the B cell context. More importantly, together with our evidence that BLNK, but not native SLP-76, is translocated into GEMs after BCR ligation, the failure of recruitment of native SLP-76 into GEMs likely causes its inability to couple Zap-70 to downstream effectors in DT40 B cells. Our conclusion is further supported by reconstitution experiments using LAT-deficient Jurkat T cells (J.Cam2; Boerth, N.J., J.J. Sadler, D. Bauer, J.L. Clements, S.M. Gheith, and G.A. Koretzy, manuscript submitted for publication). The defects of TCR function in the J.Cam2 cells can be restored by introducing a LAT/SLP-76 chimeric molecule bearing minimal sequences for targeting LAT to GEMs, demonstrating that these defects in LAT-deficient cells is presumably due to the inability of SLP-76 to be recruited to GEMs.

Consistent with important functional roles of LAT in TCR signaling (12–16, 48), our results demonstrate that LAT is essential for translocation of SLP-76 to GEMs. In the absence of LAT, SLP-76 cannot be translocated into GEMs after BCR ligation. Addition of LAT permits this translocation, albeit at very low level, even in the absence of exogenous Gads or Grb2 (Fig. 9 B). The most likely explanation is that LAT uses endogenous Grb2 for targeting SLP-76 to GEMs in this combination. In support of this explanation, addition of exogenous Grb2 enhances the translocation of SLP-76 to GEMs. The SLP-76 translocation is also augmented by addition of exogenous Gads, suggesting that Grb2 and Gads have a redundant role in translocation of SLP-76 to GEMs by bridging between SLP-76 and LAT. Grb2 and Gads, closely related adaptor molecules containing an SH3-SH2-SH3 motif, form a complex with LAT and SLP-76 via their SH2 and COOH-terminal SH3 domains, respectively (34, 35). In accord with these binding data, SLP-76 constitutively and inducibly associates with Grb2/Gads and phosphorylated LAT, respectively, in the DT40 B cells.

Although low level of translocation of SLP-76 to GEMs can be detected even in the absence of exogenous Grb2 and Gads, this level is not sufficient for allowing SLP-76 to activate downstream events such as calcium mobilization and JNK activation. Strengthening this idea, expression levels of Grb2 and Gads proteins were found to correlate with the ability of SLP-76 to couple to downstream effectors (Fig. 6). Collectively, our results demonstrate that both LAT and Grb2/Gads are required for sufficient targeting of SLP-76 to GEMs, wherein SLP-76 is able to activate downstream effectors. In this regard, it is an intriguing contrast that BLNK does not require Grb2/Grap for its targeting to GEMs and the subsequent calcium mobilization in DT40 cells. However, the dispensability of Grb2/Grap in the BLNK translocation does not eliminate the significance of Grb2/Grap in BCR signaling. In fact, we have recently provided the evidence that a redundant role of Grb2 and Grap is involved in activation of hematopoietic progenitor kinase (HPK)1, a member of the germinal center kinase family, in DT40 B cells (45).

Although translocation of BLNK and SLP-76 to GEMs is essential for propagating antigen receptor signaling, it is not entirely clear why translocation of BLNK and SLP-76 is required. This might be accounted for by the requirement for BLNK/SLP-76 in targeting the associated effector enzymes, including guanine nucleotide exchange factors Sos and Vav to GEMs. Because Ras and Rac1 are concentrated in GEMs (1, 49, 50), recruitment of Sos and Vav to GEMs is likely to be required for activating Ras and Rac1, leading to ERK and JNK activation. Indeed, Vav is reported to associate with SLP-76 and BLNK, whereas Sos binds to Grb2 (34, 35). Assuming that GEMs provide platforms to form complex multiprotein interactions required for antigen receptor signaling, the targeting of BLNK/SLP-76 to GEMs may not be simply to facilitate the localization of various signaling molecules in GEMs. Rather, after localization of BLNK/SLP-76 in GEMs, BLNK/SLP-76 may play an important role in multiprotein complex formation, which in turn is critical for proper antigen receptor signaling.

Our data indicate that even in the absence of exogenous Grb2/Gads, PLC-γ2 is phosphorylated to some extent, but not activated as determined by IP_3 generation and calcium mobilization. Because translocation of PLC-γ to GEMs is essential for its tyrosine phosphorylation and the subsequent activation in antigen receptor signaling (5, 7, 11), one explanation for this result is that the amount of tar-

geted PLC-γ2 to GEMs is small, like low level of translocation of SLP-76 to GEMs in the absence of exogenous Grb2/Gads, likely causing inefficient PLC-γ2 activation. In this regard, our IP_3 and calcium assay systems might not be sensitive enough to detect subtle PLC-γ2 activation. A second possibility is that Btk dysfunction is responsible for the low level of PLC-γ2 phosphorylation and the resulting lack of its activation, because similar PLC-γ2 abnormality is also observed in Btk-deficient DT40 B cells (40). Given that Btk associates with phosphorylated BLNK and SLP-76 via its SH2 domain (39, 51), sufficient recruitment of SLP-76 to GEMs might be required for targeting Btk to GEMs, thereby phosphorylating PLC-γ2. These two possibilities are not mutually exclusive.

Our evidence that SLP-76 and BLNK use distinct adaptor molecules for their targeting to GEMs raises questions about the translocation mechanism of BLNK after BCR ligation. On the analogy of the T cell system, one obvious possibility is that a LAT-like molecule constitutively localized in GEMs plays a role in targeting BLNK to GEMs in B cells. In contrast to requirement for Grb2/Gads in targeting SLP-76 to GEMs, BLNK can be translocated to GEMs even in the absence of Grb2, Grap, and Gads. Thus, not only a LAT-like molecule but also a Grb2-like molecule that is specifically expressed in B cells might exist. The opposite possibility is that BLNK does not require these adaptor molecules for its targeting to GEMs in B cells. For instance, direct binding of BLNK with Syk or Igα/Igβ may be sufficient for its targeting.

We would like to thank Drs. R. Goitsuka, G.A. Koretzky, L.E. Samelson, and S. Tsukada for providing us with chicken thymus cDNA library, human SLP-76 cDNA, human LAT cDNA, and FLAG-tagged chicken BLNK plasmid, respectively. We also thank to Dr. G.A. Koretzky, Dr. A. Kosugi, and N. Narumai for communicating unpublished results, advice on the GEM preparations, and technical assistance, respectively.

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan (to M. Ishiai and T. Kurosaki), the Naito Foundation (to M. Ishiai), the Toray Science Foundation, Takeda Science Foundation, Hoh-ansha Foundation, and Uehara Memorial Foundation (to T. Kurosaki), and the Human Frontier Science Program (to A.C. Chan and T. Kurosaki), and by grant R01AI42787 from the National Institutes of Health (to A.C. Chan).

Submitted: 17 May 2000
Revised: 1 August 2000
Accepted: 15 August 2000

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