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Grb2 and the Non-T Cell Activation Linker NTAL Constitute a Ca^{2+}-Regulating Signal Circuit in B Lymphocytes

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Summary

Activation of the B cell antigen receptor triggers phosphorylation of cytoplasmic and transmembrane adaptor proteins such as SLP-65 and NTAL, respectively. Specific phosphoacceptor sites in SLP-65 serve as docking sites for Ca^{2+}-mobilizing enzymes Btk and PLC-γ2. Phosphorylated NTAL recruits the Grb2 linker, but downstream signaling cascades are unclear. We now show that receptor-induced tyrosine phosphorylation of NTAL and concomitant Grb2 complex formation critically modulate the Ca^{2+} response without affecting SLP-65 and PLC-γ2 phosphorylation. Grb2 turned out to play a negative regulatory role, which appears to be eliminated upon binding to NTAL. This allows for a sustained release of intracellular Ca^{2+} and is mandatory for subsequent entry of Ca^{2+} from extracellular sources. Thus, elevation of Ca^{2+} is regulated by at least two signaling modules, the B cell-specific Ca^{2+} initiation complex comprising SLP-65, Btk, and PLC-γ2 and the more ubiquitously expressed NTAL/Grb2 complex, which acts as an amplifier by switching off inhibitory elements.

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Introduction

The precise assembly of multimolecular signaling complexes by enzymatically inert adaptor proteins is a hallmark of lymphocyte antigen receptor function (Kelly and Chan, 2000; Leo et al., 2002). The B cell antigen receptor (BCR) utilizes Syk-mediated tyrosine phosphorylation of the cytosolic adaptor SLP-65 (Wienands et al., 1998) (BLNK [Fu et al., 1998]; BASH [Goisuka et al., 1998]) for recruitment of Bruton's tyrosine kinase (Btk) and phospholipase C-γ2 (PLC-γ2) (Hashimoto et al., 1999; Ishiai et al., 1999a; Ishiai et al., 1999b; Su et al., 1999). The SH2 domains of both enzymes recognize distinct phosphotyrosine residues in SLP-65 and hence can be simultaneously recruited to a given SLP-65 molecule (Chiu et al., 2002). Indeed, the formation of such a trimolecular complex is required for sufficient PLC-γ2 activation to hydrolyze membrane phospholipids (for a review, see Kurosaki and Tsukada, 2000). The inositol-tris-phosphate (IP_3) product triggers the transient release of intracellularly stored Ca^{2+} ions by binding to and activation of ligand-gated Ca^{2+} channels in the membrane of the endoplasmic reticulum (ER). This is followed by a more sustained influx of Ca^{2+} from the extracellular medium through weakly characterized Ca^{2+}-permeable channels in the plasma membrane. Interestingly, the contribution of intra- and extracellular ions to the complete Ca^{2+} response differs depending upon the developmental stage of the B cell. Immature B cells exhibit only a weak extracellular Ca^{2+} entry, while in mature B cells, this is the dominating part of the Ca^{2+} response (Koncz et al., 2002). Two key questions are currently a matter of intense investigations. First, how is the trimolecular Ca^{2+} initiation complex translocated from the cytosol into the signaling-competent membrane microdomains, the lipid rafts (alternatively called GEM or DIG [Langlet et al., 2000; Simons and Toomre, 2001])? Second, how is the internal Ca^{2+} release coupled to the opening of plasma membrane channels to allow influx of extracellular Ca^{2+}?

Generation of the Ca^{2+} second messenger has been studied in great detail in T lymphocytes stimulated through their antigen receptor (TCR). In these cells, Ca^{2+}-regulating enzymes translocate into the lipid raft fraction by virtue of the transmembranous linker of activated T cells, LAT (Zhang and Samelson, 2000; Zhang et al., 1998a). Following TCR engagement, PLC-γ1 directly binds a specific phosphotyrosine residue of LAT, while SLP-76 and its associated Btk analog, Itk, are recruited to phospho-LAT through the Grb2 family member Gads/GrpL (for a review, see Jordan et al., 2003; Yablonski and Weiss, 2001). Whether Grb2 possesses a Ca^{2+}-regulating role in Gads-negative B cells has not been studied in detail. However, recently the LAT-related non-T cell activation linker, NTAL (Brdicka et al., 2002) (alternatively called LAB [Janssen et al., 2003]), has been identified in several tissues, including B lymphocytes and

myeloid lineage-derived cell types. Subcellularly, NTAL is targeted to lipid rafts by palmitoylation of membrane-proximal cysteine residues, and multiple tyrosine-based signaling motifs become rapidly phosphorylated upon activation of BCR and high-affinity receptors for IgG (FcγRI) and IgE (FcϵRI) (Brdicka et al., 2002; Janssen et al., 2003). Five of a total of ten consensus phosphorylation sites are of the YXN type (where X is any amino acid) and are thus potential binding sites for the SH2 domain of Grb2. Indeed, biochemical studies revealed a stimulation-dependent association between NTAL and Grb2 together with SOS, which is known to bind the two SH3 domains of Grb2. NTAL was therefore regarded to relay signals from activated immunoreceptors to the Ras/MAP kinase pathway. RNA interference studies supported this idea and suggested, moreover, a positive regulatory role of NTAL for BCR-induced Ca^{2+} mobilization (Janssen et al., 2003). The underlying mechanism for the latter function remained, however, obscure, as NTAL, in marked contrast to LAT, is devoid of PLC-γ2 binding sites and no association to SLP-65 or other known Ca^{2+}-regulating proteins could be observed. The work presented here provides genetic and biochemical evidence that the transmembrane adaptor NTAL is not a direct functional counterpart of LAT but promotes Ca^{2+} mobilization by counteracting an unexpected inhibitory role of Grb2.

Results

Grb2 Is a Negative Regulator of Ca^{2+} Mobilization in B Cells

The BCR-induced Ca^{2+} response was examined in chicken DT40 B cells and in genetic mutants in which Grb2 expression has been ablated by gene targeting (Hashimoto et al., 1998). To discriminate between mobilization of Ca^{2+} from intra- and extracellular sources, BCR stimulation was performed for 6 min in the presence of 0.5 mM EGTA to remove extracellular Ca^{2+} and to allow for the monitoring of Ca^{2+} release from ER stores only. After restoring the extracellular Ca^{2+} concentration to 1 mM, entry of Ca^{2+} through ion channels in the plasma membrane was recorded. As shown in Figure 1A, wild-type DT40 cells exhibit a monophased response with internal Ca^{2+} elevation but no subsequent Ca^{2+} influx across the plasma membrane (green line). In marked contrast, *grb2*$^{-/-}$ DT40 cells mobilize Ca^{2+} from both intra- and extracellular sources (black line). This gain of function is solely due to the absence of Grb2, as reconstitution of wild-type Grb2 expression recapitulated the partial Ca^{2+} profile of the parental cells (gray line). Hence, Grb2 is a negative regulatory element of BCR-induced Ca^{2+} mobilization. Mutational analysis of the three Grb2 interaction modules further showed that the C-terminal SH3 domain and central SH2 domain are required for the inhibitory effect, while the N-terminal SH3 domain is dispensable (Figures 1B and 1C; pink, blue, and orange lines, respectively). Equal expression of the different Grb2 proteins was controlled by anti-Grb2 immunoblotting (Figure 1D). The presence of Grb2 does not alter BCR-induced tyrosine phosphorylation of SLP-65 and PLC-γ2 (Figure 1E, left and right panels, respectively), indicating that Grb2-mediated inhibition of the Ca^{2+} response does not affect the primary Ca^{2+} initiation complex directly but may be part of an independent signaling module. Moreover, we found that the negative signaling role of Grb2 is not restricted to immature B cells like DT40, but also operates in B cells with a more mature phenotype, such as K46. As shown in Figure 2, already moderate overexpression of wild-type Grb2 in K46 B lymphoma cells strongly attenuates the prevailing influx of extracellular Ca^{2+} ions. These results establish an unexpected function of Grb2. It acts as a negative BCR effector molecule by regulating the signaling threshold for Ca^{2+} entry. Grb2, however, is expressed throughout the B cell lineage. It thus appears that mounting a complete biphased Ca^{2+} response requires inactivation of Grb2 by a molecule that may be missing or only weakly expressed in DT40 immature B cells.

Signaling Motifs of NTAL and Inducible Association with Grb2 Are Highly Conserved during Evolution

In search for an upstream regulator of Grb2, we concentrated on NTAL, which in mammalian B cells is reported to bind Grb2, positively affects the Ca^{2+} response (Brdicka et al., 2002; Janssen et al., 2003), and is expressed more strongly in mature than in immature mouse B cells purified from spleen and bone marrow, respectively (Figure 3A, lanes 1 and 2). Developmentally regulated expression of NTAL is also apparent from anti-NTAL immunoblotting of various mouse B cell lines representing mature, immature, pre-B, or pro-B cell stages (Figure 3B, lanes 1–6). In chicken DT40 cells, expression of NTAL is hardly detectable by immunoblot analysis (Figure 3B, lane 7), but *ntal* messenger RNA could be isolated from these cells by rt-PCR (accession number AY743659). The deduced amino acid sequence of avian NTAL revealed a protein of 198 amino acids with a calculated molecular mass of 22.4 kDa. Sequence comparison to murine and human NTAL shows that the previously described cytoplasmic peptide motifs for subcellular localization and signaling (Brdicka et al., 2002; Janssen et al., 2003) are also present in avian NTAL and are moreover conserved (Figure 4A). Four tyrosine-based Grb2 binding sites are especially common to NTAL proteins from different species. Indeed, the avian NTAL/Grb2 complex could be isolated by coimmunoprecipitation experiments with NTAL-positive DT40 transfectants (Figure 4B). The data indicate that NTAL expression and its phosphorylation-dependent interaction to Grb2 serves an important signaling function throughout evolution.

Lipid Raft-Anchored NTAL Is a Positive Regulator of Ca^{2+} Mobilization in B Cells

Next, we directly monitored the impact of NTAL on the BCR-induced Ca^{2+} response in various DT40 B cell lines. In this analysis, we also included DT40 mutants that were rendered completely deficient for NTAL protein expression by targeted gene disruption. The latter cells were generated (see the Experimental Procedures for details) and employed to exclude that residual expression of endogenous NTAL may dominantly affect signaling in our functional studies. As shown before, wild-type DT40 cells do mobilize Ca^{2+} from intracellular, but not extracellular, sources (Figure 5A, green line). Similarly,

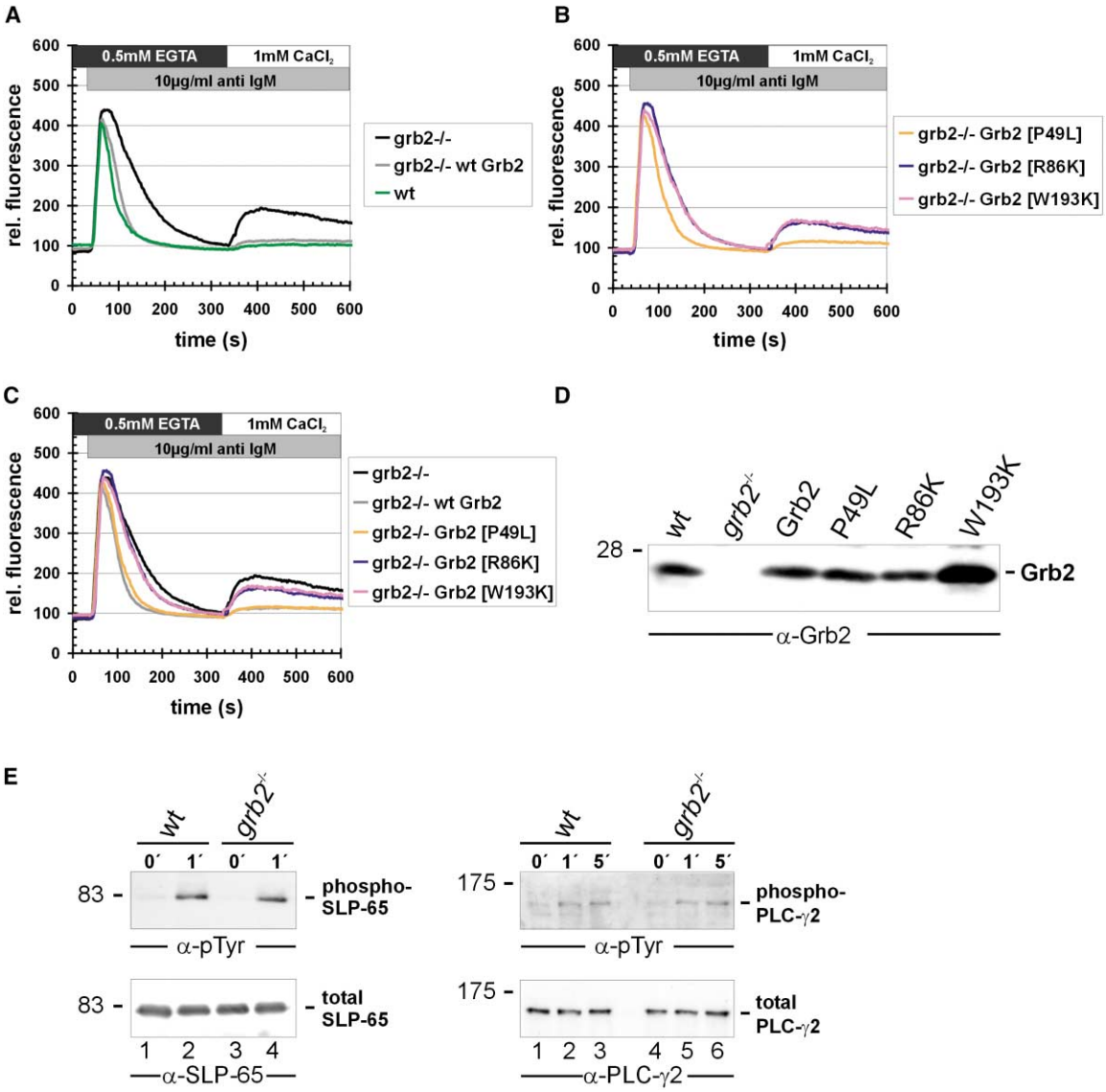

Figure 1. SLP-65- and PLC-γ2-Independent Effect of Grb2 and Its Protein Interaction Domains on the Ca^{2+} Response in DT40 B Cells

Upon loading of the indicated cells with 1.5 μM Fluo3/0.015% pluronic F127, BCR-induced release of intracellular Ca^{2+} was monitored for 6 min in the presence of 0.5 mM extracellular EGTA to remove Ca^{2+} ions from the medium. Subsequently, the extracellular Ca^{2+} concentration was restored to 1 mM, and the influx of Ca^{2+} through plasma membrane channels was recorded.

(A and B) Wild-type DT40 B cells (wt, green line), *grb2*$^{-/-}$ mutants (black line), and derivatives that upon retroviral gene transfer express wild-type Grb2 (gray line) or, in (B), express Grb2 variants with a nonfunctional N-terminal SH3 (orange), central SH2 (blue), or C-terminal SH3 domain (pink). Amino acid exchanges are described by using a single letter code and are P49L, R86K, or W193K.

(C) Overlay of (A) and (B). Equal cellular loading with the Ca^{2+}-sensitive dye was controlled by treatment of the cells with 100 nM ionomycin (data not shown).

(D) Grb2 protein expression was controlled in by anti-Grb2 immunoblot analysis of total cellular lysates.

(E) Wild-type and *grb2*$^{-/-}$ DT40 cells were left untreated or stimulated through their BCR for the indicated times, lysed, and subjected to immunoprecipitation with antibodies to SLP-65 (upper panel) or PLC-γ2 (lower panel). Purified proteins were analyzed by anti-phosphotyrosine (α-pTyr) immunoblotting (upper panels), and equal protein recovery and loading was confirmed by reprobing of the membranes with anti-SLP-65 and anti-PLC-γ2 antibodies (lower panels). The relative molecular mass of marker proteins is indicated on the left in kilodaltons.

ntal$^{-/-}$ clone N2-1 lacks an influx of extracellular Ca^{2+} (red line). In marked contrast, NTAL expression in DT40 cells leads to pronounced extracellular Ca^{2+} entry, and, also, the duration of intracellular Ca^{2+} release increases (blue line). The mobilization profile of NTAL-positive cells is almost identical to that of Grb2-negative DT40 cells (see above). The Ca^{2+} signaling function of NTAL is dependent on localization of the protein in lipid rafts, as exchanging the two critical cysteine residues within the NTAL palmitoylation motif to alanines (NTAL[C23/26A]) abrogates extracellular Ca^{2+} influx (Figure 5B). NTAL mutants, in which all four consensus Grb2 binding sites were rendered nonfunctional by tyrosine-to-phenylalanine exchanges (NTAL[4Y4F]), are also unable to pro-

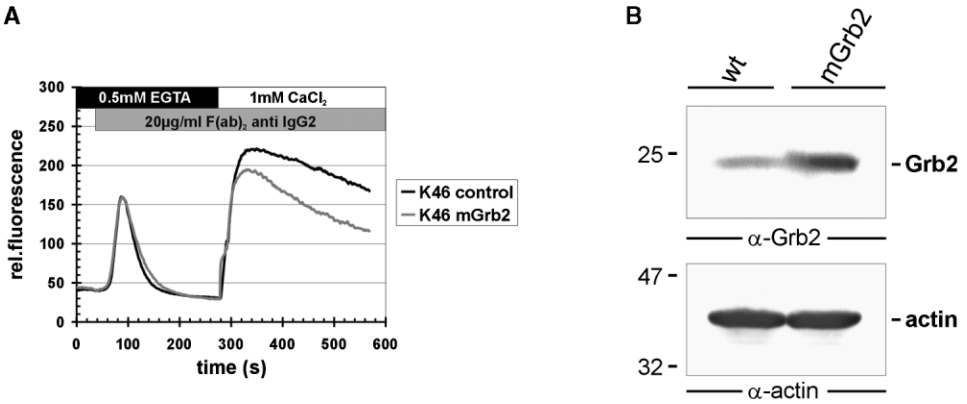

Figure 2. Effect of Grb2 on the Ca^{2+} Response in Mouse K46 B Cells

(A) The intra- and extracellular Ca^{2+} mobilization was recorded by flow cytometry (for details, see the legend to Figure 1) in wild-type K46 B cells and bulk populations of Grb2 infectants upon stimulation of the cells with $F(ab')_2$ fragments of anti-IgG2 antibodies (data represent the results of three independent infection experiments).

(B) Grb2 protein expression was analyzed by anti-Grb2 immunoblot analysis of total cellular lysates (upper panel). Anti-actin immunoblotting of the same membrane served as a loading control (lower panel). The relative molecular mass of marker proteins is indicated on the left in kilodaltons.

voke activation of extracellular Ca^{2+} influx (Figure 5B). Inactivation of distinct Grb2 binding sites by single Y-to-F exchanges reveals that the membrane-proximal binding site (Y95) is dispensable, while all three C-terminal binding sites (Y136, 155, and 184) contribute to maximal Ca^{2+} elevation. (Figure 5B). Interestingly, NTAL-related LAT as well as LAT lacking the PLC-γ binding site (LAT[Y132F]) can substitute NTAL function for Ca^{2+} mobilization in B cells (Figure 5B). Equal expression of NTAL and NTAL variants in the different transfectants was controlled by anti-HA immunoblotting (Figure 5C). Plasma membrane deposition of the NTAL[C23/26A] variant devoid of the lipid raft targeting signal was confirmed by confocal laser scan microscopy (Figure 5D). Our data show that raft-localized NTAL positively regulates the Ca^{2+} response in B cells and strongly suggest Grb2 as its downstream Ca^{2+} effector protein.

NTAL Regulates Ca^{2+} Flux in a Phosphorylation-Dependent Manner via Grb2 Binding

To further assess the correlation between the different NTAL peptide motifs and NTAL-regulated Ca^{2+} mobilization via Grb2 recruitment, we reintroduced single tyrosine residues into the NTAL[4Y4F] variant. Phosphorylation of the four add-back mutants was examined in parallel to that of wild-type NTAL, NTAL[C23/26A], NTAL[4Y4F], and human LAT by anti-phosphotyrosine immunoblotting (Figure 6A). Prominent tyrosine phosphorylation is observed for wild-type NTAL (lanes 1 and 2) and LAT (lanes 17 and 18), but not for the non-raft version NTAL[C23/26A] or the Grb2 binding-less variant NTAL[4Y4F] (lanes 5–8). Tyrosine 95 appears not to be a major phosphoacceptor site (lanes 9 and 10), which is consistent with its less prominent role for NTAL-mediated Ca^{2+} mobilization (see above). In contrast and in accordance with their important impact for Ca^{2+} mobilization, tyrosine phosphorylation is easily detectable at the single Grb2 binding sites Y136 and Y155 and is somewhat weaker at Y184 (lanes 11–16). Consistent with recent studies by Zhang and colleagues (Koonpaew et al., 2004), coimmunoprecipitation experiments shown in Figure 6B (lanes 1–6) demonstrated that phosphorylation-deficient NTAL variants (4Y4F and C23/26A) are incapable of recruiting wild-type Grb2 upon cellular

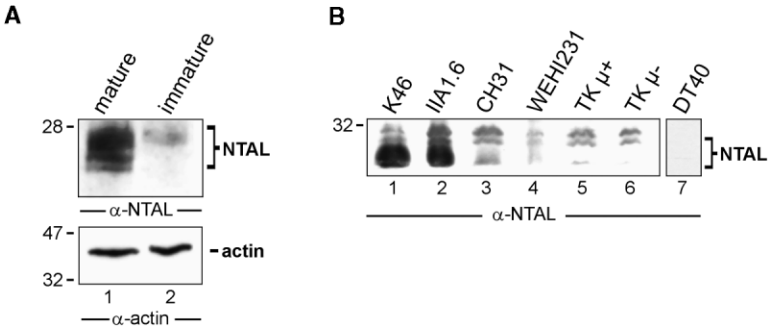

Figure 3. NTAL Protein Expression in B Cells Is Developmentally Regulated

(A) Mature (lane 1) and immature (lane 2) B cells of SV129 × C57/B6 wild-type mice were sorted from spleen and bone marrow, respectively (see the Experimental Procedures for details), and lysates were analyzed by immunoblotting with anti-NTAL (recognizing mouse and chicken proteins; also see Figure 4) or anti-actin antibodies (upper and lower panel, respectively).

(B) Total cellular lysates were prepared from approximately 1 × 10^6 cells of B cell lines representing different developmental stages and were subjected to anti-mouse (lanes 1–6) or anti-chicken NTAL (lane 7) immunoblotting. A mature B cell phenotype is represented by murine K46 (lane 1) and the A20-derivative IIA1.6 (lane 2); an immature phenotype is represented by murine CH31 (lane 3), WEHI-231 (lane 4), and chicken DT40 (lane 7), while the murine pre-B cell line TKμ^+ (lane 5) is a μm-positive variant of the pro-B cell line TKμ^- (lane 6). The relative molecular mass of marker proteins is indicated on the left in kilodaltons.

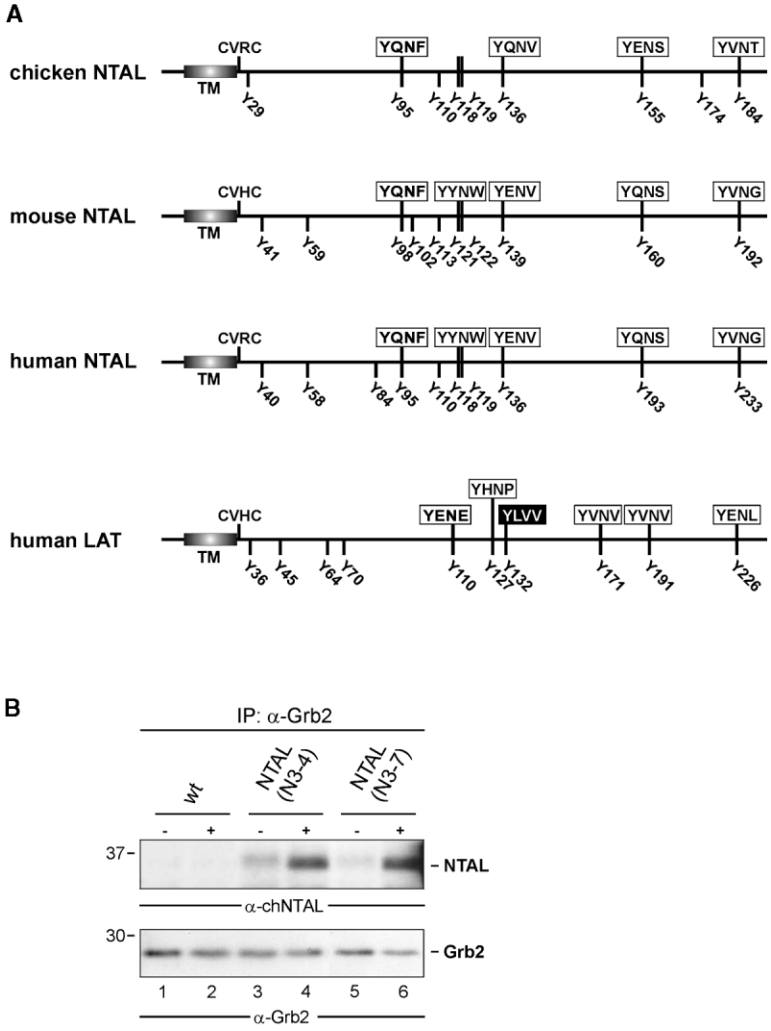

Figure 4. Evolutionary Conservation of NTAL Structure and Grb2 Complex Formation

(A) A schematic representation of NTAL from different species and a comparison to human LAT. TM, transmembrane regions. Cystein-containing palmitoylation motifs for raft localization and tyrosine-based Grb2 binding sites are indicated by using a single letter code. Note the unique PLC-γ binding site at amino acid position 132 in LAT. The avian *ntal* sequence is accessible under accession number AY743659.

(B) Anti-Grb2 immunoprecipitates derived from unstimulated (−) or BCR-stimulated (+) wild-type DT40 cells (lanes 1 and 2) and two independently obtained transfectants, N3-4 and N3-7, expressing avian NTAL (lanes 3–6) were analyzed by immunoblotting with antibodies to chicken NTAL and Grb2 (upper and lower panel, respectively).

stimulation. Conversely, an SH2 domain-defective Grb2 mutant (Grb2[R86K]) does not associate with HA-tagged NTAL (Figure 6B, lanes 7–10). Collectively, our genetic and biochemical data identify raft-localized NTAL as a phosphorylation-dependent linker between BCR stimulation and the regulation of Ca^{2+}-permeable channels in the plasma membrane. This function appears to be accomplished by SH2-mediated recruitment of Grb2 and concomitant elimination of its inhibitory activity, which is predominantly exerted by the C-terminal SH3 domain. The NTAL/Grb2 module appears to signal independently of the Ca^{2+} initiation complex, as expression of neither Grb2 (see Figure 1E) nor NTAL (data not shown) affects tyrosine phosphorylation of immediate early mediators of Ca^{2+} mobilization, i.e., SLP-65 and PLC-γ2.

Lipid Raft Localization of Grb2 Is Sufficient to Inactivate Its Negative Signaling Capacity

Our data so far suggest that BCR-induced translocation of Grb2 from the cytosol to the lipid raft plasma membrane fraction is a critical step to evoke full Ca^{2+} mobilization in mature B cells. To directly test for this hypothesis, Grb2 was equipped at its N terminus with the transmembrane region and cysteine-based acylation motif of NTAL (TM-Grb2). The TM-Grb2 fusion protein was expressed in Grb2-negative DT40 cells in the absence of NTAL and was detected in large amounts as a protein doublet of 35 and 36 kDa (Figure 7A, lane 3). In contrast to wild-type Grb2, the TM-Grb2 fusion is a permanent resident of membrane lipid rafts (Figure 7B). As controls, we analyzed in parallel our DT40 transfectants expressing either NTAL or LAT and found that these transmembrane adaptor proteins were recovered within the same fractions (no. 4–6) as chimeric Grb2 from TM-Grb2-positive cells (data not shown). Note that the appearance of multiple protein bands is a typical feature of lipid raft residents such as NTAL (see Figure 3) and LAT (Zhang et al., 1998b). Successful coimmunoprecipiation of TM-Grb2 with endogenous c-Cbl and binding to a SLP-65-derived peptide encompassing the Grb2 binding site indicate that at least the C-terminal SH3 domain of the fusion protein is properly folded and functional (data not shown). However, the Ca^{2+} response of TM-Grb2 transfectants is almost identical to that of Grb2-negative cells (Figure 7C, red and black line, respectively). Hence and in contrast to wild-type Grb2 (gray line), TM-Grb2 is incapable of suppressing Ca^{2+}

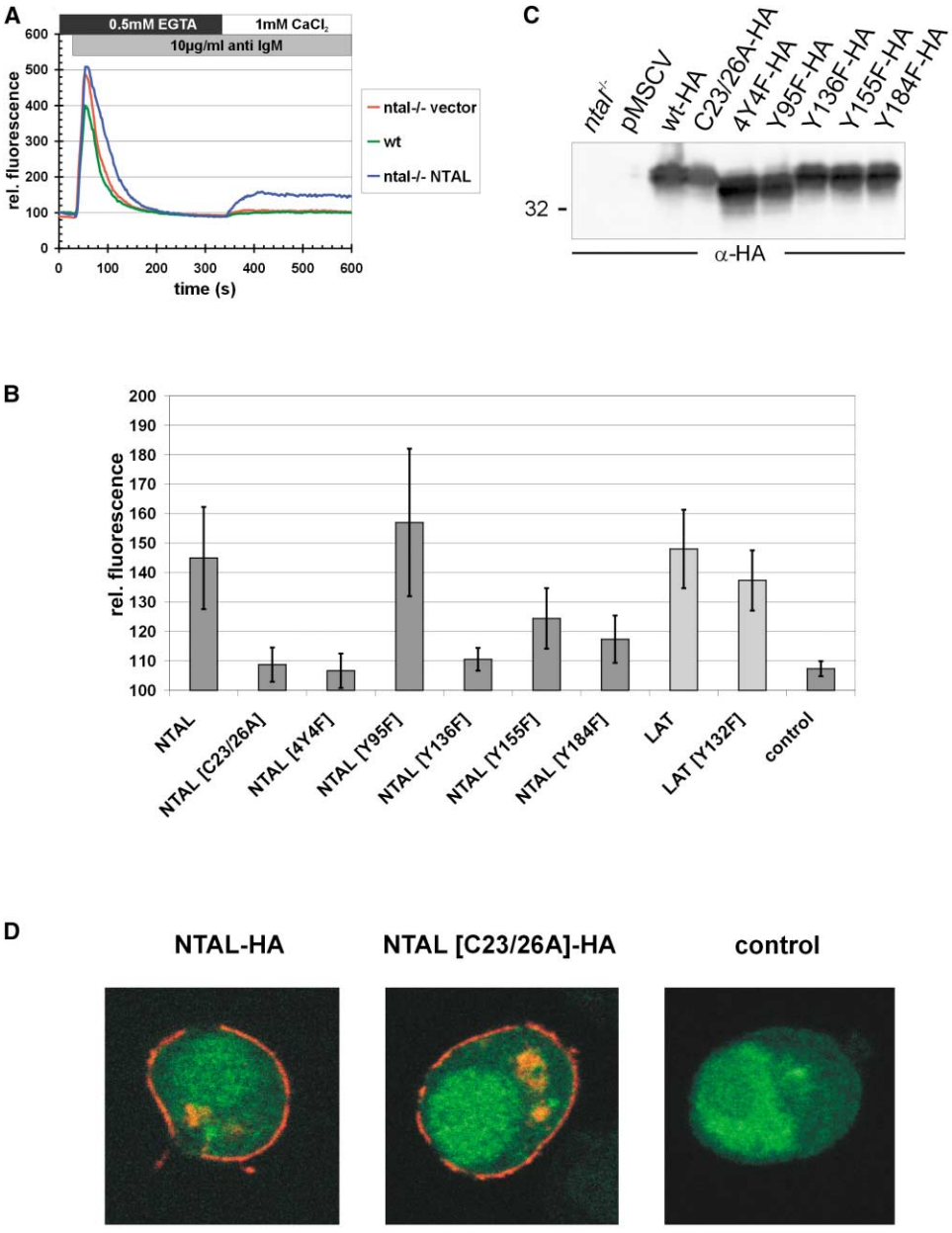

Figure 5. Effect of NTAL and Its Signaling Motifs on the Ca^{2+} Response

(A) The BCR-induced Ca^{2+} mobilization profiles were recorded by flow cytometry (for details, see Figure 1) for wild-type DT40 cells (green) and *ntal*$^{-/-}$ clone N2-1 transfected with either the empty pMSCV vector (red) or pMSCV containing a functional NTAL expression cassette (blue).

(B) A summary of intra- and extracellular Ca^{2+} mobilization in various DT40 derivatives expressing either HA-tagged wild-type NTAL, NTAL variants containing the indicated C-to-A or Y-to-F amino acid exchanges, wild-type LAT, or a LAT Y-to-F mutant lacking the PLC-γ2 binding site (LAT[Y132F]). "Control" indicates the Ca^{2+} response of empty vector control transfectants, and standard deviations ($n = 4 - 17$) were calculated for the maximal signal following addition of extracellular Ca^{2+}.

(C) Expression of HA-tagged NTAL proteins in the transfectants described in (B) was analyzed by anti-HA immunoblotting.

(D) Plasma membrane localization of the non-raft NTAL variant was monitored by confocal laser scanning microscopy of DT40 double transfectants expressing enhanced green fluorescent protein (EGFP) together with either HA-tagged wild-type NTAL (left picture) or HA-tagged C23/26A NTAL mutant (middle picture) stained in red with anti-HA plus Cy3-labeled secondary antibodies. As control, DT40 cells transfected with the EGFP expression construct and the empty NTAL vector backbone were analyzed in parallel (right picture).

flux from intra- or extracellular sources. This experiment shows that lipid raft targeting is sufficient to sequester Grb2 away from negative regulators of Ca^{2+} flux in the cytosol.

Discussion

Based on structural similarities, conservation of exon-intron boundaries, and preliminary functional studies,

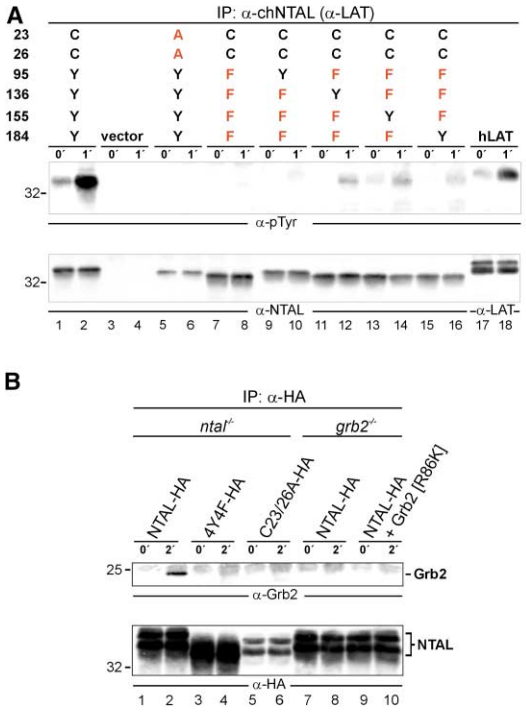

Figure 6. Tyrosine Phosphorylation and Grb2 Binding Analysis of NTAL and LAT

(A) Unstimulated (0′) and BCR-stimulated (1′) DT40 B cell transfectants expressing wild-type NTAL (lanes 1 and 2), no NTAL (empty vector control transfectants, lanes 3 and 4), indicated NTAL mutants (lanes 5–16), or wild-type human LAT (lanes 17 and 18) were subjected to immunoprecipitation with anti-NTAL (lanes 1–16) or anti-LAT (lanes 17 and 18) antibodies, and purified proteins were analyzed by immunoblotting with antibodies to phosphotyrosine (upper panel) or NTAL and LAT, respectively (lower panel). NTAL mutants lack either a functional raft targeting signal (C-to-A exchanges at positions 23 and 26, lanes 5 and 6) or one of the four Grb2 binding sites (Y-to-F exchanges at positions 95, 136, 155, or 184, respectively, lanes 7–16). Amino acid positions referring to the chicken NTAL protein (see Figure 4) are indicated on the left.

(B) Anti-HA precipitates prepared from *ntal*$^{-/-}$ (lanes 1–6) and *grb2*$^{-/-}$ (lanes 7–10) DT40 mutant cells reconstituted with HA-tagged versions of either wild-type NTAL (lanes 1 and 2 and lanes 7 and 8), 4Y4F NTAL (lanes 3 and 4), C23/26A NTAL (lanes 5 and 6), or wild-type NTAL plus SH2 domain-defective Grb2[R86K] (lanes 9 and 10) were analyzed by immunoblotting with antibodies to Grb2 and HA peptide flag (upper and lower panel, respectively). Note that background bands in the upper panel are derived from the light chain of the precipitating antibodies that almost comigrates with Grb2 and is detected by the secondary antibody. The relative molecular mass of marker proteins is indicated on the right in kDa.

the broadly expressed NTAL protein has been discussed to represent an analog of LAT (Brdicka et al., 2002; Janssen et al., 2003, 2004). Here, we have shown that indeed both proteins are intriguingly implicated in the Ca^{2+} response in antigen receptor-stimulated lymphocytes. However, the relative contributions of LAT and NTAL to Ca^{2+} elevation are distinct. While LAT has been reported to nucleate assembly of the Ca^{2+} initiation complex in lipid rafts, NTAL appears to play a pivotal role for entry of Ca^{2+} from the extracellular medium. The NTAL signaling mechanism requires no association to or altered phosphorylation of SLP-65, but a phosphorylation-dependent interaction with Grb2 at Y135, Y155, and Y184 is required. The inducible NTAL/Grb2 complex functions only when located in lipid rafts and appears to eliminate negative signaling by Grb2. This explains why the Ca^{2+} elevation profiles of NTAL/Grb2-positive and NTAL/Grb2-negative cells are identical. The importance of lipid raft localization is directly supported by our finding that the TM-Grb2 chimera has lost inhibitory signaling properties. Our Grb2 mutants further showed that Grb2 inhibits Ca^{2+} mobilization through its C-terminal SH3 domain and its central SH2 domain. Collectively, we conclude from these data that phosphorylated NTAL promotes Ca^{2+} mobilization by SH2-mediated relocalization of Grb2 in order to prevent its phosphorylation-dependent interaction with a yet to be characterized Ca^{2+} inhibitor. Thus, and in accordance with our mutational analysis, the three distally located Grb2 binding sites of NTAL act in concert to effectively compete with other Grb2 binding sites in phosphoproteins, which suppress Ca^{2+} mobilization. Interestingly, the same NTAL tyrosine residues are also critical for NTAL-mediated Ca^{2+} signaling in Jurkat T cell transfectants (Koonpaew et al., 2004). We found that in the DT40 cell system, NTAL expression is dispensable for BCR-induced activation of MAP kinases Erk2 and JNK1 (data not shown). This strongly suggests that NTAL and its interaction with Grb2 may not play a prominent role in the activation of small G proteins Ras and Rac, respectively. Indeed, recent data suggest that in lymphocytes, diacylglycerol-regulated RasGRP proteins rather than the Grb2/SOS module act as guanine nucleotide exchange factors to stimulate Ras (Dower et al., 2000; Oh-Hora et al., 2003; Quilliam et al., 2002).

How may Grb2 that is *not* associated with NTAL execute its inhibitory role on the Ca^{2+} response and how may recruitment to NTAL positively affect opening of Ca^{2+} plasma membrane channels? Different phosphatases have been implicated in signal downmodulation (for a review, see Veillette et al., 2002). The protein tyrosine phosphatase SHP-1 affects primarily intracellular Ca^{2+} release upon associating with inhibitory BCR coreceptors (Adachi et al., 2001; Ono et al., 1997). Four consensus binding motifs for the Grb2 SH2 domain are present in the SHP-1 C terminus. In our experiments, NTAL-regulated Ca^{2+} mobilization is not accompanied by altered protein tyrosine phosphorylation. However, the SHP-1/Grb2 module has been described to inhibit cytokine receptor signaling independently of SHP-1 phosphatase activity (Minoo et al., 2004). Thus, phosphorylated NTAL may effectively compete with SHP-1 for the Grb2 SH2 domain and thereby promote Ca^{2+} elevation from IP_3-sensitive stores. Upon exceeding a critical threshold level, this may trigger Ca^{2+} channels of the plasma membrane (Mori et al., 2002; Parekh and Penner, 1997). Another candidate effector protein of the NTAL/Grb2 complex is the lipid phosphatase SHIP. SHIP acts predominantly on extracellular Ca^{2+} flux, which is increased in B cells from *ship*$^{-/-}$ mice (Brauweiler et al., 2000; Kim et al., 1999; Ono et al., 1997). Moreover, SHIP has been reported to bind the C-terminal SH3 domain of Grb2 (Harmer and DeFranco, 1999; Jefferson et al., 1997; Kavanaugh et al., 1996), which we identified to be required for suppression of Ca^{2+} entry. So far, we cannot

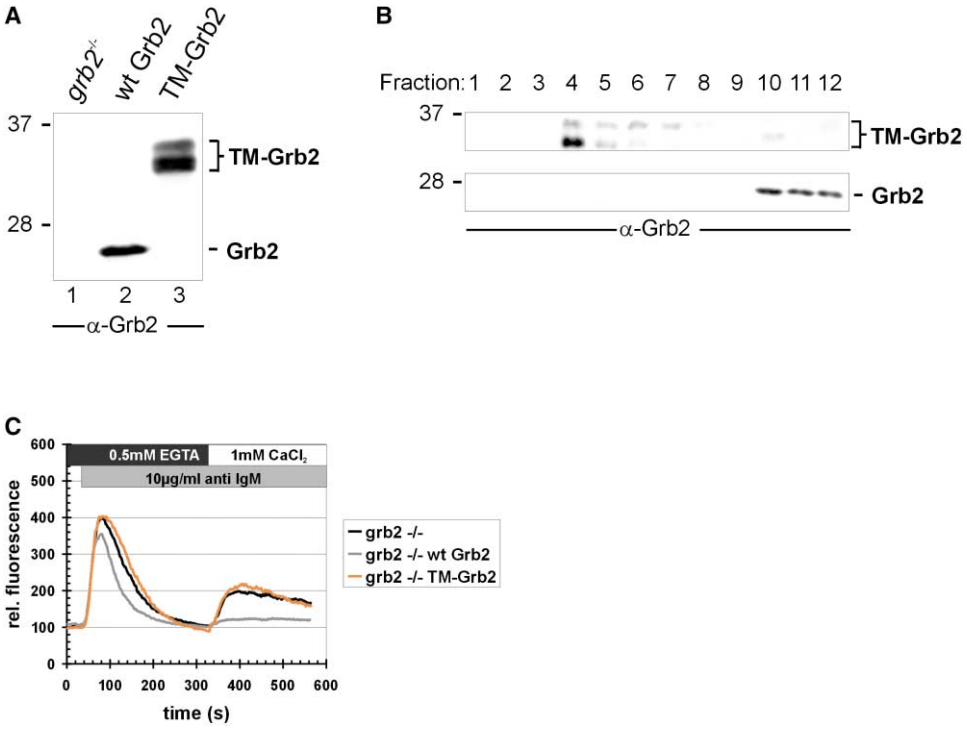

Figure 7. Subcellular Localization of Grb2 Regulates BCR-Induced Ca^{2+} Signaling

(A and B) (A) Grb2 protein expression and (B) its subcellular localization were analyzed by (A) anti-Grb2 immunoblotting of total cellular lysates and (B) isolated sucrose gradient fractions containing lipid rafts (Johmura et al., 2003) obtained from *grb2*$^{-/-}$ DT40 cells and transfectants reconstituted with either wild-type Grb2 or a membrane bound Grb2 fusion protein (TM-Grb2). Constitutive membrane anchoring of the latter is provided by the N-terminal presence of the first 94 amino acids of NTAL, which include the NTAL transmembrane region and lipid raft-targeting palmitoylation motif. The functionality of TM-Grb2 was verified by its successful coimmunopreciption with c-Cbl and its association to a biotinylated SLP-65 peptide encompassing the proline-rich binding motif (amino acids 202–214) for the C-terminal SH3 domain of Grb2 (data not shown).

(C) BCR-induced Ca^{2+} responses of the described DT40 cells were analyzed by flow cytometry (see the legend to Figure 1 for details).

distinguish between the two possible downstream effectors, which are, however, not necessarily mutually exclusive.

Based on our data, we propose a bidentated model for sustained elevation of BCR-induced Ca^{2+} mobilization. First, phosphorylated SLP-65 nucleates the formation of the Ca^{2+} initiation complex that suffices for activation of PLC-γ2. Second, phosphorylated NTAL inactivates inhibitory elements to prevent a premature stop of the Ca^{2+} signal. In support of this, SLP-65 and Grb2 independently translocate into lipid rafts (Johmura et al., 2003). While the first module is compiled by signaling proteins that are specific for the BCR, the second module involves effectors that are more ubiquitously expressed and may thus also operate in other receptor systems, for example, FcγRI or FcεRI, and, as shown for NTAL more recently, downstream of c-Kit on human mast cells (Tkaczyk et al., 2004). In the case of the TCR, both functions may be unified by LAT (Janssen et al., 2004), which assembles the Ca^{2+} initiation complex, has multiple Grb2 binding sites, and, as shown here, can indeed substitute NTAL function for prolonged Ca^{2+} flux. Thus, in cell types that are positive for the endogenous expression of *both* transmembrane adaptors, loss of one of the two may be functionally compensated by the other. Clearly, additional work is needed to verify our model. The NTAL function, however, appears to be evolutionary conserved from chicken to man and operates in B cells representing different developmental stages. It is noteworthy that there is a direct correlation between NTAL expression levels and the increasing capability of developing B cells to efficiently mount Ca^{2+} flux across the plasma membrane (Koncz et al., 2002; and this publication). Immature B cells with a low level of NTAL expression show weak Ca^{2+} entry, while mature B cells with high NTAL levels induce robust Ca^{2+} entry, leading to a sustained elevation of the intracellular Ca^{2+} concentration. The kinetics of Ca^{2+} signaling is known to control activation of distinct transcription factors implicated in cell fate decisions. Given the fundamentally different biological responses of immature and mature B cells to BCR stimulation (i.e., apoptosis versus activation), it is tempting to speculate that NTAL/Grb2-regulated Ca^{2+} signaling may be involved in tolerance induction.

Experimental Procedures

Cells, Abs, and Reagents

Chicken DT40 cells were cultured in RPMI 1640 (PAA) supplemented with 10% FCS (PAA, Invitrogen), 1% chicken serum (PAA, Sigma), 3 mM L-glutamine (PAA), 50 μM β-ME (Sigma), and antibiotics. Murine, K46, the A20-derivative IIA1.6 (Vandenherikoudijk et al., 1994), WEHI-231, CH31 (Pennell et al., 1985), and the AMuLV-trans-

formed cell lines TKμ^+ and TKμ^- (kindly provided by Dr. H.M. Jäck, Erlangen, Germany) were cultured in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 2 mM pyruvate, 50 μM β-ME, and antibiotics. Grb2-deficient DT40 cells are described in (Hashimoto et al., 1998). BCR stimulation of DT40 and K46 B cells was performed for the indicated times by using 10 μg/ml anti-chicken IgM (M4, Southern Biotechnology) or 20 μg/ml F(ab′)$_2$ fragments of goat anti-mouse IgG2 (Jackson Immuno Research Laboratories), respectively. For isolation of primary spleenic B cells from wild-type SV129 $\times$ C57/B6 mice, the Miltenyi Biotec isolation kit and AutoMACS were used. Mature B cell populations were tested for being B220$^+$/CD3$^-$/CD49b$^-$. Bone marrow cells were sorted with a FACSDiva (Becton Dickenson) gated on IgM$^+$/B220$^+$/IgD$^-$. Fluorescinated antibodies (FITC, PE, or Cy5) to CD3, CD11b, B220, IgM, IgD, and CD49b were purchased from BD Pharmingen. All cells were solubilized in NP-40 lysis buffer (10 mM Tris/HCl [pH7.5], 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM Na_2MoO_4, 1% NP40, protease inhibitors P 2714 [Sigma]). For isolation of lipid-raft-localized NTAL and NTAL-containing protein complexes, cells were solubilized by 1% laurylmaltoside lysis buffer (n-dodecyl β-D-maltoside, Sigma). Lipid rafts were isolated from cell lysates in Triton X-100 (Sigma) by sucrose density gradient ultracentrifugation as described previously (Johmura et al., 2003). Antibodies to NTAL and SLP-65 were generated by immunizing rabbits with bacterially expressed glutathione S-transferase (GST) fusion proteins encompassing the C-terminal 49 amino acids of either chicken or murine NTAL and amino acids 79–201 of chicken SLP-65 (Ishiai et al., 1999a), respectively. Antibodies to Grb2 (C-23 for immunoprecipitation), PLC-γ2 (Q-20), LAT (FL-233), and the hemaglutinin (HA) peptide flag (Y11 for immunoprecipitation) were purchased from Santa Cruz Biotechnology. The mAb anti-Grb2 3F2 and mAb rat anti-HA (both used for immunoblotting) and anti-pTyr (4G10) were purchased from Upstate Biotechnology and Roche, respectively.

Expression Constructs

Wild-type chicken and murine Grb2 cDNA and wild-type human LAT cDNA were cloned into the pCRII-TOPO vector. Using QuikChange, chicken Grb2 proline 49 was substituted for leucine, arginine 86 was substitutes for lysine, and tryptophane 193 was substituted for lysine to create SH2 or SH3 domain mutant constructs. A PCR-generated chicken cDNA fragment encoding the transmembraneous form of Grb2 encompasses NTAL codons 1–282 and full-length Grb2 at the 3′ end. Human LAT tyrosine 132 was replaced by phenylalanine to generate a PLC-γ binding site mutant construct. All cDNAs were directly cloned into pMSCVpuro (BD Biosciences, Clontech). Alternatively, the chicken cDNA encoding the Grb2 R86K mutant was cloned into p5N-M-IREShph (kindly provided by Dr. M Jücker). Wild-type NTAL cDNA was cloned into the pApuro expression vector (Takata et al., 1994) to generate NTAL expression constructs. A PCR-generated expression cassette encoding C-terminally HA-tagged chicken NTAL was inserted into pENTR/SD/D-TOPO (Invitrogen). Using QuikChange (Stratagene), the cysteine residues (23 and 26) were replaced by alanine to generate a nonraft mutant construct, and the tyrosine residues (95, 136, 155, and 184) were substituted for phenylalanine to create Grb2 SH2 domain binding site mutant constructs. The pMSCVpuro expression vector (Stratagene) was converted to a destination vector by ligating a Gateway conversion cassette (Invitrogen) into the HpaI site. Using the Gateway Cloning Technology (Invitrogen), wild-type or mutant NTAL cDNAs were inserted into the pMSCVpuro to generate appropriate expression vectors. All pApuro-based expression constructs were electroporated (550 V, 25 μF), and transfectants were selected in the presence of puromycin (0.5 μg/ml). All pMSCVpuro- and p5N-M-IREShph-based expression constructs were transduced retrovirally (see below). Expression of introduced cDNAs was confirmed by Western blot analysis.

Retroviral Transduction

Retroviral supernatants were produced in the helper cell line Plat-E (kindly provided by Dr. Toshio Kitamura, Tokyo, Japan [Morita et al., 2000]), and transduction was performed by using the Fugene transfection reagent (Roche). The MMLV was pseudotyped with VSV-G. For transduction, 10^6 cells were incubated for 20 hr with retroviral supernatant containing 3 μg/ml Polybrene (Sigma). The infection step was repeated for another 8 hr. Subsequently, cells were selected with 1 μg/ml (DT40) or 10 μg/ml (K46) puromycin (Sigma) or 1.2 mg/ml hygromycin (Invivogen).

Calcium Measurements

A total of 10^6 DT40 cells were loaded in 700 μl RPMI containing 5% FCS, 1.5 μM Fluo3-AM (Molecular Probes), and 0.015% Pluronic F127 (Molecular Probes) at 30°C for 25 min. K46 cells were loaded with 1 μM Fluo3-AM and 0.02% Pluronic F127. Subsequently, the cell suspension was diluted 2-fold with RPMI 10% FCS and were incubated for 10 min at 37°C. Cells were washed twice with Krebs Ringer solution composed of 10 mM HEPES (pH 7.0), 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose. Prior to measurements, cells were resuspended in Krebs Ringer solution that lacked $CaCl_2$ and contained 0.5 mM EGTA. After 30 s, DT40 and K46 cells were stimulated with 10 μg/ml M4 or 20 μg/ml F(ab′)$_2$ goat anti-mouse IgG2, respectively. The extracellular Ca^{2+} level was restored to 1 mM after 6 min. The changes in fluorescence intensity of Fluo3 were monitored on a FACSCalibur cytometer (Becton Dickinson). Equal loading of the samples was controlled by treatment with 100 nM ionomycin (Sigma).

Confocal Laser Scanning Microscopy

A total of 3 $\times$ 10^6 DT40 cells were resuspended in PBS and were seeded onto coverslips. After 30 min of incubation at 37°C, cells were fixed with PBS containing 1% PFA. Reactive aldehyde groups were saturated with PBS containing 100 mM glycine, and plasma membranes were permeabilized with PBS containing 0.2% Triton X-100. All of the following incubations were performed in PBS 1.5% BSA. Upon blocking for 30 min, cells were incubated with rat anti-HA (0.7 μg/ml) for 1 hr at 4°C, washed three times, stained with Cy3-labeled donkey anti-rat (Jackson ImmunoResearch Laboratories, 1:400 dilution), and mounted after three washing steps with Vectashield (Vector Labs)/glycerol (1:1) containing 2% DABCO (Sigma). Samples were examined on a Leica TCS SP2 confocal laser scanning microscope. For EGFP excitation and emission, wavelengths were 488 and 490–510 nm, respectively. For Cy3, the excitation wavelength was 543 nm, and emission was recorded at 550–570 nm.

Generation of NTAL-Deficient DT40 Cells

Based on murine *ntal* sequences, a partial cDNA sequence of chicken *ntal* was obtained by searching the expressed sequence tag (EST) database of Boardman et al. (Boardman et al., 2002) and was subsequently used to identify the *ntal* sequence in the genomic database. The GeneBank accession number of the chicken NTAL locus is AC091726. Full-length chicken NTAL cDNA was obtained by RT-PCR with RNA from DT40 cells by using 5′-ATGTGGCTGGG CCATGGCGCAG-3′ and 5′-CTTCACTTTGACAGCACGACG-3′ as forward and reverse primers, respectively. Genomic clones of NTAL were obtained by using the forward and reverse primers 5′-GCT CTCCATGCCTGGATTTCTCCTGG-3′ and 5′-CACACATCACCCTG GGTCACTTCTAC-3′, respectively, and DT40 genomic DNA was used as a template. The targeting vectors, pNTAL-neo and pNTAL-hisD, were constructed by replacing the genomic fragment containing exons corresponding to chicken NTAL amino acid residues 1–142 with neo and hisD cassettes. These cassettes were flanked by 3.2 and 2.3 kb of genomic sequence on the 5′ and 3′ sides, respectively. Targeting vectors were transfected by electroporation at 550 V, 25 μF. G418 was used at 2 mg/ml for selection of transfectants, and homologous recombination was identified by PCR and Southern blot analysis. The pNTAL-hisD was introduced into the neotargeted clone N2 and was selected with both G418 (2 mg/ml) and histidiol (1 mg/ml), generating the NTAL-deficient clones N2-1, N2-15, N2-20, and N2-23. Introduction of a single copy of each targeting vector was verified by reprobing the blots with internal neo or hisD probe.

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Accession Numbers

The GenBank accession number for the chicken NTAL cDNA sequence is AY743659.