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The N Terminus of the Non-T Cell Activation Linker (NTAL) Confers Inhibitory Effects on Pre-B Cell Differentiation[1]

Sebastian Herzog and Hassan Jumaa[2]

SLP-65 and the linker for activation of T cells (LAT) are central adaptor proteins that link the activated pre-BCR to downstream events in pre-B cells. Recently, a new transmembrane adaptor called NTAL/LAB/LAT2 (hereafter called NTAL for non-T cell activation linker) with striking functional and structural similarity to LAT has been identified in B cells. In this study, we compare the function of NTAL and LAT in pre-BCR signaling and show that, in contrast to LAT, NTAL does not induce pre-BCR down-regulation, calcium flux, or pre-B cell differentiation. To test whether differences between NTAL-mediated and LAT-mediated signaling are caused by the missing phospholipase C (PLC)-γ binding motif in NTAL, we inserted the PLC-γ1/2 binding motif of LAT into NTAL. This insertion rendered NTAL capable of activating pre-BCR down-regulation and calcium flux. Unexpectedly however, the ability of NTAL to induce calcium flux was not sufficient to promote pre-B cell differentiation, suggesting that the PLC-γ binding motif has only partial effects on NTAL-mediated pre-BCR signaling. By generating chimeric swap mutants, we identified the N terminus of NTAL as an inhibitory domain that prevents pre-B cell differentiation while allowing pre-BCR down-regulation and receptor-mediated calcium flux. Our data suggest that, in addition to the missing PLC-γ1/2 binding motif, the N terminus is responsible for the functional differences between NTAL and LAT in pre-B cells. *The Journal of Immunology,* **2007, 178: 2336–2343.**

B cell development takes place in distinct stages defined by the expression of various surface markers and the recombination status of the immunoreceptor H chain and L chain genes *HC* and *LC* (1–4). An important checkpoint in this process is the pre-B cell stage, which is characterized by the expression of a functional pre-BCR comprising a successfully rearranged μ H chain, the surrogate L chain components λ5/VpreB, and the signaling subunits Ig-α/Ig-β (5, 6). Pre-BCR signals promote survival, induce cell expansion, and allow differentiation into small pre-B cells in which the rearrangement of the *LC* gene segments takes place (7). Consecutive assembly of H and L chain genes results in expression of the BCR, which characterizes the immature B cell stage. Immature B cells leave the bone marrow and migrate into the spleen where they complete maturation (8). Providing both developmental and survival signals, a signaling-competent BCR is indispensable for the generation and maintenance of a mature B cell pool (9, 10).

Engagement of the BCR results in the activation of the tyrosine kinase Syk, which is then able to phosphorylate substrate proteins such as the adaptor SLP-65 (Src homology (SH)2 domain-containing leukocyte protein of 65 kDa) (11, 12). Phosphorylated SLP-65 provides docking sites for several SH2 domain-containing signaling molecules and links Syk and the Tec family kinase Btk to phospholipase C (PLC)[3] γ2 activation (13, 14). PLC-γ2 generates the second messengers inositol 1,4,5-trisphosphate and diacylglycerol, resulting in the elevation of intracellular calcium and the activation of downstream effectors including protein kinase C and nuclear transcription factors NF-AT and NF-κB (15). Although these signaling events have been studied mainly in the context of the BCR, a corresponding pre-BCR signalosome has been postulated (16).

The central role of SLP-65 in both pre-BCR and BCR signaling is demonstrated in mice deficient for this adaptor (17–19). Targeted deletion of SLP-65 results in partial blocks at the pre-B and the immature B cell stages and a reduction of mature B cells in the periphery. Because B cell development is not completely abrogated, an additional SLP-65-independent pre-BCR signaling pathway has been proposed. Indeed, recent data indicate a role for the T cell adaptor LAT (linker for activation of T cells) in pre-B cells where it seems to organize a complex that recruits PLC-γ1/2 to the pre-BCR and enables the generation of the second messenger, inositol 1,4,5-trisphosphate (20, 21). Reconstitution of SLP-65 or LAT in SLP-65$^{-/-}$LAT$^{-/-}$ pre-B cells restores calcium mobilization, induces pre-BCR down-regulation, and allows differentiation to immature B cells. In vivo, the block at the pre-B cell transition in SLP-65$^{-/-}$LAT$^{-/-}$ mice is even more severe compared with SLP-65$^{-/-}$ mice, suggesting that pre-BCR signaling mediated by LAT/SLP-76 can rescue SLP-65$^{-/-}$ pre-B cells (20).

With LAT being the central adaptor in T cells, much speculation has been devoted to a possible LAT-like molecule in B cells (22–24). This led to the identification of the non-T cell activation linker (NTAL; also known as LAT2 or LAB, linker for activation of B cells), a novel transmembrane adaptor molecule that is expressed in B cells, NK cells, mast cells, and monocytes (25, 26). Resembling LAT in its general organization, NTAL consists of a short extracellular N terminus, a single transmembrane domain followed by a palmitoylation site, and a large cytoplasmic tail that contains several tyrosines. Upon receptor engagement, NTAL becomes phosphorylated and organizes a downstream signaling complex involving cytoplasmatic molecules such as Grb2, Gab1, or Sos1. In contrast to LAT, NTAL lacks a binding motif for PLC-γ1/2, and no signal-induced interaction of NTAL with either SLP-76 or SLP-65 has been reported.

Reconstitution of NTAL in LAT-deficient Jurkat cells only partially rescues calcium mobilization and ERK1/2 activation upon

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[3] Abbreviations used in this paper: PLC, phospholipase C; LAT, linker for activation of T cells; NTAL, non-T cell activation linker; PLCγBM, PLC-γ binding motif; TL, tailless; WT, wild type.

TCR-stimulation (26). However, LAT$^{-/-}$ thymocytes reconstituted with NTAL are able to overcome their developmental block and develop normally into CD4$^+$ or CD8$^+$ T cells, indicating that LAT and NTAL are at least to some degree functionally redundant (25). In mast cells, NTAL seems to be a negative regulator, as disruption of the *NTAL* gene in mice renders mast cells hyperresponsive to FcεRI-stimulation in terms of calcium mobilization, ERK activation, and degranulation (27, 28). A positive role of NTAL was reported in DT40 cells, where sequestering Grb2 by tyrosine-phosphorylated NTAL enhances the calcium response upon receptor stimulation (29). In mice, deletion of NTAL alone or combined with LAT only minimally effects B cell development and function (30).

To date, the precise function of NTAL in B cells remains unclear. To answer the questions of whether NTAL is involved in pre-B cell development and whether NTAL is a functional LAT equivalent in B cells, we compared the effects of NTAL and LAT on pre-BCR signaling and pre-B cell differentiation in vitro.

Materials and Methods

Cells and cell culture

The pre-B cell line *Oct* and the SLP-65$^{-/-}$/LAT$^{-/-}$ pre-B cell line derived from the bone marrow of a SLP-65$^{-/-}$/LAT$^{-/-}$ mouse were described previously (20). Primary B cells were isolated by the preparation of cell suspensions from the bone marrow of 7–10 wk old BALB/c (wild type (WT)) and SLP-65$^{-/-}$ mice. The pre-B cell lines *Oct* and SLP65$^{-/-}$/LAT$^{-/-}$ as well as the bone marrow-derived WT and SLP-65$^{-/-}$ cells were cultured in Iscove's medium containing 10% inactivated FCS (Vitromex), 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen Life Technologies), 5×10^{-5} M 2-ME, and IL-7. IL-7 was obtained using supernatant of J558L cells stably transfected with a vector encoding murine IL-7 and was used in excess.

Retroviral constructs and transduction

The retroviral vectors pMOWS-LAT-GFP and pMOWS-GFP-SLP-65 were described previously (25, 26). The retroviral vector pMOWS-NTAL-GFP was generated by amplifying the coding region of murine NTAL from a murine spleen cDNA library using 5′-CCTTAATTAAATGAGTGCCG AGCTGGAG-3′ as the forward and 5′0CCTCTAGAGATGTTCTCTGCT GCGGC-3′ as the reverse primer. The retroviral vector pMOWS-NTAL/PLCγBM was generated through directed mutagenesis by sequential PCR steps using pMOWS-NTAL-GFP as a template, 5′-GCCTATCTAGTCGT CCTCCCTACA-3′ as the forward and 5′-TGTAGGGAGGACGACTAG ATAGGC-3′ as the reverse mutagenesis primer, and 5′-TCACTCCTTC TCTAGGCGC-3′ and 5′-GTAATCCAGAGGTTGATTG-3′ as primers flanking the cloning site. The PCR fragment encoding NTAL/PLCγBM-GFP was then subcloned into the retroviral vector pMOWS-NTAL-GFP and replaced the NTAL-GFP fragment. The pMIG-based vector pMIG/CD8 was produced by amplification of the region encoding murine CD8α lacking the intracellular tail from a murine thymus cDNA library using 5′-CACCATGGCCTCACCGTTGACCCG-3′ as the forward and 5′-GC TGTCGACTCCTCTAGTAGC-3′ as the reverse primer. The PCR fragment was cut with *Nco*I and *Acc*I and cloned into a *Nco*I/*Acc*I-digested pMIG (provided by W. S. Pear, Abramson Family Cancer Research Institute, University of Philadelphia, PA) to replace the GFP coding region. pMIG/CD8-NTAL and pMIG/CD8-LAT were generated by cloning fragments encoding full-length murine NTAL and LAT into the multiple cloning site of pMIG/CD8. For the generation of the LAT/NTAL swapping constructs, respective LAT and NTAL regions were amplified by PCR using pMOWS-LAT-GFP or pMOWS-NTAL-GFP as templates (primers and the swap mutants are available upon request). The respective fragments were cut and cloned into pMOWS. All vectors used in this study were confirmed by sequencing and Western blot analysis.

Retroviral transduction was performed as described previously (25, 26). Transfection of the Phoenix retroviral producer cell line using GeneJuice (Novagen) was done according to the manufacturer's instructions. For transduction, pre-B cells were mixed with retroviral supernatants and centrifuged at 1800 rpm at 37°C for 3 h. Ca^{2+} response and down-regulation of pre-BCR were analyzed 24–36 h posttransduction, and the differentiation assay was performed 4 days posttransduction.

Stimulation of cells and Western blotting

Transduced SLP-65$^{-/-}$LAT$^{-/-}$ cells enriched by selection with puromycin (1 μg/ml) were harvested 5 days after retroviral infection. Stimulation of 2×10^7 cells was performed for 2 min at 37°C using 20 μg/ml anti-μ Ab (Southern Biotechnology). Cell pellets were lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 1% *n*-octyl-β-D-glucopyranoside, 137.5 mM NaCl, 1% glycerol, 1 mM sodium orthovanadate, and 0.5 mM EDTA (pH 8)) supplemented with a protease inhibitor mixture (Sigma-Aldrich). GFP fusion proteins were precipitated using mouse anti-GFP Abs (Roche) and protein-G Sepharose beads (Amersham Biosciences). Purified samples were washed, boiled for 10 min, and subjected to SDS-PAGE and Western blot analysis. GFP fusions were detected by rabbit anti-GFP (Clontech) and mouse anti-phosphotyrosine (4G10, Upstate Biotechnology; either nonmodified or biotinylated) Abs, followed by incubation with HRP-coupled anti-rabbit and anti-mouse Abs or HRP-coupled streptavidin (Pierce), respectively. Immobilized HRP was visualized using the ECL system (Amersham Biosciences).

RT-PCR analysis

Total RNA was isolated from the *Oct* pre-B cell line or from bone marrow-derived WT and SLP-65$^{-/-}$ B cells after 5 days of culture in IL-7-supplemented medium. One microgram of RNA was used to perform cDNA synthesis as described (31). For the amplification of *HPRT*, 5′-GCTGGT GAAAAGGACCTCT-3′ as the forward and 5′-CACAGGACTAGAAC ACCTGC-3′ as the reverse primer were used. *NTAL* was amplified using 5′-AGTCCCCTAGGTACCAGAA-3′ as the forward and 5′-GGCCACAT CCCCATTCAC-3′ as the reverse primer, and *LAT* was amplified using 5′-ATGGAAGCAGACGCCTTGA-3′ as the forward and 5′-CCCCAGCA AGTCCAGTTTC-3′ as the reverse primer.

Flow cytometry

Aliquots of transduced cells (1×10^6) were stained for FACS analysis (FACSCalibur and LSRII; BD Biosciences) using Cy5-anti-IgM (μ-chain specific; Southern Biotechnology), PE-anti-CD8α (Southern Biotechnology), and biotin-anti-κ (Southern Biotechnology) Abs and streptavidin-Cy5 (Dianova).

Measurement of Ca^{2+} release

Transiently transfected cells (1×10^6) were incubated with 5 μg/ml indo-1 acetoxymethyl ester (Molecular Probes) and 0.5 μg/ml pluronic F-127 (Molecular Probes) in Iscove's medium supplemented with 1% FCS (Vitromex) at 37°C for 45 min. Afterward, the cell pellets were resuspended in Iscove's medium plus 10% FCS and kept on ice. The fluorometric analysis was performed at 37°C and the Ca^{2+} response was induced by the addition of 20 μg/ml goat anti-mouse μ (Southern Biotechnology). Events were recorded for 6–8 min after stimulation.

Results

NTAL is expressed in pre-B cells

The adaptor protein LAT is expressed in pre-B cells and is, similar to SLP-65, involved in connecting the pre-BCR to downstream signaling events that regulate pre-B cell differentiation (20). To test whether the newly identified adaptor protein NTAL is expressed in early B cell stages, we performed RT-PCR analyses to examine the distribution of NTAL mRNA and LAT mRNA in pre-B cells using cDNA from a pre-B cell line (*Oct*) and from primary pre-B cells cultures derived from the bone marrow of wild-type (WT) or SLP-65$^{-/-}$ mice. As previously reported (20), LAT was highly expressed in primary bone marrow-derived WT and SLP-65$^{-/-}$ pre-B cells (Fig. 1*A*). NTAL mRNA was distributed in a similar pattern as LAT, with high levels in both WT and SLP-65$^{-/-}$ bone marrow-derived pre-B cells. The amount of NTAL transcripts in the pre-B cell line *Oct* was reduced compared with that in bone marrow-derived pre-B cells.

NTAL fails to induce pre-BCR down-regulation and calcium mobilization in pre-B cells

Previous reports showed that the expression of either GFP-SLP-65 or LAT-GFP fusions in SLP-65$^{-/-}$/LAT$^{-/-}$ pre-B cells led to down-regulation of the pre-BCR, thereby limiting the proliferation of pre-B cells (20, 32). To test whether NTAL plays a role in pre-BCR

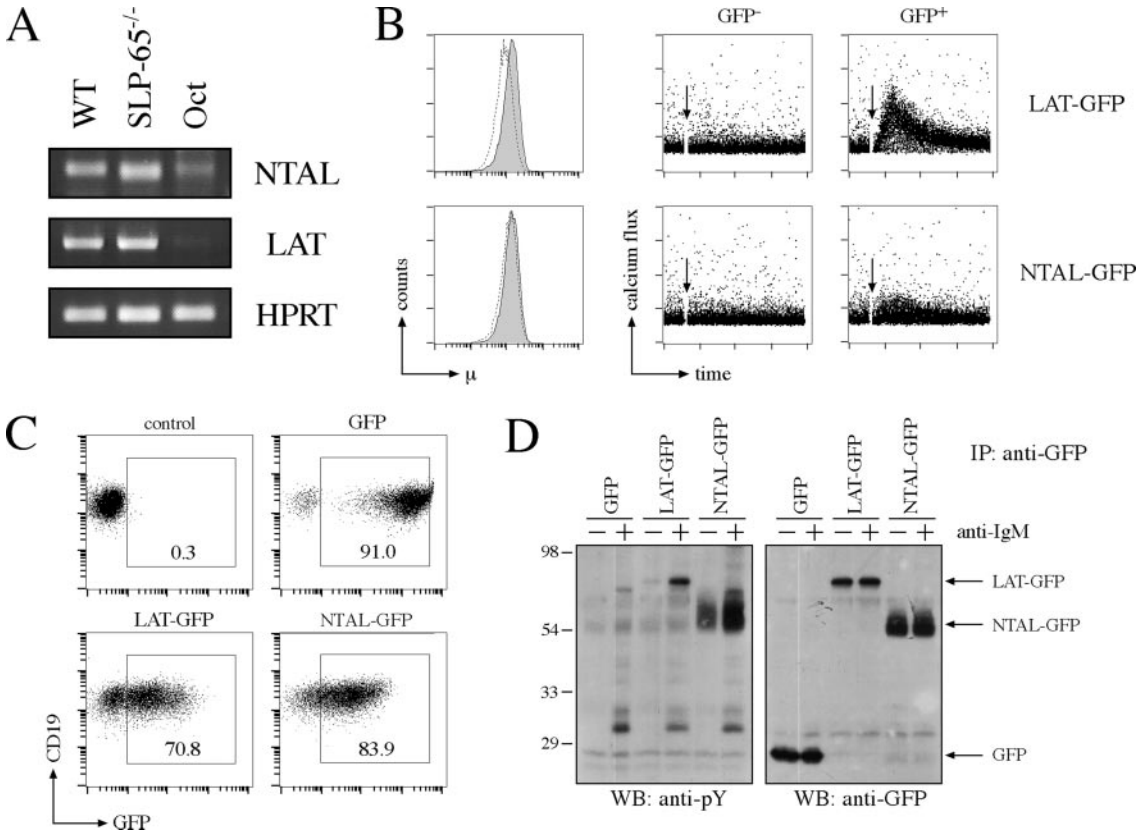

FIGURE 1. Expression of LAT and NTAL in WT and SLP-65$^{-/-}$ pre-B cells. *A*, RT-PCR analysis for LAT and NTAL expression in pre-B cells. cDNA was generated from total RNA of the *Oct* pre-B cell line or from that of bone marrow-derived WT and SLP-65$^{-/-}$ pre-B cells. The PCR was performed using specific primers for amplification of either LAT or NTAL transcripts. The housekeeping gene *HPRT* (hypoxanthine phosphoribosyltransferase) was used as a control. *B*, FACS analysis comparing pre-BCR down-regulation and calcium mobilization induced by LAT and NTAL. *Oct* pre-B cells were transduced with constructs encoding LAT-GFP or NTAL-GFP and analyzed by FACS after 24 h. Down-regulation of the pre-BCR is indicated by histograms comparing μ expression of nontransduced GFP$^-$ cells (filled histogram) with GFP$^+$ cells (dotted line). For calcium mobilization, SLP-65$^{-/-}$/LAT$^{-/-}$ pre-B cells were transduced with LAT-GFP or NTAL-GFP, stimulated with anti-μ after 24 h, and analyzed by FACS. Calcium flux profiles of the GFP$^-$ and the GFP$^+$ population within one sample are shown. Arrows mark the time points when anti-μ Ab was added. *C*, SLP-65$^{-/-}$/LAT$^{-/-}$ cells were transduced with GFP, LAT-GFP, or NTAL-GFP and selected by the addition of puromycin for 5 days. Selected cells were stimulated for 2 min with anti-μ or left unstimulated as a control. *D*, Phosphorylation of LAT and NTAL after pre-BCR stimulation. Western blot (WB) analysis was performed using immunoprecipitated (IP) GFP, LAT-GFP, and NTAL-GFP. Western blots were sequentially developed with biotinylated anti-phosphotyrosine (4G10) and anti-GFP to detect tyrosine phosphorylation and fusion proteins, respectively.

down-regulation, we expressed an NTAL-GFP fusion protein in *Oct* pre-B cells and analyzed pre-BCR expression compared with LAT-GFP expressing *Oct* pre-B cells. As expected, both LAT-GFP and NTAL-GFP fusion proteins were associated with the membrane when expressed in pre-B cells (data not shown). In contrast to LAT-GFP, however, the expression of NTAL-GFP resulted in a minor, if any, down-regulation of the pre-BCR (Fig. 1*B*).

Both SLP-65 and LAT have been shown to connect pre-BCR engagement to calcium mobilization, which seems to be indispensable for early B cell development and pre-B cell differentiation (17–20). To test whether NTAL is involved in calcium mobilization in pre-B cells, we investigated the calcium response of pre-B cells expressing either LAT-GFP or NTAL-GFP fusion proteins. For these analyses we used two different bone marrow-derived pre-B cell lines, the *Oct* pre-B cell line and a second pre-B cell line derived from SLP-65$^{-/-}$LAT$^{-/-}$ mice. In both cell lines, pre-BCR engagement induced strong calcium flux only in the pre-B cells that were reconstituted with LAT-GFP but not in those expressing NTAL-GFP or nontransduced GFP$^-$ cells (Fig. 1*B* and data not shown). This difference in the capability of LAT and NTAL to induce a strong calcium flux in pre-B cells may be explained by the lack of a canonical PLC-γ1/2 binding motif in NTAL.

NTAL is phosphorylated after pre-BCR stimulation

The inability of NTAL-GFP to down-regulate the receptor and induce a clear calcium signal after stimulation raised the question of whether NTAL is involved in pre-BCR-mediated signaling. Therefore, we tested whether, similar to LAT, the phosphorylation of NTAL is increased after pre-BCR stimulation. SLP-65$^{-/-}$LAT$^{-/-}$ pre-B cells reconstituted with LAT-GFP, NTAL-GFP, or GFP were selected with puromycin (Fig. 1*C*) and then used for stimulation experiments with anti-μ Abs to activate the pre-BCR. Anti-GFP immunoprecipitation and Western blot analyses showed that both LAT-GFP and NTAL-GFP were strongly tyrosine phosphorylated after pre-BCR engagement (Fig. 1*D*, *left blot*), indicating that NTAL participates in pre-BCR mediated signaling. Phosphorylation of NTAL-GFP after stimulation appeared to be stronger compared with that of LAT-GFP, which might be explained by the higher expression of NTAL-GFP as indicated by FACS analysis and Western blotting (Fig. 1, *C* and *D*).

NTAL does not contribute to SLP-65-mediated pre-BCR down-regulation

Because it has been proposed that the transmembrane adaptor NTAL recruits SLP-65 to the membrane, exerting a similar

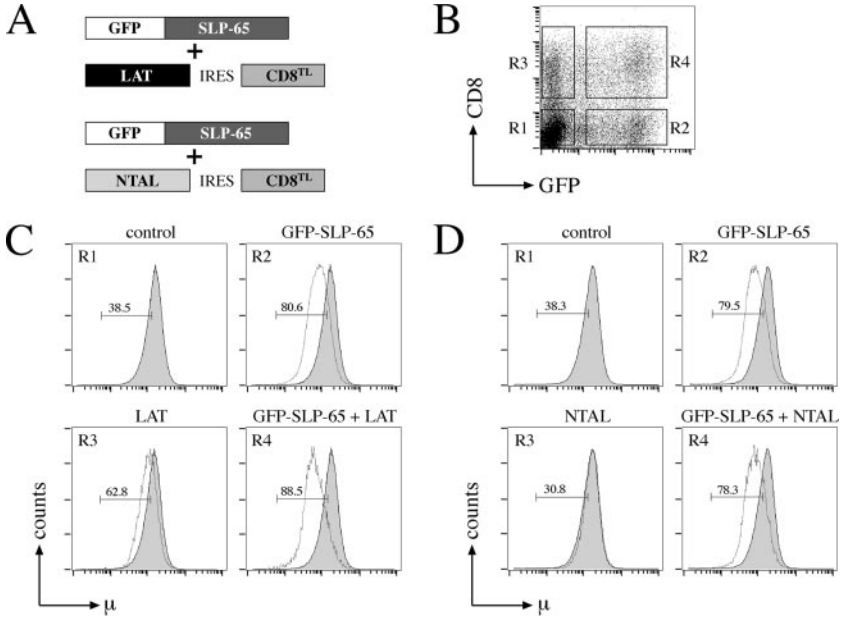

FIGURE 2. Effects of SLP-65, LAT, and NTAL on pre-BCR down-regulation. *A*, Scheme of the constructs that were coexpressed in pre-B cells. SLP-65 was expressed as an N-terminal fusion protein with GFP. LAT and NTAL were expressed using internal ribosome entry site (IRES) constructs encoding a TL CD8 as a marker for expression. *B*, Subdivision of pre-B cells into four populations after cotransduction. *Oct* pre-B cells were transduced with either GFP-SLP-65 and LAT-IRES-CD8^{TL} or with GFP-SLP-65 and NTAL-IRES-CD8^{TL}. Twenty-four hours later the cells were stained with PE-labeled anti-CD8 and Cy5-conjugated anti-μ and analyzed by FACS. Based on the staining for CD8 and the expression of GFP, living cells were classified as nontransduced (region R1), single positive expressing either GFP or CD8 (R2 and R3), and double positive expressing both GFP and CD8 (R4). *C* and *D*, Pre-BCR down-regulation of pre-B cells expressing LAT and SLP-65 (*C*) or NTAL and SLP-65 (*D*). Nontransduced, single-positive and double-positive populations were gated according to Fig. 2*B* and analyzed for μ expression. Pre-BCR down-regulation is shown by histograms comparing the μ expression of nontransduced control cells (R1, filled histogram) with either single- or double-positive cells (R2-R4; dotted line). Numbers indicate the percentage of cells in the corresponding region.

function as LAT for SLP-76, we investigated whether NTAL functionally depends on SLP-65 in pre-BCR signaling. Therefore, we compared the effects of NTAL and LAT on pre-BCR expression of both SLP-65$^{-/-}$ and SLP-65^{+} pre-B cells. For this purpose, *Oct* pre-B cells were cotransduced with vectors encoding either GFP-SLP-65 and LAT-IRES-CD8^{TL} or GFP-SLP-65 and NTAL-IRES-CD8^{TL} (Fig. 2*A*), where the expression of a tailless (TL) CD8α from the bicistronic vector served as a marker for expression of LAT and NTAL, respectively. Using this experimental system, we were able to subdivide the cells within each sample into a nontransduced population (R1) that served as a control, a GFP-SLP-65-expressing population (R2), a population positive only for CD8^{TL} (R3) indicating the expression of either LAT or NTAL, and a double-positive population (R4) expressing both GFP-SLP-65 and CD8^{TL} (Fig. 2*B*). Compared with the nontransduced control, SLP-65$^{-/-}$ pre-B cells reconstituted with either GFP-SLP-65 or LAT showed a strong down-regulation of the pre-BCR as previously reported (Fig. 2*C*, *panels R2* and *R3*) (20, 32). However, pre-BCR down-regulation was even more enhanced in cells expressing both adaptors (Fig. 2*C*, *panel R4*), indicating that SLP-65 and LAT have synergistic functions in pre-BCR signaling. In contrast to LAT, the overexpression of NTAL did not result in a significant down-regulation of the pre-BCR (Fig. 2*D*, *panel R3*). Moreover, pre-BCR down-regulation mediated by SLP-65 was not altered by coexpression of NTAL (Fig. 2*D*, *panels R2* and *R4*), making it unlikely that NTAL is required for the formation of SLP-65-dependent signaling complexes.

Introducing the LAT PLC-γ1/2 binding motif into NTAL induces pre-BCR down-regulation and calcium mobilization, but not differentiation

Despite their remarkable similarities in their overall structure and gene organization, a major difference between LAT and NTAL is their distinct ability to recruit PLC-γ1/2 upon receptor engagement. In the context of LAT, phosphorylated Y136 has been shown to effectively interact with PLC-γ1, bringing it into close proximity to Tec family and Syk/ZAP-70 family kinases and allowing its activation (22, 33). The importance of this recruitment has been demonstrated for TCR as well as pre-BCR signaling, as the Y136F LAT mutant is virtually nonfunctional in both systems (24, 34, 35). In contrast to LAT, NTAL lacks a classical binding motif for PLC-γ1/2 and shows no clear interaction with PLC-γ1/2 in immunoprecipitations (25). To test whether the recruitment of PLC-γ1/2 is the main functional difference between LAT and NTAL in pre-B cells, we mutated a stretch of four amino acids from Y_{113}VDPIP to Y_{113}LVVLP in NTAL to generate a PLC-γ1/2 binding motif identical with Y_{136}LVVLP in LAT. NTAL Y113 was chosen because it resembles LAT Y136 in respect to its position, making it likely that the resulting NTAL PLC-γ1/2 binding motif has a similar orientation in both NTAL and LAT molecules (see Fig. 4).

To test whether the NTAL mutant containing the PLC-γ1/2 binding motif (hereafter referred to as NTAL/PLCγBM) is more potent in receptor down-regulation compared with the nonmutated NTAL, we expressed both as GFP fusion proteins in pre-B cells and analyzed pre-BCR expression after 24 h. Cells expressing LAT-GFP or GFP alone were used as controls. Overexpression of NTAL-GFP resulted in a minor down-regulation of the pre-BCR compared with cells expressing LAT-GFP (Fig. 3*A*). The NTAL/PLCγBM-GFP mutant, however, had a significant effect on pre-BCR down-regulation as compared with NTAL-GFP, indicating that the introduction of a PLC-γ1/2 binding motif activated NTAL in pre-BCR signaling.

As mentioned above, the inability of NTAL to induce a strong calcium flux in B cells has been attributed to the lack of a PLC-γ1/2 binding motif. Therefore, we investigated whether the NTAL/PLCγBM mutant is able to activate calcium flux after pre-BCR stimulation in pre-B cells. In contrast to control cells expressing GFP or NTAL-GFP, pre-BCR stimulation of cells expressing

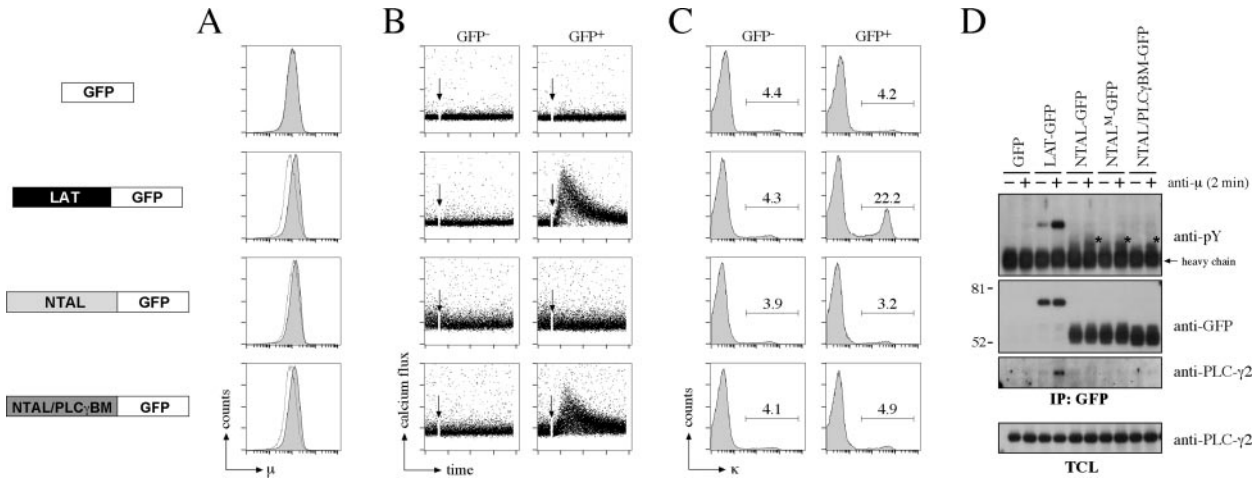

FIGURE 3. Comparing LAT, NTAL, and the mutant NTAL/PLCγBM in pre-B cells. *A*, FACS analysis comparing pre-BCR down-regulation induced by LAT, NTAL, and NTAL/PLCγBM. *Oct* pre-B cells were transduced with constructs encoding GFP, LAT-GFP, NTAL-GFP, or NTAL/PLCγBM-GFP. Twenty-four hours later, the cells were stained with Cy5-labeled anti-μ and analyzed by FACS. Down-regulation of the pre-BCR is indicated by histograms comparing the μ expression of nontransduced GFP$^-$ cells (filled histogram) with that of GFP$^+$ cells (dotted line). *B*, Calcium mobilization upon pre-BCR engagement. SLP-65$^{-/-}$/LAT$^{-/-}$ pre-B cells were transduced as described above, stimulated with anti-μ after 24 h, and analyzed by FACS. The calcium response was compared between the GFP$^-$ and the GFP$^+$ population within one sample. *C*, Differentiation capacity of pre-B cells expressing LAT, NTAL, or NTAL/PLCγBM. *Oct* pre-B cells transduced as described in *A* were cultured in medium without IL-7. After 4 days, the cells were stained with biotinylated anti-κ and Cy5-labeled streptavidin and analyzed by FACS. Differentiation of GFP$^-$ and GFP$^+$ cells of the same sample is shown in the histograms. Numbers indicate the percentage of κ^+ cells within the corresponding region. *D*, Western blot analysis using anti-GFP immunoprecipitations (IP). SLP-65$^{-/-}$/LAT$^{-/-}$ cells were transduced with GFP, LAT-GFP, NTAL-GFP, and the PLC-γ binding mutant NTAL/PLCγBM-GFP and sorted for GFP$^+$ cells after 3 days. NTAL^M-GFP is an additional NTAL mutant bearing a D115V mutation that had no effect. Sorted cells were stimulated for 2 min with anti-μ or left unstimulated as a control. GFP fusion proteins were precipitated of total cellular lysates using anti-GFP Abs. Precipitates were subjected to SDS-PAGE and analyzed for tyrosine phosphorylation, GFP expression, and association with PLC-γ2 by Western blotting using mouse anti-phosphotyrosine (anti-pY), rabbit anti-GFP, and rabbit anti-PLC-γ2 Abs. Asterisks mark the tyrosine-phosphorylated NTAL-GFP mutants that comigrate with the H chain of the precipitating mouse anti-GFP Ab. Development with anti-PLC-γ2 using the total cellular lysate (TCL) indicates equal expression of PLC-γ2 in all samples.

NTAL/PLCγBM-GFP resulted in a calcium signal almost as strong as that observed for LAT-GFP, indicating that the inserted motif is sufficient to enable NTAL-mediated calcium flux (Fig. 3*B*). In contrast to LAT, however, we were unable to detect a clear interaction of NTAL/PLCγBM-GFP with PLC-γ2 in immunoprecipitations, suggesting that other regions of LAT might be required to stabilize the interaction with PLC-γ2 (Fig. 3*D*).

Because the NTAL/PLCγBM mutant was more active compared with NTAL, we investigated the ability of NTAL/PLCγBM to promote pre-B cell differentiation. B cell development in SLP-65$^{-/-}$ mice is partially blocked at the pre-B cell stage, indicating that alternative pre-BCR signaling cascades exist and enable the progression of pre-B cells to immature and mature B cells. Indeed, reconstitution of LAT in *Oct* and other SLP-65$^{-/-}$/LAT$^{-/-}$ pre-B cells induced the differentiation of these cells to BCR$^+$ B cells in vitro after IL-7 withdrawal (20, 32). To assess the role of NTAL in these processes, we expressed GFP fusions of NTAL, LAT, and NTAL/PLCγBM in pre-B cells and monitored κ^+ cells after 4 days of culture in medium without IL-7. No induction of κ recombination above the background was observed with cells expressing either GFP or NTAL-GFP (Fig. 3*C*), whereas a strong increase in the percentage of κ^+ cells was found upon reconstitution of LAT-GFP. In contrast to LAT-GFP, pre-B cells expressing the mutant NTAL/PLCγBM-GFP showed no enhanced differentiation compared with the nontransduced control, demonstrating that calcium mobilization alone is not sufficient to induce differentiation.

Chimeric swap mutants identify the N terminus of NTAL as an inhibitory domain that prevents pre-B cell differentiation while allowing pre-BCR down-regulation and receptor-mediated calcium flux

The NTAL/PLCγBM mutant resembles LAT in terms of receptor down-regulation and calcium mobilization but fails to induce differentiation of pre-B cells upon IL-7 withdrawal. To analyze which parts of LAT are indispensable to restore LAT-like functions in pre-B cells and to what extent NTAL can substitute for LAT on the molecular level, we generated several chimeric swap mutants of both proteins and analyzed them in the respective readouts. The distinct swap domains were chosen based on the protein sequence and the exons encoding the distinct parts of NTAL and LAT (Fig. 4). The N-terminal swap domains L_1 and N_1 correspond to exons 1–6 and contain the respective transmembrane domain. The swap domains L_2 and N_2 are encoded by exons 7 and 8 and form the central part of the protein. In LAT, this domain contains the classical PLC-γ1/2 binding motif with tyrosine Y136, whereas the corresponding tyrosine Y113 in NTAL lacks such a motif. The LAT-derived C-terminal swap domain L_3 (exons 9–11) comprises the so-called distal tyrosines Y175, Y195, and Y235, which have been shown to be important for LAT function

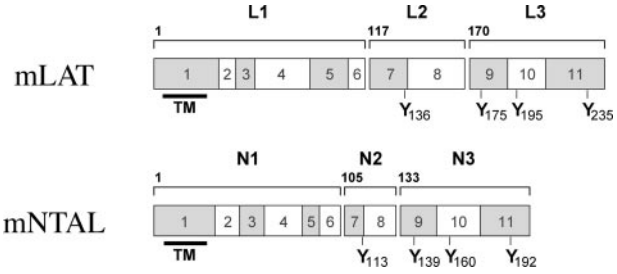

FIGURE 4. Schematic drawing of murine LAT (mLAT) and murine NTAL (mNTAL) showing the swap strategy. Each adaptor was divided into three swap domains depicted as L1, L2, and L3 and N1, N2, and N3, corresponding to coding exons 1–6, 7–8, and 9–11 for LAT and NTAL, respectively. The first amino acid in the swap domain is indicated above each adaptor. The positions of selected tyrosines implicated in adaptor function and the respective transmembrane domains (TM) are indicated.

in both B and T cells (34, 35). The corresponding tyrosines Y139, Y160, and Y192 of NTAL N_3 have been demonstrated to contribute to NTAL function in a T cell system (36).

To test the respective LAT/NTAL swap mutants for pre-BCR down-regulation, the chimeric constructs were expressed as GFP fusion proteins in pre-B cells and analyzed after 24 h (Fig. 5*A*). As expected, $L_1L_2L_3$-GFP (containing all three LAT parts) induced a strong down-regulation of the pre-BCR, and neither replacing the C-terminal ($L_1L_2N_3$-GFP) nor the N-terminal domain ($N_1L_2L_3$-GFP) interfered with this activity. Substitution of the central L_2 swap domain by N_2 to generate $L_1N_2L_3$-GFP abolished pre-BCR down-regulation (Fig. 5*A*).

We also measured calcium flux in pre-B cells that were reconstituted with chimeric constructs. We found that robust calcium flux (Fig. 5*B*) was induced with the fusions that allowed notable pre-BCR down-regulation ($L_1L_2L_3$, $L_1L_2N_3$, $N_1L_2L_3$). Surprisingly, exchanging the central L_2 domain of LAT by N_2 to generate $L_1N_2L_3$ lowered but did not completely abolish pre-BCR-induced calcium flux. The fact that cells expressing $L_1N_2L_3$ or $L_1N_2N_3$ show similar calcium flux (Fig. 5*B*) whereas cells expressing $N_1N_2N_3$ fail to mobilize calcium (Figs. 1 and 3) suggests that an important difference between LAT and NTAL is localized in the N terminus.

We further analyzed the ability of the chimeric constructs to induce differentiation of pre-B cells into BCR^+ cells. The percentage of κ^+ cells was measured after 4 days of culture without IL-7 (Fig. 5*C*). Compared with GFP, the $L_1L_2L_3$-GFP mutant clearly induced the differentiation of pre-B cells into immature B cells (Fig. 5*C*). Although replacing the C-terminal domain L_3 by N_3 ($L_1L_2N_3$) slightly reduced the differentiation capacity of the respective pre-B cells, replacing the central domain L_2 by N_2 ($L_1N_2L_3$) or both L_2L_3 by N_2N_3 ($L_1N_2N_3$) drastically reduced the ability of $L_1L_2L_3$ to induce pre-B cell differentiation. These data underline the importance of the central L_2 domain and its PLC-γ1/2 binding motif for inducing pre-B cell differentiation. Unexpectedly however, replacing the N-terminal domain L_1 by N_1 to obtain $N_1L_2L_3$-GFP induced a significant decrease, below the level of background differentiation, in the percentage of κ^+ cells (Fig. 5*C*). Further analysis showed that κ expression was reduced ~5-fold in cells expressing high amounts of $N_1L_2L_3$-GFP (GFP^{high}) compared with GFP^- cells (Fig. 6, *lower panel*). This result indicates that the N-terminal domain of NTAL exerts an inhibitory function on pre-B cell differentiation. In agreement with this, cells expressing high amounts of $N_1N_2N_3$-GFP showed drastic reduction of pre-B cell differentiation, which was restored by replacing the N_1 domain with L_1 to generate a $L_1N_2N_3$ fusion protein (Fig. 6). Importantly, GFP^{high} cells in the control transduction using only GFP show no negative effect on pre-B cell differentiation (Fig. 6, *upper panel*). Moreover, GFP^{high} cells expressing $L_1L_2L_3$-GFP show the highest differentiation capacity, suggesting that the inhibitory effect observed with $N_1L_2L_3$-GFP is specific for the N_1 domain.

Discussion

In this study we investigated the role of NTAL in pre-B cell signaling and pre-B cell development by analyzing the down-regulation of surface pre-BCR, Ca^{2+} mobilization, and pre-B cell differentiation in vitro. Similar to LAT, we found NTAL transcripts in bone marrow-derived WT and SLP-$65^{-/-}$ pro- and pre-B cells, which is in line with other studies that detected the NTAL protein in all developmental stages of B cells (29, 30). However, despite being involved in pre-B cell signaling as indicated by induced tyrosine phosphorylation, NTAL fails to promote both pre-BCR down-regulation and calcium mobilization and shows negative effects on pre-B cell differentiation compared with LAT. We also analyzed the influence of NTAL on SLP-65-mediated signaling with respect to pre-BCR down-regulation. The symmetry in initial BCR and TCR signaling, with SLP-76 being recruited to the plasma membrane by LAT in T cells, led to the assumption that SLP-65 might be targeted to the membrane by NTAL in a similar manner in B cells. If so, one would expect that the coexpression of NTAL would lead to efficient SLP-65 membrane recruitment and increased SLP-65 activity. However, we were unable to detect a positive effect of NTAL on SLP-65-mediated signaling, making it unlikely that NTAL is responsible for bringing SLP-65 to the membrane as is known for LAT and SLP-76. In contrast, our recent data

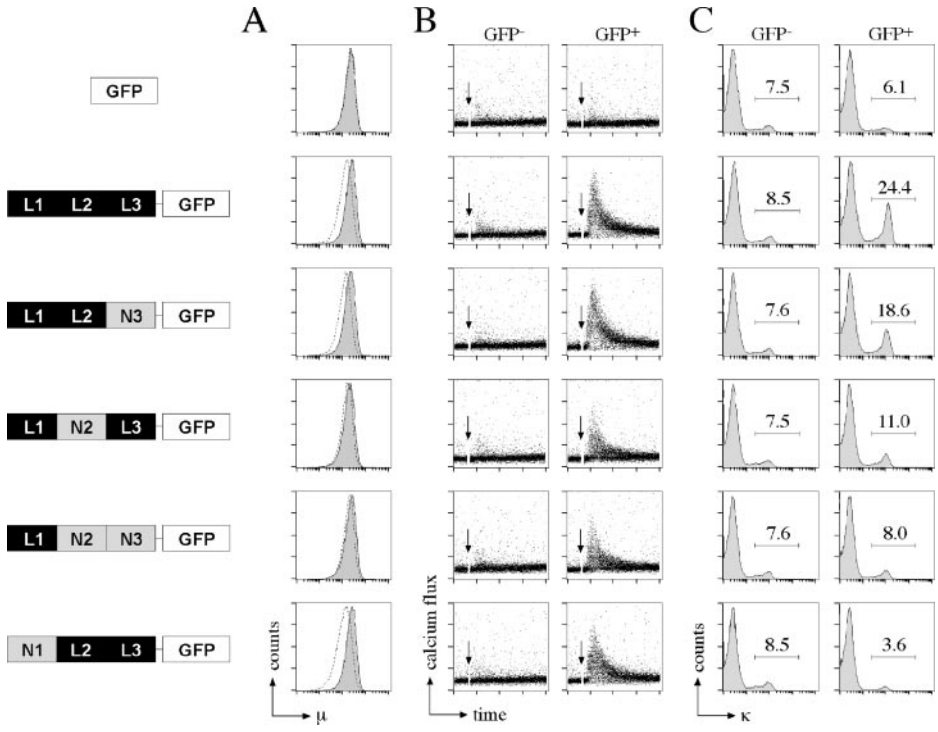

FIGURE 5. Functional analysis of LAT/NTAL swap mutants in pre-B cells. *A*, FACS analysis comparing pre-BCR down-regulation induced by different LAT/NTAL swap mutants. *Oct* pre-B cells were transduced with constructs according to the schemes depicted on the *left-hand side* (domains originating from LAT are drawn in black, domains from NTAL are drawn in gray). Twenty-four hours later cells were stained with Cy5-labeled anti-μ and analyzed by FACS. Down-regulation of the pre-BCR is indicated by histograms comparing the μ expression of nontransduced GFP^- cells (filled histogram) with that of GFP^+ cells (dotted line). *B*, Calcium mobilization upon pre-BCR engagement. SLP-$65^{-/-}$/$LAT^{-/-}$ pre-B cells were transduced as described above, stimulated with anti-μ after 24 h, and analyzed by FACS. The calcium mobilization of the GFP^- and the GFP^+ population within one sample is shown. *C*, Differentiation capacity of pre-B cells expressing the respective LAT/NTAL swap mutants. Transduced *Oct* pre-B cells were cultured in medium without IL-7 and analyzed for expression of κ by FACS after 4 days. Numbers indicate the percentage of κ^+ cells within the GFP^- and GFP^+ populations.

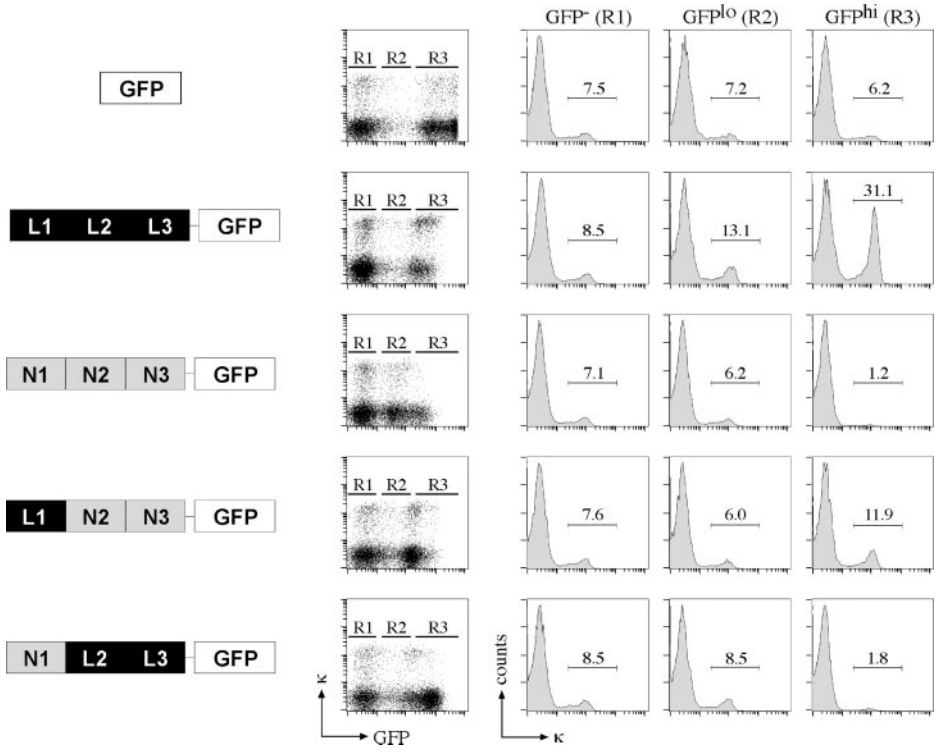

FIGURE 6. Effects of LAT/NTAL swap domains on pre-B cell differentiation. FACS analysis showing the differentiation capacity of selected LAT/NTAL-GFP swap mutants in dependence on their expression levels in pre-B cells. *Oct* pre-B cells were transduced with the constructs according to the schemes depicted on the *left-hand side*. After 4 days of culture in medium without IL-7, cells were analyzed for expression of κ and GFP by FACS. Based on the expression of GFP, living cells were classified as nontransduced (GFP^-), GFP^{low}, or GFP^{high}. Numbers indicate the percentage of κ^+ cells within the respective populations.

identified a leucine zipper motif in the N terminus of SLP-65 as the domain that mediates SLP-65 membrane recruitment (37). Several proteins that interact with this leucine zipper have been identified, although their role in membrane recruitment remains to be characterized (38).

On the molecular level, the major difference between LAT and NTAL seems to be the lack of a classical PLC-γ1/2-binding motif in the latter. To test whether differences between NTAL-mediated and LAT-mediated pre-BCR signaling are caused by this missing motif in NTAL, we inserted the PLC-γ1/2 binding motif of LAT into NTAL. This insertion rendered NTAL capable of activating pre-BCR down-regulation and calcium mobilization. However, despite its role in inducing Ca^{2+} mobilization, this NTAL/PLCγBM mutant failed to promote the differentiation of pre-B cells. Although recent experiments demonstrated that NTAL resembles a LAT mutant defective for PLC-γ1 binding in T cells, our data suggest that the PLC-γ binding motif has only partial effects on the function of NTAL in pre-B cells (39).

To elucidate the observed differences between NTAL- and LAT-mediated pre-BCR signaling on the molecular level, we generated several chimeric swap mutants of both adaptors. The C-termini of both LAT and NTAL contain three tyrosines within Grb2-binding motifs which are phosphorylated after receptor engagement and are important for cooperative binding of the adaptors Grb2 and Gads (35). Mutations in these distal tyrosines severely reduced the function of LAT in pre-B cells and T cells (34). In terms of receptor down-regulation and calcium signaling, we found that the C-terminal domain of NTAL is functionally equivalent to the LAT C terminus. The slightly reduced ability of chimeric LAT with the NTAL C terminus to promote differentiation might result from suboptimal spacing of the distal NTAL tyrosines relative to important regions in LAT such as Y136.

Substitution of the central LAT swap domain L_2 by N_2 abolished LAT-mediated pre-BCR down-regulation and decreased the activity of LAT in calcium mobilization and pre-B cell differentiation. This is in line with previous reports demonstrating the importance of LAT Y136 involving the binding motif of PLC-γ1/2 for signaling (34). The finding that inserting the PLC-γ1/2 binding motif of LAT into the central domain of NTAL improved NTAL function in pre-BCR down-regulation and calcium flux further supports the importance of this motif. However, the insertion of the PLC-γ1/2 binding motif was unable to promote NTAL-mediated pre-B cell differentiation. Surprisingly, exchanging the central L_2 swap domain by N_2 did not completely abolish calcium signaling of the resulting fusion protein. Given that the Y136F mutation in LAT completely disrupts calcium flux (34), this implies that the central N_2 swap domain, although lacking a classical PLC-γ1/2 binding motif, retains some activity in mediating or stabilizing the association with PLC-γ1/2.

Most interestingly, our data point to an unexpected inhibitory role of the NTAL N terminus in differentiation. Although the N_1 swap domain was functionally equivalent to the L_1 domain of LAT with respect to receptor down-regulation and calcium mobilization, replacing the LAT N terminus resulted in a drastic decrease of differentiation below background levels. The molecular mechanism underlying this unexpected inhibitory function of the NTAL N terminus as compared with the LAT N terminus remains to be clarified. According to recent publications, the N terminus of NTAL is not phosphorylated and does not contain any known protein interaction motifs (25, 26, 36). This makes it unlikely that NTAL N terminus recruits components that interfere with pre-B cell differentiation and may indicate that the N termini of NTAL and LAT mediate distinct localizations within the membrane. Indeed, although both LAT and NTAL are palmitoylated on their N termini and have been described to participate in lipid rafts, electron microscopic analysis of mast cells revealed that both molecules are located in distinct, nonoverlapping regions of the plasma membrane (27). In the case of the swap mutants, it is conceivable that the NTAL N terminus targets the intracellular part of the molecule to membrane areas where important signaling pathways cannot be initiated. Possibly, such mislocalization may also sequester signaling components from their normal place of function, resulting in the observed negative effect of NTAL on differentiation. However, further studies are required to characterize the exact domain and

mechanism by which the N terminus of NTAL affects NTAL and LAT function in pre-BCR signaling and pre-B cell differentiation.

The identification of the N terminus as an inhibitory element may explain the reported positive as well as negative regulatory functions of NTAL (27, 28). For instance, inactivation of the *NTAL* gene in mice renders mast cells hyperresponsive to FcεRI-stimulation (27, 28). However, combined deficiency of NTAL and LAT results in signaling defects that are more severe than in $LAT^{-/-}$ mast cells, suggesting that NTAL also plays a positive role in FcεRI signaling. Our data indicate that the negative effects are mainly mediated by the N terminus of NTAL, whereas the central part and the C terminus seem to be responsible for the positive role of NTAL. In the case of mast cells, it was suggested that NTAL and LAT compete for related signaling molecules upon receptor engagement. Because NTAL is compromised in signaling compared with LAT, NTAL may reduce the LAT-dependent signaling by sequestering important signaling molecules. Loss of NTAL releases the sequestered molecules and makes them available for LAT-mediated signaling, resulting in an enhanced response. Together, our data identify a distinctive role of the N terminus of NTAL that inhibits pre-B cell differentiation although the initial steps of pre-BCR signaling such as calcium flux and pre-BCR down-regulation are activated. Characterizing the mechanism by which this NTAL domain regulates protein function should improve both our understanding of adaptor protein function and the requirements for efficient pre-B cell differentiation.

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Disclosures

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