Published in final edited form as:
Cell Signal. 2008 January ; 20(1): 195–205.

Kit- and FcεRI-induced differential phosphorylation of the transmembrane adaptor molecule NTAL/LAB/LAT2 allows flexibility in its scaffolding function in mast cells

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Abstract

The transmembrane adaptor protein (TRAP), NTAL, is phosphorylated in mast cells following FcεRI aggregation whereby it cooperates with LAT to induce degranulation. The Kit ligand, stem cell factor (SCF), enhances antigen-induced degranulation and this also appears to be NTAL-dependent. However, Kit and FcεRI appear to utilize different mechanisms to induce NTAL phosphorylation. Thus, we examined whether the responsible kinases selectively phosphorylated distinct tyrosines in NTAL and explored the implications for downstream signaling. Whereas FcεRI required Lyn and Syk for NTAL phosphorylation, Kit appeared to directly phosphorylate NTAL. Furthermore, co-transfection studies with NTAL constructs revealed that Lyn, Syk, and Kit phosphorylate different tyrosines in NTAL. The tyrosines principally phosphorylated by Syk were recognized as Grb2-binding sites, whereas Lyn and Kit phosphorylated other tyrosines, both inside and outside of these motifs. Pull down studies revealed that PLCγ_1 associated with the two terminal Syk-phosphorylated Grb2-binding sites, which would help to explain the observed decrease in antigen-induced calcium signal and degranulation in NTAL-knock down-human mast cells. The observations reported herein support the conclusion that NTAL may be differentially utilized by specific receptors for relaying alternative signals and this suggests a flexibility in the function of TRAPs not previously appreciated.

Keywords

Mast cells; FcεRI; Kit; NTAL; Lyn; Syk; Signaling; Adaptor molecules

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1. Introduction

Activated mast cells promote allergic inflammation following the release of biochemical mediators [1]. Multiple receptors expressed on the surface of mast cells may contribute to such activation. The high affinity receptor for IgE (FcεRI), however, is undoubtedly the major receptor involved in antigen-induced responses [2]. We [3–5] and others [6–10] have demonstrated that FcεRI-mediated responses are significantly enhanced by pre- or concurrent exposure of mast cells to the Kit ligand, stem cell factor (SCF).

FcεRI and Kit generate both common and unique signals which account for their abilities to respectively induce mediator release and potentiate antigen-induced degranulation [4]. Upon aggregation, a fraction of the FcεRI translocates to defined glycolipid-enriched microdomains within the plasma membrane termed lipid rafts [11]. Thus, the FcεRIβ and γ chains are phosphorylated by the Src tyrosine kinase family member, Lyn, which is preferentially activated in these microdomains [12]. The phosphorylated γ chains recruit Syk tyrosine kinase, which is subsequently activated following auto/transphosphorylation and phosphorylation by Lyn [2]. The major initial substrate for Syk is the lipid raft-associated transmembrane adaptor protein (TRAP), LAT (linker for activation of T cells) [13]. Phosphorylated tyrosines on LAT provide docking sites for the cytosolic adaptor molecules Grb2 and GADS, and the signaling enzymes phospholipase (PL)Cγ_1 and PLCγ_2 [14]. Binding of PLCγ to LAT is also stabilized by its indirect binding to LAT-bound GADS via the cytosolic adaptor SLP-76 [15]. By this mechanism, LAT regulates FcεRI-mediated degranulation via a PLCγ_1-dependent pathway [13]. More recently, another lipid raft-associated TRAP, NTAL (non-T cell activation linker; alternatively termed LAB (linker for activation of B cells) or LAT2) [16–19], was observed to be expressed in mast cells and to be highly phosphorylated following FcεRI aggregation [3, 16,17,19–21].

NTAL possesses 10 tyrosines within its cytosolic tail, which may be potential targets for phosphorylation. Six of the tyrosines (Y) are contained in 5 putative Grb2-binding YXN motifs [16,17] However, the capacity of the other tyrosines to bind associating molecules is less well defined. Data obtained from gene knock down studies conducted in human mast cells [3] and from studies conducted in mast cells (BMMCs) derived from the bone marrows of NTAL$^{-/-}$/LAT$^{-/-}$ mice [20,21] indicated that NTAL and LAT play complementary roles in the positive regulation of FcεRI-mediated degranulation. However, studies conducted in NTAL$^{-/-}$ BMMCs suggest that NTAL may also regulate a negative pathway for this response [20,21]. How NTAL contributes to these responses remains to be delineated.

Kit has also been demonstrated to induce NTAL phosphorylation in human and mouse mast cells and, at least in human mast cells, it appears that this signal is required for the ability of Kit to enhance FcεRI-mediated degranulation [3]. However, these studies also suggested that NTAL may be differentially phosphorylated by FcεRI and Kit, though the mechanism(s) regulating this differential phosphorylation and the implications for mast cell activation remain unclear. In this study we have set out to address these issues. Herein we provide evidence to support the conclusion that whereas the FcεRI requires Lyn and Syk to phosphorylate NTAL, Kit appears to directly phosphorylate NTAL. Furthermore, we demonstrate that these kinases produce entirely different patterns of phosphorylation of the individual tyrosines on NTAL, allowing flexibility in the function of this adaptor molecule in the regulation of mast cell activation.

2. Materials and methods

2.1. Mast cells

Human mast cells (HuMCs), derived from $CD34^+$ pluripotent peripheral blood progenitor cells, were cultured as described [22] and were used after 7–9 weeks. $Lyn^{-/-}$and $Fyn^{-/-}$ mice, obtained from Taconic (Hudson, NY), were on a background of C57BL/6 and 129Sv at 5th and 6th backcross, respectively, and $Lyn^{-/-}/Fyn^{-/-}$ mice (Taconic) were on a background of C57BL/6 and 129Sv at 5th backcross. Wild type strain-matched mice were used as controls. BMMCs were developed from these animals by femur lavage, then cultured in RPMI 1640 medium containing mouse IL-3 as described [23], and used after 4–6 weeks in culture. The HMC-1 human mast cell line was maintained in RPMI 1640 medium [24].

2.2. Knock down of NTAL and LAT

Generation of the lentiviral expression plasmids and the recombination procedure were previously described [25]. The sense and antisense oligonucleotide sequences for NTAL-shRNA were as follows: sense; 5′-CAC CGA GAA CTT CAG CAA AGG AAG CAC GAA TGC TTC CTT TGC TGA AGT TCT-3′, anti-sense; 5′-AAA AAG AAC TTC AGC AAA GGA AGC ACG AAT GCT TCC TTT GCT GAA GTT CTC-3′. Packaging vector (9 μg) (ViraPower packaging mix; Invitrogen Life Technologies, Carlsbad, CA) and pLenti6/Block-iT with NTAL-shRNA or LacZ-shRNA were used to transfect 293FT packaging cells with Lipofectamine 2000 (35 μl) (Invitrogen) and viruses were collected after 48 hours. The viruses were added to infect 3–4 weeks old HuMCs (2 x 10^6 cells). Two days after infection, the medium was changed (virus-free) and, after an additional day, antibiotic selection by 2 μg/ml blasticidin was initiated. The cells were used after 3 weeks of selection. In studies in which we examined the outcome of the simultaneous knock down of NTAL and LAT, HuMCs were transfected with short inhibitory RNA (siRNA) oligonucleotides using the sequences and protocol described [3].

2.3. Cell activation

HuMCs were sensitized overnight in cytokine-free, supplemented Stem Pro culture media containing human biotinylated IgE (100 ng/ml) [26]. Following rinsing in HEPES buffer [27] containing 0.04% BSA (Sigma, St. Louis, MO), the cells were stimulated in this buffer with streptavidin (SA; 0–100 ng/ml) (Sigma) and/or human SCF (0–100 ng/ml) (PeproTech, Rocky Hill, NJ) for the times indicated. BMMCs were sensitized overnight with anti-mouse monoclonal dinitrophenyl (DNP) IgE (100 ng/ml) (Sigma) in IL-3-free RPMI medium, and then stimulated with 100 ng/ml of DNP-human serum albumin (HSA) (Sigma) or 100 ng/ml of murine SCF (PeproTech) for 2 min, unless specified. Degranulation was monitored by β-hexosaminidase release as described [28] and was calculated as the percentage of total (tissues and supernatants) content found in the supernatants following challenge.

2.4. Isolation of lipid raft fractions

Wild type mouse BMMCs (20 x 10^6 cells) obtained from Balb/c mice were activated as above and lysed as described [29] with 0.05% Triton X-100. Lipid raft fractions were isolated as described [29]. For translocation of FcεRI, BMMCs were sensitized with [^{125}I]-conjugated DNP-IgE [11] and stimulated with 300 ng/ml of DNP-HSA for 3 min. For studies examining translocation of phospho-Kit and other signaling molecules, the fractions were boiled with LDS NuPage sample buffer (Invitrogen) containing dithiothreitol (5 mM) and β-mercaptoethanol (5%) prior to immunoblot analysis as described below. The purity of the lipid raft and non-lipid raft fractions was verified by probing for flotillin-2 and CD45, respectively [30].

2.5. Constructs and transfection

The mutants used in this study (also see Fig. 3A) were generated by site-directed mutagenesis of human NTAL (in the pBSK vector), in which the tyrosines were replaced by phenylalanines using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), based on wild type NTAL (denoted as $NTAL^{10Y}$): a construct with all tyrosines mutated to phenylalanines ($NTAL^{10Y\text{-}10F}$), constructs containing single tyrosines with all others mutated to phenylalanines (denoted as Y^{40}, Y^{58}, Y^{84}, $Y^{95}XN$, Y^{110}, $Y^{118}XN$, Y^{119}, $Y^{136}XN$, $Y^{193}XN$, $Y^{233}XN$), and a construct with the tyrosines in the five Grb2-binding motifs (95, 118, 136, 193, 233) mutated with those in the five non-Grb2-binding motifs preserved (denoted as $NTAL^{5Y\text{-}5F}$).

Combined mutants (with multiple changes of tyrosines to phenylalanines) were prepared by gradual introduction of the mutations into the simpler mutants and by assembling of suitable mutated cDNA fragments using restriction endonuclease sites present in the NTAL cDNA or created during mutagenesis. All mutants and all intermediary products were sequenced and finally subcloned into the expression vector pEFBOS, which generates FLAG-tagged proteins.

Syk cloned into the pRK5 vector and Myc-tagged Lyn in pcDNA3.1 vector were kindly provided by Dr. W. Kolanus (Gene Center, Munich, Germany) and Dr. S. Watson (University of Oxford, UK), respectively. The Kit construct was a kind gift of Dr. Gunnar Nilsson (Uppsala University, Uppsala, Sweden). The Kit construct was transformed in DH5α-T1 *E. coli* (Invitrogen) and other constructs were transformed in TOP10 *E. coli* (Invitrogen) following manufacturer's instructions.

293T cells were transfected with Lipofectamine 2000 (Invitrogen) as described in the manufacturer's instructions. Briefly, 1 x 10^6 cells were plated in 6 well plates on the day before transfection. 0.7 μg of NTAL, Lyn, and Syk, and 2 μg of Kit DNA, used alone or in combination, and 10 μl of Lipofectamine 2000 were mixed in 500 μl of Opti-MEM (Invitrogen) and were added into each culture well. 293T cells were harvested 24 hours after transfection and cell lysates were immunoprecipitated with anti-NTAL mAb as described below.

2.6. Cell extraction, immunoprecipitation, pull down studies, and immunoblotting

Immunoprecipitates from the transfected 293T cells and cell lysates from HuMCs were prepared as described [3]. In all immunoprecipitation studies, cell lysates were presorbed with control antibody (mouse 3ZD IgG_1, Hezleton, Vienna, VA)-conjugated beads (control Ab-beads) for 30 min at 4 °C. Pre-sorbed lysates were immunoprecipitated with anti-NTAL antibody-conjugated beads (NTAL Ab-beads) for 3 hours at 4 °C. HuMC and BMMC immunoprecipitates were essentially prepared as above, but were lysed in the absence of NP-40. For pull down studies, cell lysates of tranfected 293T cells and HMC-1 cells were first pre-sorbed separately as above. The pre-sorbed 293T cell lysates were then incubated with NTAL Ab-beads or control Ab-beads for 3 hours at 4 °C. This allowed the binding of phosphorylated NTAL molecules from the 293T cell lysates to the NTAL Ab-beads. The resulting NTAL-loaded beads (NTAL-beads) were rinsed and then incubated with pre-sorbed HMC-1 cell lysates for 3 hours at 4 °C. This resulted in the capture of NTAL-binding proteins from the HMC-1 cell lysates. These beads were rinsed and then resuspended with LDS sample buffer (Invitrogen). Proteins were separated by electrophoresis on 4–12% NuPage Bis-Tris gels (Invitrogen) and were probed for immuno-reactive proteins utilizing the following antibodies: anti-Lyn pAb, anti-PLCγ_1 pAb, anti-OctA-Probe (FLAG) pAb, anti-Grb2 mAb (Santa Cruz Biotechnology Inc, Santa Cruz, CA), biotin-conjugated anti-phosphotyrosine mAb (clone 4G10), anti-phospho-LAT (pY191) pAb, (Millipore, Billerica, MA), anti-phospho-Kit (pY823) pAb, anti-phospho-PLCγ_1 (pY783) pAb (Invitrogen), anti-flotillin-2 mAb, and anti-CD45 mAb (BD Biosciences, Franklin Lakes, NJ). Anti-NTAL mAbs and pAbs were produced

and characterized as described [16]. All pAbs were from rabbit and mAbs were from mouse. The immuno-reactive proteins were visualized by initially probing with horseradish peroxidase-conjugated anti-mouse (Jackson Labs, West Grove, PA) or anti-rabbit IgG (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) then by ECL (Perkin Elmer, Schelton, CT). Equivalent protein loading was verified by stripping and then reprobing or alternatively probing identically loaded membranes. To quantitate changes in protein expression and phosphorylation, films were scanned with ChemiDoc XRS and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.7. Measurement of intracellular calcium

Intracellular free calcium levels were monitored using Fura-2 AM ester (Molecular Probes, Eugene, OR) as before [5]. Briefly, HuMCs were loaded with Fura-2 AM at 37 °C for 30 min then rinsed. Following resuspension in HEPES buffer containing 0.04% BSA and sulfinapyrazone (0.3 mM) (Sigma), fluorescence was measured at 340 and 380 nm excitation wavelengths and 510 nm emission wavelength. The ratio of the fluorescence readings was calculated following subtraction of the autofluorescence of the cells.

3. Results

3.1. FcεRI- and Kit-mediated NTAL phosphorylation and its requirement for degranulation

We initially confirmed that NTAL contributed to the positive regulation of degranulation from HuMCs and its potentiation by SCF. To do this, NTAL expression was stably knocked down in HuMCs by transducing NTAL-targeted shRNA using a recently described lentiviral transduction approach [25]. As a control, HuMCs were also transduced with shRNA targeting LacZ. This approach achieved a marked knockdown of NTAL protein expression in the absence of a decrease in the expression of other proteins including LAT, Lyn, and Syk (Fig. 1A). We observed little difference in the expression of these proteins and NTAL in the LacZ-shRNA-transduced cells compared to the non-tranduced cells (data not shown).

As before [3], FcεRI aggregation induced by streptavidin-crosslinking of FcεRI-bound biotinylated IgE and SCF-induced Kit dimerization promoted NTAL phosphorylation (Fig. 1B). In the case of the FcεRI, but not Kit, this phosphorylation was accompanied by an increase in the phosphorylation of LAT. The NTAL-targeted shRNA produced a substantial reduction in streptavidin- and SCF-induced NTAL phosphorylation compared to that observed in the LacZ-shRNA-transduced cells (Fig. 1B). This was observed in the absence of a similar decrease in the LAT phosphorylation observed following FcεRI aggregation. The decrease in NTAL phosphorylation associated with NTAL knock down resulted in a significant reduction in FcεRI-mediated degranulation in the NTAL-shRNA-transduced cells compared to both the LacZ-shRNA-transduced cells (Fig. 1C) and non-transduced cells (data not shown). Similarly, there was a significant inhibition of the SCF-enhanced degranulation in the NTAL-shRNA-transduced HuMCs (Fig. 1C). These data thus support our previous conclusion [3] that NTAL plays a role in the positive regulation of mast cell degranulation.

3.2. Differential phosphorylation of NTAL mediated by Kit and FcεRI

Due to its palmitoylation site, NTAL is largely resident within lipid rafts [16,17] and we observed that, following FcεRI aggregation or Kit activation, phosphorylated NTAL co-localized with a fraction of FcεRI and phosphorylated Kit, respectively, in these microdomains (data not shown). Having demonstrated that co-localization would permit both FcεRI and Kit to induce NTAL phosphorylation, we next determined the responsible tyrosine kinases. To investigate this, we utilized BMMCs from kinase-deficient mice, knowing that FcεRI and Kit phosphorylate NTAL in a similar manner in both HuMCs and mouse BMMCs [3]. These previous studies also suggested that FcεRI-mediated NTAL phosphorylation, in both cell types,

is dependent on Lyn and Syk and that Kit appeared to phosphorylate NTAL independently of these kinases. However, it is possible that Fyn, which is an important kinase for FcεRI-mediated mast cell activation [31] and which also is activated by Kit [32,33], may be responsible for the phosphorylation of NTAL induced by Kit. Thus, we examined the relative abilities of SCF to phosphorylate NTAL in $Fyn^{-/-}$ BMMCs, compared to responses of both antigen and SCF in wild type, $Lyn^{-/-}$ and $Lyn^{-/-}/Fyn^{-/-}$ BMMCs. As before [3], antigen-induced NTAL phosphorylation was substantially reduced in the $Lyn^{-/-}$ BMMCs (Fig. 2, A and B). Similarly, antigen-induced NTAL phosphorylation was defective in the $Lyn^{-/-}/Fyn^{-/-}$ (Fig. 2, C and D) but not the $Fyn^{-/-}$ BMMCs (Fig. 2, A and B). In contrast, SCF-induced NTAL phosphorylation was unaffected in the $Lyn^{-/-}$ (Fig. 2, A and B), the $Fyn^{-/-}$ (Fig. 2, A and B), and the $Lyn^{-/-}/Fyn^{-/-}$ BMMCs (Fig. 2, C and D). These data suggest that FcεRI mediates NTAL phosphorylation via Lyn and Syk [3], but not Fyn and, as previously described, Btk [3], whereas Kit phosphorylates NTAL independently of these kinases and thus would appear to directly phosphorylate NTAL.

3.3. Phosphorylation of NTAL constructs by Lyn, Syk, or Kit

As the above data indicated that FcεRI and Kit utilized different kinases to phosphorylate NTAL, we hypothesized that these kinases, in turn, may phosphorylate different tyrosine residues on NTAL. To avoid potential interpretational issues due to endogenous Lyn, Syk, and Kit, present in mast cells, and due to the lack of available phosphorylation-site-specific antibodies for NTAL, we adopted a transfection system to explore this hypothesis. A series of NTAL constructs were generated (Fig. 3A) and used to transfect 293T cells along with Lyn, Syk, or Kit constructs. The subsequent phosphorylation of specific tyrosine residues on NTAL was examined. Lyn, Syk, and Kit effectively phosphorylated wild type human NTAL ($NTAL^{10Y}$) in this system (Fig. 3B), thus confirming the data obtained from the studies conducted in the HuMCs and BMMCs. The phosphorylation of NTAL by Kit was, however, lower than that produced by either Lyn or Syk. In 293T cells transfected with NTAL in the absence of Lyn, Syk, and Kit, there was no evidence of NTAL phosphorylation (Fig. 3B), demonstrating that the phosphorylation of NTAL was entirely dependent on the co-transfected kinases.

Five of the 10 tyrosines in the cytosolic tail of NTAL are recognized as putative Grb2-binding sites, being part of a YXN motif [16,17]. It has been suggested that the terminal YXN Grb2-binding sites are critical for NTAL function [34,35]. To determine whether the Grb2-binding site tyrosines, $Y^{95}XN$, $Y^{118}XN$, $Y^{136}XN$, $Y^{193}XN$, and $Y^{233}XN$ (Fig. 3A), were primary substrates for Lyn, Syk, and Kit, these tyrosines were mutated to phenylalanines ($NTAL^{5Y\text{-}5F}$) (Fig. 3A) and this construct was used to transfect 293T cells along with Lyn, Syk, or Kit constructs. In Fig. 4, it can be seen that there was a slight reduction in the intensity of the phosphorylation of $NTAL^{5Y\text{-}5F}$ by both Lyn and Kit, when compared to the phosphorylation of $NTAL^{10Y}$. This suggests that these kinases phosphorylated both tyrosines contained in the YXN motifs as well as tyrosines outside of the YXN motifs. In contrast, the phosphorylation of $NTAL^{5Y\text{-}5F}$ by Syk was dramatically lower than that of $NTAL^{10Y}$, suggesting that the YXN putative Grb2-binding sites were the major targets for Syk. As expected, no phosphorylation of NTAL was observed with all three kinases when all 10 tyrosines were mutated to phenylalanines ($NTAL^{10Y\text{-}10F}$) (Figs. 3A and 4).

To further explore which NTAL tyrosine residues are targets for phosphorylation by Lyn, Syk, and Kit, the individual tyrosines in $NTAL^{10Y\text{-}10F}$ were mutated back to produce a series of NTAL constructs (Y^{40}, Y^{58}, Y^{84}, $Y^{95}XN$, Y^{110}, $Y^{118}XN$, Y^{119}, $Y^{136}XN$, $Y^{193}XN$, $Y^{233}XN$) (Fig. 3A), each encoding NTAL with a single tyrosine. These constructs, along with those of Lyn, Syk, or Kit, were used to transfect 293T cells and the subsequent phosphorylation of NTAL was examined. In Fig. 5, it can be seen that Lyn, Syk, and Kit produced markedly

different patterns of tyrosine phosphorylation of NTAL. As suggested by the experiments conducted with $NTAL^{5Y\text{-}5F}$, Lyn and Kit phosphorylated tyrosines outside of the YXN motifs and tyrosines contained within the YXN motifs (Fig. 5, A and B). Both kinases phosphorylated tyrosines Y^{110} and $Y^{118}XN$, whereas Lyn also phosphorylated tyrosines $Y^{95}XN$, $Y^{136}XN$, and Y^{119}. In contrast, Syk primarily phosphorylated the three terminal tyrosines contained in YXN motifs ($Y^{136}XN$, $Y^{193}XN$, and $Y^{233}XN$) (Fig. 5, A and B). With the exception of $Y^{136}XN$, which is phosphorylated by Lyn, the terminal YXN motifs appear not to be substrates for either Lyn or Kit.

3.4. Binding of phospholipase $C\gamma_1$ to phosphorylated NTAL constructs

We hypothesized that the differential pattern of tyrosine phosphorylation of NTAL produced by Lyn, Syk, or Kit may result in selective recruitment of signaling molecules to NTAL. To investigate this possibility, we conducted pull down studies. We initially generated phosphorylated NTAL by co-transfecting 293T cells with $NTAL^{10Y}$, $NTAL^{5Y\text{-}5F}$, and $NTAL^{10Y\text{-}10F}$ with Lyn, Syk, or Kit constructs. The phosphorylated NTAL was then immunoprecipitated with the NTAL Ab-beads to obtain phospho-NTAL-beads. To eliminate the possibility of non-specific interactions, we compared the responses obtained with these NTAL Ab-beads to those obtained with control Ab-beads. In addition, we also compared these responses to those obtained with $NTAL^{10Y}$ which had not been phosphorylated and with $NTAL^{10Y\text{-}10F}$ which had been transfected in the presence of Lyn, Syk, or Kit as above. The phospho-NTAL-beads and control Ab-beads were then incubated with lysates of the HMC-1 human mast cell line to capture associating molecules.

Using this approach, we determined that NTAL phosphorylation by Syk allowed the inducible association of $PLC\gamma_1$ with NTAL, whereas NTAL phosphorylated by Lyn or Kit only resulted in marginal association between these molecules (Fig. 6). This association was not observed when the lysates from the transfected 293T cells and HMC-1 cells were incubated with the control Ab-beads (Fig. 6A). Furthermore, when the 5 tyrosines associated with the Grb2-binding motifs ($NTAL^{5Y\text{-}5F}$) or all 10 tyrosines were mutated to phenylalanines ($NTAL^{10Y\text{-}10F}$), the ability of phosphorylated NTAL to bind $PLC\gamma_1$ was not observed either (Fig. 6, B and C). These data suggest that $PLC\gamma_1$ can associate with NTAL in a Syk-specific manner and that this binding is through one or more of the Grb2-binding sites following their phosphorylation by Syk. The markedly higher immunoprecipitation of Lyn with the NTAL-beads in the absence or presence of co-transfected kinases, compared to that observed using the control Ab-beads (Fig. 6A), suggests that Lyn may also constitutively associate with NTAL. The high association of Lyn observed in the third and fourth lanes was due to the greater expression of Lyn in these 293T cells.

To establish which specific phosphotyrosines within the Grb2-binding motifs of NTAL allow the association of $PLC\gamma_1$, the 10 NTAL constructs ($NTAL^{Y40}$ to $NTAL^{Y233XN}$) containing the single tyrosines were used to transfect 293T cells. To obtain maximal phosphorylation of NTAL constructs (data not shown), based on pilot studies, we used Syk alone with $Y^{136}XN$, $Y^{193}XN$, and $Y^{233}XN$, and Lyn/Syk/Kit with other mutant constructs. Following phosphorylation and immunoprecipitation, these NTAL constructs were again used to pull down $PLC\gamma_1$ from HMC-1 cell lysates. These studies revealed that $PLC\gamma_1$ primarily associates with $Y^{193}XN$ and $Y^{233}XN$ that are contained within the two terminal Grb2-binding sites (Fig. 6D). Although, as expected, we could co-immunoprecipitate Grb2 with NTAL from HuMCs triggered via aggregation of FcεRI (Fig. 7A), attempts to similarly co-immunoprecipitate $PLC\gamma_1$ with NTAL from antigen-stimulated HuMCs were unsuccessful. This may reflect the established difficulty in preserving $PLC\gamma_1$ in HuMC lysates under conditions required for immunoprecipitation [36]: a conclusion supported by our inability to detect $PLC\gamma_1$ in the cell lysates prior to capture (data not shown). Therefore, to establish that the association of

PLCγ_1 with NTAL may have relevance for mast cell function, we examined whether knock down of NTAL expression in HuMCs affected downstream calcium mobilization. As LAT has been established to directly bind PLCγ_1, we also wished to examine the calcium signal in LAT and NTAL/LAT- knock down HuMCs. To achieve this, HuMCs were transfected with NTAL- and/or LAT-targeted siRNA. This approach produced an approximately 70% decrease in NTAL expression, an approximately 60% decrease in LAT expression, and a significant decrease in antigen-induced and SCF-enhanced degranulation following both transfections [3].

As can be seen in Fig. 7B, both NTAL- and LAT-targeted siRNA markedly reduced the antigen-induced calcium signal, and the combination of both transfections produced a greater degree of inhibition than that observed with either transfection alone. In contrast, there was little difference in the SCF-induced calcium signal in the cells transfected with the LAT-targeted siRNA. The NTAL-targeted siRNA and combination of both approaches only produced a marginal reduction in this signal (Fig. 7C). However, the NTAL-targeted siRNA and the LAT-targeted siRNA, alone or in combination, attenuated the synergistic calcium response elicited by the concurrent challenge of the HuMCs with antigen and SCF (Fig. 7D). This was particularly the case in the initial phase of calcium mobilization which is PLCγ-dependent [37]. Taken together, the above data support the conclusion that NTAL regulates the antigen-induced and SCF-enhanced calcium signal required for degranulation following its association with PLCγ_1.

4. Discussion

The precise role that NTAL plays in mast cell activation remains unclear [3,16,19–21], however, our previous studies conducted in HuMCs [3] and those of others conducted in NTAL$^{-/-}$/LAT$^{-/-}$ BMMCs [20,21] have suggested a cooperative role for NTAL and LAT in the regulation of FcεRI-mediated and Kit-enhanced mast cell degranulation. Studies conducted in NTAL$^{-/-}$ mice [20,21] have suggested that NTAL can also negatively regulate antigen-dependent degranulation. Our current studies, in which we adopted a lentiviral transduction system [25] to stably knock down NTAL expression in HuMCs, do support the conclusion that NTAL is indeed required for optimal antigen-induced and SCF-potentiated degranulation in HuMCs (Fig. 1), although these studies certainly do not rule out the possibility that NTAL may also control a negative regulatory pathway for mast cell activation.

The phosphorylation of NTAL likely contributes to the activation of mast cells by providing docking sites for the recruitment of critical signaling molecules into the receptor-signaling complex resident within the lipid rafts. NTAL and LAT are targeted to these domains by virtue of their palmitoylation sites [2,16,17,38] and our studies indeed confirmed that the majority of NTAL and LAT expressed in mast cells is present within lipid rafts (data not shown). The observed co-localization of FcεRI and phosphorylated Kit with NTAL in these microdomains of activated mast cells (data not shown) would allow kinase activity regulated by these receptors to induce NTAL phosphorylation. It is clear from our studies conducted with mast cells derived from the bone marrows of Lyn$^{-/-}$, Fyn$^{-/-}$, and Lyn$^{-/-}$/Fyn$^{-/-}$ mice, however, that FcεRI- and Kit-dependent NTAL phosphorylation is regulated by different pathways (Fig. 2). Whereas FcεRI-dependent NTAL phosphorylation is mediated by Lyn (Fig. 2 and [3]) and Syk [3], but not Fyn (Fig. 2 and [21]), Kit apparently directly phosphorylates NTAL independently of these tyrosine kinases. This conclusion was supported by the co-transfection studies conducted in 293T cells, which revealed that Lyn, Syk and Kit can independently induce NTAL phosphorylation (Fig. 3B).

These studies also revealed that Lyn, Syk, and Kit differentially phosphorylated specific tyrosines within the NTAL molecule (Fig. 4). In this respect, the tyrosines phosphorylated by

Syk were primarily contained within the YXN putative Grb2-binding sites, whereas the tyrosines which are substrates for Lyn and Kit were contained both in these sites and at other sites outside of these motifs (Fig. 4). The sites within NTAL phosphorylated by Syk were mapped to the three C-terminal tyrosines (Y^{136}XN, Y^{193}XN, and Y^{233}XN) (Fig. 5) which have been demonstrated to be critical for the ability of NTAL to substitute for LAT function when transfected into LAT-deficient T cells [34]. Thus, the phosphorylation of these tyrosines by Syk is also likely to be crucial for the ability of NTAL to relay FcεRI-mediated signaling events for the promotion of degranulation in HuMCs.

By adopting a pull down approach, we determined that the tyrosine residues contained within the Grb2-binding motifs, when phosphorylated by Syk, allowed the specific association of PLCγ_1 (Fig. 6) and, furthermore, identified the two terminal Grb2-binding sites (Y^{193}XN and Y^{233}XN) as the principal sites responsible for this interaction. As these terminal Grb2-binding sites are not recognized as PLCγ_1-binding motifs, it is likely that the association of PLCγ_1 is indirect, via a cytosolic adaptor molecule linked directly to NTAL, or indirectly via Grb2. The identity of this potential adaptor molecule linking NTAL to PLCγ_1 is currently under investigation. This indirect binding may help to explain why we were unable to demonstrate co-immunoprecipitation of PLCγ_1 with NTAL in HuMCs. However, this may also be due to technical issues related to the fact that PLCγ_1 and PLCγ_2 are rapidly degraded under conditions required for co-immunoprecipitation in HuMC lysates [36]. Regardless, our demonstration of reduced calcium mobilization in the NTAL-knock down HuMCs (Fig. 7) does support a coordinated role for both NTAL and LAT in recruiting PLCγ_1 to the FcεRI-signaling complex leading to calcium mobilization, although LAT is likely to be the major TRAP that regulates this process [13,31].

In addition to PLCγ_1, our studies suggested that Lyn may also associate with NTAL but in a constitutive and non-inducible manner. NTAL possesses several RXXK motifs that, in other molecules, have been documented to bind to the SH3 domains of a number of signaling molecules including Src kinases [39–41]. Thus, Lyn may bind to NTAL in a similar manner. Finally, the abilities of Lyn, Syk, and Kit to phosphorylate Y^{110}IDP (Fig. 5), which is homologous to an auto-phosphorylation target sequence in Kit that has the potential to bind Src kinases and SHP1/2, suggest that other molecules may be recruited to NTAL following phosphorylation by Lyn, Syk, and/or Kit, but these have yet to be identified.

We can conclude from the above discussion that FcεRI-mediated phosphorylation of NTAL is regulated by a Lyn/Syk-dependent mechanism, whereas activated Kit directly phosphorylates NTAL. All three kinases phosphorylate distinctly different tyrosine residues on NTAL. The two terminal Grb2-binding sites, which are targets for Syk, allow the association of PLCγ_1, likely via an, as yet, unidentified cytosolic adaptor molecule. This interaction may explain the requirement for NTAL in the positive regulation of FcεRI-mediated degranulation. Although Lyn does not phosphorylate the two terminal Grb2-binding domains directly in vivo, it likely indirectly contributes to the phosphorylation of these residues by regulating the activity of Syk [42]. The lack of phosphorylation of these critical residues by Kit and its inability to phosphorylate LAT may help to explain why Kit does not effectively promote degranulation in the absence of antigen. The requirement of NTAL for the ability of Kit to potentiate antigen-induced degranulation may be a consequence of binding of a critical signaling molecule to phosphorylated Y^{110} or the apparent constitutive binding of Lyn to NTAL. In this respect, we have previously demonstrated that the ability of Kit to potentiate FcεRI-mediated responses is lost in $Lyn^{-/-}$ BMMCs [5].

Finally, the differential phosphorylation of NTAL by specific kinases and the selective binding of associating signaling molecules described in this study provide evidence for a flexibility in the function of TRAPs not previously appreciated. Other TRAPs such as LAT, LIME, and

PAG also possess multiple tyrosines in their cytosolic tails, which may similarly be potential targets for phosphorylation by multiple different tyrosine kinases [43]. LAT, for example, has a similar structure, and perhaps overlapping function, to NTAL. Thus, it would be of interest to examine whether LAT and other TRAPs are differentially phosphorylated by multiple kinases and to determine the manifestations of these events in cell activation. This may have important implications for cell function, as a variety of signaling molecules with different regulatory roles may be selectively recruited to lipid rafts depending on the particular kinase activated. Furthermore, the sequence of signaling events controlled by TRAPs may also be highly regulated depending on the chronology of kinase activation following receptor aggregation.

Acknowledgements

We thank Dr. Michael A. Beaven (NHLBI/NIH) for his assistance in the calcium studies and for his helpful discussions.

This work was supported by the NIAID and NIAMS Intramural Program within the National Institutes of Health and a Japan Society for the Promotion of Science research fellowship for Japanese Biomedical and Behavioral Research at National Institutes of Health to S. I.. J.S. and V.H. are supported by Academy of Sciences of the Czech Republic (AVO20550514).

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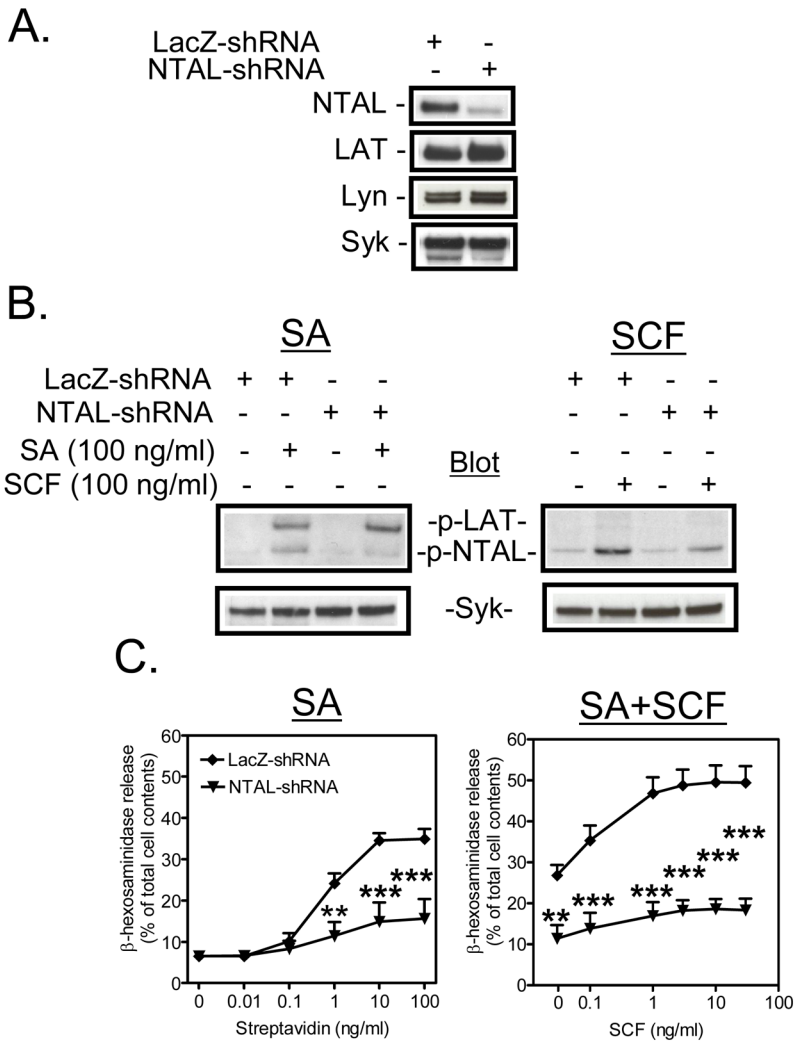

Fig. 1.

FcεRI- and Kit-mediated NTAL phosphorylation in human mast cells and its requirement for degranulation. (A) Effect of NTAL-targeted shRNA on signaling proteins expressed in HuMCs. (B) Phosphorylation of NTAL and LAT in HuMCs following FcεRI aggregation and Kit activation and inhibition of NTAL phosphorylation by NTAL-targeted shRNA. Sensitized cells were challenged with streptavidin (SA) (100 ng/ml) or SCF (100 ng/ml) for 60 s, and then cell lysates were probed with an antibody which recognizes both phosphorylated LAT and phosphorylated NTAL. (C) Effect of NTAL-targeted shRNA on FcεRI-dependent and Kit-enhanced degranulation of HuMCs. Cells were challenged with the indicated concentrations of SA and/or SCF for 30 min. The blots are representative of n=3 and the release experiments are the means +/− S.E.M. of n=4 individual experiments conducted in duplicate. The stars designate that the release observed in the NTAL-shRNA-transduced cells is significantly lower than that observed in the LacZ-shRNA-transduced cells as assessed by matched paired 2 tailed T test (**: 0.005<p<0.01; ***: p<0.005). In the above experiments, LacZ-targeted shRNA was used as a control.

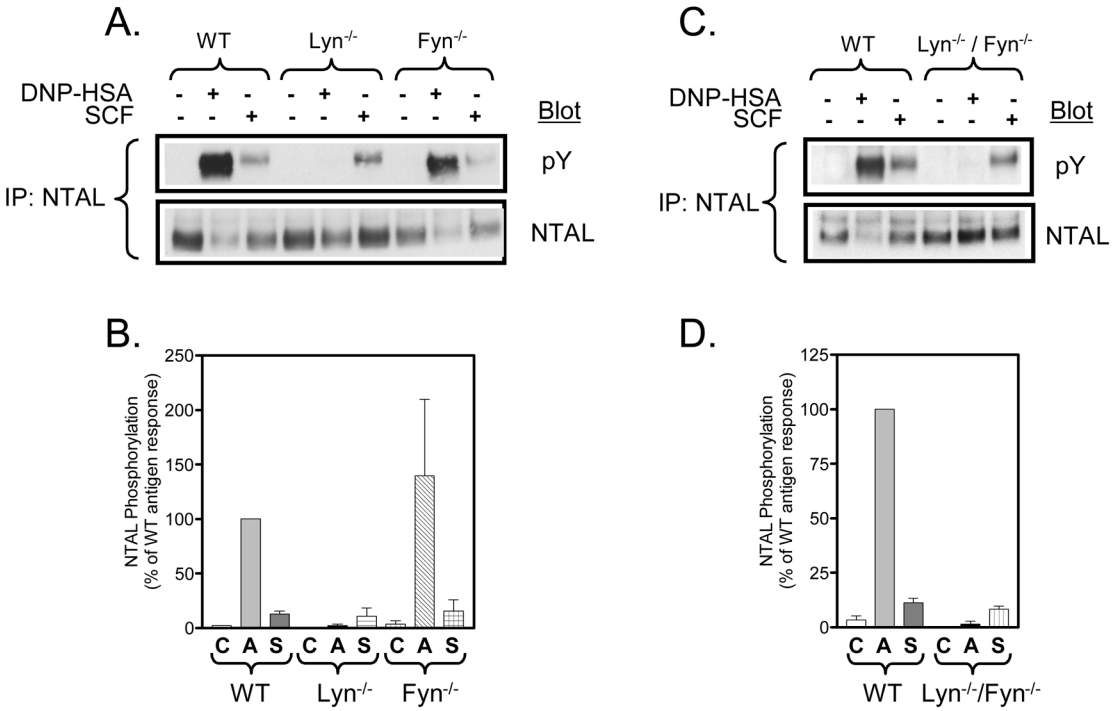

Fig. 2.

Phosphorylation of NTAL in $Lyn^{-/-}$ or $Fyn^{-/-}$ BMMCs (A and B), and $Lyn^{-/-}/Fyn^{-/-}$BMMCs (C and D) challenged with antigen (DNP-HSA; 100 ng/ml) or SCF (100 ng/ml) for 2 min. The data are either representative (A and C) or means +/− S.E. (B and D) of at least n=3. The data in (B) and (D) were generated by scanning the blots in (A) and (C) then normalizing to the NTAL blots. C: control; A: antigen; S: SCF. The lighter total NTAL bands in the heavily phosphorylated samples in this, and other figures, represents epitope masking as described [3].

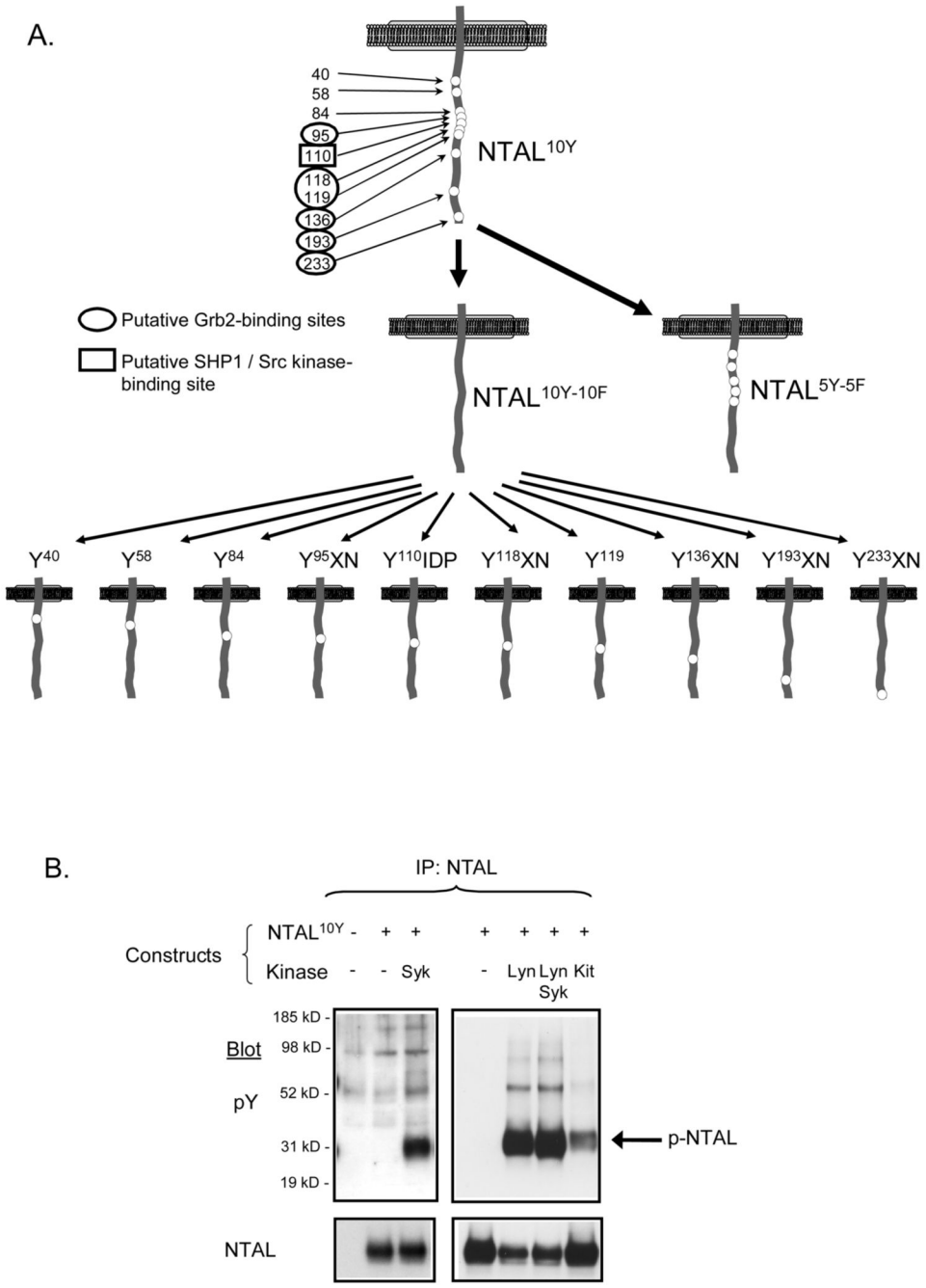

Fig. 3.

Phosphorylation of wild type NTAL constructs by Lyn, Syk, or Kit constructs following transfecting 293T cells. (A) Diagrammatic representation of the wild type and mutant NTAL constructs used in this study. (B) NTAL phosphorylation following co-transfection of 293T cells with the indicated kinases. p-: phospho-.

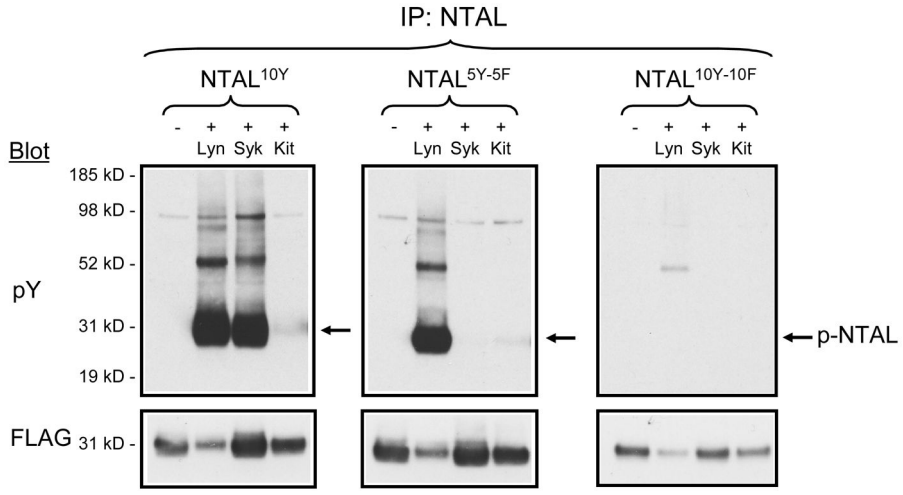

Fig. 4.

Phosphorylation of mutant NTAL constructs by Lyn, Syk, or Kit in 293T cells. The left panel shows phosphorylation of wild type NTAL ($NTAL^{10Y}$) by Lyn, Syk, or Kit; the middle panel shows phosphorylation of the NTAL construct in which the 5 tyrosines contained within the YXN putative Grb2-binding sites have been mutated to phenylalanines ($NTAL^{5Y-5F}$); and the right panel shows the lack of phosphorylation of the NTAL construct in which all 10 tyrosines have been mutated to phenylalanines ($NTAL^{10Y-10F}$). p-: phospho-. The data shown are representative of n=2–3.

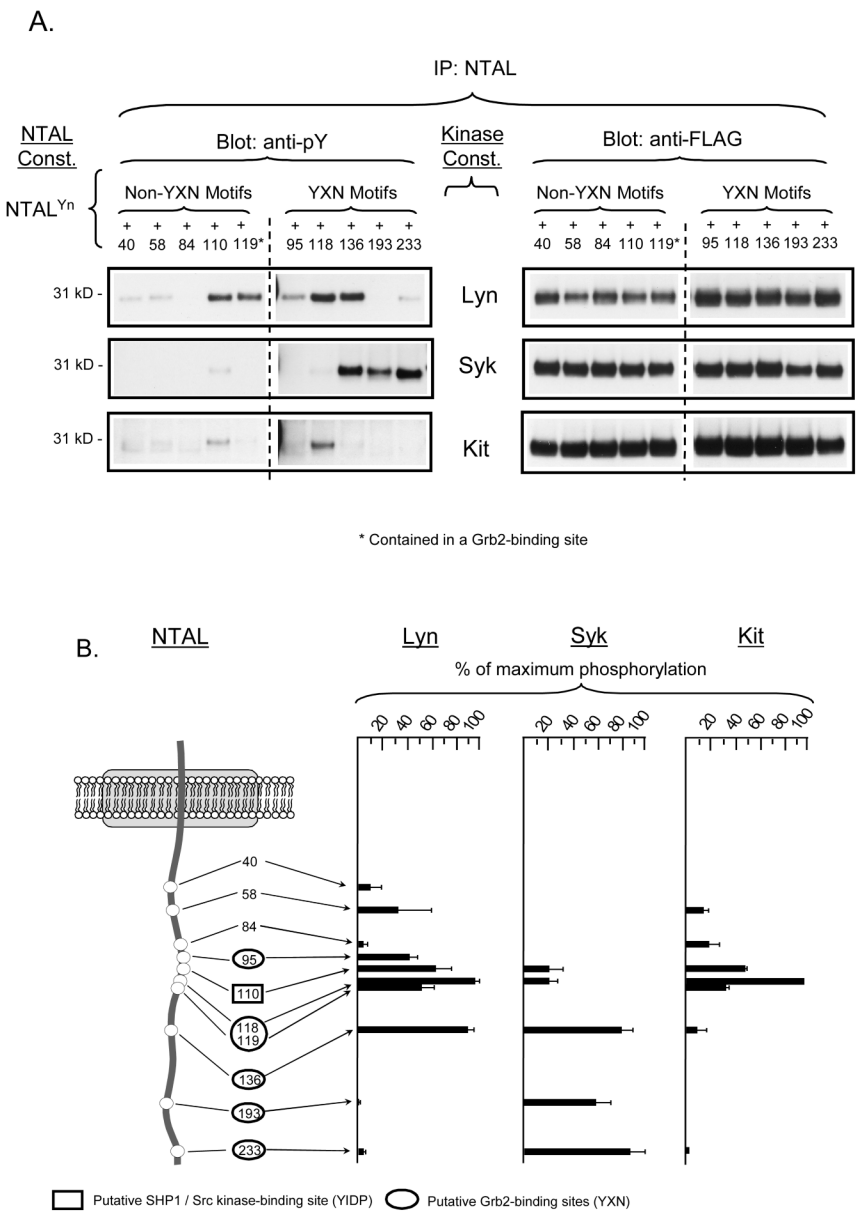

Fig. 5.

Phosphorylation of the individual tyrosines within the NTAL molecule by Lyn, Syk, or Kit. (A) These data show the phosphorylation of the constructs described in Fig. 4A in which only single tyrosines are expressed. The data presented in (B) were obtained by scanning the blots in (A), normalizing these data to the blots obtained with an anti-FLAG antibody, and then calculating as a percentage of the maximal phosphorylation. The data are either representatives (A) or means +/− S.E. (B) of n=3.

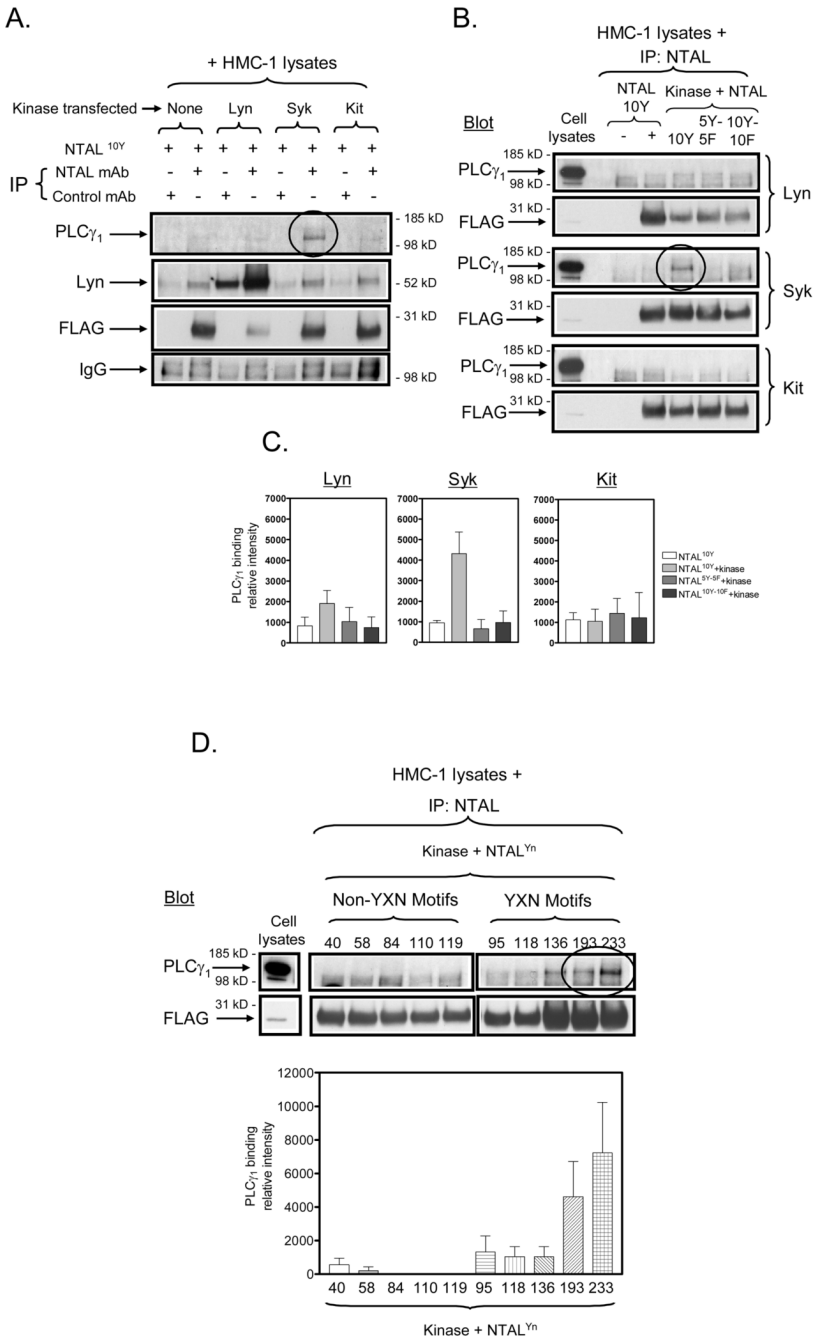

Fig. 6.

Association of human mast cell signaling molecules with NTAL. 293T cells were transfected with NTAL constructs and Lyn, Syk, or Kit constructs. Cell lysates were incubated with NTAL Ab-beads or control Ab-beads as indicated. Pull down studies were then conducted by adding HMC-1 cell lysates as described in the Materials and Methods. (A) Association of PLCγ_1 and Lyn with wild type NTAL following phosphorylation by Lyn, Syk, or Kit and then immunoprecipitation with an anti-NTAL or control Ab-beads. (B and C) Association of PLCγ_1 with indicated NTAL constructs phosphorylated by Lyn, Syk, or Kit. (D) Association of PLCγ_1 with specific phosphorylated tyrosine residues within the NTAL molecule. For optimal NTAL phosphorylation, Syk construct was used for $Y^{136}XN$, $Y^{193}XN$, and $Y^{233}XN$,

and Lyn/Syk/Kit in combination were used for other mutant constructs, based on pilot studies. The graphs in (C) and (D) were generated by scanning the blots (B and D), then normalizing the data to the FLAG blots. The data are representatives (blots) or means +/− S.E. (graphs) of n=3–4.

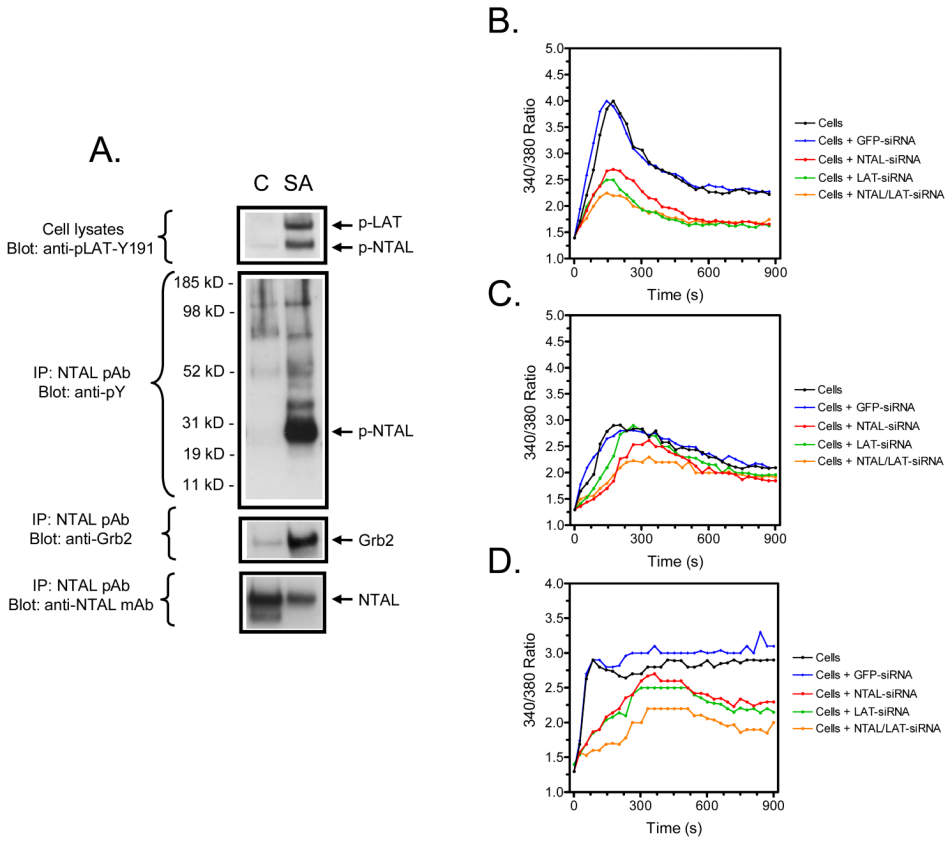

Fig. 7.

NTAL-mediated responses in HuMCs. (A) Phosphorylation and association of Grb2 with NTAL in HuMCs following antigen stimulation (SA; streptavidin; 100 ng/ml) for 30 s. (B–D) Calcium mobilization in HuMCs transfected with NTAL- and/or LAT-targeted siRNA followed by antigen (B), SCF (C), or antigen+SCF (D) stimulation. In these studies, oligonucleotides targeting GFP were used as controls. The data are representatives of n=3.