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**Regulatory functions of the transmembrane adaptor
protein NTAL in activation of mast cells**

Regulační funkce transmembránového adaptorového proteinu
NTAL při aktivaci žírných buněk

Ph.D. Dissertation in Biochemistry

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Declaration:

I hereby declare, that presented thesis has been composed entirely by myself and all used sources are quoted. Neither presented thesis nor any part of it has been submitted to obtain any academic degree.

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Declaration of co-authors:

This thesis contains three original papers published in peer-reviewed journals. I, as a corresponding author of all these papers, hereby declare, that Magda Tůmová, a first author or co-author of the publications, substantially contributed to all of them. The studies were performed in our research team and she actively participated in all stages of the work from study design, performing experiments to writing manuscript. Her contribution in the experimental part of the first article “Regulation of Ca^{2+} signaling in mast cells by tyrosine-phosphorylated and unphosphorylated non-T cell activation linker” published in The Journal of Immunology, 2007, consisted of the cloning of rat NTAL cDNA and TNF- α detection. In the second article, “Topography of signaling molecules as detected by electron microscopy on plasma membrane sheets isolated from non-adherent mast cells” published in The Journal of Immunological Methods, 2007, she was responsible for isolation and cultivation of bone marrow-derived mast cells and flow cytometric analysis of Fc ϵ RI receptor internalization. All the experience she obtained during the compilation of the two articles she used in the third publication “The transmembrane adaptor protein NTAL signals to mast cell cytoskeleton via the small GTPase Rho” published in The European Journal of Immunology, 2010, in which she is the first author. In this publication, she isolated and cultivated bone marrow-derived mast cells, analyzed cell adherence and calcium levels and participated in all other experiments apart from chemotactic assays.

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This thesis describes the work carried out at the Department of Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague during my Ph.D. study. All the obtained experimental data were summarized in three publications that were published in peer-reviewed journals.

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ABSTRACT

Mast cells originate from hematopoietic pluripotent stem cells present in bone marrow. They do not circulate, but are spread throughout a body and reside in all vascularized tissues. Mast cells as a part of innate immune system are also able to influence adaptive immune system and are effectors in IgE-mediated allergic and inflammatory diseases. Mast cells possess high affinity receptor for IgE (FcεRI). Aggregation of the receptor triggers signaling cascades which lead to the release of preformed and *de-novo* synthesized effector molecules. Early phases of mast cell activation include tyrosine phosphorylation of numerous substrates and formation of signaling assemblies (also called signalosomes). Important structural components of the signalosomes are transmembrane adaptor proteins. One of them is the transmembrane adaptor protein NTAL (Non-T cell Activation Linker), which becomes rapidly tyrosine-phosphorylated upon FcεRI triggering and serves as a positive or a negative regulator of FcεRI signaling.

To be able to study fine topography of NTAL and other plasma membrane components in mouse bone marrow-derived mast cells (BMMCs), we first developed a new method of plasma membrane sheets isolation from non-adherent cells. The method is based on adsorption of the cells to ultraclean glass cover-slips, isolation of the membrane sheets, their labeling from cytoplasmic and/or exoplasmic side with target-specific antibodies followed by gold-conjugated secondary antibodies, and examination under electron microscope. When we compared formation of FcεRI clusters after antigen-mediated activation, we found that large receptor clusters typical for rat basophilic leukemia (RBL) cells were not formed on BMMCs. Surprisingly, downstream adaptor proteins, LAT and NTAL, were localized in isolated domains in both cell types. These results suggest that care should be taken to generalize data from a particular source of mast cells.

Using RBL cells with enhanced or reduced NTAL expression we showed importance of NTAL for tyrosine-phosphorylation of several substrates and formation of signalosomes in early stages of FcεRI-mediated signaling. At later stages, we found that NTAL regulates the activity of store-operated calcium channels independently of enhanced NTAL tyrosine phosphorylation. Cells with modified levels of NTAL exhibited changes in morphology and amount of filamentous actin levels after FcεRI triggering. These observations led us to further investigate the role of NTAL in signaling towards actin cytoskeleton. We found, that NTAL-deficient mouse BMMCs exhibited reduced spreading on fibronectin, enhanced filamentous actin depolymerization in response to antigen alone or in combination with SCF, and enhanced migration towards antigen. We also analyzed activity of small GTPases Rac and Rho. We reported for the first time that adaptor protein NTAL signals to actin cytoskeleton via RhoA and ROCK in mouse mast cells in response to FcεRI activation.

SOUHRN

Žírné buňky pocházejí z kmenových buněk kostní dřeně. Tyto buňky necirkulují v krvi, ale nachází se v celé řadě tkání, kde jsou často umístěny v blízkosti cév. Žírné buňky jsou součástí přirozeného imunitního systému, ale zároveň ovlivňují i adaptivní imunitní systém a také se účastní alergických a zánětlivých reakcí. Žírné buňky mají na svém povrchu vysokoafinitní receptor pro IgE (FcεRI). Prokřížení tohoto receptoru spouští kaskádu reakcí, která vede k uvolnění předpřipravených a nově syntetizovaných efektorových molekul. Časná fáze aktivace těchto buněk zahrnuje tyrosinovou fosforylaci četných substrátů a formování signálních komplexů (v angl. "signalosomes"). Důležité strukturní prvky těchto komplexů jsou transmembránové adaptorové proteiny. Jedním takovým proteinem je adaptorový protein NTAL (Non-T cell Activation Linker), který je velmi rychle fosforylován na tyrosinových zbytcích po prokřížení FcεRI a který slouží jako pozitivní, ale i negativní regulátor signálních drah FcεRI.

Abychom byli schopni studovat podrobnou topografii proteinu NTAL a dalších molekul plazmatické membrány u žírných buněk odvozených z kostní dřeně (BMMC), nejprve jsme vyvinuli novou metodu izolace listů plazmatické membrány neadherentních buněk. Tato metoda je založena na adsorpci buněk k ultračistému mikroskopickému krycímu sklu, izolaci membránových listů, jejich značení specifickými protilátkami z vnitřní či vnější strany plazmatické membrány, následném značení sekundárními protilátkami konjugovanými s částicemi zlata a zkoumání elektronovým mikroskopem. Při porovnání velikosti shluků FcεRI po aktivaci buněk antigenem jsme u BMMC nenalezli velké shluky typické pro buňky krysí bazofilní leukemie (RBL). Avšak adaptorové proteiny LAT a NTAL, které jsou aktivovány po prokřížení FcεRI, byly lokalizovány v rozdílných oblastech u obou typů buněk. Tyto výsledky naznačují, že zobecňování výsledků dosažených zkoumáním určitého typu žírných buněk by mělo být kriticky zváženo.

Pomocí RBL buněk se zvýšenou nebo redukovanou expresí proteinu NTAL jsme ukázali, že tento protein je důležitý pro tyrosinovou fosforylaci řady substrátů a pro formování signálních komplexů v časných fázích aktivace žírných buněk způsobené prokřížením FcεRI. V pozdějších fázích aktivace reguloval NTAL aktivitu vápníkových kanálů zvaných SOC (store-operated calcium channels), ačkoliv jeho fosforylace nebyla zvýšena. Buňky s pozměněnou expresí proteinu NTAL vykazovaly morfologické změny a odlišné hladiny polymerního aktinu po aktivaci FcεRI v porovnání s původními RBL buňkami. To nás dále vedlo ke zkoumání úlohy adaptorového proteinu NTAL v signálních drahách vedoucích k přestavbě aktinového cytoskeletu. BMMC myši postrádající protein NTAL vykazovaly snížené rozprostírání se na fibronektinu a zvýšenou depolymeraci aktinových vláken po aktivaci žírných buněk antigenem nebo společným působkem antigenu a SCF. Migrace vyvolaná antigenem u těchto buněk byla zvýšena. Dále jsme analyzovali aktivitu Rac a Rho GTPáz. Tato práce je prvním dokladem, že adaptorový protein NTAL hraje klíčovou roli v signální dráze vedoucí k aktinovému cytoskeletu při aktivaci myších žírných buněk vyvolané prokřížením FcεRI a to skrze GTPázu RhoA a kinázu ROCK.

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1. INTRODUCTION

Mast cells were firstly described by Paul Ehrlich in 1879. These cells of innate immune system are 10-15 μm in diameter and contain prominent granules, which can be stained by metachromatic dyes. Their precursors are found in bone marrow and migrate into peripheral tissues where they undergo final maturation. Mast cells do not circulate, but they are spread throughout a body and reside in all vascularized tissues. Mast cells are rather long-lived cells that are able to re-enter cell cycle and proliferate after appropriate stimulation. Mast cells as a part of innate immune system are able to influence adaptive immune system and are effector cells in IgE-mediated allergic and inflammatory diseases.

1.1. MAST CELL DEVELOPMENT

1.1.1. Origin

Mast cells originate from hematopoietic pluripotent stem cells present in bone marrow (Kirshenbaum and others, 1991). Mast cell precursors migrate into wide spectrum of tissues where they undergo terminal differentiation under the local conditions. Mature mast cells normally do not circulate in the blood (Mekori and Metcalfe, 2000). The specific sites where mast cells reside include thymus, lymph nodes, spleen, skin and heart, and the mucosa of the genitourinary, respiratory, and gastrointestinal tracts. As mature mast cells settle near blood vessels, nerves, epithelia, near smooth muscle cells and mucus-producing glands (Brown and others, 2008; Galli and others, 2005a) they are strategically located to be the first cells of the immune system reacting to different environmental stimuli such as allergens and other antigens as well as to infectious pathogens (Galli and others, 2005b; Mekori and Metcalfe, 2000).

1.1.2. Mast cell precursors

Before mast cell precursors were identified, the studies of mast cells were restricted to their isolation from various tissues at low yield and with viability and functions affected by the isolation methods. More convenient methods of mast cell precursor's isolation and their culturing under specific conditions were later established.

Human mast cells precursors were first described as CD34⁺ progenitors in bone marrow (Kirshenbaum and others, 1991). However this marker was not found on precursors of murine mast cells, which were identified as Thy1^{lo} c-kit^{hi} pluripotent cells in fetal blood (Rodewald and others, 1996). The CD34 is a cell surface sialomucin widely used as phenotypic marker for immature hematopoietic stem cells (HSC). CD34 is absent on mature mast cells from human precursors. Interestingly, although mRNA for CD34 was not detected in murine progenitor cell lines, high expression levels were found on mature murine mast cells, bone marrow-derived mast cells (BMMCs) and peritoneal mast cells (Drew and others, 2005a; Drew and others, 2002). Later, function of CD34 was described to block adhesion of mast cells (Drew and others, 2005b) and thus a question arises whether the difference in its expression between human and mouse mast cells could lead for example to different localization, migration and local proliferation of these cells.

Other marker of mast cell precursors, both human and murine, is Kit (also known as c-kit). This tyrosine kinase receptor is essential for all subtypes of mast cells as it was proved by the absence of mast cells in mice that bear functionally defective Kit or its ligand - stem cell factor (Kitamura and Go, 1979; Kitamura and others, 1978). Kit is also expressed on HSC and many other progenitor cells, but its expression is lost during differentiation. Only a low number of cell types retain Kit in mature state like mast cells, dendritic cells and natural killer (NK) cells (Ray and others, 2008).

Up to day, no universal specific marker for mast cell precursors was found. A specific combination of surface markers is usually used to select the mast cell progenitors with respect to their origin e.g. expression of $\beta 7$ integrin in addition to cell-surface markers c-kit^{low} CD34⁺ Fc ϵ RI⁺ in the intestine (Hallgren and Gurish, 2007) or CD34⁺ CD13⁺ c-kit⁺ Fc ϵ RI⁻ in mouse bone marrow (Jamur and others, 2005). The problem with selection of the right phenotypic marker is complicated as documented by recent papers dealing with the heterogeneity of mast cells, their progenitors and culture condition influence on mast cell phenotype (Andersen and others, 2008; Hallgren and Gurish, 2007).

1.1.3. Mast cell subtypes

According to local microenvironment, mast cells can be very heterogeneous in biochemical composition and subsequently physiological functions. In the past, rodent mast

cells were divided into 2 subtypes, mucosal (MMC) and connective tissue type mast cells (CTMC), that can be generally distinguished according to cell localization, morphology and secretory granule content (Puxeddu and others, 2003). MMC granules contain chondroitin sulfate and little histamine, whereas CTMC granules contain heparin and large amount of histamine and serotonin. Granules of both types further consist of enzymatically active proteases that are characteristic for each subtype (Welle, 1997). Interestingly, it was shown that mucosal and connective tissue type mast cells may interchange by microenvironment alteration (Kitamura and others, 1987). Mucosal mast cells were found to be T cell-dependent as they were absent in athymic mice (Ruitenbergh and Elgersma, 1976).

Although experiments with human mast cells suggested also heterogeneity within these cells, it was not possible to distinguish the same groups as for rodent cells. The classification of human mast cells was based on protease composition. MC_T contain tryptase and MC_{TC} contain tryptase and chymase and also carboxypeptidase and cathepsin G. MC_T were described as T cell-independent and contain IL-4,5,6 in their granules whereas MC_{TC} are T cell-dependent and contain only IL-4 (Metcalf and others, 1997; Welle, 1997).

1.2. MAST CELL FUNCTION

1.2.1. Degranulation

Mast cells react to activation signals by release of effector molecules from their granules. These molecules are divided into 3 groups: preformed mediators, newly synthesized lipid mediators and cytokines.

The preformed mediators, stored in secretory granules, released within minutes after activation are histamine, serine proteases (tryptase and chymase), carboxypeptidase A, and proteoglycans - heparin and chondroitin sulfate E.

Those mediators synthesized *de novo* upon cell stimulation are lipid mediators (PAF), prostaglandins (PGI₂) and leucotrienes (LTC₄) (Prussin and Metcalfe, 2003; Stone and others, 2010).

The major cytokine produced by mast cells is TNF- α . Also other immune cells (NK, T cells, macrophages, neutrophils) can synthesize this cytokine, but only mast cells are able to store TNF- α . Therefore, mast cells secrete TNF- α immediately after activation and also

later after its synthesis (Gordon and Galli, 1990). Other cytokines produced by mast cells are IL-3, 4, 5, 6, 10, 13, granulocyte-macrophage colony-stimulating factor (GM-CSF). Mast cells also release some chemokines as macrophage inflammatory protein 1 α (MIP1 α) and IL-8 (Prussin and Metcalfe, 2003; Stone and others, 2010).

1.2.2. Interaction with other immune cells

Mast cells were accepted for long as cells of innate immune system. However, there are lots of cells of innate system that are potential to influence adaptive immune system by their content of broad spectrum of mediators or by expression of specific cell surface molecules. Mast cells have both and thus appear to be the crucial player in this role. The ambition of this chapter is not to fully describe all mast cell interactions, but to show some examples to illustrate broad spectrum of mast cell effects. The regulatory roles of mast cells are extensively studied and still are not completely understood.

Immature dendritic cells as well as mast cells are located in the barrier tissue with connection to the outside environment. It was shown, using a model of contact hypersensitivity (CHS), that activation of mast cells is essential for migration of dendritic cells. Activated mast cells are essential for the sensitization phase of CHS in which immature dendritic cells take up antigen and migrate into draining lymph nodes where they mature and subsequently activate T cells. IgE^{-/-} mice and mast cell-deficient mice, failed to display immature dendritic cells migration out of the epidermis. This observation correlated with decreased production of mRNAs encoding IL-1b, IL-6, monocyte chemoattractant protein 1 (MCP-1), mouse mast cell protease-6, and TNF- α (Bryce and others, 2004). Similar study with mast cell-deficient mice and TNF- α ^{-/-} mice showed also reduced migration of hapten-bearing skin dendritic cells into draining lymph nodes 24 h after epicutaneous administration of hapten in naive mice. Migration was repaired in mast cell-deficient mice which had been engrafted with wild type (WT) but not TNF- α ^{-/-} BMMCs, indicating that mast cell TNF- α is a key mediator (Suto and others, 2006).

Mast cell function in Experimental autoimmune/allergic encephalomyelitis (EAE) - the rodent model of multiple sclerosis was extensively studied. Mast cell-deficient W/W(v) mice exhibited significantly reduced disease incidence, delayed disease onset, and decreased mean clinical scores when compared with their WT congenic littermates (Secor and others,

2000). It was shown that peripheral CD4 and CD8 T cell activation, including IFN- γ production and increases in CD44 and CD11a expression, was attenuated in mast cell-deficient (W/W^v) mice after myelin oligodendrocyte glycoprotein 35–55 priming when compared to WT animals. Reduced infiltrates of activated T cells in the central nervous system were also observed (Gregory and others, 2005). IgE- and antigen-stimulated mast cells were also shown to enhance T cell activation through secretion of soluble TNF and direct cell-cell interactions between mast cell OX40L and T cell OX40 (Nakae and others, 2006). Piconese et al. showed direct interaction between mast cells and regulatory T cells (Treg). Mast cells reverted the anergic and suppressive phenotype of Tregs and promoted an IL-17-producing T cells-skewed immune response. This process depends on IL-6 production and OX40/OX40L interaction (Piconese and others, 2009).

Other report that mast cells have crucial regulatory role in immune system is that activation of adenosine receptors on mast cells led in production of IL-4 and IL-13 and subsequently in IgE synthesis by B cells (Ryzhov and others, 2004).

Mast cell-derived TNF- α plays a critical role in innate immunity in mice by recruiting neutrophils to sites of enterobacterial infection. *In vitro* studies revealed that mast cell activation and TNF- α release were achieved by the specific coupling of the type 1 fimbrial adhesion, FimH, on enteric bacteria with the mast cell surface molecule, CD48 - a glycosylphosphatidylinositol-anchored molecule (Malaviya and others, 1999). Later, it was discovered, using mast cell-deficient mice (W/W^v) and their normal congenic control mice, that *in vivo* mast cell-derived leukotrienes LTB₄ and LTC₄ are responsible for neutrophil influx and subsequent bacterial clearance during *E. coli*-induced peritonitis (Malaviya and Abraham, 2000).

1.2.3. Fc ϵ RI signaling

Mast cells are important effectors in IgE-dependent immune reactions. Expression of the high affinity receptor for Immunoglobulin E (Fc ϵ RI) on mast cells appears early in their development, but apparently not earlier than they undergo lineage-commitment (Rodewald and others, 1996). Cross-linking of IgE bound to the Fc ϵ RI results in activation of mast cells and culminates in release of preformed as well as newly synthesized mediators.

1.2.3.1. Receptor structure

FcεRI is a cell-surface receptor, a member of the multichain immune recognition receptor (MIRR) superfamily, which binds Fc portion of IgE with high affinity. There are two forms of FcεRI – a tetrameric and a trimeric complex. Tetrameric receptor (Figure 1) is expressed on mast cells and basophils and consists of an extracellular α-chain, which is responsible for binding of IgE, a membrane tetraspanning β-chain and a disulfide-linked homodimer of γ-chain (αβγ₂). The trimeric form of the FcεRI is found on monocytes, dendritic cells and Langerhans cells and lacks β-chain (Kinet, 1999). The particular chains are held together in the plasma membrane through a combination of hydrophobic and electrostatic noncovalent interactions involving both covalently and noncovalently bound lipids (Kinet and others, 1985a and 1985b).

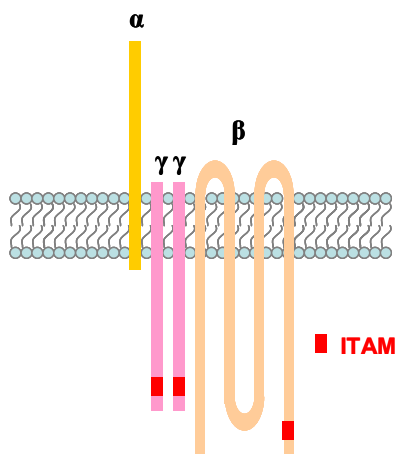


Figure 1. FcεRI structure. FcεRI is a tetrameric complex formed by an IgE-binding α-subunit, a signal-amplifying β-subunit, and a homodimer of disulfide-linked γ-subunits with immunoreceptor tyrosine based activation motifs (ITAMs) in the cytoplasmic domains.

The α-chain comprises of 2 extracellular immunoglobulin-like domains that binds single IgE, a transmembrane domain containing conserved aspartic-acid residue and a short cytoplasmic tail with no apparent signaling role. The α-chain possesses seven N-linked glycosylation sites and is heavily glycosylated. The glycosylation is required in the endoplasmic reticulum to mediate the proper interaction between the α-chain and the endoplasmic reticulum folding machinery (Kraft and Kinet, 2007).

The β-chain functions as a signal amplifier of early and late mast cell responses and FcεRI expression. On the other hand, there is a truncated β-isoform (β_T) that is produced by

alternative splicing and acts as an inhibitor of FcεRI surface expression by inhibiting α-chain maturation (Dombrowicz and others, 1998; Donnadieu and others, 2003).

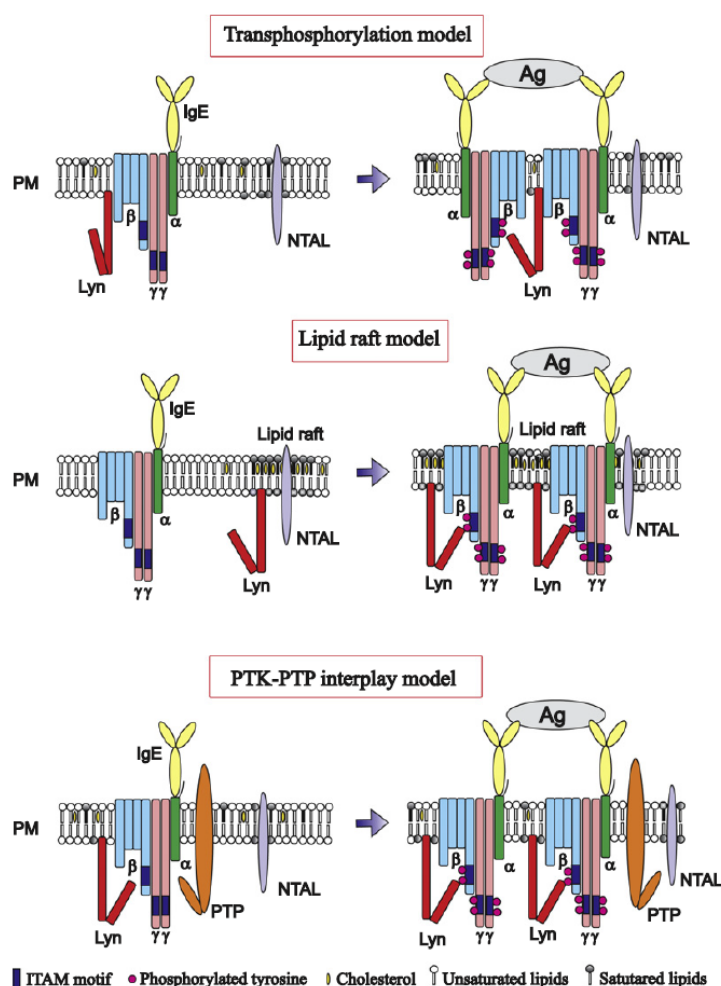
Both, β- and γ-subunits possess immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic parts, which consists of six conserved amino acid residues spaced precisely over a ~ 26-amino acid sequence. These motives are responsible for signal transduction. Upon FcεRI aggregation, receptor associated kinases phosphorylate ITAMs by transphosphorylation which is important for association with other intracellular signaling molecules and signal propagation (Cambier, 1995). There are also other antigen receptors that utilize β- and γ-chains, e.g. IgG receptor III - FcγRIIIA (Kurosaki and others, 1992).

1.2.3.2. Early signaling events

Antigen aggregation of IgE-occupied FcεRI leads to rapid tyrosine phosphorylation within ITAMs of β- and γ-subunits. The ITAM phosphorylation is dependent on Lyn (Lck/Yes-related novel tyrosine kinase) - the Src family tyrosine kinase, which is associated with FcεRI in both, non-activated and activated cells (Pribluda and others, 1994; Eiseman and Bolen, 1992). The precise mechanism of the early signaling step remains unclear. Lyn is anchored to the inner leaflet of the plasma membrane through myristate and palmitate chains. It was shown, that if Lyn lacks the fatty acylation in both, palmytoylation and myristoylation sites, it is not anchored to the plasma membrane and it is not capable to initiate FcεRI phosphorylation (Kovářová and others, 2001). The phosphorylated tyrosines in ITAMs of FcεRI β- and γ-subunits are then binding sites for phosphotyrosine recognizing Src-homology 2 (SH2) domain of additional molecules of Lyn and Syk (spleen tyrosine kinase) (Kihara and Siraganian, 1994; Sada and others, 2001). Lyn is also important for phosphorylation of Syk. It was reported, that treatment of rat basophilic leukemia cells (RBL) with PP1 – a specific inhibitor of Lyn kinase inhibited also activation of Syk and downstream signaling after aggregation of FcεRI (Amoui and others, 1997). Syk binds predominantly to the ITAM of γ-subunit of FcεRI (Shiue and others, 1995).

Immediately after FcεRI activation, another Src kinase Fyn is phosphorylated. Fyn can be coimmunoprecipitated with β-subunit of FcεRI. However, Fyn is not important for FcεRI phosphorylation. Fyn kinase signaling pathway is essential for mast cell degranulation and is not dependent on Lyn. Fyn-dependent signals and degranulation were enhanced in

Lyn-deficient mast cells, but calcium response was inhibited. Contrarily, Fyn-deficiency led to impaired degranulation, but Lyn-mediated signals and calcium were normal. This observation showed a cross-talk between Fyn and Lyn signaling pathways in the late phases at the level of protein kinase C (PKC) and calcium response (Parravicini and others, 2002). Recently, another Src family kinase Hck was found to regulate mast cell degranulation in part via suppressing negative regulatory role of Lyn kinase (Hong and others, 2007).



(Bugajev and others, 2010)

Figure 2. Models of FcεRI phosphorylation.

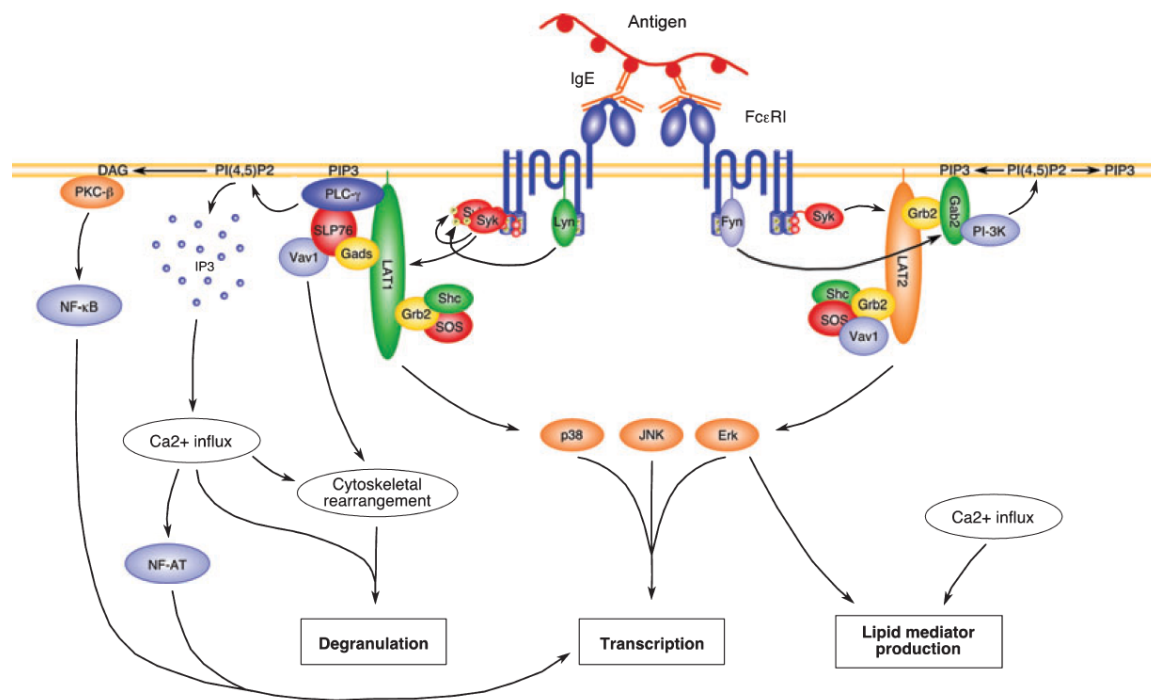
Transphosphorylation model presumes that active Lyn is constitutively associated with non-phosphorylated FcεRI; upon FcεRI aggregation, Lyn phosphorylates adjacent receptors in the aggregate. Lipid raft model postulates that FcεRI and Lyn are localized in different plasma membrane microdomains; after aggregation FcεRI is translocated to lipid rafts where it is phosphorylated by lipid raft-associated Lyn kinase. PTK-PTP interplay model assumes that PTPs set the threshold of FcεRI tyrosine phosphorylation; after FcεRI triggering there is a shift in the PTK-PTP steady state in favor of PTKs.

There are three proposed models of the early signaling in mast cells recently reviewed (Figure 2, Bugajev and others, 2010). First model is based on constitutively associated Lyn with monomeric FcεRI. After aggregation of FcεRI, Lyn phosphorylates the adjacent

receptor in the aggregate. Second model suggests that FcεRI and Lyn are situated in specific isolated microdomains in the membrane and aggregation of the receptor brings them to close proximity and thus Lyn phosphorylates the receptor. The third model depends on the idea that not only protein tyrosine kinases (PTKs) kinases but also protein tyrosine phosphatases (PTPs) are present in the signaling complex.

1.2.3.3. Downstream signaling

After binding of Syk to γ -subunit of FcεRI, Syk undergoes a conformational change which leads to phosphorylation of multiple substrates and a cascade of downstream signaling resulting in mast cell mediator release (Figure 3). A large number of proteins is downstream of Syk including phospholipase C γ 1 (PLC γ 1), PLC γ 2, LAT, NTAL, Vav, MAP kinase, JNK (Jun N-terminal kinase), the focal adhesion kinase (FAK), Pyk2, paxillin, the adhesion molecule PECAM-1(CD31), Cbl, ras-GAP, SLP-76 (SH2-domain containing leukocyte



(Alvarez-Errico and others, 2009)

Figure 3. Scheme of FcεRI signaling. FcεRI aggregation by cell surface-bound IgE antibody interaction with a multivalent antigen leads to the phosphorylation of the receptor immunoreceptor tyrosine-based activation motifs (ITAMs) by Lyn kinase. Then Syk kinase is recruited to the receptor and phosphorylates LAT (also known as LAT1) and NTAL (LAT2) adaptor proteins that are important organizers of signaling complexes necessary for diverse signaling pathways.

protein of 76 kDa) and HS-1 (Siraganian and others, 2002; Tkaczyk and others, 2004).

Receptor aggregation also leads to Fyn-dependent phosphorylation of Grb2-associated binder (Gab2) which is positive effector of phosphatidylinositol 3-kinase (PI3K) (Parravicini and others, 2002). The Gab2 and p85 subunit of PI3K interaction results in membrane localization of PI3K which catalyzes conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). After PI3K activation, phosphoinositide-dependent protein kinase 1 (PDK1) and subsequently PKC δ are activated. Fyn pathway is required for degranulation and maintenance and/or amplification of the Ca²⁺ signal (Siraganian, 2003; Kraft and Kinet, 2007).

The PIP3 attracts many proteins containing pleckstrin homology (PH) domains to the membrane, such as Btk, PLC γ 1, PLC γ 2 and PDK1. Btk (Bruton tyrosine kinase) is a kinase that phosphorylates PLC γ and enhances its activity. Tyrosine-phosphorylated PLC γ 1 and PLC γ 2 cleaves PIP3 into inositol-1,4,5-trisphosphate (IP3), and 1,2-diacylglycerol (DAG). Binding of IP3 to the membrane of endoplasmic reticulum leads to initial increase of intracellular Ca²⁺. The depletion of Ca²⁺ from intracellular stores induces opening of Ca²⁺ release-activated Ca²⁺ channels in the plasma membrane and strong Ca²⁺ influx into the cytoplasm. This is essential for mast cell degranulation – fusion of granules with membrane and actin cytoskeleton reorganization. These reactions lead to activation of many adaptors including Vav, Grb2 (Growth factor receptor-bound protein 2), SOS (Son of sevenless), activation of Rho GTPases, extracellular-signal-regulated kinase (ERK), JNK and p38 mitogen-activated protein kinase (MAPK) pathway, histamine release, synthesis of new cytokines, cytoskeleton reorganization (Siraganian, 2003; Alvarez-Errico and others, 2009).

Reversible phosphorylation of proteins is important for control of protein activity and subcellular localization and depends not only on PTKs but also partly on PTPs. SH2-containing inositol 5'-phosphatase (SHIP) binds to Fc γ RIIB (low-affinity receptor for IgG) expressed on mast cells and to β - and γ -subunits of Fc ϵ RI and thus SHIP translocates into plasma membrane and inhibits Fc ϵ RI-induced degranulation. Another possibility is that SHIP is located in the membrane through Shc/SHIP/PKC complex. SHIP plays a crucial role in inflammatory cytokine secretion by dampening of PIP3-mediated activation of NF κ B. Also adherence of mast cells to fibronectin is restrained by SHIP (Sly and others, 2003).

Transmembrane protein tyrosine phosphatase CD45 positively regulates activation of Lyn and probably other Src kinases and is crucial to antigen-induced degranulation of mast cells (Murakami and others, 2000). CD45 was also found to dephosphorylate Janus family kinases (JAKs) and thus negatively regulates JAK/STAT (Signal transducers and activators of transcription) signaling in response to multiple cytokines, including IL-3, IL-4, EPO and IFN- α . CD45-deficiency led to enhanced cytokine-triggered proliferation, differentiation and antiviral activities (Irie-Sasaki and others, 2001). Phosphatase and tensin homologue deleted on chromosome ten (PTEN) opposes PI3K activity. PTEN functions as a negative regulator of Fc ϵ RI-induced calcium flux, degranulation and cytokine production (Furumoto and others, 2006). SH2 domain-containing non-receptor PTPs, SHP-1 (Src homology region 2 domain-containing phosphatase-1) and SHP-2, have negative effect on Fc ϵ RI-mediated mast cell signaling. However, downstream function of SHP-1 may be required to activate pathways needed for gene transcription or translation. SHP-2 binds to Grb2, Gab2, and Fc ϵ RI. These two phosphatases were suggested as Syk activity regulators through dephosphorylation of tyrosine residues within ITAM motif of Fc ϵ RI γ (Gilfillan and Rivera, 2009).

1.2.4. Kit signaling

Fc ϵ RI-mediated activation of mast cells is not an isolated pathway. Not only IgE, but also SCF (Stem cell factor) was described as a potent effector of mast cell activation via its tyrosine kinase receptor - Kit. SCF is a hematopoietic growth factor that promotes survival, proliferation, and differentiation of hematopoietic cells (Broudy, 1997).

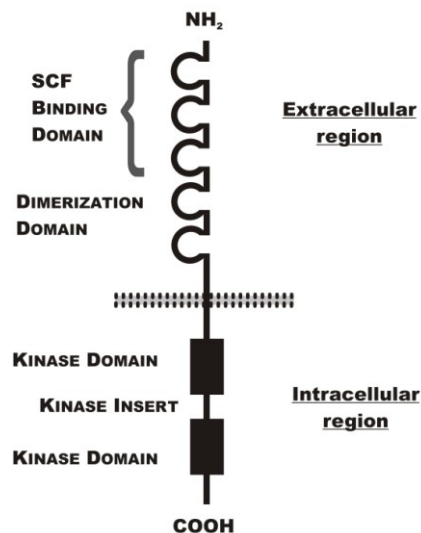


Figure 4. Kit receptor structure. Extracellular part of Kit contains five Ig-like loops, a single transmembrane domain and a split cytoplasmic kinase domain.

SCF was shown to influence mast cell chemotaxis, and adhesion and also to be an enhancer of degranulation mediated by FcεRI (Vosseller and others, 1997).

Kit is a type III transmembrane receptor protein-tyrosine kinase (RPTK) which is related to platelet-derived growth factor receptor (PDGF receptor) and the macrophage colony stimulating factor receptor (M-CSF-R). Its extracellular part comprises of five immunoglobulin-like domains. The first three domains bind SCF, the fourth is important for receptor dimerization and the fifth has unknown function. Kit has two catalytic domains separated by a kinase insert and inhibitory cytoplasmic juxtamembrane domain in the intracellular part (Figure 4). Binding of SCF to Kit leads to dimerization of the receptor and Kit becomes autophosphorylated at tyrosine residues (Tyr568 and Tyr570). Then other residues are phosphorylated and serve as docking sites for SH2 domain-containing molecules (Linnekin, 1999; Roskoski, 2005; Reber and others, 2006). Binding of specific signaling proteins to the phosphorylated tyrosine residues leads to mast cell responses (Figure 5).

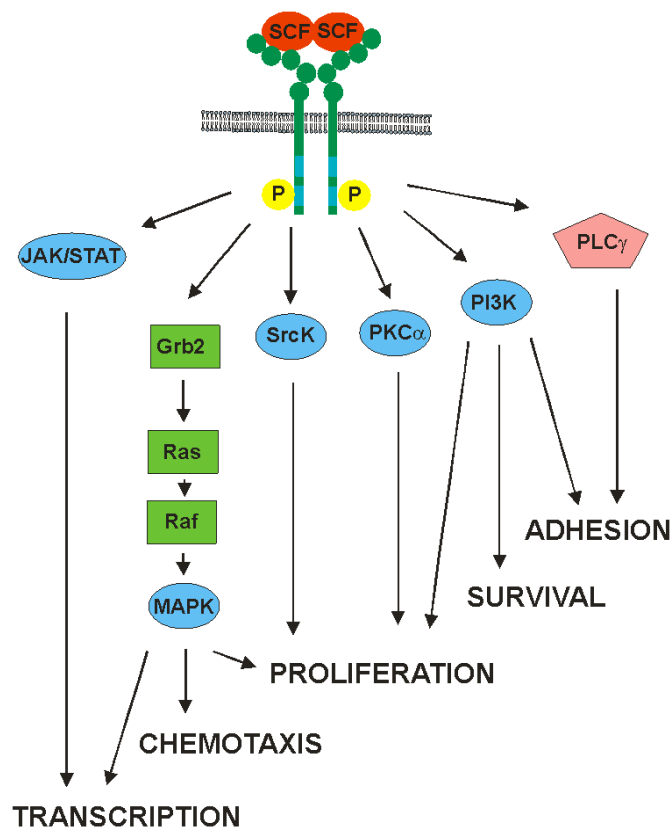


Figure 5. Scheme of Kit signaling. Binding of SCF to its receptor results in Kit dimerization and phosphorylation of specific tyrosine residues within Kit cytoplasmic domain with subsequent binding of signaling proteins and activation of different signaling pathways.

Src family kinases are widely utilized by many signaling pathways. It is not surprising that they play role also in Kit activation. It was demonstrated that Lyn is phosphorylated after Kit activation, coimmunoprecipitates with Kit and binds to the juxtamembrane region of Kit (Tyr568 and Tyr570) (Linnekin and others, 1997). Lyn contributes not only to phosphorylation of Kit but also to phosphorylation of JNKs and STAT3 (Shivakrupa and Linnekin, 2005). Another member of Src family kinases, Fyn was found to bind to tyrosine residue Tyr567 (corresponding to human Kit residue Tyr568) in mouse BMMCs (Timokhina and others, 1998). The necessity of Kit residue Tyr568 for Src kinases was shown in porcine aortic endothelial cells. There was no Src, Fyn and Yes kinase activity in cells expressing Tyr568Phe mutant Kit (Lennartsson and others, 1999). Src kinase and also phosphatidylinositol 3-kinase signaling pathways converge to activate Rac1 and JNK. This pathway is important for SCF-induced mast cell proliferation (Timokhina and others, 1998). Src family kinases mediate also Ras/MAP kinase activation and c-fos induction after Kit activation (Lennartsson and others, 1999).

Grb2 is a specific binding partner for phosphorylated Tyr703 in the kinase insert and phosphorylated Tyr936 in the C-terminal tail of Kit. This provides a link to the Ras/MAPK pathway (Thömmes and others, 1999). In addition to its positive role, Grb2 causes Kit degradation through recruitment of Cbl (protein from family of ubiquitin E3 ligases) to Kit, leading to ubiquitination of Kit followed by internalization and degradation (Sun and others, 2007).

Kit activation also leads to activation of PI3K which then generates lipid second messengers. Substitution of tyrosine residue with phenylalanine (Y719F, in the kinase insert of Kit) in BMMCs revealed importance of association of regulatory subunit p85 of PI3K with Kit and led to diminished mitogenic response and fibronectin induced adhesion (Serve and others, 1995). PI3K mediates SCF-induced proliferation via activation of Akt and subsequent phosphorylation of Bad, a pro-apoptotic molecule (Blume-Jensen and others, 1998). It was also shown using dominant negative mutants of Rac1 and JNK that PI3K signaling pathway after Kit activation leads to cell proliferation through these two molecules (Timokhina and others, 1998). PI3K was also reported to be required for survival and growth of IC2 mast cell line bearing mutant Kit (KitD814Y) both *in vitro* and *in vivo*. This mutation is found in

patients with mastocytosis, leukemia, and germ cell tumors and PI3K inhibition led to reduction of tumors (Shivakrupa and others, 2003).

Further studies presented activation of JAK/STAT pathway that transmits signals from outside the cell to the cell nucleus either to start or to repress transcription of target genes. Weiler et al. described association of JAK2 with Kit and JAK2 phosphorylation after SCF activation (Weiler and others, 1996). STAT1 is associated with Kit after binding of SCF to its receptor in hematopoietic cell lines, normal hematopoietic progenitor cells and fibroblasts transfected with Kit. This interaction is mediated by binding of the SH2 domain of STAT1 protein to specific phosphotyrosyl residues on activated Kit (DeBerry and others, 1997). Later, STAT5 was identified as STAT protein activated via SCF signaling pathway. STAT5 is a key regulator of SCF and IL-3 signaling during mast cell ontogeny. Its absence results in improper mast cell development, proliferation and survival (Brizzi and others, 1999; Shelburne and others, 2002).

Cellular proteins known to associate with Kit include also PLC γ 1 (Rottapel and others, 1991). PLC γ induces phosphatidylinositol (PI) 4,5-bisphosphate hydrolysis and production of DAG and IP3. These two messengers induce PKC activation and intracellular Ca²⁺ release, respectively (Rhee and Bae, 1997). PLC γ 1 binds to Y728 of Kit as was showed by Gommerman et al. that studied PI3K and PLC γ stimulation through Kit mutants. KitYF728 failed to stimulate PLC γ phosphorylation or mobilize Ca²⁺ in 32D myelomonocytic cells (Gommerman and others, 2000).

SHP-1 is a member of the PTP family. PTPs are known to regulate a broad spectrum of signaling pathway. SHP-1 negatively regulates Kit signaling by association with the receptor. It was also demonstrated that activated Kit is tyrosine dephosphorylated via this association (Yi and Ihle, 1993). Later, Kozlowski et al. showed Tyr569 within the Kit juxtamembrane region as the binding site for SHP-1 and also Tyr567 as the binding site for the structurally similar SHP-2 tyrosine phosphatase. Mutation of the two tyrosine residues of Kit led to hyperproliferative responses after SCF stimulation (Kozlowski and others, 1998). SHIP belongs to a family of inositol 5'-phosphatases. Comparing BMMCs from SHIP^{+/+} and SHIP^{-/-} mice suggested that SHIP prevents SCF to trigger degranulation by hydrolyzing PIP3 (Huber and others, 1998).

Another negatively regulating molecule of Kit signaling is PKC which phosphorylates Kit on serine residues Ser741 and Ser746 of the kinase insert and this phosphorylation reduced Kit kinase activity (Blume-Jensen and others, 1993).

1.2.5. Lipid rafts

It has been proposed that the critical regions for signal propagation and membrane trafficking are lipid rafts (Lingwood and Simons, 2010). Membrane regions with properties presumably corresponding to lipid rafts have been isolated as detergent resistant membranes (DRMs) or glycosphingolipid-enriched microdomains (GEMs). For isolation of DRMs, the cells are solubilized in nonionic detergents such as Triton X-100 at 4°C and glycolipid-enriched low density complexes are separated using sucrose density gradient centrifugation. It should be noted, however, DRMs isolated by this procedures are unlikely to correspond to lipid rafts found in living cells (Brown, 2006). The model of lipid rafts is based on asymmetric lipid bilayer with sphingomyelin and glycosphingolipids in the exoplasmic leaflet and glycerolipids (e.g. phosphatidylserine and phosphatidylethanolamine) in the cytoplasmic leaflet. Cholesterol is present in both leaflets (Simons and Ikonen, 1997). It has been suggested that lipid rafts selectively concentrate glycosylphosphatidylinositol-anchored proteins on their outer leaflet and proteins anchored by saturated palmitoyl or myristoyl groups and cholesterol-binding proteins on the cytoplasmic leaflet (Silveira E Souza and others, 2011). It was shown using patterned lipid bilayer technique that inner leaflet lipid-anchored proteins redistribute selectively with clustered IgE–FcεRI under conditions of cell activation and this process depends on actin polymerization. Surprisingly, outer leaflet components associated with lipid rafts do not detectably coredistribute with these inner leaflet components (Wu and others, 2004).

First suggestion of lipid raft importance for antigen-mediated activation of mast cells came from a study which showed enhanced Lyn association with these regions after FcεRI aggregation. Also FcεRI was shown to become insoluble in Triton X-100 after its cross-linking, but not in steady state (Field and others, 1995). Another approach using electron microscopic examination of gold-labeled membrane sheets also confirmed redistribution of FcεRI after its aggregation into large membrane domains (Wilson and others, 2004). However these domains did not correspond to those enriched in Lyn and other typical lipid

raft components. This suggested heterogeneity of membrane microdomains in the plasma membrane. Other experiments showed that Lyn kinase activity is higher in lipid rafts than outside of these regions (Young and others, 2003). On the other hand, there is evidence that lipid rafts are not essential for the initial steps of FcεRI pathway. Mutation of palmitoylation site of Lyn, which is still anchored in the membrane, led to reduced sublocalization to lipid rafts. However, such mutated Lyn kinase was capable to initiate FcεRI signaling (Kovářová and others, 2001). Similar results were obtained by cholesterol depletion which had small effect on FcεRI phosphorylation while Lyn was almost excluded from lipid rafts (Surviladze and others, 2001). Thus, the role of lipid rafts in initiation of FcεRI signaling remains to be determined.

Some other key players in mast cell activation, adaptor proteins, were suggested to be localized in lipid rafts where they act as scaffolding molecules recruiting crucial signaling components into the receptor complex. For example, LAT and NTAL adaptors possess palmitoylation site which targets these molecules into DRMs (Zhang and others, 1998b; Brdička and others, 2002; see also below).

After subcellular fractionation of detergent treated cells, Kit was also shown to redistribute into DRM fraction after SCF activation. This probably allows interaction with Src family kinases which were localized in this fraction already in unstimulated cells. Kit kinase activity resulted in recruitment of the regulatory subunit p85 of PI3K into DRMs where the catalytic PI3-K subunit p110 was constitutively found. Based on these results, it was suggested that Kit localization into lipid rafts is required for efficient activation of PI3K pathway and Kit-mediated proliferation (Jahn and others, 2007).

The combined data suggest that lipid rafts could play an important role in mast cell biology. Nevertheless, there are some questions about their structure and function that remain elusive (Munro, 2003).

1.2.6. Other mast cell receptors

Mast cells can directly cooperate in host defense through Toll-like receptors (TLR). Wide variety of TLR-mediated mast-cell activation events occur in response to bacterial-, fungal- and viral-associated signals and results in release of inflammatory cytokines. TLRs recognize various microbial products. The role of TLR2 and TLR4 was studied in mast cell

response to lipopolysaccharide (LPS) and peptidoglycans derived from Gram-negative and Gram-positive bacterium, respectively. TLR1 and TLR6 form functional heterodimers with TLR2, thereby enabling responses to many mast-cell activators, including peptidoglycans and lipopeptides. TLR9 produces several cytokines in response to CpG-containing oligodeoxynucleotides. TLR7 and TLR8 are crucial for responses to single-stranded RNA sequences that are associated with viral infection (Marshall, 2004).

In normal conditions, mast cells express a low-affinity receptor for IgG – FcγRII. The expression of FcγRI (the high-affinity receptor for IgG) and FcγRIII (a low affinity receptor for IgG) are induced after exposure to IFN-γ. Activation of these receptors leads to degranulation, lipid mediators, chemokines and cytokines release. The FcγRII exists in 3 different subtypes A, B and C. FcγRIIA and C bears activation motif ITAM, whereas FcγRIIB possesses an immunoreceptor tyrosine-based inhibition motif (ITIM) which attenuates ITAM signaling. Coaggregation of FcγRIIB and FcεRI recruits SHIP, SHP-2 and SHP-1 phosphatases and it results in inhibition of FcεRI-induced degranulation (Marshall, 2004; Unkeless and Jin, 1997; Siraganian, 2003).

Complement receptors on mast cells represent another way how these cells respond to pathogens. Mast cells have C3aR, C5aR, CR2, CR4 and CR5. C3a and C5a are potent chemoattractants for mast cells and C3a also induces degranulation and chemokines CCL2 and CCL5 production in human mast cells. C3a binding to C3aR was shown to inhibit FcεRI signaling through direct inhibition of phosphorylation of β-subunit of FcεRI (Dawicki and Marshall, 2007).

1.3. ADAPTOR PROTEINS

Recent observation of a new group of signaling molecules led to novel view of signal propagation during FcεRI signaling. This group is named transmembrane adaptor proteins (TRAPs). Generally, adaptor proteins are molecules without enzymatic activity. They contain multiple protein–protein and protein–lipid interacting domains. They have the capability to link proteins to other proteins, to the plasma membrane or to intracellular organelles. Thus large signaling complexes can be formed and cellular localization of signaling molecules can be determined.

1.3.1. Transmembrane adaptor proteins

The TRAPs are almost exclusively found in immunoreceptor signaling, and no homologous proteins are known in other systems (Csiszár, 2006). These proteins are characterized by a short extracellular domain, a single transmembrane domain and a cytoplasmic tail with various motifs serving as anchors for cytoplasmic signaling molecules. The common intramolecular interaction structural domains of TRAPs are SH2 and phosphotyrosine-binding (PTB) domains which recognize phosphorylated tyrosine residues, SH3 domain which binds to prolin-rich regions and pleckstrin homology (PH) domain which mediates association with specific phospholipids (Myung and others, 2000).

1.3.1.1. LAT

Linker for activation of T cells (LAT, also termed LAT1 by The Human Genome Organisation (HUGO)) was originally described as a protein of a 36 – 38 kDa rapidly phosphorylated after T cell receptor activation. LAT is a first TRAP described and also seems to be the most important. LAT possesses a typical TRAP structure - a very short extracellular part (only 4 amino acids (aa)), a 24 aa transmembrane region and a long cytoplasmic tail of 213–234 aa containing nine conserved tyrosine residues. LAT also have a conserved juxtamembrane CxxC palmitoylation motif, which directs LAT into lipid rafts. LAT is expressed not only on T cells, but also on pre-B cells, NK cells, platelets and mast cells (Hořejší, 2004).

Activation of Syk leads to phosphorylation of LAT (Zhang and others, 1998a). When phosphorylated, LAT recruits a number of signaling molecules containing SH2 domain. Grb2 and Gads (Grb2-related adaptor downstream of Shc) are known as LAT-binding proteins. After binding to LAT, Grb2 and Gads form complexes with SOS and SLP-76, respectively. Phosphorylated LAT also associates with PLC γ 1 (Zhang and others, 2000).

Importance of LAT for mast cell-signaling was described in study with BMMCs isolated from LAT-deficient mice. The mast cells lacking LAT showed normal growth and development whereas tyrosine phosphorylation of SLP-76, PLC γ 1, and PLC γ 2 and calcium mobilization were dramatically reduced. LAT-deficient BMMCs exhibited profound defects

in activation of MAPK, degranulation, and cytokine production after FcεRI cross-linking (Saitoh and others, 2000). However, this reduce was not complete as it was seen in T cells.

Mutational analysis of LAT identified critical tyrosine residues involved in the recruitment of PLCγ1 (Y136) and Grb2 and Gads (Y175, Y195 and Y235) in T cells (Zhang and others, 2000). As the mechanism by which LAT controls FcεRI signaling is thought to be similar to that in T cells, this mutational analysis was also performed in mast cells with similar results. When cells expressing mutated LAT in the four tyrosine residues (Y136F, Y175F, Y195F and Y235F) were tested, no reconstitution of the response in LAT^{-/-} mast cells is shown, indicating that function of LAT is wholly dependent on these tyrosines. Partial reconstitution is observed with other LAT mutants (Saitoh and others, 2003). Later, again studying these four tyrosine residues in mast cells not only positive but also negative regulatory role for LAT was suggested (Malbec and others, 2004). Two of these tyrosine residues were then shown as SHIP binding sites. Binding to phosphorylated Y136 and Y235 of LAT occur via the SH2 domain of SHIP (Roget and others, 2008).

1.3.1.2. NTAL

Non-T cell activation linker (NTAL, also termed LAB or LAT2) is a transmembrane adaptor protein structurally and evolutionarily related to LAT. It is the product of a previously identified *WBSCR5* gene with unknown function. Similarly to LAT, NTAL consists of a short (6 aa) extracellular region, a single transmembrane domain followed immediately by the CxxC palmitoylation motif and a cytoplasmic domain containing 10 tyrosine motifs (Figure 6). Mouse NTAL is 40 aa shorter. NTAL is expressed in B cells, NK cells, monocytes, and mast cells but not in resting T cells (Hořejší, 2004).

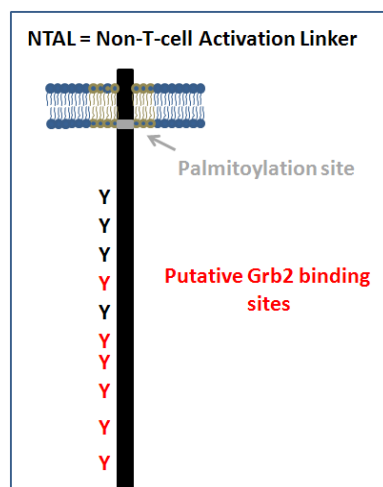


Figure 6. Structure of adaptor protein NTAL. NTAL possesses a short extracellular domain, a transmembrane domain, a juxtamembrane CXXC palmitoylation motif and a long cytoplasmic tail containing 10 potential phosphorylation sites known as tyrosine-based signaling motifs (Y). Five of the tyrosine motifs are potential Grb2 binding sites (in red).

NTAL becomes rapidly tyrosine-phosphorylated by Src- and Syk-family kinases upon cross-linking of the B cell receptor (BCR) or of high-affinity Fc γ and Fc ϵ -receptors of myeloid cells and then associates with the cytoplasmic signaling molecules Grb2, SOS1, Gab1, and c-Cbl. In contrast to LAT, association of NTAL with PLC γ or another cytoplasmic adaptor protein, SLP-76 (or its B cell analogue SLP-65) were not found. Expression of NTAL in LAT-deficient Jurkat T cells partially rescued T cell receptor (TCR)-mediated signaling (Brdička and others, 2002).

To map the tyrosine phosphorylation sites in NTAL, mutation of three membrane-distal tyrosines (Y136, Y193, and Y233) were studied. Single mutation of any residue reduced NTAL phosphorylation in T cells. Mutation of all three tyrosines virtually abolished NTAL phosphorylation, indicating a crucial role of these three tyrosines. These tyrosine residues are all within Grb2 binding sites. Mutation of any of them alone reduced the Grb2-NTAL interaction, whereas mutation of two or three abolished Grb2 binding. Wild type NTAL and single tyrosine mutants were capable of rescuing thymocyte development in LAT^{-/-} mice, but they had a low expression of TCR (Koonpaew and others, 2004).

The role of NTAL in mast cells was studied using NTAL-deficient mice. This study suggested a negative regulatory role for this adaptor molecule as antigen-mediated degranulation was increased in the absence of NTAL. Development of NTAL^{-/-} mast cells was normal under in vitro and in vivo conditions. NTAL^{-/-} mice compared with WT mice exhibited an enhanced passive anaphylactic response. Another evidence for negative regulatory role of NTAL was enhanced Fc ϵ RI-mediated tyrosine phosphorylation of Syk, LAT, ERK and PLC γ and also increased activity of PLC γ and PI3K. NTAL-deficiency was associated with enhanced calcium response after antigen-activation. This was explained by enhanced LAT accumulation in lipid rafts and stronger LAT phosphorylation resulting in more PLC γ recruited in to the signaling complex and elevated IP3 production with consequent higher calcium levels and increased degranulation (Volná and others, 2004). At the same time, other study dealing with NTAL-deficient mice was published (Zhu and others, 2004). This work confirmed hyperresponsiveness to stimulation via the Fc ϵ RI.

Interestingly, NTAL phosphorylation was also observed after SCF-induced activation of human mast cells. Unlike in antigen-induced activation of mast cells, SCF induced only

phosphorylation of NTAL, LAT phosphorylation was not detected. When SCF and antigen were added simultaneously, degranulation and NTAL phosphorylation were enhanced whereas LAT phosphorylation was not. Contrary to murine mast cells, reduction of NTAL expression via small interfering (si) RNA method led to decreased degranulation after SCF- or antigen-induced activation of human mast cells (Tkaczyk and others, 2004).

However, all studies showed that mast cells deficient in both adaptors, LAT and NTAL, evinced a more severe reduction of antigen-induced signaling than in LAT^{-/-} cells suggesting also positive regulatory role of NTAL adaptor protein. Thus both NTAL and LAT appeared to be indispensable for optimal degranulation in response to FcεRI aggregation. Other evidence that NTAL and LAT could substitute for each other was showed by the increased presence of NTAL in lipid rafts of LAT^{-/-} cells and vice versa. However, partitioning into lipid rafts in detergent-solubilized cells indicated that they differ in physical properties. Majority of phosphorylated LAT was found in high density fractions whereas majority of phosphorylated NTAL was in low density fractions of sucrose density gradient. These data suggested different localization of these two molecules in the plasma membrane, which was later confirmed by electron microscopy. NTAL was found in clusters that were similar in size to LAT clusters and were topographically separated from LAT clusters. Although NTAL and LAT were often found in the vicinity of FcεRI aggregates, they did not mix together (Volná and others, 2004; Zhu and others, 2004).

1.3.1.3. PAG, LAX, GAPT

Phosphoprotein associated with GEMs (PAG, also known as Csk-binding protein (Cbp)) is also transmembrane adaptor protein with similar structure to LAT and NTAL and it is ubiquitously expressed. PAG is also palmitoylated and this targets PAG to lipid rafts in the plasma membrane. PAG negatively regulates mast cell signaling. PAG tyrosine phosphorylation and its association with Csk (C-terminal Src kinase) are increased after FcεRI aggregation. Csk then suppresses activity of Lyn kinase. Overexpression of PAG leads to inhibition of FcεRI-mediated signaling (Ohtake and others, 2002).

The search for similar molecule like LAT in B cells in human genome database led to discovery of a novel protein named LAX. LAX is also membrane-associated protein but unlike LAT it lacks palmitoylation motif and is excluded from lipid rafts. LAX is expressed

in T cells, B cells, mast cells and other lymphoid cell types. LAX has tyrosine motifs for binding Grb2, Gads, and the p85 subunit of PI3K. As LAX-deficient mast cells were hyperresponsive to FcεRI stimulation, a negative regulatory role for LAX in mast cells is proposed. However, expression of NTAL in LAX^{-/-} cells was downregulated and thus the negative regulatory role of LAX could be at least partially explained (Zhu and others, 2002 and 2006).

Other search for new adaptor proteins in the human genome database revealed a GAPT. This shortcut stands for Growth factor receptor-bound protein 2 (Grb2)-binding adaptor protein, transmembrane. GAPT has an extracellular domain, a transmembrane domain, and a cytoplasmic tail with multiple Grb2-binding motifs. Similarly to LAT, GAPT possesses two cysteine residues which are palmitoylated in LAT. However, GAPT was not found in lipid rafts, it only associates with the plasma membrane. GAPT is expressed in B cells, NK cells, dendritic cells and mast cells. GAPT-deficiency did not significantly affect FcεRI-mediated signaling and degranulation in mast cells and tyrosine phosphorylation of GAPT after FcεRI stimulation was not detected (Liu and Zhang, 2008).

1.3.2. Cytosolic adaptor proteins

An important part of the signaling complex scaffolded by LAT is adaptor protein Gads. Gads is recruited to the LAT via its SH2 domain after FcεRI activation and main function of Gads is to bring another adaptor SLP-76 to the LAT proximity. Disruption of Gads/SLP-76 interaction led to reduced degranulation and cytokine production in mast cells. Similarly, SLP-76 deficiency in mast cells led to impaired β-hexosaminidase or IL-6 release in vitro after FcεRI engagement. Mice lacking SLP-76 are also resistant to IgE-mediated passive anaphylaxis (Pivniouk and others, 1999). SLP-76 is strongly tyrosine-phosphorylated upon FcεRI stimulation in a Syk-dependent manner. Then SLP-76 provides binding sites for Vav1, Nck, and Btk. Guanine nucleotide exchange factor (GEF) Vav1 functions as an activator for the Rho family of small G-proteins regulating cytoskeletal changes and MAPK activation, and also stabilizes the recruitment and activation of PLCγ and the regulation of Ca²⁺ responses. Recruitment and activation of Btk in a LAT/SLP-76-dependent manner also contributes to PLCγ activation in mast cells (Alvarez-Errico and others, 2009).

Grb2 (Growth-factor-receptor-bound protein 2) is an adaptor protein ubiquitously expressed in mammalian cells. Grb2 interacts with SOS and after FcεRI aggregation, SOS activates membrane-bound small G-protein Ras. Membrane localization of Grb2 is not direct but is mediated through interaction via another adaptor Shc (Jabril-Cuenod and others, 1996).

Grb2 SH3 domain plays a crucial role in bringing another adaptor Gab2 to FcεRI. Gab2 becomes tyrosine phosphorylated upon FcεRI aggregation in a Syk-dependent manner. This leads to recruitment and activation of PI3K and PIP3 production (Yu and others, 2006a). Gab2 mediates also signaling induced via SCF in mast cells. After binding of SCF to its receptor Kit, Gab2 is phosphorylated and associates with Kit and SHP-2. Gab2 is required for SCF-induced proliferation and mast cells development (Yu and others, 2006b).

2. AIMS

The major aim of this study was to better understand the role of adaptor protein NTAL in FcεRI and Kit signaling in mast cells.

The study included the following specific aims:

1. To generate mast cell lines with enhanced or reduced expression of NTAL adaptor protein.
2. To characterize FcεRI-induced signaling events in cells with increased or decreased NTAL levels.
3. To develop a new method for isolation of plasma membrane sheets from non-adherent cells and use it for high-resolution electron microscopic analysis of topography of selected plasma membrane components.
4. To compare topography of plasma membrane components in non-adherent and adherent mast cells in the course of FcεRI-induced activation.
5. To identify the role of NTAL in cell adhesion, spreading and actin polymerization.
6. To determine activity of Rho-family GTPases in activated BMMC and the role of NTAL in this process.
7. To analyze properties of NTAL-deficient BMMCs with or without NTAL restoration.

3. SUMMARY OF METHODS

All methods used to solve the particular aims of this study are described in more details in the result section in the relevant publications.

1. RBL cells, clone 2H3, were grown as monolayer cultures in complete culture medium consisting of 1:1 mixture of RPMI 1640 and minimal essential medium (MEM) supplemented with 10% fetal calf serum, extra D-glucose (2.5 mg/ml) and antibiotics. BMMCs were isolated from the femurs and tibias of the 6–10-wk-old mice. The cells were incubated for 4–8 weeks in suspension cultures in Iscove's medium containing 10% fetal calf serum, SCF (40 ng/ml) and IL-3 (20 ng/ml), and antibiotics.

2. To generate RBL cells with reduced expression of NTAL adaptor protein we had to determine amino acid sequence of rat NTAL. RNA was isolated from RBL cells using Qiagen kit, reverse transcribed in cDNA and NTAL cDNA was amplified by PCR. The PCR product was transferred into pGEM3Z vector and the insert was sequenced. Then, a pair of oligonucleotides from the rat NTAL sequence was selected and cloned into mU6pro vector. To prepare cells with enhanced NTAL expression, mouse *NTAL* cDNA was cloned into pcDNA3.1/Zeo expression vector. The plasmids were electroporated into RBL cells and clones resistant to antibiotics were selected. We also electroporated empty vectors and vector bearing the same oligonucleotides with two mismatches as negative controls.

3. To determine mast cell degranulation, the enzymatic activity of β -glucuronidase present in the supernatant of lysed cells was measured using 4-methylumbelliferyl β -D-glucuronide as a substrate. Fluorescence was detected in a microtiter plate reader using 365 nm excitation and 460 nm emission filters.

4. Protein phosphorylations were analyzed by direct immunoblotting of SDS-PAGE fractionated cell lysates or by immunoprecipitation with specific antibodies and subsequent immunoblotting with anti-pTyr antibody.

5. Filamentous actin (F-actin) content was determined by flow cytometry after binding of phalloidin conjugate to fixed and permeabilized cells.

6. Concentration of free intracellular calcium was monitored by Fura-2-AM. Probenecid was used to prevent Fura-2 leakage from the cells. The calcium levels were monitored using luminescence spectrometer with excitation wavelengths 340 and 380 nm, and with constant emission at 510 nm. The values were calculated using ICBC Calibration PerkinElmer Fluorescence WinLab software.
7. Uptake of extracellular calcium was measured in cells loaded with $^{45}\text{Ca}^{2+}$. Free $^{45}\text{Ca}^{2+}$ was separated by centrifugation through 12% BSA. Cells with bound ^{45}Ca were lysed and the radioactivity was measured in scintillation liquid in a scintillation counter with QuantaSmart software.
8. TNF- α secretion was determined with commercial ELISA kit.
9. Plasma membrane topography was investigated in cells growing on glass cover slips (adherent cells) or bound to glass cover-slips using fibronectin (non-adherent cells). The cover slips were then pressed onto electron microscopic grids and quickly side-lifted in order to get grids with plasma membrane sheets. When cell adsorption to ultraclean glass cover slips was used, the cell suspension was dropped onto grids and the cover slips were pressed onto the grids. The plasma membrane sheets were then fixed, labeled using specific antibodies and examined with electron microscope. The “GOLD” program was used for statistical evaluation of clustering and colocalization.
10. Surface Fc ϵ RI was detected by binding of FITC-conjugated antibody to Fc ϵ RI-bound IgE and analyzed by flow cytometry in fixed resting and activated cells.
11. 96-well plates were used to define cell adherence. Suspensions of Calcein-AM loaded cells were transferred into fibronectin-coated wells, activated when indicated and fluorescence of all cells was measured using fluorescent plate reader at excitation and emission wavelengths of 494 nm and 517 nm, respectively. Then, non-adherent cells were washed out and fluorescence of cells that adhered to fibronectin was detected. The contribution of particular integrins to cell adhesion on fibronectin was tested by pre-incubation with function-blocking anti-integrin antibodies.
12. Chemotaxis was assayed using 24-well Transwell chambers with 8- μm polycarbonate filters in upper well. Chemoattractants were added into the lower well and the number of cells migrated to the lower well were counted in a Bürker chamber.

13. Commercial Rac(1,2,3), Rac1 and RhoA G-LISA kits were used to assess GTPases activity. Cell lysates were prepared using G-LISA lysis buffer and snap-frozen in liquid nitrogen. Lysate aliquots were applied to wells coated with Rac- or Rho-GTP-binding protein, inactive GDP-bound forms were removed during washing steps and active forms of GTPases were determined with primary specific and secondary HRP-labeled antibodies using microplate spectrophotometer at 490 nm.

14. Microscopic evaluation of cell spreading was performed as follows. Cell suspensions were dropped onto wells of microscopic 8-well slide coated with fibronectin. Cells were left to attach and non-adherent cells were washed out. After activation, cells were fixed and stained for F-actin with phalloidin conjugated to Alexa Fluor 488 or Alexa Fluor 555 and slides were examined with fluorescence microscope. Quantitative assessment of spreading was obtained by measuring the average number of pixels per cell and also by calculating the proportion of spread cells.

15. To confirm our results, we performed experiments in which we restored NTAL expression in NTAL-deficient cells. Mouse *NTAL* gene was cloned into pEGFP-N1 vector. NTAL fusion protein was thus expressed onto N-terminus of EGFP. To transfect the construct to NTAL^{-/-} BMMCs, we used nucleofection, which is a method based on electroporation and cell specific reagents. This method is usually used for cells that are difficult to transfect and the substrate is transferred directly into the cell nucleus and the cytoplasm. We used Mouse Macrophage Nucleofactors Kit (Amaxa) according to the manufacturer's protocol.

4. RESULTS

4.1. REGULATION OF CA^{2+} SIGNALING IN MAST CELLS BY TYROSINE-PHOSPHORYLATED AND UNPHOSPHORYLATED NON-T CELL ACTIVATION LINKER

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This publication summarizes the effect of enhanced or reduced expression of NTAL in RBL cells. The enhanced expression of NTAL caused inhibition of early stages of FcεRI activation and had a positive effect on the uptake of extracellular calcium. Reduced expression of NTAL led to suppression of the uptake of extracellular calcium. The data indicate, for the first time, that NTAL regulates the activity of store-operated calcium channels through a mechanism independent of enhanced NTAL tyrosine phosphorylation.

Regulation of Ca^{2+} Signaling in Mast Cells by Tyrosine-Phosphorylated and Unphosphorylated Non-T Cell Activation Linker¹

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Engagement of the FcεRI in mast cells and basophils leads to a rapid tyrosine phosphorylation of the transmembrane adaptors LAT (linker for activation of T cells) and NTAL (non-T cell activation linker, also called LAB or LAT2). NTAL regulates activation of mast cells by a mechanism, which is incompletely understood. Here we report properties of rat basophilic leukemia cells with enhanced or reduced NTAL expression. Overexpression of NTAL led to changes in cell morphology, enhanced formation of actin filaments and inhibition of the FcεRI-induced tyrosine phosphorylation of the FcεRI subunits, Syk kinase and LAT and all downstream activation events, including calcium and secretory responses. In contrast, reduced expression of NTAL had little effect on early FcεRI-induced signaling events but inhibited calcium mobilization and secretory response. Calcium response was also repressed in Ag-activated cells defective in Grb2, a major target of phosphorylated NTAL. Unexpectedly, in cells stimulated with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} ATPase, the amount of cellular NTAL directly correlated with the uptake of extracellular calcium even though no enhanced tyrosine phosphorylation of NTAL was observed. The combined data indicate that NTAL regulates FcεRI-mediated signaling at multiple steps and by different mechanisms. At early stages NTAL interferes with tyrosine phosphorylation of several substrates and formation of signaling assemblies, whereas at later stages it regulates the activity of store-operated calcium channels through a distinct mechanism independent of enhanced NTAL tyrosine phosphorylation. *The Journal of Immunology*, 2007, 179: 5169–5180.

Aggregation of FcεRI in mast cells and basophils triggers numerous signaling steps, which eventually lead to degranulation and cytokine production. Early signaling events involve sequential activation of Src family protein tyrosine kinases Lyn and Fyn, and Syk/Zap family kinase Syk (1–4). The kinases phosphorylate several substrates, including β and γ subunits of the FcεRI and transmembrane adaptor protein linker for activation of T cells (LAT).³ Phosphorylated LAT becomes a docking site for phospholipase C (PLC) γ 1 and PLC γ 2 and some other Src homology 2 (SH2) domain containing signaling pro-

teins, namely Grb2 adaptor (5, 6). Recently two groups have identified in mast cells another transmembrane adaptor protein called NTAL (non-T cell activation linker) or LAB (linker for activation of B cells) (7, 8), a product of the Williams-Beuren syndrome gene, *Wbscr5*. This protein, also expressed in B cells and NK cells but not in resting T cells, resembles LAT in possessing a short extracellular domain, a single transmembrane region, and a cytoplasmic tail with two palmitoylation cysteine residues and evolutionary conserved motifs containing tyrosine residues. Five of these motifs are of the YXN type (where X is any amino acid), and thus are potential binding sites for the SH2 domain of the cytosolic adaptor protein Grb2. However, unlike LAT, NTAL does not possess a consensus binding motif for PLC γ 1 and PLC γ 2 (7–9).

An important role of NTAL in immunoreceptor signaling was inferred from experiments in which diminution of NTAL expression by silencing RNA oligonucleotides resulted in reduced BCR-mediated activation of MAPK in A20 cell line (8), as well as impaired degranulation in FcεRI-activated human mast cells (9). Unexpectedly, bone marrow-derived mast cells (BMMCs) isolated from NTAL-deficient mice were hyperresponsive to stimulation via the FcεRI, as evidenced by enhanced tyrosine phosphorylation of several substrates, calcium response, degranulation, and cytokine production. However, BMMCs obtained from mice lacking both LAT and NTAL had a more severe block in FcεRI-mediated signaling than BMMCs deficient in LAT alone (10, 11), suggesting

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³Abbreviations used in this paper: LAT, linker for activation of T cells; PLC, phospholipase C; SH2, Src homology 2; NTAL, non-T cell activation linker; BMMC, bone marrow-derived mast cell; RBL, rat basophilic leukemia; SOC, store-operated Ca^{2+} ; BSS, buffered saline solution; NP-40, Nonidet P-40; PIP₂, phosphatidylinositol 4,5-

bisphosphate; IP₃, inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]_i$, concentration of free intracellular Ca^{2+} ; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; TNP, trinitrophenyl.

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that under certain circumstances NTAL may exert a positive signaling role even in BMMC.

Positive regulatory role of NTAL in immunoreceptor signaling was also observed in studies with immature chicken B cell line, DT40 (12). In these cells, Grb2 negatively regulates the Ca^{2+} response through its binding to so far unidentified suppressor. It has been shown that SH2-mediated binding of Grb2 to tyrosine phosphorylated NTAL resulted in sequestering of the Grb2 inhibitory complex away from the cytosol, enhancing thus the calcium response. However, the role of NTAL in Ca^{2+} signaling is more complex as indicated by previous studies describing enhanced calcium responses in NTAL-deficient BMMCs (10, 11).

To enlighten the role of NTAL in Fc ϵ RI signaling, we investigated by genetic and biochemical approaches the properties of rat basophilic leukemia (RBL) cells with enhanced or reduced expression of NTAL, and cells defective in Grb2 alone or in combination with NTAL. Our data indicate multiple regulatory roles of NTAL in Fc ϵ RI signaling in mast cells and document for the first time that activity of the store-operated Ca^{2+} (SOC) channels could be regulated by NTAL even in the absence of its enhanced tyrosine phosphorylation.

Materials and Methods

Abs, reagents, and cell cultures

The following mAbs were used: anti-Syk (13), anti-Lyn (14), anti-LAT (15), anti-Fc ϵ RI β subunit (JRK) (16), trinitrophenyl (TNP)-specific IgE mAb (IGEL b4 1) (17), DNP-specific IgE (18), and anti-NTAL (NAP-07; Exbio). Phospho-Tyr-specific mAb (PY-20), conjugated to HRP, was purchased from Transduction Laboratories. Rabbit polyclonal Abs specific for Syk, Lyn, LAT, and NTAL were prepared by immunization with recombinant fragments of Syk (13), Lyn (14), LAT (15) or rat NTAL (aa 30–196; GenBank accession no. Q8CGL2), respectively. Rabbit anti-IgE was prepared by immunization with whole IGEL b4 1. Polyclonal Abs specific for PLC γ 1, PLC γ 2, Erk1, phospho-Erk (specific for phosphorylated Tyr²⁰⁴), Grb2, Akt1, phospho-Akt1 (specific for phosphorylated Ser⁴⁷³), and HRP-conjugated donkey anti-goat IgG, goat anti-mouse IgG and goat anti-rabbit IgG, were obtained from Santa Cruz Biotechnology. Rabbit anti-PI3K p85 subunit Ab (a mixture of equal amounts of antisera against the intact p85 subunit and the N-SH2 region of PI3K) was obtained from Upstate Biotechnology. Goat anti-mouse IgG and anti-rabbit IgG conjugated to colloidal gold particles of 10- or 5-nm were obtained from Amersham Biosciences. Fura-2/AM and ⁴⁵Ca (sp. act. 566 MBq/mg Ca^{2+}) were purchased, respectively, from Molecular Probes and MP Biomedicals. Origin of RBL cells (clone 2H3) and their culture conditions have been described (19).

Cloning of rat NTAL cDNA and its sequencing

Based on the nucleotide sequence of human *Wbscr5* (GenBank accession no. AF045555) and mouse *Wbscr5* (AF139987) we used 5' primer 5'-AAAGAAATTCGTCAGTGGTGTGGCATCAGC-3' (*Eco*RI site underlined) and 3' primer 5'-AAAAAGCTTGGGCTTCCAGTCAGCACAGTC-3' (*Hind*III site underlined) to amplify the NTAL cDNA from RBL cells by RT-PCR as described (20). The PCR product was digested with *Eco*RI and *Hind*III and ligated into pGEM3Z vector (Promega). The plasmid was amplified and the sequence of the insert was verified by DNA sequencing. All primers used in this study were obtained from Genetech.

Construction of plasmid vectors and isolation of cell lines with changes in expression of NTAL and/or Grb2

Mouse NTAL cDNA was obtained from V. Hořejší and cloned into *Eco*RI site of pcDNA3.1/Zeo vector (Invitrogen). The plasmid, pZeo-NTAL-1, was isolated and its sequence confirmed by sequencing. The plasmid or empty pcDNA3.1/Zeo vector (negative control) were transfected into RBL cells by electroporation (250 V and 750 μ F) using Gene Pulser (Bio-Rad). Colonies resistant to zeocin (300 μ g/ml) were then isolated, and clones with enhanced expression of NTAL were selected.

For production of NTAL- and Grb2-specific RNA silencing vectors, two sets of oligonucleotides, 5'-TTTGAAGCTCTACGAGAATGTGCTCGAAGCTTGGCAGCAGCATATCTCTCGTAGGAGTTTTTTT-3' and 5'-CTAGAAAAAAGCTCTACGAGAATGTGCTCGCAAGCTTCCGAGCA

CATTCTCGTAGGAGT-3' (for NTAL), and 5'-TTTGAATAGATTAC CACAGATCAACATAGCTTTTGTGTGATCTGTGGTAATCTATTTT TT-3' and 5'-CTAGAAAAAAGCTCTACGAGAATGTGCTCGAAGCTTGTATGTGATCTGTGGTAATCTATT-3' (for Grb2) were annealed and cloned into mU6pro vector as described (21). These sequences upon expression form hairpins using the loops in the middle of the sequences (underlined). The plasmids, pU6/siNTAL and pU6/siGrb2 were amplified, the sequences of the inserts were verified by DNA sequencing, and co-transfected at a ratio 10:1 with pStNeoB vector (22) into RBL cells by electroporation. In some experiments RBL cells were transfected with a mix of plasmids, pU6/siGrb2, pU6/siNTAL and pStNeoB at a ratio 5:5:1. Negative controls included empty mU6pro vector or mU6pro vector with the annealed NTAL insert as above except that two mismatches were introduced at positions 30 and 50 (pU6/NTAL-30/50). Clones resistant to antibiotic G418 (0.4 mg/ml) were isolated and analyzed by immunoblotting for NTAL and/or Grb2 expression.

Cell activation, immunoprecipitation, and immunoblotting

Cells were harvested, resuspended in culture medium at a concentration 10×10^6 cells/ml and sensitized with IgE (IGEL b4 1; ascites diluted 1/1000). After 30 min at 37°C the cells were washed in buffered saline solution (BSS) containing 20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.1% BSA, and challenged with Ag (TNP-BSA) for different time intervals. When the cells were activated with thapsigargin, the sensitization step was omitted. Toward the end of the activation period the cells were briefly centrifuged, and β -glucuronidase released into supernatant was determined as described (23) using 4-methylumbelliferyl β -D-glucuronide (Sigma-Aldrich) as a substrate. The cell pellets were lysed in an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and supplemented with 1% Nonidet P-40 (NP-40) (for Lyn, Syk, Erk), 0.2% Brij 96 (for Fc ϵ RI) or 1% NP40 plus 1% *n*-dodecyl β -D-maltoside (for NTAL and LAT). In experiments analyzing the association of proteins with large signaling assemblies, the activated or nonactivated cells were resuspended in ice-cold PBS supplemented with 0.1% saponin, 5 mM MgCl_2 and 1 mM Na_3VO_4 (permeabilization buffer). After 5 min of incubation on ice, the cells were spun down and extracted for 15 min in a lysis buffer containing 1% Triton X-100. Postnuclear supernatants were immunoprecipitated with corresponding Abs prebound to UltraLink-immobilized protein A or G (Pierce), size fractionated by SDS-PAGE and immunoblotted with PY-20-HRP conjugate or with protein-specific Abs followed by an appropriate second stage HRP-conjugated anti-mouse or anti-rabbit IgG. HRP signal was detected by the ECL reagent (Amersham Biosciences). Tyrosine-phosphorylated Erk and Akt were determined by direct immunoblotting with phosphospecific Abs. Immunoblots were quantified by Luminescent Image Analyzer LAS 3000 (Fuji Photo Film) and further analyzed by AIDA image analyzer software (Raytest). The amount of tyrosine-phosphorylated proteins was corrected for the amount of proteins immunoprecipitated as determined by densitometry of immunoblots after stripping of the membranes, followed by development with the corresponding Abs.

Flow cytometry analysis of Fc ϵ RI and F-actin

To determine the surface Fc ϵ RI, cells were exposed to 1 μ g/ml anti-TNP IgE followed by FITC-conjugated anti-mouse IgG cross-reacting with mouse IgE, and probed by flow cytometry using a FACSCalibur (BD Biosciences). The total amount of polymeric actin was measured as previously described (24, 25). In brief, 10^6 cells in 200 μ l of BSS-BSA were sensitized with IgE and stimulated or not with Ag for various time intervals. The reaction was terminated by adding 300 μ l of PBS containing 50 μ g of lysophosphatidylcholine, 6% formaldehyde, and 0.125 μ g/ml FITC-phalloidin (Sigma-Aldrich). After 10 min of incubation at 37°C, the cells were centrifuged and resuspended in 1 ml of PBS before flow cytometry analysis. The geometric mean fluorescence intensity was determined for each sample, and data points were plotted relative to the mean fluorescence intensity of nonactivated control cells.

Cytokine detection

Quantitative measurements of rat TNF- α was performed using murine TNF- α ELISA development kit (cross-reacting with rat TNF- α ; Pepro-Tech) according to the manufacturer's instructions.

Lyn kinase assay

In vitro Lyn kinase assay was performed as previously described (26). In brief, Lyn was immunoprecipitated from cells lysed by sequential

treatment with 0.1% saponin and 1% Triton X-100. Saponin/Triton X-100-extracted material was incubated with rabbit anti-Lyn Ab and the immunocomplexes were collected on protein A beads. The kinase reaction was conducted for 30 min at 37°C in kinase buffer (25 mM HEPES (pH 7.2) 3 mM $MnCl_2$, 0.1% NP-40, 100 mM Na_3VO_4 , 20 mM $MgCl_2$) containing 1 μCi [γ - ^{32}P]ATP (Amersham Biosciences), 100 μM cold ATP, and 0.5 $\mu g/\mu l$ denatured enolase as exogenous substrate. The kinase reaction products were resolved by SDS-PAGE, transferred to nitrocellulose, visualized by autoradiography, and quantified by Fuji Bio-Imaging Analyzer Bas 5000.

Electron microscopy

Plasma membrane sheets were prepared from nonactivated or activated cells and examined by electron microscopy as described (27) with some modifications (28).

Immune complex PI3K and PLC γ assay

PI3K and PLC γ enzymatic activity was measured as previously described (25). In brief, Fc ϵ RI-activated or control cells (2×10^6) were solubilized in lysis buffer supplemented with 1% Triton X-100. PI3K in postnuclear supernatant was immunoprecipitated with anti-PI3K p85 subunit Ab and immunocomplexes were collected on UltraLink-immobilized protein A. PI3K assay was initiated by addition of 25 μl of kinase buffer (20 mM HEPES (pH 7.4), 20 mM $MgCl_2$, and 0.25 mM EGTA) containing 10 μg of sonicated phosphatidylinositol (Sigma-Aldrich) and 37 kBq [γ - ^{32}P]ATP. After 30 min at 25°C, the reaction was terminated and lipids were separated on TLC Silica gel-60 plate (Merck) in a mixture of chloroform/methanol/4 M ammonium hydroxide (9:7:2, v/v/v) for 1 h. ^{32}P -labeled materials were visualized by autoradiography and quantified by Fuji Bio-Imaging analyzer Bas 5000.

To determine the PLC γ enzymatic activity, postnuclear supernatants from nonactivated or activated cells were immunoprecipitated with anti-PLC γ 1 and immunocomplexes were collected on beads of UltraLink-immobilized protein A. The beads were washed and resuspended in 25 μl of reaction buffer followed by addition of 10 μl substrate solution (25 mM sodium phosphate (pH 6.8), 50 mM KCl, 2.5% Triton X-100, 6 μg of phosphatidylinositol 4,5-bisphosphate (PIP_2)) supplemented with 1.1 kBq of [^3H]IP $_2$ (PerkinElmer Life Sciences). After 30 min at 37°C, the reaction was stopped by adding 300 μl of ice-cold 0.5% BSA in PBS. The samples were centrifuged and 300- μl aliquots of the supernatant were mixed with 100 μl of ice-cold 25% (w/v) TCA. Precipitates were removed by centrifugation, and supernatants were collected for quantification of released [^3H]inositol 1,4,5-trisphosphate (IP $_3$) by liquid scintillation counting.

IP $_3$ determination

The procedure used a commercially available [^3H]IP $_3$ radioreceptor assay kit and followed the manufacturer's protocol (PerkinElmer Life Sciences). In brief, IgE-sensitized cells (6×10^6) were stimulated or not with TNP-BSA (500 ng/ml) in 500 μl BSS-BSA. At various time intervals the reactions were terminated by adding 100 μl of ice-cold 100% TCA and the tubes were incubated on ice for 15 min. After centrifugation, supernatants were incubated for 15 min at room temperature and then mixed with a mixture of 1,1,2-trichloro-1,2,2-trifluoroethane-trioctylamin (3:1). The tubes were vortexed, centrifuged, and water phase was used for determination the radioactivity bound to IP $_3$ -binding protein.

Measurement of intracellular Ca^{2+} concentrations

Changes in the concentration of free intracellular Ca^{2+} [Ca^{2+}] $_i$ were determined using fura-2/AM as a probe as previously described (23). IgE-sensitized and control cells were resuspended in BSS-BSA supplemented with 2.5 mM probenecid and 2 μM fura-2/AM. After 40 min at 37°C, the cells were washed with BSS-BSA-probenecid and immediately before measurement briefly centrifuged and resuspended in BSS-BSA. The levels of [Ca^{2+}] $_i$ were monitored using luminescence spectrometer LS-50B (PerkinElmer Life Sciences) with excitation wavelengths 340 and 380 nm, and with constant emission at 510 nm. The values were calculated using ICBC Calibration PerkinElmer Fluorescence WinLab software (PerkinElmer Life Sciences).

Uptake of extracellular calcium

Calcium uptake was determined by a modified previously described procedure (29). Briefly, the cells (2×10^6) were resuspended in 100 μl of BSS-BSA with 1 mM Ca^{2+} , mixed with 100 μl of BSS-BSA supplemented with $^{45}Ca^{2+}$ and various concentrations of thapsigargin, and incubated for 5 min at 37°C. The reaction was terminated by placing the tubes on ice

followed by suspending 100- μl aliquots on the wall of the microtest tube separated by air space from the 12% BSA in PBS (300 μl) at the bottom. Cells with bound ^{45}Ca were separated from free $^{45}Ca^{2+}$ by centrifugation at $1200 \times g$ for 15 min at 4°C through 12% BSA. The cell pellets were recovered by freezing the tubes, slicing off the tube bottom, and solubilized with 1 ml of 1% Triton X-100. The radioactivity was measured in 10 ml of scintillation liquid (EcoLite; ICN Biomedicals) in a scintillation counter with QuantaSmart software (PerkinElmer). The efflux of calcium was determined in cells loaded with $^{45}Ca^{2+}$ after 2 μM thapsigargin-induced $^{45}Ca^{2+}$ influx for 15 min at 37°C. The cells were then washed and incubated in BSS-BSA containing 1 mM Ca^{2+} for different time intervals. The amount of cell-associated $^{45}Ca^{2+}$ was determined after removing free extracellular $^{45}Ca^{2+}$ as described above.

Results

Generation and initial characterization of mast cell lines with enhanced or reduced expression of NTAL

To prepare RNA silencing probes specific for rat NTAL, we first cloned rat NTAL (*Whscr5*) cDNA (GenBank accession no. AY170849). As shown in Fig. 1A, the predicted amino acid sequence of rat NTAL (204 aa) is one amino acid longer than its mouse ortholog (GenBank accession no. NM_020044). All tyrosine motifs as well as a potential palmitoylation site (CxxC motif) are conserved in rat and mouse NTAL sequence, suggesting identical functions.

Next, we transfected RBL cells with pZeo-NTAL-1 vector containing mouse NTAL cDNA or empty pcDNA3.1/Zeo vector, and isolated stable cell lines resistant to zeocin. We also isolated RBL cells transfected with pU6/siNTAL-1, encoding rat NTAL-specific hairpin small interfering RNA (siRNA), and pstNeoB, and isolated G418-resistant stable cell lines. As controls, G418-resistant cells after transfection with pU6/NTAL-30/50 and pstNeoB were also isolated. The expression levels of NTAL in individual clones are shown in Fig. 1B. Compared with control RBL cells, there was an up to 7.4-fold increase in the expression of NTAL in cells after transfection with pZeo-NTAL-1, and almost no detectable endogenous NTAL in cells transfected with pU6/siNTAL-1 (>95% inhibition). Lyn (Fig. 1B) and several other signaling proteins (see below) were not affected by the transfection and knock-down procedure. Cells transfected with empty pcDNA3.1/Zeo, pU6/NTAL-30/50 or pstNeoB vector did not differ in NTAL expression and their secretory response from untransfected RBL cells; only control RBL cells are therefore presented in Fig. 1B and other figures.

After stimulation of RBL cells with Ag, NTAL was phosphorylated on tyrosine residues as detected by immunoblotting with PY-20-HRP conjugate (Fig. 1C, top). We confirmed previous data (7, 8, 10–12) that phosphorylated NTAL bound the Grb2 adaptor (Fig. 1C, middle); the amount of bound Grb2 correlated with that of phosphorylated NTAL present.

The relationship between the amount of NTAL and Fc ϵ RI-mediated degranulation was estimated by the production of β -glucuronidase in individual cell lines at various time intervals after triggering with Ag (Fig. 1, D and E). In cells expressing high levels of NTAL (3B/18, 3B/17 and 2A/4), the secretory response was reduced to ~50% of that found in control RBL cells or cells transfected with empty vector (not shown). In the cell line with lower level of exogenous NTAL expression (1A/1), the secretory response was inhibited less, indicating a correlation between the inhibitory effect and the extent of NTAL overexpression. The secretory response of 2A/4 cells was reduced at all concentrations of Ag used; two other clones with high NTAL levels (3B/18 and 3B/17) exhibited similar properties. To simplify the presentation, only data from clone 2A/4 (NTAL+) are included in Fig. 1F and other figures. The secretory response was also inhibited in all cell lines with decreased amount of NTAL (Fig. 1E) at all concentrations of Ag used; only

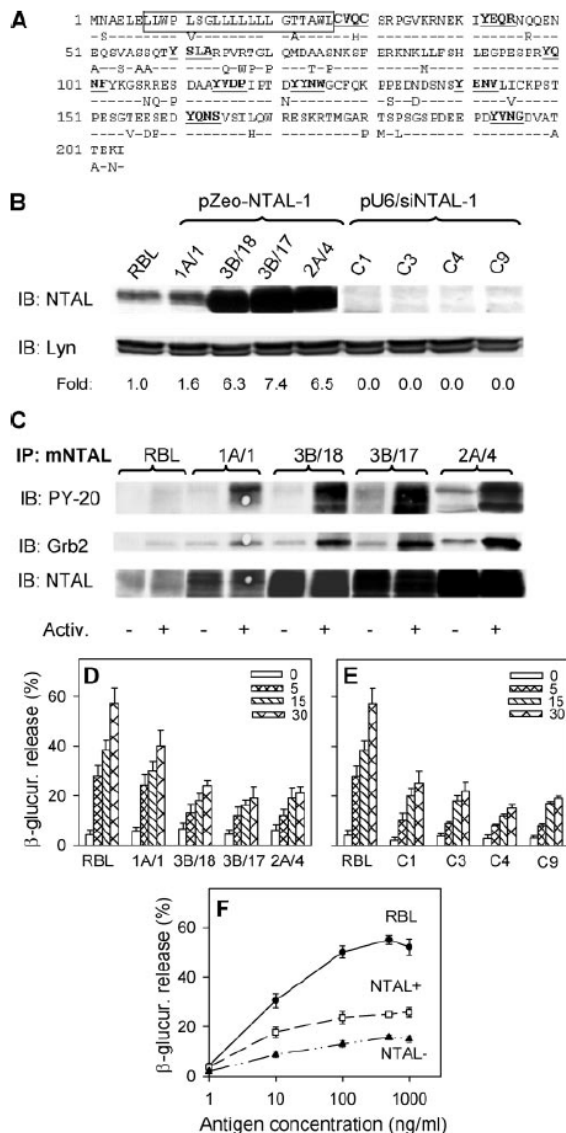


FIGURE 1. Rat NTAL cDNA sequence and initial characterization of RBL-derived cell lines with either enhanced or reduced NTAL expression. **A**, Comparison of predicted amino acid sequence of rat NTAL (top line) and mouse NTAL (bottom line; only the different amino acids are shown). In rat NTAL, the putative transmembrane region is boxed, and potential palmitoylation sequence (CVQC) and tyrosine-containing motifs are underlined in bold. **B**, RBL cells were transfected with vector pZeo-NTAL-1 or pU6/siNTAL-1, and stable clones with, respectively, enhanced or reduced NTAL expression were selected. Total cell lysates were analyzed by immunoblotting (IB) with anti-NTAL or anti-Lyn mAbs and amounts of the corresponding proteins were quantified by densitometry. NTAL expression was normalized to control RBL cells and to the amount of Lyn in individual samples (Fold). **C**, IgE-sensitized control RBL cells or individual transfectants with enhanced expression of NTAL were activated for 5 min by Ag (TNP-BSA, 100 ng/ml; +) or incubated with BSS-BSA alone (-). Lysates from 10^7 cells were immunoprecipitated (IP) with anti-NTAL mAb, and analyzed by immunoblotting with anti-pTyr-HRP conjugate (PY-20), anti-Grb2 and anti-NTAL Abs. **D** and **E**, IgE-sensitized control RBL cells or cells with enhanced (**D**) or decreased (**E**) expression of NTAL were stimulated for the indicated time intervals (0–30 min) with Ag (100 ng/ml) and release of β -glucuronidase was determined in individual

data from clone C4 (NTAL-) are shown in Fig. 1F and other figures. The finding that Fc ϵ RI-mediated secretory response was inhibited in both NTAL+ and NTAL- cells was unexpected and induced additional experiments.

Using IgE-sensitized cells and fluorescently labeled anti-IgE we found that all cell lines differing in NTAL expression exhibited comparable amount of Fc ϵ RI as detected by flow cytometry (Fig. 2A). Light microscopy of cultured NTAL+ cells (Fig. 2B) and other NTAL overexpressors (not shown) revealed their decreased adhesion to tissue culture plastic surface, a more rounded morphology and less developed processes when compared with RBL cells. In contrast, NTAL- cells had fewer but more developed processes (Fig. 2B). The observed changes in morphology in NTAL- cells were probably related to an enhanced amount of F-actin observed in nonactivated cells (Fig. 2C). After Fc ϵ RI-triggering, the amount of F-actin rose as described before (30, 31) and remained higher in NTAL- cells than RBL cells. In contrast, in NTAL+ cells activation-induced increase in F-actin was less pronounced (Fig. 2C).

Electron microscopy on membrane sheets isolated from nonactivated NTAL+ cells showed NTAL distributed in clusters, which resembled NTAL clusters present in RBL cells (Fig. 2D, a and b; 5-nm gold particles). However, the average cluster size was higher in NTAL+ cells (94.0 ± 24.8 nm; mean $\pm$ SD, $n = 3$) than in RBL cells (48.0 ± 8.1 nm, $n = 3$). As expected, the density of NTAL-bound gold particles was elevated in NTAL+ cells ($68.7 \pm 23.4/\mu\text{m}^2$) compared with RBL cells ($35.9 \pm 13.0/\mu\text{m}^2$). For control we also assessed the distribution of gold particles in NTAL- cells, where only background levels were found (Fig. 2Dc); the numbers of Fc ϵ RI β subunit were comparable in RBL, NTAL+ and NTAL- cells (Fig. 2D, a–c; 10-nm gold particles). In Ag-activated cells, Fc ϵ RI formed clusters within osmophilic regions, which were often associated, but not intermixed, with NTAL clusters; the NTAL average cluster sizes and label densities were analogous to those in nonactivated cells. Ag-induced formation of Fc ϵ RI clusters in osmophilic regions of the plasma membrane was not affected by enhanced or reduced NTAL expression (Fig. 2D, d–f), suggesting that the expression of NTAL does not interfere with receptor aggregation.

NTAL overexpression inhibits the Fc ϵ RI-induced tyrosine phosphorylation of Fc ϵ RI subunits, Syk, and LAT

Next we assessed the tyrosine phosphorylation of several proteins known to be pivotal for initial phases of Fc ϵ RI signaling. When total cell lysates from nonactivated or Ag-activated cells were analyzed by SDS-PAGE and immunoblotting with phosphotyrosine-specific mAb, NTAL+ cells, compared with the control RBL cells, exhibited significantly reduced tyrosine phosphorylation of several proteins (~ 38 , 55, 70, 97, and 115 kDa) and enhanced phosphorylation of some other proteins, including a protein of ~ 30 kDa, presumably NTAL (Fig. 3A). In contrast, NTAL- cells showed a phosphorylation pattern more similar to that in control RBL cells; some proteins (e.g., ~ 40 and 50 kDa) showed an increase, other (~ 30 kDa (presumably NTAL) and ~ 70 kDa) a decrease in phosphorylation.

To analyze individual molecules, we first immunoprecipitated Fc ϵ RI, Syk, or LAT from control and Ag-activated cells and found

clones. **F**, IgE-sensitized control RBL cells, or cells with enhanced [clone 2A/4 (NTAL+)] or decreased [clone C4 cells (NTAL-)] expression of NTAL were stimulated for 30 min with different concentrations of Ag and release of β -glucuronidase was determined. Data in **D–F** represent the average of three to four separate experiments, and are expressed as the mean $\pm$ SD.

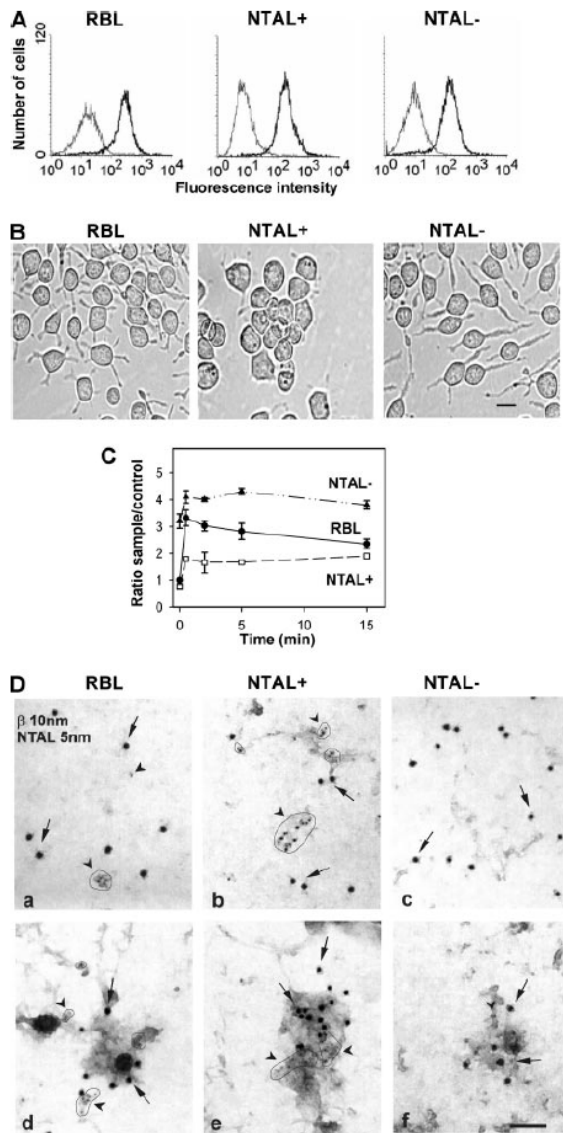


FIGURE 2. Expression of FcεRI, cell morphology, actin polymerization, and topography of NTAL on plasma membrane sheets. **A**, The cells were stained for surface FcεRI by sequential exposure to TNP-specific IgE (thick line; 1 μg/ml) or PBS alone (negative control; thin line) followed by anti-mouse IgE-FITC conjugate. The samples were analyzed by flow cytometry. **B**, Phase contrast images of cells cultured for 48 h under standard conditions. Bar, 10 μm. **C**, Actin polymerization in nonactivated or Ag-activated cells. The cells were sensitized with TNP-specific IgE (1 μg/ml) and then activated by Ag (TNP-BSA; 100 ng/ml) for the indicated time intervals. The amount of F-actin was determined by flow cytometry. Means ± SD were calculated from three independent experiments. **D**, Membrane sheets were prepared from resting cells (**a–c**) or from Ag-activated cells (DNP-BSA, 1 μg/ml, 2 min; **d–f**), and double-labeled from the cytoplasmic side of the plasma membrane for FcεRI β subunit (10-nm gold particles, arrows) and NTAL (5-nm gold particles, arrowheads). Bar, 100 nm.

decreased tyrosine phosphorylation of all of those proteins during activation of NTAL+ cells, compared with RBL cells (Fig. 3, *B–G*). In NTAL- cells, phosphorylation of FcεRI β and γ subunit, Syk and

LAT was comparable to that in RBL cells. Phosphorylation of these proteins depends on Lyn kinase activity (3); we therefore also examined the binding of Lyn to FcεRI, the first well defined intermolecular interaction step in FcεRI signaling (32), and the enzymatic activity of Lyn. Immunoprecipitation studies showed a clear activation-dependent association of Lyn with FcεRI in both control RBL and NTAL- cells (Fig. 3*B*, bottom). In contrast, no Lyn was coprecipitated with FcεRI in NTAL+ cells. To elucidate the molecular basis of this difference we assessed Lyn kinase enzymatic activity in immunocomplex kinase assay in vitro (Fig. 3*H*). Surprisingly, Lyn kinase auto-phosphorylation and phosphorylation of the Lyn substrate, enolase, was higher in NTAL+ cells than in control RBL or NTAL- cells. Thus, the observed inhibition of phosphorylation of FcεRI and several other proteins is not caused by suppressed Lyn kinase activity.

Properties of signaling assemblies depend on NTAL expression levels

To examine signaling assemblies during FcεRI-induced activation, the cells were first permeabilized with cholesterol-sequestering reagent saponin to release free cytoplasmic components. All membrane components, including those residing in lipid rafts and otherwise insoluble in nonionic detergents, were then efficiently solubilized with Triton X-100. In our previous study, we have found that this two-step solubilization procedure allows better estimation of formation of signaling assemblies in the course of cell activation (25). Using this two-step solubilization procedure we compared the signaling assemblies formed by Grb2, which is the major adaptor protein bound to tyrosine phosphorylated NTAL (7, 8). Immunoblotting analyses of Grb2 immunoprecipitates with PY-20 mAb showed that the amount of Grb2-associated and tyrosine phosphorylated proteins increased during Ag-mediated activation (Fig. 4*A*). One of the participating tyrosine phosphorylated proteins was LAT, as determined by its molecular mass (~38 kDa) and immunoblotting with LAT-specific Abs (Fig. 4, *A*, *B*, and *D*). Consistent with previous data (Fig. 3), the amount of phosphorylated LAT bound to Grb2 was reduced in NTAL+ cells and enhanced in NTAL- cells. There was only an insignificant decrease in the amount of LAT in Grb2 immunocomplexes from NTAL+ cells, compared with RBL cells (Fig. 4*D*), suggesting that reduced LAT phosphorylation did not remove all Grb2 binding sites. Another of the tyrosine phosphorylated proteins was NTAL, as determined by its molecular mass (~30 kDa) and immunoblotting with NTAL-specific Abs (Fig. 4, *A*, *C*, and *E*). As expected, the amount of Grb2-associated NTAL was enhanced in NTAL+ cells and undetectable in NTAL- cells.

Recently we and others have found that Grb2 immunocomplexes from Ag-activated RBL cells possess PI3K activity (28, 33, 34). To determine whether NTAL has any effect on the formation of these complexes, we measured PI3K activity in Grb2 immunoprecipitates. Data in Fig. 4*F* indicate that in resting cells the activity of PI3K in Grb2 immunoprecipitates was higher in NTAL- cells than in RBL and NTAL+ cells. After FcεRI triggering, higher activity of PI3K in Grb2 immunocomplexes was observed at all time intervals analyzed in RBL cells and even more in NTAL- cells, whereas in NTAL+ cells only a transient increase (2.1-fold after 0.5 min) was seen. When PI3K was directly immunoprecipitated and the immunocomplexes were tested for PI3K activity, all cell lines showed enhanced activity in response to FcεRI-mediated activation, and the dramatic differences between NTAL+ and NTAL- cells were less pronounced (Fig. 4*G*). Nevertheless, even under these conditions, PI3K activity associated with large signaling assemblies was higher in NTAL- cells than in RBL or NTAL+ cells. When NTAL was immunoprecipitated from RBL or NTAL+ cells, no PI3K activity was detected in

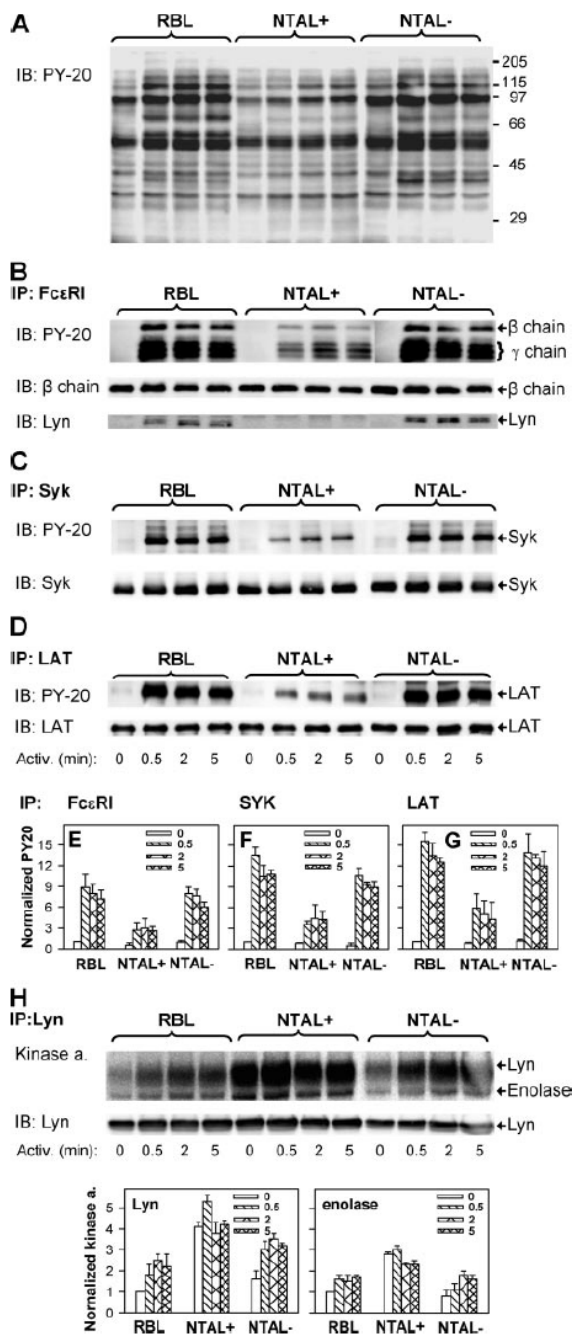


FIGURE 3. Overexpression of NTAL inhibits tyrosine phosphorylation of FcεRI, Syk, and LAT but enhances enzymatic activity of Lyn kinase. IgE-sensitized cells were stimulated with Ag (TNP-BSA, 500 ng/ml) for the indicated time intervals. **A**, Cells were lysed in 1% NP-40-containing lysis buffer and total cell lysates were analyzed for protein tyrosine phosphorylation by immunoblotting with PY-20-HRP conjugate. Numbers on the right indicate positions of molecular mass standards (in kDa). **B–D**, Cells were solubilized in 0.2% Brij 96 (**B**), 1% NP-40 (**C**) or a mix of 1% NP-40 and 1% *n*-dodecyl β-D-maltoside (**D**) and the target proteins were immunoprecipitated with Abs specific for IgE (**B**), Syk (**C**), or LAT (**D**) and analyzed by immunoblotting with PY-20-HRP conjugate. After stripping, the membranes were reblotted with protein-specific Abs as indicated. **E–G**, Densitometry analysis of phosphotyrosine immunoblots (as in **B–D**)

immunoprecipitates from either nonactivated or FcεRI-activated cells (not shown). Thus, NTAL does not form complexes possessing PI3K activity, but inhibits the formation of functional PI3K-Grb2 complexes.

Enzymatic activity of PI3K results in the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 and phosphatidylinositol 3,4-bisphosphate recruit Akt to the plasma membrane, where it is phosphorylated and activated. Phosphorylation of Akt was enhanced after FcεRI triggering in RBL cells (Fig. 4H). In nonactivated NTAL+ cells the amount of Akt and phospho-Akt associated with saponin-permeabilized cells was higher, but the changes faded out 2 and 5 min after FcεRI triggering. In accordance with the enhanced activity of PI3K in NTAL- cells (Fig. 4G), the amount of membrane-bound Akt and its phosphorylation was also enhanced. These data support the concept that NTAL regulates the formation of signaling assemblies containing PI3K and Akt.

Cytokine TNF-α production in NTAL+ and NTAL- cells

Previous experiments showed that production of several inflammatory mediators, including TNF-α, is enhanced in FcεRI-activated BMMC from NTAL-deficient mice (11). Central to regulation of cytokine gene transcription is Ras/Raf/MEK/Erk signaling pathway (35–37). As expected, enhanced tyrosine phosphorylation of Erk was indeed observed in NTAL-/- BMMC (10). In additional experiments we therefore examined phosphorylation of Erk and secretion of TNF-α in NTAL+ and NTAL- RBL cells. Immunoblotting experiments showed that the amount of tyrosine phosphorylated Erk in Ag-activated RBL cells was increased, reaching the peak 2 min after triggering (Fig. 5A). NTAL overexpression resulted in an impaired phosphorylation of Erk. In contrast, the onset of Erk phosphorylation in NTAL- cells was faster and remained higher at all time intervals tested. Inhibition of Erk phosphorylation in NTAL+ cells correlated with an inhibition of TNF-α secretion from the cells activated by two different doses of Ag (Fig. 5B). In NTAL- cells, Ag-induced secretion of TNF-α was, surprisingly, also reduced, but less dramatically than in NTAL+ cells and only at lower Ag concentration (100 ng/ml); no inhibition was observed at 500 ng/ml. These data suggest that NTAL in NTAL+ cells negatively regulates cytokine production through inhibition of Ras/Raf/MEK/Erk signaling pathway. In NTAL- cells this pathway is potentiated; the observed inhibition of TNF-α production in NTAL- cells seems therefore to reflect some positive effects of NTAL on later stages of mast cell signaling (see below).

Expression levels of NTAL modulate activity of PLCγ

Immunoblotting analyses of PLCγ1 and PLCγ2 immunoprecipitates from saponin/Triton X-100 solubilized cells showed that the amount of tyrosine phosphorylated PLCγ1 associated with signaling assemblies decreased in both NTAL+ cells and NTAL- activated cells (Fig. 6A). Recruitment of PLCγ2 and

normalized to the amount of the proteins immunoprecipitated and to their phosphorylation in nonactivated RBL cells. **H**, Lyn was immunoprecipitated and its enzymatic activity was determined by *in vitro* kinase assay using ³²P-γATP and enolase as a substrate. The kinase reaction products were quantified by autoradiography and after stripping off the membranes the amount of Lyn was determined by immunoblotting. Kinase activity of Lyn, as determined by autoradiography of ³²P-labeled Lyn and enolase, normalized to the parameters in nonactivated RBL cells and corrected for the amount of Lyn in each immunoprecipitate is also indicated. Means ± SD in **E–H** were calculated from three experiments.

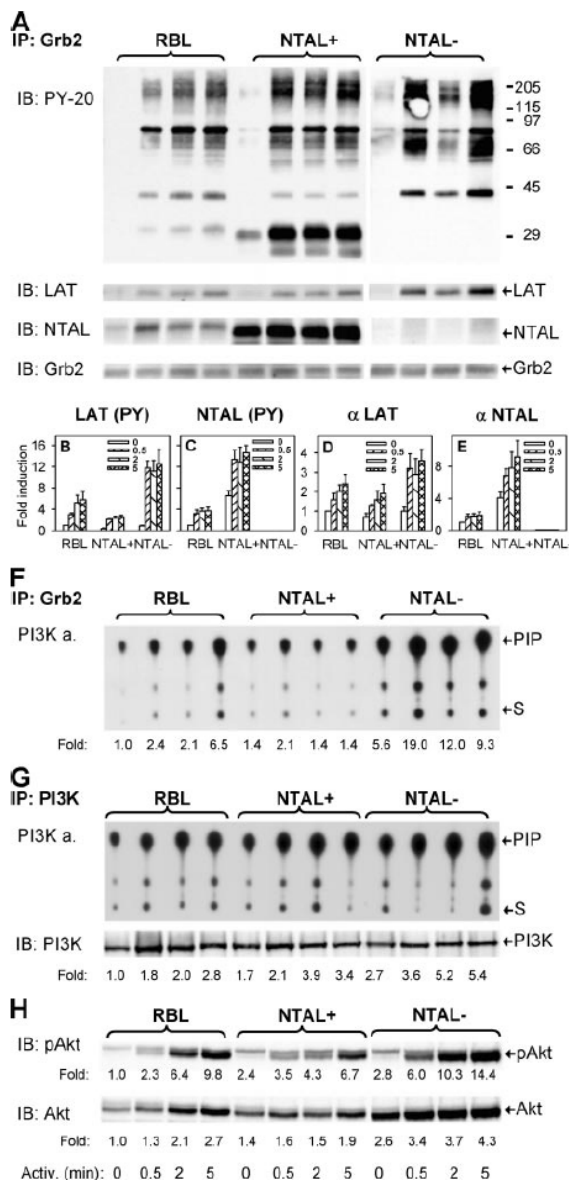


FIGURE 4. Changes in Grb2 signaling assemblies, PI3K activity and tyrosine phosphorylation of Akt. Cells were activated as in Fig. 3, solubilized with saponin/Triton X-100 procedure, and Grb2 immunocomplexes were isolated by precipitation with anti-Grb2 Ab. **A**, Grb2 immunocomplexes were analyzed by immunoblotting for the presence of total tyrosine-phosphorylated proteins (PY-20), LAT, NTAL and Grb2. **B–E**, Densitometry analysis of tyrosine-phosphorylated (PY) LAT (**B**) and NTAL (**C**), and total amount of LAT (**D**) and NTAL (**E**) in Grb2 immunocomplexes. Means $\pm$ SD in **B–E** were calculated from three to four experiments. **F**, PI3K activity associated with Grb2 immunocomplexes was estimated using [^{32} P]-ATP and phosphatidylinositol as a substrate in PI3K assay (PI3K a.). Positions of [^{32} P]PI (PIP) and start (S) are indicated by arrows. **G**, PI3K immunoprecipitates were analyzed in parallel for PI3K enzymatic activity by PI3K assay and for the amount of immunoprecipitated PI3K by immunoblotting with anti-p85 subunit of PI3K. Enzymatic activity of PI3K normalized to its levels in nonactivated RBL cells and corrected for the amount of PI3K-p85 subunit precipitated is also indicated. **H**, The cells were solubilized with the saponin/Triton X-100 procedure and postnuclear supernatants were analyzed by immunoblotting with anti-phospho-Akt, followed by stripping and immunoblotting with Akt-specific Ab. Relative

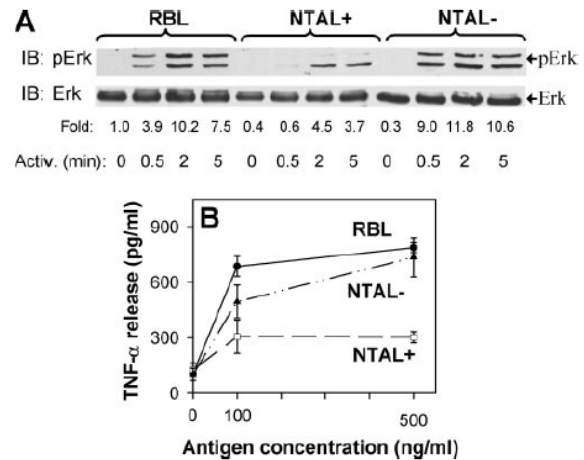


FIGURE 5. Changes in tyrosine phosphorylation of Erk and TNF- α production. **A**, Cells were activated as in Fig. 3 and then solubilized with 1% Triton X-100. Postnuclear supernatants were analyzed by immunoblotting with anti-phospho-Erk, followed by stripping and immunoblotting with Erk-specific Ab. Relative amounts of the proteins were normalized to nonactivated RBL cells. A typical result from three experiments is shown. **B**, IgE-sensitized cells were activated with different concentrations of Ag for 3 h and the amount of TNF- α released into supernatant was determined by ELISA. Data represent means $\pm$ SD from three experiments performed in triplicates.

degree of its phosphorylation were comparable in all cell lines (Fig. 6B). Enzymatic activity of PLC γ was detected by immunocomplex PLC γ assay, determining the production of [^{3} H]IP $_3$ from P[^{3} H]IP $_2$ substrate. In nonstimulated cells the activity of PLC γ was comparable in RBL, NTAL+ and NTAL- cells (Fig. 6C). After Fc ϵ RI triggering, PLC γ activity rapidly increased in all cell lines; however, in NTAL- cells, and especially in NTAL+ cells the increase was lower than in RBL cells. These findings were corroborated by direct measurements of IP $_3$ levels (Fig. 6D). In nonactivated cells IP $_3$ concentrations were similar in all cell lines under study, but in Fc ϵ RI-activated cells IP $_3$ reached higher levels in RBL cells compared with NTAL- cells, and especially to NTAL+ cells.

Intracellular Ca^{2+} mobilization and uptake of extracellular Ca^{2+} are affected by NTAL expression levels

Enhanced levels of IP $_3$ induce a release of calcium from intracellular stores, followed by calcium influx through SOC channels in the plasma membrane (38). To determine the role of NTAL in both these steps we followed the calcium response in cells under different conditions. NTAL- cells activated with Ag in the presence of extracellular Ca^{2+} showed a lower calcium response than control cells, but identical initial kinetics. In contrast, NTAL+ cells exhibited a delay in calcium response and a slower decline to baseline levels (Fig. 7A). Activation of the cells by Ag in the absence of extracellular calcium resulted in $\sim$ 30% inhibition of the calcium response in NTAL- cells relative to RBL cells, whereas in NTAL+ cells this response was dramatically inhibited and delayed (Fig. 7B). After increasing the concentration of extracellular Ca^{2+} , both RBL and NTAL- cells showed rapid increase in

amounts of the proteins were normalized to nonactivated RBL cells. Representative data from three experiments performed are shown.

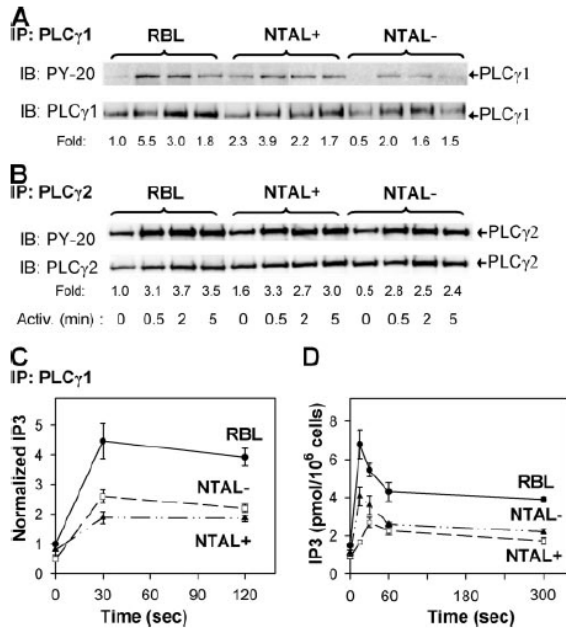


FIGURE 6. Changes in the properties of PLC γ 1 and PLC γ 2. *A* and *B*, Cells were activated as in Fig. 3, solubilized with saponin/Triton X-100 procedure, and PLC γ 1 (*A*) and PLC γ 2 (*B*) were immunoprecipitated and analyzed by immunoblotting with PY-20-HRP conjugate. After stripping the same membrane was reblotted with protein-specific Abs. Relative amounts of proteins were normalized to nonactivated cells. Data from representative experiments from at least three performed are shown. *C*, Cells were lysed in 1% Triton X-100, and enzymatic activity of the immunoprecipitated PLC γ 1 was measured by immune complex PLC γ assay. *D*, Cellular IP₃ levels were determined by ³H-labeled radioreceptor assay kit. Data in *C* and *D* represent means $\pm$ SD from three to four experiments.

[Ca²⁺]_i, with faster return to initial levels in NTAL⁻ cells, whereas NTAL⁺ cells showed only a weak response (Fig. 7*B*).

The observed dramatic decrease in calcium response in Ag-activated NTAL⁺ cells could be related to decreased activity of PLC γ and impaired production of IP₃ (Fig. 6, *C* and *D*), and/or to negative regulatory role of NTAL in Ca²⁺ mobilization at later stages. To explore the role of NTAL on Ca²⁺ mobilization independently of its effect on activity of PLC γ , cells were activated by thapsigargin, an agent that induces the release of Ca²⁺ from intracellular stores by inhibiting the endoplasmic reticulum ATPase (39). In the absence of extracellular Ca²⁺, thapsigargin induced a small increase in [Ca²⁺]_i in all cell lines, suggesting that NTAL does not interfere with the transport of thapsigargin to its target and release of Ca²⁺ from cytoplasmic stores. Interestingly, when the extracellular Ca²⁺ level was restored, 5 independent experiments showed that the maximum [Ca²⁺]_i in NTAL⁺ cells was higher (750 $\pm$ 80 nM; mean $\pm$ SD) than in RBL cells (620 $\pm$ 54 nM) and NTAL⁻ cells (580 $\pm$ 37). Furthermore, [Ca²⁺]_i reverted to baseline level more rapidly in NTAL⁻ cells than in RBL and NTAL⁺ cells (Fig. 7*C*).

These data suggested that NTAL could regulate the transport of extracellular Ca²⁺ through the plasma membrane. To test this we measured the ⁴⁵Ca uptake in thapsigargin-stimulated cells. A direct correlation was found between the amount of NTAL expressed and calcium uptake; it was high in NTAL⁺ cells, medium in RBL cells and low in NTAL⁻ cells (Fig. 7*D*). The observed correlation could imply not just an important role of NTAL in regulating Ca²⁺ uptake but also an inhibitory effect of NTAL on the release of Ca²⁺ from the cells. Next we therefore labeled the cells with ⁴⁵Ca²⁺, washed them and measured the radioactivity retained within the cells at different time intervals. Results presented in Fig. 7*E* show that all cell lines exhibited the same kinetics of ⁴⁵Ca²⁺ efflux. Together with previous findings on Ca²⁺ uptake, these data

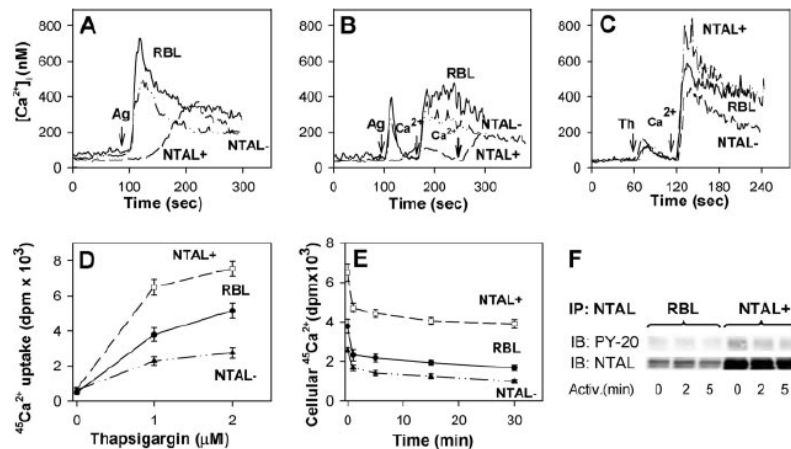


FIGURE 7. Intracellular Ca²⁺ mobilization, extracellular ⁴⁵Ca uptake and calcium release from the cells. *A–C*, Calcium responses in fura-2 loaded RBL, NTAL⁺, and NTAL⁻ cells. *A*, The cells were sensitized with IgE and stimulated in the presence of 1 mM extracellular calcium with TNP-BSA (500 ng/ml; arrow, Ag). Calcium levels were determined by spectrophotometry. *B*, Cells were stimulated with Ag in the absence of extracellular calcium (arrow, Ag) and 1 mM Ca²⁺ was added after Ca²⁺ calcium levels returned to original values (arrow, Ca²⁺). *C*, Cells were exposed to thapsigargin (1 μ M; arrow, Th) in the absence of extracellular calcium and 1 mM Ca²⁺ was added later on (arrow, Ca²⁺). *D*, The cells were activated with various concentrations of thapsigargin in the presence of extracellular ⁴⁵Ca²⁺ (1 mM). After 5 min at 37°C, the reaction was terminated and the cells were centrifuged through 12% BSA in BSS and cell-bound radioactivity was determined. *E*, The cells were loaded with ⁴⁵Ca²⁺ during 15-min activation with thapsigargin, unbound ⁴⁵Ca²⁺ was washed out and calcium efflux was determined at different time intervals. *F*, The cells were activated with thapsigargin (2 μ M) for different time intervals and solubilized in lysis buffer containing 1% NP-40 plus 1% *n*-dodecyl β -D-maltoside. NTAL was immunoprecipitated from postnuclear supernatant and analyzed by immunoblotting with PY-20-HRP conjugate. After stripping, the membrane was reblotted with NTAL-specific Ab. Data in *A–C* and *F* are representative experiments from, respectively, three and two performed. Data in *D* and *E* represent means $\pm$ SD from four experiments performed in duplicates or triplicates.

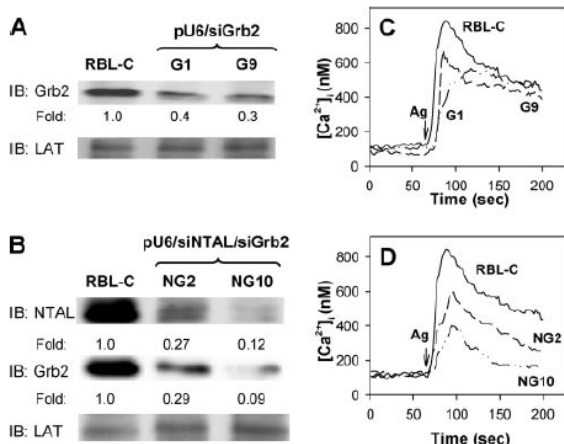


FIGURE 8. Positive regulatory role of Grb2 in Ag-induced Ca^{2+} signaling. **A** and **B**, Immunoblotting analysis of Grb2- or Grb2/NTAL-deficient cells. RBL cells transfected with empty vector (RBL-C), pU6/siGrb2 vector (clones G1 and G9) or both pU6/siNTAL-1 and pU6/siGrb2 (clones NG2 and NG10) were lysed and postnuclear supernatants were analyzed by immunoblotting for the presence of Grb2, LAT, and NTAL. Relative amounts of Grb2 and NTAL were normalized to the amount of LAT in each sample. **C** and **D**, Calcium response in fura-2-loaded control and transfected cells. The cells were sensitized with IgE and stimulated in the presence of 1 mM extracellular Ca^{2+} with TNP-BSA (500 ng/ml, arrow, Ag). Calcium levels were determined by spectrophotometry. Data are representative of at least two experiments performed.

indicate that NTAL positively regulates Ca^{2+} uptake rather than Ca^{2+} efflux.

Experiments with DT40 chicken B cells suggested that phosphorylated NTAL could regulate the Ca^{2+} uptake by a mechanism involving its binding with Grb2, a negative regulator of Ca^{2+} signaling (12). We therefore attempted to find out whether or not NTAL is phosphorylated in thapsigargin-activated cells. NTAL was immunoprecipitated from nonactivated or thapsigargin-activated RBL or NTAL+ cells and its phosphorylation was assessed by immunoblotting with PY-20-HRP. Data presented in Fig. 7F indicate that thapsigargin had no effect on NTAL tyrosine phosphorylation in either RBL or NTAL+ cells. To determine whether Grb2 could function as a negative regulator of Ca^{2+} response in RBL cells, we transfected the cells with pU6/siGrb2 plus pSTneoB and selected G418-resistant clones, G1 and G9, with decreased amount of Grb2 (Fig. 8A). We also isolated RBL-derived cells with decreased amounts of both NTAL and Grb2 after simultaneous transfection with pU6/siNTAL, pU6/siGrb2 and pSTneoB (Fig. 8B, clones NG2 and NG10). It should be noted that the amount of LAT was not affected in any transfectant (Fig. 8, A and B), confirming the specificity of the knock-down procedure. Detailed analysis showed that Ag-activated RBL cells with decreased amount of Grb2 exhibited lower increase in $[\text{Ca}^{2+}]_i$ than RBL cells transfected with empty vector (Fig. 8C). When the expression of both NTAL and Grb2 was reduced, an even deeper decrease in $[\text{Ca}^{2+}]_i$ was observed (Fig. 8D). These data indicate that Grb2 functions as a positive regulator of Ca^{2+} response in RBL cells.

Discussion

Variation of NTAL expression levels is shown to have multiple effects on FcεRI-mediated activation events in RBL cells. Permanent cell lines with low expression were isolated after transfection with U6-based expression vector producing NTAL siRNA. In con-

trast to previous studies in which the transfected RNA reduced the expression levels by ~70% (9), hairpin siRNA strategy lowered it by >95%. Stable cell lines with enhanced NTAL expression were obtained after transfection of mouse NTAL cDNA under cytomegalovirus promoter. We expected that NTAL overexpression would uncover additional signaling pathways undetectable in NTAL-deficient cells if they acted downstream of the early regulatory effects of NTAL.

Initial characterization revealed that cells with increased or decreased amount of NTAL exhibited restricted secretory response after FcεRI triggering, unrelated to differences in surface expression of FcεRI and evident at all concentrations of Ag used. NTAL levels also affected morphology of the cells; NTAL+ cells being less adherent to tissue culture plastic surface and NTAL- cells having longer processes. The observed changes in cell morphology could be related to the amount of F-actin and its formation during FcεRI signaling. Thus, NTAL- cells had more F-actin than parental RBL or NTAL+ cells, and only a weak increase in F-actin formation was induced by activation of both NTAL+ and NTAL- cells.

Previously we have examined the distribution of NTAL and LAT by electron microscopy of membrane sheets. NTAL in resting RBL cells was localized in small clusters, topographically separated from clusters of LAT and FcεRI (10), but not Thy-1 (40). When membrane sheets were isolated from NTAL overexpressors, no difference in the distribution of FcεRI and NTAL was observed except that the average cluster size and density of NTAL-bound gold particles was higher in NTAL+ than control cells. Importantly, the absence of NTAL from the FcεRI aggregates is not confined just to control RBL cells, as reported previously (10), but applies also to NTAL+ cells (this study). Together with our finding of normal redistribution of aggregated FcεRI into osmophilic regions in NTAL+ cells, these data suggest that the initial FcεRI aggregation step induced by multivalent Ag is not changed in NTAL overexpressors.

Immunocytochemical studies showed that overexpression of NTAL inhibited the tyrosine phosphorylation of FcεRI β and γ subunits, Syk and LAT in Ag-activated cells. Consequently, recruitment of PLCγ to signaling assemblies and its tyrosine phosphorylation and activation were inhibited. Reduced production of PLCγ metabolite IP3 led to a reduction in both the release of Ca^{2+} from intracellular stores and the uptake of extracellular Ca^{2+} through SOC channels. This could explain the inhibition of the secretory response in Ag-activated cells. The MAP kinase signaling pathway was also inhibited in NTAL+ cells as reflected in the impaired tyrosine phosphorylation of Erk and subsequent low secretion of TNF-α.

Decreased tyrosine phosphorylation of FcεRI subunits in NTAL+ cells suggested that the activity of Lyn kinase is inhibited. However, immunocomplex kinase assays showed Lyn kinase activity in NTAL+ cells is undiminished, implying that NTAL interferes with the accessibility of Lyn to FcεRI. This possibility is strengthened by data indicating that the amount of Lyn coprecipitated with FcεRI was higher in activated RBL cells than in NTAL+ cells. Because Lyn, like NTAL, seems to be localized in lipid rafts (7, 41), it is possible that direct or indirect interactions of Lyn with NTAL preclude the interaction between Lyn and FcεRI subunits. Although immunoprecipitation and immunocomplex kinase assays failed to show NTAL-Lyn interactions (L. Dráberová, unpublished data), it is possible that procedures used to isolate NTAL immunocomplexes destroyed these interactions.

Our results indicate that the signaling assemblies formed in NTAL+ cells are different from those formed in control or NTAL- cells. Indeed, in Ag-activated NTAL+ cells, more NTAL

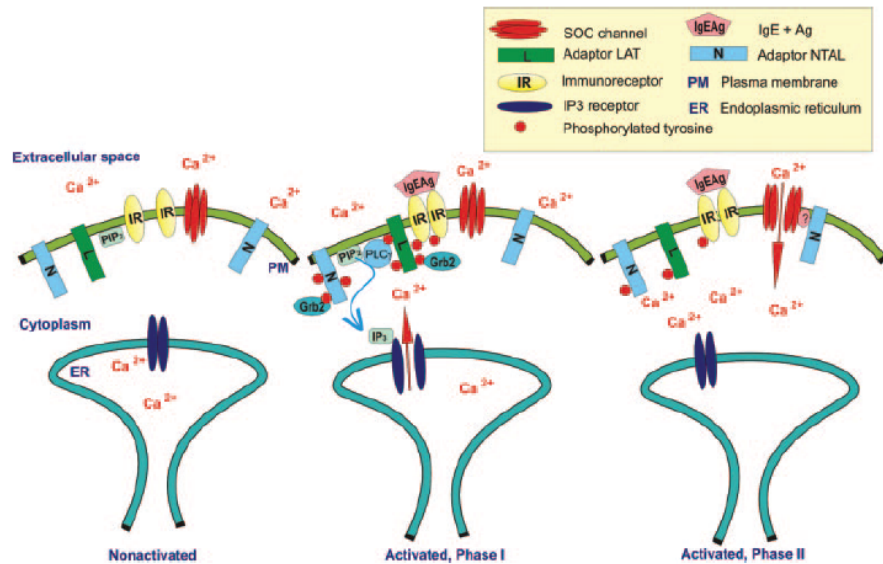


FIGURE 9. A model of NTAL function in FcεRI-mediated Ca^{2+} signaling. At early stages after FcεRI aggregation (phase I), NTAL is rapidly tyrosine phosphorylated, competing with phosphorylation of FcεRI and LAT. Phosphorylated NTAL binds Grb2 complexes and interferes with the activity of PI3K and several other signaling molecules. NTAL affects activity of PLCγ and in this way the generation of IP_3 followed by release of Ca^{2+} from internal stores. At later stages of activation (phase II), extracellular Ca^{2+} flows into the cytoplasm through SOC channels. Activity of these channels could be modulated by direct or indirect interaction of NTAL with SOC channel proteins and/or regulators of their activity. However, this effect is independent of enhanced NTAL tyrosine phosphorylation.

and less LAT is bound to Grb2, supporting the concept of competition between NTAL and LAT for Grb2 as a substrate (10). In contrast, in activated NTAL⁻ cells phosphorylation of FcεRI subunits, Syk and LAT was not inhibited. However, association of PLCγ with insoluble complexes, its phosphorylation and its enzymatic activity were less efficient and resulted in a partial inhibition of downstream events. The impaired function of PLCγ could reflect involvement of NTAL in the formation of signaling assemblies required for PLCγ enzymatic activity (42–44). As previously shown (10, 45), Grb2 forms complexes with SHP-2 phosphatase, and these complexes could bind to NTAL to regulate early signaling events. Alternatively, if PLCγ somehow interacts with NTAL, its enzymatic activity would be inhibited in NTAL⁻ cells even though the early FcεRI-activation events proceed normally. Although we were unable to coprecipitate PLCγ with NTAL (L. Dráberová, unpublished data), it remains possible that these interactions are sensitive to the solubilization and immunoprecipitation procedures.

If IP_3 signal generated by FcεRI aggregation is bypassed by thapsigargin, NTAL⁺ cells show a higher uptake of extracellular Ca^{2+} than control RBL cells. This suggests that NTAL could have a positive regulatory role in Ca^{2+} uptake. This is corroborated by complementary studies in which thapsigargin-activated NTAL⁻ cells showed a lower Ca^{2+} uptake. The role of NTAL in uptake of extracellular Ca^{2+} is unclear but could be related to NTAL-dependent Ca^{2+} -regulating signal circuit recently described in DT40 B lymphocytes (12). In these cells, Grb2 plays a negative regulatory role in Ca^{2+} uptake, which appears to be eliminated upon binding to NTAL. However, several pieces of evidence indicate that in rodent mast cells NTAL plays a different role in Ca^{2+} response. First, no inhibition in $[\text{Ca}^{2+}]_i$ was observed in Ag-activated BMMC from NTAL^{-/-} mice; rather, there was an enhancement of the Ca^{2+} response (10, 11). Second, Ag-activated NTAL^{-/-} BMMC showed no decrease, but rather an increase in

the uptake of extracellular $^{45}\text{Ca}^{2+}$; this could be related to enhanced activity of PLCγ (10, 11). Third, there was a higher Ca^{2+} response to FcεRI triggering in NTAL⁻ cells than in NTAL⁺ cells. It should be emphasized that the highest Ca^{2+} response was observed in control RBL cells, suggesting that an optimal concentration and/or topography of NTAL is required for maximum Ca^{2+} response. Fourth, after activation by Ag in the absence of extracellular Ca^{2+} and restoration of Ca^{2+} level later on, there was no dramatic difference between control and NTAL⁻ cells in initial Ca^{2+} uptake. In contrast, the response in NTAL⁺ was markedly inhibited. This inhibition reflected low levels of Ca^{2+} released from intracellular stores and consequently low Ca^{2+} influx through the SOC channels. Finally, $[\text{Ca}^{2+}]_i$ in cells activated with thapsigargin in the absence of extracellular Ca^{2+} was not dependent on NTAL. However, when Ca^{2+} was replenished, a significant increase in $[\text{Ca}^{2+}]_i$ occurred in the following sequence: NTAL⁺ cells > control RBL cells > NTAL⁻ cells. These data suggest that NTAL could play a positive role in regulating the Ca^{2+} uptake at the level of SOC channels. Our finding of correlation between $^{45}\text{Ca}^{2+}$ uptake and the amount of NTAL in thapsigargin-activated cells supports the notion. The possibility that NTAL modulated Ca^{2+} efflux rather than its influx was excluded by experiments measuring the kinetics of calcium release from activated $^{45}\text{Ca}^{2+}$ -labeled cells. Interestingly, no increase in tyrosine phosphorylation of NTAL was observed in thapsigargin-triggered cells. Thus, our data indicate that NTAL could have a novel regulatory role in Ca^{2+} uptake independent of de novo NTAL tyrosine phosphorylation.

Possible regulatory functions of NTAL at different phases of FcεRI-mediated Ca^{2+} signaling are shown in Fig. 9. At early stages of activation (phase I) NTAL serves as a substrate for protein tyrosine kinases and thus could interfere with phosphorylation of FcεRI and LAT by a competitive mechanism. Furthermore, phosphorylated NTAL binds Grb2 and other signaling molecules,

which could modulate the activity of various enzymes, including PI3K and PLC γ . Enhanced activity of PLC γ leads to increased production of IP $_3$ and consequently to elevated levels of cytoplasmic Ca $^{2+}$. At later stages of activation (phase II), NTAL could affect the function of SOC channels, reflecting its direct or indirect interactions with channel-forming proteins and/or their regulators such as Orai1 and/or Stim1 (46–49). This function of NTAL is not dependent on its enhanced tyrosine phosphorylation.

This model is based on functional analysis of cells with increased or decreased levels of NTAL. Although useful, this genetic approach has, however, its limitations. Knockdown or knockout approaches may not disclose all functions of the proteins, due to compensatory activities of other proteins. Overexpression approach, which is complementary to down-regulation approach and could reveal otherwise undetectable activities of the proteins under study, could impose indirect effects by sequestering proteins to which they bind or by targeting proteins that are not their natural targets. It is very likely that NTAL is a natural target of Lyn kinase and therefore enhanced expression of NTAL could compromise the ability of Lyn to interact with the Fc ϵ RI; this could explain the inhibition of phosphorylation of Fc ϵ RI β and γ subunits and all downstream targets in Fc ϵ RI-activated NTAL+ cells. Provided that NTAL, through its interaction with Lyn, negatively regulates phosphorylation of the Fc ϵ RI under physiological conditions, this phosphorylation should be enhanced in activated NTAL-deficient cells. Although the results of this and our previous study (10) show no clear increase in phosphorylation of the receptor in such cells, it still remains possible that enhanced Lyn-mediated phosphorylation of the Fc ϵ RI subunits in NTAL-deficient cells is compromised by enhanced activity of phosphatases. In fact, our unpublished data, showing indirect correlation between activity of the membrane-associated tyrosine phosphatases and amount of cellular NTAL, support this notion. Thus, although activities of the relevant kinases and phosphatases toward the Fc ϵ RI could be higher in NTAL-deficient than in wild-type cells, their functional equilibrium could be comparable in both cell types. When this equilibrium is shifted, e.g., by exposure of the cells to a phosphatase inhibitor, such as pervanadate, NTAL is tyrosine phosphorylated even in the absence of Fc ϵ RI aggregation (L. Dráberová, unpublished data). The combined data suggest that NTAL, through its interaction with Lyn and other signaling molecules, could contribute to setting a threshold for Fc ϵ RI-induced degranulation and cytokine response. This concept is corroborated by our findings of enhanced activity of PI3K (Fig. 4G) and enhanced association of Akt and pAkt with signaling assemblies (Fig. 4H) even in nonactivated NTAL-deficient cells.

In summary, NTAL overexpression suppresses early Fc ϵ RI-induced activation events, but has a positive effect on the uptake of extracellular Ca $^{2+}$ in thapsigargin-stimulated cells. Accordingly, inhibition of NTAL expression has no inhibitory effect on early signaling pathways but does suppress the late ones, including the uptake of extracellular Ca $^{2+}$. Expression levels of NTAL may thus regulate mast cells activation at multiple steps. Differences in interaction of NTAL with various regulatory proteins and signaling pathways (50) could explain why down-regulation of NTAL expression either inhibited (9) or enhanced (10, 11) Fc ϵ RI-induced secretory response in different mast cell types.

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Disclosure

The authors have no financial conflict of interest.

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4.2. TOPOGRAPHY OF SIGNALING MOLECULES AS DETECTED BY ELECTRON MICROSCOPY ON PLASMA MEMBRANE SHEETS ISOLATED FROM NON-ADHERENT MAST CELLS

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In this work, we present a novel versatile method of isolation of plasma membrane sheets from non-adherent cells. The isolated membrane sheets were used for investigation of fine topography of plasma membrane molecules. Using this new method we compared topography of selected plasma membrane components in adherent RBL cells and non-adherent BMMCs.

Research paper

Topography of signaling molecules as detected by electron microscopy on plasma membrane sheets isolated from non-adherent mast cells

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Abstract

Immunolabeling of isolated plasma membrane (PM) sheets combined with high-resolution electron microscopy is a powerful technique for understanding the topography of PM-bound signaling molecules. However, this technique has been mostly confined to analysis of membrane sheets from adherent cells. Here we present a rapid, simple and versatile method for isolation of PM sheets from non-adherent cells, and show its use for examination of the topography of Fcε receptor I (FcεRI) and transmembrane adaptors, LAT (linker for activation of T cells) and NTAL (non-T cell activation linker), in murine bone marrow-derived mast cells (BMMC). The data were compared with those obtained from widely used but tumor-derived rat basophilic leukemia (RBL) cells. In non-activated cells, FcεRI was distributed either individually or in small clusters of comparable size in both cell types. In multivalent antigen-activated BMMC as well as RBL cells, FcεRI was internalized to a similar extent, but, strikingly, internalization in BMMC was not preceded by formation of large (~200 nm) aggregates of FcεRI, described previously in activated RBL cells. On the other hand, downstream adaptor proteins, LAT and NTAL, were localized in independent domains in both BMMC and RBL cells before and after FcεRI triggering. The combined data demonstrate unexpected properties of FcεRI signaling assemblies in BMMC and emphasize the importance of studies of PM sheets isolated from non-tumor cells.

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Keywords: Plasma membrane; Electron microscopy; Immunolabeling; Leukocytes; Mast cells

Abbreviations: BMMC, bone marrow-derived mast cells; BSA, bovine serum albumin; BSS, buffered salt solution; DNP, dinitrophenyl; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; FcεRI, Fcε receptor I; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); HRP, horseradish peroxidase; IgE, immunoglobulin E; IL-3, interleukin 3; LAT, linker for activation of T cells; mAb, monoclonal antibody; NC, nitrocellulose; NTAL, non-T cell activation linker; PBS, phosphate buffered saline; PCCF, pair cross-correlation function; PCF, pair correlation function; PLC, phospholipase C; PLL, poly-L-lysine; PM, plasma membrane; RBL, rat basophilic leukemia; SCF, stem cell factor.

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1. Introduction

Plasma membrane (PM) is an important cellular compartment accommodating numerous proteins involved in various functions. Although it is known that immunoreceptors and other signaling molecules interact within the PM, their topographical changes in the course of cell activation are still poorly understood. Various methods, such as fluorescence energy transfer, immunofluorescence microscopy and electron microscopy (EM) have been used to locate PM-associated molecules (Lagerholm et al., 2005). Regarding resolution, EM represents a bridge between fluorescence resonance energy transfer, working at distances under 10 nm, and fluorescence microscopy with a 100-nm scale resolution. Transmission EM on ultrathin sections allows visualizing the PM components in the context of the whole cell. To provide a horizontal view, examination of PM sheets is the method of choice.

PM sheets from cells growing on glass coverslips have been isolated by attaching the dorsal side of the cells to poly-L-lysine (PLL)-coated film on EM grids, followed by separation of PM sheets connected to the EM grid from the rest of the cell. Components of the extracellular and intracellular leaflets of the PM can be labeled before or after membrane sheets isolation, respectively. As the cells must be attached to a glass coverslip prior to isolation of the PM sheets, the studies were confined mainly to adherent cells such as fibroblasts, cultured neurons, Chinese hamster ovary cells, rat basophilic leukemia (RBL) cells and baby hamster kidney cells (Sanan and Anderson, 1991; Wilson et al., 2000; Wyse et al., 2003; Choy et al., 2006). Non-adherent leukocytes have been either excluded from such studies or they had to be attached to a substrate by PLL or streptavidin in procedures taking about 45–60 min (Schade and Levine, 2002; Lillemeier et al., 2006), which itself could induce cell activation.

Mast cells, the effectors of allergic reactions, have been extensively used for studies of activation events induced by aggregation of the Fcε receptor I (FcεRI) (Metcalf et al., 1997; Gilfillan and Tkaczyk, 2006). Although FcεRI and numerous other signaling molecules involved in antigen-mediated mast cell activation have been identified, sequenced and characterized, their topography in the PM at nanometer-scale resolution remained almost unknown until the experiments performed by Bridget Wilson's group on PM sheets isolated from adherent RBL cells (Lara et al., 2001; Wilson et al., 2000, 2002, 2004). These authors proposed a model of primary and secondary signaling domains formed in the PM after FcεRI triggering. The

primary domains consist of FcεRI patches with a diameter of ~200 nm, rich in Syk kinase, phospholipase C (PLC)γ2, cytoplasmic adaptors Grb2, Gab2 and other signaling proteins. Interestingly, Lyn kinase, which phosphorylates FcεRI, was excluded from these domains. The primary signaling domains were proposed to pass the signal onto the secondary signaling domains represented by aggregates of the transmembrane adaptor LAT (linker for activation of T cells) and containing PLCγ1.

EM studies performed on PM sheets from RBL cells also contributed to understanding the topography of molecules thought to be localized predominantly in cholesterol- and sphingolipid-rich microdomains, called lipid rafts or membrane rafts (Simons and Toomre, 2000; Pike, 2006). Some molecules considered to be associated with lipid rafts, such as glycosylphosphatidylinositol (GPI)-anchored glycoprotein Thy-1 and glycosphingolipid GM1, turned out to be localized independently in the PM (Wilson et al., 2004). On the other hand, Thy-1 showed clear colocalization with palmitoylated adaptor proteins LAT and NTAL (non-T cell activation linker) (Wilson et al., 2004; Heneberg et al., 2006), even though these adaptors were found in different non-overlapping patches in both resting and activated RBL cells (Volná et al., 2004; Heneberg et al., 2006). However, RBL cells are of tumor origin, and it is therefore possible that the topography of signaling molecules in these cells differs from that found in non-tumor mast cells.

In this study we describe a new technique that is rapid, simple and versatile for isolation of PM sheets from non-adherent cells, and show its use for determination of topography of selected plasma membrane signaling molecules in murine bone marrow-derived mast cells (BMMC). Although these cells are more useful for analysis of signaling processes, compared to the widely used tumor-derived RBL cells, BMMC have been excluded from such studies because of their growth in suspension. Direct comparison of topography of several signaling molecules in BMMC and RBL cells revealed a substantial difference in the formation of FcεRI signaling spots, whereas the downstream signaling domains possessing adaptor proteins, LAT and NTAL, showed analogous distribution in both cell types.

2. Materials and methods

2.1. Antibodies and reagents

The following mouse monoclonal antibodies (mAb) were used: anti-FcεRI-β subunit (JRK, Rivera et al.,

1988), anti-NTAL (Brdička et al., 2002), anti-LAT (Tolar et al., 2001), anti-Lyn (Dráberová et al., 1996) and dinitrophenyl (DNP)-specific immunoglobulin E (IgE) (Liu et al., 1980). Horseradish peroxidase (HRP)-conjugated phosphotyrosine-specific mAb (PY20) was obtained from Becton Dickinson Biosciences (CA, USA). Rabbit polyclonal antibodies included anti-LAT (Upstate Biotechnology, NY, USA), anti-NTAL and anti-IgE (Volná et al., 2004). DNP conjugated to bovine serum albumin (BSA) was purchased from Molecular Probes (OR, USA), goat anti-mouse and anti-rabbit secondary antibodies conjugated to 5 nm or 10 nm gold particles (diluted 1/25 for intracellular and 1/5 for extracellular labeling) were from Amersham (UK), fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse Ig was from Jackson ImmunoResearch Laboratories (PA, USA), HRP-conjugated goat anti-mouse IgG was from Santa Cruz (CA, USA). Nickel EM grids (300 mesh), OsO₄ and pioloform were obtained from Christine Gröpl Elektronemikroskopie (Austria). Stem cell factor (SCF) and interleukin 3 (IL-3) were from PeproTech EC Ltd (UK). Poly-L-lysine (MW >300,000), fibronectin and other chemicals were purchased from Sigma (MO, USA). Water used for the preparation of solutions and cleaning of glass coverslips was deionized and further purified using a Milli-Q water purification system (Millipore S.A., France) and had a resistivity of $\geq 18 \text{ M}\Omega \cdot \text{cm}$.

2.2. Preparation of ultraclean glass coverslips

Round glass coverslips (#72196-15, 15-mm diameter, Electron Microscopy Sciences, PA, USA) were washed manually (using rubber gloves) one by one with a mix of ionic and non-ionic detergents, Jar (Procter & Gamble, Czech Republic). They were transferred to H₂O (Millipore-purified, 500 ml) and washed several times to completely remove the detergent. Then the coverslips were washed briefly with 96% ethanol, dried and transferred one by one to 35% HCl and kept in HCl for 16–20 h. After repeated thorough washes with H₂O the coverslips were kept in H₂O for at least 30 min and again washed several times in H₂O to remove all traces of HCl. After two brief washes with ethanol, the coverslips were stored in ethanol until use.

2.3. Cells

RBL-2H3 cells were cultured as described by Dráberová and Dráber (1991). Sixteen to eighteen hours before the experiment, the cells were transferred into fresh complete medium containing DNP-specific IgE (1 $\mu\text{g}/\text{ml}$) and allowed to settle on an ultraclean glass coverslip.

BMMC were isolated from murine femurs and tibias and cultivated in Iscove's medium containing 10% fetal calf serum (FCS), SCF (40 ng/ml) and IL-3 (20 ng/ml). Sixteen to eighteen hours before the experiment the cells were transferred into Iscove's medium without SCF, but with FCS, IL-3 and DNP-specific IgE (1 $\mu\text{g}/\text{ml}$), and incubated either in suspension, or on glass coverslips covered with fibronectin (50 $\mu\text{g}/\text{ml}$ in phosphate buffered saline (PBS) for 1 h at 37 °C, followed by washing with PBS). Murine T cells were isolated from peripheral blood as described by Smrž et al. (2007).

2.4. Isolation and staining of PM sheets

2.4.1. Preparation of EM grids

On the day of the experiment, EM grids covered with pioloform and coated with carbon were glow-discharged for 45–60 s by 300 V, then incubated with 1 mg/ml PLL in H₂O for 30 min, washed once for 5 s in H₂O and dried. Immediately before the PM sheet isolation, a round nitrocellulose (NC) membrane filter (Millipore; pores 0.45 μm) was placed onto a drop of *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer (25 mM HEPES, pH 7.0, 25 mM KCl, 2.5 mM magnesium acetate) on an ice-cold glass support. The filter had to be fully wetted, but no liquid should appear on its upper side. Two EM grids were placed with the PLL side up onto the filter (Fig. 1A and B).

2.4.2. Cells

RBL cells cultured as adherent on glass coverslips, or BMMC bound to glass coverslips covered with fibronectin (as described above), were washed with PBS and, when indicated, activated by DNP-BSA in buffered salt solution (BSS; 20 mM HEPES, pH 7.4, 5 mM KCl, 135 mM NaCl, 1.8 mM CaCl₂·2H₂O, 1 mM MgCl₂, 5.6 mM glucose) at 37 °C. In some experiments the cells were fixed for 7 min by 2% paraformaldehyde in PBS at room temperature, washed and incubated for 10–15 min with antibodies in PBS supplemented with 0.1% BSA (washing with PBS). Cells cultured in suspension were washed twice with BSS supplemented with 0.1% BSA and transferred to BSS without BSA. A 100- μl aliquot of the suspension ($\sim 10^6$ cells/ml) was applied onto an ultraclean glass coverslip for 1 min and, when indicated, processed for activation (by DNP-BSA in BSS, 37 °C) and/or extracellular labeling (as described above). Alternatively, the cells were activated in suspension.

2.4.3. Isolation of PM sheets

Coverslips with adherent RBL cells or fibronectin-bound BMMC (Fig. 1A, left) as well as with ultraclean

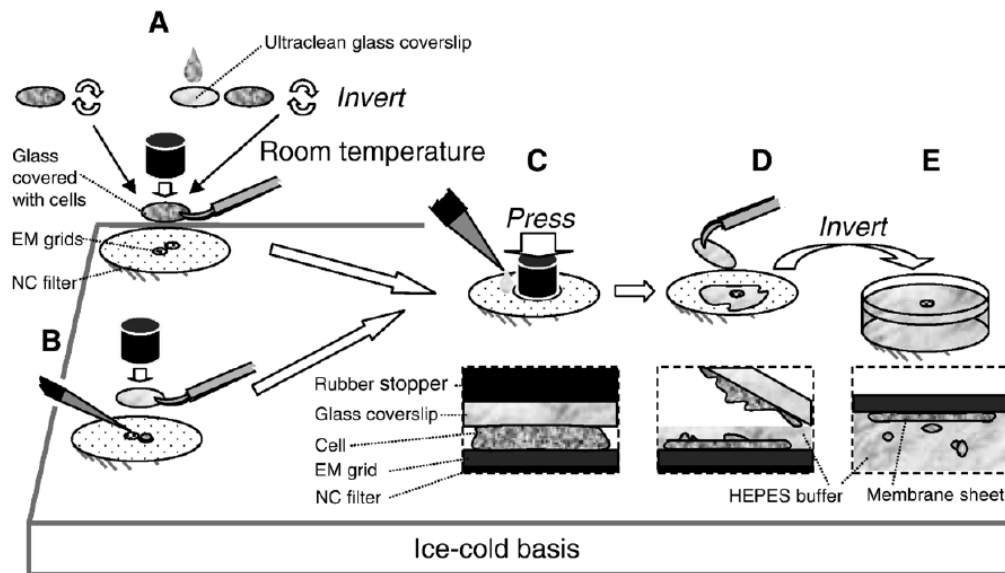


Fig. 1. Schematic drawing of the procedures used to isolate PM sheets. (A) Glass coverslip with cells growing as adherent or attached via fibronectin (left) or an ultraclean glass coverslip with cells adsorbed in protein-free buffer (right) was placed face-down onto PLL-coated EM grids laid on NC filter wetted with ice-cold HEPES buffer. (B) Alternatively, cells in suspension were dropped onto individual EM grids and covered with an ultraclean glass coverslip. (C) Immediately, a sandwich formed by the glass coverslip, cells and EM grid was pressed using an ice-cold rubber stopper. (D) The coverslip was quickly side-lifted, leaving the dorsal, Jar PM sheets bound to the EM grid. (E) The EM grid with the attached PM sheets was instantly transferred face-down onto ice-cold HEPES buffer surface for several seconds and further processed (fixation, immunolabeling and contrasting steps). Schematic transversal views of the PM sheet isolation procedure is shown at the bottom of (C)–(E) at high magnification.

glass-bound BMMC (Fig. 1A, right) were rinsed twice in PBS and once in ice-cold HEPES buffer and pressed face-down onto EM grids by firm finger pressure for 10 s using a rubber stopper (Fig. 1C); a drop of ice-cold HEPES buffer was applied to the edge of the coverslip to maintain moisture in the following step. The coverslip was quickly side-lifted, exposing the cells to shearing forces (Fig. 1D), and the grids with attached PM sheets were immediately placed face-down onto ice-cold HEPES buffer (Fig. 1E). After 5–10 s the grids were transferred onto ice-cold 2% paraformaldehyde in HEPES buffer for 10 min. The grids were then floated on PBS for 5–30 min. Alternatively, if the PM sheets were isolated from cells in suspension, 10- μ l aliquots of cells suspended in BSS (without BSA) were loaded onto each EM grid, then covered with an ultraclean glass coverslip (Fig. 1B), pressed and processed as described above (Fig. 1C–E).

2.4.4. Intracellular leaflet labeling and contrasting the specimen

Labeling of the intracellular leaflet was performed by incubation for 30 min on drops of PBS supplemented with 0.1% BSA and antibodies, followed by three 5-min

washes with PBS. Samples were post-fixed with 2% glutaraldehyde in PBS for 10 min and then washed with PBS for 10 min. The specimens were further stained for 10 min with 1% OsO₄ in cacodylate buffer, washed three times for 5 min in H₂O, incubated for 10 min with 1% aqueous tannic acid, washed three times for 5 min in H₂O, and finally stained for 10 min with 1% aqueous uranyl acetate. After a 1-min wash in H₂O, the samples were air-dried and examined by electron microscope (JEOL JEM 1200EX operating at 60 kV).

2.5. Flow cytometry analysis

IgE-primed BMMC and RBL cells were fixed by 4% paraformaldehyde in PBS at various stages of their activation. Subsequently, they were washed with PBS and immuno-labeled with FITC-conjugated anti-mouse Ig. Samples were analyzed using FACSCalibur™ (Becton Dickinson, NJ, USA).

2.6. Degranulation assay

The extent of mast cell degranulation was assessed by measuring the relative content of β -glucuronidase released

into the supernatant. Twenty microliters of supernatant was mixed with 60 μ l of 40 μ M 4-methylumbelliferyl β -D-glucuronide and incubated for 60 min at 37 °C. The reaction was stopped by adding 200 μ l of ice-cold 0.2 M glycine buffer (pH 10.5) and fluorescence was measured in a microtiter plate reader (Fluorostar, SLT Labinstruments, Austria) using 365 nm excitation and 460 nm emission filters. The total content of β -glucuronidase was evaluated in the supernatants from cells lysed by 0.1% Triton X-100.

To check for the effect of BMMC adsorption to ultraclean glass on degranulation, the cells (10^5 in 100 μ l) were dropped onto ultraclean glass coverslips and incubated for 1, 5 or 10 min at room temperature. Then the supernatant and non-adsorbed cells were centrifuged, and amount of β -glucuronidase in the cell-free supernatant was determined as described above. As a control, degranulation of cells in suspension was measured at the respective time intervals. Three independent experiments were performed.

2.7. Immunoblotting

IgE-sensitized or non-sensitized BMMC were washed twice with BSS supplemented with 0.1% BSA and transferred to BSS without BSA. A 100- μ l aliquot of the suspension (10^6 cells/ml) was applied onto an ultraclean glass coverslip for 1 min (approximately 50% of the cells adhered within this time period), rinsed in BSS and, when indicated, processed for activation by DNP-BSA in BSS at 37 °C. Then the cells were lysed for 20 min in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM Na_2VO_4 , 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 0.5% Triton X-100). Alternatively, the amount of BMMC corresponding approximately to those adherent to the glass was activated or not in suspension and lysed as described above. After lysis, cellular proteins were size-fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions and electrotransferred to NC membranes (BA85, Schleicher & Schuell, Germany). Tyrosine-phosphorylated proteins were detected by immunolabeling with HRP-conjugated PY20 mAb. To check for cell loading, the amount of Lyn kinase in the samples was detected by immunolabeling with mouse anti-Lyn mAb, followed by HRP-conjugated anti-mouse secondary antibody. The membrane-bound HRP was visualized with ECL Western blotting reagent (Amersham, UK) according to the manufacturer's instructions. Immunoblots were analyzed using a Luminescent Image Analyzer LAS-3000

(Fuji Photo Film Co., Japan) and AIDA image analyzer software (raytest Isotopenmeßgeräte GmbH, Germany).

2.8. Statistics

The GOLD computer program was used for statistical evaluation of clustering and colocalization of immunogold markers. It maps the distribution of gold particles by pair correlation function (PCF; analysis of clustering) or pair cross-correlation function (PCCF; analysis of colocalization). PCF is a ratio of the density of gold particles at a given distance from a typical particle to the average density of these particles. PCCF is a ratio of the density of particles of the first type at a given distance from a typical particle of the second type to the average density of the particles of the first type (Philimonenko et al., 2000). Data shown are means $\pm$ SD unless stated otherwise.

3. Results

3.1. New procedures for isolation of PM sheets from non-adherent cells

In pilot experiments we found that leukocytes, such as BMMC or T cells, which do not adhere to glass surfaces in media or buffers supplemented with serum, bound rapidly to ultraclean glass surfaces in protein-free buffer, and that this property can be used for isolation of PM sheets. The binding of cells to the ultraclean glass surface occurs rapidly (within $\sim$ 1 min) at room temperature and is not dependent on the actin cytoskeleton, as PM sheets were isolated with comparable efficiency from BMMC pretreated with an inhibitor of actin polymerization latrunculin B (20 μ M, 90 min, 37 °C) and from latrunculin B non-treated cells. Importantly, binding of BMMC to ultraclean glass for 1, 5 or 10 min at room temperature did not cause enhanced spontaneous degranulation when compared to cells kept in suspension for the indicated time intervals (not shown). Similarly, tyrosine phosphorylation of BMMC proteins was comparable between resting or DNP-activated cells incubated on glass or in suspension when normalized to the amount of Lyn protein (Fig. 2).

To elaborate the procedure for isolation of PM sheets by adsorption of cells to glass (Fig. 1A, right), we compared it with two other methods we developed for isolation of membrane sheets from BMMC. The first (Fig. 1A, left) makes use of the finding that BMMC under certain circumstances adhere to fibronectin, a component of the mast cell environment (Lam et al.,

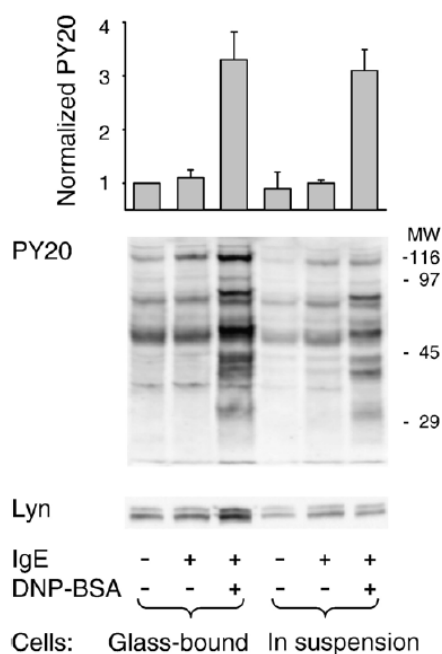


Fig. 2. Protein tyrosine phosphorylation profiles in cells bound to ultraclean glass and those incubated in suspension. The cells were cultured overnight in the presence (+) or absence (-) of DNP-specific IgE, transferred to protein-free BSS and then incubated for 1 min on ultraclean glass coverslips or in suspension. When indicated (+) they were activated with DNP-BSA (1 μ g/ml) for 5 min at 37 °C and lysed directly on glass; cells in suspension were centrifuged before lysis. Tyrosine-phosphorylated proteins were detected using HRP-labeled PY20 mAb and the signal was normalized to Lyn protein detected by immunoblotting. Means $\pm$ SD were calculated from three independent experiments.

2003), thus providing relatively physiological conditions for cell adhesion. The second method (Fig. 1B) is based on pressing the cells suspended in protein-free buffer to PLL-coated EM grids by ultraclean glass coverslip, thus reducing the length of attachment as much as possible. Further steps were identical in all isolation procedures used, and consisted of pressing the sandwich formed by the glass coverslip, cells and EM grid (Fig. 1C), followed by side-lifting the glass coverslip (Fig. 1D) and washing the membrane sheets attached to the EM grids by floating on ice-cold HEPES buffer (Fig. 1E).

PM sheets isolated from BMMC after binding to fibronectin (Fig. 3A), ultraclean glass (Fig. 3B) or pressing directly to PLL-covered EM grids (Fig. 3C) showed comparable distribution of the Fc ϵ RI- β subunit as detected by EM after intracellular leaflet double-step immunolabeling. Furthermore, no difference in the amount of receptor or in the PM structure was noticed.

When PM sheets were isolated 2 min after exposure to antigen (DNP-BSA), a comparable decrease in the amount of Fc ϵ RI- β subunit in PM was observed for all three procedures (Fig. 3D). Therefore, we used only the method based on cell adsorption to ultraclean glass in subsequent studies.

As the density of Fc ϵ RI- β subunits on PM sheets was approximately twice as high for RBL cells (prepared by the technique of Sanan and Anderson, 1991) than for BMMC (Fig. 3E), we checked whether this difference was attributable to spreading of RBL cells on the glass surface, and thus possibly exposing the Fc ϵ RI on the dorsal membrane. PM sheets isolated from RBL cells in suspension using the same procedure as from BMMC (1 min adhesion to ultraclean glass coverslips, without spreading) showed an amount of detectable Fc ϵ RI- β (24.91 ± 2.97 particles/ μ m², $n=3$, 60 μ m²) comparable to that detected on PM sheets of glass-grown RBL cells (23.96 ± 2.66 particles/ μ m², $n=3$, 60 μ m²).

3.2. Topography of Fc ϵ RI in the course of IgE-dependent BMMC activation

Aggregation of Fc ϵ RI by IgE and multivalent antigen leads to Fc ϵ RI internalization by a dynamin-dependent mechanism in RBL cells (Fattakhova et al., 2006). We found that both RBL cells and BMMC show a similar decrease in detectable Fc ϵ RI on PM sheets when stimulated by DNP-BSA at concentrations optimal for degranulation (1 μ g/ml for RBL, 0.1 μ g/ml for BMMC). No obvious difference was observed in the amount of detectable Fc ϵ RI on BMMC stimulated by either 1 or 0.1 μ g/ml DNP-BSA (Fig. 3E).

It has been reported that activation of RBL cells by multivalent antigen leads to formation of Fc ϵ RI patches several hundreds of nanometers in size (Wilson et al., 2000). To find out whether similar patches are also formed in BMMC, we analyzed the topography of Fc ϵ RI in resting and activated cells. To detect both Fc ϵ RI- α and Fc ϵ RI- β subunits on the same PM sheets, the IgE bound to Fc ϵ RI- α was detected by two-step immunolabeling from the extracellular side, PM sheets were isolated, and Fc ϵ RI- β was labeled from the intracellular side. As expected, in resting cells both immunogold markers colocalized in the PM and were distributed in small clusters (Fig. 4A and C). Surprisingly, in antigen (DNP-BSA)-activated BMMC, no dramatic changes in Fc ϵ RI topography were observed and no large patches of Fc ϵ RI in osmiophilic regions were formed. In fact, the number of Fc ϵ RI clusters in activated BMMC was lower than in resting cells; this

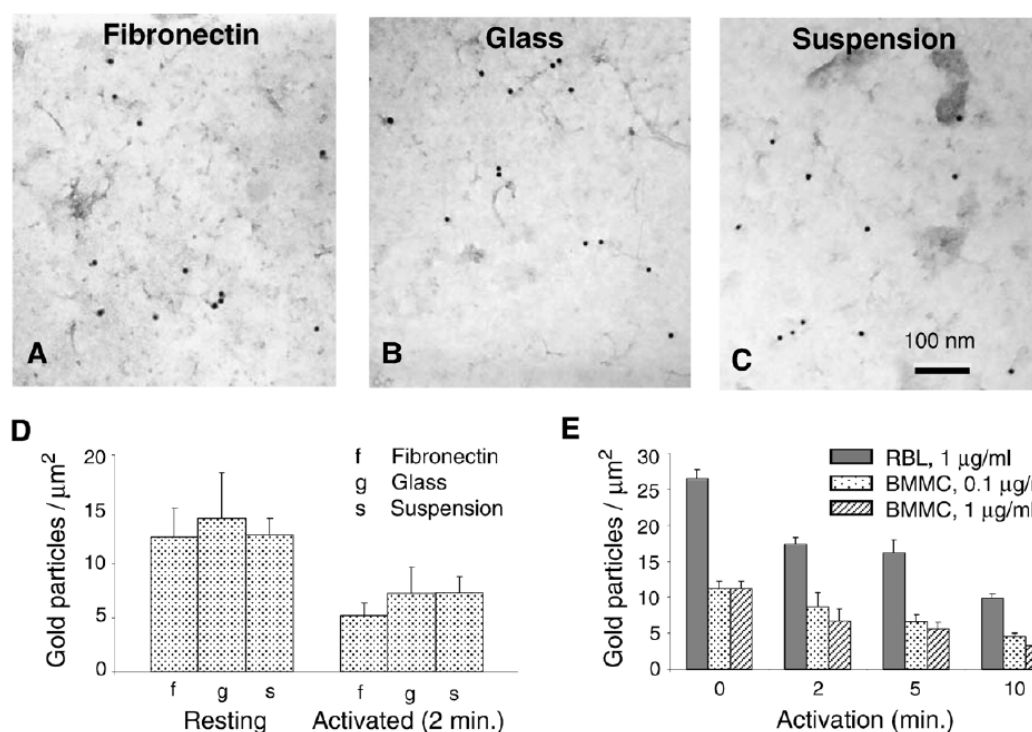


Fig. 3. Properties of PM sheets isolated by various procedures. (A)–(C) Examples of PM sheets isolated from resting BMMC by adhesion to fibronectin (A), adsorption to ultraclean glass coverslips (B), or direct pressing in suspension to EM grids (C). The Fc ϵ RI- β subunit was labeled on the cytoplasmic side with JRK mAb followed by goat anti-mouse IgG–10 nm gold conjugate. (D) The density of 10 nm gold immuno-labeled Fc ϵ RI- β on plasma membrane sheets isolated from resting or activated (2 min, 37 °C, 0.1 $\mu\text{g/ml}$ DNP-BSA) BMMC by adhesion to fibronectin (f), adsorption to ultraclean glass coverslips (g), or pressing in suspension to EM grids (s). (E) The density of 10 nm gold immuno-labeled Fc ϵ RI- β on PM sheets isolated from adherent RBL cells or ultraclean glass-adsorbed BMMC in the course of their activation with DNP-BSA at 37 °C. Means and SD from three independent experiments are shown; each experiment covers approximately 30 μm^2 of the PM.

was probably caused by internalization of aggregated Fc ϵ RI. The clusters that remained on PM after BMMC activation were slightly smaller than those before activation (Fig. 4A–D). As expected, analogous treatment of RBL cells led to formation of large (~200 nm) Fc ϵ RI aggregates (Fig. 4E–H). Fc ϵ RI clusters were of comparable size on resting BMMC and RBL cells, but they were more numerous in RBL cells. Thus the difference in Fc ϵ RI content of BMMC and RBL cell PM reflects differences in the number of Fc ϵ RI domains, but not in the Fc ϵ RI content of the individual domains.

Further experiments showed that no large Fc ϵ RI patches were formed at any time interval after BMMC triggering, either with low (0.1 $\mu\text{g/ml}$; Fig. 5A) or high (1 $\mu\text{g/ml}$; Fig. 5B) concentrations of antigen. When RBL cells were analyzed under comparable conditions, the formation of large Fc ϵ RI aggregates was clearly

observed 2 min after triggering (Fig. 5C). Thus, although Fc ϵ RI aggregates in BMMC are internalized to the same extent as in RBL cells (Fig. 3E), large patches of aggregated Fc ϵ RI are observed only in RBL cells.

Quantitative changes in the total amount of PM Fc ϵ RI in the course of activation were detected by flow cytometry. Data in Fig. 6 show that both BMMC and RBL cells rapidly internalized Fc ϵ RI during DNP-mediated stimulation (approximately 40% internalized within 2 min) and to a comparable extent, and that there was no significant difference between BMMC activated by 0.1 and 1 $\mu\text{g/ml}$ DNP-BSA. These results are in agreement with data obtained by EM on PM sheets.

In all previous experiments cells were activated at 37 °C. It has been shown that decreasing the temperature to 4 °C delays all signaling events including

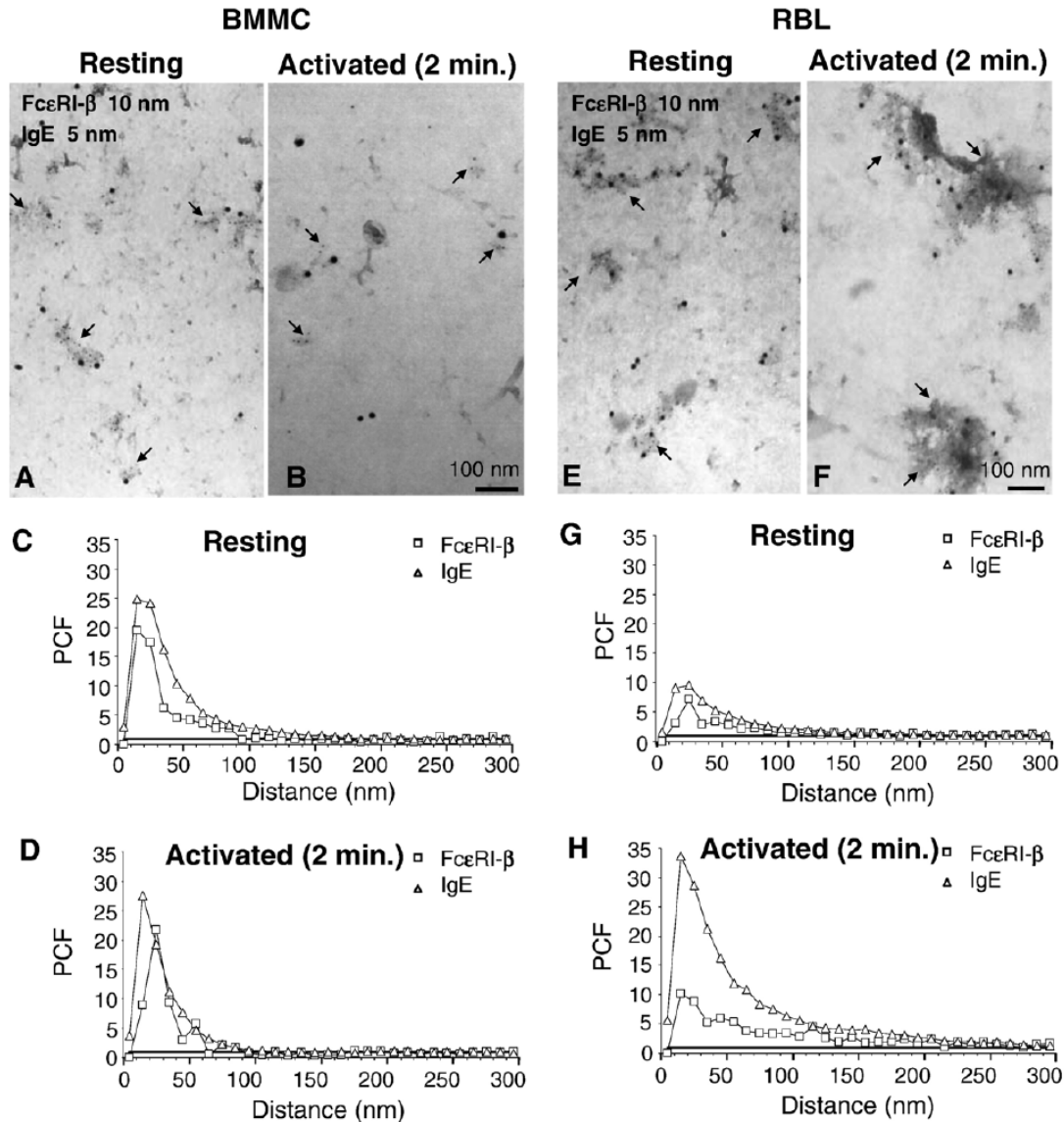


Fig. 4. Different topography of FcεRI in activated BMMC and RBL cells. (A, B) Topography of intracellularly labeled FcεRI-β subunit (10 nm gold particles) and FcεRI-α subunit-associated extracellular IgE (5 nm gold particles, indicated by arrows) in PM sheets isolated from resting (A) or activated (B; 2 min; 37 °C; 0.1 μg/ml DNP-BSA) BMMC. (C, D) Clustering of IgE (triangles) and FcεRI-β (squares) in resting (C) and activated (D) BMMC. Clustering is indicated at respective distance from a typical particle, when the pair correlation function (PCF) exceeds 1. Ideal random distribution of gold markers (PCF=1) is indicated by a solid line. The distance at which the peak of the PCF value falls to 1 indicates the size of the immuno-labeled aggregates of the respective membrane antigen (the real aggregates are, however, smaller, as the size of antibody sandwiches must be taken into consideration). The height of the peak indirectly reflects the density of these aggregates in PM (the higher the number of distinct aggregates within a certain area, the lower is the PCF peak) but it also reflects saturation of labeling within the aggregates (5 nm immunogold label provides a more intense signal than 10 nm immunogold label). Each graph represents data from two independent experiments covering approximately 60 μm². (E, F) As a control, topography of the FcεRI-β subunit and FcεRI-α subunit-associated extracellular IgE in resting (E) and activated (F) RBL cells is shown. (G, H) Clustering of IgE (triangles) and FcεRI-β (squares) in resting (G) and activated (H) RBL cells was calculated as above.

internalization, and preserves the formation of larger signaling assemblies (Holowka et al., 2000). We therefore assessed the formation of FcεRI patches in

cells activated at 4 °C. Under these conditions, enhanced FcεRI clustering in a time-dependent manner was found in both RBL cells and BMMC (Fig. 7).

3.3. Independent distribution of LAT and NTAL in BMMC

We have reported previously that the transmembrane adaptor proteins, LAT and NTAL, are localized in independent regions of the PM sheets isolated from resting RBL cells as well as after activation via Fc ϵ RI or Thy-1 (Volná et al., 2004; Heneberg et al., 2006). This was an unexpected finding because LAT and NTAL exhibit structural similarities, including submembrane localization of their palmitoylation sites and association with detergent-resistant membranes. To exclude the possibility that the observed independent distribution of the adaptors is connected with the tumor origin of RBL cells or their adhesive properties, we analyzed the topography of LAT and NTAL in PM sheets isolated from BMMC. In initial control experiments, LAT (Fig. 8A and D) or NTAL (Fig. 8B and E) were labeled with two different antibodies directed against the same target antigen. As anticipated, this resulted in colocalization of the immunogold markers on PM in both BMMC and RBL cells, and was reflected in enhanced values of the pair cross-correlation function (PCCF; Fig. 9A and B, black squares and black diamonds). This is an important control, excluding the possibility that localization of various proteins in different domains is caused by segregating activity of the antibodies. In contrast, when LAT and NTAL were labeled on the same membrane, both proteins were accumulated in separate domains not only in RBL cells but also in BMMC (Fig. 8C and F). Analysis of colocalization confirmed the topographical independence of LAT and NTAL in the PM sheets isolated from resting or activated BMMC (Fig. 9A) and RBL cells (Fig. 9B). These data prove that LAT and NTAL are clustered in different domains in non-tumor mast cells.

4. Discussion

Examination of isolated PM sheets by high-resolution EM is an established and powerful method for topographical analyses of various PM molecules. Originally, this method was developed for studies of cells growing adherent to a glass surface in the presence of culture media (Sanan and Anderson, 1991); however, such cells are rare among non-transformed leukocytes. Later, the method was modified to allow isolation of PM sheets from non-adherent T cells (Schade and Levine, 2002; Lillemeier et al., 2006). The cells were attached to the glass surface by interaction with immobilized immuno-ligands or by binding to PLL at 37 °C or 4 °C. Alternatively, biotinylated T cells were bound to glass coverslips covered with streptavidin. In these

methods, the immobilization step itself was lengthy (45–60 min). Here we report a new procedure for isolation of PM sheets from non-adherent leukocytes that is based on non-specific adhesion of cells to ultraclean glass in protein-free buffer. Compared to previous methods for isolation of membrane sheets from non-adherent cells, this new method has several advantages. First, it is very rapid, requiring only ~1 min for settling the cells onto ultraclean glass coverslips. This reduces the risk of artificial rearrangement of PM components during interaction of the cell with the substrate. Second, it is independent of binding of the cells to PLL, which has been reported to cause mast cell activation (Benyon et al., 1987). It should be noted that the mere binding of BMMC to ultraclean glass did not cause their degranulation or enhanced tyrosine phosphorylation of cellular proteins. Third, this method does not require preincubation of the cells at low temperature, which causes changes in organization of the PM (Magee et al., 2005). The only step required to be done at 4 °C before paraformaldehyde fixation, i.e. the plasma membrane ripping off procedure, takes no more than several seconds and is almost identical with the well-established procedure of isolation of plasma membrane sheets from adherent cells (Sanan and Anderson, 1991; Wilson et al., 2000). Fourth, the method can be performed in the presence of actin polymerization inhibitors such as latrunculin B, because it does not require active cytoskeleton-driven adsorption. This will facilitate future studies on the role of cytoskeletal components in the topography of various PM molecules.

In control experiments, we analyzed PM sheets prepared from BMMC bound to fibronectin-coated surfaces, a process dependent on cell sensitization. Alternatively, PM sheets were isolated from suspended cells pressed directly onto PLL-covered EM grids. As the topography of Fc ϵ RI and the quality of the isolated PM sheets did not differ among these two controls and the cells adsorbed to ultraclean glass, the technique based on adsorption to glass was used in subsequent studies. It is fast, can be used universally, and enables high effectivity even of extracellular leaflet labeling.

Changes in the topography of PM signaling molecules in the course of Fc ϵ RI-mediated activation have previously been examined by high-resolution EM almost exclusively in RBL cells (Lara et al., 2001; Wilson et al., 2000, 2002, 2004; Dráberová et al., 2004; Heneberg et al., 2006). Wilson et al. (2002) proposed the existence of primary signaling domains, possessing aggregated Fc ϵ RI in ~200 nm patches, and secondary signaling domains, enriched with the transmembrane adaptor protein LAT. Here we present for the first time

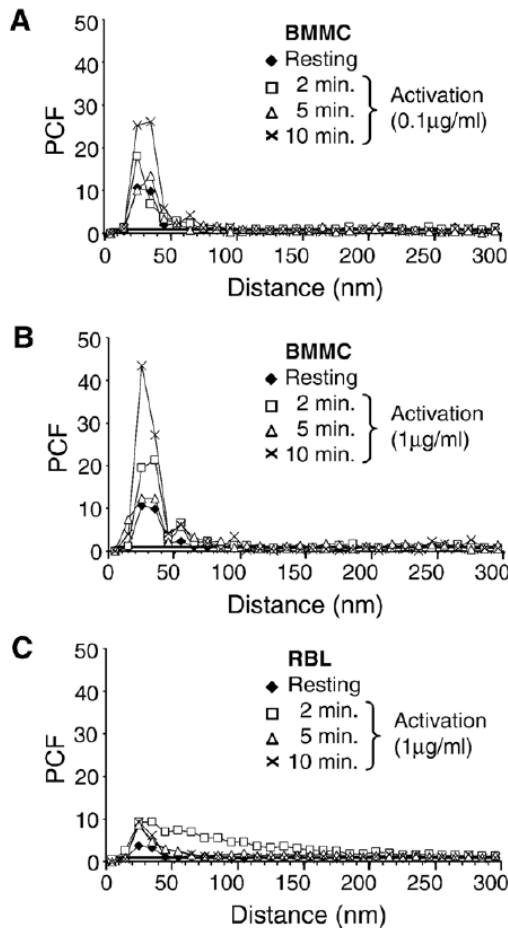


Fig. 5. Clustering of 10 nm immunogold-labeled Fc ϵ RI- β in the course of BMMC and RBL cell activation. BMMC were stimulated with 0.1 μ g/ml (A) or 1 μ g/ml (B), RBL cells with 1 μ g/ml (C) DNP-BSA at 37 °C. Clustering is plotted as described in Fig. 4. Each graph represents data from two independent experiments covering approximately 60 μ m².

the topography of Fc ϵ RI and downstream adaptors in PM sheets isolated from BMMC. In resting BMMC, the Fc ϵ RI was distributed in small clusters of comparable size to those observed in RBL cells, but the clusters were less numerous than in RBL cells. The overall amount of Fc ϵ RI- β subunits per μ m² was reduced almost to half in BMMC compared to RBL cells. The possibility that the enhanced amount of Fc ϵ RI- β in RBL cells is caused by transfer of the receptor from the ventral to the dorsal side during adhesive growth of the cells was excluded by experiments in which the membrane sheets from RBL cells were isolated in the same way as from BMMC and the difference still remained.

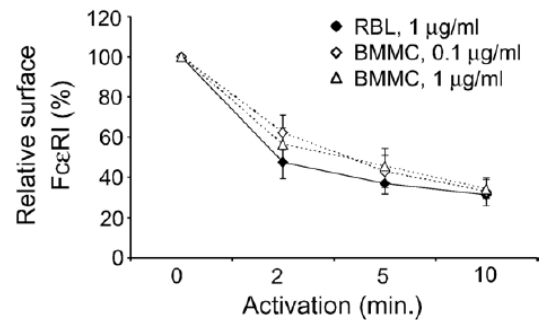


Fig. 6. Relative concentration of surface Fc ϵ RI on BMMC and RBL cells in the course of their activation by DNP-BSA as detected by flow cytometry. Fc ϵ RI-bound IgE was labeled by FITC-conjugated donkey anti-mouse Ig on fixed resting or activated cells. Means $\pm$ SD from three independent experiments are shown.

Unexpectedly, activation of IgE-sensitized BMMC with DNP-BSA at concentrations which are either optimal (0.1 μ g/ml) or supraoptimal (1 μ g/ml) for degranulation did not lead to formation of large Fc ϵ RI signaling domains. However, this treatment caused a decrease in the amount of detectable Fc ϵ RI- β on the

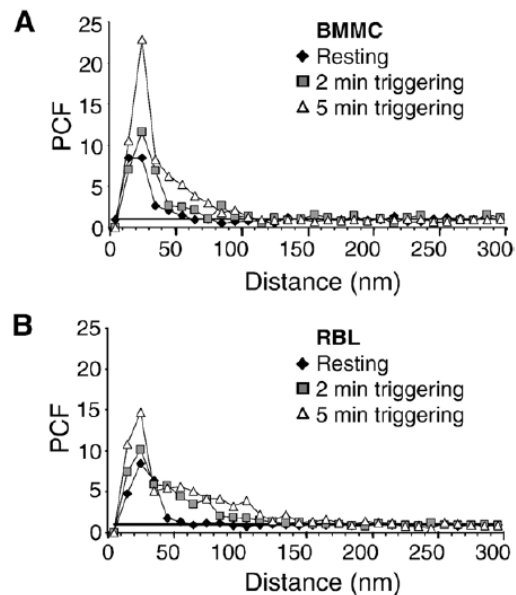


Fig. 7. Enhanced Fc ϵ RI clustering in BMMC stimulated at low temperature. IgE-sensitized BMMC (A) or RBL cells (B) were incubated for 10 min on ice and Fc ϵ RI was then triggered for 2 or 5 min with DNP-BSA on ice. PM sheets were isolated and Fc ϵ RI- β was labeled with JRK mAb followed by goat anti-mouse IgG–10 nm gold conjugate. Clustering is plotted as described in Fig. 4. Each graph represents data from two independent experiments covering approximately 60 μ m².

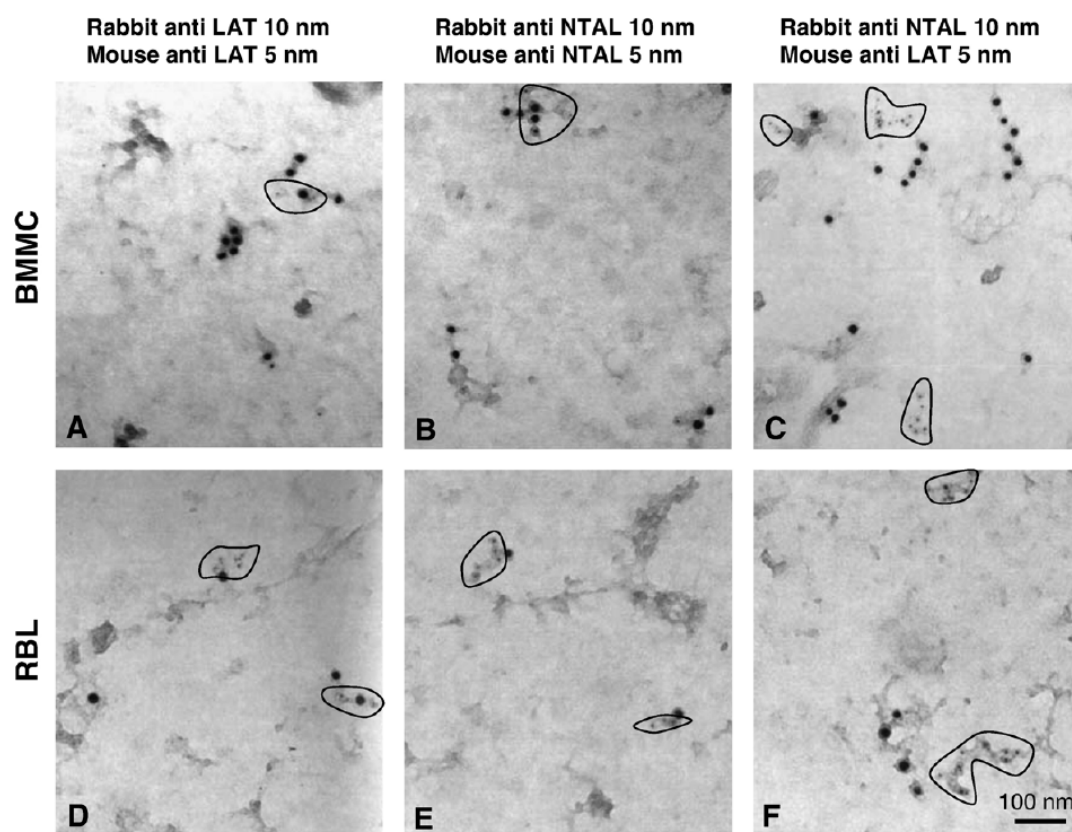


Fig. 8. Topography of LAT and NTAL on the PM sheets from resting cells. PM sheets were isolated from BMMC (A–C) or RBL cells (D–F). LAT (A, D) or NTAL (B, E) were labeled by two different antibodies or they were labeled together on the same membrane (C, F). Clusters of 5 nm gold are circled.

cytoplasmic side of the membrane sheets isolated from both BMMC and RBL cells. The observed decrease was probably caused by receptor internalization as shown using flow cytometry, which demonstrated an activation-dependent loss of cell surface FcεRI. The combined data indicate that activation of BMMC by multivalent antigen-IgE complexes leads to a rapid FcεRI internalization from multiple small patches of FcεRI aggregates. Thus, formation of large receptor aggregates, comparable in size to those formed in RBL cells activated under similar conditions, are not required for initiation of BMMC signaling events. The observed difference in size of the antigen-induced FcεRI patches between both mast cell types is likely to reflect different local dynamics, favoring formation of more numerous but smaller signaling domains in BMMC compared to RBL cells. When FcεRI triggering occurred at 4 °C, the differences between the two cell types disappeared. These data, together with our previous results that RBL cells can be activated by mAb-mediated dimerization of

the FcεRI without formation of detectable receptor clusters (Dráberová et al., 2004), suggest that primary signaling domains are much smaller than previously thought; their size in BMMC is comparable to that of FcεRI clusters observed in the PM isolated from resting cells. This calls for reconsideration of the view of initiation of mast cell signaling, thereby stressing subtle and dynamic processes, as well as the importance of further studies on other mast cell types.

FcεRI-mediated activation leads to rapid phosphorylation of the transmembrane adaptor proteins, LAT and NTAL, which serve as scaffolds for downstream signaling. Both adaptors are very similar in structure, including two acylation sites which are likely to cause enhanced resistance to solubilization in non-ionic detergents. Our previous EM studies on isolated PM sheets revealed that LAT and NTAL were localized in separate non-overlapping domains from both non-activated and FcεRI-activated RBL cells. When the GPI-anchored protein Thy-1 (whose aggregation causes

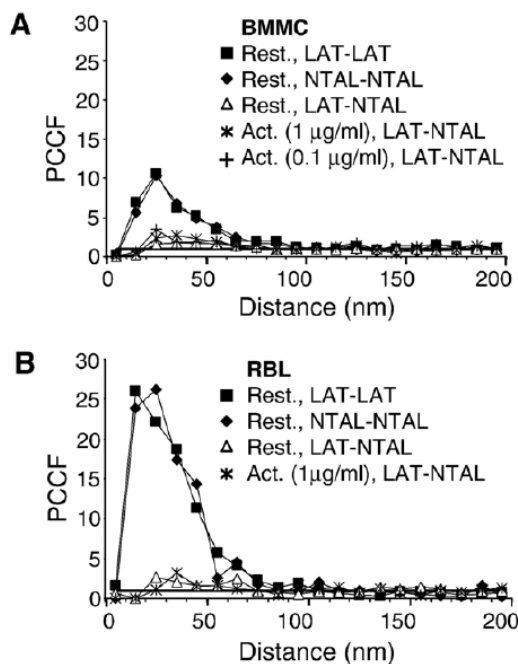


Fig. 9. Quantitative analysis of the topographical relationship between LAT and NTAL. The distribution of gold particles on PM sheets isolated from BMMC (A) or RBL cells (B) was mapped by pair cross-correlation function (PCCF). Co-localization is indicated at the respective distance from a typical particle when the PCCF exceeds 1. The ideal independent distribution of both gold markers (PCCF=1) is indicated by a solid line. The maximal width of the peak above value 1 reflects the distance at which both antigens are selectively closer to each other than would be in the case of random distribution. The height of the peak corresponds to the tendency of both antigens to co-localize and it is reduced when the overall label density is higher. The black squares and black diamonds show LAT or NTAL simultaneously labeled with rabbit and mouse antibodies against the same adaptor (positive controls). Other markers show PCCF for LAT and NTAL labeled together on resting or DNP-BSA-activated cells as indicated. Each graph represents data from two independent experiments covering approximately $60 \mu\text{m}^2$.

mast cell degranulation (Dráberová, 1989)) were labeled in fixed state or aggregated by mAb, both adaptors colocalized with the clustered Thy-1, but again, no mixed adaptor clusters were formed (Volná et al., 2004; Heneberg et al., 2006). Experiments with BMMC from knock-out mice revealed that LAT and NTAL had different functions (Volná et al., 2004; Zhu et al., 2004). Because the topography of these adaptors in BMMC has not yet been studied, we made use of the method of isolation of PM sheets described above to address this issue. To exclude the objection that separation of both adaptors may be induced secondarily by the antibodies used for detection, each of the adaptor proteins was

labeled with different (rabbit and mouse) antibodies together. This resulted in strong colocalization of both markers, indicating that the antibodies are not by themselves responsible for separation of the proteins. However, when the same antibodies were used for simultaneous detection of LAT and NTAL, they clearly showed different domains for both adaptors not only in RBL cells, but also in BMMC. Thus, sequestration of LAT and NTAL does not seem to be an artifact observed only in the immortalized RBL cell line, and does not reflect the experimental procedures.

Why should these two structurally similar proteins form separate domains in the PM? Perhaps differences in the amino acid sequence of their transmembrane domains could cause association with different lipids within the membrane rafts. Alternatively, cytoplasmic domains may interact with different proteins leading to formation of variant signalosomes. The latter possibility is supported by findings that in T cells, LAT associates with CD2 coreceptor and tyrosine kinase Lck in discrete PM regions, which depend on protein–protein interactions mediated through LAT phosphorylation sites but not on interactions with lipid rafts (Douglass and Vale, 2005). As the repertoire of LAT and NTAL cytoplasmic docking sites is not identical (Brdička et al., 2002), the specific protein–protein network might result in separation of both adaptors. However, the finding that NTAL and LAT are sequestered into discrete domains even before cell triggering and therefore in the absence of their tyrosine phosphorylation, suggests that other mechanisms are also involved.

Although restricted to fixed specimens, high-resolution EM combined with immunogold labeling is a powerful technique that has brought about important findings on PM organization (Wilson et al., 2002, 2004; Prior et al., 2003; Heneberg et al., 2006; Lillemeier et al., 2006). Extension of the method to rapid and simple isolation of PM sheets from non-adherent cells will contribute to its wider use and thus to better understanding of signaling events in various immune system cells which are mostly non-adherent.

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4.3. THE TRANSMEMBRANE ADAPTOR PROTEIN NTAL SIGNALS TO MAST CELL CYTOSKELETON VIA THE SMALL GTPASE RHO

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In previous studies (see section 4.1) we obtained evidence, that NTAL affects formation of F-actin in RBL cells. In this study we investigated morphology, actin cytoskeleton, adhesion, migration and the activities of small Rho GTPases following antigen and/or SCF activation of WT and NTAL-deficient BMMCs. We provide first evidence that NTAL signals via RhoA to cytoskeleton in mast cells after FcεRI triggering.

The transmembrane adaptor protein NTAL signals to mast cell cytoskeleton via the small GTPase Rho

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The transmembrane adaptor protein NTAL (non-T-cell activation linker) participates in signalosome assembly in hematopoietic cells, but its exact role in cell physiology remains enigmatic. We report here that BM-derived mast cells from NTAL-deficient mice, responding to Ag alone or in combination with SCF, exhibit reduced spreading on fibronectin, enhanced filamentous actin depolymerization and enhanced migration towards Ag relative to WT cells. No such differences between WT and NTAL^{-/-} BM-derived mast cells were observed when SCF alone was used as activator. We have examined the activities of two small GTPases, Rac and Rho, which are important regulators of actin polymerization. Stimulation with Ag and/or SCF enhanced activity of Rac(1,2,3) in both NTAL^{-/-} and WT cells. In contrast, RhoA activity decreased and this trend was much faster and more extensive in NTAL^{-/-} cells, indicating a positive regulatory role of NTAL in the recovery of RhoA activity. After restoring NTAL into NTAL^{-/-} cells, both spreading and actin responses were rescued. This is the first report of a crucial role of NTAL in signaling, via RhoA, to mast cell cytoskeleton.

Key words: Cell activation • Cytoskeleton • Mast cells



Supporting Information available online

Introduction

Mast cells in an adult organism originate from HSC-derived progenitors in BM. Their recruitment into connective tissues requires cell adhesion, spreading and migration; these functions are crucial for mast-cell retention, proliferation, differentiation, survival, priming and activation, and depend on transduction of signals from cell surface receptors to the cytoskeleton [1]. Mast cells possess the high affinity IgE receptor (FcεRI), whose aggregation triggers a sequence of signaling events that

eventually lead to release of pro-inflammatory mediators. Events immediately following FcεRI aggregation are the phosphorylation of tyrosines in the ITAM of the receptor, and subsequent assembly of signaling complexes [2]. Transmembrane adaptor proteins play an essential role in the organization of such assemblies; after their phosphorylation, they serve as recruitment sites for numerous signaling molecules [3]. The transmembrane adaptor protein non-T-cell activation linker (NTAL), also known as linker for activation of B cells or T cells (LAT)2, is structurally related to LAT. Uniquely, both NTAL and LAT are expressed in mast cells and may be involved in complementary signaling pathways. Both adaptors become rapidly tyrosine-phosphorylated upon FcεRI

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aggregation. Phosphorylated NTAL is known to associate with cytoplasmic signaling molecules such as Grb2, Sos1 and Vav [4–6].

After Ag stimulation, BM-derived mast cells (BMMC) from NTAL-deficient mice exhibit an increase (relative to WT cells) in tyrosine phosphorylation of several substrates and in the activities of phospholipase C γ 1 and 2 and other enzymes. This leads to an increase in concentration of free cytoplasmic Ca²⁺ [Ca²⁺]_i and degranulation [7, 8]. Not much is known about the effects of NTAL deficiency on the responses to SCF, a cytokine important for mast cell proliferation and differentiation. SCF acts as a mast cell chemoattractant via c-kit, a tyrosine kinase receptor, and induces and/or potentiates mast cell degranulation [9]. After c-kit receptor engagement, NTAL becomes phosphorylated. In human and murine mast cells, Fc ϵ RI- and SCF-mediated NTAL phosphorylations are regulated differentially by Src family kinases and c-kit, respectively [10].

An indication that NTAL may be involved in signaling to actin cytoskeleton has been obtained in rat basophilic leukemia cells: changes in expression levels of NTAL led to alterations in the amount of filamentous actin (F-actin) in resting as well as Ag-activated cells [11]. However, molecular mechanism of the cross-talk between NTAL and actin has not been studied. In this study we examined morphology, actin cytoskeleton, migration and the activities of Rac and Rho, small Rho GTPases regulating actin cytoskeleton [12], following activation of WT and NTAL^{-/-} BMMC by Ag and/or SCF. Our results provide the first evidence of a link between NTAL and the small GTPase RhoA and of its importance for regulation of mast-cell spreading and migration via actin polymerization.

Results

Reduced spreading of NTAL^{-/-} mast cells in response to Ag

In accordance with a previous study [13], resting BMMC derived from WT mice adhered to fibronectin-coated glass poorly and did not spread, but after Ag challenge their adhesion increased and the cells spread. F-actin staining of resting BMMC revealed a strong subplasmalemmal actin cortex and some microvilli, while Ag-activated cells exhibited a more complex staining and ruffling (Fig. 1A). BMMC derived from NTAL^{-/-} mice also exhibited an increased adhesion after Ag triggering, but their spreading, compared with WT cells, was noticeably reduced; a large subpopulation of unspread cells was present (Fig. 1B, Ag). Contrary to the Ag response, SCF induced comparable spreading and ruffling in both WT and NTAL^{-/-} cells (Fig. 1B, SCF). When cells were activated with both Ag+SCF, spreading of WT cells was extensive, but that of NTAL^{-/-} cells was again reduced (Fig. 1B, Ag+SCF).

Quantitative assessment of spreading was obtained by measuring the cell area (the average number of pixels per cell). WT cells began to spread within 5 min following Ag stimulation,

while the response was delayed in NTAL^{-/-} cells (Fig. 1C, Ag). These data were supported by calculating the proportion of spread cells (Fig. 1D, Ag).

Evaluation of SCF-induced spreading confirmed that NTAL^{-/-} and WT cells respond to SCF in a similar manner (Fig. 1C and D, SCF), indicating that SCF-induced cell spreading is NTAL-independent. However, when cells were stimulated with Ag and SCF simultaneously, the difference between spreading responses of WT and NTAL^{-/-} cells was even more pronounced. Relative area of Ag+SCF-stimulated WT cells was significantly higher ($p < 0.012$ at 20 min) than the relative area of WT cells stimulated with Ag alone (Fig. 1C, Ag+SCF and Ag). On the other hand, spreading of Ag+SCF-stimulated NTAL^{-/-} cells remained similar to that observed after Ag alone. When counting the proportion of spread cells, the same trends were observed (Fig. 1D). Even when spread subpopulations only were evaluated (Fig. S1), areas of Ag and Ag+SCF-activated NTAL^{-/-} cells were smaller than those of WT cells. These data suggest that Fc ϵ RI-induced cell spreading is dependent on NTAL.

Integrin-dependent increase in cell adherence

Cell spreading is preceded by an increase in cell adhesion, a process dependent on integrins [13]. We investigated any differences in adhesiveness to fibronectin between WT and NTAL^{-/-} cells (Fig. 2A). After Ag challenge, the proportion of adherent cells increased in both cell types; a small yet significant reduction in adhesive response of NTAL^{-/-} cells was apparent. In contrast, the responses to SCF were the same for both cell types. Stimulation with Ag+SCF led to a substantially lower adhesiveness in NTAL^{-/-} cells than in WT cells.

Ag- or SCF-induced increases in adherence were significantly inhibited by Ab specific for β 1 integrin (Fig. 2B and C) while those specific for integrins α 4, α 5, β 2 and β 7 gave no significant inhibition. Because Ag-induced cell spreading (Fig. 1C and D) was affected by NTAL deficiency much more noticeably than cell adherence, it is likely that events downstream of integrins, such as actin polymerization, are responsible for the defective spreading of NTAL^{-/-} cells after Ag and Ag+SCF.

Enhanced depolymerization of F-actin in Ag-stimulated NTAL^{-/-} cells

Next we compared actin responses of WT and NTAL^{-/-} cells stimulated with Ag, SCF or combination of the two. In WT cells, Ag induced an increase in F-actin content peaking at 3–5 min, followed by a gradual return to basal levels (Fig. 2D). In contrast, NTAL^{-/-} cells exhibited a strong loss of F-actin, with the lowest level at 3 min, followed by a slow recovery. On the other hand, SCF induced an increase in F-actin content in both cell types (Fig. 2E), reaching a peak within 5 min. These results indicate a different role of NTAL in actin-regulatory pathways initiated by Fc ϵ RI and c-kit. The combination of Ag and SCF, however,

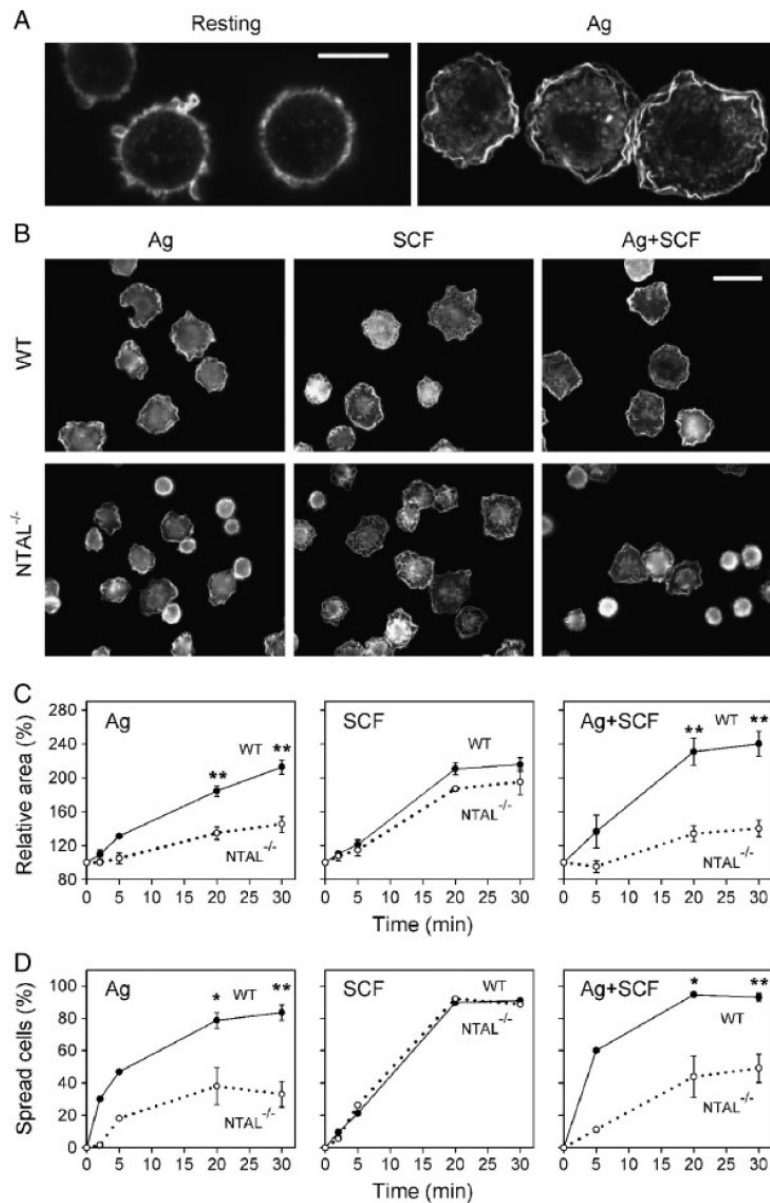


Figure 1. Positive regulation of cell spreading by NTAL in Ag-activated cells. (A) IgE-sensitized WT BMMC were attached to fibronectin-coated glass surface and then stimulated with Ag (Ag) or not (Resting). After 20 min, cells were fixed and stained for F-actin. The images are confocal micrographs taken at equatorial planes. Scale bar, 10 μ m. (B) IgE-sensitized WT or NTAL^{-/-} BMMC were activated for 20 min with Ag, SCF or both (Ag+SCF) and stained for F-actin. Scale bar, 20 μ m. (C and D) WT (full line) and NTAL^{-/-} (dotted line) BMMC were treated as in (B), fixed at indicated times and stained for F-actin. Cell area (C) was determined and normalized to nonactivated cells. Proportion of spread cells (D) was calculated from the total number of stained cells; remaining cells were rounded or ambiguous. At least 200 cells were evaluated in each experiment. Means and SE were calculated from five independent experiments, except for the 5-min interval (two experiments). Significance of differences at given times between WT and NTAL^{-/-} cells is shown (* $p < 0.05$; ** $p < 0.005$; Student's *t*-test).

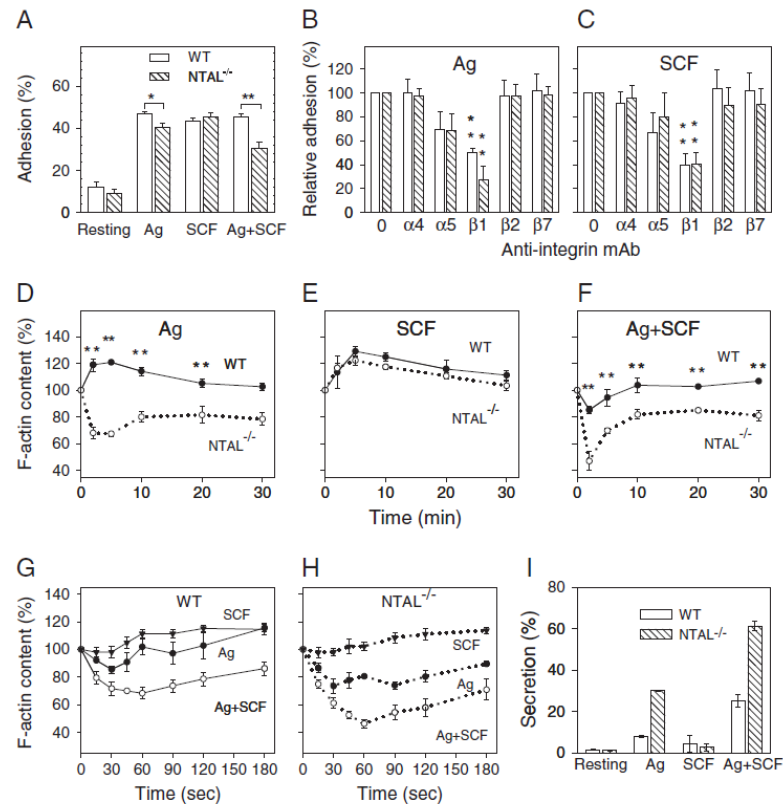


Figure 2. Ag- and/or SCF-induced cell adherence, actin polymerization and secretion. (A) IgE-sensitized and calcein-loaded WT (open columns) or NTAL^{-/-} (hatched columns) BMMC were transferred into fibronectin-coated wells and left nonactivated (Resting) or activated (45 min at 37°C) with Ag, SCF or Ag+SCF. Fluorescence was determined before (100%) and after washing out the non-adherent cells, and percentages of adherent cells were calculated. Values indicate means ± SE (n = 13). (B and C) Calcein-loaded WT (open columns) and NTAL^{-/-} (hatched columns) BMMC were incubated with function-blocking anti-integrin antibodies (20 µg/mL) before their transfer into fibronectin-coated wells. Adherence was detected as above after activation of the cells with Ag (B) or SCF (C). Values (means ± SE, n = 6) indicate percentages of adherent cells in the presence of blocking Ab, normalized to adherence in the absence of the Ab. (D–F) IgE-sensitized WT (full line) and NTAL^{-/-} (dotted line) BMMC were challenged with Ag (D), SCF (E) or Ag+SCF (F). At the indicated times, cells were fixed, stained for F-actin and analyzed by flow cytometry. Data (means ± SE, n = 3–6 independent experiments performed in triplicates) were normalized to the fluorescence of resting cells (similar in both cell types). (G and H) Detailed analysis of F-actin content for the first 3 min after activation with Ag (closed circles), SCF (closed triangles) or Ag+SCF (open circles). Cells were treated and analyzed as in (D–F). (I) IgE-sensitized WT (open columns) and NTAL^{-/-} (hatched columns) BMMC were activated for 15 min with Ag, SCF or Ag+SCF. Secretion was determined and expressed as the percentage of β-glucuronidase in supernatants. Values indicate means ± SE (n = 4). Significant differences between WT and NTAL^{-/-} cells are shown in A and D–F. Significance of differences in adherence of the cells in the absence or presence of blocking Ab is shown in B and C (*p < 0.05; **p < 0.005; Student's t-test).

enhanced F-actin depolymerization; the effect was apparent even in WT cells (Fig. 2F).

WT cells challenged with Ag alone responded with a small decrease in F-actin content in the first minute, before the onset of actin polymerization (see Fig. 2G, note the time scale). This initial decrease became more pronounced when Ag and SCF were applied together. Secretion was higher after Ag+SCF challenge compared with Ag alone (Fig. 2I). Thus, enhanced secretion seems to correlate with higher F-actin depolymerization. SCF alone did not induce F-actin depolymerization (Fig. 2E, G and H) and failed to cause degranulation (Fig. 2I).

Drug-induced F-actin depolymerization in WT and NTAL^{-/-} cells

To investigate whether the greater F-actin depolymerization in Ag-stimulated NTAL^{-/-} cells causes their increased secretory responses, we examined BMMC pre-treated with latrunculin B (LB), a drug which inhibits actin polymerization by sequestering actin monomers [14]. Figure 3A shows that both WT and NTAL^{-/-} cells exhibited similar sensitivity to LB: a 50% decrease in F-actin was achieved by ~0.3 µg LB/mL. In the presence of LB up to ~0.5 µg/mL, WT cells were still able to increase their

F-actin content in response to Ag; in contrast, LB-treated NTAL^{-/-} cells responded to Ag by a further decrease in their F-actin content (Fig. 3B). These results again indicate a weakened signal for actin polymerization in NTAL^{-/-} cells.

Depolymerization of F-actin prior to Ag activation did not eliminate the differences in secretion from WT and NTAL^{-/-} cells; they persisted at all LB concentrations tested (Fig. 3C). Thus the data indicate that the greater depolymerization of F-actin in NTAL^{-/-} cells is not the main reason for their greater secretion. The bell-shaped secretory responses to increasing LB concentrations are compatible with the reported stimulatory effects of LB at low concentrations [15] and the inhibitory effects at higher concentrations [16].

Differences in calcium mobilization do not explain the potentiating effect of SCF on F-actin depolymerization

To find out whether the extent of F-actin depolymerization correlates with the strength of calcium signals, Fura-2-loaded BMMC were used. The typical stronger and more sustained response [7] was seen in Ag-stimulated NTAL-deficient cells (Fig. 4A). In contrast, after SCF stimulation, an increase in $[Ca^{2+}]_i$ was similar for WT and NTAL^{-/-} cells except for the short time interval (~1 min after triggering) when the response of WT cells was higher than that of NTAL^{-/-} cells (Fig. 4B). The more sustained calcium response of NTAL-deficient cells was also seen after activation by Ag+SCF (Fig. 4C). However, the combination did not induce enhanced calcium responses (relative to Ag alone; compare Fig. 4A and C); in fact they were significantly decreased ($p < 0.05$ at interval 131–201 s). Therefore, increased $[Ca^{2+}]_i$ cannot be responsible for the enhanced F-actin depolymerization and for the potentiation of secretion.

Regulatory role of NTAL in chemotactic response

Further experiments explored the role of NTAL in Ag- and/or SCF-induced cell migration. In transwell migration assays,

NTAL^{-/-} BMMC exhibited significantly faster migration towards Ag than WT cells (at least twofold, Fig. 4D). In contrast, no difference between WT and NTAL^{-/-} cells was observed in SCF gradient; both cell types migrated much faster towards SCF than towards Ag. WT cells responding to a mixture of Ag+SCF exhibited migration that was slower than that towards SCF alone, but faster than that towards Ag alone. Migration of NTAL^{-/-} cells towards Ag+SCF was also slower than that towards SCF alone, but similar to that towards Ag. Thus, NTAL is a negative regulator of chemotaxis towards Ag but has no apparent role in chemotaxis towards SCF.

Activation of Rac and inactivation of Rho in cells responding to Ag and/or SCF

Since Rho-family GTPases are the major regulators of actin dynamics [12], we examined changes in the state of activation of two members of this family, Rac(1,2,3) and RhoA, both of which are involved in regulation of cell spreading, migration and exocytosis [17–19]. Levels of active GTPases were examined by G-LISA assays, specific for Rac(1,2,3) (pan Rac), Rac1 or RhoA. Figure 5A–C shows that after stimulation with Ag, SCF or both, Rac(1,2,3) activity rapidly increased in both cell types. Similarly, 3 min activation with Ag or SCF increased activity of Rac1 in both cell types, with no meaningful differences between WT and NTAL^{-/-} cells (Fig. 5G).

On the other hand, Ag activation induced a decrease in the activity of RhoA that was significantly more pronounced in NTAL^{-/-} cells (Fig. 5D). RhoA inactivation was rapid, occurred within the first minute and was followed by a slow recovery. The combination of Ag+SCF induced even more intense and longer-lasting decline in RhoA activity in both cell types (Fig. 5F) and again, RhoA inactivation was stronger in NTAL^{-/-} cells. SCF alone also induced a decrease, more pronounced in NTAL^{-/-} cells (Fig. 5E), but its extent was lower than that induced by Ag±SCF.

We also examined the effects of a Rho kinase (ROCK) inhibitor, Y-27632, and observed a significant decrease of cell spreading in both Ag- and Ag+SCF-activated WT cells (Fig. 6A and C). In

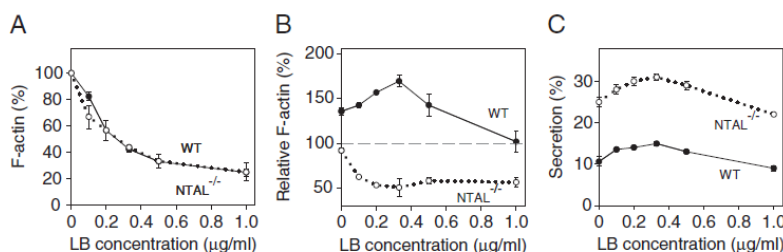


Figure 3. Effects of LB on actin polymerization and secretion. (A–C) IgE-sensitized WT (full line) and NTAL^{-/-} (dotted line) BMMC were pre-treated for 1 h at 37°C with the indicated concentrations of LB. (A) Cells were fixed, stained for F-actin and analyzed by flow cytometry. Data were normalized to the fluorescence of cells in the absence of LB. (B) The cells were challenged with Ag for 15 min and analyzed as in (A). Data represent F-actin content of activated cells normalized to that of resting cells at the appropriate LB concentration (dashed line). (C) Secretion of the cells activated as in B was assayed 15 min after triggering. Values indicate means ± SE ($n = 4$).

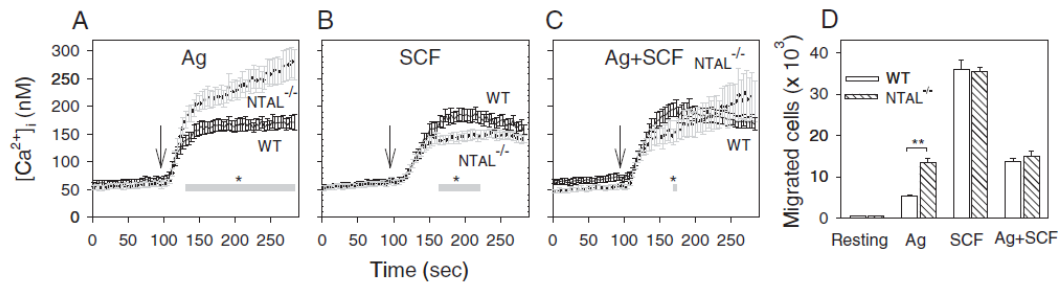


Figure 4. Calcium and chemotactic responses. (A–C) IgE-sensitized WT and NTAL^{-/-} cells were loaded with Fura-2 and stimulated (arrows) with Ag (A; 250 ng/mL TNP-BSA), SCF (B; 40 ng/mL) or both (C). $[Ca^{2+}]_i$ was monitored by spectrofluorometry. Values indicate means $\pm$ SE ($n = 9$). (D) Chemotactic assays were performed with IgE-sensitized WT or NTAL^{-/-} BMMC using medium alone (Resting) or the indicated chemoattractants (at concentrations as above) in the lower wells. Each group consisted of BMMC from three different WT or NTAL^{-/-} mice; means and SE are shown. Significant differences (Student's *t*-test) between WT and NTAL^{-/-} cells are indicated by grey lines below the curves (A–C; * $p < 0.05$) or over the column (D; ** $p < 0.005$).

contrast, no significant inhibition of (already reduced) spreading was observed in Ag- or Ag+SCF-activated NTAL^{-/-} cells. Moreover, Y-27632 did not inhibit spreading of either cells activated with SCF alone (Fig. 6B). Thus NTAL, via RhoA and ROCK, plays an important role in Ag- but not SCF-induced spreading.

RhoA activity is negatively regulated by phosphorylation/activation of p190RhoGAP [20, 21]. To determine whether NTAL regulates RhoA activity through p190RhoGAP pathway we immunoprecipitated p190RhoGAP from nonactivated or Ag-activated WT and NTAL^{-/-} cells and analyzed its tyrosine phosphorylation by immunoblotting. Data presented in Fig. S2A and B indicate that both WT and NTAL^{-/-} cells exhibited enhanced tyrosine phosphorylation of p190RhoGAP after stimulation with Ag. However, no significant difference between the two cell types was observed.

Localization and content of Rac1 and RhoA

Localization of the GTPases was studied by immunofluorescence using Ab specific for RhoA and Rac1. In resting BMMC, both GTPases appeared mainly cytosolic; only a small fraction of fluorescence was cortical and colocalized with F-actin (Fig. S3A and B, resting). After Ag activation, enhanced colocalization with F-actin on the plasma membrane was apparent, especially in the case of RhoA, but the bulk of staining was still in the cytosol for both GTPases (Fig. S3A and B; activated). Using ImageJ program and manually selecting the cell membrane regions, we estimated percentage of the plasma membrane-associated RhoA as $15.2 \pm 1.6\%$ ($n = 17$) and $13.1 \pm 1.6\%$ ($n = 17$) in WT and NTAL^{-/-} Ag-activated cells, respectively, the difference being insignificant.

To determine the overall content of the GTPases, cell lysates from resting cells were used for immunoblotting. The total content of Rac1, about 50 ng/10⁶ cells, was similar in both WT and NTAL^{-/-} cells (Table S1). G-LISA assays have shown the presence of about 3 ng of active Rac1/10⁶ cells. The overall content of RhoA was 53.9 ± 4.8 ng and 41.2 ± 6.0 ng per 10⁶ WT

and NTAL^{-/-} cells, respectively. Notably, NTAL^{-/-} cells contained significantly higher proportion of active RhoA (53%) than WT cells (22%; $p < 0.005$; Student's *t*-test).

The overall actin content was similar in both cell types ($\sim 10 \mu\text{g actin}/10^6$ cells). With the cell diameter of about 13 μm for both WT and NTAL^{-/-} cells, the intracellular concentrations of actin could be estimated at $\sim 200 \mu\text{M}$. The calculated concentrations of total RhoA and Rac1 were both about 2 μM . Thus, molar ratios of both RhoA and Rac1 to actin are estimated as about 1:100 (Table S1).

Phenotype reversion of NTAL^{-/-} BMMC

Finally, to confirm the role of NTAL in cell spreading and actin polymerization, we prepared NTAL-enhanced GFP (EGFP) construct and transfected it into NTAL^{-/-} BMMC. Subsequent sorting of the transfected cells into EGFP⁺ and EGFP⁻ populations allowed evaluation of Ag-induced spreading and actin responses in NTAL-deficient cells following successful or unsuccessful restoration of NTAL. Three separate experiments were performed, each conducted with two independent cultures of each cell type. Figures 7A and B are images of a typical experiment in which NTAL^{-/-} cells were transfected with NTAL-EGFP and visualized for F-actin and EGFP. It is obvious that cells that are EGFP⁺ exhibit more spreading than those that are EGFP⁻. NTAL-EGFP was localized mostly on the plasma membrane (Fig. 7B).

Figure 7C shows evaluation of Ag-induced spreading of the controls and transfected cells. As expected, activated WT cells have more than doubled their cell area. Nontransfected NTAL^{-/-} cells exhibited a greatly reduced spreading relative to WT cells, confirming the above results. Transfection of NTAL^{-/-} cells with NTAL-EGFP and subsequent cell sorting showed that NTAL^{-/-}EGFP⁺ cells exhibited significantly higher increase in their cell area than NTAL^{-/-}EGFP⁻ cells and NTAL^{-/-} cells. Insertion of NTAL-EGFP into NTAL^{-/-} cells has also restored their defect in Ag-induced actin polymerization (Fig. 7D). While EGFP⁺ cells behaved

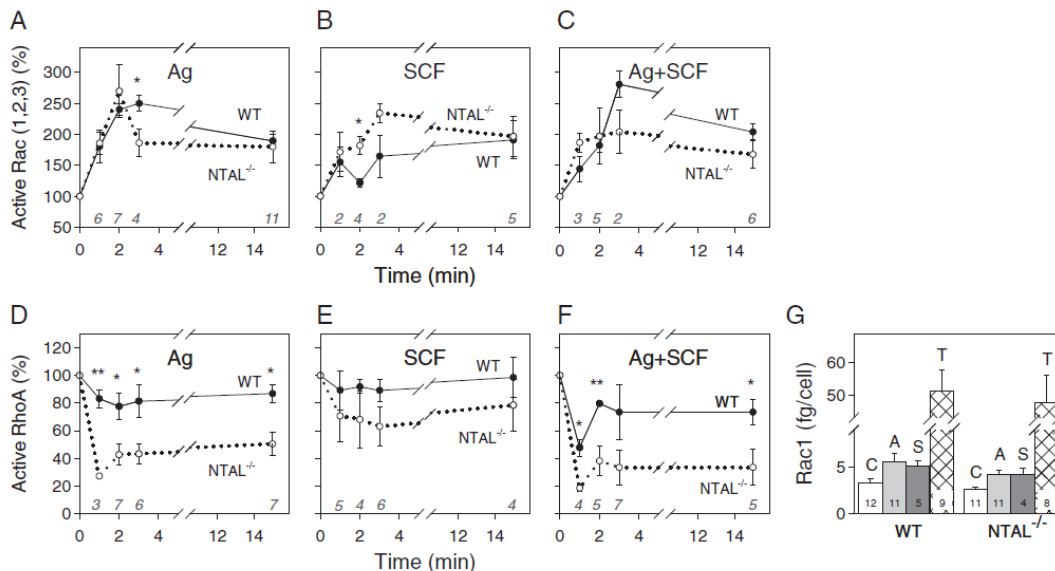


Figure 5. Activities of Rac and Rho GTPases. (A–F) IgE-sensitized WT (full line) and NTAL^{-/-} (dotted line) BMMC were activated with Ag (A and D), SCF (B and E) or Ag+SCF (C and F) at concentrations as in Fig. 4. At given times, active Rac(1,2,3) (A–C) or RhoA (D–F) were determined by G-LISA assays. Data were normalized to the values obtained in resting cells (see Table S1). Values indicate means $\pm$ SE, n values for each time point are shown above the horizontal axes. (G) Lysates of WT and NTAL^{-/-} cells were prepared and examined by immunoblotting for total Rac1 (T, crosshatched columns). Amounts of active Rac1 in resting cells (C) or cells activated with Ag (A) or SCF (S) for 3 min were determined by G-LISA assays. Values indicate means $\pm$ SE; n values are shown at the bottom of the columns. Significant differences between WT and NTAL^{-/-} cells are shown (* p < 0.05; ** p < 0.005; Student's t -test).

as WT cells, EGFP⁻ cells exhibited loss of F-actin comparable to control NTAL^{-/-} cells. Actin responses of WT cells, transfected with empty pEGFP vector alone, were similar to those seen in nontransfected WT cells (data not shown). Thus, the impact of NTAL restoration was very strong, confirming the crucial role of NTAL in mast-cell signaling to actin cytoskeleton.

Discussion

We have found that in response to Ag stimulation, NTAL-deficient BMMC spread on fibronectin less than WT cells. Hence we have examined the role of NTAL in signaling from Fc ϵ RI and c-kit to mast cell cytoskeleton. As reported previously [13], adherence of resting WT BMMC to fibronectin was weak, but increased after Ag or SCF triggering. Ag-induced increase in adherence of NTAL^{-/-} cells was reduced only by $\sim$ 15% relative to WT cells, insufficient to account for their substantial defect in Ag-induced spreading.

Ag, however, induced disparate actin responses in WT and NTAL^{-/-} cells, suggesting that the deficiency in actin polymerization in NTAL^{-/-} cells is responsible for their inefficient spreading. Previous work has shown higher Ag-induced degranulation from NTAL-deficient BMMC and this trend was associated with an increased phosphorylation of the adaptor LAT [7]. Enhanced and more persistent calcium responses in NTAL-defi-

cient cells were also reported [7, 8]. However, we did not find any simple correlation between secretion, [Ca²⁺]_i and F-actin depolymerization. Although higher secretion from NTAL^{-/-} cells was associated with both greater F-actin depolymerization and stronger calcium response, the correlation did not always stand, indicating that additional factors must be involved. For example, LB-induced F-actin depolymerization did not eliminate the differences in secretion from the two cell types. Moreover, simultaneous activation with Ag and SCF resulted in both enhanced secretion and F-actin depolymerization, but calcium levels were not higher than those induced by Ag alone. Similar lack of correlation between the extents of Ag-induced secretion and calcium signals was reported for Lyn-deficient cells [22].

In contrast to the different responses of WT and NTAL^{-/-} cells to Ag, reactions to SCF were similar. Calcium responses to SCF were slower than those to Ag, and no secretion was apparent for SCF concentrations $\leq$ 40 ng/mL. Although NTAL is phosphorylated after SCF stimulation, its phosphorylation is weaker and more transient than that after Ag ([23] and our unpublished data). This could, at least partially, explain the lack of NTAL requirements for SCF responses. Lam *et al.* [24] found that adhesion to fibronectin of SCF- but not Ag-activated BMMC does not require increase in [Ca²⁺]_i. Our data confirm that signaling pathways to cell spreading are different in c-kit- and Fc ϵ RI-activated cells.

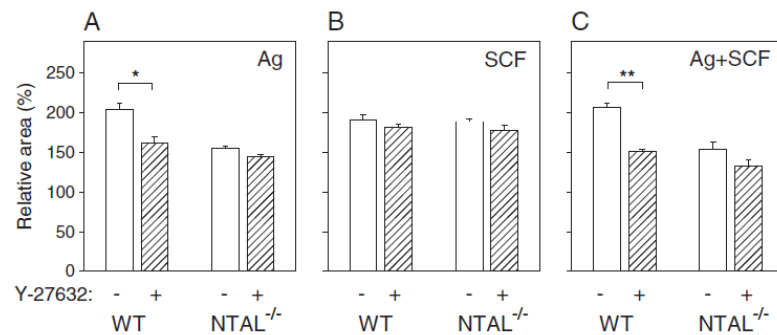


Figure 6. Effects of Rho kinase inhibitor on spreading of BMMC. IgE-sensitized WT or NTAL^{-/-} cells were incubated 15 min without (empty columns) or with (hatched columns) Rho kinase inhibitor Y-27632 (10 μ M), then placed on fibronectin, activated for 30 min with Ag (250 ng/mL; A), SCF (50 ng/mL; B) or both Ag+SCF (C) and stained for F-actin. Cell area was determined and normalized to nonactivated cells. At least 400 cells were evaluated in each experiment. Means $\pm$ SE were calculated from three independent experiments. Significance of differences between WT and NTAL^{-/-} cells is shown (* p <0.05; ** p <0.005; Student's t -test).

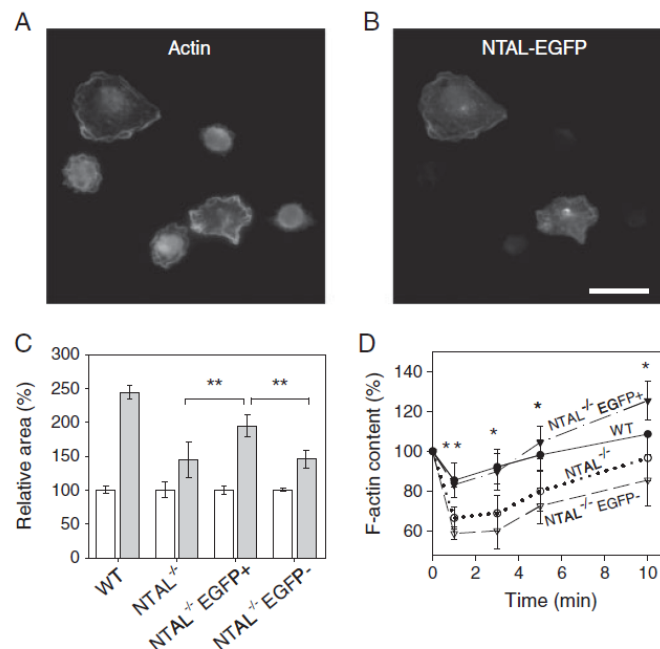


Figure 7. Phenotype rescue of NTAL^{-/-} BMMC. (A and B) NTAL^{-/-} cells were transfected with NTAL-EGFP, left to recover for 12 h and then sensitized with IgE for further 12 h. Cells were then attached to fibronectin-coated glass, stimulated with Ag for 20 min and stained for F-actin. Typical micrograph of the transfected cells showing phalloidin (A) and EGFP (B) fluorescence. Scale bar, 10 μ m. (C) WT and NTAL^{-/-} cells were treated as above. Mean cell area of resting (open columns) and Ag-activated (grey columns) WT and non-transfected NTAL^{-/-} cells as well as that of NTAL-EGFP-transfected NTAL^{-/-} cells was evaluated. NTAL^{-/-} EGFP⁺ and NTAL^{-/-} EGFP⁻ cell populations were distinguished by setting the threshold to basal EGFP fluorescence. (D) Suspensions of WT (full line), NTAL^{-/-} (dotted line) and NTAL-EGFP-transfected NTAL^{-/-} BMMC were activated with Ag, fixed at the indicated times and stained with Alexa Fluor 555-phalloidin. F-actin content was determined by flow cytometry. NTAL-EGFP-transfected NTAL^{-/-} BMMC were separated into EGFP⁺ (dash-dot line) and EGFP⁻ (dashed line) subpopulations as above. In (C) and (D), at least 500 cells for each cell population were evaluated; the data were normalized to resting cells. Mean values $\pm$ SE are shown. Statistical significances of the indicated intergroup differences (C) and differences between NTAL^{-/-} EGFP⁺ and NTAL^{-/-} EGFP⁻ (D) are shown (* p <0.05; ** p <0.005; Student's t -test).

The presence of SCF amplified the differences observed between Ag-activated NTAL^{-/-} and WT cells. Although SCF alone induced an increase in cellular F-actin, its combination with Ag shifted the balance of signals towards a more extensive F-actin depolymerization. This effect was evident in both cell types, but was more prominent in NTAL^{-/-} cells. Presence of Ag suppressed migration of mast cells towards SCF (Fig. 4D). Sawada *et al.* [25], on the other hand, reported that SCF suppresses chemotactic migration of mast cells towards Ag. Our observation of Ag+SCF-induced F-actin depolymerization may be relevant to these observations. The interplay between SCF- and Ag-induced signals is obviously complex and our results indicate that NTAL may have a crucial role in it. With regard to secretion, the effects of c-kit and FcεRI signals are synergistic, but with regard to spreading and migration, they are antagonistic. Molecular mechanisms of these diverse processes are unknown, but could be related to different modes and qualities of NTAL tyrosine phosphorylation. Thus, c-Kit can phosphorylate NTAL directly, whereas FcεRI only indirectly through Lyn or Syk kinase. Importantly, Syk and c-Kit phosphorylate different tyrosines in NTAL, which then serve as direct or indirect binding sites for different kinases, phosphatases and other enzymes [10]. Different ways of cell activation could lead to formation of different NTAL signalosomes and therefore different performance of the cells in such complex processes as migration, spreading and degranulation.

Rac1 and RhoA have been shown to control both secretion and actin organization in mast cells [18, 26]. We have found that after stimulation with Ag, SCF or Ag+SCF, the increases in the proportion of active Rac(1,2,3) or Rac1 were similar in WT and NTAL^{-/-} cells. This indicates that NTAL is not crucial in the signaling pathway from FcεRI or c-kit to Rac. Surprisingly, RhoA activity decreased after cell activation with Ag, SCF or both, and the inactivation of RhoA was significantly greater in Ag or Ag+SCF-activated NTAL^{-/-} cells. In all three cases, RhoA inactivation was followed by a slow recovery, and it is possible that NTAL is essential for this recovery step. The greater and longer lasting decrease in RhoA activity could be due to the reduced recruitment of accessory proteins required for maintaining RhoA in active state. One such protein could be the guanine nucleotide exchange factor Vav [27] shown to be associated with NTAL in activated B cells [6]. Another possibility is overactivation of p190RhoGAP [20]. Both proteins are known to be activated by phosphorylation, but we have not found any indication of differences in Tyr phosphorylation of total immunoprecipitated p190RhoGAP (Fig. S2) or Vav (our unpublished data) in WT and NTAL^{-/-} cells. Potential mechanisms for Rho regulation are complex and will require further studies.

While the respective proportions of active Rac1 in resting WT and NTAL^{-/-} cells were only 6 and 5%, those of active RhoA were relatively large, 22 and 53%. It is likely that the significantly larger proportion of active RhoA in NTAL^{-/-} cells is relevant to their greater secretory responsiveness [7, 8]; this is in line with the reported requirements for RhoA in secretion from mast cells [18]. Active RhoA may be necessary to initiate the secretory process (perhaps by activating a fusogen), and its immediate subsequent inactivation may be required for cell attachment [28, 29] and/or F-actin disassembly [26]. The later increase in

RhoA activity would then be required for the formation of newly polymerized actin serving an inhibitory function in exocytosis and promoting cell spreading and migration [29]. The role of NTAL in the recovery of RhoA after its initial inhibition was supported by the differential effects of ROCK inhibition on spreading of WT and NTAL^{-/-} cells. Regarding cell migration, the role of RhoA depends on specific cell types and conditions [30]. Our transwell chemotactic assays have shown faster migration of NTAL^{-/-} cells towards Ag, but not towards SCF, suggesting negative role of NTAL in Ag-induced migration.

In conclusion, our study provides evidence that in mouse mast cells FcεRI signaling, adaptor protein NTAL, in a pathway via RhoA and ROCK to the cytoskeleton, serves as a positive regulator of actin polymerization and cell spreading and a negative regulator of chemotaxis.

Materials and methods

Reagents and Ab

Calcein-AM and 1- α -lysophosphatidylcholine were purchased from Fluka (Buchs, Switzerland). Fura-2-AM and Alexa Fluor-phalloidin conjugate were from Probes (Eugene, OR, USA). BD Cell-Tak™ Cell and Tissue Adhesive were from BD Biosciences (San Jose, CA, USA). Mouse mAb against RhoA and Rac1 were obtained from Cytoskeleton (Denver, CO, USA) and anti-p190RhoGAP (p190; clone 30/p190) was from BD Biosciences. Function blocking mAb specific for mouse integrins $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$ and $\beta 7$ were obtained from BD Pharmingen (San Diego, CA, USA). Origin of other antibodies has been previously described [7]. If not stated otherwise, all other reagents were purchased from Sigma-Aldrich (Prague, Czech Republic).

Cell cultures and activation

BMMC were isolated from WT and NTAL^{-/-} mice as previously described [7]. Unless otherwise stated, BMMC were sensitized in SCF- and IL-3-free culture medium supplemented with IGE1 b4 1 mAb (1 μ g/mL). After 16 h the cells were washed in buffered saline solution-BSA, containing 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 5.6 mM glucose, 2 mM MgCl₂ and 0.1% BSA, and activated with Ag (TNP-BSA conjugate, 15–25 mol TNP/mol BSA; 100–1000 ng/mL, depending on batch) and/or SCF (20–40 ng/mL, depending on batch) at concentrations giving maximum degranulation and/or cells spreading. The extent of secretion was determined by measuring the release of β -glucuronidase [11].

Microscopy and image analysis

Wells on eight-well multitest slides (ICN Biomedicals, Aurora, OH, USA) were coated overnight at 4°C with 13 μ L of the solution

containing 50 mM NaHCO₃ and 50 µg/mL fibronectin. To enhance adhesiveness of BMMC, a cell adhesive BD Cell-Tak (8 µL/mL) was combined with fibronectin. Coated wells were washed with buffered saline solution-BSA and 15×10^3 cells was added *per* well. Cells were allowed to attach for 1 h at 37°C, washed and then activated. After indicated times, cells were fixed for 20 min at room temperature with 3% paraformaldehyde in glutamate/EGTA buffer (GBE; 137 mM K-glutamate, 2 mM MgCl₂, 3 mM EGTA and 20 mM NaOH-PIPES, pH 6.8), supplemented with 4% polyethylene glycol (MW 3200). For F-actin staining, cells were washed with 50 mM glycine in GBE and then exposed to phalloidin conjugated with Alexa Fluor 488 or Alexa Fluor 555, diluted 1:250 in GBE supplemented with L-α-lysophosphatidylcholine (120 µg/mL). After 30 min, cells were washed, mounted and examined with an Olympus A70 Provis microscope equipped with KAF 1400 cooled CCD camera. In some cases, Olympus Scan^R system was utilized. Confocal images were obtained using Leica TCS SP microscope.

Cell adherence assay

Wells of a 96-well plate were coated overnight with fibronectin (10 µg/mL PBS), blocked with 4% BSA in PBS (1 h at 37°C) and washed twice with PBS. IgE-sensitized BMMC were loaded with Calcein-AM (4 µM) for 30 min at 37°C, washed and transferred into the fibronectin-coated wells (2.5×10^4 cells/well). Fluorescence was determined using fluorescent plate reader FLUOstar-P (BMG Lab. Technologies, Offenburg, Germany) at excitation and emission wavelengths of 494 and 517 nm, respectively.

F-actin content

Cells ($1-2 \times 10^5$ /well) in 96-well plate were fixed and stained with GBE supplemented with paraformaldehyde, 4% polyethylene glycol, lysophosphatidylcholine (200 µg/mL) and 1000 × diluted Alexa Fluor 488- or Alexa Fluor 555-phalloidin. After 1 h at room temperature, an equal volume of GBE was added and the plates were centrifuged. The pelleted cells were resuspended in 50–100 µL of GBE and analyzed on BD LSRII flow cytometer.

Determination of [Ca²⁺]_i

BMMC were loaded with Fura-2-AM, and [Ca²⁺]_i was determined as previously described [7].

Chemotactic response

Chemotactic responses were assayed using 24-well Transwell chambers (Corning, Corning, NY, USA) with 8-µm polycarbonate filters in upper wells. Chemoattractants were added to the lower wells in 0.6 mL chemotactic medium (RPMI-1640 supplemented with 1% BSA and 20 mM HEPES, pH 7.4). IgE-sensitized BMMC

(0.2×10^6 cells in 150 µL chemotactic media) were added into each upper well. Cells migrating into lower wells during 8-h incubation (37°C, 5% CO₂ in air) were counted in a Bürker chamber.

Active GTPase assays

GTPase activation assays were performed using Rac(1,2,3), Rac1 and RhoA G-LISA kits (Cytoskeleton). Cell lysates ($\sim 1.5 \times 10^5$ cells/25 µL) were prepared using G-LISA lysis buffer, snap-frozen in liquid nitrogen and processed within 2 wk after preparation. For Rac(1,2,3), Rac1 and RhoA assays, lysate aliquots, corresponding to, respectively, 2.5×10^4 , 1.2×10^5 and 1.5×10^5 cells, were applied to wells coated with Rac- or Rho-GTP-binding protein. Active, GTP-bound GTPases bound to the wells were detected with the corresponding specific Ab. One ng of constitutively active Rac1 or RhoA was used as a standard.

NTAL-EGFP fusion construct and cell transfection

Mouse *ntal* gene without stop codon was cloned from pZeo-NTAL-1 vector [11] into BamHI and EcoRI restriction sites of pEGFP-N1 vector (BD Biosciences Clontech) using PCR. NTAL fusion protein was thus expressed onto N-terminus of EGFP. The NTAL-EGFP construct was transfected into NTAL^{−/−} BMMC using Mouse Macrophage Nucleofector[®] Kit (Amata Biosystems, Cologne, Germany) according to the manufacturer's protocol.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: BMMC: BM-derived mast cell · BSS: buffered saline solution · [Ca²⁺]_i: concentration of free cytoplasmic Ca²⁺ · EGFP: enhanced GFP · F-actin: filamentous actin · GBE: glutamate/EGTA buffer · LAT: linker for activation of T cells · LB: latrunculin B · NTAL: non-T-cell activation linker

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Supporting information

for

The transmembrane adaptor protein NTAL signals to mast cell cytoskeleton via the small GTPase Rho

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Supplementary methods

In general, experiments were performed as described in the Materials and methods section of the manuscript. Additional methods used only for the supplementary experiments are described below.

Protein content in total cell lysates

Amounts of Rac1, RhoA, and actin were determined by SDS-PAGE and immunoblotting using the corresponding antibodies. Twenty ng of Rac1 or 15 ng of RhoA (both from Cytoskeleton) and 300 ng of actin (Sigma Aldrich) were loaded onto the gel as standards. Protein content was quantified by Luminescent Image Analyzer LAS 3000 (Fuji Photo Film Co.) and normalized to the cell number or to 1 µg of actin.

Immunofluorescence

Cells were sensitized and attached to multiwell slides as described above, washed with BSS-BSA and, where indicated, stimulated with Ag. After fixation, cells were washed with 50 mM glycine in GBE, then permeabilized and blocked for 30–60 min with GBE containing 120

μg/ml lysophosphatidylcholine, 5% goat serum and 250 μg/ml succinyl-Con A. Cells were then stained for 1 h with primary Abs (anti-RhoA or anti-Rac1) diluted 1:150 in GBE containing 5% goat serum. Cells were washed 4x with GBE before applying Alexa Fluor 488-phalloidin (1:250) together with secondary Ab (IgG1-specific goat anti-mouse Ab conjugated with Cy3, Jackson Immuno, 1:400) in GBE-5% goat serum. After 1 h, cells were washed 4x with GBE, mounted and examined with a Leica TCS SP microscope. Controls with the secondary Ab alone or with primary Abs blocked with the corresponding recombinant proteins were negative.

IP and immunoblotting

Cells were lysed for 30 min on ice in the RIPA buffer (50 mM Tris-HCl, pH 7.2, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM MgCl₂, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.57 mM PMSF, 1 mM Na₃VO₄), followed by clearing of the lysate by centrifugation (15.000 x g; 5 min, 4°C). Supernatants were directly used for analysis by SDS-PAGE, or IP for 90 min at 4°C followed by 2 h incubation with protein G UltraLink Resin (Thermo Scientific, Rockford, IL, USA). Immunocomplexes bound to the beads were washed with lysis buffer, resolved on 7.5% SDS-PAGE, and transferred to nitrocellulose membrane (Protran, BA85, Whatman, Dassel, Germany). The blots were probed with the indicated antibodies, followed by detection using chemiluminescence. Immunoblots were quantified by Luminescent Image Analyzer LAS-3000 (Fuji Photo Film Co., Tokyo, Japan).

Supplementary figures

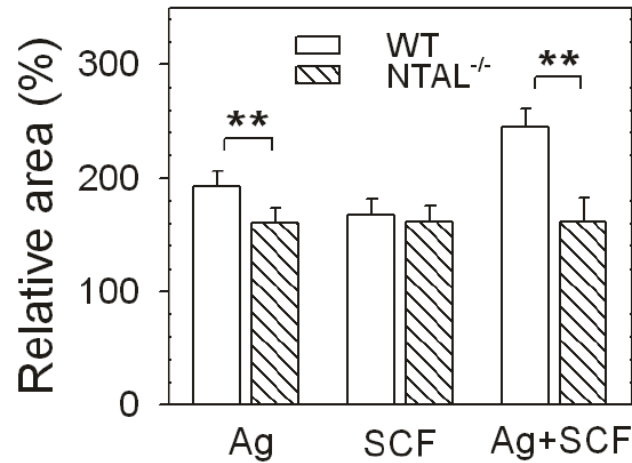


Figure S1. Impaired spreading of NTAL^{-/-} BMMCs activated by Ag or Ag+SCF. IgE-sensitized WT or NTAL^{-/-} BMMCs were attached to fibronectin, activated for 20 min with Ag, SCF or both (Ag+SCF) and stained for F-actin. Relative cell area was determined as in Fig. 1C except that only spread cells were evaluated. Data were normalized to non-activated cells. Means and SE were calculated from 4 independent experiments, each involving at least 100 WT or NTAL^{-/-} cells. Significance of differences between WT and NTAL^{-/-} cells is shown (** $p < 0.005$; Student's t-test).

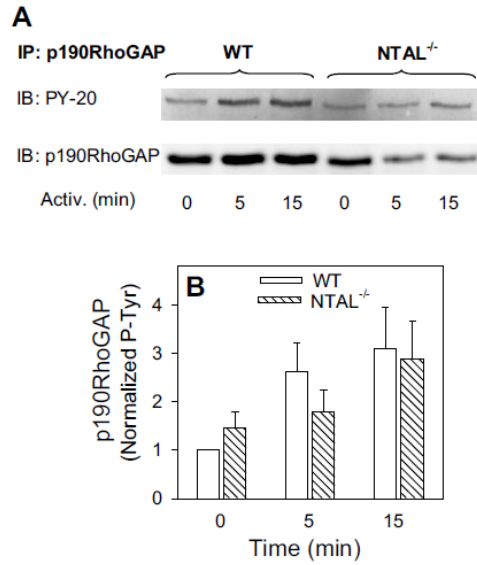


Figure S2. Tyrosine phosphorylation of p190RhoGAP. (A) IgE-sensitized WT or NTAL^{-/-} cells were activated for different time intervals with Ag (TNP-BSA; 250 ng/ml) and solubilized in RIPA buffer. p190RhoGAP was immunoprecipitated followed by immunoblotting for phosphotyrosine with PY-20 mAb-HRP conjugate. The amount of immunoprecipitated p190RhoGAP was determined with anti-p190 mAb after stripping procedure. (B) Quantitative analysis of p190RhoGAP tyrosine phosphorylation. Immunoblots prepared as in (A) were analyzed by densitometry and the data were normalized to nonactivated cells and to the amount of p190RhoGAP in immunoprecipitates. Means and SE were calculated from 3 independent experiments.

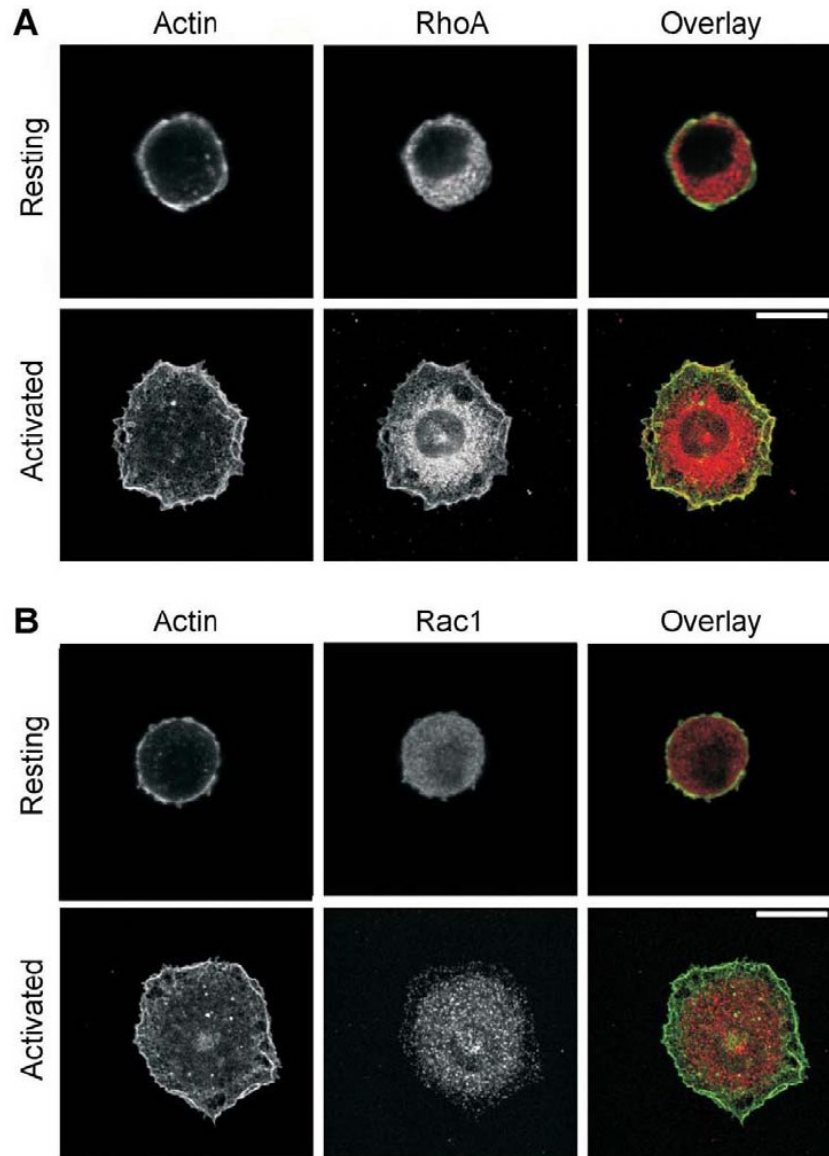


Figure S3. Different localization of RhoA and Rac1 in resting and activated cells. IgE-sensitized WT BMMCs were attached to fibronectin-coated glass and activated with Ag or not (Resting). After 30 min, cells were fixed, permeabilized, and stained with Alexa Fluor 488-phalloidin and with anti-RhoA (A) or anti-Rac1 (B) Abs. Cy3-labeled goat anti-mouse IgG1 was used as a secondary Ab. Equatorial confocal slices are shown. Scale bar, 10 μ m.

Table S1. Content of RhoA, Rac1, Rac(1,2,3) and actin in resting BMMCs.

Protein content (ng/10⁶ cells)	WT		NTAL^{-/-}	
	mean ± SE	n	mean ± SE	n
Total RhoA	53.9 ± 4.8	13	41.2 ± 6.0	10
Total Rac1	51.3 ± 6.6	9	47.8 ± 8.5	8
Active RhoA	12.0 ± 1.7	12	21.9 ± 3.0	11
Active Rac(1,2,3)	17.0 ± 2.4	16	12.9 ± 1.9	16
Active Rac1	3.3 ± 0.5	12	2.6 ± 0.2	11
Actin	11 100 ± 873	12	9 980 ± 839	11
Proportion of active GTPase*				
Active RhoA/total RhoA	0.22		0.53	
Active Rac1/total Rac1	0.06		0.05	
Intracellular concentrations (μM)**				
Actin	203.3 ± 16.0	12	188.7 ± 15.9	11
Total Rho A	2.0 ± 0.2	13	1.6 ± 0.2	10
Total Rac1	1.9 ± 0.2	9	1.9 ± 0.3	8

* No anti-pan Rac Ab is available to estimate total Rac(1,2,3) content.

**Calculations are based on cell diameters of $13.45 \pm 0.04 \mu\text{m}$ and $13.28 \pm 0.20 \mu\text{m}$ for WT and NTAL^{-/-} cells, respectively. Diameters were measured using Vi-CELL XR – cell viability analyzer (Beckman Coulter) on 15 different BMMC cultures.

5. DISCUSSION

Recently discovered adaptor protein NTAL structurally resembles to another previously described adaptor protein LAT. The major role for LAT was found in activation of T cells. Because LAT was not found in mature B cells, it was suggested that similar protein should function in B cells and that the protein is NTAL. The first characterization of NTAL in mast cell signaling came from two simultaneous studies (Vohná and others, 2004; Zhu and others, 2004). Both studies showed that mast cells derived from bone marrow of NTAL-deficient mice are hyperresponsive after FcεRI aggregation. These data indicated that NTAL is negative regulator of FcεRI-mediated signaling. Contrarily, antigen-induced degranulation in LAT-deficient mast cells was partially reduced when compared to WT cells. Interestingly, the absence of both adaptor proteins, NTAL and LAT, led to further decrease in FcεRI signaling, suggesting that NTAL is also a positive regulator. In other studies using siRNA approach human mast cells with reduced NTAL expression exhibited decreased degranulation (Tkaczyk and others, 2004) supporting the concept that NTAL in human cells is mainly positive regulator of FcεRI signaling. To throw more light on NTAL function, we prepared permanent cell lines with (1) low expression of NTAL (NTAL-) using hairpin siRNA method, and (2) enhanced expression of NTAL (NTAL+) after transfection with a vector bearing mouse NTAL cDNA. We expected that some so far unrevealed pathways directly downstream of NTAL could be uncovered as all the works dealt with reduced amount of NTAL in cells and thus these pathways would be impaired or masked.

Interestingly, modification of NTAL levels led to changes in cell morphology. We found decreased adhesion to tissue culture plastic and less developed processes in NTAL+ cells and longer processes in NTAL- cells. These changes could be related to increased amount of F-actin in resting NTAL- cells and a weak increase of F-actin after cell activation in both NTAL+ and NTAL- cells compared to parental RBL cells. Both, NTAL+ and NTAL- cells showed reduced degranulation. This indicates that NTAL must be present at optimal concentration to obtain maximal degranulation response in these cells.

Both, F-actin polymerization and degranulation are influenced by calcium concentration. Activation of FcεRI leads to calcium release from intracellular stores which is followed by extracellular calcium entry. The calcium response to antigen in NTAL- cells was

slightly decreased. The response of NTAL⁺ cells also did not reach the response of control cells and was significantly delayed. When we checked calcium response in the absence of extracellular calcium, we obtained similar results. When the extracellular calcium was restored, NTAL⁻ and control cells showed rapid increase, but NTAL⁺ cells responded weakly. The defects in calcium mobilization could be due to the observed decreased association of tyrosine phosphorylated PLC γ 1 with signaling assemblies, lower enzymatic activity of PLC γ and lower amount of IP3 produced after activation in both cell lines, NTAL⁺ and NTAL⁻. To test the effect of NTAL on the extracellular calcium influx with no influence of PLC γ , we used thapsigargin – a drug which inhibits an intracellular calcium pumps with subsequent raise in intracellular calcium and extracellular calcium influx. The calcium uptake in these experiments directly correlated with the amount of NTAL. These experiments suggested different roles of NTAL in the release of calcium from intracellular stores and also in the influx of extracellular calcium through the plasma membrane.

Adaptor protein LAT possesses PLC γ -binding site (Zhang and others, 2000). NTAL phosphorylation by Syk leads to its indirect association with PLC γ 1 (Iwaki and others, 2008). Thus, the observed impaired phosphorylation of Syk and LAT in activated NTAL⁺ cells could be a reason for inhibition of subsequent Fc ϵ RI-mediated signaling events in these cells, including degranulation and Ca²⁺ response.

We also observed decreased phosphorylation of the Fc ϵ RI subunits in NTAL⁺ cells. However, it is unclear how NTAL overexpression could influence phosphorylation of Fc ϵ RI, when NTAL, Syk and LAT phosphorylation are downstream of Fc ϵ RI. It is possible that overcrowding of the membrane with NTAL interfere with tyrosine phosphorylation of the receptor.

Using modified method for isolation of plasma membrane sheets in combination with immunogold labeling and electron microscopy we also studied fine topography of NTAL and Fc ϵ RI in NTAL⁺ cells. We observed typical distribution of these molecules in separated clusters. The density and size of NTAL clusters were higher in cells expressing increased amount of NTAL. Redistribution of Fc ϵ RI clusters after activation was comparable in WT and NTAL⁺ cells suggesting an unchanged early phases of activation. A possible mechanism for impaired Fc ϵ RI subunit phosphorylation could be through association of NTAL signaling complex with phosphatases. For example overexpression of SHP-1 in RBL cells led to

decreased FcεRI aggregation-induced tyrosine phosphorylation of the β- and γ-subunits of the receptor and also Syk phosphorylation (Xie and others, 2000). Another cytoplasmic phosphatase SHP-2 was found to directly bind to the β-subunit of FcεRI after receptor aggregation (Kimura and others, 1997). Grb2 recruits SHP-2 to the plasma membrane in activated T cells and thus brings the phosphatase into close proximity of its potential substrates (Tailor and others, 1996). As NTAL was found to be a major binder of Grb2, it is possible that NTAL signaling complex possesses SHP-2 activity. Furthermore, NTAL was described to decrease recruitment of SHIP by the tyrosyl-phosphorylated LAT upon FcεRI engagement (Roget and others, 2008). PTP20, a phosphatase of PTP-PEST family with unclear biological relevance, is expressed on several tumor-derived cell lines including RBL cells (Heneberg and Dráber, 2002). It is probable that cell origin is determining the content of signaling molecules and that the spectrum of phosphatases is different in different cell lines. I should also mention that model mast cell line RBL is popular for studies on FcεRI signaling as these cells are easy to culture in routinely used media without interleukins and they show a very rapid and strong response to receptor triggering. Furthermore, RBL cells, in contrast to e.g. BMMC are amenable to transfection studies. On the other hand, RBL cells are tumor-derived and possess point mutation in *Kit* gene; this leads to constitutive activation of Kit (Tsujimura and others, 1995). With the recent observation that activation of Kit enhances FcεRI-induced signaling events and that activated Kit can directly phosphorylate NTAL (Tkaczyk and others, 2004; Iwaki and others, 2008), it is likely that RBL cells are less suitable for studies aimed at understanding the role of NTAL function than cells derived from animals or humans.

Nishida and colleagues showed that adaptor protein Gab2 is a key player in the Kit-induced activation and mast cell development (Nishida and others, 2002). Another study described similar results and new findings that this effect is dependent on SHP-2 (Yu and others, 2006b). NTAL associates with Grb2, which binds to Gab2 and anchors this cytoplasmic adaptor to the membrane. SCF, as the major factor for adhesion of mast cells, is capable of changing mast cell morphology through its receptor Kit (Kim and others, 1998). Taken these data together, it is likely that different levels of NTAL could bring altered levels of Gab2 to the membrane and thus signaling from Kit to cell morphology could be changed,

and our observation of changed cell morphology of NTAL⁺ and NTAL⁻ cells could be explained.

In addition to the *Kit* gene mutation present in RBL and several other mast cell lines, there are also studies stating opposite results of NTAL function, which differ in cell origin and used methods. For example, we observed an increased degranulation in BMMCs from NTAL-deficient mice after FcεRI activation (Volná and others, 2004), whereas diminution of NTAL expression by siRNA method in human mast cells resulted in a reduction of FcεRI-mediated degranulation (Tkaczyk and others, 2004). Furthermore, there is a study showing an importance of threonine residue of LAT (Thr155) in TCR signaling, but this residue is present only in human LAT (Fuller and others, 2011). Taken together, it is necessary to be cautious when generalizing data obtained from particular mast cell source.

The requirement to study fine topography of signaling molecules in plasma membrane sheets from BMMCs, led us to develop a new method for isolation of plasma membrane sheets. All described methods based on isolated membrane sheets use cells growing on cover slips or attached to the cover slips by poly-L-lysine or other adhesive compounds, which however could induce cell activation or non-physiological changes. As BMMCs are non-adherent, we decided to test three different procedures to attach BMMCs to cover-slips. We found that plasma membrane sheets isolated from BMMCs after binding to (1) fibronectin, (2) ultraclean glass cover slips or (3) by pressing the cells directly to poly-L-lysine-covered electron microscopic grids gave similar results. For further studies we have selected the method based on non-specific adhesion of the cells to ultraclean glass in protein-free buffer. The procedure is very rapid and cell attachment is attained within 1 min. This is much faster when compared to 45 – 60 min in previous methods, which could lead to inducible plasma membrane changes. Our new protocol does not require coating with poly-L-lysine, which in mast cells causes histamine release (Benyon and others, 1987). Importantly, the binding occurs at room temperature and there is no need for preincubation at low temperature causing changes in the topography of plasma membrane constituents (Magee and others, 2005). Attachment of the cells to ultraclean glass was inhibited by an inhibitor of F-actin polymerization, latrunculin B. This suggests that the procedure can be used as a useful tool for studies on the role of cytoskeleton in the topography of plasma membrane components.

In our experiments, we showed for the first time the topography of FcεRI and adaptor proteins LAT and NTAL in BMMCs and their comparison with RBL cells. We found, that the density of FcεRI clusters in non-activated RBL cells is twofold higher than in non-activated BMMCs. Activation of RBL cells led to formation of large FcεRI aggregates. In contrast, there was no significant increase in FcεRI cluster size in the course of BMMCs activation. Thus, it is likely that early stages of BMMCs activation do not require formation of large signaling assemblies observed in RBL cells. The observed difference in size of the antigen-induced FcεRI patches between both mast cell types is likely to reflect different local dynamics, favoring formation of more numerous but smaller signaling domains in BMMCs compared to RBL cells. When we activated cells at 4 °C, the size of FcεRI clusters were comparable in both cell types. Previous study (Dráberová and others, 2004) showed that binding of anti-FcεRI monoclonal antibody, which causes only receptor dimerization, but no aggregation, is also potent to trigger activation of RBL cells with no large cluster formation. These results together suggest that the formation of primary signaling domains as large clusters containing FcεRI receptors described previously on RBL cells (Wilson and others, 2001) is possibly not universal mechanism of mast cell activation.

Adaptor proteins LAT and NTAL form signaling assemblies after FcεRI triggering. Although they are structurally similar and both possess palmitoylation motif that determines their presence in DRMs, they were found in separated domains in resting and also activated RBL cells (Vohná and others, 2004). We have found that the two adaptors are also localized in different membrane regions in BMMCs and thus confirmed the results obtained in RBL cells. We propose that LAT and NTAL interact with different cytoplasmic proteins and form different signaling complexes that could be responsible for their separation into different domains. LAT and NTAL also probably associate with different lipid microenvironment within the plasma membrane and this could contribute to their separation even in the resting cells. Plasma membrane-associated proteins are clustered in cholesterol-enriched domains. These structures are connected to the actin cytoskeleton, which is at least in part involved in their formation and maintenance (Lillemeier and others, 2006). As we found that NTAL can influence actin polymerization, it is also possible that separation of those adaptors is dependent on actin cytoskeleton. The methods investigating fine topography of plasma membrane components are important in elucidating possible functional links of particular

membrane proteins. This can not be attained by routinely used biochemical techniques which use membrane disruption and detergent extraction.

The data suggesting a cross-talk between NTAL and actin cytoskeleton in RBL cells, led us to investigate the role of NTAL in signaling to F-actin in BMMCs. First, we measured cell spreading on fibronectin. We noticed that spreading of NTAL-deficient cells on fibronectin is significantly reduced when compared to parent cells. Interestingly, when we checked adherence to fibronectin, the antigen-induced adherence of NTAL^{-/-} BMMCs was reduced only by ~ 15% compared to NTAL^{+/+} cells. Such a small difference is unlikely to account for the observed defect in cell spreading. Nevertheless, formation of actin filaments in response to antigen was clearly different in the presence or absence of NTAL. F-actin content in WT cells showed transient decrease in the first three minutes, subsequent increase with return to basal levels, whereas NTAL^{-/-} cells exhibited rapid loss of F-actin followed by a slow recovery. Thus, the decrease in spreading in the absence of NTAL could be caused by the defect in actin polymerization.

Recently, a function of F-actin as a physical barrier was described (Deng and others, 2009). In resting cells, F-actin in cellular cortex serves as a physical barrier to the access of secretory vesicles to the plasma membrane, and thus blocks the membrane fusion. Upon activation of FcεRI, the F-actin molecules, originally distributed evenly under the plasma membrane, reorganize into a more heterogeneous network structure. This effectively breaks the original F-actin barrier and allows temporary fusion to occur. In this way, the higher degranulation of NTAL-deficient cells could be explained by decrease in actin polymerization after activation with antigen.

Previous work with permeabilized rat peritoneal mast cells has shown a great sensitivity of cellular F-actin to calcium (Sullivan and others, 1999). Consistent to this finding, enhanced calcium responses were found in cells lacking NTAL compared to WT cells. However, we were not able to find any simple correlation between degranulation, calcium response and F-actin depolymerization. When NTAL^{-/-} cells were activated with antigen and SCF together, the secretion was higher, F-actin content was further decreased, but calcium response was not increased compared to response to antigen alone. Also latrunculin B, F-actin disrupting drug, did not eliminate the differences in degranulation between WT and NTAL^{-/-} cells.

In contrast to the different responses of WT and NTAL^{-/-} cells to antigen, reactions to SCF were similar. It may reflect very weak and transient phosphorylation of NTAL after SCF activation (Tkaczyk and others, 2004; and our unpublished data). This confirms different signaling pathways to cell spreading in response to SCF or antigen.

When the cells were activated simultaneously with both antigen and SCF the differences in secretion, spreading, and adhesion between NTAL^{-/-} and WT cells were amplified. Although SCF alone induced an increase in cellular F-actin, combination of antigen and SCF shifted the balance of signal towards a more extensive F-actin depolymerization. This effect was evident in both cell types, but was much more prominent in NTAL^{-/-} cells.

The only difference in migration between WT and NTAL^{-/-} cells was towards to antigen alone. SCF ± antigen caused migration similar in both cell types suggesting a negative regulatory role of NTAL in chemotaxis towards antigen, but no apparent role in chemotaxis towards SCF.

Rac1 and RhoA have been shown to control both secretion and actin organization in mast cells (Price and others, 1995; Norman and others, 1996). We found that NTAL is not critical player in the signaling pathway initiated by antigen or SCF leading to Rac1, 2 or 3 activation as reflected by similar increases in Rac activity in responses to antigen and SCF in WT and NTAL^{-/-} cells. However, we found reduced RhoA activity more pronounced in the absence of NTAL. The significant difference was observed in response to antigen or antigen + SCF between WT and NTAL^{-/-} cells. RhoA inactivation was followed by a slow recovery. NTAL seems to be essential for both, the initial drop and also for recovery of RhoA activity. It is probable that NTAL signalosome contains some RhoA activity controlling proteins. One possible candidate could be Vav - the guanine nucleotide exchange factor, which was found to be recruited by phosphorylated NTAL in B cells (Malhotra and others, 2009). Another possibility is overactivation of p190RhoGAP which suppresses RhoA activity and enhances spreading and chemotaxis as it was shown in fibroblasts (Arthur and Burridge, 2001). Unfortunately, we were not able to detect any differences in the phosphorylation of these two molecules between WT and NTAL^{-/-} cells. We also examined the effect of Rho kinase (ROCK) inhibitor on cell spreading. ROCK is a major downstream effector of RhoA GTPase. We found reduced spreading of ROCK inhibitor treated WT cells in response to

antigen and antigen + SCF. NTAL-deficient cells, already reduced in spreading, did not show further decrease. Further candidate which could regulate RhoA activity in BMMCs is SWAP-70. It resembles proteins of the Dbl-family of guanine nucleotide exchange factors for small Rho GTPases. Upon activation of B cells, SWAP-70 translocates from the cytoplasm to the membrane, where it associates with the BCR. It is also strongly expressed in mast cells. SWAP-70-deficient mast cells were found to be impaired in development and FcεRI-induced degranulation. Key FcεRI activation events like phosphorylation of LAT, activation of Akt/PKB and of p38 MAP kinase were also reduced in SWAP-70^{-/-} BMMCs, but ERK was strongly hyperactivated (Gross and others, 2002; Sivalenka and others, 2008). Role of SWAP-70 in Kit-induced signaling in mast cells was also reported. Using SWAP-70-deficient mast cells, SWAP-70 was determined as an important regulator of Kit pathways including mast cell activation, migration, and cell adhesion (Sivalenka and Jessberger, 2004).

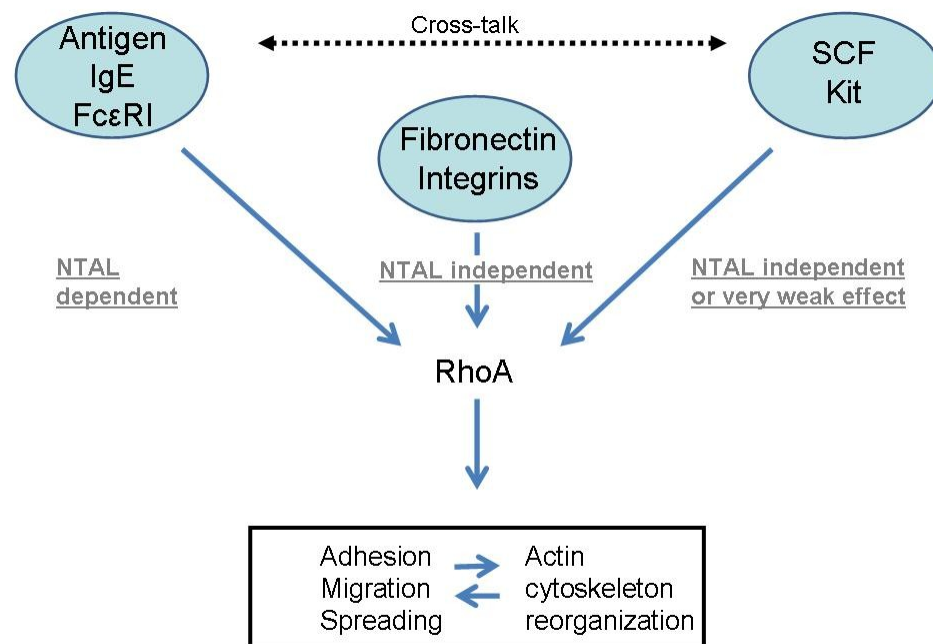


Figure 7. Role of NTAL in signaling to actin cytoskeleton in BMMCs. Integrin stimulation induces cell adhesion, spreading and migration that require actin cytoskeleton reorganization. Antigen or SCF-induced activation of mast cells costimulates cell adhesion on fibronectin. Any of these three signaling pathways are not isolated in cells and all the pathways have common downstream players. The central molecule that mediates cross-talk between these pathways could possibly be RhoA. Adaptor protein NTAL affects these pathways only when FcεRI receptor is engaged.

Recently, SWAP-70, hitherto known to bind F-actin and Rac, was found to bind also RhoA-GTP. SWAP-70 negatively regulated RhoA preventing premature RhoA activation in dendritic cells (Ocana-Morgner and others, 2009). The relation between SWAP-70 and NTAL has not been studied yet and remains to be investigated in the future. Figure 7 summarizes our results concerning the role of NTAL in BMDCs signaling to actin cytoskeleton.

In conclusion, our study provides for the first time evidence that NTAL adaptor protein in mouse mast cells serves as a positive regulator of actin polymerization and cell spreading and a negative regulator of chemotaxis after FcεRI triggering in a pathway via RhoA and ROCK to the cytoskeleton.

6. CONCLUSIONS

1. NTAL cDNA was cloned from RBL cells and based on the sequence obtained siRNA probes were designed. RBL cells were transfected with (1) expression vector containing NTAL cDNA to get cell line with enhanced NTAL expression and (2) with vector containing rat NTAL-specific hairpin siRNA to obtain cell line with reduced NTAL expression. Stable cell lines were selected together with appropriate control cell lines.
2. Cells expressing high levels of NTAL showed reduced degranulation, lower TNF- α secretion, inhibition of tyrosine phosphorylation of Fc ϵ RI subunits, Syk kinase and LAT and inhibition of calcium response induced by Fc ϵ RI aggregation. The degranulation, TNF- α secretion and calcium mobilization of the cell line with reduced NTAL expression was also inhibited. NTAL overexpressors also exhibited changes in morphology and adhesion properties. The cells were more rounded and less adhered to tissue culture plastic. In cells pretreated with thapsigargin, NTAL expression directly correlated with uptake of extracellular calcium although NTAL phosphorylation was not observed. These results show that NTAL regulates Fc ϵ RI-mediated signaling at different stages of activation through different mechanisms.
3. A new method for isolation of plasma membrane sheets from non-adherent cells was developed. The method is based on cell adsorption to ultraclean glass.
4. Antigen-activated adherent RBL cells and also non-adherent BMMCs showed similar rate of Fc ϵ RI internalization. Whereas large Fc ϵ RI clusters were formed after activation of RBL cells, no large clusters were seen after BMMCs activation. LAT and NTAL clusters were topographically independent in resting and activated cells in both cell types.
5. Antigen-activated NTAL-deficient BMMCs, when compared to WT cells, were characterized by reduced adhesion and spreading on fibronectin, enhanced F-actin depolymerization and increased migration. In the event of adhesion, spreading and F-actin

depolymerization, the differences between WT and NTAL-deficient cells were even more pronounced when simultaneously activated by both antigen and SCF.

6. When NTAL-deficient BMMCs or WT cells were activated with antigen, SCF or both antigen and SCF, activity of GTPases Rac1, 2, 3 together or Rac1 alone increased similarly in both cell types. However, antigen-stimulated RhoA GTPase activity was decreased and this reduction was stronger in NTAL-deficient cells than in WT cells. SCF potentiated this decrease in both cell types, but again the absence of NTAL caused more dramatic decline. RhoA kinase (ROCK) inhibitor caused impaired spreading in WT cells in response to antigen or antigen and SCF. In the absence of NTAL, further decrease of cell spreading was not induced with the inhibitor. Spreading of SCF-activated cells was not affected by the inhibitor in any cell line. These data suggest a cross-talk between NTAL and RhoA/ROCK in antigen-activated mast cells.

7. NTAL-EGFP construct was prepared, electroporated into NTAL-deficient cells, and spreading and F-actin responses of cells with restored NTAL expression were evaluated. These experiments confirmed an important role of NTAL in signaling from membrane receptors towards actin cytoskeleton.

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8. ABBREVIATIONS

BMMCs: Bone marrow-derived mast cells
Btk: Bruton tyrosine kinase
CD: Cluster of differentiation
CHS: Contact hypersensitivity
Csk: C-terminal Src kinase
CTMC: Connective tissue type mast cells
DAG: 1,2-diacylglycerol
DRM: Detergent resistant membrane
EGFP: Enhanced green fluorescent protein
ERK: Extracellular-signal regulated kinase
F-actin: Filamentous actin
FcεRI: High affinity receptor for IgE
Gab2: Grb2-associated binder 2
Gads: Grb2-related adaptor downstream of Shc
GAPT: Grb2-binding adaptor protein, transmembrane
GEM: Glycosphingolipid-enriched microdomain
Grb2: Growth factor receptor-bound protein 2
HSC: Hematopoietic stem cells
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
IP3: Inositol 1,4,5-trisphosphate
ITAM: Immunoreceptor tyrosine-based activation motif
ITIM: Immunoreceptor tyrosine-based inhibition motif
JAK: Janus family kinase
JNK: Jun N-terminal kinase
LAT: Linker for activation of T cells
Lyn: Lck/Yes-related novel tyrosine kinase
MAPK: Mitogen-activated protein kinase

MMC: Mucosal mast cells
NK: Natural killer
NTAL: Non-T cell activation linker
PAG: Phosphoprotein associated with glycosphingolipid-enriched microdomains
PDK1: Phosphoinositide-dependent protein kinase 1
PH: Pleckstrin homology
PI3K: Phosphatidylinositol 3-kinase
PIP3: Phosphatidylinositol 3,4,5,-trisphosphate
PKC: Protein kinase C
PLC γ : Phospholipase C γ
PTEN: Phosphatase and tensin homologue deleted on chromosome ten
PTK: Protein tyrosine kinase
PTP: Protein tyrosine phosphatase
RBL: Rat basophilic leukemia
ROCK: Rho-associated protein kinase
SCF: Stem cell factor
SH: Src homology
SHIP: SH2-domain containing inositol 5'-phosphatase
SHP: SH2-domain containing phosphatase
siRNA: small interfering RNA
SLP-76: SH2-domain containing leukocyte protein of 76 kDa
SOS: Son of sevenless
STAT: Signal transducers and activators of transcription
Syk: Spleen tyrosine kinase
TCR: T cell receptor
TLR: Toll-like receptor
TNF- α : Tumor necrosis factor α
TRAP: Transmembrane adaptor protein
Treg: Regulatory T cells
WT: Wild type