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Review

What precedes the initial tyrosine phosphorylation of the high affinity IgE receptor in antigen-activated mast cell?

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ABSTRACT

An interaction of multivalent antigen with its IgE bound to the high-affinity IgE receptor (FcεRI) on the surface of mast cells or basophils initiates a series of signaling events leading to degranulation and release of inflammatory mediators. Earlier studies showed that the first biochemically defined step in this signaling cascade is tyrosine phosphorylation of the FcεRI β subunit by Src family kinase Lyn. However, the processes affecting this step remained elusive. In this review we critically evaluate three current models (transphosphorylation, lipid raft, and our preferential protein tyrosine kinase–protein tyrosine phosphatase interplay model) substantiating three different mechanisms of FcεRI phosphorylation.

1. Introduction

Activation of mast cells and basophils through the high-affinity IgE receptor (FcεRI) leads to the release of pro-inflammatory mediators involved in inflammation and allergy disorders [1]. FcεRI, which belongs to the multichain immune recognition receptor (MIRR) family is a tetrameric complex formed by an IgE-binding α-subunit, a signal-amplifying β-subunit, and a homodimer of disulphide-linked γ-subunits. Each FcεRI β- and γ-subunit contains one immunoreceptor tyrosine-based activation motif (ITAM) which, after tyrosine phosphorylation, serves as a docking site for other signaling molecules such as Src homology 2 (SH2) domain-containing Src family kinase Lyn or Syk/Zap family kinase Syk [1]. Several models have been proposed to explain mechanistically how the FcεRI becomes phosphorylated by protein tyrosine kinase (PTK) Lyn. The models differ in their perception of the spatiotemporal relationship between Lyn kinase and FcεRI, and of the role of protein tyrosine phosphatases (PTPs) at initial tyrosine phosphorylation of the receptor.

2. Models of FcεRI phosphorylation

2.1. Transphosphorylation model

This model assumes that FcεRI in quiescent cells is non-covalently associated through its non-phosphorylated β subunit with Lyn kinase which is unable to phosphorylate its carrier receptor (Fig. 1). Aggregation of the IgE–FcεRI complexes with multivalent antigen initiates Lyn-dependent phosphorylation of the neighboring receptors within the aggregate [2]. This process is known as transphosphorylation and was suggested to play a role in the signal transduction in several other systems [3]. It is corroborated by several experimental findings. First, when biochemically crosslinked, at least 3–4% of the non-aggregated FcεRI was found to be directly associated with Lyn [4]. Second, Vonakis et al. [5] showed that Lyn binds through its unique domain to β subunit of FcεRI, irrespective of phosphorylation of the ITAM. This binding does not involve Lyn SH2 domain which interacts with phosphorylated ITAM, serving to amplify the initial activation signal. Third, transfection of Lyn unique domain into rat basophilic leukemia (RBL) cells inhibited antigen-induced phosphorylation of FcεRI β and γ subunit; this

Abbreviations: FcεRI, high-affinity IgE receptor; MIRR, multichain immune recognition receptor; ITAM, immunoreceptor tyrosine-based activation motif; SH2, Src homology 2; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; RBL, rat basophilic leukemia; EGFP, enhanced green fluorescent protein; DRM, detergent-resistant membrane; MBCD, methyl-β-cyclodextrin; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; BMMC, bone marrow-derived mast cell; LAT, linker of activated T cell; PLC, phospholipase; NTAL, non-T cell activation linker

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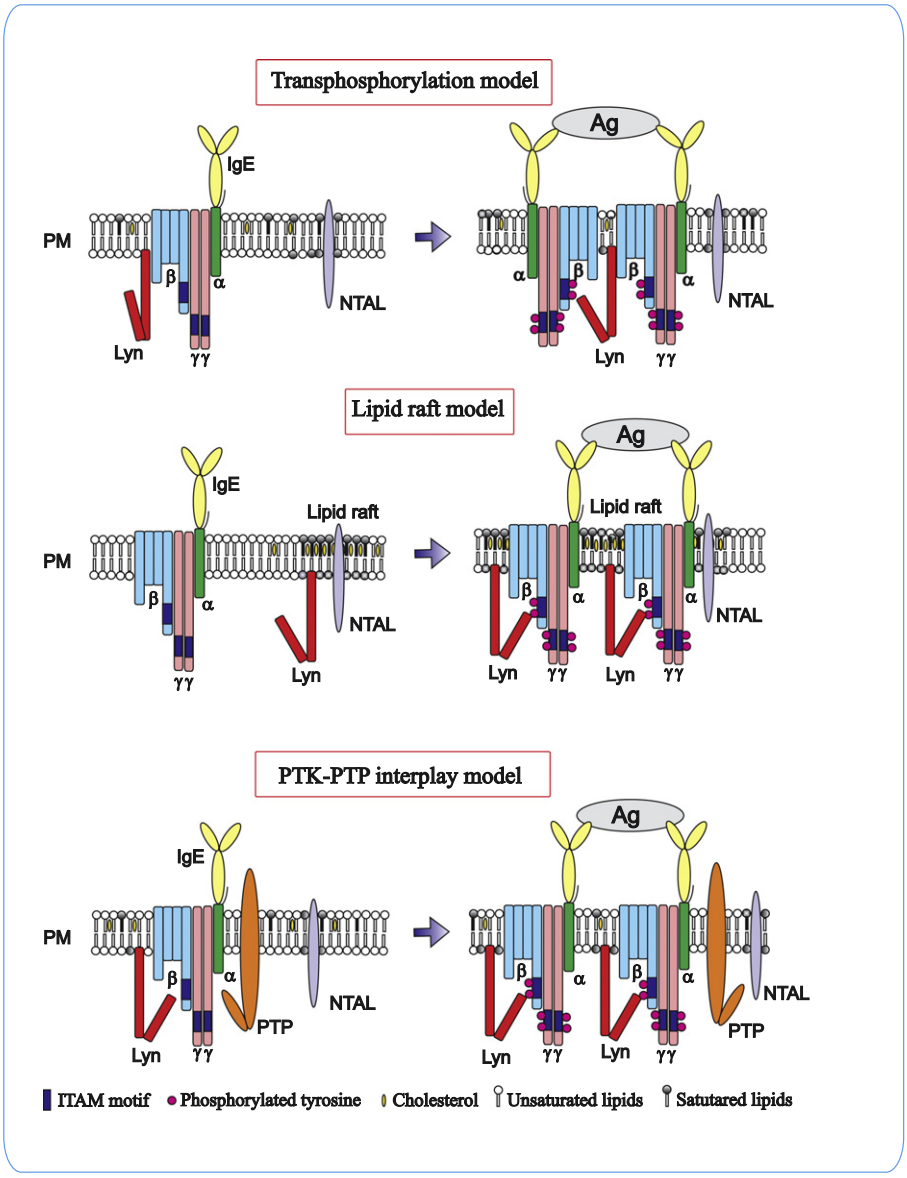

Fig. 1. Models of FcεRI phosphorylation. Transphosphorylation model presumes that active Lyn is constitutively associated with non-phosphorylated FcεRI; upon FcεRI aggregation, Lyn phosphorylates adjacent receptors in the aggregate. Lipid raft model postulates that FcεRI and Lyn are localized in different 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.

suggests a competition for FcεRI as a substrate between endogenous Lyn and the transfected Lyn unique domain [6]. Fourth, studies with Chinese hamster ovary fibroblasts expressing FcεRI and various amounts of Lyn showed a direct correlation between amount of Lyn and tyrosine phosphorylation of the FcεRI. Detailed mathematic analysis demonstrated that one Lyn molecule is capable of phosphorylating numerous adjacent FcεRIs [7]. Fifth, Lyn was found to colocalize with FcεRI as detected by transmission electron microscopy on plasma membrane sheets isolated from RBL cells. In non-stimulated cells about 25% of randomly dispersed FcεRI was associated with one or a few Lyn molecules [8]. Sixth, Lyn kinase in quiescent mast cells is enzymatically active and its activity is not further enhanced after FcεRI triggering [9]. A mutation of the C terminal tyrosine, responsible for inhibition of Lyn kinase activity, led to a weak constitutive tyrosine phosphorylation of several substrates including β and γ subunits of FcεRI and Syk kinase in non-stimulated cells [9]. Seventh, aggregation of FcεRI with trivalent ligands bound in defined distance to a symmetric DNA scaffold, resulted in phosphorylation of the FcεRI β and γ subunits depending on the length of DNA spacer; more potent trivalent ligands were those with shorter spacer [10]. Similarly, it has been suggested that dominant factor affecting signaling capacity of various anti-FcεRI monoclonal antibodies is the orientational element [11]. Eight, simultaneous observation of FcεRI marked with various quantum dot-labeled IgE revealed that aggregates of at least three crosslinked FcεRI remained mobile, showed prolonged diffusion and exhibited phosphorylation of the FcεRI β subunit and β-glucuronidase release [12]. Finally, fluorescence correlation spectroscopy of Lyn-enhanced green fluorescent protein (EGFP) and fluorescently labeled IgE bound to FcεRI confirmed that a small fraction of FcεRI in non-activated cells colocalized with Lyn [13]. Taken together, the results from the studies applying different methods provide a strong support for the hypothesis that a fraction of Lyn binds to unphosphorylated FcεRI, and strengthen the notion that transphosphorylation could be the first step in FcεRI signaling. However, some data are in conflict with this hypothesis. For exam-

ple, aggregated FcεRI becomes tyrosine phosphorylated even in the absence of β subunit [14]. Furthermore, chimeric receptors containing only the γ cytoplasmic tail become tyrosine phosphorylated upon their aggregation [15]. Alternative models had been therefore proposed.

2.2. Lipid raft model

Lipid raft membrane microdomains were conceived as a mechanism for intracellular trafficking of lipids and lipid-anchored proteins. The model assumes that biological membranes possess microdomains having a lipidic structure with properties of lipid order phase. These domains are rich in cholesterol and sphingomyelin, and possess a limited set of proteins, including those anchored to the membrane via glycolipids (e.g. glycophosphatidylinositol) or acyl groups (e.g. palmitoyl or myristoyl), and also some acylated proteins with transmembrane domain. They are surrounded by membranes with lipid structure resembling lipid disorder phase possessing phosphatidylinositols and a majority of the transmembrane proteins [16]. Cholesterol plays a crucial role in the formation of lipid rafts, and cholesterol-depletion experiments have been used in lipid raft function studies. Membrane domains with properties of lipid rafts are often isolated as membranes resistant to solubilization with non-ionic detergents, such as Triton X-100 at low temperature. Thus, the lipid raft hypothesis is in part based on studies presuming that detergent-resistant membranes (DRMs) correspond to lipid rafts, an assumption which is probably incorrect [16]. On the other hand, the lipid raft model is supported by experiments showing that aggregation of FcεRI and some other membrane proteins leads to a decreased detergent solubility as can be documented by density gradient ultracentrifugation. From the FcεRI perspective, lipid raft model postulates that in quiescent cells FcεRI is physically separated from active Lyn residing in lipid raft domains [17]. After aggregation, FcεRI is translocated into these domains and is phosphorylated there by Lyn (Fig. 1). Decreased detergent solubility was observed not just in extensively aggregated FcεRI, but even upon FcεRI dimerization, although the activation was delayed and more sustained [18]. Furthermore, it has been shown that cholesterol depletion with methyl-β-cyclodextrin (MBCD) reduced phosphorylation of the FcεRI subunits as well as other proteins. The tyrosine phosphorylation of FcεRI was rescued when cholesterol was added [19]. These data were taken as evidence that FcεRI cannot be phosphorylated in the cells in which lipid rafts are destroyed. Removal of cholesterol with MBCD, however, led to a decreased expression of FcεRI and had some other effects on mast cell physiology [20]; therefore, these experiments cannot simply be interpreted as supporting the role of lipid rafts in FcεRI signaling.

Recent studies based on advanced microscopy methods revealed that membrane domains in quiescent cells are highly dynamic and small in size (less than 10 nm). They can transiently coalesce after ligand-induced aggregation of membrane proteins and form larger domains [21]. In antigen-stimulated mast cells, transient increase of FcεRI in specialized cholesterol-rich domains, peaking at 5 min after receptor aggregation has been described [22]. However, it remains to be determined whether these changes reflect either an association of FcεRI with cholesterol-enriched lipid rafts possessing sequestered Lyn, or rather changes leading to removal of aggregated FcεRI. In fact, experiments with N-palmitoylation-site deficient Lyn show that anchor of the Lyn to the plasma membrane but not to DRMs is important for proper tyrosine phosphorylation of the FcεRI [23]. Furthermore, Lyn-EGFP mobility within the plasma membrane decreased upon FcεRI triggering, and association of the kinase with FcεRI was increased. However, no change in association of aggregated FcεRI with EGFP anchored to the plasma membrane through Lyn acylation site was observed [13]. Thus, lipid raft localization of Lyn is not sufficient for its interaction with aggregated FcεRI.

2.3. PTK–PTP interplay model

The key player in the transphosphorylation and lipid raft models in antigen-activated mast cells is the PTK Lyn utilizing FcεRI ITAM as a substrate. However, the extent of phosphorylation of the immunoreceptors and other substrates depends not only on enzymatic activity of the PTKs, but, at least partly, also on the activity of PTPs. As an attempt to explain some conflicting experimental data which were inconsistent with lipid raft model, the PTK–PTP interplay model was coined (Fig. 1). Initially, the model was elaborated in studies of immunoreceptor signaling in B cells. Reth and co-workers [24] showed that the B cell receptor, belonging to the MIRR family, forms a complex with PTKs and PTPs; this complex is indispensable for reactive oxygen species (ROS)-induced phosphorylation. Also, ROS encompassing superoxide or hydrogen peroxide are known inhibitors of PTPs and are capable of initiating early FcεRI-induced signaling events in mast cells. Despite a growing list of confirmed PTPs present in mast cells, their contribution to cell signaling regulation is still poorly understood [25,26]. One possible way of regulation of the PTP activity is reversible oxidation of Cys residue in a conserved signature motif (I/

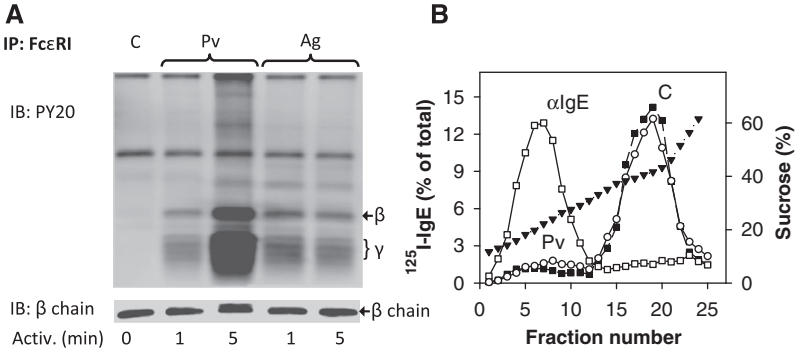

Fig. 2. Phosphorylation of FcεRI does not require its translocation into lipid rafts. (A) IgE-senzitized BMMCs were non-activated (C, 0 min) or activated for 1 or 5 min with pervanadate (Pv) or antigen (Ag). The cells were solubilized and the FcεRI complexes were immunoprecipitated (IP) and analyzed by immunoblotting (IB) with antibodies specific for phosphotyrosine (PY20) or FcεRI β chain (loading control). Positions of FcεRI β and γ chains are indicated. (B) Density gradient fractionation of cell lysates. The cells were sensitized with ^{125}I-IgE and then left non-activated (C; filled squares) or activated with pervanadate (Pv; open circles) or anti-IgE (αIgE; open squares). After 5 min the cells were solubilized and fractionated by sucrose density gradient ultracentrifugation. Radioactivity in individual fractions was determined and expressed as percentage of the total radioactivity recovered. Percentage of sucrose in individual fractions is also shown (filled triangles). Methodological details can be found elsewhere [32].

V)HCxxGxxR(S/T) in the catalytic domain. The proximity of Arg in the signature motif makes it possible for an invariant Cys to form a thiolate anion at physiological pH (p*K*a <6.0) that can act in nucleophilic attack on the phosphate group. However, the same Cys residue can be oxidized by ROS to catalytically inactive Cys-sulphenic acid, a mechanism that is fully reversible by action of glutathione or other thiols [27]. ROS are produced under physiological conditions by the activity of specialized enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase [28,29]. Strong oxidants like pervanadate (a mixture of vanadate and H_2O_2) irreversibly inactivate PTPs by the formation of Cys-sulphonic acid residues [30]. Exposure of mast cells to pervanadate leads to activation events which resemble those induced by physiological activators [31,32].

The PTK–PTP interplay model is supported by several lines of evidence. First, exposure of RBL or bone marrow-derived mast cells (BMMCs) to pervanadate or H_2O_2 induced robust tyrosine phosphorylation of the FcεRI subunits in the absence of receptor migration into DRMs, as determined by solubilization of the cells in Triton X-100 followed by sucrose density gradient ultracentrifugation. This was in sharp contrast to antigen- or anti-IgE antibody-induced activation which led to the movement of FcεRI into DRMs (Fig. 2). Such data indicate that association of FcεRI with DRMs is not required for receptor phosphorylation. Second, electron microscopy studies on isolated plasma membrane sheets showed that FcεRI in pervanadate-activated cells was distributed in the same way as the receptor in non-activated cells. Thus, enhanced receptor clustering is not required for tyrosine phosphorylation

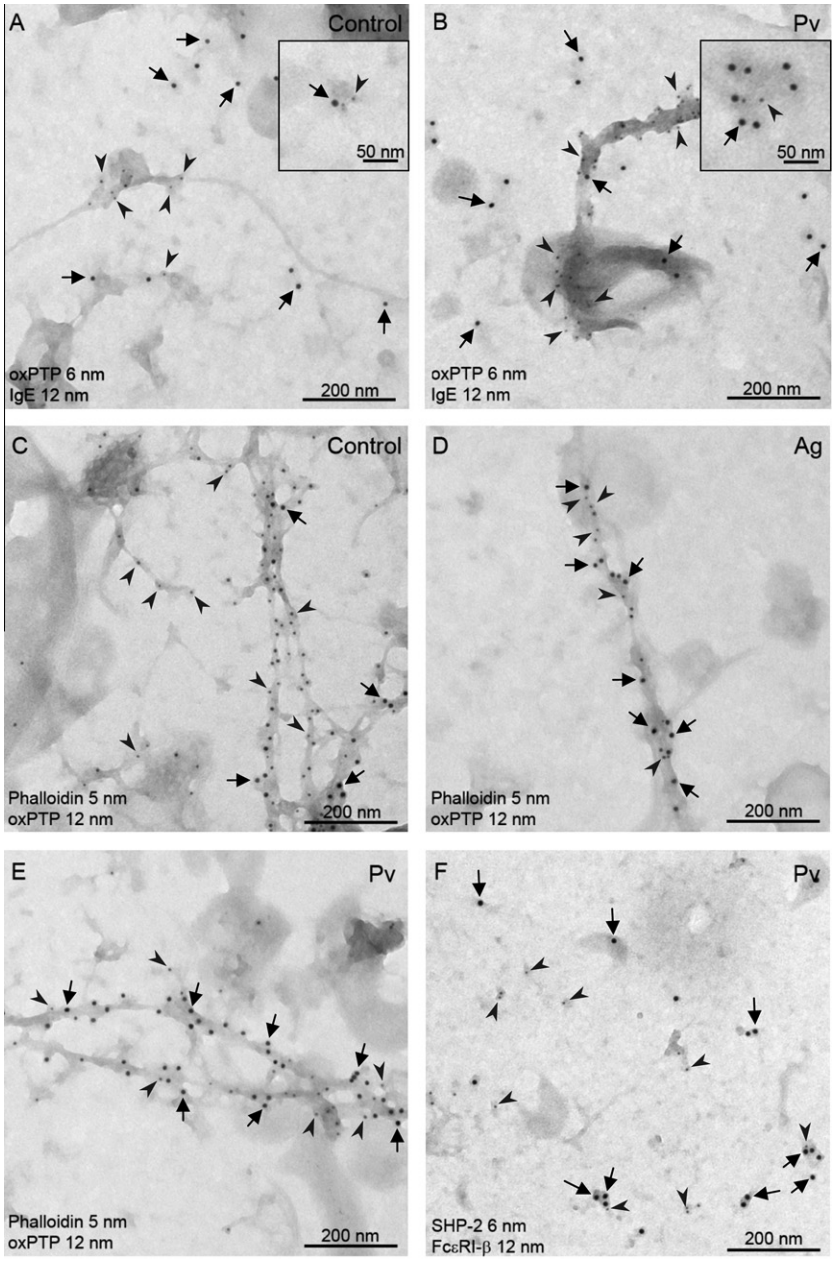

Fig. 3. Membrane topography of phosphatases, actin and FcεRI. Plasma membrane sheets were prepared from non-activated (Control; A and C), pervanadate-activated (Pv; B, E, and F) or antigen-activated (Ag; D) BMMCs and after two-step labeling procedure were analyzed by electron microscopy. FcεRI was labeled extracellularly using IgE (12 nm gold; A and B) or from cytoplasmic side using anti-FcεRI β chain (12 nm gold; F). Oxidized phosphatases were labeled with oxPTP monoclonal antibody marked with 6 nm (A and B) or 12 nm gold particles (C–E). F-Actin or SHP-2 were labeled with phalloidin (5 nm gold; C–E) or anti-SHP-2 antibody (6 nm gold; F), respectively. Gold particles of 5–6 nm are indicated by arrowheads and those of 12 nm by arrows. Inlets in A and B show higher magnification. Experimental procedure details have been described [32].

of FcεRI [32]. Third, using a monoclonal antibody (oxPTP) recognizing oxidized Cys residue in catalytic domain of PTPs, enzymatically inactive PTPs were detectable in a time- and dose-dependent manner in cells activated with pervanadate, H_2O_2 or antigen [32]. Fourth, most of PTK substrates are not phosphorylated if pervanadate is added to cell lysates instead of intact cells [24]. Similarly, lysates prepared from non-activated mast cells and treated with H_2O_2 or pervanadate exhibited a lower amount of oxidized PTPs than lysates prepared from cells activated with H_2O_2 or pervanadate before lysis [32]. These data suggest that solubilization of the cells with detergents destroys preformed signaling assemblies containing PTKs, PTPs, and their substrates. Fifth, pretreatment of activated cells with saponine, leading to cell permeabilization and release of the majority of free cytoplasmic components, resulted in association of the majority of oxidized PTPs with cellular ghosts [32]. This indicates that oxidized PTPs are not freely moving in cytosol, but rather form large complexes. In fact, FcεRI on plasma membrane sheets isolated from non-activated (Fig. 3A) or activated (Fig. 3B) cells occasionally colocalizes with oxidized PTPs. Interestingly, fraction of oxidized PTPs was associated with membrane-bound actin cytoskeleton, especially after activation (Fig. 3C–E). Finally, activation of the cells with antigen leads to decreased enzymatic activity of HePTP (PTPN7) and some other PTPs, and exposure of the cells to H_2O_2 leads to a substantial decrease in overall phosphatase activity [32].

An important task is the identification of PTPs directly involved in regulating the tyrosine phosphorylation of the receptor. Using a modified immunoprecipitation procedure with oxPTP antibody, followed by mass spectrometry analysis of the immunoprecipitated proteins, we found that several PTPs are oxidized in activated

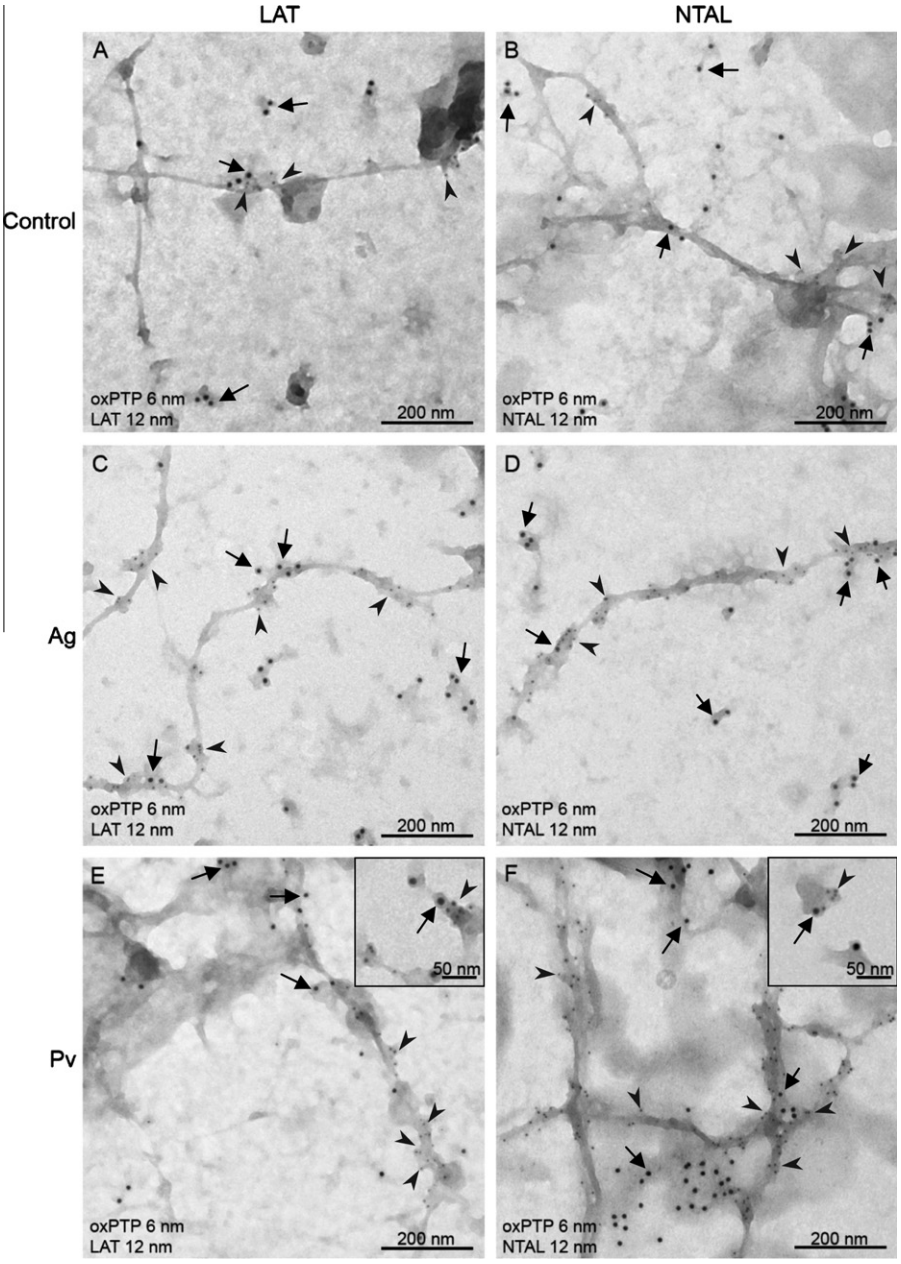

Fig. 4. Topography of oxidized phosphatases and transmembrane adaptor proteins. Plasma membrane sheets were prepared from non-activated (Control; A and B), antigen-activated (Ag; C and D) and pervanadate-activated (Pv; E and F) BMMCs and labeled for oxidized PTPs with 6 nm gold (A–F) or for LAT (A, C, and E) or NTAL (B, D, and F) with 12 nm gold. Gold particles of 6 or 12 nm in size are indicated with arrowheads or arrows, respectively. Inlets in E and F show higher magnification. Experimental procedure details have been described [32,38].

mast cells; they include SHP-1 (PTPN6), SHP-2 (PTPN11), PTP-MEG2 (PTPN9) and HePTP. However, we failed to co-immunoprecipitate the PTPs with FcεRI and we found no binding motifs in FcεRI β and γ subunits for the phosphatases, except for the phosphotyrosine motifs. Interestingly, it has been found that SHP-2 occasionally colocalizes with FcεRI (Fig. 3F), and that FcεRI is pre-associated in non-activated cells with SHP-1 [33]; these phosphatases exhibited enhanced enzymatic activity after FcεRI triggering [32]. The finding that a fraction of these phosphatases was reversibly oxidized in their active sites [32], suggests that there are spatiotemporal differences in enzymatic activity of the phosphatases. It is possible that the enzyme molecules in the vicinity of the receptor in submembraneous region have different enzymatic activity immediately after receptor triggering than the rest of molecules in the cell. Another PTP, the HePTP, also oxidized in activated mast cells, exhibited a decreased enzymatic activity after FcεRI triggering, and could thus contribute to an enhanced phosphorylation in activated cells.

How could the PTPs become inactivated under physiological conditions? Production of ROS either intracellularly [28] or by adjacent macrophages [34] influences mast cell activation. It has been shown that ROS produced by NADPH oxidase is crucial for phosphorylation of many substrates [linker of activated T cells (LAT), phospholipase (PLC)γ1 and (PLC)γ2], but NADPH oxidase alone is dependent on the activity of Src family kinases and PI3-kinase [28]. In one recent study, however, abrogation of FcεRI-dependent ROS production had no effect on degranulation or cytokine production [29]; this would suggest that antigen-induced conformational changes in FcεRI and resulting impaired access of PTPs to their substrates could be more important for enhanced FcεRI β and γ subunits phosphorylation than inhibition of enzymatic activity of the PTPs by ROS. Conformational changes in activated MIRRs have already been described [35,36]. It is possible that a similar change could lead to a shift in PTK–PTP steady state phosphorylation level of FcεRI after mast cells triggering. The combined data suggests, that non-activated mast cells possess preformed signaling assemblies containing immunoreceptors, and PTKs and PTPs which are in steady state. The binding of a multivalent antigen could cause a change in conformation of FcεRI which would be followed by a change in the steady state in favor of PTKs.

3. Topography of signaling molecules

Electron microscopy studies showed that most of the plasma membrane-bound molecules are associated with osmiophilic regions of the membrane [37]. Interestingly, molecules which were expected to be localized in lipid rafts, such as non-T cell activation linker (NTAL) and LAT, were localized individually or in small clusters and were not intermixed [38]. This suggests that lipid rafts, if they exist, are smaller than previously thought, and could be restricted to individual proteins or their clusters and surrounding lipids. Small size of signaling domains was documented in studies in which FcεRI dimers were capable of initiating signaling cascades without any dramatic changes in their redistribution in the plasma membrane [12,18]. Signalosomes of dimerized FcεRI persisted at the membrane longer than large aggregates formed by binding of multivalent antigen to IgE–FcεRI complexes. Thus, formation of large FcεRI aggregates is important for more potent removal of the membrane signalosomes at later stages of signaling.

Interesting data were obtained in studies analyzing the topography of membrane-bound PTPs. Antibody specific for oxidized phosphatases reacted mostly with cytoskeleton-like structures in non-activated mast cells [32]. Enhanced association of oxidized phosphatases with these structures was observed in cells activated by antigen and especially by pervanadate. The same structures were labeled with phalloidin (Fig. 3C–E), indicating that oxidized phosphatases are preferentially associated with actin cytoskeleton. These data suggest that actin cytoskeleton is involved in early signaling events by regulating the topography of phosphatases. Alternatively, actin could play a role in sequestering and/or scavenging irreversibly oxidized PTPs. In this connection it should be mentioned that actin probably plays a role in sequestering Lyn kinase to the periphery of large FcεRI aggregates which are consequently internalized [8].

Further studies showed that PTPs could also play a role in activation-induced tyrosine phosphorylation of the transmembrane adaptor protein NTAL and LAT, which in phosphorylated state serve as docking sites for molecules involved in FcεRI signaling [1]. Although both LAT and NTAL are detergent-resistant proteins, they exhibited enhanced colocalization with oxidized PTPs after FcεRI triggering and even more so after treatment with pervanadate (Fig. 4). Nevertheless, oxidized phosphatases have not been found in DRMs [32]. Similarly, Syk was detected in FcεRI clusters on plasma membrane sheets isolated from activated mast cells [8], in spite of the fact that only FcεRI, was associated with DRMs [39]. Moreover, experiments with disaggregated FcεRI clusters showed that the rate of their dephosphorylation was the same as that of other proteins [39]. These data indicate that proteins in DRMs are not protected from phosphatase activity and that even typical DRM proteins could be phosphorylated as proposed by the PTK–PTP interplay model.

4. Conclusions

We evaluated three models which have been proposed to explain the molecular mechanism whereby aggregation of the FcεRI initiates the first biochemically defined step in the antigen-mediated signaling in mast cells – tyrosine phosphorylation of the FcεRI β subunit by Lyn kinase. Although the transphosphorylation and lipid raft models are supported by numerous studies that are not limited to immunoreceptor signaling, they do not satisfactorily explain all activation events, such as tyrosine phosphorylation by ROS. This necessitated the introduction of PTK–PTP interplay model based on an assumption that PTKs and PTPs are in steady state in quiescent cells and activation disturbs this steady state in favor of kinases. Considering the fact that the turnover rate of active PTPs is 100- to 1000-times higher than that of PTKs [40], one can hypothesize that the PTK–PTP steady state in quiescent cell is set to override PTK, a process that could serve as a threshold of FcεRI signaling. Local ROS production or conformational changes of FcεRI leading to inactivation of the enzymatic activity of PTPs or their inaccessibility to the substrate could precede tyrosine phosphorylation of the FcεRI.

New approaches and methods are required to understand in detail the initiation of FcεRI phosphorylation and spatiotemporal coordination of the FcεRI signalosome formation. Better understanding of these events is expected from the introduction of new real-time methodologies with nanoscale resolution. Promising are approaches analyzing immunoreceptor signaling in cell-free systems with defined composition of lipid bilayers and properly accommodated signaling molecules. Genetic approaches will no doubt play a major role in deciphering signaling events in a complex cellular environment. Detailed analysis of properties of cells with knock-out of selected genes and restored with various mutant forms of the gene (knock-in approach) could still lead to the discovery of new players involved in initial stages of FcεRI signaling. These, in turn, could become targets for new drugs, and thus contribute to new rational approaches to therapy of mast cell mediated diseases.

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