Reconstitution of Regulated Phosphorylation of FcεRI by a
Lipid Raft-excluded Protein Tyrosine Phosphatase

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SUMMARY

To examine the exquisite regulation of IgE-FcεRI tyrosine phosphorylation by Lyn kinase that is stimulated by antigen-mediated crosslinking, we utilized co-expression of FcεRI and Lyn in Chinese hamster ovary (CHO) cells that results in high basal levels of Lyn kinase activity and spontaneous phosphorylation of FcεRI. We find that co-expression of a lipid raft-excluded transmembrane tyrosine phosphatase, PTPα, suppresses Lyn kinase activity and markedly reduces the level of spontaneous phosphorylation of FcεRI, while facilitating its antigen-stimulated phosphorylation. Other tyrosine phosphatases, including SHP-1, CD45, and a lipid raft-preferring chimeric version of PTPα fail to reconstitute antigen-dependent FcεRI phosphorylation. We conclude that both substrate specificity and submembrane location are critical to phosphatase-mediated regulation of Lyn kinase activity that supports activation of FcεRI.
INTRODUCTION

FcεRI, the high affinity receptor for IgE, is a member of the family of multichain immune recognition receptors (MIRR) including T cell and B cell receptors for antigen, certain NK cell receptors, and other Fc receptors on various hematopoietic cells. All of these receptors contain at least one ITAM or ITIM sequence in the cytoplasmic segment of one or more subunit (1,2). In every case, receptor-mediated signaling is initiated when tyrosine residues in these sequences are phosphorylated by a Src family tyrosine kinase. For FcεRI in mast cells, ITAM phosphorylation of both its β and γ subunits is catalyzed principally by the Src kinase Lyn, and this is stimulated by antigen-mediated crosslinking of two or more IgE-receptor complexes (3,4). ITAM phosphorylation of this and other MIRR family members recruits and activates the tyrosine kinase Syk (or Zap70 in T cells), leading to a cascade of downstream signaling and cell activation processes (5,6).

The mechanism by which IgE-FcεRI crosslinking initiates receptor tyrosine phosphorylation has been extensively studied. The β subunit of FcεRI has been shown to bind Lyn weakly in the absence of ITAM phosphorylation (7), and a transphosphorylation model has been developed from this and other studies (3). In addition, FcεRI β has been shown to serve an amplifying role for FcεRI γ2 ITAM phosphorylation and consequent Syk activation, most probably by the binding of the SH2 domain of Lyn to phosphorylated FcεRI β (4). However, FcεRI β is dispensable for signal initiation and downstream cell activation (8,9), and a role has been demonstrated for ordered regions of plasma membrane, commonly called lipid rafts, in facilitating signal initiation by this receptor (10,11) and other MIRR family members (12).
To understand the role of lipid rafts in FcεRI phosphorylation by Lyn, we investigated the effects of this ordered lipid environment on Lyn tyrosine kinase activity in RBL mast cells. We found that rafts protect the active site tyrosine residue of Lyn from dephosphorylation, thereby enhancing the specific activity of Lyn kinase in lipid rafts relative to that in more disordered regions of the plasma membrane (13). As crosslinking of IgE-FcεRI causes association of these receptors with lipids rafts (10,14), we postulated that the raft environment serves principally to permit receptor phosphorylation by active Lyn in the same environment and to protect these proteins from dephosphorylation by a phosphatase that is excluded from lipid rafts (13).

To test this hypothesis, we utilized FcεRI stably expressed in a CHO cell line that exhibits an unusually high level of basal (unstimulated) FcεRI tyrosine phosphorylation due to co-expressed Lyn (15). We compared the capacity of different tyrosine phosphatases to regulate both basal and stimulated FcεRI tyrosine phosphorylation in this situation. We find that expression of the transmembrane phosphatase PTPα is highly effective in this role. Furthermore, we show that conversion of PTPα to a lipid-anchored, raft-preferring phosphatase abolishes its capacity to facilitate antigen-stimulated FcεRI tyrosine phosphorylation. Our results are consistent with a general role for protein segregation by lipid rafts in the regulation of enzymatic and other signaling processes.

EXPERIMENTAL PROCEDURES

DNA constructs - Lyn was amplified by PCR from Lyn-pcDM8, provided by Dr. Henry Metzger (NIH, Bethesda, MD) with primers (fs) 5' -
TATTGTGGATCCGCCACCATGGGATGTAAATCAA-3' and (rs) 5' -
ATTGTTCGAGCGATGGCGTGCCTGATGACT-3' and subcloned into pcDNA3 with BamH I and Xho I. Tyr to Phe Lyn mutants were generated using the Quickchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). PTPα-pcDNA3 was supplied by Dr. David Shalloway (Cornell University, Ithaca NY) and SHP-1-pRc-CMV by Dr. Katherine Siminovitch (University of Toronto, Toronto ON). PM-PTPα is a construct of the first 21 amino acids of Lyn attached to the cytoplasmic portion of PTPα with a 2 amino acid linker (KL), created using PCR to generate a fragment containing the first 63 nucleotides of Lyn with a 5' BamH I and a 3' Hind III site using the same forward primer as above and (rs) 5'-TGTATAGCTTAGTCATCATCTACTCCATC-3' for the Lyn fragment. The cytoplasmic portion of PTPα with a 5' Hind III and a 3' Xho I site was created by PCR with primers (fs) 5'-TATGTAAAGCTTAGGTTTAAGAAATACAAGC-3' and (rs) 5'-ATTGTTCGAGCTTTGCTGATGACT-3'. The fragments were ligated together in pcDNA3. Human CD45RO-Neo3 was provided by Dr. Arthur Weiss (University of California at San Francisco).

Transfections - CHO cells stably transfected with FcεRI (CHO-FcεRI cells) (16) were transiently transfected in 6-well plates using Geneporter (GTS, San Diego, CA): for each well, 10 µl Geneporter and 1 µg of Lyn-pcDNA3 and 1 µg phosphatase construct DNA (or 0.5 µg of PM-PTPα and 0.5 µg pcDNA3), or equivalent amounts of empty pcDNA3 vector for controls, were added to 100 µl Optimem (Invitrogen, Grand Island, NY), incubated for 15 minutes and added to cells plated over with 1 ml Optimem, then incubated for 6 hours at 37°C with 5% CO2, and finally washed and plated with CHO medium containing 1 µg/ml mouse IgE.
Antibodies – Mouse IgE specific for DNP-BSA was prepared as previously described (Posner et al. 1992). Antibodies used include: mouse anti-Lyn H6 mAb and polyclonal rabbit anti-Lyn 44 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-phosphotyrosine mAb (4G10) and rabbit anti-SHP-1 (Upstate Biotechnology, Lake Placid, NY), mouse anti-CD45 mAb (GAP8.3 (ATCC), rabbit anti-pY508 (anti-phospho-Lyn (Y507) (Cell Signaling Technologies, Beverly, MA), rabbit anti-IgE (17), and FITC-goat anti-mouse IgG2a (Southern Biotechnology, Birmingham, AL)

Examination of phosphorylation - To examine whole cell tyrosine phosphorylation, transfected CHO-FcεRI cells, or RBL-2H3 cells (18), were suspended at 2x10^6 cells/ml in buffered saline solution (BSS (11)) containing bovine serum albumin (Sigma) and equilibrated to 37°C. Cells were lysed with non-reducing SDS sample buffer before and after 2 or 5 minutes of stimulation with 0.9 µg/ml DNP-BSA (14). Samples were separated by electrophoresis on 12% acrylamide SDS gels and quantitative immunoblotting was performed as previously described (13). For immunoprecipitation of FcεRI, cells were suspended at 8 x 10^6 cells/ml and lysed in RIPA buffer (0.5% TX-100, 0.5% deoxycholate, 0.05% SDS), which has been shown to solubilize all membrane components (13). Anti-IgE immunoprecipitations used 10 µg of anti-IgE for 0.5 ml of cell lysate, and immune complexes were pulled down with 35 µl of protein A beads (Pierce). Determination of Lyn in vitro kinase activity from Lyn was immunoprecipitated with mouse anti-Lyn mAb from RIPA or TX-100 post nuclear supernatants of CHO-FcεRI cells using α-casein as the substrate was carried out as previously described (13).

Measurement of in vitro phosphatase activity for expressed PTPα and PM-PTPα, anti-HA immunoprecipitated from CHO cell lysates, was carried out as previously described (41).
RESULTS

CHO cells expressing FceRI and Lyn exhibit poorly regulated Lyn kinase activity.

Torigoe and Metzger reported high levels of spontaneous FceRI tyrosine phosphorylation in CHO cells stably expressing FceRI and the Src family kinase Lyn (15). In their study, spontaneous FceRI β subunit phosphorylation was ~17 times greater than in RBL-2H3 mast cells. Antigen stimulation only modestly increased FceRI phosphorylation in CHO cells. Similarly, we find high levels of basal tyrosine phosphorylation on FceRI stably expressed in CHO cells when Lyn kinase is transiently expressed. As shown in Figure 1A (left panel) anti-phosphotyrosine western blotting of whole cell lysates (WCL) reveals high levels of spontaneous FceRI β and γ2 subunit phosphorylation for these transfected CHO cells. Additionally, there are elevated levels of phosphorylation of Lyn and several other proteins. In contrast, the same number of RBL cell equivalents exhibits no detectable tyrosine phosphorylation in the absence of antigen stimulation, but there is a robust increase in phosphorylation on multiple proteins, including FceRI, in response to antigen. We verified that the bands of ~35 kDa and ~25-30 kDa on anti-phosphotyrosine western blots of CHO-FceRI WCLs are FceRI β and γ2, respectively, by confirming their co-migration with those bands precipitated by anti-IgE antibody (Fig. 1A, right panel), and by selective immunodepletion of these bands from WCLs (data not shown). Note that the lower level of stimulated phosphorylation of FceRI β and γ2 in CHO-FceRI cells, compared with that in RBL cells, is expected because of lower levels of FceRI expression in the CHO cells (15 and data not shown).
To investigate the basis for the high basal tyrosine phosphorylation observed in Lyn-transfected CHO cells, we more closely examined Lyn kinase. As a first step we immunoprecipitated Lyn kinase from these cells and from RBL cells and compared their specific activities using a-casein as previously described (13). Figure 1B shows that Lyn kinase expressed in CHO cells has ~3-fold higher specific activity than Lyn from RBL cells, consistent with the higher levels of basal phosphorylation in the CHO-FcεRI cells.

Lyn kinase activity is positively regulated by phosphorylation near its active site, Tyr397 (Lyn A notation), and negatively regulated by phosphorylation on its C-terminal tail, Tyr508. Our previous study in RBL cells showed that Lyn activity is largely determined by phosphorylation at Tyr397, whereas C-terminal phosphorylation is very low in resting cells (13). To assess the relative phosphorylation of these sites for Lyn in the CHO cells, we compared various Lyn mutants, using both 4G10 anti-phosphotyrosine and an antibody specific for the phosphorylated C-terminus of Lyn (anti-pY508) by western blot analysis. As shown in Figure 1C, we find that wt Lyn expressed in CHO cells has several times more net tyrosine phosphorylation than Lyn from RBL-2H3 cells (top panel), whereas C-terminal phosphorylation of Lyn from these two different cell types are more similar (middle panel). Mutation of Tyr397 to Phe results in dramatic reduction of tyrosine phosphorylation detected by 4G10 on Lyn expressed in CHO cells (top panel). Conversely, mutation of Tyr508 to Phe results in only a modest reduction in 4G10-detected phosphorylation (top panel) and complete elimination of the anti-pY508 phosphorylation (middle panel). Taken together, these results indicate that Lyn expressed in CHO cells is hyperphosphorylated at Tyr397, and this is likely to account for its high specific activity.
Co-expression of protein tyrosine phosphatase PTPα with Lyn in CHO cells reconstitutes antigen-dependent FcεRI phosphorylation.

The large amount of Lyn-dependent basal phosphorylation in CHO cells suggests that these cells lack sufficient phosphatase activity to regulate exogenously expressed Lyn kinase. PTPα is a ubiquitously expressed transmembrane tyrosine phosphatase that has been found to regulate Src kinase activity (19). Figure 2A shows that transient co-expression of this phosphatase with Lyn in CHO-FcεRI cells dramatically reduces basal levels of Lyn phosphorylation and the accompanying spontaneous phosphorylation of FcεRI β and γ2 subunits. Notably, co-expression of PTPα permits antigen-stimulated tyrosine phosphorylation of both receptor subunits, which is maximal between two and five minutes and declines thereafter, as observed for stimulation in RBL-2H3 cells (20). As reported by Vonakis and Metzger, CHO-FcεRI cells have a poorly detectable endogenous Src-family kinase activity which mediates variable amounts of FcεRI β and γ2 phosphorylation in response to antigen (16), and this is apparent in the vector control, lanes 1 and 2 of Figure 2A.

INSERT FIGURE 2 HERE

Quantification of basal and stimulated FcεRI β and γ2 phosphorylation from multiple experiments is summarized in Figure 2B. For FcεRI β, Lyn expression causes a significant increase in both basal and stimulated phosphorylation. Co-expression of PTPα reduces basal phosphorylation of FcεRI β to very low levels while maintaining elevated levels of stimulated phosphorylation. For FcεRI γ2, Lyn expression causes substantial increases in basal and stimulated phosphorylation. Co-expression of PTPα reduces basal phosphorylation by ~10-fold, while reducing stimulated phosphorylation
by less than 3-fold. These results demonstrate that co-expression of PTPα with Lyn in CHO-FcεRI cells controls spontaneous phosphorylation while permitting antigen-stimulated phosphorylation of FcεRI by Lyn.

Reconstitution of signaling in CHO cells is dependent on the location of PTP activity.

We showed in Figure 1 that Lyn expressed in CHO cells is highly active, most likely because it is strongly phosphorylated at its active site. Likewise, the activity of endogenous Lyn in RBL cells is controlled by phosphorylation at the active site (13). In that study, we found that Lyn specific activity is substantially higher in lipid rafts than in disordered regions of the plasma membrane. We hypothesized that rafts exclude transmembrane phosphatases that dephosphorylate and thereby inactivate Lyn located outside of lipid rafts. In Figure 3A, sucrose gradient analysis of TX-100-lysed CHO-FcεRI cells transfected with PTPα shows that greater than 99% of this transmembrane protein is found in the soluble, non-raft portion of the gradient. To test the role of raft exclusion in the reconstitution of stimulated phosphorylation, we created a raft-associating form of PTPα, named PM-PTPα, by fusing the cytoplasmic portion of PTPα with the N-terminal 21 amino acids of Lyn, which target Lyn to rafts by virtue of post-translationally added palmitoylation and myristoylation (21). PM-PTPα and Lyn have a similar localization to lipid rafts in transfected CHO cells, with >60% of these proteins in the low density raft fractions (Fig. 3A). To evaluate the role of this differential raft targeting for PTPα and PM-PTPα, we compared the effects of similar co-expression of each phosphatase with Lyn. As shown in Figure 3B (top panel), co-expression of PM-PTPα leads to a further decrease in Lyn phosphorylation than for PTPα co-expression. Importantly, little antigen-stimulated phosphorylation of FcεRI is detected in the presence of PM-PTPα in this and in three other experiments of similar design. In contrast, PTPα suppresses spontaneous
FcεRI phosphorylation but allows stimulated FcεRI phosphorylation, similar to results in Figure 2.

In these experiments, quantitative analysis of anti-PTPα blots indicates that PM-PTPα was expressed at 1.9 +/- 0.3 (Std. Error, n=6) -fold higher levels than PTPα. To attempt to evaluate whether this modest difference in expression could account for the more profound suppression of stimulated FcεRI phosphorylation due to PM-PTPα, we compared in vitro phosphatase activities of PTPα and PM-PTPα immunoprecipitated from equal numbers of transfected CHO cells. In two separate experiments, the ratios of PM-PTPα/PTPα phosphatase activities were 1.22 and 0.67. These results suggest that net phosphatase activity per cell equivalent due to PM-PTPa is not significantly different from that due to PTPα, and this is not likely to account for their qualitatively different effects on stimulated FcεRI phosphorylation.

Lyn expressed in CHO cells is highly active (Fig. 1B), and we next examined if the effects of PTPα on whole cell phosphorylation are mediated through control of Lyn activity. Figure 3C compares the specific activities of Lyn solubilized and immunoprecipitated from CHO cells with or without PTPα or PM-PTPα co-expressed. PTPα causes a substantial decrease in specific activity of Lyn. This reduction in Lyn kinase activity may be sufficient to account for the control of basal FcεRI phosphorylation in CHO cells expressing exogenous Lyn. The specific activity of Lyn solubilized from CHO-FcεRI cells co-expressing Lyn and PM-PTPα is more severely reduced than that with PTPα (Fig. 3C). This further reduction in activity correlates with a reduction of Lyn activity regardless of its raft environment in PM-PTPα transfected cells, consistent with
the dephosphorylation of Lyn at its active site tyrosine by PM-PTPα in both the raft and non-raft environment. Because PM-PTPα co-localizes with Lyn in lipid rafts, it has continual access to this substrate, whereas transmembrane PTPα is restricted to non-raft portions of the plasma membrane and is unable to inactivate raft-associated Lyn.

**Effects of other PTPs on FcεRI phosphorylation by Lyn in CHO cells.**

To ascertain the selectivity of PTPα regulation of FcεRI phosphorylation by Lyn, we compared its effects to those of two other tyrosine phosphatases, SHP-1 and CD45. SHP-1 is a hematopoietic phosphatase shown to negatively regulate MIRR signaling, including FcεRI signaling in mast cells (22, 23). It is located primarily in the cytoplasm, but it is recruited to the plasma membrane during antigen stimulation (22). Figure 4A shows a representative experiment in which co-expression of SHP-1 with Lyn in CHO-FcεRI cells is compared to co-expression of PTPα. SHP-1 efficiently inhibits Lyn autophosphorylation and suppresses FcεRI stimulation, both before and after stimulation, reminiscent of the effects of PM-PTPα (Fig. 3B). Active SHP-1 binds to protein substrates via its SH2 domains and is probably not restricted from Lyn in lipid rafts. In contrast, expression of CD45 in CHO cells causes small increases in tyrosine phosphorylation levels of multiple cellular proteins, including Lyn and FcεRI, both before and after stimulation (Fig 4B). Because CD45 is difficult to detect by immunoblotting, we used flow cytometry analysis that showed relatively low levels of CD45 in transfected CHO cells compared to the highly abundant expression of this phosphatase on Jurkat T cells (data not shown). Like PTPα, CD45 is a type 1 transmembrane protein that is excluded from lipid rafts (24), so it is unlikely that its plasma membrane location distinguishes its effects from those of PTPα. CD45 is often implicated in positive regulation of immune cell signaling by dephosphorylation of the C-terminal negative
regulatory tyrosine of Src family kinases such as Lck (25), and our results are consistent with this role.

To investigate the mechanism by which CD45 expression leads to an increase in basal phosphorylation in CHO-FcεRI cells we compared tyrosine phosphorylation of Lyn expressed in the presence or absence of PTPα, SHP-1 or CD45. Figure 4C summarizes the quantitative comparisons for both total tyrosine phosphorylation and Tyr508 phosphorylation, normalized separately to equivalent amounts of Lyn. We find that PTPα and SHP-1 both dephosphorylate Lyn exclusively at Tyr397, and that SHP-1 expression actually leads to an increase in Tyr508 phosphorylation through an unknown mechanism. In contrast, CD45 expression results in a small net dephosphorylation of Lyn at Tyr508 that can account for the modest increase in cellular tyrosine phosphorylation we observe in CD45 expressing CHO cells (Fig. 4B). Thus, among these three phosphatases, only PTPα has the necessary combination of plasma membrane location (exclusion from lipid rafts) and substrate specificity (Lyn Tyr397) that allow it to reconstitute effective regulation of Lyn kinase activity and the early events of FcεRI signaling in CHO cells.

DISCUSSION

Our present findings show that a transmembrane tyrosine phosphatase, PTPα, plays an essential role in the regulation of FcεRI phosphorylation by Lyn when this kinase and receptor are co-expressed in CHO cells. Lyn expressed in CHO cells has a
substantially higher specific activity than Lyn in RBL cells due to high levels of phosphorylation at its active site Tyr397 (Fig. 1). Enhanced Lyn kinase activity in the CHO cells leads to spontaneous phosphorylation of multiple cellular proteins, including FcεRI (Fig 1). Co-expression of PTPα results in marked dephosphorylation of Lyn in these cells, thereby suppressing basal phosphorylation of several substrates while enabling stimulated phosphorylation of FcεRI (Fig. 2).

Our previous studies revealed that active Lyn in RBL mast cells is largely sequestered in lipid rafts, and this Lyn is active due to phosphorylation at its active site (13). In light of this, we investigated whether segregation of Lyn from a transmembrane phosphatase by lipid rafts is important for the regulation of Lyn activity. CHO-FcεRI cells provide a useful vehicle to test this possibility because Lyn in these cells is less effectively regulated than in RBL cells. We determined that PTPα is excluded from lipid rafts, and thus is unable to gain access to and inactivate Lyn within the ordered raft environment. In clear contrast, targeting chimeric PM-PTPα to lipid rafts abolishes stimulation-dependent FcεRI phosphorylation by inactivating Lyn both within and outside of the raft environment (Fig. 3). These results support a model in which lipid rafts act both to protect Lyn from phosphatases, which keeps raft-associated Lyn active, and to inhibit productive interactions of FcεRI with active Lyn until crosslinking by multivalent antigen brings them together by driving raft association of FcεRI. Crosslinking FcεRI in this manner may also serve to stabilize ordered membrane domains containing active Lyn.

Metzger and colleagues have argued against a role for lipid rafts in protecting FcεRI from dephosphorylation in studies that utilized the reversal of antigen crosslinking by monovalent hapten to observe rapid dephosphorylation of FcεRI and other phosphorylation substrates (26,27). However, reversal of crosslinking causes a rapid
reversal of FcεRI lateral immobilization (28) and the loss of its lipid raft association (10), so that dephosphorylation of FcεRI due to hapten addition probably occurs outside of lipid rafts and leads to dephosphorylation of more downstream substrates. Furthermore, the role of lipid rafts in protecting Lyn kinase from dephosphorylation was not evaluated by Metzger and colleagues, and this is probably at least as important as protection of FcεRI from dephosphorylation based on the current results.

Torigoe and Metzger originally described spontaneous phosphorylation of stably expressed FcεRI by stably co-expressed Lyn in CHO cells (15). These authors attributed this to differential glycosylation of FcεRI between CHO and RBL cells after detecting no other differences in physical and biochemical properties of FcεRI or Lyn in these two cell types. Using a different assay for Lyn kinase activity, we find substantial differences in immunoprecipitated Lyn activity (Fig. 1B). In addition, we have characterized tyrosine phosphorylation in whole cell lysates, which shows high basal phosphorylation of multiple proteins independent of FcεRI expression (Figs. 1A, 2A, and data not shown).

Previous studies on PTPα largely focused on its positive regulation of Src kinase activity in fibroblasts due to dephosphorylation of Src C-terminal tyrosine (19). Our present findings demonstrating negative regulation of Lyn kinase activity by dephosphorylation of its active site tyrosine were unanticipated by these studies. Ng et al. reported little phosphatase activity of PTPα relative to CD45 towards a phosphorylated peptide mimic of the C-terminus of Fyn in vitro, and they found that PTPα had a lower K_m towards phosphorylated peptide mimics of the active site versus the C-terminus of Src in vitro (29), suggesting that PTPα is not generally restricted to dephosphorylation of the negative regulatory site. Our preliminary results indicate that PTPε, which is highly homologous to PTPα, also reconstitutes antigen-stimulated signaling in CHO cells by negatively regulating Lyn (data not shown). Other
transmembrane phosphatases may likewise negatively regulate Lyn kinase outside of lipid rafts in a redundant fashion, suppressing spontaneous signaling and complementing the negative role of C-terminal tyrosine phosphorylation in different cell types and for different Src family members (30).

SHP-1 and CD45, two common hematopoietic phosphatases, do not reconstitute regulated FcεRI phosphorylation in CHO cells (Fig. 4). Both phosphatases are often implicated for regulating signaling through FcεRI and other members of the MIRR family, and SHP-1 is reported to negatively regulate FcεRI signaling in RBL mast cells (23). Similar to PTPα, we found that SHP-1 dephosphorylates the active site tyrosine of Lyn expressed in CHO-FcεRI cells (Fig. 4), consistent with results previously reported in B cells (31). Unlike PTPα, SHP-1 is a cytosolic phosphatase and is not excluded from lipid rafts, allowing it to dephosphorylate Lyn regardless of its membrane environment, thus preventing stimulated FcεRI phosphorylation in the CHO-FcεRI cells, similar to results with raft-targeted PM-PTPα (Fig. 3). These results are consistent with a model in which SHP-1 generally suppresses spontaneous receptor phosphorylation by Lyn in resting cells (32). Following cell activation, SHP-1 is recruited to phosphorylated ITIM sequences by its SH2 domains, where it has a more targeted role in dephosphorylating ITAMs on FcεRI or at the active site of Lyn (33). Its strong suppression of stimulated FcεRI phosphorylation in the present experiments may be the result of relatively high expression levels, but reduced expression of this phosphatase also did not confer a differential effect on stimulated vs. unstimulated FcεRI phosphorylation as seen with PTPα (data not shown).

In contrast, CD45 is largely excluded from lipid rafts (24), like PTPα, but its expression in CHO cells causes small increases in spontaneous and stimulated FcεRI phosphorylation (Fig. 4). We find that CD45 preferentially dephosphorylates Lyn at its
negative regulatory site, Tyr508, which likely leads to an increase in Lyn activity, causing enhanced FcεRI phosphorylation. This suggests that CD45 is primarily a positive regulator of Lyn activity, in agreement with evidence showing that high levels of CD45 expression in RBL cells leads to increased degranulation following antigen stimulation (34). Previous studies showed that CD45 is not required for FcεRI signaling in RBL mast cells (35, 36), and our results are consistent with these findings.

In other studies, expression of lipid raft-targeted chimeric phosphatases from either SHP-1 or CD45 were used to investigate the role of tyrosine phosphatase segregation from lipid rafts in T cell receptor signaling (37-39). All of these studies reported inhibition of downstream signaling due to the raft-targeted chimeric phosphatases, and, in one study, inhibition of stimulated receptor phosphorylation was observed (39). These studies, together with our results, suggest that exclusion of membrane associated tyrosine phosphatases from the ordered lipid environment of lipid rafts may be a general mechanism for the regulation of immunoreceptor signaling.

Scharenberg et al. showed that regulated FcεRI phosphorylation could be reconstituted in NIH3T3 cells upon co-expression of Lyn (40). These authors concluded that non-hematopoietic phosphatases present in NIH3T3 cells sufficiently regulate FcεRI signaling. PTPα is ubiquitously expressed, and may be relevant to this regulation in NIH3T3 cells (41). We have detected endogenous PTPα in both the CHO-FcεRI and RBL-2H3 cells by immunoblotting; its endogenous expression in the CHO-FcεRI cells is generally less than that in RBL cells or when this phosphatase is transfected into CHO cells (Fig. 3B and data not shown). Although it is possible that transfected PTPα is expressed to levels that overcome normal limits to its substrate specificity, we find that transfected PTPα functions consistently in reconstituting regulated FcεRI phosphorylation over a wide range of expression levels in the CHO-FcεRI cells, including
levels approaching those of endogenous PTPα in these cells (data not shown).

Regulation of PTPα activity by dimerization (42) or other mechanisms may also influence its functional capacity in addition to effects of differential expression.

The role of PTPα, or related transmembrane tyrosine phosphatases, in FcεRI signaling in mast cells remains to be established. From this study it is clear that PTPα can regulate Lyn kinase in a manner that enables robust tyrosine phosphorylation of FcεRI in response to crosslinking by antigen. Our results demonstrate that phosphatase location in the plasma membrane and its substrate specificity are both important to the control of signal initiation via FcεRI. Unlike SHP-1 and CD45, PTPα fulfills both criteria, but it may be only one of several such phosphatases responsible for regulating Lyn kinase activity in mast cells and other hematopoietic cells in which Lyn plays important physiological roles.

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REFERENCES

FIGURE LEGENDS

FIGURE 1: CHO-FcεRI cells expressing Lyn kinase have constitutively phosphorylated FcεRI and Lyn kinase. A) Left side: anti-phosphotyrosine and anti-Lyn western blotting of whole cell lysates from RBL-2H3 cells and CHO-FcεRI cells transiently transfected with Lyn kinase with or without stimulation by 0.9 μg/ml DNP-BSA stimulation. Both panels were from the same blot. Right side: Western blot of anti-IgE immunoprecipitates from Lyn-expressing CHO-FcεRI cells before or after antigen stimulation. B) in vitro kinase analysis of the specific activity of Lyn immunoprecipitated from RIPA post nuclear supernatants of CHO or RBL-2H3 cells (n=6, error bars show SE). C) WT and Tyr mutants of Lyn B expressed in CHO-FcεRI cells or endogenous Lyn from RBL cells were blotted with 4G10 (upper panel), anti-pY508-Lyn (middle panel) and anti-Lyn (lower panel).

FIGURE 2: Co-expression of transmembrane tyrosine phosphatase PTPα with Lyn reconstitutes regulated tyrosine phosphorylation of FcεRI and Lyn in CHO-FcεRI cells. A) Anti-phosphotyrosine western blot (upper panel) of whole cell lysates obtained from CHO-FcεRI cells with or without Lyn B and PTPα stimulated with DNP-BSA. Lower panels shows a reprobe detecting Lyn kinase. B) Quantitation of FcεRI β and γ2 tyrosine phosphorylation before and after 2 minutes of stimulation with 0.9 μg/ml DNP-BSA. Data are averaged from six independent experiments, including that shown in 2A (error bars show SE).

FIGURE 3: Reconstitution of stimulated FcεRI phosphorylation by PTPα in CHO-FcεRI cells is lipid raft dependent. A) Western blot analysis of sucrose gradient fractions from
TX-100 lysed CHO cells. The upper and middle panel were probed with anti-PTPα and the lower panel was probed with anti-Lyn. B) Western blot analyses of reduced whole cell lysates of CHO-FcεRI-Lyn cells co-expressing either wt PTPα or PM-PTPα before and after stimulation by DNP-BSA. The upper panel is a 4G10 anti-phosphotyrosine blot, the middle panel shows relative levels of wt and PM-PTPα expression (anti-PTPα blot) and the lower panel shows relative Lyn expression (anti-Lyn blot). C) *in vitro* kinase analysis of the specific activity of Lyn immunoprecipitated from TX-100 lysates of CHO-FcεRI cells expressing Lyn with or without either wt PTPα or PM-PTPα (n=4, error bars show SE).

FIGURE 4: Effects of hematopoietic cell PTPs on Lyn and FcεRI tyrosine phosphorylation in CHO cells. A) 4G10 (upper panel) and anti-Lyn (middle panel) western blot analysis of WCLs from CHO-FcεRI cells expressing Lyn B and PTPα or SHP-1 (lower panel). B) 4G10 anti-phosphotyrosine (upper panel) and anti-Lyn (lower panel) analysis of WCLs from CHO-FcεRI cells expressing Lyn B, PTPα or CD45. C) Quantitation of western blot analysis of the relative levels of both total tyrosine and Tyr 508 phosphorylation on Lyn in unstimulated CHO cells with no PTP, PTPα, SHP-1 or CD45 (n=4, error bars show SE).
Figure 1

A. RBL and CHO-FceRI (w/ Lyn) WCL and Anti-IgE IP

B. Relative Lyn Specific Activity

C. CHO-FceRI

IP: Anti-Lyn
WB: 4G10
WB: Anti-pY508
WB: Anti-Lyn
Figure 2

A. 

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<td>0 2 0 2 5 10</td>
<td>0 2 5 10</td>
<td>Min. of Stimulation</td>
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WB: 4G10

97 - 66 - 45 - 30 - Lyn B

WB: Lyn

B. 

Relative FcεRI β phosphorylation

Relative FcεRI γ2 phosphorylation

- - - - + + + Ag. Stim.

- - Lyn B Lyn B & PTPα
FIGURE 3

A. 

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WB: PTPα

WB: PTPα

WB: Lyn

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WB: 4G10

WB: PTPα

C.

Relative Lyn Specific Activity

no PTP | PTPα | PM-PTPα

WB: PTPα

WB: Lyn

Lyn B

FcεRI β

wt PTPα

PM-PTPα
Figure 4

A. Lyn B

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WB: 4G10

- Lyn
- FcεRI β
- FcεRI γ2

WB: Anti-Lyn

WB: Anti-SHP-1

B. Lyn B

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WB: 4G10

- Lyn
- FcεRI β
- FcεRI γ2

WB: Anti-Lyn

C.

Relative Lyn Phosphorylation

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Reconstitution of regulated phosphorylation of FcεRI by a lipid raft-excluded protein tyrosine phosphatase
Ryan M. Young, Xinmin Zheng, David Holowka and Barbara Baird
J. Biol. Chem. published online November 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408339200

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