Requirement for a Negative Charge at Threonine 60 of the FcRγ for Complete Activation of Syk*

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Aggregation of FcεRI on mast cells results in the phosphorylation of the FcεRIγ chain on tyrosine and threonine residues within the immunoreceptor tyrosine-based activation motif. In the present study we sought to identify the site of threonine phosphorylation in FcεRIγ and investigate its functional importance. We found that threonine 60 was phosphorylated in vitro and in vivo. Expression of a mutated FcεRIγ (T60A), in either FcεRIγ-deficient or γ-null mast cells, resulted in a delay of FcεRI endocytosis, inhibition of TNF-α mRNA production, and inhibition of degranulation but did not affect FcεRI-induced cell adhesion. Tyrosine phosphorylation of the T60A mutant γ chain was normal, but Syk phosphorylation was dramatically reduced in these transfectants. This correlated with reduced co-immunoprecipitation of FcεRIγ with Syk. Substitution of an aspartic charged amino acid residue at position 60 of the FcεRγ chain was demonstrated to be mediated by receptor-associated ITAM containing signaling chains; PKCδ, protein kinase C δ isoform; TNF-α, tumor necrosis factor α; FITC, fluorescein isothiocyanate; BMMC, bone marrow-derived mast cells; DNP, dinitrophenylated; HSA, human serum albumin; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); ITAM, immunoreceptor tyrosine-based activation motif; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorter; HPLC, high pressure liquid chromatography.

The high affinity receptor for IgE, FcεRI, is an important initiator of type I allergic reactions (1) and is associated with the development of chronic disease because its expression is significantly increased in atopic individuals (2, 3). FcεRI is a tetramer consisting of α, β, and two disulfide-linked γ chains (4, 5). The γ chain is required for FcεRI signal transduction and is also involved in the signaling pathways of Fcγ and T cell receptors (6, 7). In addition, FcεRIγ is required for cell surface expression of FcεRI, and thus mast cells from γ-null mice do not degranulate nor produce interleukin-4 to an IgE-mediated stimulus (8). Furthermore, the Fc receptor γ chain (FcεRIγ) is important to the pathological consequences of immune complex deposition and inflammation (9).

The importance of tyrosine phosphorylation of the FcεRIγ to downstream signaling events is well established (10, 11). After FcεRI aggregation, a Src family tyrosine kinase phosphorylates the tyrosines within the immunoreceptor tyrosine-based activation motif (ITAM) of the γ chain. Tyrosine phosphorylation of the ITAM tyrosines leads to recruitment of SH2-containing proteins which are involved in subsequent signaling steps (reviewed in Ref. 12). One such protein is Syk kinase (13) whose activity is critical to mast cell responses (14–16).

In mast cells, tyrosine phosphorylation of both FcεRIβ and γ is observed upon FcεRI engagement (10). In addition, the FcεRIβ is also phosphorylated on serine residues, whereas the FcεRIγ is phosphorylated on threonine residues (10). In a mast cell line (RBL-2H3), threonine phosphorylation of the FcεRIγ chain was demonstrated to be mediated by receptor-associated protein kinase Cδ (PKCδ) (17). The function of threonine phosphorylation of the FcεRIγ is not known but has been the subject of speculation. Bingham et al. (18) reported that threonine phosphorylation of the FcεRIγ preceded its tyrosine phosphorylation and that both threonine and tyrosine phosphorylation were delayed in a low secretory variant. This suggested the possible functional correlation of threonine phosphorylation with degranulation. In addition, we previously found a correlation between threonine phosphorylation and the rate of FcεRI endocytosis as a specific PKC inhibitor inhibited both processes (17).

In the present study we sought to identify the site of threonine phosphorylation in the FcεRIγ and to assess the functional significance of this phosphorylation event by transfecting RBL-2H3-derived γ-deficient cells (19) or γ-null bone marrow-derived mast cells (BMMC) with either wild type or variant γ chains. Using this approach we found that threonine 60 was phosphorylated in vivo and that insertion of a negatively charged amino acid residue at position 60 of the FcεRIγ substituted for phosphorylation at this site, resulting in the FcεRI-dependent activation of Syk.

**EXPERIMENTAL PROCEDURES**

Immunoglobulins and Reagents—Anti-dinitrophenyl (DNP)-specific mouse monoclonal IgE (20) was purified as described (21). Dinitrophenylated human serum albumin (DNP₃₀₋₄₀-HSA) was from Sigma. The mouse monoclonal (4G10) antibody to phosphotyrosine conjugated to...
horseradish peroxidase and a polyclonal rabbit antibody to Shc were from Upstate Biotechnology Inc. (Lake Placid, NY). The mouse monoclonal antibody to PKCδ was from Transduction Laboratories (Lexington, KY). The mouse monoclonal antibody to Syk was as described (22), and a rabbit polyclonal antibody to Syk was a kind gift from U. Blank (Institut Pasteur, Paris, France). A rabbit polyclonal antibody to MyD88 was kindly provided by R. P. Siraganian (NIDCR, National Institutes of Health), and a chicken antibody to the FcRy was as described previously (16). FcRy peptides used in the in vitro studies were obtained from Quality Controlled Biochemicals (Hopkinton, MA) or were produced in-house (George Poy, NIDDK, National Institutes of Health) and analyzed by mass spectrometry and for amino acid composition by Harvard Microchemistry (Cambridge, MA). 3,3-Dithiobis(sulfosuccinimido)propionate (DTSSP) was purchased from Pierce. Polyvinylidene difluoride membranes, Tricine and Tris-glycine sample buffers, Tris-glycine, and Tricine SDS-polyacrylamide gels were from Novex (San Diego, CA).

**Cells and Activation**—RBL-2H3 and the RBL-2H3-derived γ-cell lines were cultured as a monolayer in stationary flasks essentially as described (23, 24). Stable transfectants were generated by electroporation of the FcRy or mutants thereof as described previously (25). To obtain cell populations expressing approximately equal numbers of receptors, stable transfectants were labeled with FITC-IgE and sorted for 10% of the cells providing the highest fluorescent signal. Cloning of the inserts was avoided to minimize the potential clonal variation (26). FcRy-null BMMC were grown in interleukin-3-containing medium essentially as described (27). Cells were used at the fourth to sixth week where cultures were greater than 90% mast cells. For degradation assays, transfected BMMC were stimulated with 20 ng of DNP-HSA for 10 min at 37 °C, and then the percent release of β-hexosaminidase was determined (21). For endocytosis and protein tyrosine phosphorylation, cells were stimulated with 100–400 ng of DNP-HSA for the indicated times.

**Site-directed Mutagenesis and Constructs**—Individual mutations of the threonine residues at positions 52 and 60 to alanine or aspartic acid were by PCR using mismatched primers and Extend™ high fidelity polymerase (Roche Molecular Biochemicals). For the T52A mutation the internal 5′-end was avoided to minimize the potential clonal variation. (26). FcRy-null BMMC were grown in interleukin-3-containing medium essentially as described (27). Cells were used at the fourth to sixth week where cultures were greater than 90% mast cells. For degradation assays, transfected BMMC were stimulated with 20 ng of DNP-HSA for 10 min at 37 °C, and then the percent release of β-hexosaminidase was determined (21). For endocytosis and protein tyrosine phosphorylation, cells were stimulated with 100–400 ng of DNP-HSA for the indicated times.

**In Vitro Phosphorylation Assays**—A peptide encoding the cytosolic region of the FcRy was synthesized, and in addition other FcRy peptides were synthesized in which either all of the threonines were converted to alanine or threonine-alanine substitutions were limited to defined positions. Protein kinase Cδ was prepared by P. Acs (NCI, National Institutes of Health) and used as described previously (17). After an in vitro kinase reaction using described conditions (17), phosphorylated peptide was isolated by reverse phase HPLC. We found that the background of the assay was significantly reduced if polyethylene glycol was substituted for stainless steel in the HPLC system tubing and column jacket. Radioactivity in the collected peaks was measured by Cerenkov counting, and relevant fractions were then subjected to phosphoamino acid analysis (17).

**[32P]Orthophosphate Labeling of FcRy, Phosphoamino Acid Analysis, and Peptide Maps**—Cells were metabolically labeled with [32P]orthophosphate essentially as described (10). Cells were then sensitized with anti-DNP-specific IgE at a concentration of 5 μg/ml for 1 h. Radiolabeled, IgE-sensitized cells (2.1 × 10⁶ in 0.7 ml) were stimulated with 400 ng/ml DNP-HSA for 3 min at 37 °C and processed to recover IgE-occupied FcRy as described previously (17). Recovered proteins were resolved, and phosphoamino acid analysis was as described previously (17).

Peptide maps were generated as described (35). Briefly, the identified FcRy was excised from the polyvinylidene difluoride membrane and incubated with polyvinylpyrrolidone 360 kDa (PVP-360, Sigma) to block nonspecific binding of the protease. The excised membrane was then washed with 50 mM ammonium bicarbonate and subsequently incubated with 10 μg of Glu-C V8 protease at 37 °C. This was followed by a second addition of Glu-C V8 protease and an overnight incubation at 37 °C. The soluble peptides were recovered by lyophilization and were resuspended in electrophoresis buffer for two-dimensional peptide mapping as described (35).

**Chemical Cross-linking, Immunoprecipitations, and Western Blots**—Chemical cross-linking conditions, immunoprecipitations, and Western blots were previously described (36). In some experiments antibody to phospho-Syk (clone 4G10; Upstate Biotechnology) was used to immunoprecipitate FcRy, as a measure of Syk activation (an indication of its translocation), and the presence of FcRy under reducing conditions. In other experiments proteins were identified directly with their respective antibodies.

**Transient Expression of FcRy in BMMC from FcRy Null Mice and Degranulation Assay**—Generation of recombinant Semliki Forest virus was described previously (29). For infection of BMMC, 4.0 to 7.0 × 10⁵ cells from FcRy-null mice were infected with a 1.25 titer of Semliki Forest virus in the presence of polyethylene glycol as described (29). After a 30-min incubation, the virus was removed, and cells were incubated for an additional 8 h. During this time one-tenth of the cells were separated and incubated with FITC-IgE (1 μg), whereas the remaining cells were incubated with 5 μg of unlabeled IgE. Cells were then washed with phosphate-buffered saline to remove unbound IgE and either analyzed by FACs (FITC-IgE labeled cells) or in a degranulation assay. Degranulation (β-hexosaminidase release) was measured in either 250- or 500-μl volumes using 20 ng/ml of DNP-HSA for 10 min (maximal release) at 37 °C in Tyrodes buffer (32).
RESULTS

Phosphorylation of FceRIγ on Threonine 60—To define the site of threonine phosphorylation of the FceRIγ, the in vitro studies took advantage of the previous observation that PKCδ was uniquely able to phosphorylate the FceRIγ (17). Purified peptides corresponding to the entire cytosolic region of the FceRIγ (wild type or variants) were incubated with PKCδ in an in vitro kinase reaction. After incubation, the phosphorylated peptides were isolated by HPLC. The results confirmed our previous study (17) that showed that PKCδ phosphorylates the wild type FceRIγ peptide mainly on threonine (Fig. 1) with a minor incorporation of label to serine. Substitution of alanine for all threonines (T48A, T52A, T57A, T60A) in the FceRIγ peptide resulted in a significant reduction in the overall extent of phosphorylation, and phosphoamino acid analysis indicated that only serine was phosphorylated (Fig. 1A). FceRIγ peptides in which the threonine residues were sequentially added (T52A, T57A, T60A) showed minimal to no threonine phosphorylation (Fig. 1, A and B). In contrast, the FceRIγ peptide variant (T48A, T52A, T57A) that contained only threonine 60, the threonine closest to the C terminus, incorporated similar amounts of radiolabel to that seen for the wild type peptide (Fig. 1B). Furthermore, the overall level of phosphorylation was far greater than that seen for the other threonine-to-alanine variants used in these experiments. In addition, as with the wild type peptide, the amount of threonine phosphorylation was far greater than the amount of serine phosphorylation. Thus, we conclude from these in vitro studies that the target of PKCδ activity is the threonine closest to the C terminus (Thr60) on the FceRIγ chain.

Based on the aforementioned in vitro results, we limited our in vivo analysis to the mutation of T60A but also considered a T52A mutation that was proposed in a previous report by Pribluda et al. (37) as the possible site of threonine phosphorylation. Stable transfectants were generated, in the previously described RBL FceRIγ-deficient cell line (24), that expressed wild type, T52A, and T60A FceRIγ. As shown in Fig. 2A, the transfected cell populations expressed receptor numbers similar to those of RBL cells. As compared with the parental RBL-γ cell line, receptor expression increased approximately 10-fold with all transfectants expressing between 2 and 3 × 10^5 receptors/cell. Tyrosine phosphorylation of FceRIγ was analyzed by
immunoprecipitation of phosphorylated proteins with antibody to phosphotyrosine followed by immunoblotting with antibodies to FcεRIγ or to Shc (Fig. 2B). The Shc immunoblot served as a control for protein loading as Shc tyrosine phosphorylation is unaffected by FcεRI aggregation if the cells were incubated in the presence of serum (38). Although no significant difference in the tyrosine phosphorylation of FcεRIγ wild type (γWT) and the FcεRIγ T60A (γT60A) was observed, a dramatic increase in the tyrosine phosphorylation of the FcεRIγ T52A (γT52A) was found even in nonstimulated cells (Fig. 2B). Phosphorylation of FcεRIβ was not dramatically different among transfectants, although a 30% increase was observed in the FcεRIγ T52A (γT52A) transfectant (data not shown). To determine what effect the mutations of the FcεRIγ had on threonine phosphorylation, we isolated the FcεRIγ from FcεRI-activated transfectants that were labeled with [32P]orthophosphate and phosphoamino acid analysis was performed. As shown in Table I, threonine phosphorylation of FcεRIγ was still present in the T52A mutant. In fact, an increase in the ratio of threonine to tyrosine was observed. In contrast, the T60A mutation resulted in a dramatic decrease of threonine phosphorylation, although low levels of threonine phosphorylation were detected consistent with the expression of low levels of wild type FcεRI in the transfectants derived from γ cells (Fig. 2A). Two-dimensional peptide mapping (using Glu-C V8 protease) of the γWT and γT60A showed a dramatic reduction in the presence of one radiolabeled peptide that co-migrated with the expected peptide standard (Fig. 2C). We did not expect, nor did we find, complete absence of this phosphorylated peptide, given that low levels of wild type FcεRIγ are present in these transfectants (Fig. 2A). From the collective data we conclude that threonine 60, which is found in the Y+2 position of the FcεRIγ ITAM C-terminal YXXL motif, is the sole threonine modified by phosphorylation in these cells.

Effects of FcεRIγ T60A Mutation on Mast Cell Function—We previously reported that the specific PKC inhibitor Ro 31-7549 was able to inhibit both the FcεRI-stimulated threonine phosphorylation of the FcεRIγ and the rate of FcεRI endocytosis (17). We revisited this topic to determine whether the T60A mutation would mimic our previous results. Fig. 3A shows that mutation of T60A, but not T52A, had a significant effect on the rate of FcεRI endocytosis. As much as 50% inhibition of endocytosis was found at early times (5 and 10 min) following FcεRI aggregation. However, at later times (40 and 60 min) no significant difference in FcεRI endocytosis was observed (Fig. 3A). These results were almost identical to those observed in our
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Previous study where the use of the PKC inhibitor, Ro 31-7549, inhibited the initial rate of endocytosis by as much as 80% (17). In contrast, the FcεRI endocytosis of the T52A mutant showed kinetics similar to that of the wild type transfectant, but the overall extent of endocytosis was increased when compared with the wild type transfectant. Because the γT52A has a greater level of tyrosine phosphorylation (Fig. 2B), these results are consistent with the importance of tyrosine phosphorylation to endocytosis of FcεRI-containing receptors (39).

Increased adhesion of RBL cells in response to FcεRI stimulation has been reported (reviewed in Ref. 40). The increased adhesion can be demonstrated by binding of these cells to fibronectin-coated surfaces (41). We analyzed the adhesion based on an integrin-fibronectin interaction, because we could inhibit up to 95% of the FcεRI-stimulated adhesion by the concurrent addition of the competing RGD peptide with the cells to the fibronectin-coated wells (data not shown). We utilized this approach to determine whether the T60A mutation would have an effect on FcεRI-mediated adhesion. Fig. 3B shows the fibronectin-specific adhesion of FcεRI-stimulated γ-, γWT, γT52A, and γT60A cell lines. Results were normalized to the FcεRI-stimulated response of the γ cells where little difference in adhesion was observed when compared with nonstimulated cells. Although reconstitution of the γ cells with FcεRIγ led to increased adhesion (~1.5-fold), no significant difference was observed among the transfectants. This suggested that the FcεRI-stimulated adhesion to fibronectin was unaffected by increased threonine and tyrosine phosphorylation (T52A) or decreased threonine phosphorylation (T60A) of the FcεRIγ.

Similar results were obtained when phorbol ester was used as a stimulus for adhesion (data not shown). Collectively, the results demonstrated that some FcεRI-dependent events were unaffected by mutation of the threonines in FcεRIγ.

We also analyzed the effect of the T60A mutation on the stimulation of TNF-α mRNA production (21, 42). The results shown in Fig. 3C were normalized to the FcεRI-stimulated response of the γ cells. Both the γWT and γT52A transfectants showed an additional increase of TNF-α mRNA levels of up to 50% following FcεRI stimulation when compared with the γ cell response. In contrast, the γT60A transfectant showed no increase in response, and in most experiments TNF-α mRNA levels were below those of the γ cells (Fig. 3C). The reduced response was a consistent observation regardless of the concentration of antigen used for stimulation (data not shown). Thus, the threonine phosphorylation of the FcεRIγ is important to the FcεRI-mediated induction of TNF-α mRNA.

We analyzed the effect of threonine mutation (T60A) of FcεRIγ on degranulation in BMMC derived from FeγR-null mice because the threonine transfectants, like the parental γ cells, secreted poorly, and prior studies showed that reconstitution with wild type FcεRIγ did not induce a degranulation response comparable to the RBL cell line (24). Table II shows that in four independent experiments, reconstitution of FcεRI expression with either γWT or γT60A showed that expression of the latter inhibited degranulation (11–49%). Although all experiments showed inhibition, variable levels of secretion (15 to 38%) and
inhibition (11 to 49%) were observed. This is likely due to the inability to control the levels of receptor assembly and expression among experiments because of transient expression of the FcεRIγ. Regardless, expression of the γT60A showed inhibition of degranulation in all experiments.

The FcεRIγT60A Mutation Inhibits Syk Interaction and Activation—Because FcεRI-mediated endocytosis (43), TNF-α production (44), and degranulation (15) have been demonstrated to be dependent on the activation of Syk, we analyzed Syk activation in FcεRIγ transfectants by measuring its tyrosine phosphorylation, because we previously demonstrated that tyrosine phosphorylation is an accurate measure of Syk activity (16). As shown in Fig. 4A, cells expressing the γT60A showed a significant defect in the activation of Syk. In contrast, both γWT and γT52A expressing cells effectively activated Syk with the latter showing increased Syk phosphorylation consistent with the increased levels of tyrosine phosphorylated receptors in this transfectant (Fig. 2B). In some experiments up to a 3-fold increase in Syk activation was observed in the γT52A expressing cells when compared with the wild type control, whereas a 60–90% inhibition of Syk activation was observed in the γT60A transfectants (Figs. 4A and 5). However, the observed inhibition did not result from a generalized defect in FcεRI signal transduction because, as shown in Fig. 4B, the tyrosine phosphorylation of PKCδ in response to FcεRI stimulation was unaffected by either the γT52A or γT60A mutations. Because tyrosine phosphorylation of PKCδ requires its translocation to the plasma membrane (45, 46), these results demonstrated that PKCδ translocation is independent of threonine phosphorylation of FcεRIγ. Thus, threonine phosphorylation of the FcεRIγ is primarily important for the activation of Syk and

| Experiment | Inhibition of stimulated degranulation [%] |
|------------|-------------------------------------------|
| 1          | 48.8                                      |
| 2          | 20.6                                      |
| 3          | 10.8                                      |
| 4          | 32.5                                      |

a Percentage of inhibition was calculated from net values of γWT and γT60A.

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| TABLE II | Mutation of threonine 60 of FcεRIγ inhibits mast cell degranulation |
|----------|---------------------------------------------------------------------|
| FcεR-null BMMC were transfected with γWT or γT60A constructs by infection with Semliki Forest virus as described under “Experimental Procedures.” Four independent experiments from two independent cultures of BMMC are shown. Cells were stimulated with 20 ng/ml DNP-HSA for 10 min at 37 °C. Percentage of stimulated degranulation was calculated from the level of hexosaminidase detected in the medium, and the total hexosaminidase of cells expressing FcεRI (as determined by FITC-IgE binding and FACS analysis). This was corrected for the levels detected in nonstimulated samples. For the γWT percent net degranulation ranged from 15 to 38%. |
downstream effector functions.

We explored the possible mechanism by which the γT60A mutation inhibited the activation of Syk. Because Syk activation in mast cells was demonstrated to be dependent on its interaction with the activated FcεRI (47, 48), we investigated whether the γT52A or the γT60A mutations had any effect on the interaction of FcεRIγ with Syk in activated cells. In this series of experiments we immunodepleted Syk from chemically cross-linked (DTSSP) nonactivated and activated cell lysates and determined whether FcεRIγ co-immunoprecipitated with immunoblotting. This approach was used because after chemical cross-linking the antibody to FcεRIγ was not as efficient in immunoprecipitation as the antibody to Syk. As demonstrated in Fig. 5, immunoprecipitation of Syk from chemically cross-linked cell lysates showed that in cells expressing γT60A Syk activation was inhibited, whereas those expressing γT52A showed increased Syk activation. When immunoblots were probed for co-immunoprecipitated FcεRIγ its presence was detected following FcεRI stimulation in both the γWT and γT52A transfectants with the latter showing greater levels of FcεRIγ. In addition, in most experiments we could detect trace amounts of phosphorylated Syk and FcεRIγ in association with Syk in nonstimulated γT52A transfectants (Figs. 4A and 5). In contrast, only minimal amounts of FcεRIγ were found to co-immunoprecipitate with Syk isolated from the activated γT60A transfectants (Figs. 5 and 6A). Thus, the results suggested that the absence of threonine phosphorylation of the FcεRIγ in the γT60A transfectants inhibited FcεRI interaction with Syk.

**A Negative Charge at FcεRIγ Threonine 60 Is Required for High Affinity Syk Interaction, Efficient Syk Activation, and the TNF-α mRNA Response**—We investigated the possible requirement for phosphorylation at threonine 60 in the interaction and activation of Syk. Sequence analysis of receptor ITAMs that interact with Syk revealed the presence of an amino acid residue, at the equivalent location in the ITAMs, that could be phosphorylated or was negatively charged (Fig. 6A). We postulated that a negative charge at threonine 60 would be the equivalent of phosphorylation of threonine and would result in complete activation of Syk in response to FcεRI engagement. To test this hypothesis a γT60D transfectant was generated that contained a hybrid ITAM (YTGLX−YEDL; throughout, dashes in sequences represent introduced gaps) with features of both the B cell receptor Igα/β (YEGLX−YEDI) and the FcγR (YTGLX−YETL). As shown in Fig. 6A, the γT60D transfectant showed normal levels of Syk activation, and the association of FcεRIγ with Syk was comparable with that of the γWT transfectant. In these chemical cross-linking experiments we used 6-fold more cells (3.0 × 10⁶) than in the experiments shown in Fig. 5. Thus, we could detect FcεRIγ associated with Syk in nonstimulated cells (Fig. 6A), and in darker exposures of the shown phosphorytrosine blot Syk phosphorylation was also detected (data not shown). These results clearly demonstrated that mutation of threonine 60 to a negatively charged aspartic acid, rather than to the more physiochemically related alanine, completely reconstituted FcεRIγ interaction with Syk leading to the activation of the latter. Thus, we would expect that the γT60D transfectant would show a TNF-α mRNA response similar to that of the γWT transfectant. Fig. 6B shows that the mutation T60D reconstituted the TNF-α mRNA response to levels comparable with that of wild type FcεRIγ. This demonstrates the requirement of a negative charge at threonine 60 for high affinity interaction of Syk with the FcεRIγ ITAM and activation of Syk to levels that result in "normal" mast cell responses.

**DISCUSSION**

In this study, we present direct evidence identifying the site of threonine phosphorylation on the FcεRIγ. Specifically, results from both our in vitro and in vivo experiments demonstrated that threonine 60 is the site of phosphorylation on the FcεRIγ and that phosphorylation at threonine 60 is important for complete activation of Syk. In addition, our findings provide evidence of a general mechanism for Syk activation because we found that a negative charge at the equivalent position of threonine 60 substitutes for threonine phosphorylation at this site and is in common to all ITAMs interacting with and activating Syk (Fig. 6A). This requirement is likely to extend to other cells like platelets, where collagen activation of Syk requires the complexing of glycoprotein VI (collagen receptor) with FeRγ (49, 50).
**A** ITAMs that interact with Syk

| Syk ITAMs | FcR ITAMs |
|-----------|-----------|
| FcRγ | ADIASREKDAM--YTLG-NTRQOE-YEYL-KHE |
| rFcRIγ | AAASREKADAV--YTLG-NTXSQT-YEYL-KHE |
| m/cIt | AATSYEKDOV--YTLG-STRQNE-YEYL-KHE |
| hFcRIγ | AATSYEKDOV--YTLG-STRQNE-YEYL-KHE |

**B** IP: Anti-Syk

Agn: - - - + + + IB:

PY

\(\gamma^{wt} \gamma^{Y60A} \gamma^{T60A} \gamma^{T60D} \gamma^{Y60A} \gamma^{T60D} \)

**FIG. 6.** Mutation of FcεRIγ threonine 60 to aspartic acid reconstitutes Syk interaction, Syk activation, and the TNF-α mRNA response. A, sequence alignment of ITAMs that interact with Syk (dashes represent introduced gaps), introduced mutations, and reconstitution of Syk activation and interaction with FcεRIγ in the γT60D mutant. IgE-sensitized FcεRIγWT, γT60A, and γT60D transfectants (3.0 x 10⁶ cells) were stimulated (+) or not (−) with 300 ng of DNP-HSA (Agn). Cells were lysed, and postnuclear lysates were treated with DTSSP. Syk was immunoprecipitated (IP: Anti-Syk), and resolved proteins were identified as Syk and FcεRIγ by immunoblotting (IB). Because of the cell numbers used, we could detect the presence of FcεRIγ with Syk in nonstimulated cells. As compared with Fig. 5, only a short exposure to film was required for detection. PY, phosphotyrosine. B, FcεRIγT60D reconstitutes the TNF-α mRNA response to the level of wild type FcεRIγ. TNF-α mRNA assay is described under “Experimental Procedures.” The PCR amplified products were resolved on agarose gels and normalized to the internal standard (see “Experimental Procedures”). Quantitation was by densitometry. The TNF-α mRNA response of the FcεRIγT60A was normalized to 1.0 to allow comparison between experiments. Data are from two experiments.

Pribluda and colleagues (37) used HPLC and peptide standards to identify products of protease reactions with in vivo labeled FcεRIγ. The evidence presented suggested that threonine 52 was a site of phosphorylation. In our hands, mutation of threonine 52 to alanine resulted in a “gain of function” because more FcεRIγ was tyrosine phosphorylated, and high levels of FcεRIγ phosphorylation occurred in nonstimulated cells. This resulted in increased endocytosis (Fig. 3A), and in several experiments higher levels of nonstimulated degranulation were observed (data not shown). Furthermore, an increased ratio of threonine to tyrosine phosphorylation was observed in the γT52A transfectants (Table 1), clearly demonstrating that a site for threonine phosphorylation was still present. A possible explanation for the observed increased phosphorylation is that a conformational change of the FcεRIγ is induced by the T52A mutation that allows receptor-associated Lyn and PKCδ to phosphorylate both cis- and trans-molecularly (51). In contrast, mutation of threonine 60 led to a “loss of function” and ablated threonine phosphorylation with no effect on tyrosine phosphorylation. The importance of in vivo phosphorylation of threonine 60 was demonstrated by the finding that substitution of a negatively charged amino acid provided full functionality in Syk activation.

It has been reported that ITAMs are not functionally redundant and that specificity of binding to SH2 domains is based on the residues surrounding the tyrosines (52). Furthermore, tandem SH2 domains showed an increased binding specificity to their biologically relevant ITAM such that another ITAM can bind a tandem SH2 domain but with up to 10,000-fold weaker affinity (53). Because the linker region (where threonine 52 resides) between paired YXXL motifs does not significantly contribute to the binding affinity of the Syk SH2 domain tandem with the FcεRIγ ITAM (54), this suggested that residues in the paired YXXL motifs might contribute to the high affinity interaction and specificity. Binding of both Syk SH2 domains to the FcεRIγ ITAM is required for high affinity interaction (54, 55), and the N-terminal SH2 domain of Syk binds the γTAM C-terminal phosphotyrosine (54). Our findings that the threonine 60 localized in the C-terminal YETL motif was solely phosphorylated in response to FcεRIγ stimulation prompted us to look at the effects of its mutation (T60A) on Syk activation and downstream events. The loss of Syk binding with the FcεRIγT60A ITAM and its recovery with the FcεRIγT60D ITAM demonstrated the importance of threonine phosphorylation in the FcεRIγ ITAM for high affinity interaction. Because the T60D mutation (YED60L) mimics the dual negative charge that would result from phosphorylation of Thr⁶⁰ in the YETL motif, this suggests that a dual negative charge is important for the high affinity interaction of Syk. This dual negative charge pre-exists in the equivalent motif of the B cell receptor Igα/β (YEDD).

The crystal structures of both the C-terminal Syk SH2 by itself and the tandem SH2 domains complexed with phosphotyrosine ITAM peptides are available (56, 57). However, the information provided by resolving the N-terminal SH2 domain interaction with a C-terminal phosphotyrosine of the CD3ε ITAM does not likely reflect the interaction of Syk with the B cell receptor Igα/β or with FcεRIγ ITAMs, because binding of the latter two occurs with at least a log fold higher affinity (54, 58). Nevertheless, some insights are gained from these studies with regard to the interaction of a FcεRIγ ITAM with the N-terminal SH2 domain of Syk. First, it appears that the C-terminal Y+2 amino acid residue (the threonine equivalent, but a glycine residue in the CD3ε ITAM) does not make significant contacts with Syk. For the FcεRIγ ITAM, a phosphorylated threonine would likely provide contact with a highly con-
erved histidine that is present in both the N- and C-terminal SH2 domains of Syk and ZAP-70. In addition, provided that the interactions of the FcεRIγ ITAM with Syk might result in a slightly different conformation than that of the CD3ε ITAM, a highly conserved arginine residue (which is also found in the N-terminal SH2 domain of ZAP-70) could also provide potential charge interactions. Thus, these possible interactions may fulfill the requirements for a high affinity interaction. In vitro, threonine phosphorylation does not appear to be a requirement for Syk activation because tyrosine biphosphorylated ITAMs can activate Syk and in vitro measurement of affinities demonstrated a high affinity interaction (54, 55). We also found (in vitro experiments) that the introduction of a phosphorylated threonine in the tyrosine biphosphorylated FcεRIγ ITAM peptide had no effect on the affinity of interaction with the Syk SH2 domain tandem (data not shown). Although at the moment we cannot explain the difference in requirements for in vivo versus in vitro activation of Syk, it is interesting to note that the mutation of T52A also did not affect the affinity of interaction of the tyrosine biphosphorylated ITAM with Syk. However, in vitro this mutation results in a gain of function (including phosphorylation of the FcεRIγ in the absence of its aggregation) presumably from a conformational change that increases phosphorylation of FcεRIγ and thus Syk interaction (Fig. 5). Although to date there is no direct evidence for an in vivo structural conformation of the FcεRIγ cytoplasmic domain, this possibility is presently raised and has been suggested in a prior study (59).

Of physiological interest is the finding that even small amounts of activated Syk are sufficient to drive low levels of mast cell responses. In particular, degranulation was not observed histidine that is present in both the N- and C-terminal SH2 domains of Syk and ZAP-70. In addition, provided that the interactions of the FcεRIγ ITAM with Syk might result in a slightly different conformation than that of the CD3ε ITAM, a highly conserved arginine residue (which is also found in the N-terminal SH2 domain of ZAP-70) could also provide potential charge interactions. Thus, these possible interactions may fulfill the requirements for a high affinity interaction. In vitro, threonine phosphorylation does not appear to be a requirement for Syk activation because tyrosine biphosphorylated ITAMs can activate Syk and in vitro measurement of affinities demonstrated a high affinity interaction (54, 55). We also found (in vitro experiments) that the introduction of a phosphorylated threonine in the tyrosine biphosphorylated FcεRIγ ITAM peptide had no effect on the affinity of interaction with the Syk SH2 domain tandem (data not shown). Although at the moment we cannot explain the difference in requirements for in vivo versus in vitro activation of Syk, it is interesting to note that the mutation of T52A also did not affect the affinity of interaction of the tyrosine biphosphorylated ITAM with Syk. However, in vitro this mutation results in a gain of function (including phosphorylation of the FcεRIγ in the absence of its aggregation) presumably from a conformational change that increases phosphorylation of FcεRIγ and thus Syk interaction (Fig. 5). Although to date there is no direct evidence for an in vivo structural conformation of the FcεRIγ cytoplasmic domain, this possibility is presently raised and has been suggested in a prior study (59).

Of physiological interest is the finding that even small amounts of activated Syk are sufficient to drive low levels of mast cell responses. In particular, degranulation was not observed.
Threonine Phosphorylation of FcεRIγ Enhances Syk Activation

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