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TULA-2 Protein Phosphatase Suppresses Activation of Syk through the GPVI Platelet Receptor for Collagen by Dephosphorylating Tyr(P)^{346}, a Regulatory Site of Syk*

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Protein-tyrosine phosphatase TULA-2 has been shown to regulate receptor signaling in several cell types, including platelets. Platelets are critical for maintaining vascular integrity; this function is mediated by platelet aggregation in response to recognition of the exposed basement membrane collagen by the GPVI receptor, which is non-covalently associated with the signal-transducing FcRγ polypeptide chain. Our previous studies suggested that TULA-2 plays an important role in negatively regulating signaling through GPVI-FcRγ and indicated that the tyrosine-protein kinase Syk is a key target of the regulatory action of TULA-2 in platelets. However, the molecular basis of the down-regulatory effect of TULA-2 on Syk activation via FcRγ remained unclear. In this study, we demonstrate that suppression of Syk activation by TULA-2 is mediated, to a substantial degree, by dephosphorylation of Tyr(P)^{346}, a regulatory site of Syk, which becomes phosphorylated soon after receptor ligation and plays a critical role in initiating the process that yields fully activated Syk. TULA-2 is capable of dephosphorylating Tyr(P)^{346} with high efficiency, thus controlling the overall activation of Syk, but is less efficient in dephosphorylating other regulatory sites of this kinase. Therefore, dephosphorylation of Tyr(P)^{346} may be considered an important "checkpoint" in the regulation of Syk activation process. Putative biological functions of TULA-2-mediated dephosphorylation of Tyr(P)^{346} may include deactivation of receptor-activated Syk or suppression of Syk activation by suboptimal stimulation.

Proteins of the two-member UBASH3/STS/TULA family featuring the histidine phosphatase domain in their structure act as regulators of signaling and other cellular processes in several experimental systems (1–11) (reviewed in Refs. 12–14). TULA-2, also termed p70, STS-1, or UBASH3B, has been shown to be an active protein-tyrosine phosphatase (PTP)[2] (15–17) capable of suppressing receptor signaling in T lymphocytes (1, 7, 9, 15), mast cells (18), osteoclasts (8), and platelets (6, 19, 20).

Platelets play a key role in maintaining vascular integrity. Platelets circulate in the bloodstream in a quiescent discoid shape until they interact with collagen, an essential part of the extracellular matrix, which becomes exposed upon blood vessel injury. This interaction triggers signaling, which activates platelets and causes their aggregation, thus developing a plug to arrest bleeding (21, 22). However, this trigger-like response can also have deleterious consequences if left unchecked. Therefore, negative regulation of platelet signaling is critical for maintaining a balance between hemostasis and thrombosis (23).

Our previous studies suggested that TULA-2 plays an important role in negatively regulating platelet signaling through GPVI, a receptor for collagen non-covalently associated with FcεR1γ (denoted FcRγ throughout), and FcγRIIA, an IgG Fc receptor, both of which transmit signals through the immunoreceptor tyrosine-based activation motifs (ITAM) (6, 19). Thus, the lack of TULA-2 or a decrease in its level correlates with the significant facilitation of platelet activation, including phosphorylation of multiple proteins involved in ITAM-triggered receptor signaling and Ca^{2+} flux as well as platelet aggregation and ATP secretion (6, 19, 20). Furthermore, animal model data indicate that the lack of TULA-2 or modulation of its level exerts a significant effect on *in vivo* functions that are dependent on platelet activation. Thus, the lack of TULA-2 greatly enhances artery occlusion caused by the chemically induced damage of the endothelial layer, a process initiated by the exposure of basement membrane collagen to circulating platelets (6). In agreement with this finding, up-regulation of TULA-2

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[2] The abbreviations used are: PTP, protein-tyrosine phosphatase; EGFP, enhanced green fluorescent protein; ITAM, immunoreceptor tyrosine-based activation motif(s); PTK, protein tyrosine kinase; CVX, convulxin; CRP, collagen-related peptide; SH2, Src homology 2; PRP, platelet-rich plasma; MBP, maltose-binding protein; PEI, polyethyleneimine; ANOVA, analysis of variance.

protects FcγRIIA-transgenic mice against thrombosis induced by platelet activation through FcγRIIA (19). Finally, an inverse correlation exists between TULA-2 expression and platelet reactivity in a population of normal donors; high expression of TULA-2 correlates with low platelet aggregation (19). Therefore, TULA-2 appears to act as a down-regulator of receptor signaling, which exerts effects on platelet biological responses both *in vitro* and *in vivo*.

Furthermore, platelets represent an excellent system for studying the functions of TULA-2. Although expressed ubiquitously (24), TULA-2 is present in platelets at a level greatly exceeding that in other cells, whereas the second protein of this family, TULA, is not detectable (6). Thus, the effects of TULA-2 in platelets are profound and not masked by the effects of TULA.

Our previous studies with purified proteins and GPVI-activated platelets indicated that TULA-2 reduces phosphorylation of Tyr^{519}-Tyr^{520} (the numbers of amino acid residues throughout this work correspond to the long isoform of mouse Syk) (6, 17, 20), the site located in the activation loop of Syk protein-tyrosine kinase (PTK). This site is known to be critical for Syk signaling functions and to a large extent for its *in vitro* kinase activity and hence is used as an activation marker of this PTK (25–28). Considering that Syk is a key PTK of the ITAM-triggered signaling in platelets (22, 29–31), our results were consistent with the idea that TULA-2 down-regulates platelet signaling and responses by suppressing the kinase activity of Syk. However, our data on the TULA-2 enzymatic specificity indicated that Syk Tyr(P)^{519}-Tyr(P)^{520} lacks the substrate specificity determinants and, thus, is a very poor target for TULA-2 (20).

Therefore, the molecular basis of the down-regulatory effect of TULA-2 on platelet activation remained to be elucidated. We hypothesized that the suppressive effect of TULA-2 on GPVI signaling is mediated primarily by down-regulation of Syk activity as a result of dephosphorylation of the regulatory phosphotyrosine sites of Syk. The Tyr(P)^{346} site of Syk was considered a possible candidate for this role for two major reasons. First, our data indicate that among the substrate sites for TULA-2 found previously in cellular proteins, Syk Tyr(P)^{346} is dephosphorylated by TULA-2 with the highest kinetic efficiency k_{cat}/K_m (20). Second, Syk Tyr(P)^{346} has been shown together with Tyr(P)^{342} to play an important role in the activation of Syk in several cell types; the relative importance of the two Tyr(P) sites varies depending on the experimental system used, but both of these sites are clearly involved in the regulation of Syk activity (32–37). Structure-function studies supported the notion that Tyr(P)^{342} and Tyr(P)^{346} act by preventing Syk and Zap-70, a PTK of the same family, from folding into the autoinhibited conformation and that both Tyr^{342} and Tyr^{346} have to be phosphorylated for the kinase domain of Syk to become fully activated (37, 38).

In this study, we elucidated the molecular basis of the down-regulatory effect of TULA-2 on GPVI-mediated signaling. Our results indicate that Tyr(P)^{346} appears to be a Syk Tyr(P) site whose dephosphorylation mediates the down-regulatory effect that TULA-2 exerts on the GPVI-mediated activation of Syk and the entire GPVI signaling pathway.

Results

Our previous study of the role of TULA-2 in the regulation of the GPVI-mediated platelet activation had been carried out using KO mice lacking both TULA and TULA-2 (6). The use of this system was justified by the fact that TULA is not detectable in platelets by immunoblotting (6). This result was consistent with the original observations demonstrating primarily lymphoid expression of TULA (1, 2). However, to rule out the possibility that the effects observed in our experiments were at least partially due to the lack of TULA, which can be present in platelets at a low level, we analyzed the effects of the single TULA and TULA-2 KOs on platelet responses to two strong agonists of the GPVI collagen receptor, convulxin (CVX), a snake venom protein (Fig. 1*A*), and the synthetic collagen-related peptide (CRP) (Fig. 1*B*), both mimicking collagen, which is a natural ligand of GPVI. These experiments showed that only TULA-2 KO, and not TULA KO, affected platelet aggregation and secretion. Effects of TULA-2 KO and TULA KO on GPVI signaling in platelets, which was assessed by phosphorylation of Syk in experiments examining its time course (Fig. 1*C*) or ligand concentration dependence (Fig. 1*D*), were in agreement with their effects on physiological responses; activation-induced Syk-phosphorylation in TULA KO platelets was not significantly different from that in WT platelets, whereas activation-induced Syk phosphorylation in TULA-2 KO platelets was significantly different from that in WT and TULA KO platelets. These results provide evidence that TULA-2 is sufficient for exerting the signaling-suppressive effect previously observed in platelets and argue that TULA does not play an appreciable role in the down-regulation of GPVI signaling in platelets.

We have previously shown that Syk and several other proteins are more phosphorylated in platelets lacking TULA-2 (6, 20). To evaluate the effect of TULA-2 on other proteins involved in GPVI signaling and their multiple phosphotyrosine sites, we stimulated WT and TULA-2 KO platelets with CVX and examined protein phosphorylation using immunoblotting. These experiments indicated that at least three Tyr(P) sites of Syk and Tyr(P) sites of SLP-76, a major biological substrate of Syk; PLCγ2, a key member of the GPVI pathway; and ERK, one of its key downstream targets, are significantly more phosphorylated in response to GPVI ligation in TULA-2 KO platelets than in WT platelets (Fig. 2). This result indicates that the entire GPVI-triggered pathway is affected by TULA-2 and is consistent with the notion that TULA-2 exerts its effect by down-regulating Syk, a key receptor-proximal PTK of this signaling pathway; the lack of TULA-2 would prevent normal down-regulation of Syk, thus elevating phosphorylation levels of all Tyr(P)-containing proteins located in the GPVI-mediated pathway downstream of Syk.

Based on the previous findings that TULA-2 dephosphorylates Syk (14, 17, 18) and Zap-70 (1, 9, 15), a PTK of the same family, and that Syk Tyr(P)^{346} represents an excellent substrate for TULA-2 (20), we hypothesized that Syk is the major target of TULA-2 in platelets. We addressed the issue of specificity of the TULA-2 effect on the GPVI-mediated signaling pathway utilizing [H380C/E490Q]TULA-2, a substrate-trapping mutant of this PTP (39), to pull down proteins bound to this form of

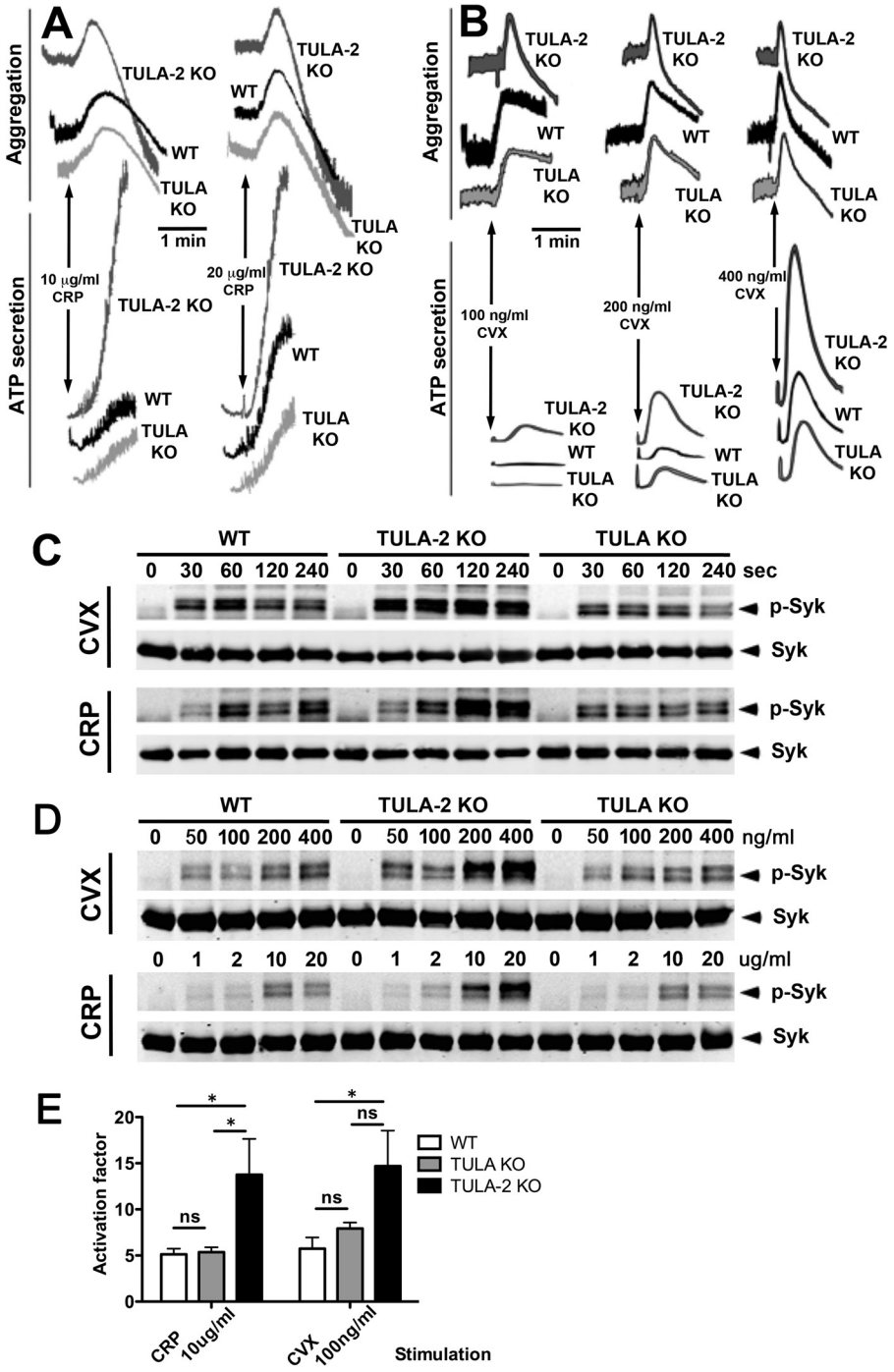


FIGURE 1. **Effect of TULA KO and TULA-2 KO on platelet responses and Syk phosphorylation.** Mouse platelets were stimulated with CRP (*A*, *C*, and *D*) or CVX (*B–D*), and platelet responses (*A* and *B*) or Syk phosphorylation (*C* and *D*) was measured. Traces represent WT, TULA KO, or TULA-2 KO platelets and show aggregation (*top rows* in *A* and *B*) or ATP secretion (*bottom rows* in *A* and *B*). Concentrations of agonists and incubation time in *A* and *B* are indicated. In *C*, CVX and CRP were added to platelets at 100 ng/ml or 10 μg/ml, respectively, for the time indicated. In *D*, CVX and CRP were added to platelets for 120 s at the concentrations indicated. The reaction was stopped by adding perchloric acid, and samples were prepared as described under "Experimental Procedures." Proteins were separated using SDS-PAGE, and immunoblotting was carried out to probe for Tyr(P)^{519}-Tyr(P)^{520} Syk and Syk, as indicated. The positions of protein standards and their molecular masses in kDa are indicated to the *left* of the corresponding *panels*. Representative experiments are shown in *A–D*. *E*, ratios of Syk phosphorylation in the samples of platelets activated with 10 μg/ml CRP or 100 ng/ml CVX for 120 s to the corresponding non-stimulated controls (activation factor) for each type of platelets are plotted as means ± S.E. (*error bars*) ($3 \leq n \leq 4$ for various groups). ANOVA followed by Bonferroni's post-hoc test was used to compare the mean values (*, $p < 0.05$; *ns*, not significant).

TULA-2 from the lysate of CVX-stimulated human platelets, because such binding is considered a key criterion of the PTP substrate identification (40). The precipitated proteins were then analyzed using immunoblotting with the antibodies to several individual Tyr(P) sites that are relevant for GPVI signaling along with the corresponding total proteins. This experiment revealed that only Syk, and not other proteins examined, was found in association with the substrate-trapping TULA-2 (Fig. 3*A*). Proteins pulled down with [H380C/E490Q]TULA-2 were also examined for total tyrosine phosphorylation. These

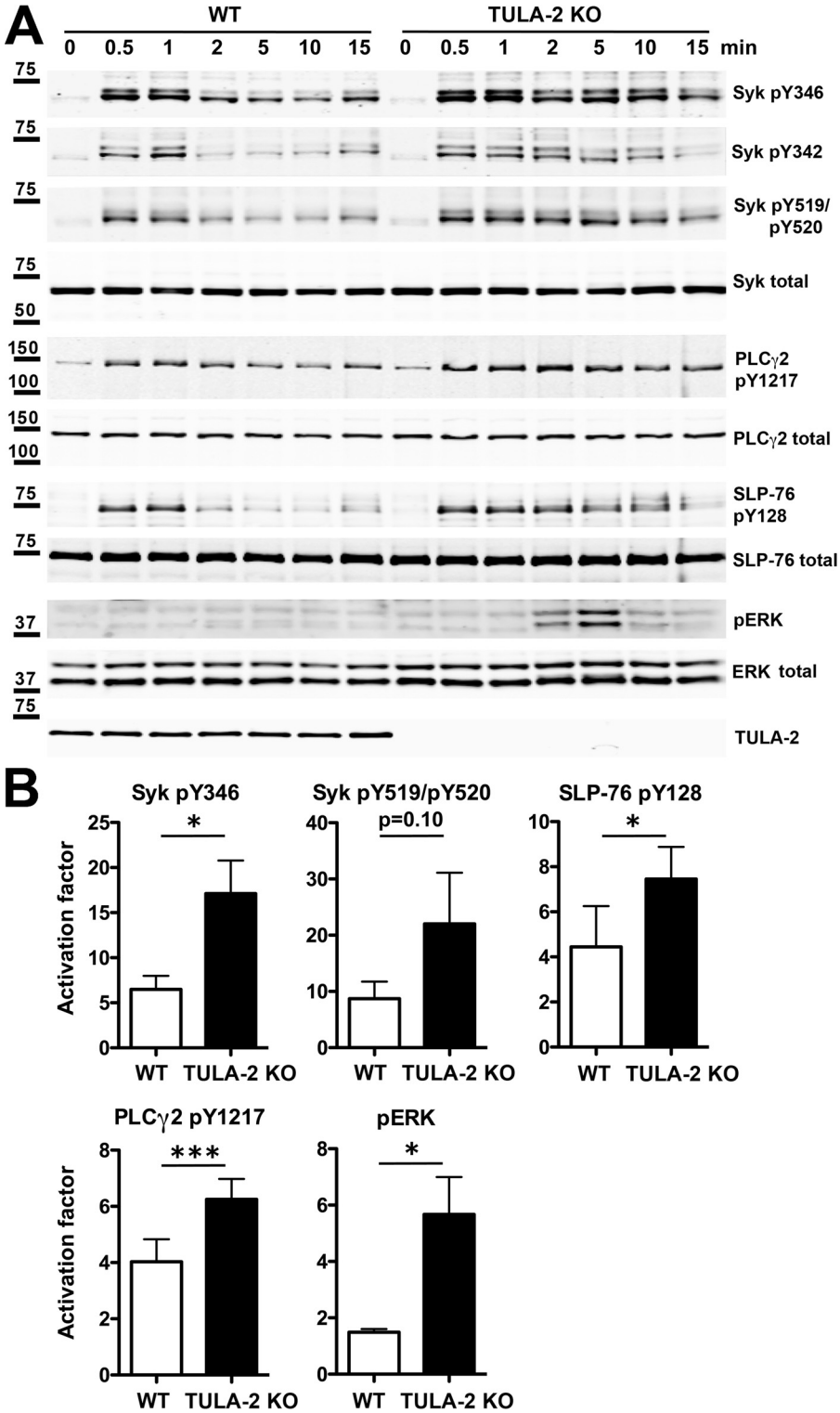


FIGURE 2. **Phosphorylation of signaling proteins in WT and TULA-2 KO platelets.** WT and TULA-2 KO mouse platelets were stimulated with 50 ng/ml CVX for the time indicated, the reaction was stopped, and samples were prepared as described under "Experimental Procedures." Proteins of the obtained samples were separated using SDS-PAGE, and tyrosine phosphorylation of multiple proteins was assessed using immunoblotting with antibodies to specific Tyr(P) sites as shown. A total protein *panel* corresponding to the Tyr(P) site analyzed is located *below* each Tyr(P) *panel*. Immunoblotting of TULA-2 is shown to confirm mouse genotypes. The positions of protein standards and their molecular masses in kDa are indicated to the *left* of the corresponding *panels*. A representative experiment of five independent experiments is shown in *A*. Quantification of the results is shown in *B*. Activation factor (see legend to Fig. 1) is plotted for individual phosphosites in WT and TULA-2 KO platelets as means ± S.E. (*error bars*) ($3 \leq n \leq 5$ for various groups). Paired *t* test was used to compare the mean values for each phosphosite (*, $p < 0.05$; ***, $p < 0.001$; $p = 0.10$ for the difference between Syk Tyr(P)^{519}-Tyr(P)^{520} in WT and TULA-2 KO platelets, albeit not significant, indicates a trend similar to all other phosphosites examined). The differences were analyzed at 2 min post-stimulation for all phosphosites shown except for phospho-ERK (*pERK*), which was analyzed at 5 min post-stimulation.

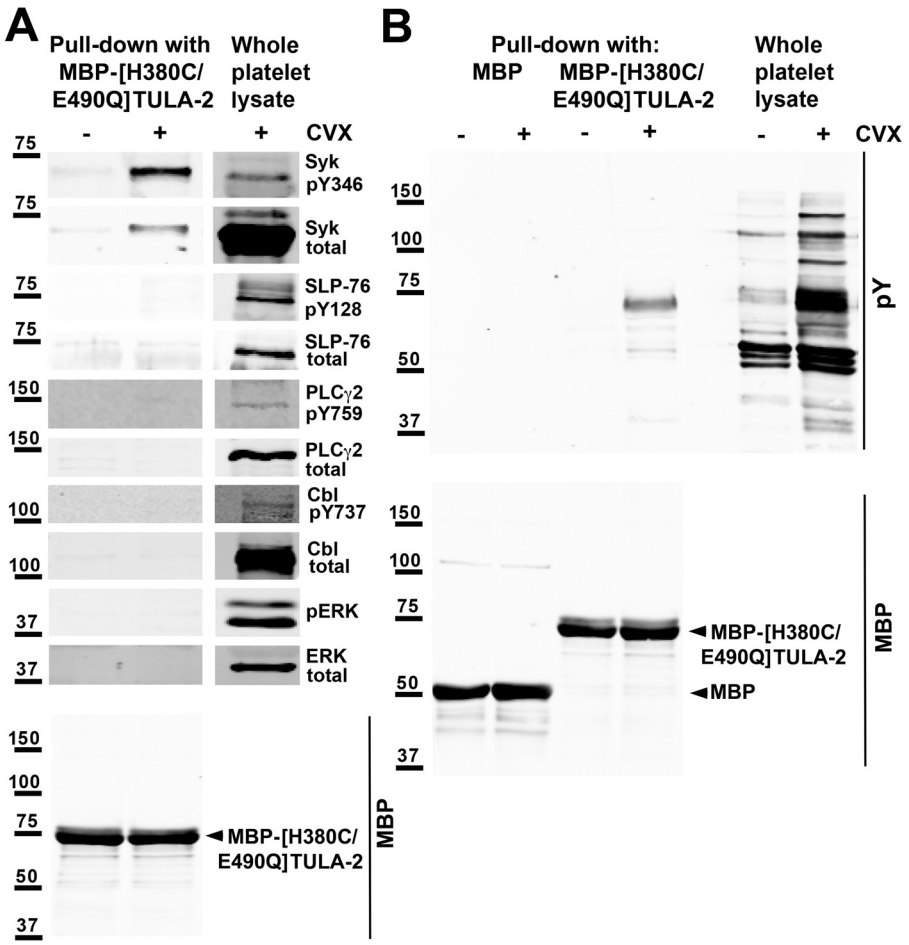


FIGURE 3. **Binding of phosphorylated proteins to substrate-trapping TULA-2.** Human platelets were stimulated with 100 ng/ml CVX for 1 min (+) or left unstimulated (−) and lysed with Nonidet P-40-containing buffer as described under "Experimental Procedures." *A*, proteins bound to the MBP-fused [H380C/E490Q]TULA-2 phosphatase domain incubated with the obtained lysates were separated using SDS-PAGE and probed with specific antibodies to candidate proteins and their individual Tyr(P) sites as indicated. Lysates of stimulated platelets were analyzed in parallel as positive control. The *bottom panel* represents the results of immunoblotting with anti-MBP in the pull-down samples carried out to verify the presence of MBP-[H380C/E490Q]TULA-2 in both samples in equal amounts. The positions of protein standards and their molecular masses in kDa are indicated to the *left* of the corresponding *panels*. A representative experiment of four independent experiments is shown. *B*, proteins were pulled down with the MBP-TULA-2 substrate-trapping reagent and free MBP as negative control, and total protein tyrosine phosphorylation was assessed using anti-Tyr(P) immunoblotting. Whole platelet lysates were analyzed as controls. The presence of MBP was evaluated as outlined in *A*. A representative experiment of three independent experiments is shown.

experiments confirmed that a major species among them corresponded to Syk, whereas the amount of other Tyr(P) proteins binding to the substrate-trapping reagent was very low (Fig. 3*B*). Similar results were obtained using murine platelets (data not shown). Together with the data on TULA-2 substrate specificity, this result argues that Syk is the major substrate of TULA-2 in this system and supports the idea that TULA-2 down-regulates the entire GPVI-triggered signaling pathway by dephosphorylating Syk, a key upstream PTK of this pathway.

The results shown above, together with our previously published data (6, 20), indicate that the lack of TULA-2 increases phosphorylation of the Syk activation loop site Tyr^{519}-Tyr^{520}, which is essential for the function of Syk, thus representing a reliable marker of Syk activity (25–28, 36). Furthermore, we have previously shown that the lack of TULA-2 increases Syk activity *in vitro* (6). The activation of Syk is expected to increase phosphorylation of its direct targets, such as the adaptor protein SLP-76, followed by all downstream elements of the GPVI signaling pathway. An increase in the tyrosine phosphorylation of SLP-76 and other proteins is thought to lead to the assembly of protein-protein complexes that play a key role in GPVI signaling (41). However, Syk phosphorylation/dephosphorylation might affect protein complex formation not only by up-regulating the enzymatic activity of Syk, but by directly enhancing protein-protein interactions of this PTK; thus, binding of Syk to PLCγ1 was shown to be $Tyr(P)^{346}$-dependent (42). To determine the effect of TULA-2 on this complex formation and to assess the mechanism of this effect, we examined the interactions between PLCγ1/2, Syk, and other phosphoproteins in response to GPVI-mediated activation of platelets.

First, we confirmed that Syk binds to the tandem SH2 domains of PLCγ1, SH2(NC), as well as to its SH2(C) domain, but not to the SH2(N) domain in agreement with the data published earlier (42) (data not shown). Also in agreement with Ref. 42, proteins binding to PLCγ1/2 SH2(NC) were greatly enriched with Syk phosphorylated on Tyr^{346}; the fraction of $Tyr(P)^{346}$-phosphorylated Syk pulled down with the reagents used was much higher than the pull-down fraction of total Syk (Fig. 4, *A* and *B*). Notably, binding of human platelet Syk, but not that of SLP-76, to the SH2(NC) of PLCγ2, the major func-

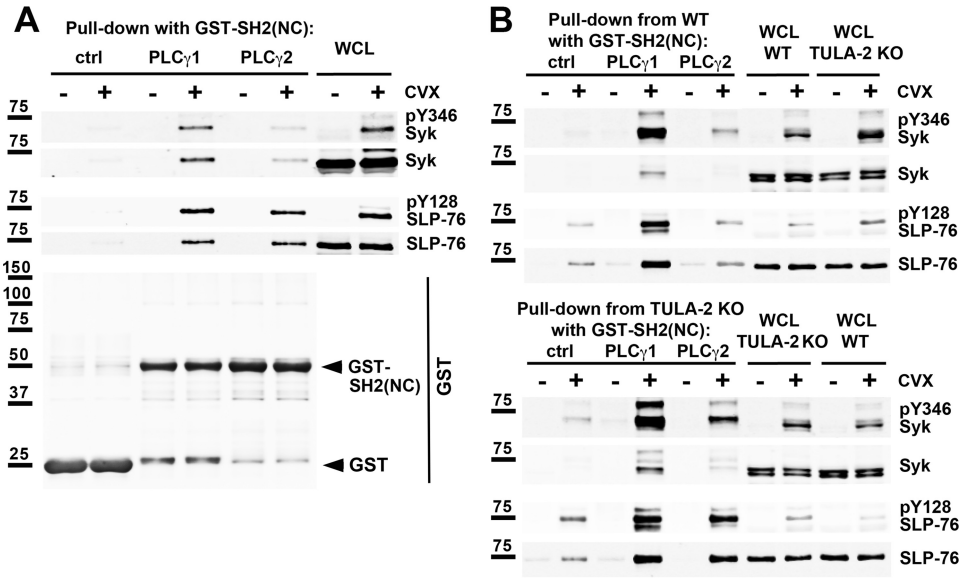


FIGURE 4. **Binding of phosphorylated proteins to the SH2 domains of PLCγ.** Human (*A*) and mouse (*B*) platelets were stimulated with 100 ng/ml CVX for 1 min (+) or left without stimulation (−) and lysed with Nonidet P-40 buffer as described under "Experimental Procedures." The obtained lysates were used to carry out pull-downs with the GST-fused tandem SH2 domains of PLCγ1 or PLCγ2, as indicated. Proteins bound to GST-SH2 domains and whole cell lysates (*WCL*) were analyzed using immunoblotting as described in the legend to Fig. 3. GST and GST-SH2 domains were detected in the same samples using immunoblotting with anti-GST to confirm the presence of equal amounts of pull-down reagents (shown only in *A*). The positions of protein standards and their molecular masses in kDa are indicated to the *left* of the corresponding *panels*. A representative experiment of three independent experiments is shown for each *panel*.

tional form of PLCγ in platelets (43, 44), was much weaker than its binding to the SH2(NC) of PLCγ1 (Fig. 4*A*). When we examined binding of Syk and SLP-76 from the lysates of CVX-treated WT and TULA-2 KO platelets to PLCγ SH2(NC), we found that TULA-2 KO facilitates binding of both Syk and SLP-76 to SH2(NC) of both PLCγ1 and PLCγ2 isoforms (Fig. 4*B*). However, the binding of SLP-76 to PLCγ2 SH2(NC) was increased by TULA-2 KO approximately to the level of its binding to PLCγ1 SH2(NC), whereas Syk binds to PLCγ2 SH2(NC) much more weakly than to PLCγ1 SH2(NC) even in TULA-2 KO platelet lysates (Fig. 4*B*). Overall, TULA-2 KO did facilitate complex formation between proteins involved in GPVI signaling, but its effect on the binding of Syk to PLCγ2 SH2(NC) was much lower than its effect on the binding of SLP-76 to PLCγ2 SH2(NC). This finding does not support the idea that the effect of TULA-2 KO is caused primarily by the direct enhancement of interactions between Syk and other members of signaling complexes and is more consistent with the idea that interactions of the proteins involved in GPVI signal transduction are facilitated as a consequence of a general increase in their tyrosine phosphorylation.

As stated above, our results suggested that TULA-2 regulates Syk by targeting the Tyr(P) site(s) critically important for the activity of Syk and that Tyr(P)^{346} is a very likely candidate for being such a site. To assess the possibility of Tyr(P)^{346} and Tyr(P)^{342} acting as the regulatory sites of Syk in platelets, we examined the dynamics of phosphorylation of Tyr^{342}, Tyr^{346}, and Tyr^{519}-Tyr^{520} in response to GPVI ligation in order to determine whether or not phosphorylation of Tyr^{346} and Tyr^{342} precedes that of Tyr^{519}-Tyr^{520}, which is the minimal requirement for Tyr(P)^{342} and Tyr(P)^{346} being critical for the activation of Syk.

Under conditions typically used in such experiments, all of the sites of Syk examined show detectable phosphorylation already at very early time points. To better resolve time courses of phosphorylation for the Tyr^{342}, Tyr^{346}, and Tyr^{519}-Tyr^{520} sites of Syk, we carried out activation of platelets at reduced temperatures; previously, this approach has been successfully used for assessing tyrosine phosphorylation of Zap-70, a Syk family PTK, and its substrates, including SLP-76 and c-Cbl, in T cells at very early time points of receptor-mediated activation (45). The results obtained at 4 °C indicated that 30 s after GPVI ligation, activation-dependent phosphorylation of the Tyr(P) sites examined demonstrates the order, Tyr(P)^{346} > Tyr(P)^{342} ≥ Tyr(P)^{519}-Tyr(P)^{520} (Fig. 5), although the observed difference was statistically insignificant (Fig. 5*B*). An activation-induced increase in Tyr(P)^{346} grew to become significantly higher than that for Tyr(P)^{342} and Tyr(P)^{519}-Tyr(P)^{520} at 60 s postactivation, whereas the difference between the latter two sites did not gain statistical significance (Fig. 5*B*). Notably, the time points examined in these experiments correspond to an activation time of several seconds at +37 °C, a temperature typical for our experiments. Overall, these results indicate that among the Syk Tyr(P) sites examined, Tyr(P)^{346} is the earliest one formed in response to GPVI ligation.

The experiments with mouse platelets demonstrated similar dependence (data not shown). These results are consistent with the notion that Tyr(P)^{342} and Tyr(P)^{346} may play a regulatory role in the activation of Syk.

Expression of the Syk mutant forms incapable of phosphorylation at the relevant sites is potentially a powerful tool for the assessment of the role of individual Tyr(P) sites in the functions of Syk, but the transfection-based approach cannot be used in platelets. Hence, a model system has been developed for these

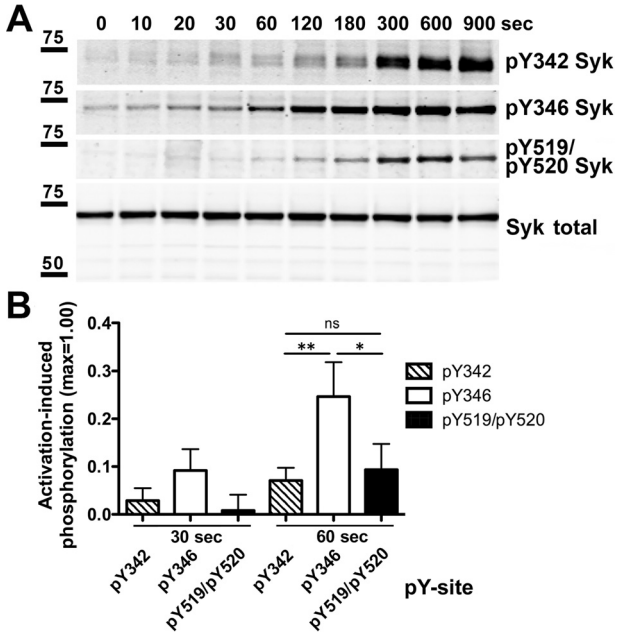


FIGURE 5. **Phosphorylation of Syk at early time points.** *A*, human platelets were stimulated with 100 ng/ml CVX at 4 °C for the time indicated. Reactions were terminated with perchloric acid, and samples were prepared as described under "Experimental Procedures." Sample proteins were separated using SDS-PAGE, and Syk and its individual Tyr(P) sites were immunoblotted as described in the legend to Fig. 2. A representative experiment of five independent experiments is shown. Quantification of the results is shown in *B*. Activation-induced phosphorylation is a difference between phosphorylation of an individual site at the indicated time postactivation and at $t = 0$ s divided by its phosphorylation maximum. The latter occurred under these conditions at ~5 min postactivation for all Tyr(P) sites. The results are plotted as means ± S.E. (*error bars*) ($n = 5$). Repeated measures ANOVA followed by Bonferroni's post-hoc test was used to compare the mean values (*, $p < 0.05$; **, $p < 0.01$; *ns*, not significant). No significant difference between Tyr(P) sites was observed at 30 s postactivation.

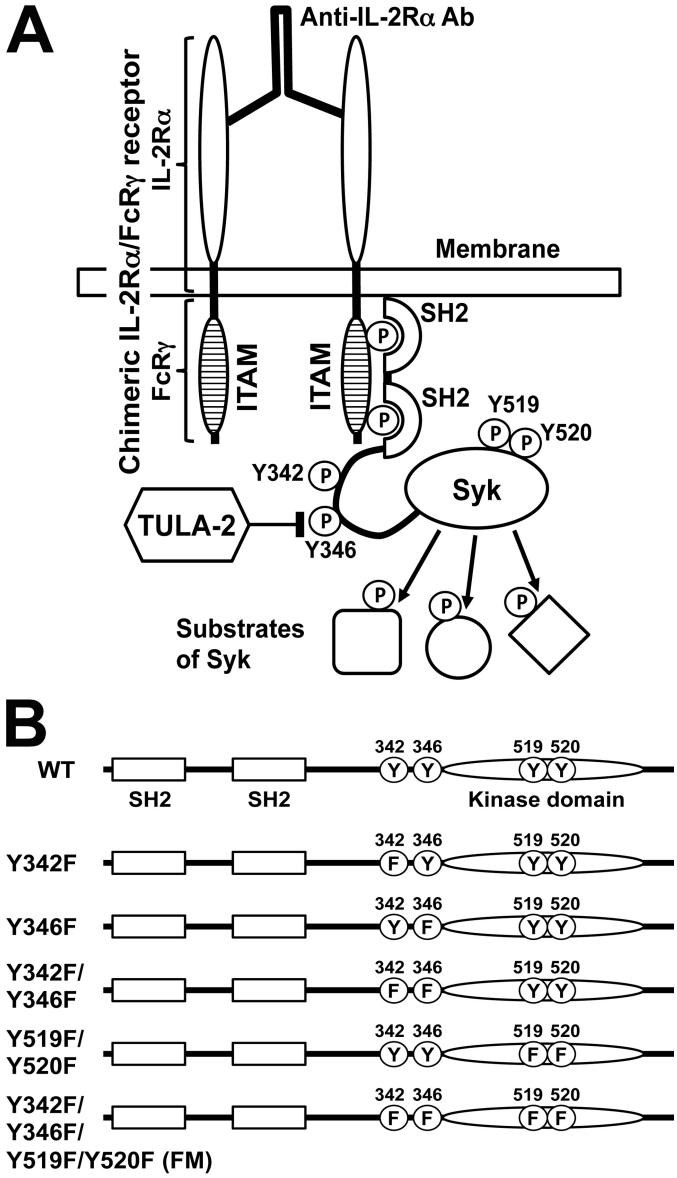


FIGURE 6. **HEK293T experimental system.** *A*, cells are transfected to express the IL-2Rα/FcRγ chimeric receptor, Syk (WT or mutant), and TULA-2. Empty vector is used for transfections when required as negative control. Cross-linking of the receptor with an anti-IL-2Rα antibody triggers signaling similar to that mediated by the Fcγ-chain in platelets. Phosphorylation of Syk and total protein tyrosine phosphorylation are used to assess Syk function. Comparison of Tyr → Phe Syk mutants with WT Syk is used to identify Syk Tyr(P) sites targeted by TULA-2 and evaluate their role in Syk regulation. *B*, the Syk mutants employed in this study are shown. All forms of Syk are fused to EGFP via their C termini (omitted here).

studies; the chimeric IL-2Rα/FcRγ receptor has been made, and HEK293T cells were transfected to express it along with various Syk mutants (Fig. 6). In this system, we could activate Syk through the ITAM-containing cytosolic tail identical to that of the GPVI·FcRγ receptor complex upon cross-linking of the chimeric receptor with an anti-IL-2Rα antibody (Fig. 6*A*). Phosphorylation of recombinant Syk caused by this cross-linking was dependent on the presence of the FcRγ chain sequence in the receptor, and the total tyrosine phosphorylation of cellular proteins induced by the cross-linking was dependent on the presence of recombinant Syk. Notably, we detected only recombinant Syk fused to enhanced green fluorescent protein (EGFP) and its phosphorylation in these experiments, whereas endogenous Syk or its phosphorylation remained undetectable (data not shown).

To evaluate the effects of Tyr^{342} and Tyr^{346} on the activation of Syk in this system, we first expressed only Syk, WT or mutant, and the chimeric receptor. Cells were activated with anti-IL-2Rα, and the phosphorylation of recombinant Syk on Tyr^{342}, Tyr^{346}, and Tyr^{519}-Tyr^{520} was examined. These experiments indicated that phosphorylation of Tyr^{519}-Tyr^{520}, a marker of Syk activity, is significantly, yet only partially, inhibited by a single mutation of either Tyr^{342} or Tyr^{346} but is fully blocked by the Y342F/Y346F double mutation (Fig. 7). Total tyrosine phosphorylation of cellular proteins, another readout for the activity of Syk in this system, was consistent with the level of Syk Tyr(P)^{519}-Tyr(P)^{520} (data not shown).

These results support the notion that both Tyr^{342} and Tyr^{346} play a critical role in Syk activation; at least one of these two sites should be phosphorylated to support the activity of Syk. Furthermore, our results show that a Tyr → Phe mutation of either the Tyr^{342} or Tyr^{346} site, rendering the mutant incapable of being phosphorylated, negatively affects phosphorylation of the second site, thus suggesting that phosphorylated Tyr^{342} and Tyr^{346} promote phosphorylation of a neighboring site, whereas non-phosphorylated ones do not support phosphorylation of the neighbor. It should be noted, however, that the Y342F

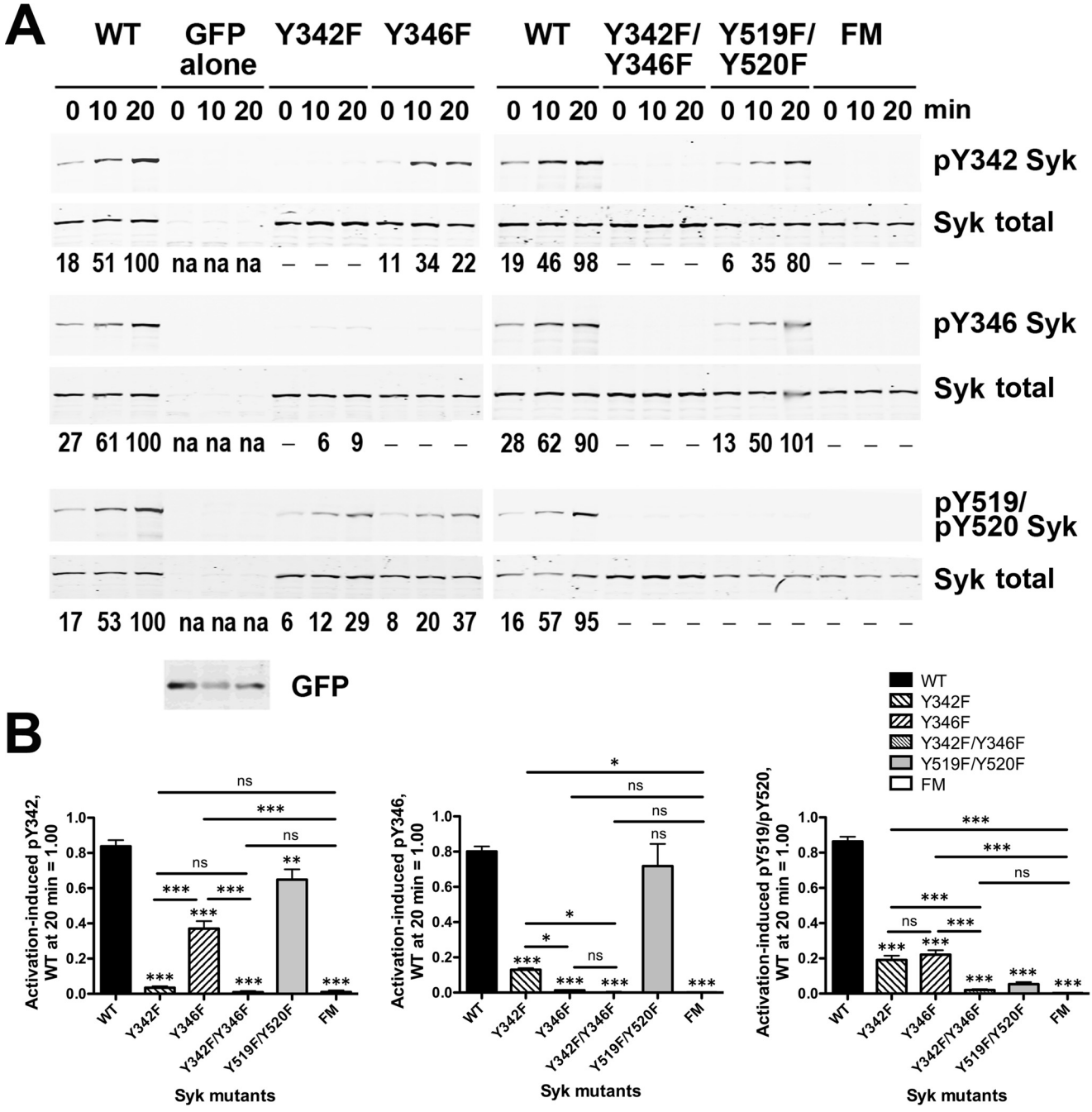


FIGURE 7. **Effects of Syk Tyr → Phe mutations on its activity.** HEK293T cells transfected to co-express Syk-EGFP and the IL-2Rα/Fcγ receptor were stimulated with rabbit anti-IL-2Rα followed by anti-rabbit IgG for the time indicated and lysed with Nonidet P-40-containing TNE buffer as described under "Experimental Procedures." An individual representative experiment is shown in *A*. Syk mutations are indicated at the *top* of each *panel*; full mutant (*FM*) is Syk with all of the sites examined mutated (Y342F/Y346F/Y519F/Y520F). Phosphorylation of Tyr^{519}-Tyr^{520}, Tyr^{342}, and Tyr^{346} was assessed using immunoblotting with the respective antibodies and normalized to total Syk (each *panel* showing an individual Tyr(P) site is paired with a corresponding total Syk *panel below*). Phosphorylation levels of the bands are shown *below* the respective total Syk *panels*. –, values not exceeding 5%. Samples of WT Syk-transfected cells were loaded on both membranes to confirm the reproducibility of visualization. Phosphorylation of each Tyr(P) site in WT Syk at 20 min on the first membrane is assigned a value of 100%. *NA*, not applicable; only EGFP was present in the cells transfected with an empty vector, and the presence of EGFP was confirmed by probing with anti-GFP (*bottom*). *B*, activation-induced phosphorylation levels for each Tyr(P) site examined were defined as a difference of its phosphorylation level at 20 min postactivation and in unstimulated cells. The activation-induced phosphorylation of each Tyr(P) site was determined for all mutant forms of Syk expressed and is plotted as the mean ± S.E. (*error bars*) ($4 \le n \le 10$ for various groups). ANOVA followed by Bonferroni's post-hoc test was used to compare the mean values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant). *Symbols* shown at the *top* of each *bar* of the graph indicate a difference between the corresponding mutant and WT. The *symbols* indicating differences between various mutant forms containing Y342F and/or Y346F are shown *above* the *lines* connecting the corresponding *bars*.

mutation suppresses the phosphorylation of Tyr^{346} much more profoundly than Y346F inhibits the phosphorylation of Tyr^{342} (Fig. 7).

In contrast, phosphorylation of Tyr^{342} and Tyr^{346} remained mostly unaffected in [Y519F/Y520F]Syk, a mutant lacking the activation loop Tyr(P) site (Fig. 7). This result supports the idea

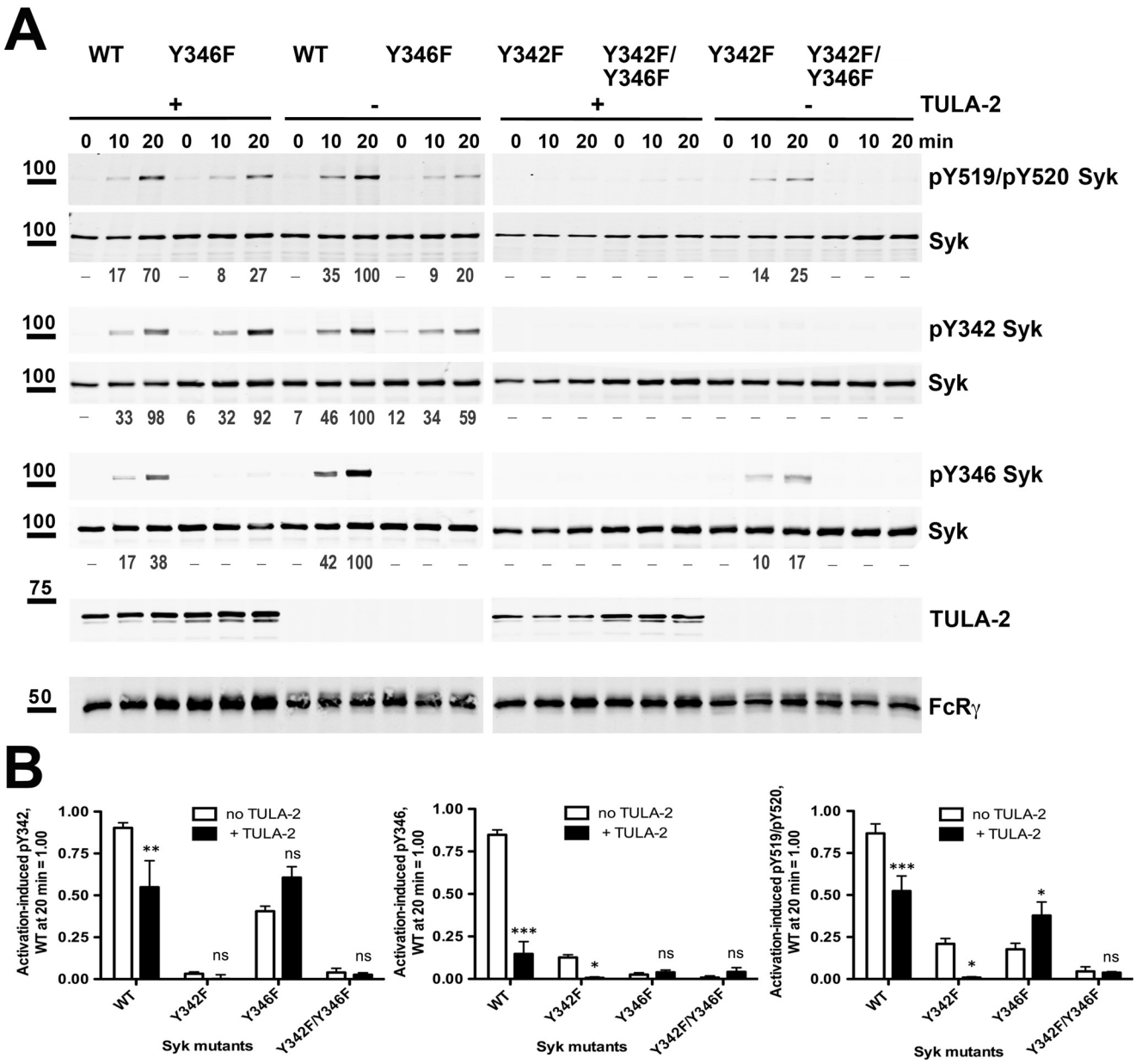


FIGURE 8. **Effect of TULA-2 on activity of WT and mutant Syk.** The experiment was carried out as described in Fig. 7, except that along with the chimeric receptor and Syk (WT and Tyr^{342} and/or Tyr^{346} mutants), cells were co-transfected with a WT TULA-2 expression plasmid (+) or the matching empty vector (−). Cells were activated and lysed, and the lysates were analyzed as described in the legend to Fig. 7. *A*, *panels* showing phosphorylation of the sites examined are paired with the corresponding total Syk *panels*. Phosphorylation levels are indicated *below* the corresponding total Syk *panels*. The level corresponding to WT Syk at 20 min for each Tyr(P) site is assigned a value of 100%. −, values not exceeding 5%. Immunoblots of FcRγ and TULA-2 are shown to demonstrate their expression in the appropriate samples. An individual representative experiment is shown. *B*, activation-induced phosphorylation levels for each Tyr(P) site examined were defined as indicated in the legend to Fig. 7. The activation-induced phosphorylation of each Tyr(P) site was determined for all mutant forms of Syk expressed and is plotted as the mean ± S.E. (*error bars*) ($4 \leq n \leq 6$ for various groups). ANOVA followed by Bonferroni's post-hoc test was used to compare the mean values for each mutant with and without TULA-2 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant).

that $Tyr(P)^{342}$ and $Tyr(P)^{346}$ regulate phosphorylation of Tyr^{519}-Tyr^{520}, which is the Tyr(P) site that needs to be formed to fully activate Syk. In contrast, $Tyr(P)^{519}$-$Tyr(P)^{520}$ does not substantially regulate phosphorylation of sites located upstream of it in the Syk activation sequence. A significant but very modest change observed for $Tyr(P)^{342}$ (Fig. 7*B*) probably reflects an overall decrease in activity of the [Y519F/Y520F]Syk mutant.

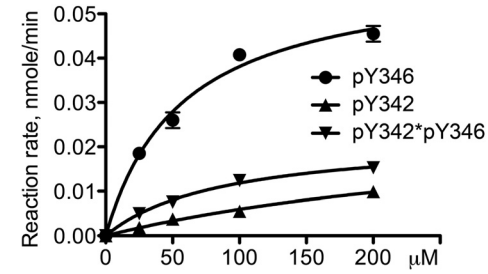
To evaluate the effect of TULA-2 on the activation of Syk, we co-expressed Syk and IL-2Rα/FcRγ with TULA-2 in the same system, activated Syk by cross-linking the receptor, and assessed the phosphorylation of Syk. These experiments indicated that $Tyr(P)^{519}$-$Tyr(P)^{520}$ was much lower in WT Syk in the presence of overexpressed TULA-2 (Fig. 8*A*), thus providing further evidence of down-regulation of Syk by TULA-2. The activation-induced increase in Syk $Tyr(P)^{519}$-$Tyr(P)^{520}$ in the presence of TULA-2 was reduced by ~40% as compared with the empty plasmid-transfected control cells (Fig. 8*B*). The effect of TULA-2 on $Tyr(P)^{519}$-$Tyr(P)^{520}$ in [Y342F]Syk was even more profound than this effect in WT Syk; the activation-induced increase in $Tyr(P)^{519}$-$Tyr(P)^{520}$ in [Y342F]Syk, while much lower than that in WT Syk, was reduced by TULA-2

almost completely (Fig. 8). In contrast, the activation of [Y346F]Syk was resistant to TULA-2; instead, a modest increase was observed in TULA-2-transfected cells (Fig. 8). As expected based on the results shown in Fig. 7, the level of Tyr(P)^{519}-Tyr(P)^{520} in [Y342F/Y346F]Syk in these experiments remained low, and TULA-2 exerted no effect on it (Fig. 8). Likewise, the total tyrosine phosphorylation in this system revealed the same dependence on the presence of TULA-2 as did the level of Syk Tyr(P)^{519}-Tyr(P)^{520} (data not shown). Overall, our results were consistent with the view that both Tyr^{342} and Tyr^{346} regulate the activity of Syk and that TULA-2 down-regulates it by dephosphorylating Tyr(P)^{346}.

The dependence of the levels of Syk Tyr(P)^{342} and Syk Tyr(P)^{346} on TULA-2 is consistent with the results on Syk Tyr(P)^{519}-Tyr(P)^{520}. Thus, TULA-2 co-expression reduces the activation-induced increase in the level of Tyr(P)^{346} in WT Syk and [Y342F]Syk by ~80 and ~95%, respectively (Fig. 8*B*). In contrast to this dramatic suppression, TULA-2 reduces the activation-induced increase in Tyr(P)^{342} modestly if at all. The TULA-2-mediated decrease in Tyr(P)^{342} in WT Syk is merely 40%, and this decrease is probably due to the loss of Tyr(P)^{346}, which appears to affect phosphorylation of Tyr^{342} (see Fig. 7). Furthermore, TULA-2 fails to suppress the activation-induced increase in Tyr(P)^{342} in the [Y346F]Syk mutant; a slight statistically insignificant increase is observed instead (Fig. 8*B*). The last effect mirrors an increase in Tyr(P)^{519}-Tyr(P)^{520} in [Y346F]Syk in the presence of TULA-2 (Fig. 8*B*), consistent with a positive role of Tyr(P)^{342} in the activation of Syk (see Fig. 7).

To confirm that the differential effects of TULA-2 on Syk Tyr(P)^{342} and Syk Tyr(P)^{346} in this system directly depend on dephosphorylation of these Tyr(P) sites by TULA-2 and not by TULA-2-mediated dephosphorylation of other regulatory Tyr(P) sites, we examined dephosphorylation of Syk Tyr(P)^{342} and Syk Tyr(P)^{346} using an *in vitro* phosphatase assay with synthetic peptides corresponding to the region surrounding these Tyr(P) sites. These experiments showed that Tyr(P)^{346} is a significantly better substrate of TULA-2 than Tyr(P)^{342}; k_{cat}/K_m, a constant characterizing the activity of an enzyme toward a particular substrate, is ~13-fold higher for Tyr(P)^{346} than for Tyr(P)^{342} (Fig. 9). The observed difference approximately corresponds to the difference between k_{cat}/K_m of Tyr(P)-containing peptides possessing the TULA-2 specificity determinants, on one hand, and lacking them, on the other (20). Importantly, the result obtained using *in vitro* phosphatase assays is consistent with the differential effects of TULA-2 on Syk Tyr(P)^{342} and Syk Tyr(P)^{346} in the cellular system, where Syk Tyr(P)^{346} is highly sensitive to the co-expression of Syk with TULA-2, whereas Syk Tyr(P)^{342} is not (see Fig. 8).

Enzyme kinetic studies were also used to study the possible effect of Tyr(P)^{342} on dephosphorylation of Tyr(P)^{346}; because Tyr^{342} is a key specificity determinant for Tyr(P)^{346} as a substrate site of TULA-2, phosphorylation of Tyr^{342} may affect dephosphorylation of Tyr(P)^{346}. To assess this effect, the peptide containing both Tyr(P)^{346} and a non-hydrolyzed analogue of Tyr(P)^{342} was analyzed along with those containing only Tyr(P)^{342} or Tyr(P)^{346}. These experiments indicated that the presence of a phosphate group at Tyr^{342} worsens significantly



	k_{cat},s^{-1}	K_m,μM	k_{cat}/K_mx10^{-5},M^{-1}s^{-1}
pY346	178+/-43	62+/-11	31.8
pY342	87+/-12	390+/-63	2.45
pY342*pY346	61+/-9.2	94+/-25	7.21

FIGURE 9. **Dephosphorylation of synthetic peptides containing Syk Tyr(P)^{342} and/or Syk Tyr(P)^{346} sites by TULA-2.** Kinetic parameters were determined using recombinant TULA-2 and synthetic Tyr(P) peptides *in vitro*. *pY342* and *pY346*, peptides containing Tyr(P) corresponding to positions 342 and 346; *pY342*pY346*, a peptide containing Tyr(P) in the position corresponding to Tyr^{346} and a non-hydrolyzable analogue of Tyr(P) in the position corresponding to Tyr^{342}. Representative concentration dependence curves are shown for each substrate; mean ± S.D. (*error bars*) is calculated from the results of at least three individual experiments for each substrate. The calculated values of kinetic constants are significantly different when each substrate is compared with the other two substrates with $p < 0.01$ (determined using ANOVA followed by Bonferroni's post-hoc test).

the substrate properties of Tyr(P)^{346}. However, even after this modification, Tyr(P)^{346} remains more sensitive to dephosphorylation by TULA-2 than Tyr(P)^{342} (see Fig. 9).

Discussion

Our previous studies demonstrated that TULA-2 down-regulates GPVI-mediated signaling in platelets and platelet responses *in vitro* and *in vivo* (6, 20). They have also indicated that TULA-2 down-regulates the activity of Syk (6, 17, 20) and that Syk Tyr(P)^{346}, a Tyr(P) site induced by GPVI receptor activation (see Fig. 2), is likely to be a good substrate for TULA-2 (20). These findings suggested that TULA-2 down-regulates Syk and the entire GPVI pathway by dephosphorylating Tyr(P)^{346}. The study reported here provides data strongly supporting this notion.

It was shown in several experimental systems that Tyr(P)^{346} and Tyr(P)^{342}, two Tyr(P) sites located in the linker between the SH2 domains and the kinase domain of Syk, play an important role in the process of activation of this PTK (32–37). The results of our experiments in platelets were in agreement with the purported role of Tyr(P)^{342} and Tyr(P)^{346} in other systems; (i) Tyr^{346} and Tyr^{342} were strongly phosphorylated in response to GPVI agonists, and this phosphorylation correlated with a profound increase in the phosphorylation of multiple proteins involved in the GPVI-mediated signaling pathway and in their phosphorylation-dependent interactions (see Figs. 2–4), and (ii) the phosphorylation of Tyr^{346} and, possibly, Tyr^{342} preceded that of Tyr^{519}-Tyr^{520}, a Syk activation marker (see Fig. 5). The subsequent findings directly supported the idea of Syk Tyr(P)^{346} being a major target of TULA-2 mediating the regulatory function of this PTP in GPVI-triggered signaling. First of all, Syk is by far the major phosphorylated protein binding to the substrate-trapping TULA-2 mutant (see Fig. 3). These results argue that TULA-2 down-regulates GPVI signaling not by dephosphorylating multiple elements of the corresponding

signaling pathway, but by specifically targeting Syk, activation and inactivation of which affects the entire GPVI signaling pathway.

Second, the activation of Syk induced through the ITAM-containing cytoplasmic tail of FcRγ is reduced by TULA-2 in WT Syk and [Y342F]Syk, but not in [Y346F]Syk (see Fig. 8). Therefore, the ability of TULA-2 to down-regulate the activity of Syk depends on the presence of Tyr(P)^{346} in the molecule of Syk. Moreover, the activity of [Y342F]Syk appears to be more sensitive to TULA-2 than the activity of WT Syk. This finding is consistent with the fact that in the absence of Tyr(P)^{342}, as occurs in [Y342F]Syk, there is only one Tyr(P) site in the SH2-kinase domain linker supporting the activation of Syk, Tyr(P)^{346}, and this site is very sensitive to dephosphorylation by TULA-2. In contrast, the activation of [Y346F]Syk, whose sole regulatory Tyr(P) site of the SH2-kinase domain linker is Tyr(P)^{342}, lacking critical specificity determinants required for being a substrate of TULA-2 (20), is not suppressed by TULA-2 co-expression, but modestly increased instead (see Fig. 8).

In agreement with the finding that the inhibition of Syk activity by TULA-2 depends on the presence of Tyr^{346} in the structure of this PTK, the effects of TULA-2 on the Tyr(P)^{342} and Tyr(P)^{346} sites differ significantly. The activation-induced increase in Tyr(P)^{346} drops precipitously in the presence of TULA-2 in both WT Syk and [Y342F]Syk (see Fig. 8), albeit in [Y342F]Syk the level of Tyr(P)^{346} is substantially lower than in WT Syk, because the lack of Tyr^{342} substantially reduces formation of Tyr(P)^{346} (see Fig. 7). In contrast, the activation-induced Tyr(P)^{342} increase in WT Syk is reduced by TULA-2 to a significantly lower extent than the Tyr(P)^{346} increase. Moreover, TULA-2 actually facilitates an activation-induced increase in the level of Tyr(P)^{342} in [Y346F]Syk (see Fig. 8).

The results obtained using the *in vitro* phosphatase assay with synthetic Tyr(P) peptides fully support the findings of the experiments in the cell system; the Tyr(P)^{346} site of Syk has been determined to be a much more preferential substrate of TULA-2 than Syk Tyr(P)^{342} (see Fig. 9). Taken together, these results are consistent with the notion that the suppressive effect of TULA-2 on the activity of Syk is dependent on the presence of Tyr(P)^{346} in this PTK but mostly independent of Tyr(P)^{342}.

Overall, the down-regulatory effect of TULA-2 on the activity of Syk appears to take place at the moment immediately after the initial events of GPVI-mediated signaling. These are phosphorylation of the ITAM tyrosine residues of FcRγ by Src family PTKs, most likely Fyn and Lyn (46–48), and binding of Syk to the double-phosphorylated ITAM via its tandem SH2 domains. As soon as Syk Tyr^{346} is phosphorylated in response to GPVI ligation, TULA-2 is likely to become capable of dephosphorylating it.

Dephosphorylation of Tyr(P)^{346} by TULA-2 appears to directly suppress further activation of Syk by inhibiting its phosphorylation at Tyr^{519}-Tyr^{520}. It is unlikely that TULA-2 dephosphorylates Syk Tyr(P)^{342} at a high rate, because this site lacks important substrate specificity determinants (20) (see Fig. 9). Furthermore, the phosphorylation of Tyr^{342} is likely to inhibit the activity of TULA-2 toward the neighboring Tyr(P)^{346} (see Fig. 9). Therefore, once Syk Tyr(P)^{342} is formed, the process of Syk activation becomes facilitated, because the ability of TULA-2 to control it diminishes.

The possibility cannot be ruled out that the effect of TULA-2 on Syk involves Tyr(P) sites that have not been evaluated in this study. Thus, it has been shown that the Tyr(P) sites Tyr^{130} and Tyr^{623}-Tyr^{624}-Tyr^{625} are also involved in regulating the function of Syk (49–53). In all, up to 15 Tyr(P) sites have been identified in Syk upon its autophosphorylation *in vitro* or receptor-induced activation in the cell (54, 55), and some of these sites might exhibit discernable properties as substrates of TULA-2. Furthermore, it is possible that dephosphorylation of a particular regulatory Tyr(P) site by TULA-2 influences phosphorylation or dephosphorylation of other sites, as appears to be the case for Tyr(P)^{342} and Tyr(P)^{346}, making the overall effect of TULA-2 on the activity of Syk very complex. Hence, although the role of TULA-2 in regulating the activity of Syk is now apparent, further elucidation of its mechanisms is warranted.

Several studies published in the last decade have shown that TULA-2 down-regulates receptor signaling in multiple systems, including T cells, mast cells, osteoclasts, and platelets (1, 6–9, 15, 18–20). In platelets, the effect of TULA-2 is much more profound than in other systems due to a very high expression level of TULA-2 as compared with other cells types (6) and to the strong specificity of TULA-2 toward Syk Tyr(P)^{346} (20), a key regulatory site of Syk, a PTK playing a crucial role in GPVI signaling (22, 29–31).

The exact biological function of TULA-2 in the regulation of GPVI-dependent platelet signaling and activation remains to be elucidated further; TULA-2 might facilitate deactivation of the GPVI-activated Syk, perhaps recycling Syk molecules for their use in subsequent waves of platelet activation through other receptors, such as integrins. The role of TULA-2 in deactivating Syk was outlined in a mathematical modeling study (56), which suggested that the activation-induced binding of TULA-2 to Syk, probably facilitated by the interaction between ubiquitin and the ubiquitin-associated domain of TULA-2 (2, 3), might be of importance for this function.

Another possible physiological function of TULA-2 is to suppress the activation of Syk in response to suboptimal stimulation, thus preventing unneeded and even dangerous "all-out" platelet responses to marginal stimuli. This idea is supported by the data indicating that TULA-2 is more effective in controlling the initial step of Syk activation than its subsequent steps (see Figs. 8 and 9).

Taken together, the results reported in this study indicate that down-regulation of the FcRγ-induced activity of Syk by TULA-2 is at least in part dependent on dephosphorylation of the Tyr(P)^{346} site of Syk by this PTP. Thus, dephosphorylation of Syk Tyr(P)^{346} appears to be a key event in TULA-2-mediated suppression of platelet activation via the collagen receptor, which exerts an important regulatory effect on biological and pathological events mediated by platelet activity.

Experimental Procedures

Reagents and Antibodies—All reagents were from Thermo Fisher Scientific unless stated otherwise. CVX was purchased from Dr. Clemetson (University of Bern). Chronolume (for

detection of secreted ATP) was from Chrono-log Corp. (Havertown, PA). CRP was purchased from Dr. Farndale (University of Cambridge). Amylose resin was from New England Biolabs (Ipswich, MA). Glutathione-agarose 4B was purchased from Machery Nagel (Bethlehem, PA). Ni^{2+}-Sepharose was from GE Healthcare (Princeton, NJ). The Biomol Green kit was from Biomol (Plymouth Meeting, PA). Nitrocellulose membrane Whatman Protran® was purchased from Whatman (Dassel, Germany). Odyssey blocking buffer and secondary antibodies IRDye 800CW goat anti-rabbit (catalog no. 926-3221) and IRDye 680LT goat anti-mouse (catalog no. 926-68020) were from LI-COR (Lincoln, NE). Anti-Syk Tyr(P)^{352} (catalog no. 2701), anti-Syk Tyr(P)^{525}-Tyr(P)^{526} (catalog no. 2710), anti-PLCγ2 Tyr(P)^{759} (catalog no. 3874), and anti-Cbl Tyr(P)^{731} (catalog no. 3554) were from Cell Signaling Technology (Beverly, MA). Anti-Syk Tyr(P)^{348} (TA325923) was from Origene (Rockville, MD). (Note that the numbering of Tyr(P) sites is shown for human sequences as originally listed; numbering may be different between mouse and human proteins.) Anti-SLP76 Tyr(P)^{128} (catalog no. 55837) was from BD Pharmingen (San Jose, CA). Syk (sc-517030), SLP-76 (sc-9062), PLCγ2 (sc-5283), and anti-GFP (sc-9996) were from Santa Cruz Biotechnology, Inc. (Dallas, TX). c-Cbl (catalog no. 610442) was from BD Transduction (San Jose, CA). Anti-CD25/IL-2Rα mouse monoclonal antibody was from R&D Systems (Minneapolis, MN). Anti-TULA-2 rabbit polyclonal antibody was described previously (5). Antibody to FcRγ was purchased from Millipore (catalog no. 06-727; Temecula, CA). Anti-actin (A 5316) was from Sigma. Anti-maltose-binding protein (MBP) (AB 3596) and anti-GST (MAC 111) were purchased from EMD Millipore (Billerica, MA).

DNA Expression Constructs—Syk expression plasmids were based on pEGFP-N2 (Clontech, Palo Alto, CA) in which murine Syk cDNA was inserted in-frame upstream of EGFP cDNA, resulting in the expression of Syk fused via its C terminus to EGFP (57). The plasmids encoding for WT Syk and [Y346F]Syk were a generous gift of Dr. R. Geahlen (Purdue University, West Lafayette, IN). Other mutations have been introduced into the Syk cDNA using site-directed mutagenesis with a QuikChange II XL mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's recommendations. Mutagenesis primers were synthesized by IDT (Coralville, IA) to introduce an A→T substitution into an appropriate TAC codon to generate a Tyr → Phe substitution at a protein sequence level. To generate the chimeric receptor expression construct, the cDNA corresponding to FcRγ intracellular sequence was amplified using Platinum *Taq* High Fidelity polymerase (Thermo Fisher Scientific) with pSVL-FcRγ plasmid originally generated by Dr. J. Kinet and purchased from Addgene (Cambridge, MA; catalog no. 8372) as a template and primers 5′-TTCAAGCTTCGACTCAAGATCCA-3′ (upper) and 5′-GCTCTAGACTACTGGGGTGGT-3′. The product was purified using the QIAQuick gel extraction kit from Qiagen (Valencia, CA) and digested with HindIII and XbaI restriction endonucleases. The backbone was prepared by digesting pCMV-IL2R/hE-cadherin-cytotail plasmid (Addgene catalog no. 45773, originally generated by Dr. B. Gumbiner) with HindIII and XbaI to remove the E-cadherin cDNA insert, and FcRγ cDNA was ligated into the backbone instead of the removed E-cadherin insert using TaKaRa ligation kit 2.1 (Takara Bio, Shiga, Japan). The resulting cDNA encoded the chimeric protein containing residues 1–261 of the IL-2Rα subunit followed by the residues 45–86 of FcRγ, which were connected by the Lys-Leu linker. All resulting constructs were verified by sequencing. TULA-2 expression plasmid was based on pCMV-Tag2B (Agilent Technologies) (17), and pCMV-Tag2B was used as the matching negative control.

Mice—All mice used in this study were of the C57/BL6 genetic background. KO mice lacking either TULA or TULA-2 were described previously (7). All animal work was conducted according to the guidelines established by the Temple University Institutional Animal Care and Use Committee in the specific pathogen-free environment of the Temple University Central Animal Facility.

Isolation of Murine Platelets—Mouse blood was collected from the anesthetized mice. Blood was drawn via cardiac puncture into one-tenth volume of 3.8% sodium citrate. Blood was spun at 100 × *g* for 10 min at room temperature, and the platelet-rich plasma (PRP) was separated. Red blood cells were mixed with 400 μl of 3.8% sodium citrate and spun for a further 10 min at 100 × *g*. Resulting PRPs were combined, 1 μM PGE1 was added, and samples were centrifuged for 10 min at 400 × *g*. Platelet-poor plasma was removed, and the platelet pellet was resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, and 0.2 unit/ml apyrase, pH 7.4). Platelet counts were determined using a Hemavet 950FS blood cell counter (Drew Scientific Inc., Dallas, TX) and adjusted to 1.5–2.0 × 10^8 platelets/ml.

Isolation of Human Platelets—Blood was drawn from informed healthy volunteers according to a protocol approved by the Institutional Review Board of Temple University in accordance with the Declaration of Helsinki into one-sixth volume of acid-citrate-dextrose (85 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid). PRP was isolated by centrifugation at 230 × *g* for 20 min and incubated with 1 mM aspirin for 30 min at 37 °C. Platelets were obtained by centrifugation for 10 min at 980 × *g* and resuspended in Tyrode's buffer as described above. Platelet counts were adjusted to 2 × 10^8 platelets/ml or 1 × 10^9 for pull-down assays.

Aggregation and ATP Secretion—Platelet aggregation was measured using a lumi-aggregometer (Chrono-log Corp.) at 37 °C under stirring conditions. Aliquots (250 μl) of washed platelets were stimulated with different agonists, and the change in light transmission was measured. Platelet-dense granule secretion was simultaneously determined by measuring the release of ATP by adding luciferin/luciferase reagent.

Platelet Sample Preparation and Western Blotting Analysis—Platelets were stimulated with agonists for the appropriate time under stirring conditions at 37 °C, and the proteins were precipitated by the addition of $HClO_4$ to a final concentration of 0.6 N. The protein pellets were washed one time with water and solubilized in sample buffer containing 0.1 M Tris base, 2% SDS, 1%(v/v) glycerol, 0.1% bromphenol blue, and 100 mM DTT and boiled for 10 min. Proteins were resolved using SDS-PAGE and transferred to Whatman Protran membrane. Membranes were blocked with the Odyssey blocking buffer for 1 h at room tem-

perature, incubated overnight at 4 °C with the desired primary antibody, and washed four times with Tris-buffered saline-Tween (TBS-T; 25 mM Tris, pH 7.4, 137 mM NaCl, 0.1% Tween 20) before incubation with appropriate infrared dye-labeled secondary antibody for 1 h at room temperature. Washed membranes were examined with a LI-COR Odyssey infrared imaging system.

Substrate Trapping—The substrate-trapping mutant of TULA-2 phosphatase domain fused to MBP was produced in *Escherichia coli* and purified from bacterial lysates on amylose resin as described previously (39). The amount of MBP-[H380C/E490Q]TULA-2 in the obtained slurry was determined using Coomassie staining of SDS-polyacrylamide gels, and the volumes of slurry used for pull-downs were adjusted to equalize the amount of the purified protein between pull-down samples. Human or mouse platelets were prepared as above, and their count was adjusted to 1×10^9/ml or 4×10^8/ml, respectively. Their aliquots (500 μl) were equilibrated at 37 °C and treated with vehicle or 100 ng/ml CVX for 1 min with stirring. The reactions were terminated by the addition of an equal volume of 2× Nonidet P-40 lysis buffer (final concentrations were 25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, and Pierce Halt protease and phosphatase inhibitor mixture). Lysates were sheared to break up any large aggregates, and the samples were rotated at 4 °C for 30 min. Lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants containing 1 mg of total protein were rotated with 50 μg of the substrate-trapping TULA-2 fused to MBP or MBP control at 4 °C for 2 h. The beads were collected by centrifugation at 800 × *g* for 2 min, washed three times with lysis buffer, and boiled in 100 μl of SDS-PAGE sample buffer to elute the captured proteins. Eluted proteins were separated using SDS-PAGE and analyzed using immunoblotting as specified in the figure legends.

PLCγ Pull-down—pGEX expression plasmids encoding for GST-fused SH2 domains of PLCγ1 and PLCγ2 originally generated by Dr. B. Mayer were purchased from Addgene. All GST-PLCγ SH2 domains and free GST were produced in *E. coli* and purified using glutathione-agarose as described previously (58, 59). Human and mouse platelets were prepared, activated with 100 ng/ml CVX, and lysed as described above for substrate-trapping experiments. GST-PLCγ1 SH2 domains (single N- or C-terminal SH2 designated N and C, respectively, or tandem SH2 designated NC), GST-PLCγ2 SH2 domains (NC), or GST alone (20 μg) were added to the lysates (1 mg of protein) and rotated overnight at 4 °C. The beads were centrifuged at 800 × *g* for 2 min, washed three times with lysis buffer, and boiled in 100 μl of SDS-PAGE sample buffer. Eluted proteins were resolved using SDS-PAGE and analyzed using immunoblotting with the antibodies as specified in the figure legends.

Cell Culture, Transfection, and Lysis—Human embryonal kidney 293T cells were grown in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer. Cells were seeded in 6-well plates 1 day before transfection to reach ~40% of their confluent density on the day of transfection. Linear polyethyleneimine (PEI) (catalog no. 23966, Polysciences, Warrington, PA) was used for transfection. To transfect cells in one well of a 6-well plate, PEI (15 μg) was mixed with the appropriate plasmids (0.5 μg of each) in 400 μl of serum-free DMEM and incubated at room temperature for 15 min. Then complete DMEM (1.6 ml) was added to the PEI/DNA mix, and the resultant transfection medium was added to a cell layer. Six hours later, the transfection medium was replaced with complete DMEM, and 48 h post-transfection, cells were harvested. Based on microscopic detection of GFP fluorescence, ~60–70% of cells were transfected under these conditions. Cells were lysed in TNE buffer (50 mM Tris, pH 7.6, 150 mM sodium chloride, 2 mM EDTA, and 1% Nonidet P-40) supplemented with the Halt inhibitor mixture, and the lysates were precleared by centrifugation at 14,000 × *g* for 10 min at 4 °C. Proteins were separated using SDS-PAGE and immunoblotted as described above.

Phosphatase Assay—Recombinant His_6-tagged TULA-2 phosphatase domain was produced in *E. coli* and purified on Ni^{2+}-Sepharose as described previously (60). Concentration of the obtained TULA-2 was determined using BCA reagent and Coomassie staining on SDS-PAGE, and the protein was stored at −20 °C in 50% glycerol. The enzyme assay was performed with synthetic peptides (Bio-Synthesis, Lewisville, TX) corresponding to the 337–351 region of mouse Syk: Tyr(P)^{342}, MDTEV(pY)ESPYADPEE; Tyr(P)^{346}, MDTEVYESP(pY)ADPEE; Tyr(P)^{342*}Tyr(P)^{346}, MTDEV(p*Y)ESP(pY)ADPEE. All three peptides contain Tyr(P) (pY) at the respective position; Tyr(P)^{342*}Tyr(P)^{346} also contains 4-phosphomethyl-L-phenylalanine (p*Y), a non-hydrolyzable analogue of phosphotyrosine, at position 342. Peptides were diluted to the indicated concentrations in phosphatase assay buffer (20 mM MOPS, pH 7.4, 100 mM NaCl, 4 mM EDTA, 100 μg/ml BSA, and 0.01% Triton X-100) at 25 °C, and TULA-2 was added at a final concentration of 3.6 ng/ml. The reaction was stopped after 20 min. The dephosphorylation rate was determined by the release of inorganic phosphate using malachite green. The enzyme reaction rate was measured within the linear region of the reaction progress curves to determine the initial velocity. Pilot experiments conducted previously confirmed that this time point is situated in the linear region. The initial velocity was plotted against the substrate concentration to determine the kinetic parameters of TULA-2 (k_{cat}, K_m, and k_{cat}/K_m) using Prism software.

Statistics—All densitometric analyses were performed using Odyssey Image Studio version 2 software. The Prism software package (GraphPad Software, La Jolla, CA) was used for statistical analysis and graphic representation of data.

Author Contributions—K. R., C. A. D., and D. H. T. conducted the experiments. J. G. generated and tested the expression constructs. N. C. generated the KO mice and the substrate-trapping mutant. A. Y. T. designed and coordinated the study, interpreted the data, and drafted the manuscript. S. P. K. and S. E. M. were involved in the design of experiments and interpretation of data and critically reviewed the manuscript. C. A. D. edited and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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