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TULA Proteins Regulate Activity of the Protein Tyrosine Kinase Syk

Rachana Agrawal,[1] Nick Carpino,[2] and Alexander Tsygankov[1,3]*

[1]Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania
[2]Department of Molecular Genetics and Microbiology, State University of New York at Stony Brook, Stony Brook, New York
[3]Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania

Abstract TULA belongs to a two-member family: TULA (STS-2) is a lymphoid protein, whereas STS-1/TULA-2 is expressed ubiquitously. TULA proteins were implicated in the regulation of signaling mediated by protein tyrosine kinases (PTKs). The initial experiments did not fully reveal the molecular mechanism of these effects, but suggested that both TULA proteins act in a similar fashion. It was shown recently that STS-1/TULA-2 dephosphorylates PTKs. In this study, we analyzed the effects of TULA proteins on Syk, a PTK playing an important role in lymphoid signaling. First, we have shown that TULA-2 decreases tyrosine phosphorylation of Syk in vivo and in vitro and that the intact phosphatase domain of TULA-2 is essential for this effect. We have also shown that TULA-2 exhibits a certain degree of substrate specificity. Our results also indicate that inactivated TULA-2 increases tyrosine phosphorylation of Syk in cells co-transfected to overexpress these proteins, thus acting as a dominant-negative form that suppresses dephosphorylation of Syk caused by endogenous TULA-2. Furthermore, we have demonstrated that phosphatase activity of TULA is negligible as compared to that of TULA-2 and that this finding correlates with an increase in Syk tyrosine phosphorylation in cells overexpressing TULA. This result is consistent with the dominant-negative effect of inactivated TULA-2, arguing that TULA acts in this system as a negative regulator of TULA-2-dependent dephosphorylation. To summarize, our findings indicate that TULA proteins may exert opposite effects on PTK-mediated signaling and suggest that a regulatory mechanism based on this feature may exist. J. Cell. Biochem. 104: 953–964, 2008.

Key words: TULA; STS; Syk; protein tyrosine kinase; tyrosine phosphorylation; dephosphorylation; phosphatase

We have identified TULA as a c-Cbl-associated protein from human T-lymphoblastoid cells [Feshchenko et al., 2004]. A mouse orthologue of TULA (STS-2) was identified independently [Carpino et al., 2004]. The gene encoding for this protein was originally studied in humans and termed UBASH3A [Wattenhofer et al., 2001]. TULA belongs to a two-member family: TULA (STS-2) is a lymphoid protein, whereas the second protein of this family (STS-1/TULA-2) is expressed ubiquitously [Carpino et al., 2002, 2004; Feshchenko et al., 2004]. (In this report we use the term TULA for consistency.) TULA proteins contain the N-terminal UBA domain, the centrally positioned SH3 domain and a region, which was initially termed HCD and which has a homology to phosphoglyceromutases (PGM) [Wattenhofer et al., 2001; Carpino et al., 2004; Feshchenko et al., 2004; Kowanetz et al., 2004] (Fig. 1).

TULA proteins were implicated in the regulation of signaling mediated by protein tyrosine kinases (PTKs). Thus, they were reported to increase the activity of receptor PTKs by inhibiting c-Cbl-dependent downregulation of their activated forms [Feshchenko et al., 2004; Kowanetz et al., 2004]. In these events, TULA

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Rachana Agrawal's present address is University of Virginia, Charlottesville, VA.

*Correspondence to: Alexander Tsygankov, Department of Microbiology and Immunology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140. E-mail: alexander.tsygankov@temple.edu

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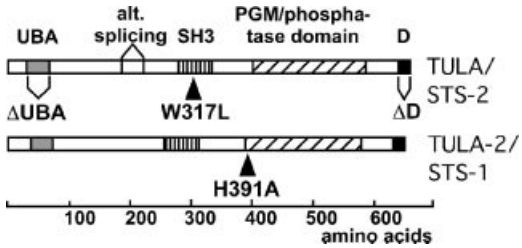

Fig. 1. Schematic view of the STS/TULA family. Major functional domains are shown, including ubiquitin-associated domain (UBA), Src-homology domain 3 (SH3), PGM/phosphatase domain, and dimerization domain (D). The mutations are shown at the family member that was utilized in the corresponding mutated form. Two alternative splice forms are shown for TULA. The predominant short form was utilized in this study. Protein length and mutation numbering are shown for the long form; W317L corresponds to W279L in the short form.

proteins appeared to act by disrupting interactions between ubiquitylated forms of activated PTKs and proteins recruiting them to the degradation pathway and, possibly, by decreasing the level of c-Cbl [Feshchenko et al., 2004; Kowanetz et al., 2004]. However, it was also shown that the lack of both proteins of the TULA/STS family in knockout mice resulted in hyper-reactivity of T lymphocytes correlated with an increase in the activity of Zap-70, thus arguing that these proteins negatively regulate PTK-mediated signaling. This effect appeared to be independent of c-Cbl [Carpino et al., 2004]. In apparent contrast to the results obtained in double-knockout mice, RNAi-mediated depletion of TULA in T-lymphoblastoid cells suppressed activation of Zap-70 [Feshchenko et al., 2004].

Considering that the initial results indicating that TULA regulates PTK-mediated signaling through UBA–ubiquitin interactions were obtained in experimental systems based primarily on EGFR and PDFGR [Feshchenko et al., 2004; Kowanetz et al., 2004], PTKs that are not highly expressed in lymphoid cells (reviewed in Tsygankov [2003]), we were prompted to elucidate the molecular basis of TULA's regulatory effects, using a PTK that is likely to interact with and be regulated by TULA under physiological conditions. We selected Syk, because it plays a crucial role in lymphoid cell signaling ([Kurosaki et al., 1994; Saouaf et al., 1994; Cheng et al., 1995; Turner et al., 1995; van Oers et al., 1995], reviewed in Tsygankov [2003]) and is regulated through c-Cbl-driven ubiquitylation [Lupher et al., 1998; Rao et al., 2001]. Considering that in lymphoid cells TULA is co-expressed with the second protein of its family, we were compelled to examine the effects of both TULA proteins on Syk.

These studies indicated that TULA proteins exert distinct effects on the activity of Syk in the cell; TULA increases it, whereas TULA-2 decreases it. The molecular basis of these effects appears to lie in the ability of TULA-2 to dephosphorylate Syk and in the ability of TULA to act as a "natural dominant-negative form" of TULA-2 due to the negligible phosphatase activity of TULA towards Syk.

MATERIALS AND METHODS

Plasmids

cDNAs encoding for wild-type Syk (splice isoform B) and its mutants [Chu et al., 1996] (kindly provided by Dr. B. Rowley, Bristol-Myers Squibb, Princeton, NJ) were cloned into the pAlterMAX vector (Promega) using restriction digestion/ligation. Expression plasmids encoding wild-type and mutant TULA as V5-tagged proteins were based on pAlterMAX and described previously [Feshchenko et al., 2004]. Expression plasmids encoding these forms of TULA as FLAG-tagged proteins were based on pFLAG-5a (Sigma) and described previously [Collingwood et al., 2007]. The C-terminal deletion of TULA encompassing the sequence homologous to the dimerization site of TULA-2 [Mikhailik et al., 2007] (amino acids 601-623 of the short TULA isoform, [ΔD]) was introduced into pFLAG5a-TULA as described [Collingwood et al., 2007]. Human TULA-2 cDNA purchased from the IMAGE Consortium (ID: 2958242) was amplified using the Expand High Fidelity PCR System (Roche Applied Science) and inserted into the pCMV-Tag2B expression plasmid (Stratagene) in-frame with the FLAG-encoding sequence. [H391A]TULA-2 mutant was generated as described previously [Collingwood et al., 2007]. Expression plasmids encoding HA-tagged c-Cbl in the pAlterMAX vector were described previously [Feshchenko et al., 1998]. Neo-CD8-ζ plasmid encoding the chimeric protein CD8-ζ [Lupher et al., 1997] was a kind gift of Dr. H. Band (Feinberg School of Medicine, Evanston, IL). A GFP-encoding expression plasmid (pEGFP-C2, Clontech) was co-transfected to each sample to assess the level of transfection.

Cells and Cell Lysis

Human embryonal kidney 293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, and 20 mM HEPES buffer. Cells were transfected using Lipofectamine-2000 or DMRIE-C (Invitrogen) and lysed either in TNE buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40) or, for native electrophoresis, in 100 mM Tris, pH 7.5, 20% Glycerol, 0.1% Triton X-100 supplemented in both cases with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 10 mM NaF, and 1 mM Na_3VO_4. Cell lysates were pre-cleared by centrifugation at 14,000*g* for 10 min at 4°C.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were carried out as described [Feshchenko et al., 1998, 2004] with some modifications for native electrophoresis [Niepmann and Zheng, 2006]. In this case, cell lysates were mixed with Serva Blue G (0.5% final concentration) before being loaded. Serva Blue G was also added to the Tris-histidine cathode buffer (0.002% final concentration). Samples were run in a 5–30% gradient gel under non-denaturing, non-reducing conditions. Gels were destained in transfer buffer, and proteins were transferred to nitrocellulose. The membrane was extensively washed with TBS, pH 7.6, 0.05% Tween-20 until there was no more visible Serva Blue G in the buffer and then subjected to immunoblotting.

Antibodies

Antibodies against c-Cbl (C-15), Syk (N19), Syk (4D10), Syk (SYK-01), GFP (B-2), normal rabbit IgG, normal mouse IgG, horseradish peroxidase-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were purchased from Santa Cruz Biotechnology. Anti-Syk N19 antibody was used for Western blotting, while 4D10 and SYK-01 were used for immunoprecipitation. Anti-phosphotyrosine 4G10 antibody was purchased from Upstate Biotechnology. Anti-phospho-Tyr525/526 antibody was purchased from Cell Signaling Technology. Anti-V5 was purchased from Invitrogen. Anti-FLAG M2 antibody was purchased from Sigma. Anti-CD3ζ antibody was purchased from BD Pharmingen. Anti-TULA antibody was custom made by Proteintech Group (Chicago, IL) and described previously [Collingwood et al., 2007]. Anti-GST antibody was raised in rabbits and described previously [Tsygankov et al., 1994]. Anti-GAPDH antibody was purchased from Research Diagnostic (Flanders, NJ).

In Vitro Phosphatase Assays

All PTKs were produced in insect cells as GST-fusion proteins: Syk was purchased from Invitrogen, Src kinases were purified as described previously [Spana et al., 1993]. The PGM/phosphatase domains (corresponding to TULA-2 amino acids 369–638) were produced in *E. coli* [Mikhailik et al., 2007]. SHP-1 was purchased from Upstate Biotechnology (Invitrogen). Kinases were mixed with phosphatases on ice at final concentrations of 0.5 and 1 μg/ml, respectively. Reaction buffer contained 25 mM HEPES, pH 7.2, 50 mM NaCl, 2 mM EDTA, 5 mM DTT, 200 μg/ml BSA, and 0.01% Triton X-100. Reaction was started by transferring reaction mixes to 37°C and was stopped by adding SDS–PAGE sample buffer and boiling.

RESULTS

TULA Proteins Bind to Syk and Modulate Its Tyrosine Phosphorylation

Consistent with our initial notion that TULA proteins may activate Syk by protecting it from c-Cbl-driven ubiquitylation and subsequent degradation, we studied the effects of TULA on Syk in a system that was previously utilized to demonstrate the effect of ubiquitylation on the activity of Syk [Lupher et al., 1998]. In 293T cells, which express no endogenous TULA, Syk was co-expressed with the CD8-ζ chimera, in order to facilitate tyrosine phosphorylation of Syk, and c-Cbl, in order to induce ubiquitylation of activated Syk. Since any effect of TULA on Syk is likely to depend on their physical interaction, we first analyzed binding of TULA to Syk, using their co-immunoprecipitation. These experiments demonstrated that TULA is present in immune complexes of Syk (Fig. 2A) and vice versa (Fig. 2B), thus indicating that TULA and Syk are associated.

To address the question of whether or not TULA regulates the activity of Syk, we next analyzed the effect of TULA on tyrosine phosphorylation of Syk, which is indicative of its kinase activity [Hutchcroft et al., 1991; Kurosaki et al., 1994; Saouaf et al., 1994; Rowley

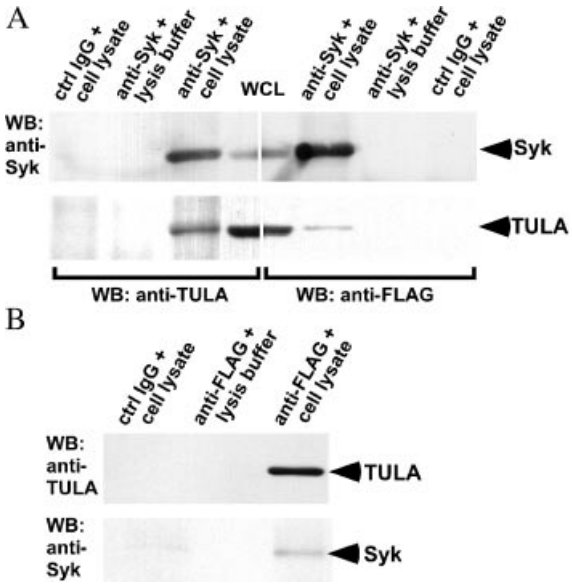

Fig. 2. Co-immunoprecipitation of TULA and Syk. 293T cells were co-transfected with expression plasmids encoding FLAG-TULA (3 μg), Syk (5 μg), c-Cbl (3 μg), and CD8-ζ (2 μg) as indicated. (All doses are shown per 100-mm Petri dish.) **A**: Syk was immunoprecipitated as indicated, and Syk immune complexes and whole cell lysate (WCL) were probed with anti-Syk and either anti-TULA or anti-FLAG as indicated. **B**: TULA was immunoprecipitated as indicated, and its immune complexes were analyzed using anti-TULA and anti-Syk immunoblotting.

et al., 1995]. Considering that our experimental system is characterized by multiple interactions of participating proteins—TULA binds to c-Cbl and Syk, whereas Syk binds to c-Cbl, TULA, and CD8-ζ (see Fig. 2 and [Bu et al., 1995; Johnson et al., 1995; Ota et al., 1996; Lupher et al., 1998; Feshchenko et al., 2004; Kowanetz et al., 2004]), we co-expressed them in various combinations to determine the minimal set of proteins required for the effect of TULA, if observed. These experiments indicated that TULA substantially increased tyrosine phosphorylation of Syk regardless of whether or not c-Cbl and CD8-ζ were co-expressed with TULA and Syk in this system (Fig. 3A). (These and multiple subsequent experiments indicated that TULA causes a three- to tenfold increase in the tyrosine phoshorylation of Syk, with variability being attributed to the use of transient transfection.) Likewise, binding of TULA to Syk was not substantially affected by co-expression of c-Cbl or CD8-ζ (Fig. 3A). To further elucidate the effect of TULA on Syk, we analyzed its dependence on the cellular level of TULA in the absence or in the presence of CD8-ζ. Co-expression of CD8-ζ upregulated tyrosine phosphorylaton of Syk, as expected, but TULA clearly showed the ability to increase this phosphorylation both in the absence and in the presence of CD8-ζ (Fig. 3B).

Next, the effect of TULA-2 on Syk was analyzed. Our results showed that TULA-2 binds to Syk (Fig. 4A), but its effect on Syk tyrosine phosphorylation was opposite to that of TULA (Fig. 4B). Like the effect of TULA, the effect of TULA-2 was dose-dependent (Fig. 4C). The results shown in Figure 4C demonstrated that TULA-2 is capable of exerting its effect without c-Cbl co-transfection both in the presence and in the absence of CD8-ζ, indicating that the effect of TULA-2 on Syk, albeit opposite to that of TULA, did not require co-expression of either c-Cbl or CD8-ζ.

The finding that the effects of TULA and TULA-2 on Syk were opposite was in sharp contrast to the previous results indicating that both proteins similarly protect receptor PTKs from c-Cbl-driven ubiquitylation-mediated downregulation [Kowanetz et al., 2004]. Indeed, c-Cbl, albeit crucial for the effects of TULA proteins on receptor PTKs [Feshchenko et al., 2004; Kowanetz et al., 2004], did not modify the effect of TULA on Syk (see Fig. 3). Finally, multiple attempts to demonstrate the role of TULA proteins on ubiquitylation of Syk in our system demonstrated no significant effect (data not shown). Thus, the effects of TULA proteins on Syk differ from those observed in the receptor PTK-based systems, in which TULA proteins appeared to protect activated PTK from c-Cbl-driven downregulation in a UBA- and SH3-dependent fashion [Feshchenko et al., 2004; Kowanetz et al., 2004]. Taken together, these findings compelled us to focus our attention on other domains of TULA proteins.

Effects of TULA Proteins on the Cellular Tyrosine Phosphorylation of Syk Are Consistent With Their Phosphatase Activity

Since the HCD/PGM domain is homologous to PGM [Carpino et al., 2004; Kowanetz et al., 2004], while PGM exhibit homology to phosphatases [Jedrzejas, 2000], we considered the possibility that the effects of TULA proteins on Syk phosphorylation are mediated by the PGM domain. To determine whether the effect of TULA proteins on Syk in our experimental system is mediated by phosphatase activity, we

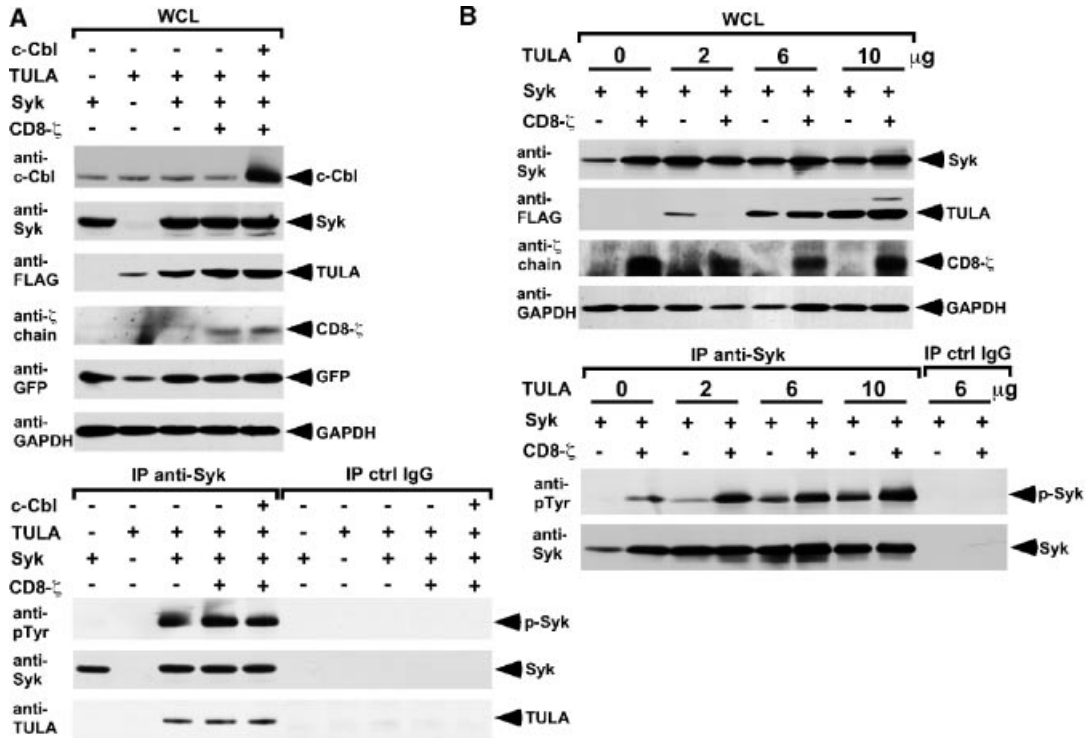

Fig. 3. TULA increases tyrosine phosphorylation of Syk. 293T cells were co-transfected with expression plasmids encoding TULA, Syk, c-Cbl and CD8-ζ, and their lysates were analyzed as indicated. Empty pAlterMAX plasmid was added to keep the concentration of DNA constant. Ctrl IgG = control IgG. **A**: 293T cells were co-transfected with the corresponding plasmids at a single dose as described in the legend to Figure 2. **B**: 293T cells were co-transfected with the expression plasmids encoding Syk and CD8-ζ as described in the legend to Figure 2 and with the TULA expression plasmid at the doses indicated.

compared the effects of wild-type TULA-2 and its mutant, corresponding to H391A in human TULA-2, which lacks the residue shown to be essential for catalytic activity of homologous enzymes [Jedrzejas, 2000]. The experiments indicated that this mutant not only failed to decrease Syk tyrosine phosphorylation, but caused its increase (Fig. 5A), suggesting that inactive TULA-2 acts as a dominant-negative form. To confirm these results and to further characterize the cellular effects of TULA proteins on Syk, we incubated baculovirus-produced Syk with recombinant C-terminal fragments of TULA proteins, containing their HCD/PGM domains. These experiments indicated that TULA-2 dephosphorylates Syk as does SHP-1, a protein phosphatase known to dephosphorylate and regulate Syk [Dustin et al., 1999; Maeda et al., 1999] (Fig. 5B). Phosphatase activity of TULA-2 was sensitive to vanadate, a known inhibitor of protein phosphatases [Leis and Kaplan, 1982] (Fig. 5B). In agreement with the results obtained in transfected cells, in vitro studies demonstrated that both catalytic histidine-mutated TULA-2 and wild-type TULA lack detectable phosphatase activity (Fig. 5B). Considering that the major autophosphorylation site of human Syk is Tyr525/Tyr526 (corresponding to Tyr519/Tyr520 in rodents), which is located in the activation loop and plays a critical role in Syk activation [Kurosaki et al., 1995; Rowley et al., 1995; Chu et al., 1996], we analyzed the effect of TULA proteins on phosphorylation of this site and demonstrated that TULA-2 dramatically reduces tyrosine phosphorylation of Tyr525/Tyr526 both in vitro and in vivo (Fig. 5B,C). Wild-type TULA and inactivated TULA-2 did not affect phosphorylation of Tyr525/Tyr526, consistent with their general inability to dephosphorylate Syk (Fig. 5B).

To characterize the specificity of TULA-2 as a phosphatase, we compared its effects on Syk and various Src-family PTKs. These experiments indicated that TULA-2 is capable of

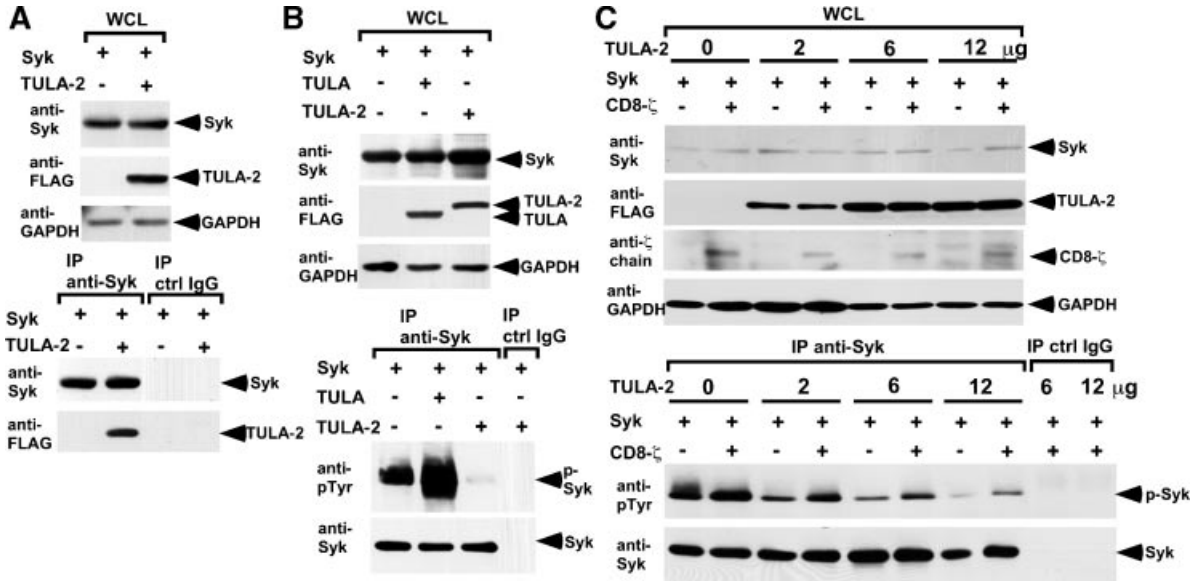

Fig. 4. TULA-2 decreases tyrosine phosphorylation of Syk. 293T cells were co-transfected with expression plasmids encoding TULA-2, Syk and CD8-ζ as shown. Cell lysates were analyzed as indicated. pAlterMAX was added to keep the dose of DNA constant. Ctrl IgG = control IgG. 293T cells were co-transfected with the indicated plasmids either at a single dose (Syk, 2 μg; TULA, 10 μg; TULA-2, 1 μg in (**A**); Syk, 2 μg; TULA-2, 3 μg in (**B**)) or with a single dose of Syk (5 μg) and CD8-ζ (2 μg) and the indicated dose of TULA-2 (**C**).

dephosphorylating not only Syk, but also Src-family PTKs (e.g., Fyn; Fig. 5D).

TULA Acts as an Inhibitor of TULA-2

Taken together, our findings were consistent with the notion that phosphatase-deficient TULA proteins act as dominant-negative inhibitors of endogenous TULA-2. This notion was supported by the finding that co-expression of TULA with TULA-2 significantly reduces the activation effect of TULA on Syk (Fig. 6A).

To explain the negative effect of TULA on TULA-2-mediated inhibition of Syk we considered two possible mechanisms. First, TULA may displace TULA-2 from Syk, thus disrupting the interaction between a phosphatase and its substrate. Second, TULA proteins are known to dimerize through their C-terminal domains [Kowanetz et al., 2004; Mikhailik et al., 2007], so it could be speculated that TULA may form a heterodimer with TULA-2, thus inhibiting its phosphatase activity. To determine whether TULA is capable of displacing TULA-2 from its complex with Syk, we co-expressed Syk with each TULA protein separately and with both TULA and TULA-2 together and examined Syk phosphorylation and binding to TULA proteins. The results of these experiments indicate that TULA is capable of disrupting the Syk-TULA-2 complex, although this effect did not correlate with an increase in Syk phosphorylation (Fig. 6B).

To determine whether TULA may exert its effect on Syk through a dimerization-mediated mechanism, we first confirmed that TULA dimerizes through the same C-terminal domain as TULA-2 does [Mikhailik et al., 2007] by co-immunoprecipitating V5-tagged wild-type TULA with either Flag-tagged wild-type or Flag-tagged [ΔD]TULA, lacking the site homologous to the TULA-2 dimerization site (Fig. 7A). As expected, [ΔD]TULA was unable to co-precipitate with TULA (Fig. 7A). To provide further support to these results, we carried out electrophoresis of wild-type and [ΔD]TULA under conditions preserving protein oligomerization and showed that [ΔD]TULA, while lacking only 4% of its sequence, migrates much faster than full-length TULA (Fig. 7B). However, the effect of [ΔD]TULA on Syk phosphorylation was comparable to that of wild-type TULA. Both forms of TULA increased Syk phosphorylation approximately fourfold (Fig. 7C), consistent with our previous observations. This result indicates that the effect of TULA is independent of its dimerization.

A straightforward explanation of the TULA-dependent increase in Syk tyrosine phosphorylation that would be an alternative to the

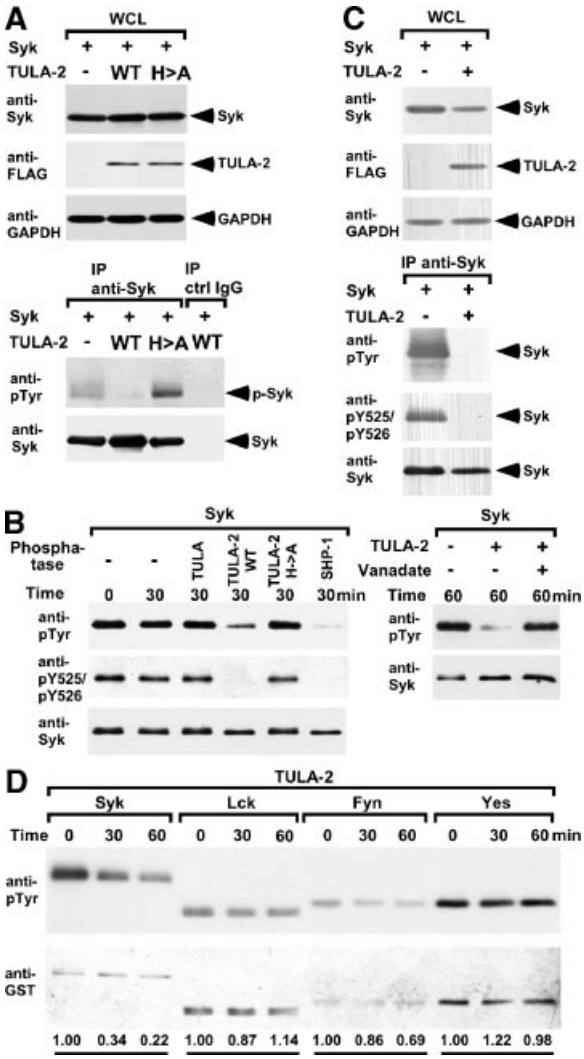

Fig. 5. Effects of TULA and TULA-2 on Syk correlate with their phosphatase activity. **A**: 293T cells were transfected with plasmids encoding for Syk (5 μg), wild-type TULA-2 or its H391A mutant (denoted as H->A; 2 μg). pAlterMAX was used to keep DNA concentration constant. Cell lysates were analyzed as indicated. Ctrl IgG = control IgG. **B**: GST-Syk was incubated with the proteins indicated for a specified time. Vanadate was added (1 mM) where indicated. Reaction mixtures were analyzed using immunoblotting. **C**: Effects of TULA-2 on total phosphorylation of Syk and phosphorylation of the Tyr525/526 site were examined as described above in (A). **D**: Specificity of the effect of TULA-2 on various PTKs in vitro was examined as described above in (B). The ratio of tyrosine phosphorylation to the amount of the respective PTK is shown at the bottom of the panel.

counteracting effect of TULA on the TULA-2-dependent dephosphorylation of Syk may be provided by a hypothetical adaptor action of TULA, which can facilitate interactions of Syk with an active PTK. To examine this possibility we compared the effect of TULA on wild-type and several mutant forms of Syk and demonstrated that TULA fails to increase tyrosine phosphorylation of Syk mutants that are catalytically inactive (KD) or retain only a small fraction of activity (YYFF), whereas TULA increases tyrosine phosphorylation of catalytically active wild-type and double SH2-defective (RR) Syk (Fig. 8A). These results indicate that the TULA-facilitated increase in the tyrosine phosphorylation of Syk is dependent on Syk autophosphorylation. Similarly, the mutational approach was employed to determine the interactions mediated by the domains of TULA that are essential for the positive effects of TULA on Syk tyrosine phosphorylation. These experiments indicated that SH3-mediated interactions are dispensable, while UBA-mediated interactions are essential for the observed effect of TULA (Fig. 8B).

Biological Consequences of the Effects of TULA Proteins

To examine possible biological consequences of the effects of TULA proteins on Syk, we analyzed how they influence total tyrosine phosphorylation and demonstrated that TULA-2 decreases tyrosine phosphorylation not only of Syk, but also of various cellular proteins, especially in Syk-overexpressing cells (Fig. 9A), while TULA exerts no effect (data not shown). Since the latter appeared to be in contrast with the activating effect of TULA on Syk, we examined the effect of TULA on phosphorylation of c-Cbl, a well-characterized substrate of Syk. As expected, tyrosine phosphorylation of c-Cbl in Syk-overexpressing cells was significantly increased in the presence of TULA (Fig. 9B). Mutation of all the major tyrosine phosphorylation sites [Feshchenko et al., 1998] greatly decreased phosphorylation of c-Cbl, but even the residual phosphorylation was enhanced by TULA (Fig. 9B), thus indicating that the effect of TULA is not restricted to particular tyrosine phosphorylation sites on c-Cbl. Since TULA may affect Syk-induced phosphorylation of c-Cbl through two mechanisms: by activating Syk and by facilitating c-Cbl-Syk interactions as an adaptor capable of binding to both c-Cbl and Syk, we compared the effects of wild-type and SH3-deficient TULA, which is incapable of binding to c-Cbl, on c-Cbl tyrosine phosphorylation. The effect of SH3-deficient TULA was substantially reduced, although not abrogated completely (Fig. 9C).

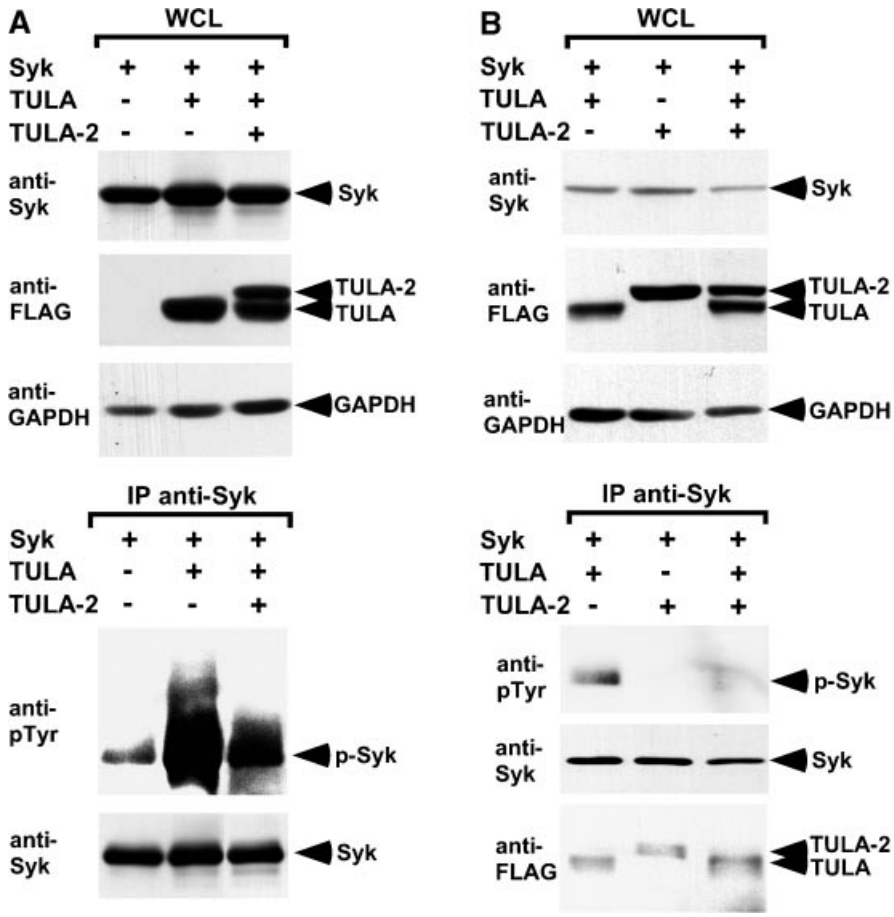

Fig. 6. TULA and TULA-2 compete in their effects on Syk. 293T cells were transfected with expression plasmids encoding for Syk (A, 5 μg; B, 1 μg), TULA (10 μg), and TULA-2 (A, 3 μg; B, 1 μg). pAlterMAX was used to keep the concentration of DNA constant. Cell lysates were analyzed as indicated. **A**: The effect of TULA-2 on the activation of Syk by TULA and (**B**) the displacement of TULA-2 from its complex with Syk by TULA were studied.

This result argued that the observed increase in the tyrosine phosphorylation of c-Cbl is dependent on both overall activation of Syk and facilitation of c-Cbl-Syk interactions.

DISCUSSION

Our results indicate that TULA-2/STS-1 acts as a phosphatase capable of dephosphorylating Syk, a PTK expressed in multiple cell types and playing a critical role in lymphoid signaling. Phosphatase activity of TULA-2 towards Syk is close to that of SHP-1, which has previously been shown to dephosphorylate and regulate Syk (Fig. 5B). Although Syk contains multiple tyrosine phosphorylation sites [Furlong et al., 1997], it is clear that TULA-2 dephosphorylates the activation loop of Syk (Fig. 5B,C), which plays a crucial role in the kinase activity of Syk [Kurosaki et al., 1995; Rowley et al., 1995; Chu et al., 1996]. The phosphatase activity of TULA-2 underlies its ability to decrease Syk tyrosine phosphorylation in vivo (Figs. 4 and 5A,C). These results are consistent with recent findings that STS-1/TULA-2 is a phosphatase active towards *para*-Nitro-phenyl-phosphate, synthetic phosphotyrosine-containing peptides, immunoprecipitated PTKs, such as Zap-70 and Src, in vitro and decreases tyrosine phosphorylation of Zap-70 and Src in vivo [Mikhailik et al., 2007]. They are also consistent with the finding that an insect protein homologous to the PGM/phosphatase region of TULA proteins is a steroid phosphatase [Davies et al., 2007].

Our results indicate that TULA-2 exhibits some degree of selectivity, dephosphorylating

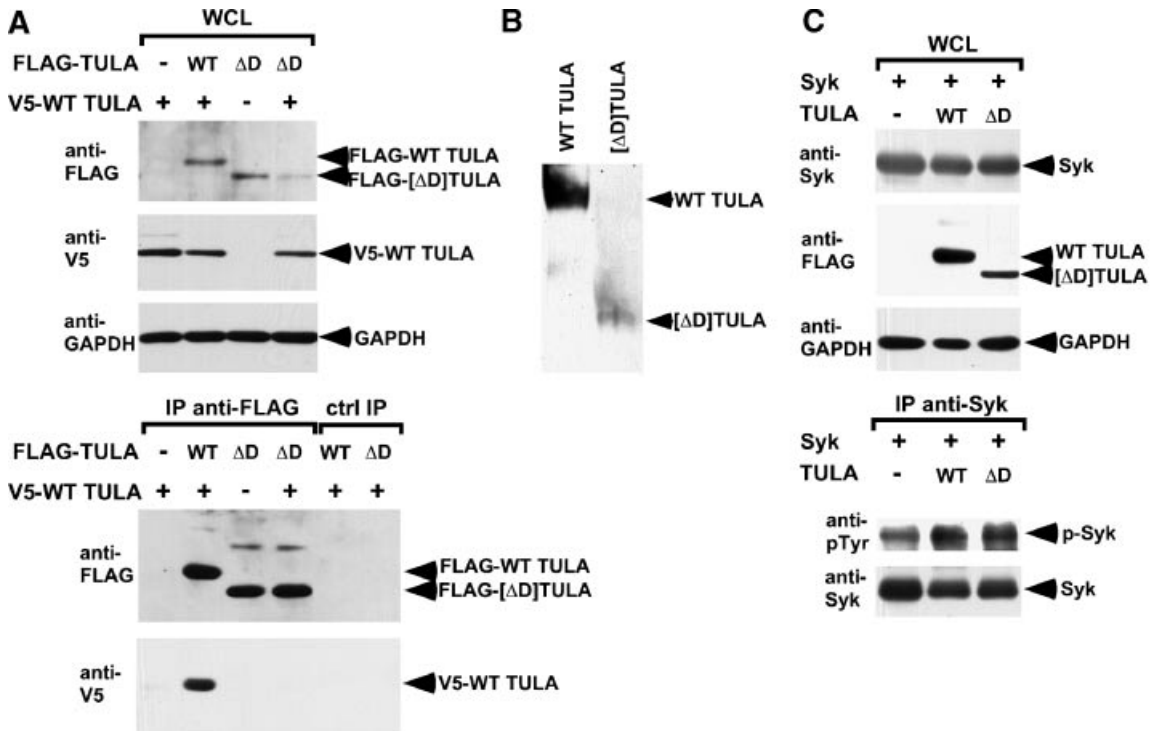

Fig. 7. Dimerization of TULA plays no role in its effect on Syk. **A**: 293T cells were transfected with expression plasmids encoding for FLAG- or V5-tagged wild-type (WT) TULA (3 μg of each) and FLAG-tagged [ΔD]TULA (10 μg) as indicated. Cell lysates were analyzed as indicated. **B**: 293T cells were transfected with expression plasmids encoding for WT TULA (1 μg of each) or [ΔD]TULA (3 μg) as indicated. Cell lysates were separated using native electrophoresis and analyzed using anti-TULA immunoblotting. **C**: 293T cells were transfected to express Syk (5 μg) and TULA (either 8 μg of WT or 15 μg of ΔD) as described above in (A) and analyzed as indicated. pAlterMAX was added to keep the concentration of DNA constant in all cases.

Syk faster than the Src-family PTKs tested in our experiments, among which Fyn appears to be a better substrate than Lck or Yes (Fig. 5D). This specificity may be based on differential affinity of TULA-2 to various PTKs or on differences in the structure of phosphorylation sites of these PTKs. Regardless of the molecular basis of the observed specificity, our results described here and another recent report [Mikhailik et al., 2007] clearly indicate that Syk-family PTKs are highly sensitive to the phosphatase activity of TULA-2.

In contrast, TULA, the second member of this protein family, lacks detectable phosphatase activity towards Syk (Fig. 5B), in agreement with the recent findings indicating that the phosphatase activity of TULA is 200-fold lower than that of TULA-2 [Mikhailik et al., 2007]. Although the reasons for this dramatic difference remain to be understood, the lack of phosphatase activity in TULA undoubtedly correlates with a substantial activating effect of TULA on Syk in vivo (Figs. 3 and 5B). Considering that [H391A]TULA-2, an inactivated form of TULA-2, exerts an effect similar to that of TULA (Fig. 5A), it is likely that TULA influences tyrosine phosphorylation of Syk by acting as a dominant-negative form diminishing the inhibitory effect of TULA-2. Similarly, a positive effect of inactivated TULA-2/STS-1 on TCR-mediated signaling was observed [Mikhailik et al., 2007].

Interestingly, these findings allow us to explain an apparent contradiction between the results indicating that the lack of both TULA/STS proteins leads to an increase in the activity of Zap-70 in T cells [Carpino et al., 2004; Mikhailik et al., 2007] and those indicating that shRNA-mediated depletion of TULA in Jurkat T-lymphoblastoid cells suppresses Zap-70 activation [Feshchenko et al., 2004]: depletion of TULA is likely to shift the balance in this system towards inactivation of Zap-70.

The molecular basis of the dominant-negative effect of TULA may be due to the competition of TULA and TULA-2 for Syk. Indeed, co-immunoprecipitation of Syk with TULA-2 was greatly decreased in the cells co-expressing Syk

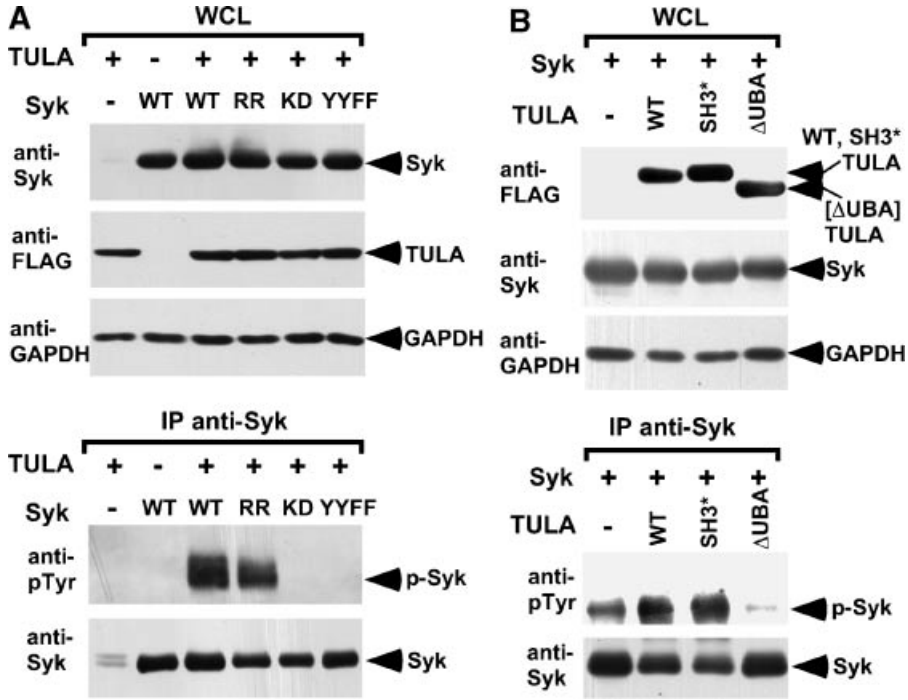

Fig. 8. Role of functional sites of Syk and TULA in the effects of TULA on Syk. 293T cells were transfected with expression plasmids encoding for (**A**) wild-type TULA (10 μg) and various forms of Syk (5 μg of WT or YYFF, 10 μg of RR or KD) or (**B**) wild-type Syk (5 μg) and various forms of TULA (8 μg of WT or 10 μg of mutant). Cell lysates were analyzed using immunoprecipitation and immunoblotting as indicated. WT = wild-type Syk, RR = Syk with both SH2 domains inactivated, KD = kinase-dead Syk, YYFF = Syk with the tyrosines of the autophosphorylation loop replaced with Phe, SH3* = TULA with inactivated SH3.

with both TULA and TULA-2 as compared to that in the cells co-expressing Syk with TULA-2 alone (Fig. 6B). Although phosphorylation of Syk remains suppressed even in the absence of detectable co-immunoprecipitation with TULA-2 (Fig. 6B), it cannot be ruled out that binding of TULA-2 to Syk, while weak and undetectable using co-immunoprecipitation, may be sufficient for the inhibitory effect of TULA-2. In contrast, it is clear that dimerization of TULA is not essential for its effect, since both wild-type and dimerization-deficient TULA activate Syk (Fig. 7C). Furthermore, it appears that the positive effect of TULA on Syk tyrosine phosphorylation requires TULA UBA (Fig. 8B). This finding suggests that TULA has to interact with ubiquitin and/or ubiquitylated proteins to exhibit the observed effects. Notably, ubiquitylated Syk is unlikely to be one of these proteins, because only ubiquitin-free Syk was detected in TULA immune complexes (Fig. 2 and data not shown). Instead, TULA may interact with ubiquitylated proteins acting as co-factors in its effects. For example, binding of TULA to Syk may be indirect and mediated by a ubiquitylated protein that directly binds to both TULA UBA and Syk. The search for such proteins remains to be conducted.

Earlier studies indicated that TULA proteins exert stabilizing effects on receptor PTKs and that these effects are likely to be dependent on the interference of the TULA UBA domains with ubiquitin-mediated interactions essential for downregulation of receptor PTKs [Feshchenko et al., 2004; Kowanetz et al., 2004]. The effects described in this report are different from these earlier studies, being mediated by the phosphatase activity of TULA-2 and the dominant-negative action of TULA. This allows us to conclude that TULA proteins are likely to be multi-functional regulators, which act through several routes, and that these routes may be differentially involved in the effects of TULA proteins on various PTKs. The multiple functional effects of TULA proteins correlate well with the multi-domain nature of these proteins, which may be especially important in the effects of TULA proteins involving both phosphatase activity and binding to a specific substrate, recruitment platform or regulator.

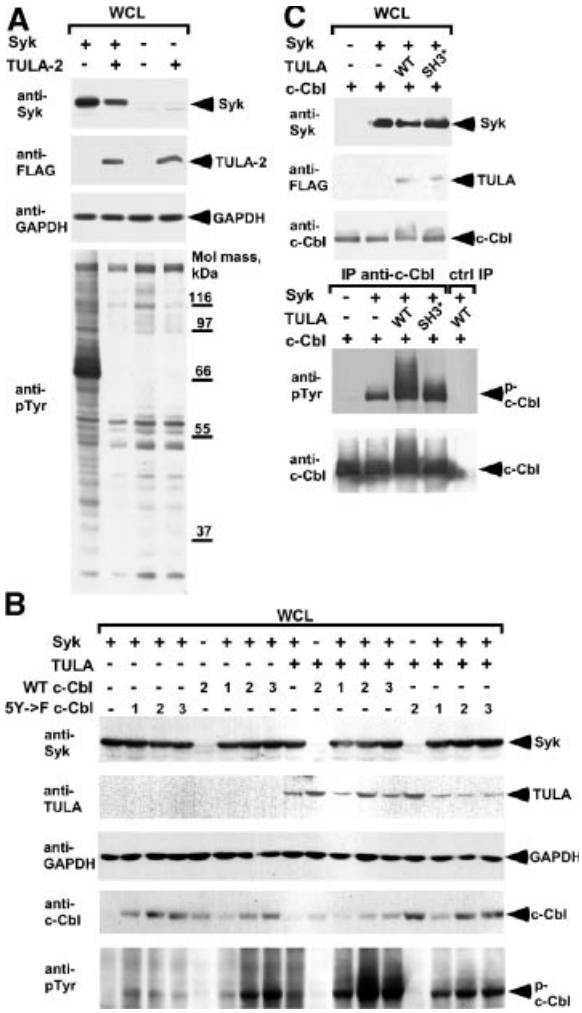

Fig. 9. Biological consequences of the effects of TULA proteins. 293T cells were co-transfected with plasmids encoding for Syk (5 μg), TULA (10 μg) and c-Cbl: (B, 2 μg; C, as indicated). Whole cell lysates were examined for total (**A**) or c-Cbl (**B**) tyrosine phosphorylation. **C**: The effects of WT and SH3* TULA on c-Cbl tyrosine phosphorylation were compared using anti-c-Cbl immunoprecipitation. Ctrl IP = control immunoprecipitation.

To sum up, we have characterized an important novel family of cellular regulators exerting complex effects on PTK-mediated cell signaling. It appears that these effects regulate not only Syk, but also Src-family PTKs. Likewise, they may contribute to regulation of other PTKs, thus being an integral part of the cellular signaling regulation network. Considering the opposite directions of the effects of TULA and TULA-2 on PTKs, a system of PTK regulation may exist that is based on changes in the expression and post-translational modifications of these two proteins.

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