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Insights into the suppressor of T-cell receptor (TCR) signaling-1 (Sts-1)-mediated regulation of TCR signaling through the use of novel substrate-trapping Sts-1 phosphatase variants

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Abstract

High affinity substrate-trapping protein tyrosine phosphatases have been widely used both to investigate the endogenous targets of many phosphatases and to address questions of substrate specificity. Herein, we extend the concept of a substrate-trapping phosphatase to include an enzyme of the histidine phosphatase superfamily. This is the first description of substrate-trapping technology applied to a member of the histidine phosphatase family. The phosphatase suppressor of T-cell receptor signaling (Sts)-1 has recently been reported to negatively regulate signaling downstream of the T-cell receptor. We generated high-affinity substrate-trapping variants of Sts-1 by mutagenesis of key active site residues within the phosphatase catalytic domain. Mutation of both the nucleophilic His380 and the general acid Glu490 yielded Sts-1 enzymes that were catalytically inactive but showed high affinity for an important tyrosine kinase in T cells that Sts-1 is known to regulate, Zap-70. Sts-1 substrate-trapping mutants isolated tyrosine-phosphorylated Zap-70 from lysates of activated T cells, validating Zap-70 as a possible substrate for Sts-1 and highlighting the efficacy of the mutants as substrate-trapping agents. Inhibition of the Zap-70 interaction by vanadate suggests that the substrate-trapping effect occurred via the Sts-1 phosphatase active site. Finally, overexpression of Sts-1 substrate-trapping mutants in T cells blocked T-cell receptor signaling, confirming the inhibitory effect of Sts-1 on Zap-70.

Keywords

histidine phosphatase superfamily; suppressor of T-cell receptor signaling (Sts) proteins; T-cell receptor (TCR) signaling

Introduction

The T-cell receptor (TCR) signaling system is broadly regulated by tyrosine kinases and protein tyrosine phosphatases (PTPs) [1,2]. For example, the Src family kinase Lck initiates TCR signal transduction by phosphorylating tyrosines on conserved immune-receptor tyrosine-based activation motifs within TCR subunits. This process creates binding sites for the kinase Zap-70, which is brought to the TCR complex to be phosphorylated and activated by Lck. Zap-70 subsequently phosphorylates and activates a number of downstream cytoplasmic targets [3,4]. Dephosphorylation of any of these signaling components

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generally inactivates TCR signaling. For example, dephosphorylation of Zap-70 and loss of its interactions with the immune-receptor tyrosine-based activation motifs have been shown to return the kinase to an autoinhibited conformation, thereby shutting down proximal TCR signaling [5].

Suppressor of TCR signaling (Sts)-1 and Sts-2 negatively regulate proximal TCR signaling events, particularly at the level of Zap-70 phosphorylation and activation [6]. T cells lacking Sts-1 and Sts-2 are hypersensitive to TCR stimulation, and show enhanced phosphorylation and activation of Zap-70 [7,8]. Both Sts-1 and Sts-2 contain a C-terminal phosphatase catalytic domain, termed a PGM domain, that shares sequence homology with enzymes of the 'phosphoglycerate mutase' subbranch within the histidine phosphatase (HP) superfamily [9]. The molecular mechanisms by which Sts-1 and Sts-2 regulate the state of Zap-70 phosphorylation are still unclear, although they appear to require PGM domain phosphatase activity, because wild-type Sts-1 efficiently restored the hypersensitive phenotype in *Sts-1/2*$^{-/-}$T cells, whereas a mutant with a catalytically inactive PGM domain failed to restore the hypersensitive phenotype [7]. We recently characterized the phosphatase activity associated with the Sts-1 PGM domain (Sts-1_{PGM}) and the Sts-2 PGM domain (Sts-2_{PGM}), and found significant differences. Sts-1_{PGM} showed substantially higher catalytic efficiency than Sts-2_{PGM} towards a range of model phosphatase substrates [7,10], and also showed phosphatase activity towards Zap-70 and other phosphotyrosine proteins. In contrast, Sts-2_{PGM} has not been observed to target Zap-70 *in vitro*, although it does appear to regulate Zap-70 *in vivo* [8]. In order to further address the question of whether Zap-70 could be a genuine Sts-1 target, we sought to develop high-affinity 'substrate-trapping' variants of Sts-1 and determine whether these mutants could interact stably with Zap-70.

Substrate-trapping techniques have been widely used to identify target substrates of PTPs [11]. They involve the use of mutant PTPs in which the catalytic cysteine that serves as a nucleophile and/or the proton-donating aspartate found within the WPD loop have been changed to a serine or an alanine, respectively [12]. These mutants are catalytically inactive, but retain the ability to bind their native substrates [13,14]. Substrate-trapping can also serve as an innate regulatory mechanism for sequestering specific components away from signaling circuits, as in the case of the pseudo- phosphatase MK-STYX targeting an effector for stress granule formation, Ras-GTPase-activating protein-binding protein [15].

In this study, we used the development of high-affinity Sts-1 mutants to investigate the possibility of Zap-70 being a substrate for Sts-1. Our results suggest that Sts-1 can directly target Zap-70 in T cells.

Results

Development of Sts-1 substrate-trapping mutants

With the aim of generating a catalytically inactive Sts-1 phosphatase as a substrate-trapping mutant, we targeted three residues in the active site of Sts-1_{PGM} for mutation: the nucleophilic His380, and two additional basic residues that are proposed to undergo critical electrostatic interactions with the substrate's phosphate moiety, Arg462 and His565 (Fig. 1A). We hypothesized that altering these residues could yield catalytically inactive phosphatase enzymes that would nonetheless interact stably with substrates. Speculating that removal of an acidic residue within the active site might decrease the electrostatic repulsion of the incoming phosphate group and thus increase the substrate-binding affinity, we also targeted Glu490 for mutation. A series of single and compound mutations were introduced into the Sts-1_{PGM}, to generate a total of 15 candidate high-affinity mutants (Fig. 1A). To evaluate catalytic activity, we expressed wild-type and mutant Sts-1_{PGM} as Flag-tagged proteins in HEK293T cells, and performed immune complex phosphatase activity assays on

anti-Flag immunoprecipitates. Although not all of the Sts-1_{PGM} mutants were expressed well (Fig. 1B: H565A/E490A, H380C/H565A, R462A/ H565A, and H380C/E490Q/ H464A), those that were lacked measurable catalytic activity (Fig. 1B).

To identify potential substrate-trapping Sts-1_{PGM} variants, we took advantage of two observations: first, the T-cell tyrosine kinsase Zap-70 has been identified as a potential in vivo Sts-1 substrate; and second, Sts-1_{PGM} can efficiently dephosphorylate Zap-70 and Zap-70-derived phosphopeptides in vitro [7,8]. We began by assessing the interaction between a Zap-70-derived phosphopeptide (GSVYESPpYSDPEEL) and the different Sts-1_{PGM} mutants described above. Pulldown assays were performed in which phosphopeptide-coupled beads were added to lysates prepared from cells expressing wild-type or mutant Sts-1_{PGM}, and bound proteins were assessed by immunoblot analysis. We observed that four Sts-1_{PGM} mutants interacted stably with the bound phosphopeptide, as shown by the fact that they could be isolated from the lysate (Fig. 1C). Interestingly, the four mutants appeared to interact with the phosphopeptide-coupled beads with substantially different affinities. Specifically, the Sts-1_{PGM} H380C (C) single mutant showed very weak interaction with the phosphopeptide-coupled beads, the Sts-1_{PGM} H380C/E490A (CA) and Sts-1_{PGM} H380C/E490Q (CQ) double mutants appeared to have the strongest affinity, and the Sts-1_{PGM} H380C/R462A/E490Q triple mutant was precipitated to an intermediate degree. Full-length Sts-1 variants also showed similar binding characteristics (data not shown), with Sts-1_{PGM} CA and Sts-1_{PGM} CQ showing better binding capabilities than Sts-1_{PGM} C. These results suggest that Sts-1_{PGM} CA and Sts-1_{PGM} CQ could serve as significantly better substrate-trapping enzymes than Sts-1_{PGM} C.

High-affinity interaction between a nonspecific small-molecule substrate and Sts-1_{PGM} mutants

We have recently characterized the phosphatase activity of Sts-1_{PGM} towards the artificial substrate 3-*O*-methyl-fluorescein phosphate (OMFP) and showed that active site residues are essential for OMFP hydrolysis [8]. To determine whether the high-affinity Sts-1_{PGM} mutants bound other phosphorylated molecules in addition to signaling proteins isolated from stimulated T-cell lysates, we employed isothermal titration calorimetry (ITC). A control binding reaction, isothermal titration of OMFP into maltose-binding protein (MBP), showed the heat of dilution throughout the course of the ITC assay (Fig. 2, left panel). In contrast, titration of OMFP into a solution of Sts-1_{PGM} CQ generated a typical binding isotherm, with upward peaks that signify an endothermic reaction (Fig. 2, right panel). A total of 27 injections were made, and by the 10th injection the system reached saturation, after which the isotherm showed only the heat of dilution. Curve fitting of the binding isotherm yielded an affinity constant (K_a) of Sts-1_{PGM} CQ for OMFP of $1.8 \times 10^5\ {}_{M}{}^{-1}$. The K_a of Sts-1_{PGM} C for OMFP was determined to be $3.8 \times 10^4\ {}_{M}{}^{-1}$, approximately five-fold lower than that of Sts-1_{PGM} CQ (Fig. 2, middle panel). The shape of the titration curve for Sts-1_{PGM} CQ suggests moderate binding affinity for OMFP, whereas the binding isotherm of Sts-1_{PGM} C suggests low binding affinity for OMFP [16]. These results demonstrate that introducing combined mutations at His380 and Glu490 in the PGM domain active site generates a catalytically inactive, high-affinity Sts-1_{PGM} mutant that binds the nonspecific substrate OMFP.

Sts-1 high-affinity mutants stably interact with Zap-70

To further evaluate the ability of Sts-1_{PGM} CA and Sts-1_{PGM} CQ to stably interact with a potential substrate, we turned to a cell-based system in which full-length Zap-70 is expressed in a nonlymphoid cell line under conditions that promote its tyrosine phosphorylation [17] (Fig. 3A). In this system, coexpression of wild-type Sts-1_{PGM} resulted in reduced phosphorylation of Zap-70, suggesting direct dephosphorylation of Zap-70 by

Sts-1_{PGM} (Fig. 3B). The stable interaction between Zap-70 and Sts-1_{PGM} mutants was evaluated by coimmunoprecipitation analysis. As shown in Fig. 3C, Sts-1_{PGM} CA and Sts-1_{PGM} CQ coimmuno-precipitated with a 70-kDa tyrosine-phosphorylated protein, which was confirmed to be Zap-70 by immu-noblot analysis. In contrast, Sts-1_{PGM} C and Sts-1_{PGM} H380C/R462A/E490Q very weakly coprecipitated with Zap-70. Likewise, Sts-1_{PGM} R462A/H565A, which tested negative in the peptide pull-down assay, also failed to coprecipitate with Zap-70. The converse analysis yielded similar results, namely Zap-70 coprecipitated with the substrate-trapping Sts-1_{PGM} CA and Sts-1_{PGM} CQ but not wild-type Sts-1_{PGM} (Fig. 3D). These results indicate that Sts-1_{PGM} CA and Sts-1_{PGM} CQ undergo stable, high-affinity interactions with full-length phosphorylated Zap-70. The fact that these binding reactions involve the minimal PGM domain of Sts-1 and not the protein-interacting UBA and SH3 domains present in the full-length protein suggests that the interaction could be mediated by the Sts-1 catalytic site (see below).

Sts-1 high-affinity mutants isolate Zap-70 and tyrosine-phosphorylated proteins from T-cell lysates

To further evaluate Sts-1_{PGM} CA and Sts-1_{PGM} CQ as high-affinity substrate interactors, we performed *in vitro* pulldowns from lysates of T cells. Wild type Sts-1_{PGM}, Sts-1_{PGM} C, Sts-1_{PGM} CA and Sts-1_{PGM} CQ were expressed as MBP fusion proteins and immobilized on amylose beads. Then, beads were incubated with lysates prepared from a T-cell line that had been treated with pervanadate to induce a generalized increase in the levels of tyrosine-phosphorylated intracellular proteins. In contrast to wild-type Sts-1_{PGM} and Sts-1_{PGM} C, Sts-1_{PGM} CA and Sts-1_{PGM} CQ precipitated numerous tyrosine-phosphorylated proteins from lysates of stimulated T cells (Fig. 4A top), with the strongest signal being detected from a 70-kDa band (marked by an asterisk). This band probably represents Zap-70, because a 70-kDa band corresponding to Zap-70 was detected in the lane containing proteins precipitated by Sts-1_{PGM} CQ (Fig. 4A, bottom left). Furthermore, serial depletion of Zap-70 prior to the Sts-1_{PGM} CQ pulldown significantly reduced the level of recovered Zap-70 (Fig. 4A, bottom right). When Jurkat T cells were stimulated with antibodies against CD3 to more specifically activate TCR signaling pathways, significantly lower levels of tyrosine-phosphorylated proteins than those in pervanadate-treated cells were found (data not shown). Nonetheless, when the binding capabilities of the Sts-1_{PGM} mutants were evaluated, Sts-1_{PGM} CA and Sts-1_{PGM} CQ again associated with phosphorylated Zap-70, whereas no binding was observed with wild-type Sts-1_{PGM} or Sts-1_{PGM} C (Fig. 4B). Additionally, when lysates from TCR-stimulated primary T cells were utilized, a number of tyrosine-phosphorylated proteins, including phosphorylated Zap-70, were observed to associate preferentially with Sts-1_{PGM} CA and Sts-1_{PGM} CQ relative to wild-type Sts-1_{PGM} (Fig. 4C). In order to determine whether individual tyrosine phosphorylation sites of Zap-70 are targeted equivalently by Sts-1, we compared the abilities of Sts-1_{PGM} CQ to interact with two mutants of Zap-70, Zap-70 Y319F and Y493F. As demonstrated in Fig. 4D, mutation of Tyr319 abrogated the ability of Sts-1 to interact with Zap-70, whereas mutation of Tyr493 had no significant effect. This suggests that Sts-1 does not target all phosphorylation sites of Zap-70 equally, and implies substrate specificity on the part of Sts-1 (Fig. 4D).

Inhibition of binding by a competitive inhibitor

To determine whether the catalytic pocket of the Sts-1_{PGM} mutants mediated the high-affinity interaction, we utilized a phosphate analog competitive inhibitor of the Sts phosphatase reaction. The crystal structure of a pentavalently coordinated vanadate molecule within the active site of an Sts family member has been presented [18]. In addition, vanadate has been used as a competitive inhibitor to verify the binding of substrates to the catalytic sites of substrate-trapping PTP mutants [11]. When increasing levels of vanadate were titrated into the binding reaction between Sts-1_{PGM} CQ and T-cell phosphorylated proteins,

we observed an inverse relationship between vanadate levels and the ability of Sts-1_{PGM} CQ to stably associate with phosphorylated Zap-70 (Fig. 5). This result suggests that phosphorylated Zap-70 interacts with Sts-1_{PGM} CQ through the PGM domain catalytic site.

Sts-1 substrate-trapping mutants block a TCR-induced signaling pathway

We then investigated the functional consequences of overexpressing high-affinity Sts-1 molecules, using a nuclear factor of activated T cells (NFAT)-driven luciferase reporter assay. Within T cells, NFAT activation occurs downstream of proximal TCR signaling events [19]. Consistent with the known function of wild-type Sts-1 as a negative regulator of proximal TCR signaling pathways, its overexpression in T cells reduced TCR-induced NFAT activation levels by 40% (Fig. 6). Conversely, the catalytically inactive Sts-1_{PGM} C failed to reduce NFAT activation levels. When the high-affinity Sts-1_{PGM} CA and Sts-1_{PGM} CQ were expressed, they reduced NFAT activation to levels below those observed in the presence of wild-type Sts-1 (Fig. 6A). However, when NFAT was activated through non-TCR-dependent pathways, via phorbol 12-myristate 13-acetate (PMA) and ionomycin, expression of wild-type and high-affinity mutant forms of Sts-1 had no effect on its activation level (Fig. 6B). These results suggest that high-affinity Sts-1_{PGM} mutants form stable complexes with proximal TCR signaling molecules such as Zap-70, and impair activation of downstream pathways by reducing the effective level of Zap-70 available to transmit downstream signals, thereby reducing the activation of downstream effectors.

Discussion

The substrate-trapping approach was originally devised to aid in the identification and characterization of native targets of PTPs [12]. In this study, we extended the substrate-trapping technique to enzymes of the HP superfamily. Specifically, Sts-1, a negative regulator of signaling pathways downstream of TCR and a member of the HP superfamily by virtue of a C-terminal PGM domain, was converted to a high-affinity substrate-interacting enzyme through combined mutation of the catalytic nucleophile and a proton-donating acidic residue within the PGM domain. Interestingly, whereas many PTPs can acquire trapping capacity following single mutation of either the catalytic cysteine or the signature aspartate within the WPD loop, Sts-1 clearly required dual mutation of both residues to function optimally as a high-affinity stable interactor.

By altering two catalytic residues within the active site of Sts-1_{PGM}, His380 and Glu490, we generated Sts-1 mutants that were catalytically inactive but retained strong substrate-binding affinities. In addition to interacting directly with Zap-70 in an HEK293T cell overexpression system, the high-affinity Sts-1 mutants Sts-1_{PGM} CA and Sts-1_{PGM} CQ isolated tyrosine-phosphorylated Zap-70 directly from complex lysates prepared from three types of activated T cell: pervana-date-treated Jurkat T cells, TCR-stimulated Jurkat T cells, and activated primary mouse T cells. We also observed that Sts-1_{PGM} CA and Sts-1_{PGM} CQ formed stable complexes with additional phosphorylated proteins, as shown by the presence of the latter in the pulldown complexes. It is unclear whether these interactions result from an ability to interact nonspecifically with a variety of tyrosine phosphoproteins. Nonetheless, our results strengthen the notion that Sts-1 can directly dephosphorylate Zap-70 in T cells. Together with the previously published observation that the Sts proteins bind directly to c-Cbl ubiquitin ligase, a protein that is known to be recruited into the TCR complex [20], these results support a model in which Sts-1 downregulates TCR signaling following its recruitment into the activated receptor complex. In such a model, Sts-1 would contribute directly to the inactivation of Zap-70. Among the Zap-70 phosphorylation sites that are probably targeted by Sts-1, Tyr319 could predominate, although it is important to remember that other positive and negative regulatory sites, including Tyr292, Tyr319, and Tyr493, each show enhanced phosphorylation in T cells lacking Sts-1 or both Sts-1 and Sts-2 [8].

Other enzymes recognized to be involved in the regulation of Zap-70 include three PTPs: Src homology phosphatase-1, low molecular weight PTP, and PTP, nonreceptor type 22 (lymphoid). Src homology phos-phatase-1 was found to participate in the negative feedback regulation of Zap-70, showing low phosphatase activity in its native state but increased phosphatase activity when bound to phosphorylated Zap-70 [21]. Low molecular weight PTP appears to activate Zap-70 by direct dephosphorylation of a negative regulatory tyrosine (Tyr292), in turn causing an increase in Zap-70 phosphorylation on other sites, presumably Tyr493 [22]. Interestingly, PTP, nonreceptor type 22 (lymphoid) has been found to dephosphorylate Zap-70 at the activating sites Tyr394 and Tyr493, but not Tyr319 [23]. Tyr319 is a critical regulatory tyrosine that is autophosphorylated upon TCR stimulation [24]. Phosphorylation at this site has been proposed to contribute to the destabilization of the autoinhibited conformation of Zap-70, and thus promote the extended, active conformation along with tyrosine phosphorylation of Tyr315, Tyr492, and Tyr493 [5]. The modulation of Zap-70 phosphorylation by multiple regulatory mechanisms that include three PTPs and both Sts proteins underscores the critical signaling role of Zap-70 downstream of the TCR. Further delineating the specificity shown by Sts-1_{PGM} for other Zap-70 tyrosine phosphorylation sites is an important future aim.

The Sts-1 high-affinity mutants reported here may interest those looking into the role of Sts-1 in other signaling pathways. Sts-1 has been implicated in the dephosphorylation of the tyrosine kinase Syk, in glyco-protein VI signaling in platelets [25,26], and in FcεRI signaling in mast cells [27]. We also aim to use the substrate-trapping approach in our studies with the hematopoietic cell-specific Sts-2, to investigate the mechanisms by which it regulates Zap-70 phosphorylation in T cells. The approach reported here may also be of use to those studying other HPs involved in cellular signaling. One interesting example is the mitochondrial phosphoglycerate mutase 5, which belongs to the PGM branch of the HP superfamily and has recently been characterized as a serine/threonine phosphatase regulating mitogen-activated protein kinase pathways [28]. Phosphoglycerate mutase 5 has also been implicated in mammalian oxidative stress responses involving the adaptor Keap1 [29], and multiple necrotic death pathways involving the serine/threonine kinases receptor-interacting protein 1 and receptor-interacting protein 3 [30].

Collectively, our data validate a molecular mechanism by which Sts-1 negatively regulates TCR signaling pathways: through dephosphorylation of Zap-70 mediated by its C-terminal PGM domain. How the UBA and SH3 domains affect Sts-1_{PGM} activity towards Zap-70 are interesting areas for future study.

Experimental procedures

Cloning and site-directed mutagenesis of Sts-1_{PGM} and full-length Sts-1

To generate candidate substrate-trapping mutants, Sts-1_{PGM} coding sequences previously cloned in the pProEx HTb vector [31] were subcloned into pBluescript and used as DNA templates for two-step PCR-based mutagenesis. This procedure was performed for all of the single mutants (Fig. 3B), except Sts-1_{PGM} C, which was generated in a single-step PCR with a forward primer that incorporated the mutation and a T7 reverse primer. The two-step PCR method was repeated to introduce each additional mutation in the double and triple mutants of Sts-1_{PGM}, with specific single-mutant and double-mutant constructs as PCR templates, respectively. All constructs were sequenced to verify that no additional mutations were introduced. Mammalian expression constructs encoding Flag-tagged wild-type and mutant Sts-1_{PGM} were generated by PCR amplification with a forward primer that contained the Kozak sequence (5′-CTCAGTTCTAGAGAATTCGCCGCCACCATGGGA CCCCAGAAGCGATGA-3′) and a reverse primer that encoded a stop codon and Flag epitope coding sequences. The Kozak–Sts-1_{PGM}–Flag cDNAs were then subcloned via an

*Afe*I site into a pmaxGFP vector (Lonza Group) that was modified to encode an internal ribosome entry site–green fluorescent protein (GFP) coding sequence in place of GFP coding sequences. Full-length Sts-1 mutants were subcloned by appropriate restriction site engineering.

Recombinant protein expression and purification

Bacterial expression constructs of wild-type Sts-1_{PGM}, Sts-1_{PGM} C, Sts-1_{PGM} CA and Sts-1_{PGM} CQ were subcloned into pMAL-c4X plasmids (New England Biolabs). MBP–Sts-1_{PGM} fusion proteins were expressed in *Escherichia coli* K12 cells, and purified by affinity column chromatography, as previously described [8].

Mammalian cell culture and transient transfections

HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/ streptomycin. Expression constructs were transiently transfected into HEK293T cells by calcium phosphate-mediated transfection or with Lipofectamine. Jurkat T cells were routinely cultured in RPMI-1640 (Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. At least 2 days prior to transfection, Jurkat cell cultures were switched to antibiotic-free high-glucose RPMI medium (Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum, and maintained at low cell densities ($0.2–0.5 \times 10^6$ cells-mL^{-1}). The cells were transfected by use of the Amaxa Cell Line Nucleofector Kit V (Lonza Group), according to the manufacturer's protocol.

T-cell stimulation for substrate-trapping pulldowns

Jurkat T cells were stimulated with antibodies against CD3 as described previously [7]. Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM sodium orthovandate, 1% NP-40, and 10% glycerol, and lysates were cleared by centrifugation at 12 000 *g*, for 20 min. Supernatants containing phosphotyrosyl proteins were stored at −80 °C. For pervanadate stimulation, 60×10^6 Jurkat T cells were resuspended in fresh medium supplemented with 1 mM pervanadate and incubated at 37 °C for 15 min. Primary mouse T-cell cultures were established and stimulated with antibodies against CD3 as previously described [6]. Animals from which T cells were obtained were housed and bred under specific pathogen-free conditions according to institutional guidelines. All animal work was conducted under guidelines established and approved by the Stony Brook Institutional Animal Care and Use Committee.

Immunoprecipitation and immunoblotting

For immunuprecipitations, cleared lysates were rotated at 4 °C with specific antibody for 1 h and 50 μL of protein A-Sepharose beads for another hour, and washed three times with lysis buffer. Bound proteins were eluted with Laemmli sample buffer. Immunoprecipitates were resolved by SDS/PAGE, and then transferred to nitrocellulose membranes with a semidry transfer apparatus (Bio-Rad Laboratories). Blots were blocked with 3% BSA in NaCl/Tris (pH 8), incubated with primary antibody for 1 h at room temperature or overnight at 4 °C, washed with NaCl/Tris, incubated with IRDye-conjugated secondary antibody, and detected with the ODYSSEY Infrared Imaging System (LI-COR Biosciences). The antibodies used were: antibody against Flag (Sigma), antibody against pTyr (4G10; Millipore), mAb against Zap (1E7.2; Santa Cruz Biotechnologies), antibody against Zap-70 pY-319 and antibody against Zap-70 pY-493 (Cell Signaling Technology), and antibody against MBP (NEB).

In vitro phosphatase assays

Flag-M2 immunoprecipitates from HEK293T cell lysates were immobilized on protein A-Sepharose beads, washed three times with assay buffer [25 mM Hepes (pH 7.2) or 50 mM Mes (pH 6.0), 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA, 0.1 mg mL^{-1} BSA], and mixed with 100 μL 0.5 mM fluorescein diphosphate (FDP). The end-point assays were conducted at 37 °C for the durations specified in the figure legends. Reactions were stopped by incubation on ice for 5 min. After brief centrifugation at 12 000 *g*, supernatants were collected and transferred to 96-well flat-bottomed plates. Absorbances of the supernatants at 477 nm were measured with a SpectraMax 190 Microplate reader (Molecular Devices) to quantify phosphate hydrolysis. Laemmli sample buffer was added to the beads to elute the anti-Flag immunoprecipitates, and levels of Sts-1_{PGM} in the assays were determined by anti-Flag immunoblotting. Assays were performed at least twice, and either empty vector transfections or no-enzyme controls were used as blanks.

Phosphopeptide pulldown assay

A 14-residue phosphorylated peptide derived from Zap-70 (GSVYESPpYSDPEEL, synthesized by Celtek Bioscience, LLC) was reconstituted to a concentration of 15 mg mL^{-1} in Affi-Gel buffer (20 mM Mes, pH 6.0, 0.5 mM EDTA, 150 mM NaCl, 2 mM β-mercaptoethanol, 10% glycerol, 0.05% Triton X-100). Then, the phosphopeptide was cross-linked to Affi-Gel 15 affinity medium (Bio-Rad Laboratories) by mixing 1 mL of the phosphopeptide solution with 500 μL of gel, according to the manufacturer's protocol. Beads crosslinked to the phosphopeptide or blocked with ethanolamine were resuspended to 33% slurries with Affi-Gel buffer, and stored at 4 °C. Phosphopeptide pulldowns were performed by mixing 50 μL of the slurries with 1 mL of HEK293T cell lysates containing Flag-tagged Sts-1_{PGM} or full-length Sts-1 constructs, for 2 h at 4 °C with rotation. Samples were then washed three times with lysis buffer or Affi-gel buffer, and boiled in 30–60 μL of sample buffer to elute captured proteins.

ITC experiments

Purified MBP–Sts-1_{PGM} fusion proteins were dialyzed overnight against ITC buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2% glycerol, 1 mM β-mercaptoethanol); OMFP was prepared in the same buffer. Final protein concentrations were determined by resolving the purified MBP proteins by SDS/PAGE alongside BSA concentration standards. Isothermal titrations were conducted at 25 °C in a VP-ITC microcalorimeter (MicroCal, LLC), with 10 μM each purified protein against 0.4 mM OMFP. Twenty-seven injections of the OMFP titrant were made at 5-min intervals: an initial 2- μL injection followed by 26 10- μL injections. Binding parameters were determined by fitting the data to a one-site binding model with MICROCAL ORIGIN.

Substrate-trapping pulldowns

Bacterial lysates containing MBP fusion proteins were mixed with 100 μL of amylose resin for 2 h with rotation at 4 °C in order to immobilize the MBP–Sts-1_{PGM} fusion proteins on beads. The beads were collected by centrifugation at 800 *g* for 2 min, washed three times with column buffer, resuspended with 200 μL of column buffer to make a 33% slurry, and stored at 4 °C.

To perform substrate-trapping pulldowns, the amounts of MBP fusion protein in the slurries were first evaluated with SDS/PAGE and Coomassie staining; the volumes of slurry used for pull-downs were adjusted accordingly to equalize the amount of bait between samples. Bait slurries were mixed with activated T-cell lysates in microcentrifuge tubes for 2 h with rotation at 4 °C. Beads were then collected by centrifugation at 800 *g* for 2 min, washed three to six times with lysis buffer, and boiled in 60 μL of sample buffer to elute the

captured proteins. Bound proteins were resolved with SDS/PAGE and analyzed with immunoblotting, as specified in the figure legends. For depletion analysis (Fig. 4A), lysates were depleted of Zap-70 by successive immunoprecipitation with anti-phospho Zap-pY319/pY493 antibodies prior to pulldown analysis.

Luciferase assays

Jurkat T cells (5×10^6 cells) were transfected with 5 μg of an NFAT–luciferase reporter construct and 10 μg of empty vector (pCDNA3), wild-type Sts-1, Sts-1_{PGM} C, Sts-1_{PGM} CA or Sts-1_{PGM} CQ plasmid. As a transfection efficiency control, a *Renilla* luciferase plasmid was included at a concentration 50 times lower than that of the experimental reporter. Twenty-four hours post-transfection, cells were plated on 96-well plates coated with antibody against CD3 at a density of 1×10^6 cells mL^{-1} in a final volume of 200 μL. After 8 h of stimulation, cells were harvested, and lysates were assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. Luminescence readings were carried out in a Lumat LB 9507 luminometer (EG&G Berthold). For stimulation with PMA and ionomycin, transfected Jurkat T cells were resuspended at a density of 1×10^6 cells mL^{-1} in medium containing 50 ng mL^{-1} PMA and 1 μM ionomycin, and plated on 96-well plates, also in a final volume of 200 μL per well. After 6 h of stimulation, cells were harvested, and lysates were assayed for luciferase activity as described.

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Abbreviations

C	H380C
CA	H380C/E490A
CQ	H380C/E490Q
FDP	fluorescein diphosphate
GFP	green fluorescent protein
HP	histidine phosphatase
ITC	isothermal titration calorimetry
MBP	maltose-binding protein
NFAT	nuclear factor of activated T cells
OMFP	3-*O*-methylfluorescein phosphate
PMA	phorbol 12-myristate 13-acetate
PTP	protein tyrosine phosphatase
Sts	suppressor of T-cell receptor signaling
Sts-1_{PGM}	suppressor of T-cell receptor signaling-1 PGM domain
Sts-2_{PGM}	suppressor of T-cell receptor signaling-2 PGM domain
TCR	T-cell receptor

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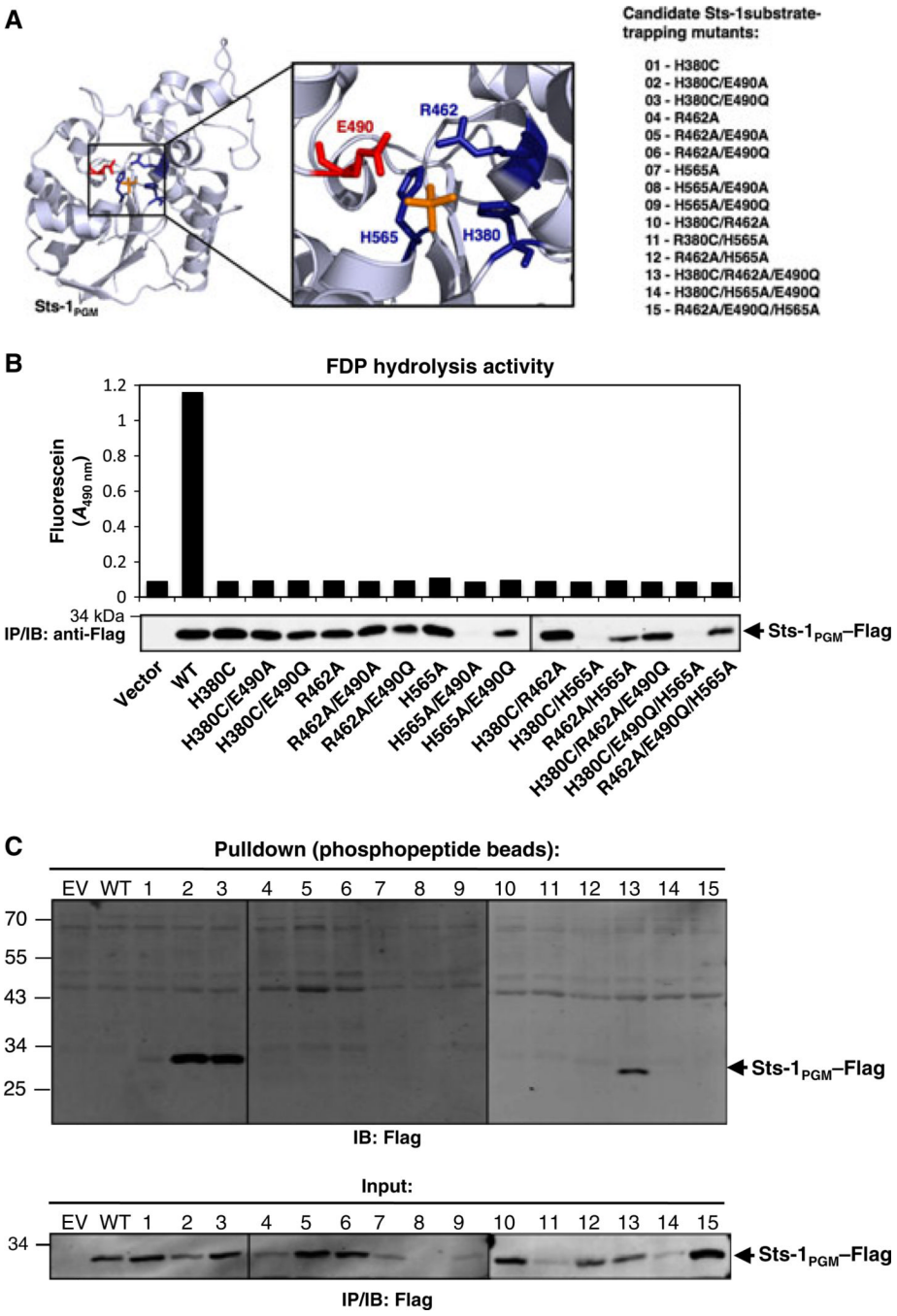

Fig. 1.

Development of Sts-1 high-affinity mutants. (A) Representation of conserved active site residues in Sts-1_{PGM}, generated in PYMOL with the crystal structure of Sts-1_{PGM} complexed with phosphate (Protein Data Bank ID: **2IKQ**). A total of 15 candidate Sts-1 substrate-trapping mutants were generated by site-directed mutagenesis of His380 to cysteine (H380C), Arg462 to alanine (R462A), and His565 to alanine (H565A), singly or in combination with mutations targeting the proton donor Glu490 (E490A or E490Q). (B) Phosphatase activity assay of candidate substrate-trapping mutants. Flag-tagged wild-type (WT) or mutant Sts-1_{PGM} was expressed in HEK293T cells, immunoprecipitated with antibody against Flag, immobilized on protein A–Sepharose beads, and evaluated for FDP

phosphatase activity. The FDP assay was carried out at 37 °C for 15 min with 0.5 m_M FDP, and this was followed by detection of the fluorescein product in the supernatants by measuring the absorbance at 490 nm. Sts-1_{PGM} expression levels were evaluated by immunoblotting (IB) with mAb against Flag. (C) A phosphopeptide pulldown assay with lysates of HEK293T cells transfected with empty vector (EV), wild-type Sts-1_{PGM} (WT), or mutant Sts-1_{PGM}s corresponding to the list in (A) (lanes 1–15). Protein complexes were resolved by SDS/PAGE and immunoblotted with antibodies against Flag. The level of Sts-1_{PGM} in the input was determined by analysis of a fraction of the lysate used in the pulldown assay (lower blot). As controls, empty beads (agarose blocked with ethanolamine) were used against an equal volume of the HEK293T lysates; they showed no interaction (not shown). IP, immunoprecipitation.

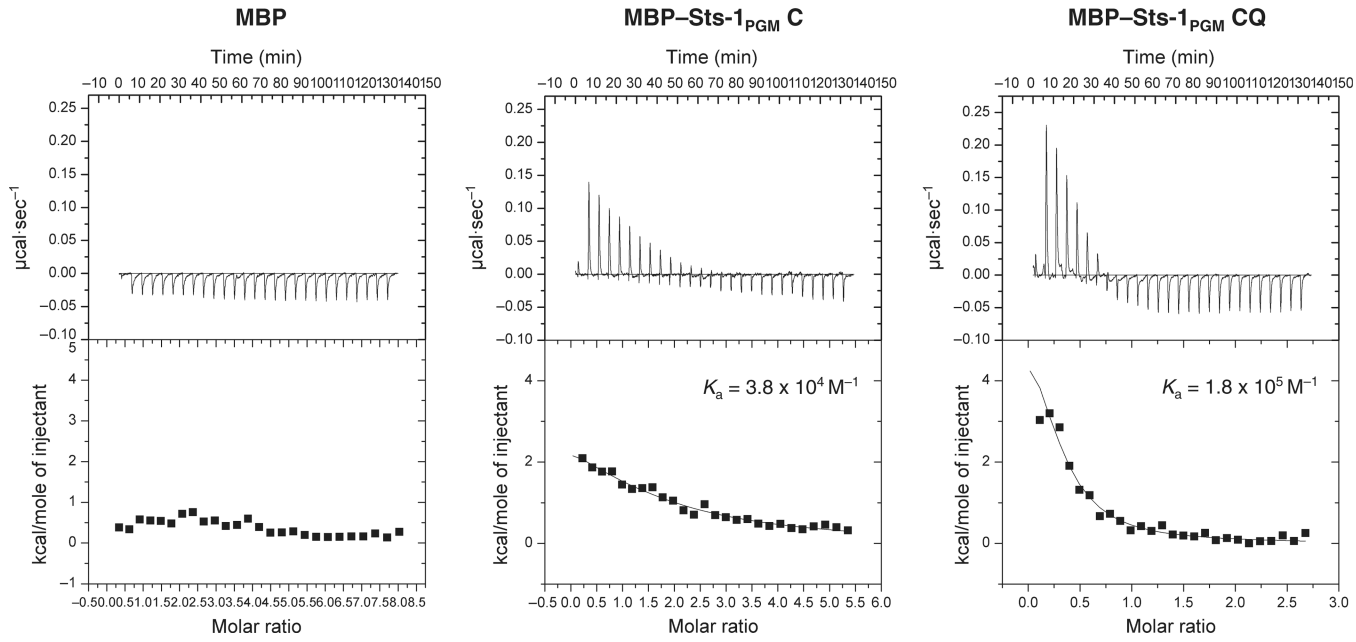

Fig. 2.

Sts-1_{PGM} CQ stably interacts with a small-molecule substrate. Calorimetric titrations of purified recombinant MBP (left), MBP–Sts-1_{PGM} C (middle) and MBP–Sts-1_{PGM} CQ (right) with OMFP. The binding affinities obtained from this analysis are indicated: Sts-1_{PGM} C, $K_a = 3.8 \times 10^4\ _M{}^{-1}$; Sts-1_{PGM} CQ, $K_a = 1.75 \times 10^5\ _M{}^{-1}$.

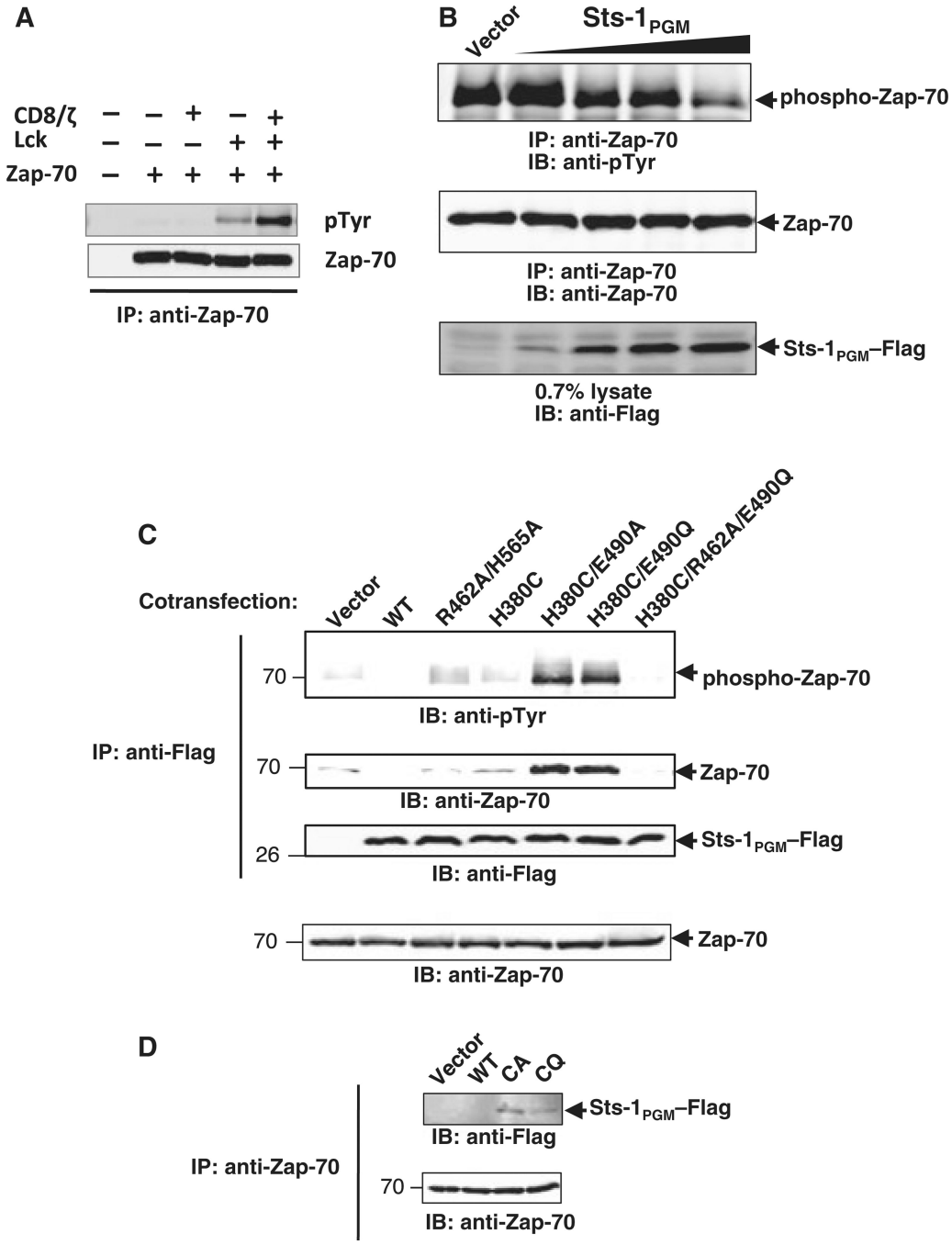

Fig. 3.

Interaction of Sts-1_{PGM} high-affinity mutants and the T-cell tyrosine kinase Zap-70. (A) HEK293T cells were transfected with cDNA encoding Zap-70, singly or in combination with constructs encoding Lck and CD8/ζ (a chimeric protein containing the extracellular and transmembrane domains of the CD8 coreceptor and the cytoplasmic domain of TCRζ). Anti-Zap-70 immunoprecipitates were immunoblotted against mAb against pTyr (4G10) to determine phosphorylation levels, and against antibody against Zap-70 to verify equal Zap-70 expression in cells. (B) Expression plasmids for Zap-70, CD8/ζ and Lck were cotransfected into HEK293T cells along with either empty vector or constructs encoding Sts-1_{PGM} at increasing plasmid DNA concentrations. Anti-Zap-70 immunoprecipitates were

analyzed by immunoblotting to determine Zap-70 tyrosine dephosphorylation by Sts-1_{PGM}. (C) HEK293T cells were transfected with Zap-70, Lck, and CD8/ζ, along with Flag-tagged wild-type Sts-1_{PGM} or mutants. Twenty-four hours post-transfection, cells were lysed, and protein complexes were assessed by immunoprecipitation (IP)/immunoblot (IB) analysis. Flag immunoprecipitates were immunoblotted with antibodies against pTyr and Zap-70 to detect Zap-70, and with antibodies against Flag to assess Sts-1_{PGM} levels. Levels of Zap-70 were assessed by IP/IB analysis (lower panel). (D) The converse IP analysis detected Sts-1_{PGM} coprecipitating with antibodies against Zap-70.

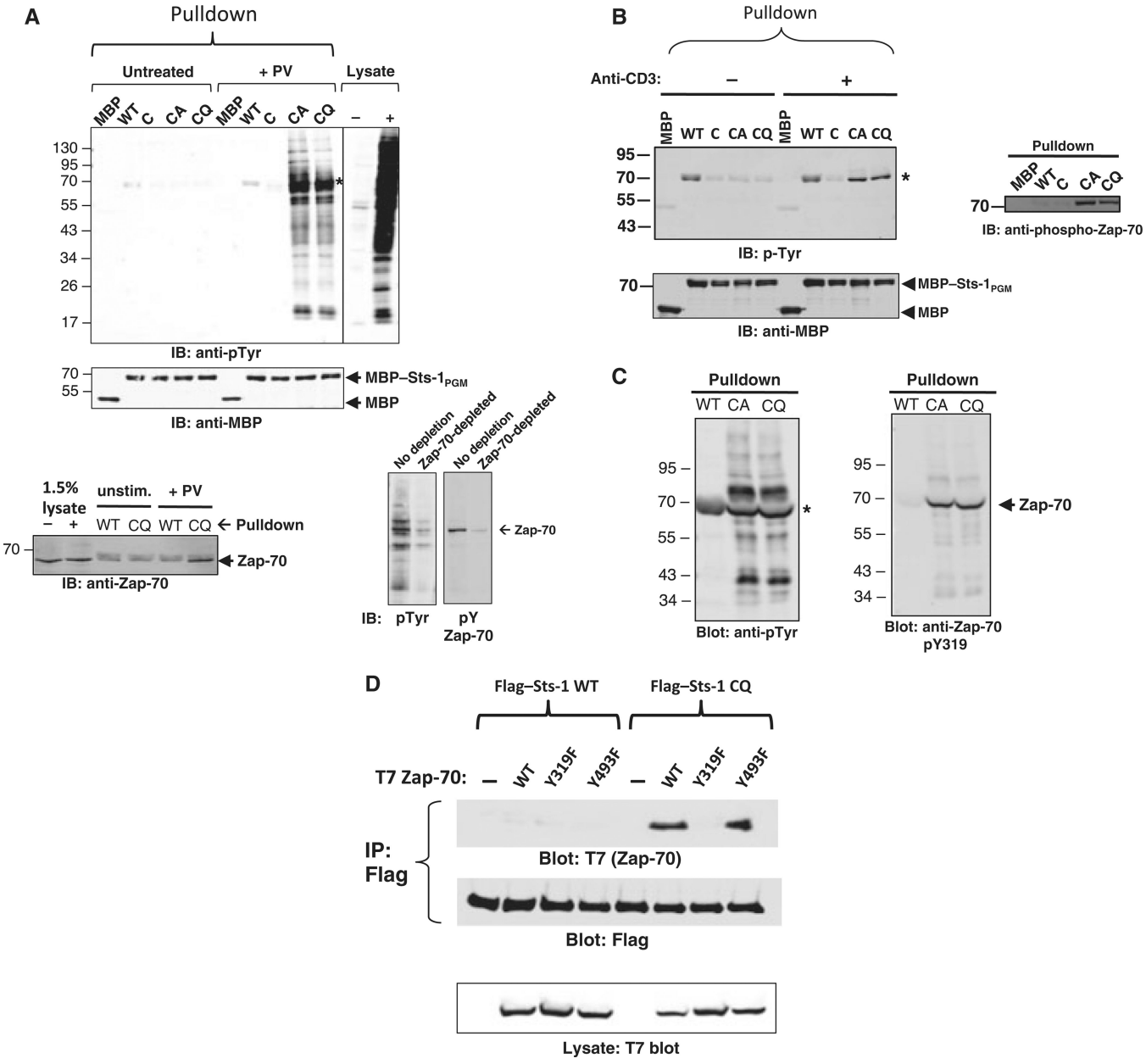

Fig. 4.

Isolation of tyrosine-phosphorylated proteins by high-affinity Sts-1_{PGM} mutants. (A) Lysates of Jurkat T cells treated with pervanadate (+PV) or left untreated were incubated with equal levels of MBP or MBP fusions of wild-type Sts-1_{PGM} (WT), Sts-1_{PGM} C, and the high-affinity mutants Sts-1_{PGM} CA and Sts-1_{PGM} CQ that had been immobilized on amylose beads. Bound-tyrosine phosphorylated proteins were analyzed by anti-pTyr immunoblotting (IB). The last two lanes contain 12.5% of untreated (−) and treated (+) Jurkat lysates. The prominent 70-kDa tyrosine-phosphorylated band that binds to Sts-1_{PGM} CA/Sts-1_{PGM} CQ contains Zap-70, as revealed by IB analysis with specific antibodies (bottom left), and depletion analysis in which Zap-70 was depleted from the lysate prior to Sts-1_{PGM} CQ pulldown (bottom right). (B) Similar analysis as in (A), performed with lysates derived from TCR-stimulated Jurkat T cells. Phosphorylated Zap-70 was recognized with a cocktail of phosphorylation site-specific antibodies recognizing Zap-70 pTyr319,

pTyr292, and pTyr493. (C) Similar analysis as in (A), performed with lysates derived from TCR-stimulated primary mouse T cells isolated from *Sts-1/2*$^{-/-}$ mice. (D) Interaction of Sts-1_{PGM} CQ is abrogated in the absence of Zap-70 Tyr319 phosphorylation. Full-length wild-type Sts-1 and Sts-1_{PGM} CQ were coexpressed with the indicated Zap-70 mutants, and evaluated for trapping abilities. The data presented are representative of three independent experiments. IP, immunoprecipitation.

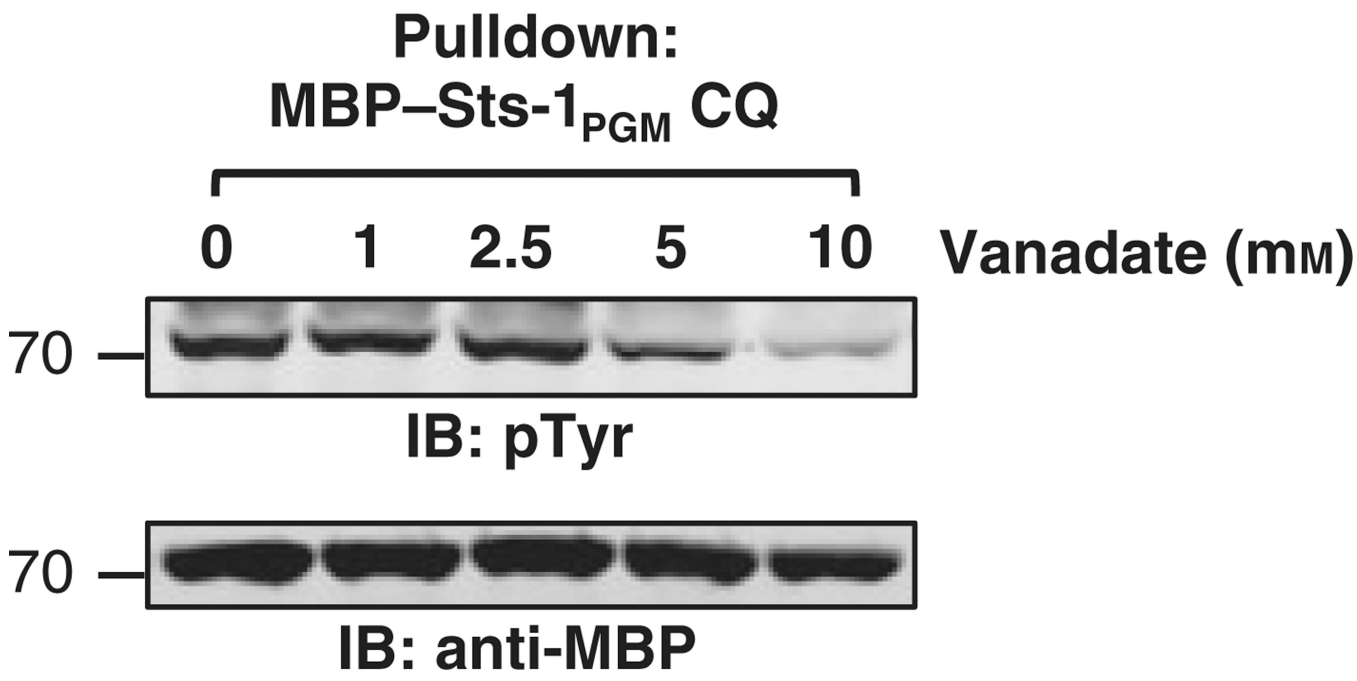

Fig. 5.

Vanadate inhibits the stable interaction of Sts-1_{PGM} CQ with phosphorylated Zap-70. Complex formation between the high-affinity Sts-1_{PGM} mutant and phosphorylated Zap-70 in the presence of increasing concentrations of vanadate was assessed by pulldown assay. Samples were analyzed by immunoblotting (IB) with antibodies against pTyr and MBP.

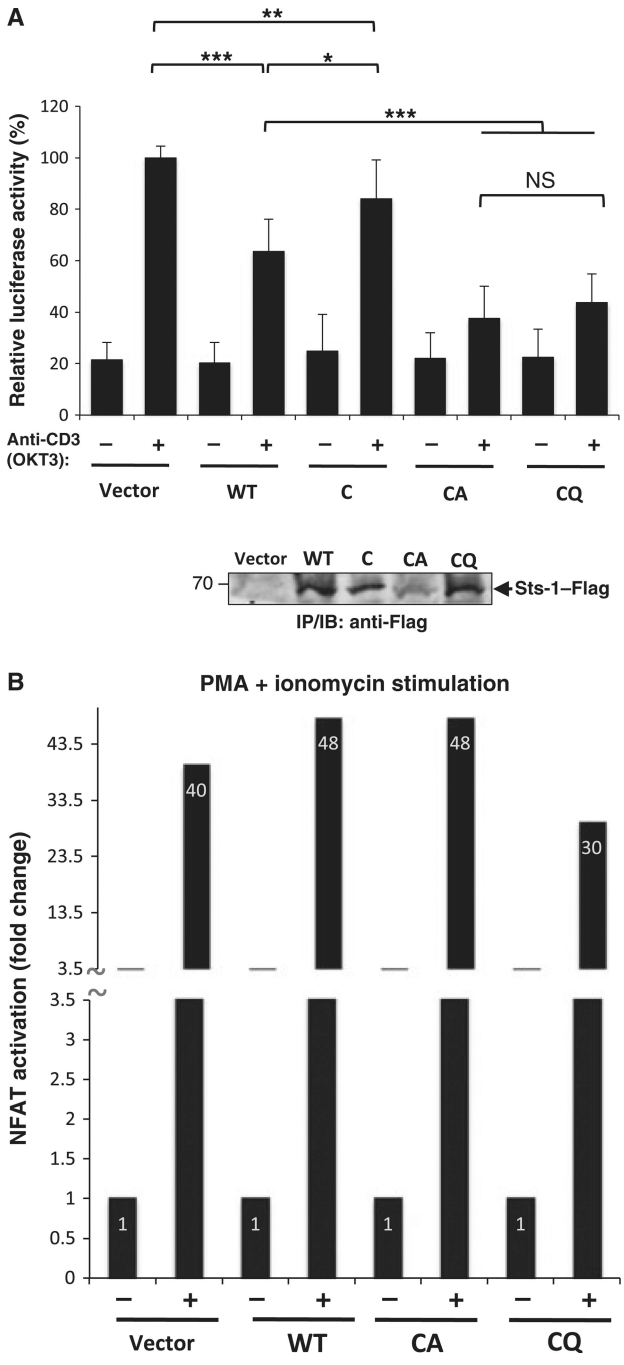

Fig. 6.

High-affinity substrate-trapping mutants inhibit TCR signaling. (A) Jurkat T cells were cotransfected with an NFAT-luciferase reporter construct, and plasmids encoding either Flag-tagged, full-length wild-type Sts-1 (WT), Sts-1_{PGM} C, Sts-1_{PGM} CA, or Sts-1_{PGM} CQ. Following 8 h of TCR stimulation, cells were harvested, and reporter activity was determined. Error bars are standard deviations from three to four independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$, where P is the probability associated with a Student's paired *t*-test, with a two-tailed distribution. NS, not significant. (B) High-affinity Sts-1 mutants fail to regulate signaling when the TCR is bypassed. Jurkat cells were

transfected with a firefly luciferase reporter construct under the control of NFAT binding sequences, a *Renilla* luciferase reporter construct under the control of a constitutive promoter, and plasmids encoding Flag-tagged, full-length wild-type Sts-1 (WT), or the substrate-trapping mutants Sts-1_{PGM} CA or Sts-1_{PGM} CQ. At least 20 h post-transfection, the cells were stimulated with PMA (50 ng mL^{-1}) plus 1 μ_M ionomycin. Levels of NFAT reporter activation were analyzed by luciferase assay. Bar graphs represent luciferase activities relative to unstimulated Jurkat transfections.