New insights into the catalytic mechanism of histidine phosphatases revealed by a functionally essential arginine residue within the active site of the Sts phosphatases

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Abstract

Sts (suppressor of T-cell receptor signalling)-1 and Sts-2 are HPs (histidine phosphatases) that negatively regulate TCR (T-cell receptor) signalling pathways, including those involved in cytokine production. HPs play key roles in such varied biological processes as metabolism, development and intracellular signalling. They differ considerably in their primary sequence and substrate specificity, but possess a catalytic core formed by an invariant quartet of active-site residues. Two histidine and two arginine residues cluster together within the HP active site and are thought to participate in a two-step dephosphorylation reaction. To date there has been little insight into any additional residues that might play an important functional role. In the present study, we identify and characterize an additional residue within the Sts phosphatases (Sts-1 Arg^{383} or Sts-2 Arg^{369}) that is critical for catalytic activity and intracellular function. Mutation of Sts-1 Arg^{383} to an alanine residue compromises the enzyme's activity and renders Sts-1 unable to suppress TCR-induced cytokine induction. Of the multiple amino acids substituted for Arg^{383}, only lysine partially rescues the catalytic activity of Sts-1. Although Sts-1 Arg^{383} is conserved in all Sts homologues, it is only conserved in one of the two sub-branches of HPs. The results of the present study highlight an essential role for Sts-1 phosphatase activity in regulating T-cell activation and add a new dimension of complexity to our understanding of HP catalytic activity.

Keywords

histidine phosphatase; phosphoglycerate mutase; suppressor of T-cell receptor signalling (Sts); T-cell receptor signalling (TCR signalling)

INTRODUCTION

Within the mammalian immune system, T-cells play a central role in the recognition and elimination of invasive microorganisms [1]. They fight foreign pathogens both directly, by eliminating cells that harbour intracellular microbes, or indirectly, by mobilizing and activating other cellular components of the immune system through the production of

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numerous cytokines [2]. However, they also pose a potential danger to their host in that many of the properties that make them effective mediators of the immune response can be turned inadvertently against host tissue [3–5]. The potent immunosuppressive properties of T-cells are activated following stimulation of the TCR (T-cell receptor). The TCR activates numerous biochemical pathways within T-cells that can ultimately lead to T-cell proliferation, cytokine secretion and cytolytic activity [6]. Not surprisingly, T-cells have evolved a variety of mechanisms to ensure that the TCR-mediated targeting of foreign microbes occurs with remarkable fidelity and selectivity [7,8]. One such mechanism involves multiple overlapping levels of positive and negative regulation within the biochemical signalling networks downstream of the TCR [9–11]. Disruption of this regulation has been linked to numerous pathologies, ranging from immune-deficiencies to autoimmune disorders [12,13]. Developing targeted therapeutics that can ameliorate immune disorders requires fully understanding the many regulatory mechanisms that underlie the control of T-cell activation thresholds.

Two homologous proteins, Sts (suppressor of T-cell receptor signalling)-1 and Sts-2, have been shown to negatively regulate TCR signalling pathways in a redundant fashion [14]. The importance of the Sts proteins in regulating lymphocyte responses is shown by the fact that mice lacking both Sts-1 and Sts-2 have hyper-reactive T-cells and an increased susceptibility to autoimmunity in a mouse model of the human disease multiple sclerosis [15]. Several genome-wide association studies have also underscored the connection between the Sts proteins and a variety of different autoimmune disorders, such as diabetes, arthritis, vitiligo and coeliac disease [16–19]. Although the exact intracellular mechanism(s) of action of the Sts proteins have yet to be resolved, it has become clear that the Sts proteins are members of a superfamily of enzymes known as HPs (histidine phosphatases) [20]. The Sts phosphatase domain is located in the C-terminal portion of each molecule, with Sts-1 having a significantly higher *in vitro* phosphatase activity than Sts-2 towards artificial substrates [21,22]. The Sts proteins are distinct among other HPs in having unique protein–protein interaction domains that contribute to their function. The current models of Sts function suggest these domains play key roles in localizing the Sts proteins to specific intracellular regions or in targeting the phosphatase domain to specific intracellular substrates [14,23].

HPs comprise a large superfamily of diverse enzymes whose origins are evolutionarily ancient [20]. The superfamily is divided into two branches, with Branch 1 consisting of PGM (phosphoglycerate mutase)-like enzymes and Branch 2 consisting of enzymes known as AcPs (acid phosphatases) [24]. The Sts proteins themselves are in Branch 1 of the HPs, with such diverse enzymes as PGM (EC 5.4.2.1), fructose-2,6-bisphosphatase (EC 3.1.3.46), glucose-1-phosphatase (EC 3.1.3.10), lysophosphatidic AcP (EC 3.1.3.2) and prostatic AcP (EC 3.1.3.48) all being included within the superfamily [20]. Despite large differences in their primary amino acid sequence, overall tertiary structure and substrate specificity, all HPs possess four invariant amino acids. In particular, two histidine and two arginine residues adopt a signature conformation within the phosphatase active site and are required for enzyme activity. One histidine residue serves as the nucleophile during the first step of the reaction, whereas the second histidine and the two invariant arginines cluster together around the nucleophile [25]. Among other functions, the latter three amino acids are thought to play an important role in correctly orientating the substrate for hydrolysis and interacting with water molecules within the active site [26]. To date, studies characterizing the mechanism of HP catalytic activity have focused on the role of these four residues. Indeed, there have been few published studies describing additional amino acids within the phosphatase active site that are essential for catalytic activity and intracellular function. In the present study, we describe an essential role for an additional arginine residue found within the Sts active sites, Sts-1Arg^{383} and Sts-2 Arg^{369}. This arginine residue is conserved

in all Sts homologues and in Branch 2 AcPs, but is absent in the vast majority of Branch 1 enzymes. Interestingly, we find that although mutation of Sts-1 Arg[383] reduces, but does not eliminate, *in vitro* phosphatase activity, the presence of Arg[383] is absolutely required for the negative regulatory function of Sts-1 in T-cells. These results provide further insight into the Sts-mediated regulation of the T-cell immune response and add a new dimension to our understanding of HP catalytic activity.

EXPERIMENTAL

Cells, cDNA mutants and transfection

HEK (human embryonic kidney)-293 cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin. All cDNA mutants were constructed by PCR mutagenesis and sequenced to confirm the presence of only the desired mutation. Expression plasmids were transfected into HEK-293 cells using calcium phosphate-based transfection. Jurkat cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. At least 2 days before transfection, the Jurkat cells were switched to an antibiotic-free high-glucose RPMI 1640 medium (Invitrogen A10491) containing 4.5 g/l D-glucose, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM Hepes and 300 mg/l L-glutamine, supplemented with 10% FBS, and grown at a density of 0.5×10^6 cells/ml. The Jurkat cells were transfected using the Amaxa Cell Line Nucleofector Kit V (Lonza) following the manufacturer's protocol.

Immunoprecipitation and immunoblotting

For immunoprecipitation, transfected HEK-293 cells were lysed in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA and 1% Triton X-100] and cleared by centrifugation for 15 min at 20000 *g*. Lysates were mixed with an anti-FLAG antibody (Sigma) for at least 1 h and Protein A–Sepharose beads for another hour. Beads were washed three times with ice-cold lysis buffer and three times with room temperature (23°C) phosphatase assay buffer. Immunoprecipitates were then used for phosphatase assays using pNPP (*p*-nitrophenyl phosphate), OMFP (3-*O*-methylfluorescein phosphate) or a Zap-70 (ζ-associated protein of 70 kDa)-derived phosphopeptide (GSVYESPpYSDPEEL; Celtek Peptides) as substrates. For immunoblotting, lysates or immunoprecipitates were resolved by SDS/PAGE (10% gels) and electrotransferred on to nitrocellulose membranes. After blocking with 3% BSA in Tris-buffered saline, the blots were probed with anti-FLAG antibodies followed by infra-red dye-conjugated secondary antibodies. Signals were detected using the Odyssey Infrared Imaging System (LI-COR).

In vitro phosphatase assay

FLAG-tagged Sts-1 or Sts-2 proteins transiently expressed in HEK-293 cells were immunoprecipitated and immobilized on Protein A–Sepharose beads. The immunoprecipitates were incubated in a 100 μl reaction mixture containing 25 mM Hepes (pH 7.2), 50 mM NaCl, 5 mM DTT (dithiothreitol), 2.5 mM EDTA, 0.1 mg/ml BSA and 0.5 mM of the substrate (pNPP, OMFP or Zap-70 phosphopeptide) for 15 min (Sts-1) or 2 h (Sts-2) at 37°C. Phosphate hydrolysis of pNPP and OMFP were determined by measuring absorbance at 405 nm and 477 nm respectively. Phosphopeptide hydrolysis was determined using the Malachite Green Phosphatase Assay Kit (Echelon Biosciences) following the manufacturer's protocol. Continuous assays using a recombinant Sts-1 HP domain (Sts-1$_{HP}$) fused to MBP (maltose-binding protein) were performed as described previously [22]. The results of the present study are all representative of multiple experiments with two or three replicates per condition per experiment.

Zap-70 dephosphorylation assay

To assess Zap-70 dephosphorylation *in vivo*, 15 μg of Sts-1 expression constructs were co-transfected with plasmids (also 15 μg each) encoding Lck (lymphocyte-specific protein tyrosine kinase), Zap-70 and a CD8-ζ chain chimaera into HEK-293 cells. Zap-70 was immunoprecipitated from the lysates and the level of Zap-70 tyrosine phosphorylation was determined by immunoblotting with the 4G10 antibody (Millipore).

Luciferase assay

Jurkat cells were transfected with Sts-1 expression plasmids, a firefly luciferase expression construct driven by NFAT (nuclear factor of activated T-cells)-binding sequences and a *Renilla* luciferase construct for normalization. At 24 h post-transfection, cells were stimulated by plating on to OKT3-coated 96-well plates (Biolegend) at a density of 0.2×10^6 cells per well for 8 h, after which luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega).

T-cell culture, retroviral infection and intracellular cytokine analysis

Naïve peripheral T-cells were obtained from *Sts-1/2^{−/−}* mice. The mice were housed and bred under specific pathogen-free conditions according to institutional guidelines. The animal work was conducted under guidelines established and approved by the Stony Brook IACUC (Institutional Animal Care and Use Committee). To obtain T-cells, dissected spleens were crushed in PBS containing 2% FBS, the red blood cells were lysed by the addition of ACK lysis buffer (pH 7.2) and the debris was removed by straining through a 70 μM filter (Becton Dickinson). Splenocytes were cultured for 24 h in the presence of 1 μg/ml anti-CD3 antibody (145-2C11; BD Biosciences) and 1 unit/ml IL-2 (interleukin 2; Peprotech) and then spin-infected with a retrovirus carrying a bicistronic cassette expressing the gene of interest and GFP (green fluorescent protein) downstream of an IRES (internal ribosome entry site). Infected T-cells were allowed to grow for 48 h in the presence of IL-2, and 1×10^6 cells were plated and then stimulated with the indicated amount of anti-CD3 antibody. Following 4 h of stimulation in the presence of 0.1 μg/ml Brefeldin A cells were processed for intracellular IFNγ (interferon γ) staining using a Cytofix/Cytoperm Fixation/Permeabilization Kit (Becton Dickinson Biosciences) according to the manufacturer's instructions. GFP^{+} cells were analysed for IFNγ expression using a Becton Dickinson FACSCalibur flow cytometer.

RESULTS

Upon aligning the Sts proteins with different members of the HP superfamily, we noted the presence of a conserved arginine residue that is found in Branch 2 AcPs, but absent in most Branch 1 enzymes (Figure 1A and Supplementary Figure S4 at http://www.biochemj.org/bj/453/bj4530027add.htm) [20]. The conserved arginine residue is adjacent to the invariant RHG motif and occupies position 383 and 369 within Sts-1 and Sts-2 respectively. Inspection of the Sts-1 catalytic pocket reveals the side chain of Arg[383] is positioned alongside the well-characterized core catalytic residues Arg[379], His[380], Arg[462] and His[565] (Figure 1B and Supplementary Figure S1A at http://www.biochemj.org/bj/453/bj4530027add.htm) [21]. Together, the three arginine residues form a triad of highly electropositive residues with their guanidinium-capped side chains projected directly into the cavity that defines the phosphatase active site. Sts-2 Arg[369] is positioned identically, adjacent to Sts-2 Arg[365], His[380], Arg[448] and His[551] (Figure 1B) [27]. Structural models of several Branch 2 enzymes indicate that the corresponding arginine residue adopts a similar conformation (Supplementary Figure S1B) [28–30]. The location of this arginine residue within the active site and its high degree of conservation suggest a functional role in Sts catalytic activity, despite the absence of a corresponding residue in the Branch 1 enzymes.

To determine whether Arg[383] plays a role in Sts-1 catalytic activity, we generated a mutant Sts-1 in which the alanine residue was substituted for Arg[383], and evaluated its phosphatase activity in three separate assays. First, Sts-1 R383A was expressed in HEK-293T cells and evaluated for enzymatic activity in an immune complex phosphatase reaction [21]. In contrast with wild-type Sts-1, but similar to the Sts-1 R379A and R462A mutants, Sts-1 R383A failed to hydrolyse the model substrate pNPP (Figure 2A, left-hand panel). Sts-1 R383A was also inactive towards additional diverse substrates that were hydrolysed readily by wild-type Sts-1. These include the phosphorylated fluorescein derivative OMFP (Figure 2A, middle panel) and a tyrosine phosphorylated peptide derived from a putative Sts-1 intracellular target, the T-cell tyrosine kinase Zap-70 (Figure 2A, right-hand panel). Secondly, we generated recombinant proteins and compared the *in vitro* activity of the HP domain of wild-type Sts-1 (Sts-1$_{HP}$, 10 nM) and R383A$_{HP}$ (10 nM–1 μM). Low concentrations of R383A$_{HP}$ displayed minimal hydrolytic activity, but the hydrolytic activity of R383A$_{HP}$ was evident when excessive concentrations (1 μM) of the enzyme were used (Figure 2B and Supplementary Figure S2 at http://www.biochemj.org/bj/453/bj4530027add.htm). The K_m value for pNPP of R383A$_{HP}$ (1 μM) was determined to be 3.3 mM, in contrast with a value of 1.1 mM for Sts-1$_{HP}$ (Supplementary Figure S2). Finally, to evaluate the role of Sts-1 Arg[383] within a cellular context, we utilized a cell-based Zap-70 dephosphorylation assay. Wild-type or mutant Sts-1 was co-expressed with Zap-70 in HEK-293T cells under conditions leading to the latter's tyrosine phosphorylation [31]. We observed that unlike wild-type Sts-1, but similar to the inactive Sts-1 mutants R379A and R462A, Sts-1 R383A failed to dephosphorylate Zap-70 (Figure 2C). To determine whether conservation of the equivalent arginine residue is required for the catalytic activity of Sts-2, we generated the Sts-2 R369A mutant and evaluated its activity by immune complex phosphatase reaction. The results mirrored those obtained for Sts-1. Specifically, we observed that Arg[369] was required for Sts-2 phosphatase activity (Figure 2D). Altogether, the results of the present study demonstrate a requirement for Sts-1 Arg[383] and Sts-2 Arg[369] for efficient Sts enzymatic activity. Thus, in addition to the well-defined quartet of two histidine and two arginine residues that has heretofore defined the motif required for HP activity, Sts phosphatase activity requires the presence of an additional arginine residue within the active site.

To gain further insight into the characteristics of Sts-1 Arg[383] required for enzyme activity, we generated mutant Sts-1 proteins in which Arg[383] was replaced with a variety of alternative amino acids. To mimic the active-site configuration of Branch 1 enzymes we generated mutants that replaced Arg[383] with the two predominant amino acids found at the equivalent position in Branch 1 enzymes, glycine and serine (see Figure 1A) [20]. We also generated mutants in which lysine and methionine residues were substituted for Arg[383], the former to evaluate the need for a basic side chain and the latter to investigate the need for a bulky aliphatic residue. Each mutant was expressed in HEK-293T cells, isolated by immunoprecipitation and evaluated for phosphatase activity relative to wild-type Sts-1. The mutants in which Arg[383] was replaced with a glycine, serine or methionine residue displayed little or no activity towards pNPP, OMFP or a phosphopeptide (Figure 3A). In contrast, when lysine was substituted for an arginine residue at position 383, significant *in vitro* hydrolytic activity toward the three substrates was observed (Figure 3A).

To more precisely define the effects of an arginine compared with a lysine residue at position 383, we utilized recombinant protein and evaluated the rate of the phosphatase reaction at a defined enzyme concentration and different substrate concentrations (1–20 mM). The initial reaction velocities at each substrate concentration were calculated and plotted as a function of the substrate concentration. We observed that the substitution of Arg[383] with a lysine residue was not as deleterious as the alanine substitution, with reaction velocities of R383K approximately one quarter of the reaction velocities of wild-type

Sts-1$_{HP}$ (Figure 3B). Finally, we addressed the unique requirement for Arg^{383} for the interaction between Sts-1 and a tyrosine-phosphorylated protein in a cellular context. As in the case with the alanine substitution, replacing Arg^{383} with glycine, serine or methionine residue rendered Sts-1 unable to target tyrosine-phosphorylated Zap-70 (Figure 4A). However, it was evident that Sts-1 R383K possessed limited protein phosphatase activity. Specifically, we observed a reduction in Zap-70 tyrosine phosphorylation to levels that were intermediate between those observed in the presence of active wild-type Sts-1 and inactive Sts-1 R383A. However, increasing the level of expression of Sts-1 R383K led to more substantial dephosphorylation of Zap-70 (Figure 4). Thus substituting Arg^{383} with another basic amino acid, albeit one whose side chain has a lower pK_a value, is not as deleterious as neutral or acidic amino acid substitutions. Cumulatively, the correlation between the basicity of residue 383 and the apparent enzyme activity of Sts-1$_{HP}$ suggest that one of the primary functions of Sts-1 Arg^{383} is to provide additional localized positive charge within the active site.

Having established a critical role for Arg^{383} in Sts-1 *in vitro* phosphatase activity we then addressed the requirement for Arg^{383} in Sts-1 signalling functions. Within T-cells the Sts proteins negatively regulate proximal signalling pathways downstream of the TCR [15]. To assess the functional importance of Sts-1 Arg^{383} in inhibiting TCR signalling, we employed two functional assays. First, we utilized an NFAT-responsive luciferase reporter assay. NFAT is a transcription factor whose level of activation following TCR engagement is critically and directly dependent on the overall strength of activation of signalling pathways downstream of the TCR [32]. Overexpression of wild-type Sts-1 in T-cells leads to a reduced NFAT response following TCR stimulation. In contrast, overexpression of Sts-1 R383A failed to inhibit levels of NFAT activation (Figure 5), suggesting an important role for Arg^{383} in Sts-1 signalling functions. To assess further the role of Arg^{383}, we employed a T-cell reconstitution assay in which Sts-1 is introduced into primary T-cells derived from *Sts-1/2*$^{-/-}$ mice. T-cells that lack the Sts proteins hyper-proliferate in response to T-cell stimulation and secrete excessive levels of cytokines, including IFNγ [15]. Reconstitution of mutant T-cells with wild-type Sts-1 suppresses the hyper-responsive phenotype [21]. Therefore the ability of wild-type and Sts-1 R383A to down-regulate IFNγ production was compared. Both proteins were expressed at similar levels in mutant T-cells (Supplementary Figure S3 at http://www.biochemj.org/bj/453/bj4530027add.htm). However, unlike wild-type Sts-1, Sts-1 R383A failed to efficiently suppress IFNγ production (Figure 6). The inability of Sts-1 R383A to suppress IFNγ production by the same amount as wild-type Sts-1 was evident over a wide range of stimulatory antibody concentrations (Figure 6A). These results confirm that the HP activity associated with the C-terminal catalytic domain of Sts-1 plays an essential role in Sts-1 function, and suggest the phosphatase activity of the Sts-1 phosphatase domain requires Arg^{383} to interact and hydrolyse its *in vivo* substrate(s).

DISCUSSION

The Sts proteins belong to the superfamily of HPs by virtue of a C-terminal catalytic domain. All HPs possess an evolutionarily conserved quartet of two arginine and two histidine residues that are required for catalytic activity, and the Sts proteins are no exception [20]. Arg^{379}, His^{380}, Arg^{462} and His^{565} of Sts-1 adopt a signature conformation within the active site, with His^{380} being the nucleophile. Mutation of any of these residues yields an inactive or severely impaired enzyme [21]. To date, there have been few attempts to characterize additional residues that are important for HP catalytic activity. However, in the present study we identify and characterize an additional arginine residue that plays a critical role in Sts phosphatase activity. Located at position 383 in Sts-1, the arginine residue clusters together with Arg^{379} and Arg^{462} in the active site of Sts-1. The results of the present study demonstrate that Sts-1 Arg^{383} is required for both the *in vitro* catalytic activity toward

model substrates and intracellular catalytic activity. In addition, we demonstrate that Arg[383] is essential for Sts-1 to function as a negative regulator of TCR signalling pathways.

Sts-1 Arg[383] is noteworthy for several reasons. First, it is conserved in all Sts orthologues. Indeed, the corresponding residue of Sts-2 (Arg[369]) is also required for Sts-2 activity (Figure 2D). Secondly, Sts-1 Arg[383] closely neighbours the nucleophilic histidine residue and its side chain projects prominently into the cavity that defines the active site. Structural data indicate that it could make hydrogen bonds directly with the phosphate moiety of the incoming substrate, as do the two other active-site arginine residues, Arg[379] and Arg[462] (Figure 1B) [21,27,33]. As the two latter residues are thought to stabilize the interaction between the enzyme and substrate, Sts-1 Arg[383] could likewise support the formation of an enzyme–substrate complex. However, we also cannot discount the hypothesis that it stabilizes an enzyme–phosphate-leaving group structure. Alternatively, because Sts-2 Arg[369] has been shown to be involved directly in the interaction between phosphorylated His[366] and a vanadate moiety, in a complex that mimics the transition state structure for phospho-transfer reactions [33], Arg[383] could facilitate the formation of a transition state structure by neutralizing build-up of negative charges on the substrate. These roles are also consistent with our biochemical data. Interestingly, a homologous arginine residue within an AcP has also been proposed to function within the second step of the proposed HP catalytic reaction. Namely, Arg[62] of *Aspergillus* phytase has been hypothesized to be involved in guiding the release of inorganic phosphate from the active site following substrate hydrolysis [34]. In addition to interacting with the incoming phosphorylated substrate or phosphate-leaving product, Sts Arg[383/369] also likely forms ionic interactions and/or hydrogen bonds with neighbouring side chains or backbone atoms, thereby helping to maintain the requisite conformation of the active-site residues. Indeed, structural data indicate an interaction via hydrogen bonding between Arg[383/369] and Asp[385/371] (see Figure 1B). Finally, structural data also indicate hydrogen-bond interactions between Arg[383] and active site water molecules. Thus Sts-1 Arg[383] probably contributes to the myriad of ionic interactions that occur within the active site while also being directly involved in the Sts-1 catalytic reaction.

By clustering together with Arg[379/365] and Arg[462/448] to form a trio of tight-knit arginine residues that together form a ring around His[380/366], Arg[383/369] contributes to the remarkably electropositive nature of the Sts active site. We surmise that its presence is intimately connected with both the nature of Sts substrates and the manner in which the Sts proteins interact with their substrates. Currently, there is circumstantial evidence that they may target tyrosine kinases, such as Zap-70 and Syk (spleen tyrosine kinase), that participate in signalling pathways downstream of antigen receptors [21,35,36]. However, the *bona fide* Sts *in vivo* substrates have not been established definitively. Undoubtedly, definitive identification will probably enhance our understanding of Arg[383/369] function in the context of the Sts catalytic reaction.

Curiously, an arginine residue corresponding to Sts Arg[383/369] is conserved within the AcP branch of the HPs, but is absent within the vast majority of Branch 1 enzymes [20,24]. This dichotomy suggests that there might be significant underlying differences between the reactions catalysed by the two individual sub-branches, despite the presence of the same core catalytic residues in each sub-branch. This notion is strengthened by the observation that Branch 1 enzymes rely on a conserved glutamate residue to serve as a general acid during the catalytic reaction, whereas in AcP enzymes a conserved aspartate that is positioned entirely differently is thought to fulfil the same function [20,24]. Interestingly, the Sts proteins possess both the invariant glutamate residue found in all Branch 1 enzymes and the extra active site arginine that uniquely characterizes all Branch 2 enzymes. To our knowledge, the only other Branch 1 enzymes that possess an Arg[383/369] equivalent are found in various species of cyanobacteria (both filamentous and unicellular), some plant fungi and

an anaerobic parasitic protozoan (*Entamoeba histolytica*) (see Supplementary Figure S4). It is currently unclear why both the Sts phosphatases and a number of putative enzymes found in evolutionarily ancient organisms simultaneously possess two features which otherwise individually help define the separate and unique branches of the HPs. It is probable that further investigation into the underlying requirement for Arg$^{383/369}$ in Sts enzyme activity and intracellular function could reveal the answer to this question. In turn, it could also yield insights into the mechanism by which the Sts proteins regulate the threshold of activation of T lymphocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

AcP	acid phosphatase
FBS	fetal bovine serum
GFP	green fluorescent protein
HEK	human embryonic kidney
HP	histidine phosphatase
IFNγ	interferon γ
IL-2	interleukin 2
NFAT	nuclear factor of activated T-cells
OMFP	3-*O*-methylfluorescein phosphate
PGM	phosphoglycerate mutase
pNPP	*p*-nitrophenyl phosphate
Sts	suppressor of T-cell receptor signalling
TCR	T-cell receptor
Zap-70	ζ-associated protein of 70 kDa

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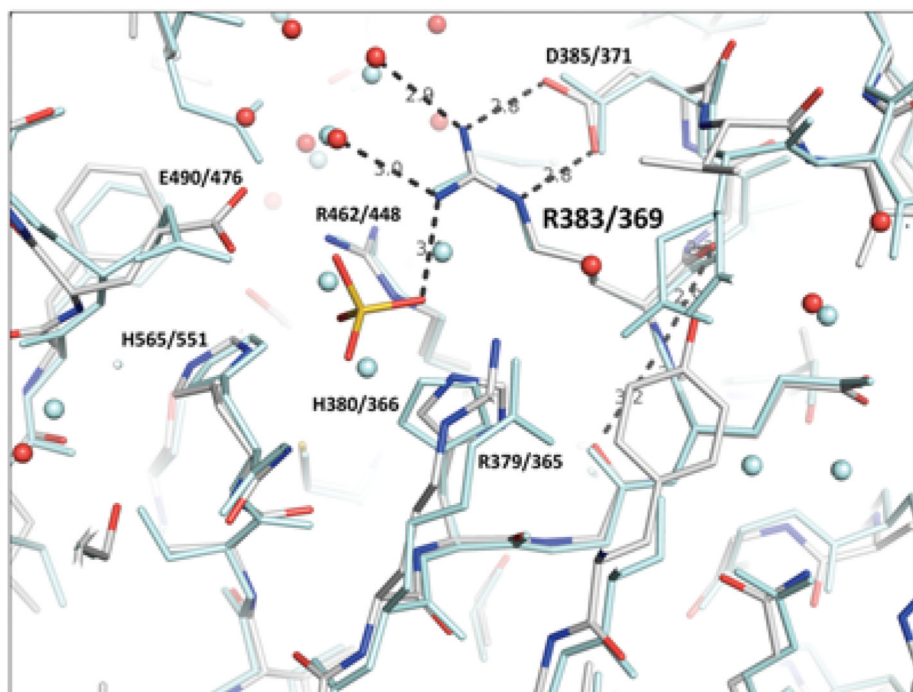
A


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hBPGM:    ...IMLRHGEGA---------VLNRSIH-----LNERH------LISAHGNSS...
Ec dPGM:  ...VLVRHGESQ---------VLKRAIH-----LNERH------IIAAHGNSL...
hF26BP:   ...YLCRHGESE--------QLKRTIQ-----LNEID------LVISHQAVM...

hSts1:    ...FVCRHGERM--------PSLRCVQ-----LFEWT------LIVAHASSL...
hSts2:    ...LVVRHGERV--------PALRCVQ-----IFEWT------LIVSHGSTL...

hPAP:     ...LVFRHGDRS---------DVDRTLM---------------MYSAHDTTV...
Ec G1P:   ...MMSRHNLRA--------SLQRTVA---------------VLVGHDSNI...
Af phyt:  ...VLSRHGARY-------GSDRVIA---------------VDFSHDNSM...
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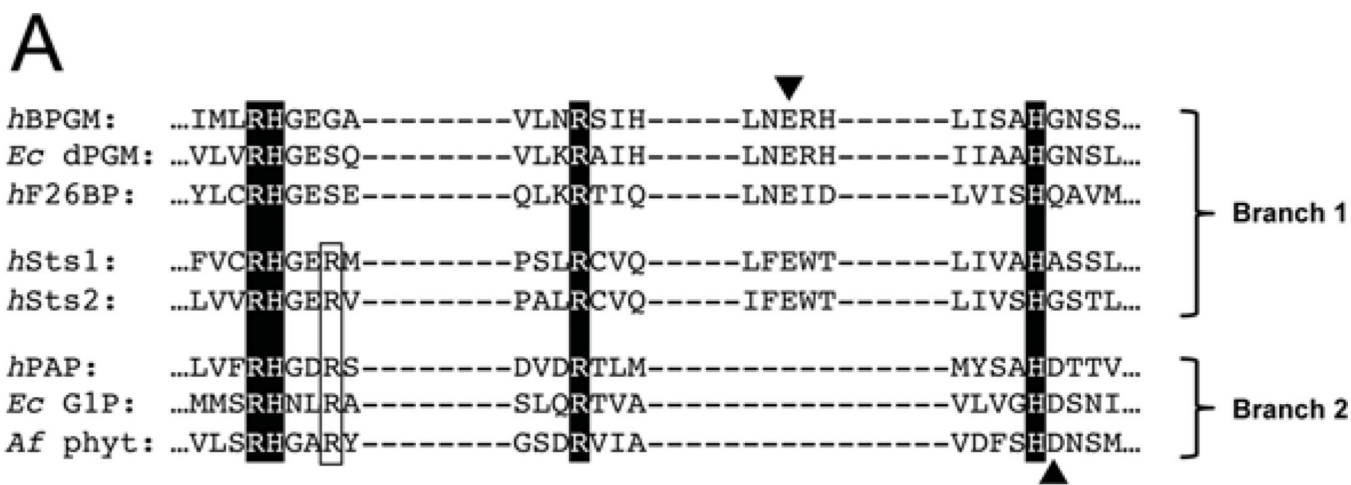
Branch 1

Branch 2

B

Figure 1. Presence of a conserved arginine residue alongside the catalytic quartet in the Sts-1 active site

(**A**) Sts phosphatase domain sequences were aligned with sequences of enzymes representing the PGM and AcP branches of the HP superfamily: human bisphosphoglycerate mutase (hBPGM), *Escherichia coli* cofactor-dependent PGM (*Ec* dPGM), human fructose-2,6-bisphosphatase (hF26BP), human prostatic acid phosphatase (hPAP), *E. coli* glucose-1-phosphatase (*Ec* G1P) and *Aspergillus fumigatus* phytase (*Af* phyt). The invariant catalytic quartet residues are highlighted and the acidic residues that are thought to act as proton donors during the second step of the catalytic reaction are indicated with arrowheads. The conserved arginine residue that is the subject of this study is outlined. (**B**)

Superimposition of Sts-1 and Sts-2 active sites, illustrating hydrogen-bond interactions between Arg[383/369] (Sts-1/Sts-2) and elements within the catalytic pocket. The phosphate molecule is derived from phosphate buffer in which crystals were soaked. Sts-1 residues are rendered in colour and those of Sts-2 are in pale blue.
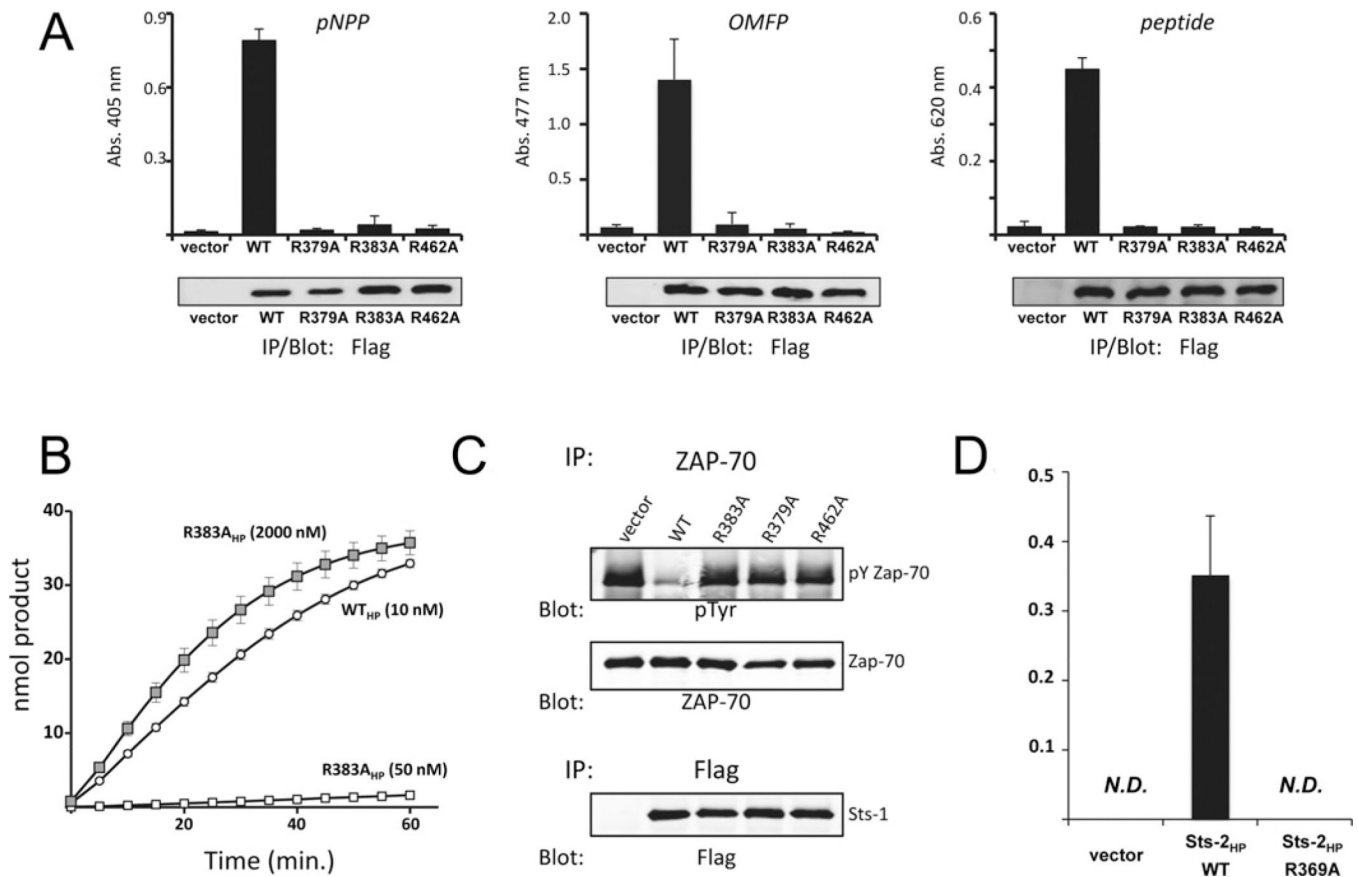
Figure 2. Requirement for Arg^{383}/Arg^{369} in Sts-1/Sts-2 catalytic activity
(**A**) Immune complex phosphatase activity assays of wild-type (WT) Sts-1$_{HP}$ and the indicated mutants using as substrates pNPP (left-hand panel), OMFP (middle panel) or a phospho-tyrosine phosphopeptide (right-hand panel) demonstrate the requirement for Sts-1 Arg^{383} for catalytic activity. Sts-1 R379A and Sts-1 R462A serve as negative controls. Results are means ± S.D. representative of multiple experiments with two replicates for each condition throughout. Abs., absorbance. (**B**) Phosphatase activity time course with recombinant wild-type Sts-1$_{HP}$ (WT$_{HP}$) and Sts-1$_{HP}$ R383A at the indicated concentrations demonstrates impairment of Sts-1$_{HP}$ R383A. The concentration of pNPP was 1 mM. (**C**) Co-expression of Zap-70 in HEK-293 cells with the indicated Sts-1 constructs indicates Sts-1 R383A is unable to dephosphorylate full-length Zap-70. The levels of Zap-70 phosphorylation were assessed by anti-phospho-tyrosine (pY/pTyr) immunoblotting. The loading controls are indicated. (**D**) OMFP hydrolysis activity of Sts-2 R369A is impaired relative with wild-type Sts-2, as demonstrated in an immune complex phosphatase assay. Results are means ± S.D. IP, immunoprecipitation; N.D., not determined.
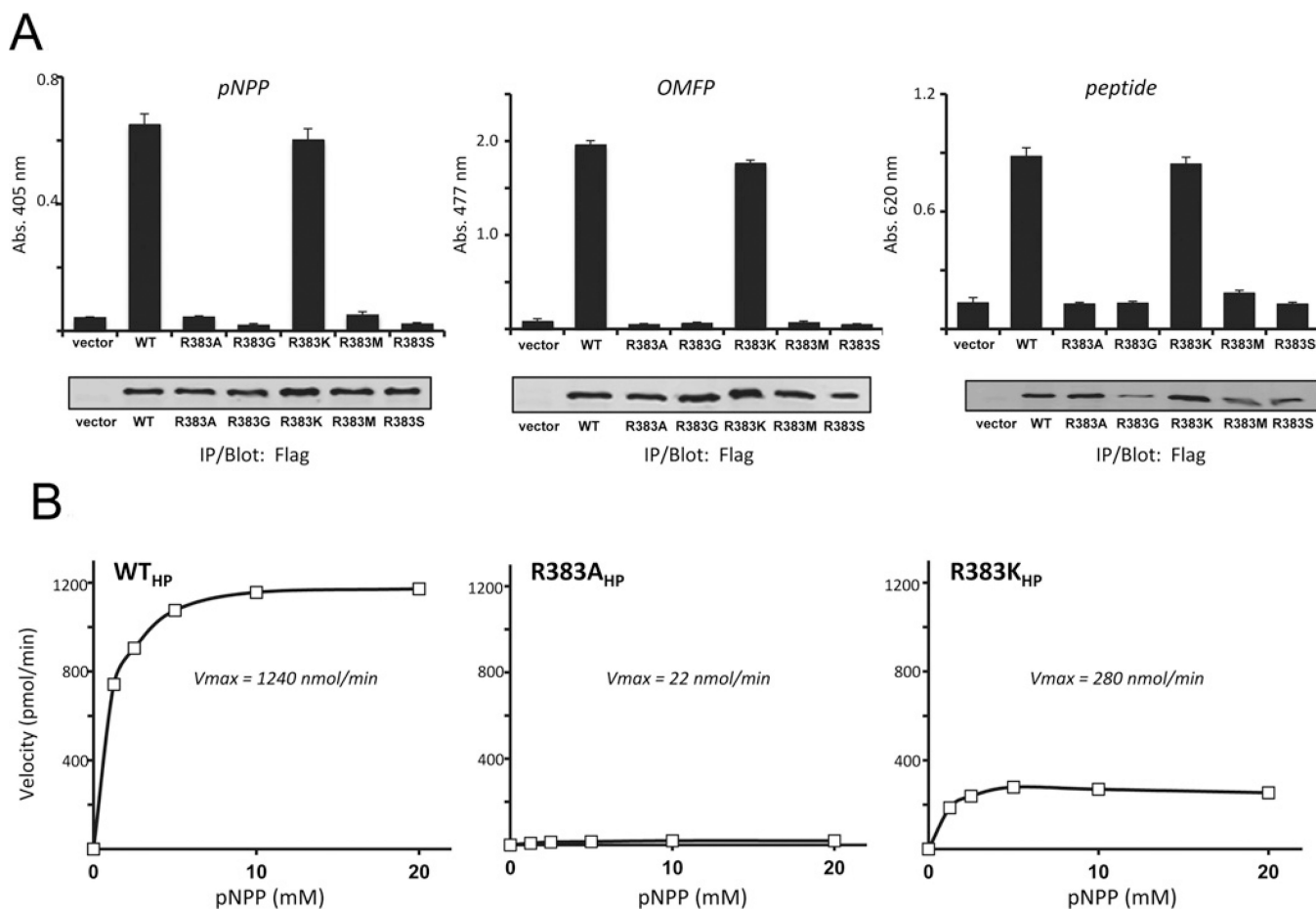
Figure 3. Lysine partially rescues Sts-1 R383A activity towards model substrates

(**A**) Substitution of Sts-1 Arg[383] with a lysine residue yields a partially active enzyme towards pNPP (left-hand panel), OMFP (middle panel) and a peptide (right-hand panel), unlike substitution with glycine, serine or methionine. Results are means ± S.D. representative of duplicate experiments with two replicates for each condition throughout. Abs. absorbance; IP, immunoprecipitation; WT, wild-type. (**B**) Substrate saturation analysis with purified recombinant wild-type Sts1$_{HP}$ (WT$_{HP}$), R383A$_{HP}$ and R383K$_{HP}$ against various concentrations of pNPP (1.25, 2.5, 5, 10 and 20 mM) reveals the contribution of the lysine residue to catalytic activity. Initial velocities were obtained from the slopes of the time course of the hydrolysis reactions and plotted against substrate concentration. Kinetic parameters were calculated using SigmaPlot 11 (Systat Software) using a dynamic curve-fitting function to a one-site saturation ligand-binding equation $y = B_{max} x/(K_D + x)$.
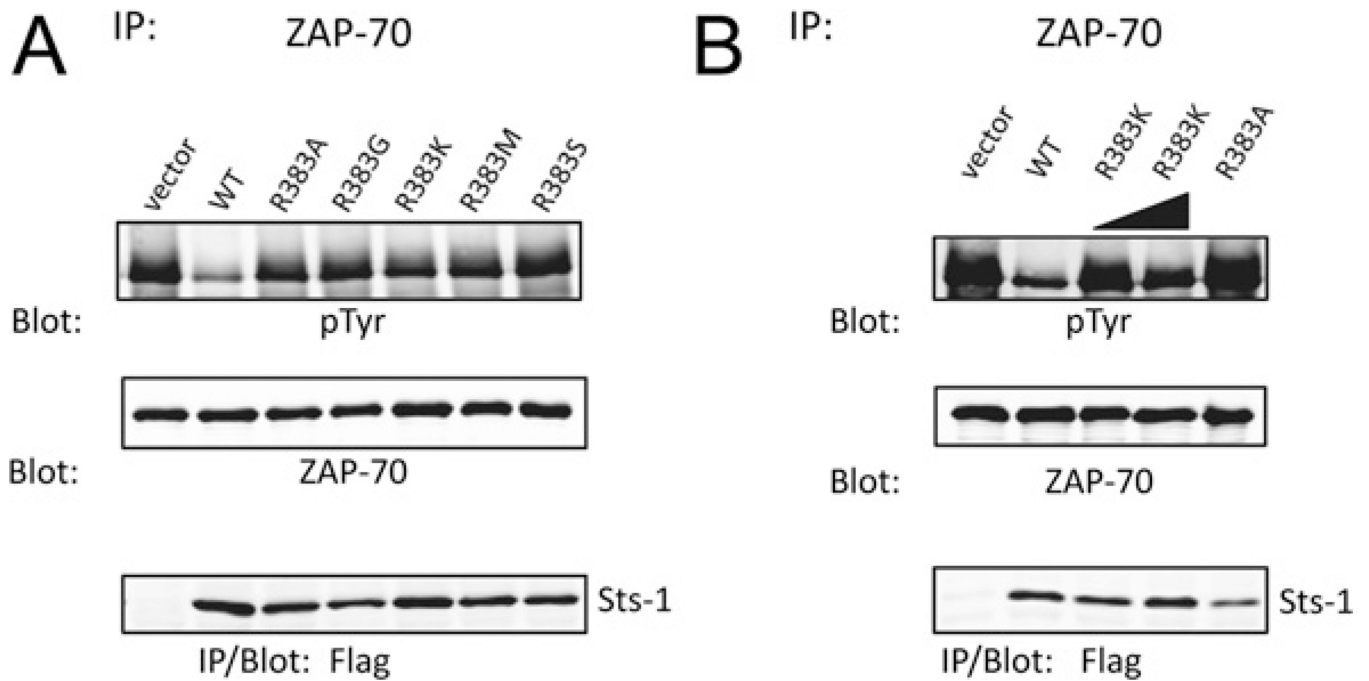
Figure 4. Lysine partially rescues Sts-1 R383A phosphatase activity towards Zap-70
Substitution of Sts-1 Arg[383] with a lysine residue yields a partially active enzyme toward
Zap-70, unlike substitution with glycine, serine or methionine. The indicated mutants were
co-expressed with Zap-70 in HEK-293 cells (**A**) and a titration of Sts-1 R383K was
evaluated (**B**). IP, immunoprecipitation; pTyr, phospho-tyrosine; WT, wild-type.
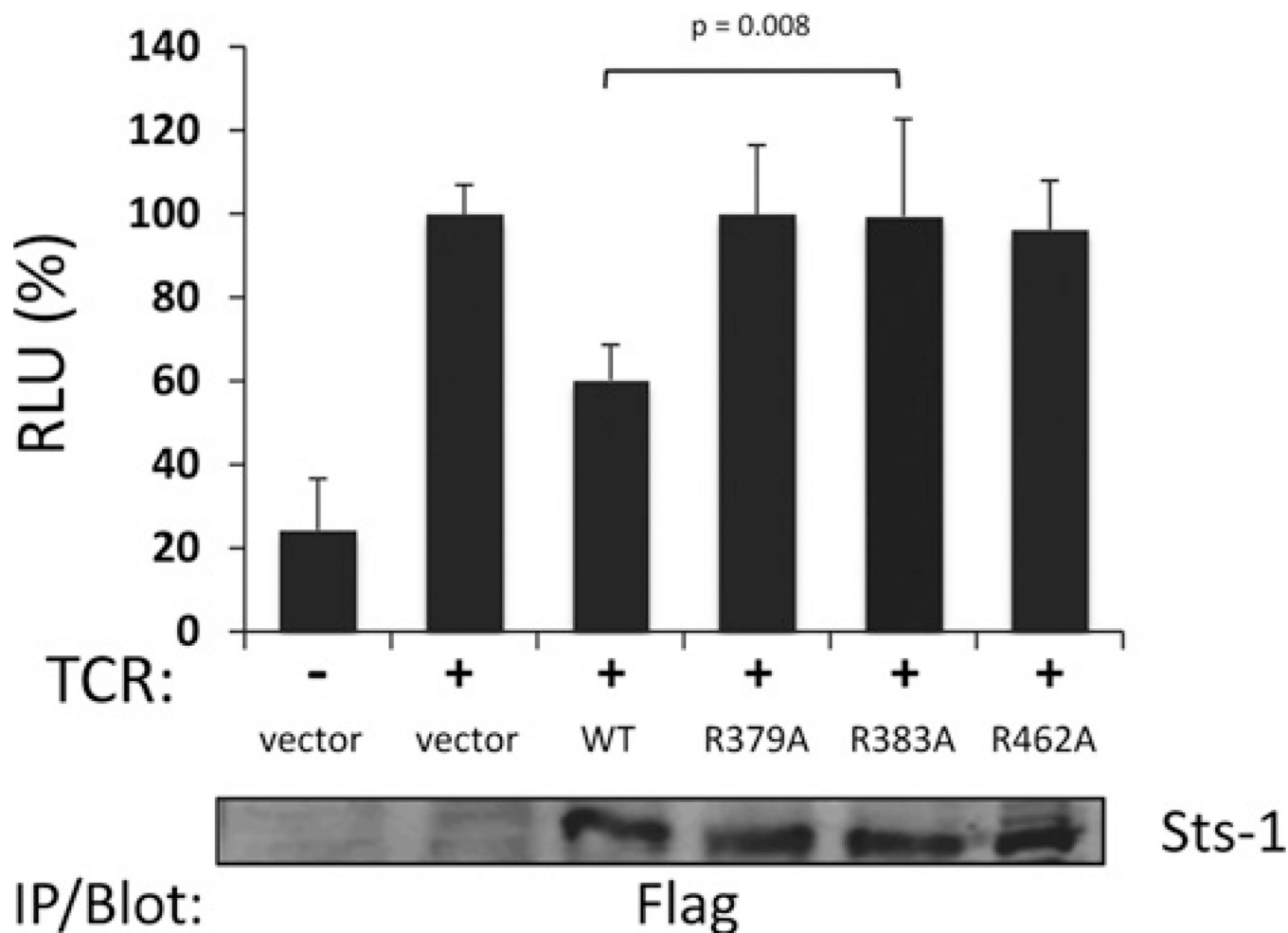
Figure 5. Requirement for Sts-1 Arg[383] in the regulation of TCR signalling pathways
Sts-1 R383A displays impaired regulation of TCR-induced NFAT activation. Jurkat cells were transfected with a firefly luciferase reporter construct under the control of NFAT binding sequences, a *Renilla* luciferase reporter construct under a constitutive promoter and plasmids encoding wild-type Sts-1 (WT) or the mutants R379A, R383A and R462A. The cells were then stimulated by placing on anti-TCR-coated plates. The histogram presents luciferase activity relative to TCR-stimulated empty-vector transfections. The illustrated results are combined from three separate experiments, each with three replicates. Error bars are S.D. from the three independent experiments. The *P* value comparing the wild-type and R383A is illustrated with the *P* values between the wild-type and other conditions being less than 0.05. IP, immunoprecipitation; RLU, relative luciferase units.
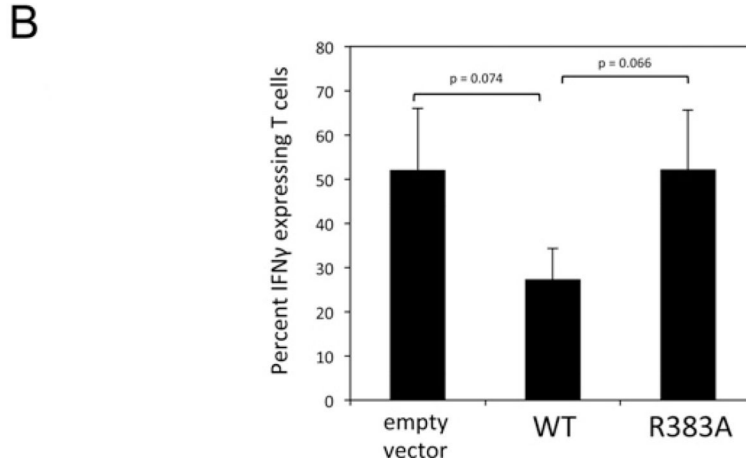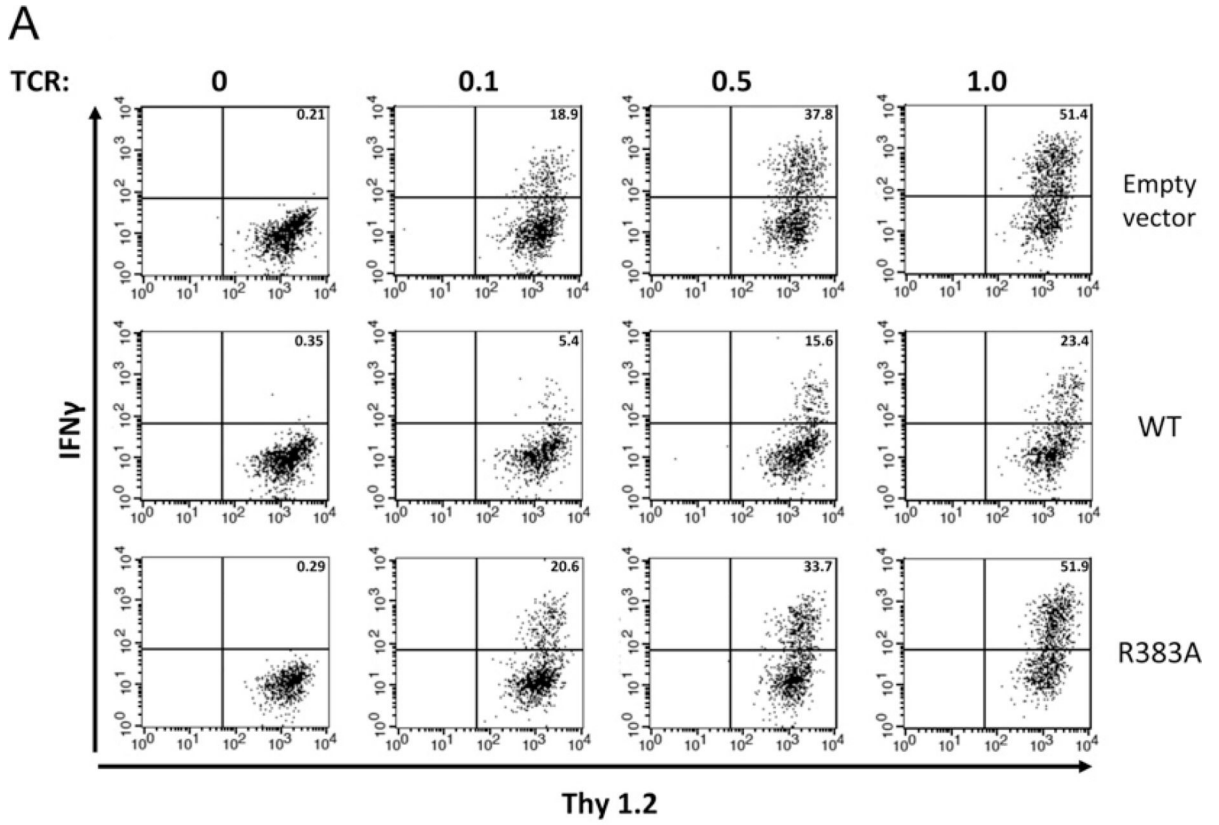
Figure 6. Requirement for Sts-1 Arg[383] in the regulation of IFNγ expression in primary T-cells
Primary T-cells were infected with retrovirus expressing empty vector, wild-type Sts-1 (WT) or Sts-1 R383A. Following stimulation with the indicated concentrations of anti-TCR stimulatory antibody, reconstituted T-cells were stained with fluorochrome-labelled antibodies to Thy1.2 (T-cell marker) and IFNγ to assess the levels of IFNγ expression. Cells were analysed by flow cytometry. Cytokine-expressing T-cells are visible in the upper right-hand quadrant with the percentage of cells indicated. Illustrated are representative data (**A**) for the average of three independent experiments ± S.D. (**B**) with 1 μg/ml stimulation. The *P* values between relevant datasets are indicated.