Modulation of Lck function through multi-site docking to T cell specific adapter protein

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Running title: Multi-site docking of Lck to TSAd

T cell specific adapter protein (TSAd) encoded by the SH2D2A gene, interacts with Lck through its C terminus and thus modulates Lck activity. Here we mapped Lck phosphorylation and interaction sites on TSAd and evaluated their functional importance. The three C-terminal TSAd tyrosines Y280, Y290 and Y305 were phosphorylated by Lck and functioned as docking sites for the Lck SH2-domain. Binding affinities of the TSAd pY280 and pY290 phosphopeptides to the isolated Lck-SH2 domain were similar to that observed for the Lck pY505 phosphopeptide; whereas the TSAd pY305 peptide displayed a 10 fold higher affinity. The proline rich Lck SH3-binding site on TSAd as well as the Lck-SH2 domain were required for efficient tyrosine phosphorylation of TSAd by Lck. Interaction sites on TSAd for both Lck SH2 and Lck SH3 were necessary for TSAd mediated modulation of proximal TCR signaling events. We found that 20-30% of TSAd molecules are phosphorylated in activated T cells, and that the proportion of TSAd:Lck molecules in such cells is approximately 1:1. Therefore, in activated T cells, a considerable number of Lck molecules may potentially be engaged by TSAd. In conclusion, Lck binds to TSAd prolines, and phosphorylates and interacts with the three C-terminal TSAd tyrosines. We propose that through multivalent interactions with Lck, TSAd diverts Lck from phosphorylating other substrates, thus modulating its functional activity through substrate competition.
In peripheral T cells, expression of the T cell specific adapter protein (TSAd), encoded by the SH2D2A gene, is rapidly induced upon T-cell activation (12-15). TSAd (or Lad/RIBP) interacts with a number of intracellular signaling molecules expressed in T cells, including the tyrosine kinases Lck (16-18) and Itk (19), the serine/threonine kinase MEKK2 (20), the GTP exchange factor rasGAP (21), the adapter protein Grb2 (22) and the molecular chaperone valosine-containing protein, VCP (23). TSAd is important for proper activation of murine T cells (19;24). Lack of TSAd expression in mice leads to hypoproliferation of T cells (19) and development of systemic autoimmune disease (25). When over expressed in Jurkat T cells, TSAd inhibits proximal signal-transduction events by modulating Lck activity (17;18).

Lck is regulated by phosphorylation of its Y394 and Y505 tyrosines (26). Csk mediated phosphorylation of Lck Y505 results in an intramolecular interaction between phosphorylated Y505 and its SH2 domain (27-29), whereby Lck is kept in an inactive conformation due to blockade of its substrate binding site. Moreover, the SH3 domain of Lck interacts with the linker between the SH2- and kinase-domain, which further blocks the kinase activity (30;31). Dephosphorylation of pY505 by the CD45 phosphatase (32) and Lck autophosphorylation of Y394 activates Lck (33-35). The intramolecular interactions controlling Lck activity are of low affinity (36), allowing specific ligands with stronger affinity for the Lck-SH2 or -SH3 domains to compete out the intramolecular interactions and thus activate Lck (37).

Here we dissected the TSAd-Lck interaction by mutational analysis in transfected cell lines as well as by chemical analysis using isothermal calorimetry (ITC) and in vitro kinase assays. We show that Lck phosphorylates and interacts with all three C-terminal tyrosines of TSAd. There is a hierarchy of affinities for the phosphorylated TSAd peptides to the Lck SH2-domain, where the lowest affinity observed was in the same range as the Lck-Y505 peptide and the highest was ten fold higher. The tyrosine-phosphorylation level of TSAd in vivo is dependent on the Lck-SH2 domain, where the lowest affinity observed was in the same range as the Lck-Y505 peptide and the highest was ten fold higher. The tyrosine-phosphorylation level of TSAd in vivo is dependent on the Lck-SH2 domain, where the lowest affinity observed was in the same range as the Lck-Y505 peptide and the highest was ten fold higher. 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**EXPERIMENTAL PROCEDURES**

**Plasmids**—The TSAd full length and the TSAd Δ239-334 cDNAs were cloned into the pEF-HA expression vector as previously reported (14;18). Point mutations in TSAd cDNA was introduced into the pEF/HA-TSAd vector using QuickChange (Stratagene, Santa Clara, CA). cDNA with encoding TSAd deleted for aa239-56 was generated using mega primer PCR. The pGEX-6P1 Lck-SH3 domain construct was previously described (17). The pEF-Lck, pEF-Lck R154K, and pGEX-3T Lck-SH2 constructs were generous gifts from Dr. Tomas Mustelin. TSAd sequence aa236-312 was subcloned by PCR into the pGEX-6P1 vector (GE Healthcare), and various mutations in the sequence were introduced with QuickChange PCR (Stratagene). All constructs were verified by sequencing.

**Antibodies and reagents**—The following monoclonal antibodies (mAbs) were used: anti-Zap-70 (Transduction Laboratories, Lexington, KY), anti-HA (Babco, Richmond, CA), anti-human CD3ε (OKT3, American Type Culture collection, Manassas, VA), anti-TCR (C305, a generous gift from Art Weiss) anti- phosphotyrosine (clone 4G10, Upstate Biotechnology, Lake Placid, NY), anti-Lck (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Lck (clone IF6, a generous gift from Dr. Joseph B. Bolen). The following polyclonal antibodies were used: anti-LAT, and anti pY319 Zap-70 (Santa Cruz Biotechnology), anti-pY394-Lck (anti-Tyr416-Src Family) (Cell Signaling Technology, Beverly, MA), anti-GST (GE Healthcare, Piscataway, NJ), Normal Rabbit Serum (NRS) and anti-TSAd antibodies were raised against synthetic peptides of TSAd (12;17) or recombinant C terminus of TSAd (15). As secondary antibodies we used horse peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Recombinant active His-tagged Lck was from Upstate Biotechnology. The kinase inhibitor PP2 were purchased from Sigma-Aldrich.

**Expression and purification of GST-fusion proteins**—GST-fusion proteins of TSAd aa 236-312 as well as the Lck-SH2 domain and the Lck-SH3 domain were produced in BL21...
Codon plus bacteria (Stratagene), and purified on glutathione Sepharose beads (GE Healthcare) according to the instructions of the manufacturer. For calorimetric analysis of peptide binding to the Lck SH2-domain, isolated Lck-SH2 domain was also excised from the glutathione Sepharose beads using thrombin.

**Cell cultures and transfections**—293T cells and Jurkat E6.1 (American Type Culture Collection) or TAg cells (38) were cultured in RPMI 1640, 5-10% foetal calf serum (FCS) supplemented with 1mM sodium pyruvate, 1mM non-essential amino acids (all from GIBCOBRL®, Life Technologies™, The Netherlands) and antibiotics. Transfections of 5-20 x 10⁶ Jurkat T cells in RPMI 1640 with 5% FCS with 5-30 µg plasmid DNA were performed using a BTX electroporator (Genetronix, San Diego, CA) at 200 V, 70 ms or the Amaxa nucleofector with the cell line nucleofector™ kit (#VCA-1003), using either program S18 or I-10. Transient transfectants were cultured for 16-48 hours. 293T cells (2x 10⁶) were washed with Optimem 1 medium (GIBCOBRL®) before transfection with a mixture of 0.5-3 µg DNA and 25 µl Lipofectin in Optimem 1 medium. Transfections were terminated after 5-8 h by addition of 1:1 ml of RPMI 1640 with 20 % FCS. The cells were further propagated for 16-24 h. Human peripheral blood CD4+ T cells were positively or negatively selected from healthy blood donors as previously described (15) and cultured in either RPMI 1640 or hTC culture medium (Amaxa Biosystems, Cologne, Germany) with 5-10% FCS.

**Cell stimulation, lysis, immune-precipitation and Western blot**—TSAd protein expression was induced in Jurkat T cells and in peripheral CD4+ T cells by stimulation for various time points with OKT3 (5 µg/ml) coated wells, or in peripheral CD4+ T cells by stimulation with anti-CD3/CD28 Dynabeads® (1 bead pr. cell). Jurkat cells were washed with PBS, resuspended and stimulated with 5-10 µg/ml anti-CD3 (OKT3) or 1:200 actives anti-TCR (C305) mAbs for specific time points. Cells were lysed by addition of an equal volume of 2 x lysis buffer (1 x lysisbuffer: 20 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 0.25-1% Igepal, 12.5-50 mM n-octyl-β-D-glucoside and 10µg/ml of the protease inhibitors; leupeptin, pepstatin A, chymostatin and antipain (all from Sigma Aldrich, St. Louis, MO). 293T cells were washed with PBS and lysed by addition of 1 x lysis buffer. Lysates were precleared 2-3x for 45 minutes with protein A/G Sepharose™ (Amersham Pharmacia Biotech, Uppsala, Sweden) or Dynabeads® protein G (Invitrogen, Oslo, Norway), followed by incubation for 1h with anti-TSAd or normal rabbit serum (NRS) followed by 1 h incubation with protein A/G Sepharose™ or for 1 h with protein G Dynabeads® precoated with anti-pY antibodies as described by the manufacturer. Pull-down experiments with GST-Lck SH2 or GST-Lck SH3 were performed in precleared lysates as described (18). After immunoprecipitation or pulldown, beads were washed 3x in 1 x lysis buffer, and isolated proteins were separated by 7.5-12.5% SDS-PAGE. Gels were blotted onto a polyvinylidine difluoride membrane (PVDF) (BioRad Laboratories, Hercules, CA) or directly stained in Coomassie blue solution. Blots were probed with the indicated antibodies in Tris buffered saline (TBS, pH 7.4) with 0.1 % Tween (Sigma Aldrich)/ 3% bovine serum albumin (BSA) (Biotest, Dreieich, Germany) or 3% skimmed milk (Sigma Aldrich). Bound antibodies were visualized by incubation with secondary HRP-labeled antibodies and Super Signal® west Pico Stable Peroxide Solution (Pierce, Rockford, IL) (18). In some experiments the intensities of the bands of interest were quantitated by the use of Kodak Image Station 2000R.

**Phosphoamino acid peptides**—Peptides corresponding to HMT pY324 (PQ[pY]EEI), Lck pY505 (GQ[pY]QPQ, TSAd pY280 (PI[pY]NEP) and pY290 (AF[pY]AMG) were synthesised by GenScript Corporation (Piscataway, NJ, USA) to more than 95% purity HPLC grade. The peptide corresponding to TSAd pY305 (NI[pY]VEV) was made by Pepscan systems (Lelystad, NL), also more than 95% pure. All the peptides contained a free N- and C-terminus.

**Isothermal titration calorimetry (ITC)**—ITC was performed using the VP-ITC MicroCal instrument (MicroCal, Northampton, MA, USA) (39). Recombinant Lck-SH2 domain was made as described and the concentration was determined using the A₂₈₀ value, with extinction coefficient 9650M⁻¹cm⁻¹. The concentration of Lck-SH2 domain in the reaction cell typically was 10 mM, and the concentration of the phosphotyrosine peptides in the ITC syringe were 0.5 mM, all dissolved in a modified MBS-buffer (50mM MOPS, 50mM NaCl, 2mM DTT, pH 6.8) (36). All experiments were performed at
25°C. Initiation delay was set to 60 seconds and aliquots of 5 μL of peptide solution were injected into the reaction cell at 120 s intervals with a stirring speed of 260 rpm. Typically 20-25 injections were used to complete the titration. ITC data were collected automatically using the ITC Origin v.7.0 software (MicroCal, Northampton, MA, USA) accompanying the VP-ITC system. The data was fitted to a single binding site mechanism using the ITC fitting algorithm included with the instrument after correcting for the heat of dilution. All data from the ITC binding reactions fitted well to a single-site binding model yielding the stoichiometry (n), equilibrium binding association constant (K_a), and the enthalpy change (ΔH) of the reaction. The value of n was found to be between 0.9 and 1.1 for all reactions. The changes in reaction free energy (ΔG) and entropy (ΔS), as well as the dissociation constant (K_d) were calculated using the relation ΔG = ΔH − TΔS = −RTlnK_a = RTlnK_d. Errors in ΔH, ΔS, and K_d were obtained as standard deviations of three or more experiments.

Mass spectrometry—Recombinant TSAd proteins were subjected to in-solution tryptic digestion as indicated in the results section. Therefore, 0.5 μg of recombinant proteins were digested by 25 ng trypsin in 50 mM NH_4HCO_3 (37 °C, 3 hours). The kinase buffer was not removed prior to digestion. Approximately 75 ng of digested recombinant protein were desalted by using C18-stop and go extraction tips (C18-stage tips) and analysed by MALDI-TOF mass spectrometry in the positive and reflector mode (Ultraflex II, Bruker Daltonics). The observed masses were compared to an artificial tryptic digest of the recombinant TSAd proteins. MS/MS experiments were performed to confirm the peptide sequences and to verify the phosphorylation sites. To further confirm the identity of the detected phosphopeptides, an aliquot of the tryptic digest (150 ng recombinant TSAd protein) was treated with alkaline phosphatase (ALP; 2 units) in 50 mM NH_4HCO_3 (37 °C, 30 minutes). Half of the sample was desalted and analyzed as described. As ALP-mediated dephosphorylation results in a mass shift of ~80 Da per phosphorylation site, this treatment proves the presence and number of phosphogroups in the given peptides.

Kinase assay—In vitro kinase assays were performed using various GST-TSAd constructs diluted in kinase buffer containing 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 75 mM MgCl_2, 15 mM DTT and 1-2 μM ATP before recombinant Lck was mixed into the samples. The samples were then immediately put on a heating block pre-warmed to 30 °C, and aliquots were withdrawn at specific time points and the kinase assays were stopped by adding SDS-PAGE loading buffer and incubation at 100 °C for 5 minutes. Purified, recombinant GST-TSAd constructs were used at an estimated final concentration in the range of 0.175-20 μM, whereas concentrations between 7.5 to 200 nM full-length, active Lck has been used per kinase assay reaction.

RESULTS

TSAd is tyrosine phosphorylated by Lck on several tyrosines—TSAd has ten potential tyrosine phosphorylation sites (Fig. 1A and supplementary table 1). We previously reported that TSAd is tyrosine phosphorylated in stimulated peripheral blood mononuclear cells (PBMC) (12) and Jurkat T cells (17). Co-expression with Lck is required for phosphorylation of TSAd upon tyrosine in 293T cells (17;18) and Fig. 1B, lane 3 and 4. The C terminus of TSAd aa 239-334 harbors docking sites for both the Lck-SH2 and -SH3 domains, and this part of TSAd is also required for Lck dependent phosphorylation of TSAd (18) and Fig 1B, lane 5 and 6.

To determine which of the TSAd tyrosines that could be phosphorylated, we made single Y to F TSAd mutants for all ten tyrosines and expressed them in 293T cells together with Lck. Immunoprecipitates (IPs) of TSAd from these cells were examined for phosphorylation by immunoblotting using anti-phosphotyrosine (pY) antibody. Two distinct bands corresponding to phosphorylated TSAd were detected when TSAd IPs were resolved by SDS-PAGE. As shown in Fig. 1C, all ten TSAd tyrosine (Y) to phenylalanine (F) mutants were tyrosine phosphorylated in the presence of Lck, showing that more than one tyrosine were phosphorylated. A slower migrating band appeared only in the presence of Y290 (Fig. 1C, lane 11). All single Y to F mutants interacted with Lck SH2 in a GST pull-down assay using the same lysates as described in Fig. 1C (Fig. 1D). These data indicate that Lck is able to phosphorylate and interact via its SH2 domain with more than one of the TSAd tyrosines.

Lck phosphorylates and interacts with the highly conserved Y280, Y290 and Y305 of...
TSAd—Murine TSAd displays 68% sequence identity to human TSAd, with the SH2 domain showing the highest degree of homology (19). However, also in the C terminus there are highly conserved elements. The region encompassing aa 236-312 has a proline rich motif, as well as the three C-terminal tyrosines of TSAd, all of which have essentially the same flanking sequences in human, chimpanzee, cow, mouse and rat (Fig. 2A). The only other tyrosine in the TSAd sequence which has equally well conserved flanking sequence is Y117 which is part of the conserved phosphotyrosine binding pocket of the SH2 domain (Supplementary table 1). This degree of conservation of the aa236-312 in TSAd strongly indicates that this region has functional importance. Accordingly, we and others previously showed that TSAd mutated for its four or three C-terminal tyrosines is not phosphorylated by Lck (18;24).

Since single mutations of any of the three C-terminal tyrosines were not sufficient to abolish Lck mediated TSAd phosphorylation, nor Lck-SH2 domain binding to TSAd, we made double or triple Y to F mutants of the four C-terminal tyrosines. All double Y to F mutants were both phosphorylated in the presence of Lck and interacted with Lck SH2 (lane 9, 10, 11). As shown in Fig. 2B, when co-expressed with Lck in 293T cells, the triple TSAd mutant lacking Y280, Y290 and Y305 was only weakly tyrosine phosphorylated and the Lck-SH2 domain did not bind to this mutant (lane 8). TSAd mutants with either Y280, Y290 or Y305 intact were still phosphorylated and interacted with the Lck-SH2 domain (lane 5-7). Taken together, these data indicate that Lck phosphorylates, and may interact through its SH2 domain with all three C-terminal tyrosines of TSAd.

None of the ten tyrosines in TSAd (supplementary table 1) are preferred Lck substrates. Only at low stringency search using the Scansite algorithm (40) the Y280 and Y305 are identified as potential Lck phosphorylation sites. To confirm that Lck is able to phosphorylate the three C-terminal tyrosines in TSAd, we therefore expressed the conserved C-terminal region (aa 236-312) of human TSAd (depicted in Fig. 2A) fused to GST (hereafter referred to as TSAd-PY-YYY, Fig. 2C). The intact protein TSAd-PY-YYY includes the Lck-SH3 recognition site (aa 239-56 i.e. P in the abbreviation PY-YYY) (18) and the four C-terminal tyrosines of TSAd (i.e. Y-YYY). In the mutated TSAd-PY-FFF protein, the three C-terminal tyrosines had been mutated to phenylalanine (Fig. 2C). These two recombinant GST-TSAd proteins were subjected to an in vitro kinase assay using a 1:10 molar ratio of recombinant active Lck to TSAd. Mass spectrometry analysis of tryptic peptide digests of the reaction mixtures revealed that all four TSAd tyrosines were phosphorylated (Fig. 2D and E, Table 1).

Although Y260 was phosphorylated by Lck in vitro, Y260 seems to be neither a major Lck tyrosine-phosphorylation site in vivo in 293T cells, nor an interaction site for the Lck-SH2 domain (Fig. 2B). Moreover, the sequence flanking the Y260 is less well conserved in evolution than the three C-terminal tyrosines (Fig 2A and supplementary table 1). Therefore we chose to focus primarily on elucidating the interaction of Lck with the three C-terminal tyrosines.

A hierarchy of binding affinities for TSAd phosphopeptides to the Lck SH2-domain—Interactions of SH2 domains with their phosphorylated tyrosine ligands are generally weak (36). The specificity and affinity of a particular SH2 domain for its ligands are known to be dependent on the amino acids immediately C terminal to the tyrosine (41). TSAd Y305 and TSAd Y280 were identified as putative Lck-SH2 domain ligands through high or medium stringency motif scanning, respectively, using Scansite (40). In contrast, TSAd Y290 was not predicted to be an Lck-SH2 ligand, even at low stringency search. To further probe the significance of the results obtained by the Lck-SH2 pull-down analysis (Fig. 2B), we determined the binding affinities of the three C-terminal TSAd tyrosines to the Lck SH2-domain. Hexameric polypeptides representing each of the three phosphorylated TSAd tyrosines were synthesized, and their binding affinities for isolated Lck-SH2 domain were assessed by ITC. In accordance with previous studies (36) the high affinity ligand HMT pY324 peptide displayed a 100 fold higher affinity than the Lck pY505 peptide for the isolated Lck-SH2 domain (Table 2), providing a quality control for our assay. The TSAd phosphopeptides pY280 and pY290 had affinities for Lck SH2 in the same range as that observed for Lck pY505, whereas the TSAd phosphopeptide pY305 bound to Lck SH2 with approximately tenfold higher affinity (Table 2). This result indicates that all three TSAd phosphopeptides, and in particular the TSAd pY305 peptide, might be able to compete...
out the Lck pY505 intramolecular binding to the Lck-SH2 domain and thus unlock the inhibitory conformation of Lck.

**TSAd association with Lck requires only Lck-SH2 or -SH3 interaction sites on TSAd**—We have previously shown that TSAd interacts with Lck in primary T cells as well as in Jurkat T cells transfected with TSAd (17;18). In order to assess whether TSAd interacts with Lck also in the absence of either the Lck-SH2 or Lck-SH3 interaction sites, we performed co-immunoprecipitation experiments in Jurkat cells transfected with intact TSAd or TSAd Y280,290,305F or TSAd Δ239-56. In all cases Lck co-precipitated with TSAd, however the amount of associated Lck was clearly reduced in the absence of either the SH2 or the SH3 interaction sites (Fig. 3A). This result show that interaction of TSAd with Lck through either the Lck-SH3 or -SH2 domain is sufficient for stable association of TSAd with Lck and that both interactions occur in vivo.

**TSAd is phosphorylated by Lck in a processive manner**—Multi-site phosphorylation by a single kinase may occur in a distributive or processive manner. Distributive phosphorylation implies that phosphorylation of substrates with multiple sites requires multiple hits between the kinase and its substrate. By contrast, processive phosphorylation involves only one hit between the kinase and the substrate for phosphorylation of multiple sites (42). Non-receptor tyrosine kinases typically contain both SH2- and SH3 domains. Binding of the kinase to the substrate via the kinase SH3 domain may allow for processive phosphorylation of the substrate (43;44). Moreover, SH2 domains of non-receptor tyrosine kinases typically have specificity for tyrosine motifs phosphorylated by the same kinase, and once phosphorylated these sites may contribute to phosphorylation of the substrate through binding to the SH2 domain of the kinase (44;45).

Since TSAd includes multiple SH2-interaction sites as well as an SH3-interaction site for Lck, it is possible that Lck phosphorylates TSAd through a processive mechanism. We tested this hypothesis by monitoring phosphorylation of different mutated and truncated versions of the TSAd-PY-YYY constructs (Fig. 2C) in our in vitro Lck-kinase assay. Phosphotyrosine levels of recombinant TSAd proteins were assessed by SDS-PAGE followed by immunoblotting with anti-pY and anti-GST antibodies.

For substrates phosphorylated by a distributive mode, the level of phosphorylation will be dependent on the kinase concentration in the reaction mixture. For substrates that are phosphorylated by a processive mode, the concentration of the kinase is less important once the kinase has bound to the substrate. Substrates that are phosphorylated through a processive mechanism should therefore be less sensitive to changes in substrate and kinase concentrations after the initial encounter between the kinase and the substrate. In agreement with this, two fold dilutions of Lck and TSAd-PY-YYY after initiation of the kinase assay did not affect the level of TSAd phosphorylation (Fig. 3B). In contrast, the TSAd-dY-YYY protein lacking the Lck SH3-interaction site (aa239-56), was less tyrosine phosphorylated at low Lck concentrations, indicating a distributive mode of TSAd phosphorylation in the absence of its Lck-SH3 ligand (Fig. 3B).

Since processive phosphorylation of multiple tyrosines in a substrate only requires one hit between the substrate and the kinase, the kinetics of phosphorylation should be more rapid than for distributive phosphorylation of multiple sites, which requires repeated hits between the substrate and the kinase (42). We therefore monitored the level of tyrosine phosphorylated GST-TSAd protein in the in vitro Lck kinase assay over time. As seen in Fig. 3C, phosphorylation of GST-TSAd-PY-YYY could be detected at an earlier time point than the GST-TSAd-dY-YYY where the phosphorylation was clearly delayed. This suggest that interaction of the Lck-SH3 domain with TSAd contributes to phosphorylation efficiency, and thereby give further support to the notion that Lck phosphorylates TSAd in a processive manner.

Because all three C-terminal tyrosines of TSAd may bind to Lck SH2, interaction of Lck SH2 to any of the TSAd phosphotyrosines could contribute to the processive phosphorylation of the other tyrosines. In order to address whether the Lck-SH2 domain contribute to processive phosphorylation of TSAd we took advantage of our observation that TSAd Y260 is phosphorylated by Lck in vitro (Table 1 and Fig. 2E), but does not interact with Lck-SH2 (Fig. 2B). Compared to the GST-TSAd-PY-YYY, GST-TSAd-PY-FFF displayed a clearly delayed kinetics (Fig. 3D, compare lane 1 and 2).
If Lck-SH2 domain docking onto one of the TSAd phosphotyrosines promotes phosphorylation of the other tyrosines, recombinant TSAd proteins carrying Y260 combined with any of the three C-terminal tyrosines should display a more rapid rate of tyrosine phosphorylation than GST-TSAd-PY-FFF carrying Y260 alone. In agreement with this notion, we found that the recombinant protein including both Y260 and Y280 or Y305 displayed clearly a higher level of tyrosine phosphorylation than the protein including Y260 only (Fig. 3D, compare lane 2, 3 and 5). By contrast, inclusion of Y290 showed only a weak increase in the overall rate of phosphorylation compared to the GST-TSAd Y260 construct (Fig. 3D, compare lane 2 and 4). Since pY280 and pY290 have essentially the same affinity for Lck SH2 (Table 2), the difference in phosphorylation efficiency conferred by the two tyrosines could be due to differences in Lck kinase preference, as residue Y280 and Y305 but not Y290 were preferred Lck substrates as predicted by Scansite (40) (Fig. 3D). This notion was supported by the observation that the phosphotyrosine peptide representing TSAd pY305 promoted accelerated activation of Lck as indicated by the level of Y394 phosphorylation after one minute co-incubation of Lck with the phosphopeptide in kinase buffer (Fig. 3E). By contrast, addition of either the TSAd pY280 or the TSAd pY290 peptide to the kinase buffer did not significantly alter the kinetics of Y394 autophosphorylation, showing that TSAd pY280 is not more potent than TSAd pY290 in promoting Lck tyrosine phosphorylation.

Taken together, the in vitro kinase experiments indicate that interaction with the Lck-SH3 domain contributes to processive phosphorylation of TSAd. However, to what extent the Lck-SH2 domain also contributes to processive phosphorylation of TSAd could not be determined. We therefore asked whether interaction of Lck with TSAd through the Lck-SH2- and/or the Lck SH3-domains determines the extent of TSAd tyrosine phosphorylation also in vivo.

Maximal tyrosine phosphorylation of TSAd is dependent both on Lck SH2- and SH3-domain interactions—TSAd IPs from these cells, were assessed for level of tyrosine phosphorylation by immunoblotting (Fig. 4A) and quantitation of chemiluminesence signals (Fig. 4B). When co-expressed together with Lck, TSAd was found to be highly tyrosine phosphorylated (here referred to as 100 % tyrosine phosphorylation). Disruption of the Lck-SH3 interaction site on TSAd resulted in a 40 % reduction of TSAd tyrosine phosphorylation. A 60 % reduction in tyrosine phosphorylated TSAd was evident when intact TSAd was expressed in the presence of Lck lacking a functional SH2 domain (LckR154K). TSAd molecules lacking the Lck-SH3 ligand co-expressed with Lck lacking a functional SH2 domain displayed less than 10% of the TSAd tyrosine phosphorylation level observed when co-expressing full length TSAd and Lck.

This result shows that interaction of TSAd to both the Lck-SH3 and -SH2 domains is necessary for maximal tyrosine phosphorylation of TSAd, and indirectly indicate that the Lck SH2 domain also contribute to processive phosphorylation of TSAd.

TSAd dependent modulation of Lck activity is dependent on intact SH2- and SH3 ligands on TSAd—We have previously shown that TSAd modulates multiple proximal signaling events in T cells (14). Upon TCR stimulation, the Src kinase family members Lck and Fyn are the major protein tyrosine kinases to become activated and initiate intracellular signaling events (47). Reconstitution studies of the Lck-deficient T-cell line (JCaM1) with either Lck or Fyn reveal that only Lck fully restores tyrosine phosphorylation and activation of Zap-70 (48). Moreover, the Zap-70 Y319 is phosphorylated by Lck upon TCR triggering (5-7). When Lck activity is abolished by siRNA knock down (49) or by the Src family kinase inhibitor PP2 (Fig 5A), phosphorylation of Zap-70 Y319 is attenuated. Phosphorylation of Zap-70 Y319 can thus be viewed as a marker for Lck activity in vivo. TCR stimulated Jurkat T cells transfected with the wild type (WT) TSAd cDNA displayed reduced Zap-70 Y319 phosphorylation, whereas the TSAd-Δ239-56 and the TSAd Y280,290,305F mutants displayed a level of Zap-70 pY319 similar to that observed in control cells transfected with empty vector (φ) (Fig. 5B). This effect was not due to altered phosphorylation kinetics, as expression of intact TSAd reduced the level of...
phosphorylation of Zap-70 Y319 both after 1, 2.5 and 5 minutes of stimulation (Fig. 5B).

We previously reported that phosphorylation of both Lck Y394 and Y505 is increased in Jurkat T cells stably expressing TSAd (17). This is in accordance with Marti et al., who showed that intact TSAd promotes phosphorylation of Lck Y394 in primary mouse CD4+ T cells (24). Here we found that the level of Lck pY394 in Jurkat T cells transiently transfected with intact or mutated TSAd was similar to that observed in Jurkat T cells transfected with empty vector (Fig. 5C). In sum, these results indicate that attenuation of Zap-70 pY319 levels in T cells expressing intact TSAd can not be explained by altered activation status of Lck as evidenced by level of pY394 autophosphorylation.

20-30 % of TSAd is tyrosine phosphorylated in TCR-activated T cells—Our results strongly indicate that interaction of TSAd with Lck-SH3 and SH2 domains results in processive phosphorylation of TSAd as well as modulation of Lck activity. Our data thus suggests that tyrosine phosphorylation of TSAd is a prerequisite for modulation of Lck.

TSAd is phosphorylated both in resting and activated Jurkat T cells transiently expressing TSAd (17;18) and in PHA stimulated PBMC (12). To explore to what extent TSAd is tyrosine phosphorylated under physiological conditions, we immunoprecipitated TSAd from human CD4+ T cells stimulated for 72 hours with anti-CD3/CD28 coated Dynabeads to induce TSAd expression (14). In these activated CD4+ T cells, TSAd was already tyrosine phosphorylated and restimulation of the cells resulted in a minor increase in phosphotyrosine level (Fig. 5D). To estimate the proportion of tyrosine phosphorylated TSAd molecules in activated T cells, we then induced endogenous TSAd expression in Jurkat T-cells by anti-CD3 stimulation for 16 hours, and performed immunodepletion of tyrosine phosphorylated proteins using an anti-phosphotyrosine antibody.

Immunoblotting of lysates from resting or CD3 stimulated cells before and after phosphotyrosine immunodepletion revealed that tyrosine phosphorylated TSAd constitute approximately 20% of the total amount of TSAd expressed in CD3 stimulated Jurkat T-cells (Fig. 5E). In cells transiently expressing TSAd, the tyrosine phosphorylated pool of TSAd was somewhat higher (30%), whereas the fraction of tyrosine phosphorylated TSAd mutated for the three C-terminal tyrosines, or the proline rich region 239-56 was virtually zero (Fig. 5F panel i and ii). Fig 5F panel iii shows that there was no gross difference in overall level of protein tyrosine phosphorylation in the lysates from the transfected cells before and after phosphotyrosine immunodepletion which could not explain this result. Thus, also in T cells both the Lck-SH3 and Lck-SH2 ligands are necessary for TSAd to be strongly tyrosine phosphorylated. Taken together, these results indicate that 20-30 % of TSAd is phosphorylated in vivo.

In order to put our results into a physiological context, we enumerated TSAd and Lck molecules in activated CD4+ T cells. To this end, we first determined that the polyclonal anti-TSAd antibody 1715T (15) contains two major epitopes, one of which accounted for 30% of the anti-TSAd reactivity and which was contained in the GST-TSAd-PY-YYY protein (Suppl. Fig. 1). As a result, the signal from intact TSAd in cell lysates was 10:3 (i.e. 3.3) fold stronger per molecule than the signal obtained from the recombinant TSAd-PY-YYY protein. By comparing the immunoblot signals from known amounts of recombinant Lck and GST-TSAd-PY-YYY proteins with the immunoblot signals of lysates from a defined number of cells, we found that peripheral CD4+ T cells activated with anti-CD3 antibodies for 24 hours express 20 000-50 000 TSAd and 50 000 Lck molecules pr cell. In comparison, anti-CD3 stimulated Jurkat T cells express in the order of 200 000 Lck and 200 000 TSAd molecules per cell (Fig. 5G). Hence in activated T cells expressing TSAd, the relative amount of TSAd to Lck is in the order of 1:1. Taken together our data suggests that a considerable number of Lck molecules may at any given time be engaged by TSAd in activated T cells.

We therefore propose a model whereby TSAd through multivalent interactions with Lck, diverts Lck kinase activity away from other substrates (Fig. 6A). When bound to TSAd, Lck may eventually phosphorylate other proteins that are brought into the vicinity of the TSAd-Lck complex (Fig. 6B) as we recently have demonstrated for Itk (Berge et al, submitted).

DISCUSSION

The main conclusion from this study is that TSAd phosphorylation is dependent on its interaction with both Lck-SH3 and -SH2
domains, and that it proceeds in a processive, rather than a distributive manner. We propose that through multi-site interactions with Lck, TSAd modulates Lck activity by sequestering Lck from some of its substrates, i.e. Zap-70.

Displacement of the intramolecular binding of Lck SH3 to prolines in the linker between the SH2 and the kinase domains, is known to partially activate Src family kinase (SFK)s (31). TSAd molecules lacking the Lck-SH3 ligand (aa 239-56) have reduced level of tyrosine phosphorylation compared to intact TSAd both in vitro and in vivo. Thus, the initial event during TSAd phosphorylation is probably the interaction between Lck-SH3 and the proline-rich region of TSAd.

Our model for how TSAd interacts with, and becomes phosphorylated by Lck, may explain how TSAd can mediate modulation of proximal tyrosine-phosphorylation events upon TCR triggering. The multivalent interaction between Lck and TSAd, where TSAd remains in complex with Lck through multiple rounds of phosphorylation, sequesters Lck away from other substrates. Recently, a crucial role for TSAd in activating Lck was reported (24). Marti et al showed that both the Lck-SH3 interaction site and the three C-terminal tyrosines of murine TSAd were required for TSAd to activate Lck in an in vitro kinase assay. Our data are in agreement with theirs; however, our interpretations of the data differ. It is well established that SH3 or SH2 ligands of SFK increases the activity of the kinase (31;37). Thus, when recombinant TSAd molecules are added to Lck in vitro, the net result may be increased activity of Lck (24). Also, if TSAd is a general and crucial activator of Lck, we would expect that transient expression of TSAd in Jurkat T cell lines would lead to increased amounts of tyrosine-phosphorylated proteins similar to what is observed for instance in Jurkat cells expressing hyperactive Lck mutated for the regulatory Y505. However, this is not the case. On the contrary, we regularly observe reduced tyrosine phosphorylation of Lck-dependent substrates when TSAd is over expressed in Jurkat cells (14;17;18). Moreover, some proteins display increased tyrosine phosphorylation level when TSAd is expressed in Jurkat T cells, for example see Fig.5G panel iii. Upon disruption of either the TSAd Lck-SH3 or -SH2 domain interaction sites, TSAd is no longer able to inhibit phosphorylation of Zap-70 Y319 (Fig. 5A). Both of these sites were also necessary for the reported activation of Lck (24). Collectively, this indicates that the Lck-SH3 and -SH2 interactions with TSAd work in synergy to exert the modulatory effect TSAd has on Lck activity in vivo.

The phenotype of mice lacking TSAd implies that TSAd is required for proper activation of T cells. TSAd is expressed only at low levels in resting naïve T cells, but is induced during the first few hours after triggering of the T cell (15). This suggests that TSAd is not essential for the initial triggering of the T cells, but plays a role later during the activation. The exact role of TSAd in T-cell activation is yet poorly defined. However, we recently found that TSAd promotes Lck-mediated phosphorylation of Itk (Berge et al, submitted). Moreover, a number of other interaction partners, including VCP (23), MEKK2 (50) and Grb2 (22) have been reported, indicating that TSAd may participate in several signaling pathways.

The presence of three combined Lck tyrosine-phosphorylation sites and Lck-SH2 ligands on TSAd within a distance of only 25 residues is analogous to the ITAMs of the T-cell receptor complex, where tyrosine motifs typically spaced between 10 to 12 amino acid residues from each others are phosphorylated by Lck and serves as docking sites for Zap-70 SH2 domains (51). The conserved nature of these three C-terminal tyrosines strongly indicate that these tyrosines are of importance for the function of TSAd. It is therefore highly likely that one or several of the C-terminal tyrosines serve as docking sites for other signaling molecules, that when bound to TSAd may eventually become phosphorylated by Lck.

Our observations of the Lck-TSAd interaction may have a biological significance extending above and beyond that related to the role of TSAd in T-cell activation. Although processive phosphorylation as a phenomenon is described in the literature, the concept and its possible consequences is not often alluded to. Many SFK substrates also harbor interaction sites for the SH2 and SH3 domains of the kinase. Indeed, the adapter protein Crk-associated substrate (Cas) has been shown to be phosphorylated in a processive manner by Src (52). The Cas family member Sin was recently reported to modulate activation of T cells through regulation of Fyn availability. In resting cells, Sin is constitutively phosphorylated by Fyn, which remains in complex with Sin until the latter becomes transiently dephosphorylated.
upon TCR triggering (53). Processive phosphorylation of SFK substrates may be an important mechanism not only to ensure proper phosphorylation of a given substrate, but also to ensure that the kinase activity is contained within a certain molecular micro environment. In the absence of its SH2 or SH3 domain, SFK may become hyperactive and oncogenic (54), which can be explained based on lack of intramolecular regulatory interactions. But another not mutually exclusive consequence of lacking SFK SH2- or SH3-domains could be inappropriate tyrosine phosphorylation of substrates which normally are not tyrosine phosphorylated by SFKs.

In conclusion, we have found that the three C-terminal tyrosines of TSAd are phosphorylated by Lck through a processive mechanism, involving TSAd interactions with both the Lck-SH2 and -SH3 domains. The multivalent interactions between TSAd and Lck are also necessary for TSAd mediated modulation of proximal signaling observed in TSAd expressing T-cell lines. We thus propose that processive phosphorylation where the kinase docks onto the substrate through protein interaction domains is an important mechanism not only to ensure proper phosphorylation of kinase substrates with multiple phosphorylation sites, but also to keep kinase activity confined to particular molecular micro environments within the cell.

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**FOOTNOTES**

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*The abbreviations used are: TSAd, T cell specific adapter protein; aa, amino acid(s); SH2, Src homology domain 2; SH3 Src homology domain 3, GST, glutathione-S-transferase; PAGE, polyacrylamide gel electrophoresis; mAbs, monoclonal antibodies; pY, phosphotyrosine; ITAM, immunoreceptor tyrosine-based activation motif; TCR, T cell receptor; Zap-70, zeta associated protein of 70 kD; SLP-76, leukocyte specific protein of 76 kD; LAT, linker of activated T cells; Lad, Lck adapter molecule; RIBP, Rlk-Itk binding protein; Lck, lymphoid cell kinase; IP, immunoprecipitate; GST, glutathione S-transferase; SFK, Src family kinase; ITC, Isothermal calorimetry.*
FIGURE LEGENDS

Figure 1. **TSAd is phosphorylated on more than one tyrosine.*** A. Schematic presentation of TSAd. Y= tyrosine, SH2=Src homology 2 domain. B and C. TSAd IPs from 293T cells transiently transfected with the indicated cDNAs (φ=empty vector) were immunblotted with the indicated antibodies (Abs) D. GST-Lck-SH2 pulldowns from 293T cells transiently transfected with the same constructs as in C. The precipitates and lysates were immunoblotted with anti-HA. Results in B are representative of at least three experiments, whereas results in C and D are representative of at least two experiments.

Figure 2. **The three evolutionary conserved C-terminal tyrosines of TSAd are substrates for Lck and ligands for the Lck SH2-domain.*** A. Sequence alignment of the C-terminal region of TSAd molecules from the indicated species. Amino acid numbering refers to the human TSAd sequence (NP_003966). B. TSAd IPs and GST-Lck-SH2 pulldowns from 293T cells transiently expressing Lck together with the indicated HA-tagged TSAd constructs were immunoblotted with the indicated Abs. Results are representative of two to five experiments. C. Schematic representation of GST-TSAd fusion protein described in the text. D and E. Mass spectrometry analysis of tryptic digests of GST-TSAd-PY-YYY and GST-TSAd-PY-FFF fusion proteins incubated with recombinant Lck *in vitro*. D shows peaks representing non-phosphorylated and phosphorylated Y280, Y290 and Y305 peptides from the TSAd-PY-YYY protein, whereas E shows non-phosphorylated and phosphorylated TSAd-Y260 peptide from both the TSAd-PY-YYY and the TSAd-PY-FFF protein. Results are representative of two experiments.

Figure 3. **The highly conserved C-terminal region of TSAd is phosphorylated by Lck in a processive manner.*** A. TSAd IPs and control IPs (NRS) from cell lysates of resting (+) or anti-CD3 stimulated (−) Jurkat T cells transiently expressing the indicated HA-tagged TSAd proteins were immunoblotted with the indicated Abs. B. Equal amounts of protein subjected to *in vitro* kinase assay (5 minutes incubation at 30°C) of a 100:1 mixture of TSAd:Lck (14 μM TSAd-PY-YYY and 140 nM Lck) diluted in two fold steps in kinase buffer as indicated, were immunoblotted with anti-pY (upper panel) or stained with Coomassie blue (lower panel). C. Immunoblotting with anti-pY (upper panel) and anti-GST (lower panel) of a 1000:1 mixture of TSAd:Lck (20 μM TSAd and 20 nM Lck) incubated over a 10 minutes time course. D. A 250:1 mixture of TSAd:Lck (5 μM TSAd and 20nM Lck) was incubated in kinase buffer over a 20 minutes time course and immunoblotted as in C. E Immunoblot with anti-pY416 Src (i.e. Lck pY394, upper panels) or anti-Lck (lower panel) of a 10:1 mixture of TSAd peptide:Lck (100nM peptide and 10 nM Lck) incubated in kinase buffer for 1 or 7.5 minutes. Results are representative of at least three (B, C and D) or two experiments (A and E).

Figure 4. **Maximal tyrosine phosphorylation of TSAd is dependent both on Lck SH2- and SH3-domain interactions.*** A. TSAd IPs and control IPs (NRS) from cell lysates of resting (+) or anti-CD3 stimulated (−) Jurkat T cells transiently expressing the indicated HA-tagged TSAd proteins were immunoblotted with the indicated Abs. B. Equal amounts of protein subjected to *in vitro* kinase assay (5 minutes incubation at 30°C) of a 100:1 mixture of TSAd:Lck (14 μM TSAd-PY-YYY and 140 nM Lck) diluted in two fold steps in kinase buffer as indicated, were immunoblotted with anti-pY (upper panel) or stained with Coomassie blue (lower panel). C. Immunoblotting with anti-pY (upper panel) and anti-GST (lower panel) of a 1000:1 mixture of TSAd:Lck (20 μM TSAd and 20 nM Lck) incubated over a 10 minutes time course. D. A 250:1 mixture of TSAd:Lck (5 μM TSAd and 20nM Lck) was incubated in kinase buffer over a 20 minutes time course and immunoblotted as in C. E Immunoblot with anti-pY416 Src (i.e. Lck pY394, upper panels) or anti-Lck (lower panel) of a 10:1 mixture of TSAd peptide:Lck (100nM peptide and 10 nM Lck) incubated in kinase buffer for 1 or 7.5 minutes. Results are representative of at least three (B, C and D) or two experiments (A and E).

Figure 5. **In vivo TSAd tyrosine phosphorylation and in vivo modulation of Lck activity are both linked to Lck SH3 and tyrosine phosphorylation/Lck SH2 interaction sites on TSAd.*** A. Immunoblots with the indicated Abs of lysates from Jurkat T cells untreated or treated with the Src family kinase inhibitor PP2 (5μM) for 30 minutes prior to anti-TCR ligation (C305) for 2.5 minutes. B. Immunblots with the indicated Abs of lysates from Jurkat T cells transiently expressing the indicated HA-tagged TSAd proteins and stimulated (+) with anti-TCR or not (-) for 1, 2.5 or 5 minutes. C. Similar to B. Jurkat T cells were stimulated for 2.5 minutes with anti-TCR D. TSAd IPs from human CD4+ peripheral T-cells stimulated for 72 hours with CD3/CD28 ligation (Dynabeads) were immunoblotted with biotinylated anti-TSAd or anti-phosphotyrosine (pY) Abs respectively. (-) cells...
after removal of beads and washing of cells, (+) cells treated as (-) and then restimulated for 20 min
with anti-CD3/CD28 coated Dynabeads. E. Phospho-immunodepletion of lysates from Jurkat T cells
stimulated with plate-bound anti-CD3 for 16 hours. i) Anti-TSAd immunoblots of the starting lysate
and the depleted lysate (+/− pY depleted) (upper panel) and of the three consecutive anti-pY IPs (lower
panel). Anti-TSAd immunoblot of control is first pY-IP from resting Jurkat T cells. ii) Relative
amount of TSAd protein before and after phospho-immunodepletion was estimated by quantification
of the chemiluminescence signals from anti-TSAd immunoblots shown in i). Level of TSAd in
stimulated cells before immunodepletion is set to 100. Median of three experiments with SD is shown.
F. Phospho-immunodepletion of lysates from Jurkat T cells expressing the indicated HA-tagged TSAd
constructs. Prior to lysis, cells were stimulated with anti-CD3 Abs for 2.5 minutes. i) Chart shows
median values ± SD from three experiments of relative amount of TSAd before and after phospho-
immunodepletion., ii) Anti-TSAd immunoblot of the starting lysate and the depleted lysate (+/− pY
depleted) (upper panel) and of the first pY IP from each TSAd transfectant (lower panel). iii) Anti-pY
immunoblots of lysates from each TSAd transfectant prior to and after the last round of phospho-
immunodepletion. G. Enumeration of TSAd and Lck molecules in peripheral CD4+ T cells and Jurkat
T cells. Immunoblots with the indicated Abs of lysates from CD4+ T cells and Jurkat T cells
stimulated with plate-bound anti-CD3 for the indicated time points, and of known amounts of
recombinant Lck and TSAd. TSAd signal from endogenous protein is 3.3 times stronger per molecule
than that of the recombinant TSAd protein (Suppl. figure 1). Results in B, C, and E are representative
of at least three experiments, whereas results in A, F and G are representative of at least two
experiments.

Figure 6. Model for TSAd mediated sequestrering of Lck activity. A. Lck phosphorlates TSAd and
is engaged by TSAd via its SH2 and SH3 domains. Protein X is not phosphorylated by Lck, since Lck
is sequestered by TSAd. B. TSAd engages both Lck and a membrane bound protein Y, which
simultaneously binds protein X. In this situation, TSAd has brought Lck into the vicinity of protein X,
which may become phosphorylated by Lck.
Table 1: Detection of phosphorylated peptides from GST-TSAd-PY-YYY by mass spectrometry

| TSAd-WT (PY-YYY) (aa position) | Tyrosines | No of phospho | Mass    | Lck | ALP\(^a\) |
|-------------------------------|-----------|---------------|---------|-----|----------|
|                               |           | tyrosines     |         |     |          |
| 238EPSQLLRPKPPIPAPKQLPPEVYTIPVR\(^{266}\) | Y260      | 0             | 3257.88 | +++| -        |
|                               |           | 1             | 3337.88 | -   | +++      |
| 267 HRPA_RPKPSPNI_YNPIEPIA_FYAMGR\(^{294}\) | Y280, Y290| 0             | 3220.61 | (+)| -        |
|                               |           | 1             | 3300.61 | -   | (+)      |
|                               |           | 2             | 3380.61 | -   | +++      |
| 295 GSPGEAPSNI_YVEVEDG\(^{312}\) | Y305      | 0             | 1848.81 | +++| +        |
|                               |           | 1             | 1928.81 | -   | +++      |

\(^a\): ALP: Alkaline phosphatase

\(^b\): +++: Clear signal, +: low signal, (+): trace signal, -: no signal
Table 2. Isothermal calorimetry titration of phosphopeptide hexamers binding to isolated Lck SH2 molecules

| Peptide | Sequence | Kd (µM) | ΔH (kcal/mol) | ΔG (kcal/mol) | -TΔS (Kcal/mol) |
|---------|----------|---------|---------------|---------------|----------------|
| TSAd pY280 | PpYNpEP | 3.47 ± 0.57 | -2.37 ± 0.35 | -7.58 | -5.21 |
| TSAd pY290 | AFpYAMG | 3.99 ± 0.17 | -2.35 ± 0.40 | -7.48 | -5.14 |
| TSAd pY305 | NLpYVEV | 0.36 ± 0.02 | -3.38 ± 0.07 | -8.94 | -5.55 |
| HMT pY324 | PQpYEEI | 0.05 ± 0.01 | -7.48 ± 0.41 | -10.10 | -2.62 |
| Lck pY505 | GQpYQQPQ | 5.58 ± 0.30 | -1.22 ± 0.26 | -7.28 | -6.07 |
Figure 1

A

B

C

D
Figure 4

A

203T cells
TSAd Δ239-56  -  -  +  +  +
TSAd  +  -  +  +  +  -
LckR154K  -  -  -  -  +  +
Lck  +  +  +  +  +  +

WB:
TSAd IP

anti-pY
anti-HA

Lysate

anti-HA
anti-Lck

B

Relative phospho-
ytrosine level of

TSAd
TSAd Δ239-56

Lck
LckR154K

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Figure 6

A

Cell membrane

Protein X

Lck

TSAd

SH2

SH3

SH2

Kinase

B

Activated receptor Y

TSAd

SH2

SH2

Kinase

Lck

pY
