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PHOSPHORYLATION AT SERINE 318 IS NOT REQUIRED FOR INHIBITION OF T CELL ACTIVATION BY ALX

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Abstract

The activation of T cells and the initiation of an immune response is tightly controlled by both positive and negative regulators. Two adaptors which function as negative regulators of T cell activation are ALX and LAX. ALX constitutively associates with LAX in T cells, and T cells from mice deficient in ALX and LAX display similar hyper-responsiveness upon T cell receptor (TCR)/CD28 stimulation, including increased production of interleukin-2. During T cell activation, ALX is inducibly phosphorylated, however the site of ALX phosphorylation had not been previously identified and the role of phosphorylation in the inhibitory function of ALX was not known. Here, using mass spectrometry, we demonstrate that ALX is phosphorylated on a serine at position 318. Substitution of alanine for serine at this position (ALX S318A) leads to an abrogation of the mobility shift in ALX induced upon TCR/CD28 stimulation. However, ALX S318A retained the ability to bind to and stimulate tyrosine phosphorylation of LAX. In addition, overexpression of ALX S318A inhibited RE/AP activation upon TCR/CD28 stimulation to a similar extent as wild type ALX. Therefore, although ALX is inducibly phosphorylated upon TCR/CD28 stimulation, this phosphorylation is not required for ALX to inhibit T cell activation.

Keywords

T cell; adaptor; ALX; phosphorylation

Introduction

Activation of T cells required the integration of signals from a minimum of two receptors: in naïve T cells, these signals are generally provided by an antigen-dependent signal through the T cell receptor (TCR) [1] combined with an antigen-independent signal through CD28 [1]. If TCR signals are received in the absence of costimulatory signals, T cells are not activated and instead enter an anergic state, in which they are refractory to further stimulation even when both TCR and CD28 signals are subsequently given together. Anergy of peripheral T cells is one mechanism that prevents autoimmunity by insuring that T cells activation and initiation

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[1]**Abbreviations:** TCR, T cell receptor; IL-2, interleukin-2; WT, wild type; PAGE, polyacrylamide gel electrophoresis; LC, liquid chromatography; MS, mass spectrometry;

of the immune response occurs only within an appropriate context in which costimulation is provided by professional antigen-presenting cells. TCR/CD28 signaling initiates a cascade of events, starting with tyrosine phosphorylation of CD3/ζ chains by Src family kinases and recruitment of Syk family tyrosine kinases [2]. Downstream pathways are subsequently triggered, leading to events including activation of mitogen-activated protein kinases, and the transcription factors NF-κB, AP-1 and NFAT leading to alteration in gene expression, including inducing cytokines such as interleukin-2 (IL-2). Within the IL-2 promoter, the key transcriptional element which is the site of signal integration between TCR and CD28 is the RE/AP element [3]. Unlike the NFAT and AP-1 sites in the IL-2 promoter which can be activated by TCR signaling alone, TCR stimulation has no effect on RE/AP activation unless paired with concurrent CD28 costimulation [3]. Thus, understanding the signaling pathways that regulate TCR/CD28-mediated activation of the RE/AP element from the IL-2 promoter will give insight into the molecular mechanisms of signal integration between TCR and CD28 that regulate T cell activation.

Previously, we identified a 352 amino acid adaptor molecule designated ALX (also known as HSH2) [4], which is a member of a larger family of adaptor molecules also including TSAd/RIBP, SH2D4A and SH2D4B (reviewed in [5]). Expression of ALX is limited to T cells and other cell types within the hematopoetic lineage. ALX lacks any intrinsic enzymatic activity, but contains several motifs for protein-protein interactions, including a phospho-tyrosine binding SH2 domain as well as four polyproline sequences for association with SH3/WW domains which are conserved evolutionarily among ALX orthologs. Overexpression of ALX in the Jurkat T cell line inhibited the activation of RE/AP, but not AP-1 reporters, upon TCR/CD28 stimulation, suggesting that ALX functions as a negative regulator of T cell activation [4]. Its negative role was confirmed when ALX-deficient mice were generated and examined; ALX-deficient T cells demonstrated enhanced IL-2 production and proliferation upon TCR/CD28 stimulation [6]. Mutational analysis demonstrated that the ALX SH2 domain was required for negative regulation of T cell activation [7]. The T cell phenotype in ALX-deficient mice was similar to that observed in mice lacking the transmembrane adaptor LAX [8,9], and a physical interaction between ALX and LAX was subsequently discovered [6]. This association is specific for LAX, as ALX does not associate with LAT or a third related adaptor LAB (also known as LAT2 or NTAL) [6,10–12]. The association between ALX and LAX occurs constitutively via a mechanism independent of both tyrosine phosphorylation of LAX and the SH2 domain ALX [13]. Coexpression of ALX and LAX in Jurkat T cells increased tyrosine phosphorylation of LAX. ALX-driven LAX phosphorylation required the ALX SH2 domain and was absent in the Lck-deficient J.CaM1 Jurkat cell line. The ALX SH2 domain was found to associate directly with Lck, suggesting that ALX functions, at least in part, by recruiting Lck to LAX [13].

Upon TCR/CD28 stimulation, ALX is inducibly phosphorylated [7], however the site of ALX phosphorylation had not been previously identified and the role of phosphorylation in the inhibitory function of ALX during T cell activation was not known. Here, using mass spectrometry, we demonstrate that ALX is phosphorylated on a serine at position 318. Substitution of alanine for serine at this position (ALX S318A) leads to the abrogation of the mobility shift in ALX induced upon TCR/CD28 stimulation. However, ALX S318A retained the ability to bind to and stimulate tyrosine phosphorylation of LAX. In addition, overexpression of ALX S318A inhibited RE/AP activation upon TCR/CD28 stimulation to a similar extent as wild type (WT) ALX. Therefore, although ALX is inducibly phosphorylated upon TCR/CD28 stimulation, this phosphorylation is not required for the inhibition of T cell activation by ALX.

Materials and Methods

Expression plasmids and cell lines

The myc-tagged LAX expression plasmid, myc-HIS-tagged ALX expression plasmid, untagged ALX expression plasmid and RE/AP luciferase reporter have been previously described [3,4,13]. The ALX S318A mutant was generated by PCR mutagenesis (primers available upon request). Jurkat T cells were provided by Art Weiss (University of California, San Francisco), and cultured in RPMI with 5% Fetal Calf Serum. Jurkat T cells which stably express myc-His-tagged wild type (WT) ALX were previously described [4].

Purification of ALX for mass spectrometry

10^8 Jurkat cells or Jurkat cells expressing myc-His-tagged ALX were stimulated with anti-TCR and anti-CD28 for 45 minutes. ALX was immunoprecipitated from NP-40 cytoplasmic lysates using anti-myc coated protein A beads, and eluted using excess myc peptide as previously described [6]. The resulting eluates were run on SDS-PAGE and stained with Simply Blue (Invitrogen). A band that corresponded to the expected size for myc-His-tagged WT ALX and that was absent in the control Jurkat immunoprecipitation was excised from the gel.

Digestion of Protein

The excised band was cut into 1 mm^3 pieces. Gel pieces were destained by adding 50% Acetonitrile (Burdick and Jackson), 200 mM Ammonium Bicarbonate (Sigma) and incubating at 37 °C for 30 minutes. Samples were reduced with 20mM TCEP (Pierce) in 50 mM Ammonnium Bicarbonate (Sigma) at 56 °C for 45 minutes followed by alkylation with 40 mM iodoacetamide (Sigma) in 50 mM Ammonium Bicarbonate (Sigma) for 45 minutes at room temperature. Gel pieces were rinsed twice with 25 mM Ammonium Bicarbonate, once with 50% Acetonitrile/25 mM Ammonium Bicarbonate and dried by vacuum centrifugation. Proteolysis was initiated by the addition of 20 ng/μL of sequencing grade GluC (Sigma) and allowed to proceed overnight at 37 °C. Supernatant was removed and placed in a separate tube. Gel pieces were incubated for another 30 min at 37 °C with 20 mM Ammonium Bicarbonate. This supernatant was combined with the previous. Gel pieces were then incubated in 50% Acetonitrile/5% Formic Acid (Pierce) for 30 minutes at 37 °C and this supernatant was combined with the previous two. The final extraction step was repeated once more and the combined supernatant was reduced to a 20 μL volume by vacuum centrifugation.

Peptide analysis

5 μL of peptide digest was analyzed by liquid chromatography-mass spectrometry/mass spectrometery (LC-MS/MS) using an Eksigent 2D nano LC system coupled to a Thermo LTQ Orbitrap XL mass spectrometer. Sample was loaded onto a C_{18} trap column (75 μm i.d. × 15 mm; Proteopep 2, New Objective, Woburn MA) isocratically in 2% Acetonitrile Burdick and Jackson)/1% Methanol (Burdick and Jackson)/0.1% Formic acid (Thermo) at a flow rate of 1 μL per minute. The trap was then switched in line with the analytical column (75 μm i.d. × 150 mm; Proteopep 2, New Objective, Woburn MA). A linear gradient was then initiated at a flow rate of 300 nL per minute (3% – 20%B over 90 minutes). Buffer A was 0.1% Formic acid (Thermo)/1%Methanol (Burdick and Jackson) and Buffer B was 80%Acetonitrile (Burdick and Jackson)/1% Methanol (Burdick and Jackson)/0.1% Formic acid (Thermo). Data were collected using a data dependent top 5 method with a minimum signal count of 1500 and dynamic exclusion with a repeat count of 2, repeat duration of 15s, exclusion size of 500 and duration of 60s.

Database Searching

All MS/MS samples were analyzed using Sorcerer™-SEQUEST® (Sagen) (Thermo Fisher Scientific, San Jose, CA, USA; version v.27, rev. 11). Sequest was set up to search a database comprising protein sequences from human, mouse, rat, rabbit, bovine and guinea pig entries (Uniprot Swissprot December 3,2008, 171404 entries) assuming the digestion enzyme V8. SEQUEST® was searched with a fragment ion mass tolerance of 1 Da and a parent ion tolerance of 50 ppm. Iodoacetamide derivative of cysteine was specified in SEQUEST®as a fixed modification. Oxidation of methionine was specified in SEQUEST®as a variable modification.

Criteria for protein identification

Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [14]. Protein identifications were accepted if they could be established at greater than 90.0% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [15]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. ALX (Q96JZ2) was identified with 14 unique peptides, 16 unique spectra with 36% sequence coverage. The MS/MS spectrum of the phospho-peptide VTPGDRSWHQMVVRALSSQE was manually annotated. Fragment ions observed in three separate runs of the sample indicated phosphorylation at the c-terminal serines, however none could distinguish between the two.

Transfections and luciferase assays

Transfections, stimulations and luciferase assays were performed as previously described [3, 16]. Briefly, 15×10^6 Jurkat cells were transfected with 10 μg of reporter and various amounts of expression plasmid (as denoted in each figure legend) by electroporation using a Gene Pulser II (BioRad). The following day, live cells were counted by trypan blue exclusion (Bio-Whittaker), and 1×10^6 live cells per sample were either left unstimulated or stimulated with antibodies to TCR (C305, 1:1000 final dilution) and CD28 (1 μg/ml) for 7 hours before luciferase assays were performed [16].

Analysis of ALX/LAX association by immunoprecipitation

As previously described, cells were transfected with untagged ALX and myc-tagged LAX expression plasmids, lysed in NP-40 buffer and subject to immunoprecipitation with anti-myc antibody followed by elution with myc peptide [13]. Cell lysates and immunoprecipitates were loaded on gels and analyzed by western blotting with rabbit antibodies to Myc (Cell Signaling) and ALX [4] and with anti-phosphotyrosine 4G10 (Millipore).

Results

Mass spectrometry identifies a site of serine phosphorylation at the c-terminal end of ALX

To understand the role of phosphorylation in ALX function during T cell activation, myc-his tagged human ALX was immunoprecipitated from Jurkat T cells that had been stimulated with anti-TCR and anti-CD28. Immunoprecipitated proteins were resolved by SDS-PAGE, stained, and the ALX band was excised and subjected to Glu-C digestion. MS/MS spectrum analysis of the digest revealed a phospho-peptide VTPGDRSWHQMVVRALSSQE corresponding to amino acids 302–321 of human ALX. The phosphorylation site was mapped to the c-terminal di-serines (at positions 318 and 319, figure 1A). However, this analysis could not determine at which serine the phosphorylation occurred. A comparison of ALX amino acid sequences

from different species revealed that only the serine at position 318 is conserved evolutionarily; murine ALX contains an alanine at position 319 (figure 1B). As we had previously demonstrated that endogenous ALX from human and murine T cells undergoes a shift in mobility upon TCR/CD28 stimulation [6,7], it seemed most likely that the site of phosphosphorylation was at the conserved serine 318. Also, serine 318 lies within a conserved RxxS motif that matches the consensus for a site of PKC phosphorylation [17]. We previously demonstrated that PMA, a potent activator of PKC, also induces a shift in ALX mobility similar to that observed upon TCR/CD28 stimulation [6]. TCR/CD28 signaling is a potent inducer of PKC. Therefore, the identification of a phospho-peptide containing a conserved PKC phosphorylation motif was not unexpected based on previous work.

Mutation of serine 318 to alanine abrogates the shift in ALX mobility upon TCR/CD28 stimulation

In Jurkat T cells, TCR/CD28 stimulation induces a shift in the mobility of ALX in SDS-PAGE attributed to phosphorylation as it occurs with kinetics paralleling that of orthophosphate incorporation [7]. To determine whether phosphorylation of serine 318 is responsible for the shift in ALX mobility observed upon TCR/CD28 stimulation, we mutated this serine to alanine (ALX S318A mutant). Upon transient transfection in to Jurkat T cells, wild type (WT) ALX displayed a mobility shift upon TCR/CD28 stimulation (figure 2). However, no shift in the mobility of ALX S318A was observed. Therefore, this indicates, in agreement with the mass spectrometry data, that the primary site in ALX which is inducibly phosphorylated upon TCR/CD28 stimulation is serine 318.

ALX S318A retains the ability to bind LAX and promote LAX tyrosine phosphorylation

ALX constitutively associates with the transmembrane adaptor LAX and promotes tyrosine phosphorylation of LAX [13]. Although the stimulation of LAX phosphorylation depends on the SH2 domain of ALX, which associates with the LCK tyrosine kinase, the SH2 domain is not required for LAX and ALX to associate. In fact, an ALX mutant containing only amino acids 136–352 associates with LAX [13]. As serine 318 is found within this segment, phosphorylation at this position might be required for the association of ALX with LAX. To address this possibility, Jurkat T cells were transfected with vector control or WT LAX, along with either vector control, WT ALX or ALX S318A. As shown in figure 3, WT ALX and ALX S318A bound to LAX to a similar extent. Additionally, co-expression of ALX S318A also lead to tyrosine phosphorylation of LAX, as measured by 4G10, to a similar extent as WT ALX. Therefore, although mutation of serine 318 leads to abrogation of ALX phosphorylation, it does not alter the ability of ALX to associate with LAX or promote LAX tyrosine phosphorylation.

ALX phosphorylation is not required for inhibition of RE/AP during T cell activation

Although phosphorylation of ALX at S318A is not required for it to associate with LAX, this phosphorylation could influence the ability of ALX to negatively regulate T cell activation through a different mechanism. To test functionally whether ALX S318A can inhibit T cell activation, Jurkat T cells were transiently transfected with an RE/AP luciferase reporter, along with either empty vector or increasing amounts of either WT ALX or ALX S318A expression plasmids. The following day, transfected cells were either left unstimulated, or stimulated with anti-TCR/anti-CD28. As shown in figure 4A, RE/AP was inhibited to a similar extent by ALX S318A as compared to WT ALX (differences not statistically significant by student's t test). To eliminate the possibility that differences in potency were masked by alterations in protein expression, ALX protein expression was examined in whole cell lysates. As shown in figure 4B, WT ALX and S318A were expressed at similar levels at either plasmid concentration. Therefore, a mutated version of ALX that does not undergo inducible phosphorylation upon

TCR/CD28 stimulation completely retains the ability to inhibit RE/AP activation, demonstrating that phosphorylation of ALX is not required for it to inhibit T cell activation.

Discussion

Previously, we identified ALX as an adaptor molecule that negatively regulates T cell activation downstream of TCR/CD28 stimulation. Overexpression of ALX in Jurkat T cells leads to inhibition of the transcriptional activation of the RE/AP element from the IL-2 promoter, and T cells deficient in ALX are hyper-responsive and produce increased IL-2 upon TCR/CD28 stimulation. Upon stimulation, ALX is inducibly phosphorylated, however the contribution of ALX phosphorylation to its function was not known. Here, we describe the identification by mass spectrometry of one site of in vivo phosphorylation of ALX, serine 318. Although mutation of serine 318 to alanine abrogated the shift in mobility of ALX observed upon T cell activation, loss of this phosphorylation did not alter the ability of ALX to inhibit RE/AP during T cell activation, or to associate with and promote tyrosine phosphorylation of LAX. Therefore, phosphorylation of serine 318 is not required for ALX-mediated inhibition of T cell function.

Our previous work demonstrated that ALX is inducibly phosphorylated upon TCR/CD28 stimulation, as measured by orthophosphate incorporation, and also undergoes two distinct shifts in mobility in SDS-PAGE (figure 2 and [7]). The first, which occurs with kinetics paralleling orthophosphate incorporation, results in a mobility change in nearly the entire population of ALX molecules between 5 and 15 minutes. This first shift requires TCR signaling, but was shown to be independent of CD28. However, the second shift, which occurs later and to a lesser extent, was found to be dependent on CD28 costimulation. The second shift was also found to be dependent on the ALX SH2 domain, as it was eliminated by an arginine to lysine mutation that abrogates the ability of the SH2 domain to bind to tyrosine phosphorylated proteins. Here, we show that the first shift is abrogated in the ALX S318A mutant, confirming that it is due to phosphorylation. In the ALX S318A mutant, we also do not see any evidence of the second, later shift that is observed in WT ALX. Either this second event is dependent on prior phosphorylation at serine 318, or it does not produce, by itself, a change in mobility that is apparent on SDS-PAGE. The identity of this second site of modification is under investigation.

Serine 318 is located within a consensus RxxS phosphoryaltion motif for PKC family members [17]. In T cells, the primary PKC family member that is activated upon TCR/CD28 stimulation is PKCθ. PKCθ is critical for activation of mature T cells, including induction of IL-2 [18]. Therefore, the most likely PKC family member to phosphoryates ALX is PKCθ. We have transiently transfected constitutively activated PKCθ (A148E mutant [19]) with ALX and find that it leads to ALX phosphorylation in the absence of TCR/CD28 stimulation (data not shown). However, PKCθA148E overexpression also led to phosphorylation of ALX S318A, which is not inducibly phosphorylated upon TCR/CD28 stimulation. It may be that overexpression of PKCθA148E results in phosphorylation at sites in addition to those normally phosphorylated in vivo; however, we can not yet unambiguously conclude that PKCθ directly phosphorylates ALX at S318.

While S318 phosphorylation is not required for ALX-mediated inhibition of T cell activation, the possibility that it contributes to a different function of ALX can not be excluded. Although deletion of ALX did not significantly alter B cell development [6], ALX is expressed in B cells and its expression is dynamically regulated. Expression is upregulated upon stimulation with pro-survival stimuli including CD40 and BAFF/BlyS and downregulated upon pro-apoptotic stimulation with IL-21 [20,21]. Thus, there may be additional functions for ALX in the B cell lineage for which phosphorylation of S318 may be critical.

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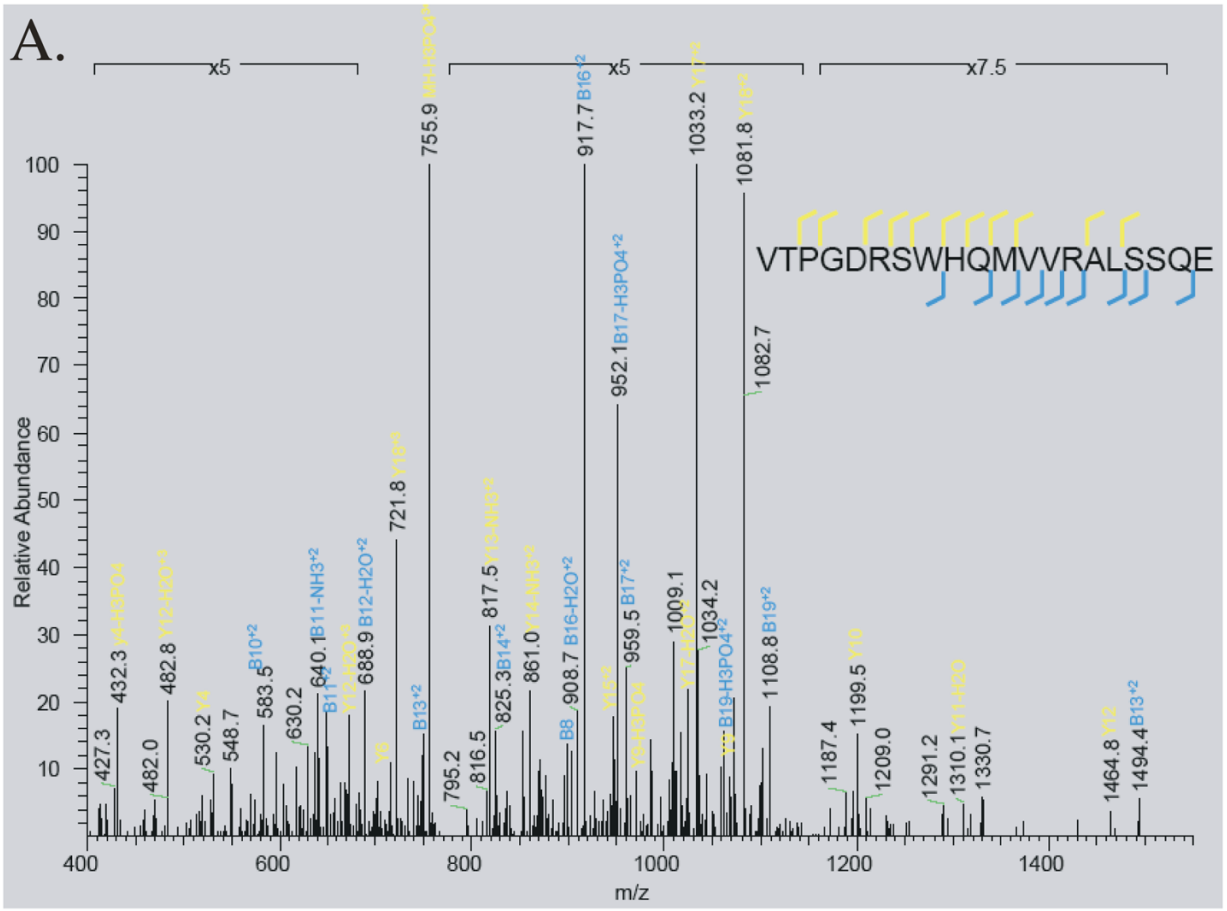

B.

human ALX	QMVVRALSSQE
murine ALX	GKVVRALSAQE
chimp ALX	QMVVRALSSQE
canine ALX	QVVVRALSSKV
bovine ALX	QTVVRVLSLQA
consensus PKC	RxxS

Figure 1. Mass spectrometry identifies an in vivo site of ALX phosphorylation

A. MS/MS spectrum of the phospho-peptide VTPGDRSWHQMVVRALSSQE from a Glu-C digest of ALX. Fragment ions observed in each of three separate runs of the sample indicate phosphorylation at the c-terminal serines, however none can distinguish localization between the two.

B. Sequence alignment of ALX protein from different species, demonstrating that S318 is conserved across species while S319 is not. Note that S318 lies within a conserved RxxS PKC phosphorylation motif.

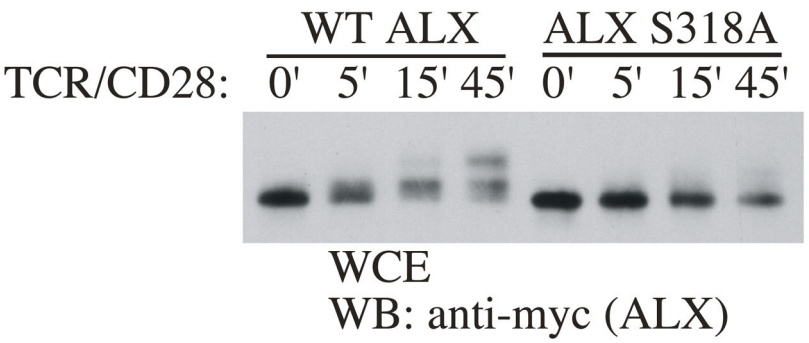

Figure 2. The shift in ALX mobility upon TCR/CD28 activation is abrogated by mutation of serine 318 to alanine

Jurkat T cells were transfected with either myc-HIS-tagged WT or S318A ALX expression plasmids. The following day, the transfected cells were stimulated with anti-TCR (C305) and anti-CD28 for the times indicated. Whole cell lysates were generated, and examined by western blotting with anti-myc to monitor ALX mobility.

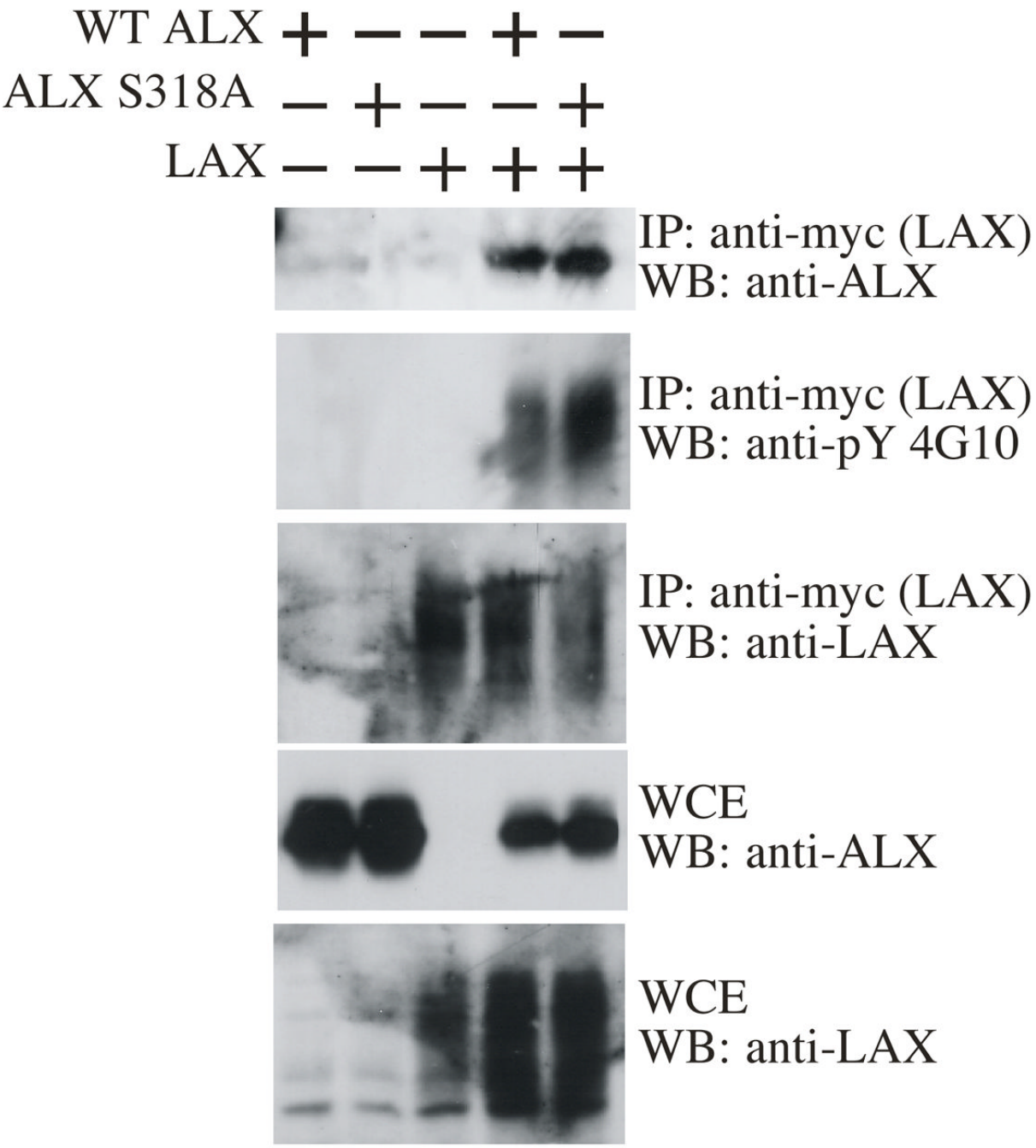

Figure 3. ALX S318A retains the ability to bind to LAX and induce LAX tyrosine phosphorylation

Jurkat T cells were co-transfected with either a vector control or an expression plasmids for myc-tagged LAX and with expression plasmids for either vector control, WT ALX or ALX S318A (without epitope tags). LAX was immunoprecipitated with anti-myc, and immunoprecipitates (IP, top three panels) and whole cell extracts (WCE, bottom two panels) were then examined by western blotting (WB) with antibodies to either ALX, LAX or phosphotyrosine (4G10).

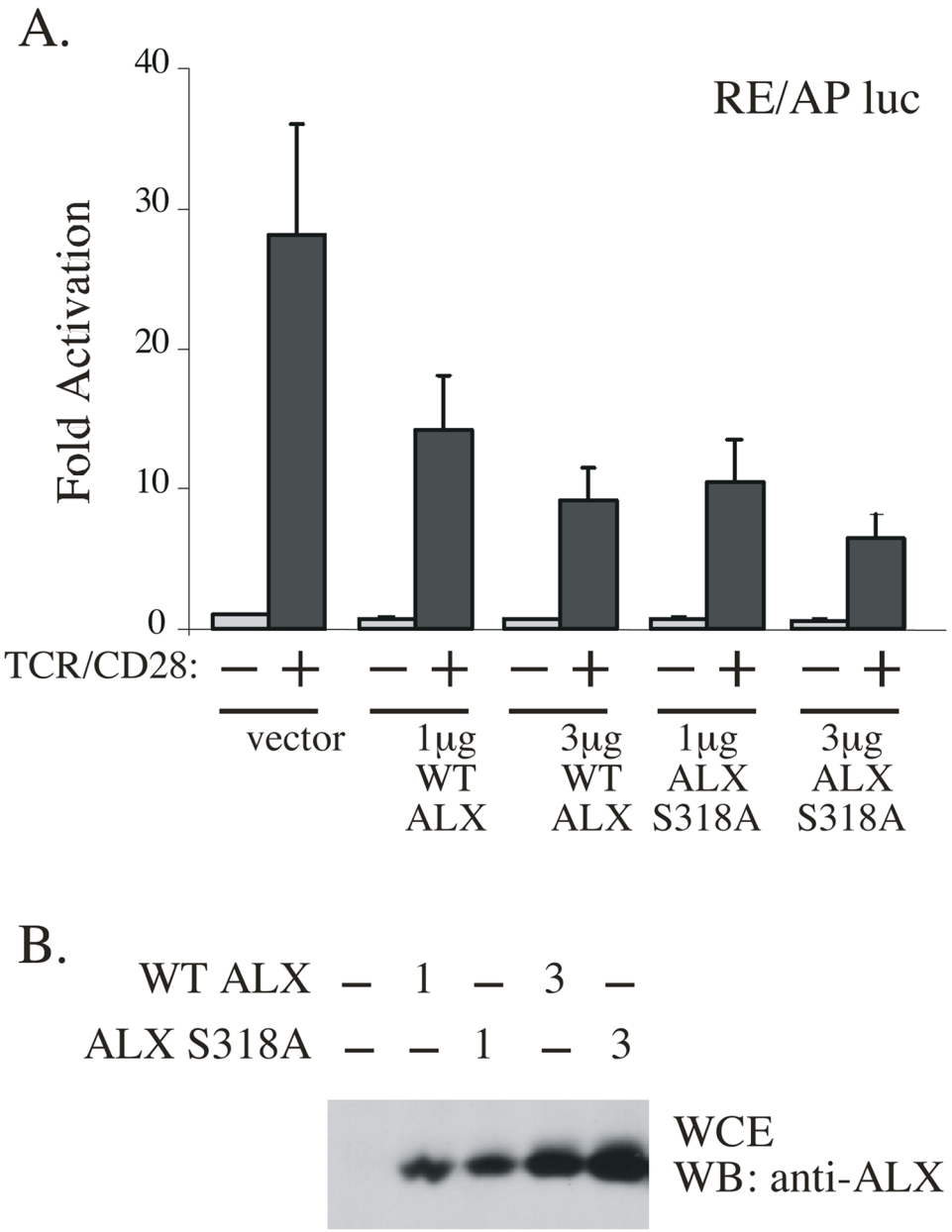

Figure 4. ALX S318A is expressed at similar levels to WT ALX and is able to inhibit activation of RE/AP in response to TCR/CD28 signaling

A. Jurkat T cells were transfected with 10μg of an RE/AP luciferase reporter with either 1μg or 3μg of either WT ALX or ALX S318A expression plasmids. The total amount of DNA transfected in each sample was standardized using empty vector. The following day, cells were left unstimulated or stimulated with anti-TCR/CD28 antibodies for 7 hours. Data is shown as fold activation relative to unstimulated Jurkat T cells transfected with empty vector only (=1). Error bars reflect SEM from three independent experiments.

B. Jurkat T cells transfected in (A) were lysed directly in SDS sample buffer and ALX expression analysed by Western blotting using antibodies against ALX. Note that the lane order is different than in (A) to emphasize that there is no difference in expression between WT ALX and ALX S318A with either quantity of plasmid.