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Negative Regulation of TCR Signaling by Linker for Activation of X Cells via Phosphotyrosine-Dependent and -Independent Mechanisms

Michael J. Shapiro,*† Chau T. Nguyen,* Haig Aghajanian,* Weiguo Zhang,‡ and Virginia Smith Shapiro2*†

The activation of T cells and the initiation of an immune response is tightly controlled through the crosstalk of both positive and negative regulators. Two adaptors that function as negative regulators of T cell activation are adaptor in lymphocytes of unknown function X (ALX) and linker for activation of X cell (LAX). Previously, we showed that T cells from mice deficient in ALX and LAX display similar hyperresponsiveness, with increased IL-2 production and proliferation upon TCR/CD28 stimulation, and that these adaptors physically associate. In this study, we analyze the nature of the association between ALX and LAX. We demonstrate that this association occurs in the absence of TCR/CD28 signaling via a mechanism independent of both tyrosine phosphorylation of LAX and the SH2 domain of ALX. Cotransfection of ALX with LAX resulted in LAX tyrosine phosphorylation in the absence of TCR/CD28 stimulation. ALX-mediated LAX phosphorylation depends upon the ALX SH2 domain, which functions to recruit Lck to LAX. We also show that LAX, like ALX, can inhibit RE/AP reporter activation. However, in contrast to its inhibition of NFAT, the inhibition of RE/AP by LAX is independent of its tyrosine phosphorylation. Therefore, it can be concluded that inhibition of signaling events involved in T cell activation by LAX occurs through mechanisms both dependent on and independent of its tyrosine phosphorylation. The Journal of Immunology, 2008, 181: 7055–7061.

T cell activation occurs via the integration of signals from cell surface receptors. Minimally, two signals are required for T cell activation: an Ag-dependent signal generated through the TCR and an Ag-independent costimulatory signal primarily provided by CD28 in naive T cells (1). If only TCR signals are received in the absence of costimulatory signals, T cells become anergic rather than activated. 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 MAPK and the transcription factors NF-κB, AP-1, and NFAT. One of the outcomes of appropriate T cell activation via TCR/CD28 is the transcriptional activation of IL-2 (3). The IL-2 promoter contains several transcription factor binding sites, including those for NFAT and AP-1. However, these elements can be activated by TCR signaling alone in reporter assays. A composite element from the IL-2 promoter designated RE/AP, which requires CD28 signals for activation, is the site at which costimulatory signals act, leading to IL-2 up-regulation (4, 5).

Adaptor proteins regulate several critical steps of T cell activation. For example, the transmembrane adaptor linker for activation of T cells (LAT)3 and cytoplasmic adaptor SLP-76 organize key regulators of TCR signaling into a “signalosome.” In mice deficient for either adaptor, T cells fail to develop, and cell lines deficient in either fail to transduce TCR signals leading to IL-2 (6, 7). Adaptors also can have negative functions in T cells. Previously, we identified an adaptor in T cells designated adaptor in lymphocytes of unknown function X (ALX; also known as HSH2) (8). 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 it functioned as a negative regulator of T cell activation. 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 (9). Analysis of signaling pathways in ALX-deficient T cells demonstrated no alterations in the level or kinetics of proximal induction of tyrosine phosphorylation, calcium flux, ERK, JNK, IκB kinase, or Akt in response to TCR/CD28 stimulation. However, ALX-deficient splenocytes exhibited constitutive activation of the p38 map kinase, through the classical MKK3/6 pathway, that was not further enhanced by TCR/CD28 stimulation.

The T cell phenotype in ALX-deficient mice was similar to that observed in mice lacking the transmembrane adaptor linker for activation of X cells (LAX) (10), and a physical interaction between ALX and LAX was subsequently discovered (9). Overexpression of LAX has been shown to result in inhibition NFAT and AP-1 upon TCR activation, and this inhibition is dependent on four sites of tyrosine phosphorylation (11). However, ALX overexpression results in inhibition of RE/AP activation, with little effect on...
either an NFAT or AP-1 reporter (8). If the association between ALX and LAX is important, LAX should also inhibit RE/AP reporter activation as well, which may depend on the association with ALX. In this study, we demonstrate that the association between ALX and LAX occurs constitutively, and is unaffected by TCR/CD28 stimulation via a mechanism independent of both tyrosine phosphorylation of LAX and the SH2 domain ALX. 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 associates directly with Lck and recruits Lck to LAX. We also show that LAX, like ALX, can inhibit RE/AP. However, in contrast to its inhibition of NFAT, the inhibition of RE/AP by LAX is independent of its tyrosine phosphorylation and correlates with its ability to bind to the ALX C-terminal fragment. Therefore, inhibition of signaling events involved in T cell activation by LAX occurs through mechanisms both dependent on and independent of its tyrosine phosphorylation, and ALX may play a part in both of these activities.

Materials and Methods
Expression plasmids
Full-length wild-type (WT) LAX and the 4YF mutant have been previously described (11). LAX truncations were generated by PCR cloning. The amino acids contained in the LAX truncations are as follows: LAX D1, aa 1–344; LAX D2, aa 1–293; LAX D3, 1–233; LAX D4, aa 1–169; LAX D5, 1–111; and LAX TL, aa 1–67. All LAX expression constructs were subcloned into the pET6 myc-His A vector (Invitrogen), which introduces a C-terminal myc-epitope tag. Untagged WT ALX and the ALX R/K mutant, in which arginine 59 is replaced by lysine, were described previously (12). All ALX truncation mutants used herein contain a portion of ALX fused to the amino terminus of yellow fluorescent protein (YFP). The ALX ΔC construct contains the residues C-terminal to the ALX SH2 domain, aa 136–352. The other ALX truncation mutants have been previously described (12). The NFAT and RE/AP luciferase reporters have been previously described (4, 13).

Jurkat transfections and luciferase assay
Transfections, stimulations, and luciferase assays were performed as previously described (4, 13). In brief, 15 × 10⁶ Jurkat T cells were washed once and resuspended in 0.4 ml of serum-free RPMI 1640. A total of 10 μg of reporter or various amounts of expression plasmid (figure legend) were added. Electroporation was performed using a Gene Pulser II (Bio-Rad) at 250 volts, 950 μF. Cells were resuspended in 10 ml of RPMI 1640 with 5% FCS (Invitrogen). The following day, live cells were counted by trypan blue exclusion (Bio-Whittaker), and 1 × 10⁶ cells per sample were stimulated as denoted in the figures. Cells were left unstimulated or stimulated with Abs to TCR and CD28, and LAX was subsequently immunoprecipitated with Abs to the myc tag. Jurkat T cells were stimulated for 2 min, which was previously shown to be the optimal time point for LAX phosphorylation (11). As shown in Fig. 1A, similar to previous work (9), ALX coprecipitated with LAX. This association was constitutive and not increased upon TCR/CD28 stimulation. Efficacy of stimulation was confirmed by blotting whole cell extracts (WCE), as well as LAX immunoprecipitations, with an Ab to phosphotyrosine (4G10; Fig. 1A, bottom panel). Interestingly, mutating the ALX SH2 domain (ALX R/K) did not alter association with LAX (Fig. 1A). This R/K mutation was previously shown to abrogate binding of tyrosine phosphorylated proteins from Jurkat cell extracts (12), implying that the association of ALX and LAX occurred via a mechanism that did not involve LAX tyrosine phosphorylation. Taken together, these results suggest that the association of ALX and LAX does not depend on TCR activation or phosphotyrosine recognition by the ALX SH2 domain.

To confirm that the association between ALX and LAX is phosphotyrosine independent, experiments were performed with a LAX mutant in which four tyrosine residues (Y193, Y268, Y294, and Y373) are changed to phenylalanine. Previously, these substitutions were shown to eliminate TCR-induced phosphorylation of LAX as well as abrogating the ability of LAX to inhibit activation of NFAT, NF-κB, or AP-1 reporters (11). As shown in Fig. 1B, WT ALX coprecipitated with LAX 4YF. In addition, the ALX R/K SH2 domain mutant also associated with the nonphosphorylated LAX-4YF mutant. Similarly, the associations of WT and ALX R/K with LAX 4YF are independent of TCR/CD28 stimulation. Therefore, ALX and LAX associate independent of LAX tyrosine phosphorylation or phosphotyrosine recognition by the SH2 domain of ALX.

Although the ALX SH2 domain does not function to recruit ALX to LAX, it was found to be important for LAX tyrosine phosphorylation. As shown in Fig. 1A (panel 3), LAX is inducibly tyrosine phosphorylated upon TCR/CD28 stimulation. Surprisingly, the extent of LAX tyrosine phosphorylation in unstimulated cells was substantially increased upon cotransfection of WT ALX.

GST precipitations
A fusion protein composed of the SH2 domain of ALX fused to GST (GST-SH2) as well as unfused GST were expressed in bacteria and purified as described previously (12). Jurkat cells were stimulated and lysed with Nonidet P-40 buffer as described for immunoprecipitation experiments. Approximately 20 μg of GST or GST-SH2 protein bound to glutathione sepharose beads were added to each lysate, which was then rotated for 2 h at 4°C. Beads were washed five times in Nonidet P-40 buffer and then loaded on denaturing gels for Western blotting with Abs to Lck or to GST (Amersham Biosciences).

Results
It was previously shown that four tyrosine residues in the cytoplasmic tail of the transmembrane adaptor LAX are inducibly phosphorylated upon TCR activation (11). Since ALX and LAX associate, and T cells deficient in either adaptor exhibit similar hyperresponsiveness to TCR/CD28 stimulation, we hypothesized that the SH2 domain of ALX may bind to phosphotyrosines in LAX. This model would suggest that TCR-induced phosphorylation of LAX would increase its association with ALX and that an ALX mutant unable to bind phosphotyrosines would not associate with LAX. To test these predictions, Jurkat T cells were transfected with WT ALX, myc-epitope tagged LAX, or both, along with vector controls. The cells were left unstimulated or treated with Abs to TCR and CD28, and LAX was subsequently immunoprecipitated with Abs to the myc tag. Jurkat T cells were stimulated for 2 min, which was previously shown to be the optimal time point for LAX phosphorylation (11). As shown in Fig. 1A, similar to previous work (9), ALX coprecipitated with LAX. This association was constitutive and not increased upon TCR/CD28 stimulation. Efficacy of stimulation was confirmed by blotting whole cell extracts (WCE), as well as LAX immunoprecipitations, with an Ab to phosphotyrosine (4G10; Fig. 1A, bottom panel). Interestingly, mutating the ALX SH2 domain (ALX R/K) did not alter association with LAX (Fig. 1A). This R/K mutation was previously shown to abrogate binding of tyrosine phosphorylated proteins from Jurkat cell extracts (12), implying that the association of ALX and LAX occurred via a mechanism that did not involve LAX tyrosine phosphorylation. Taken together, these results suggest that the association of ALX and LAX does not depend on TCR activation or phosphotyrosine recognition by the ALX SH2 domain.

Luciferase assays were performed as previously described (13).
and was not further enhanced by TCR/CD28 stimulation. However, no increase in LAX phosphorylation was observed upon co-transfection of wild-type ALX or an ALX SH2 domain mutant incapable of phosphotyrosine binding (ALX R/K) along with either (A) WT LAX or (B) a LAX mutant (LAX 4YF) in which the four sites of tyrosine phosphorylation were replaced. Empty vector (V) was used to standardize total plasmid transfected between samples. The cells were left unstimulated (−) or stimulated for 2 min (+) with Abs to TCR and CD28. WCE were made and subject to immunoprecipitation (IP) with Abs to a myc-epitope present on LAX. Western blotting (WB) was then performed with the indicated Abs to analyze expression of LAX and ALX in the WCEs and immunoprecipitations. Note that LAX appears as a "smear" on immunoblots, which is likely an effect of glycosylation, as is often found in transmembrane proteins. The minor, lower m.w. bands observed in LAX immunoblots are likely incompletely modified forms of the protein. Anti-phosphotyrosine blotting with 4G10 confirmed TCR/CD28 stimulation, as well as LAX phosphorylation. The arrowhead indicates the position of a tyrosine-phosphorylated band present in unstimulated cells only upon transfection with both WT ALX and LAX. This band is likely LAX, based on m.w., and consistent with the substantial increase in phosphorylated LAX in the immunoprecipitates observed upon cotransfection with ALX.

**FIGURE 1.** The association of ALX with LAX occurs independently of phosphotyrosine recognition. Jurkat cells were cotransfected with expression plasmids for either WT ALX or an ALX SH2 domain mutant incapable of phosphotyrosine binding (ALX R/K) along with either (A) WT LAX or (B) a LAX mutant (LAX 4YF) in which the four sites of tyrosine phosphorylation were replaced. Empty vector (V) was used to standardize total plasmid transfected between samples. The cells were left unstimulated (−) or stimulated for 2 min (+) with Abs to TCR and CD28. WCE were made and subject to immunoprecipitation (IP) with Abs to a myc-epitope present on LAX. Western blotting (WB) was then performed with the indicated Abs to analyze expression of LAX and ALX in the WCEs and immunoprecipitations. Note that LAX appears as a "smear" on immunoblots, which is likely an effect of glycosylation, as is often found in transmembrane proteins. The minor, lower m.w. bands observed in LAX immunoblots are likely incompletely modified forms of the protein. Anti-phosphotyrosine blotting with 4G10 confirmed TCR/CD28 stimulation, as well as LAX phosphorylation. The arrowhead indicates the position of a tyrosine-phosphorylated band present in unstimulated cells only upon transfection with both WT ALX and LAX. This band is likely LAX, based on m.w., and consistent with the substantial increase in phosphorylated LAX in the immunoprecipitates observed upon cotransfection with ALX.

**FIGURE 2.** ALX promotes phosphorylation of LAX dependent on the tyrosine kinase Lck. A. Cells were transfected and subject to immunoprecipitation without TCR stimulation as in Fig. 1 except that, as indicated, Jurkat or JCam1 Jurkat mutant (Lck deficient) cell lines were used. Each cell line was transfected with LAX alone or in combination with ALX. Transfection of an expression construct (+) or substitution of empty vector (−) is indicated. Immunoprecipitates and WCE were analyzed by Western blot with Abs to phosphotyrosine (4G10) to assess LAX phosphorylation, as well as LAX to confirm effective immunoprecipitation and ALX to confirm association. WCE were analyzed with Abs to LAX and ALX to assess expression and against Lck to confirm the deficiencies in JCam1. B, Examination of the requirement for ZAP-70 in ALX-mediated phosphorylation was examined in p116 ZAP-70-deficient Jurkat T cell line, as was done with JCam1 in part A above. C. Extracts were made from either Jurkat or JCam1 cells that were either unstimulated (−) or stimulated for 2 min (+) with Abs to TCR and CD28. Extracts were incubated with either GST or a recombinant protein composed of GST and the SH2 domain of ALX (GST ALX SH2), both bound to beads. The beads were precipitated and washed before being analyzed by Western blotting. The anti-Lck blot shows that Lck bound specifically to the SH2 domain of ALX (upper panel). Western blotting with Abs to GST demonstrates the presence of equivalent amounts of fusion protein in each precipitate (lower panel).
did not grossly alter the pattern of tyrosine phosphorylated proteins in Jurkat WCEs (Fig. 1, A and B, bottom panel). In fact, the only increase in tyrosine phosphorylation in the WCEs appears to be LAX itself (Fig. 1A, marked by arrow in bottom panel), as a band of appropriate m.w. was only present when WT LAX but not LAX 4YF was cotransfected (compare lane 7 of bottom panels of Fig. 1, A and B). Therefore, the phosphotyrosine-independent recognition of LAX by ALX promotes the phosphorylation of LAX and, presumably, phosphotyrosine-dependent interactions between LAX and additional partners involved in regulating T cell activation.

TCR engagement results in the activation of the tyrosine kinase Lck, initiating a cascade of events leading to activation of downstream pathways. Lck has been implicated in LAX tyrosine phosphorylation: Lck overexpression leads to LAX constitutive phosphorylation, and LAX is not inducibly phosphorylated by TCR in the J Cam1 Jurkat cell line, which is deficient in Lck (11, 14). Thus, ALX-driven LAX phosphorylation may depend on Lck recruitment. To test this model, ALX and LAX cotransfections were repeated using J Cam1 cells. As shown in Fig. 2A, cotransfection of ALX with LAX resulted in substantial constitutive LAX phosphorylation in Jurkat but almost none in J Cam1, although association of ALX with LAX was similar in both cell lines. ALX cotransfection stimulated LAX phosphorylation in p16 cells, which lack the tyrosine kinase ZAP-70 (Fig. 2B, 15), and in J Cam2 cells that lack LAT, an adaptor critical to TCR signaling (data not shown) (16). Absence Lck, ZAP-70 and LAT in these cell lines was confirmed by Western blot (Fig. 2, A and B, and data not shown). These results suggest that upon association with LAX, ALX stimulates its phosphorylation in an Lck-dependent manner. To determine whether the ALX SH2 domain directly bound to Lck, GST pull-down experiments were performed using a fusion protein containing only the SH2 domain of ALX. As shown in Fig. 2C, Lck was effectively pulled down from Jurkat extracts with a GST ALX SH2 domain fusion protein, but is not with GST alone. J Cam1 extracts were used as a negative control. The association between the ALX SH2 domain and Lck was constitutive, and was not altered by TCR/CD28 stimulation. We have thus far been unable to demonstrate an interaction between ALX and Lck in coimmunoprecipitation experiments (data not shown), though this does not eliminate the possibility that the association is weak but yet sufficient for transient recruitment of Lck to LAX resulting in LAX tyrosine phosphorylation. Together, the above data suggests that ALX recruits Lck to LAX via its SH2 domain, resulting in LAX tyrosine phosphorylation.

To map the sites within ALX required for the association with LAX, cotransfections were repeated using a series of ALX truncations mutants (shown schematically in Fig. 3A). 293T cells were used for these experiments, rather than Jurkat T cells, since 293T cells lack Lck and LAX is not phosphorylated in these cells (data not shown) (11). This eliminates the potential for indirect associations between ALX and LAX mediated either by Lck or proteins that associate with tyrosine phosphorylation sites within LAX. As shown in Fig. 3B, the isolated SH2 domain of ALX lacked any ability to interact with LAX. This same ALX truncation has been shown to inhibit RE/AP activation upon overexpression, and a purified fusion protein containing the SH2 domain was able to bind phosphotyrosine containing proteins from Jurkat extracts (12). Hence, it is unlikely that the lack of association of the ALX SH2
domain with LAX is due to a structural change in the SH2 domain when it is expressed in isolation. Thus, combined with the experiments using ALX R/K, it can be concluded that the SH2 domain of ALX is neither necessary nor sufficient for the association with LAX. ALX truncations lacking either the N- or C-terminal segments (ALX N and ALX C, respectively) retained the ability to bind to LAX. Since the ALX SH2 domain does not associate with LAX, this implies that ALX contains two separate binding sites for LAX, located both N- and C-terminal to the SH2 domain. The C-terminal segment in isolation (ALX C mutant) was also shown to associate with LAX (Fig. 3B).

Based upon the association results, if LAX and ALX function together to inhibit T cell activation, then the LAX 4YF mutant may retain some ability to negatively regulate T cell activation. Previously, it has been shown that the LAX 4YF mutation resulted in a loss of ability to inhibit AP-1 and NFAT reporters (11). However, ALX inhibits the activation of the RE/AP composite element from the IL-2 promoter, but does not substantially inhibit TCR-mediated activation of either NFAT or AP-1 (8). Therefore, we examined the ability of the both WT LAX and the 4YF mutant to inhibit activation of RE/AP. As shown in Fig. 4A, Jurkat T cells were transfected with an RE/AP luciferase reporter and either vector or increasing amounts of WT LAX or LAX 4YF. TCR/CD28 stimulation activated the RE/AP reporter 25-fold when cotransfected with vector control. Overexpression of LAX, as previously observed with ALX, caused a decrease in the activation of the RE/AP element of 85% (p < 0.01, by student’s t test). Interestingly, overexpression of LAX 4YF mutant inhibited the activation of RE/AP by 70% (p < 0.01, by student’s t test), nearly as well as WT LAX. WT LAX and the LAX 4YF mutant were expressed at similar levels (Fig. 4C). The small but significant (p < 0.01, by student’s t test) difference between inhibition of RE/AP by WT LAX and LAX 4YF indicates that LAX phosphorylation may also contribute toward RE/AP inhibition. As a control, the effect of LAX on an NFAT reporter was also examined (Fig. 4B). Consistent with previous results (11), overexpression of WT LAX blocked activation of NFAT upon TCR/CD28 stimulation by 80% (p < 0.02, by student’s t test), and this ability was almost entirely abrogated by the 4YF mutation. Therefore, these results demonstrate that LAX can inhibit different signaling pathways in T cells in distinct ways: a phosphotyrosine-dependent pathway primarily responsible for NFAT inhibition and a separate pathway.
or orientation between the proteins is not critical, all LAX truncations that can associate with WT ALX should inhibit RE/AP and only LAX TL would lack an inhibitory effect. Alternatively, if the orientation between ALX and LAX is critical, then the LAX D4, D5, and TL truncations, which fail to bind ALX C, should not inhibit RE/AP activation. As shown in Fig. 6A, Jurkat T cells were transfected with an RE/AP luciferase reporter, along with expression constructs for WT LAX and all the LAX truncations. WT LAX, LAX D1, D2, and D3 inhibited TCR/CD28-induced activation of the RE/AP reporter to similar extents. However, LAX D4, D5, and TL had no inhibitory effect on RE/AP. Therefore, the inhibition of RE/AP by LAX does not simply correlate with an ability to bind to ALX, but the orientation assumed by ALX with the cytoplasmic tail of LAX may be important.

The inhibition of NFAT by LAX is abrogated in the 4YF mutant, indicating that at least one of the four tyrosines is crucial for this inhibition. We used the truncation mutants described above to determine which tyrosine is important for the inhibition of NFAT by LAX. As diagramed in Fig. 5A, the D1, D2, D3, and D4 LAX truncations serve to eliminate, respectively, one, two, three, and all four tyrosines from LAX. As shown in Fig. 6B, Jurkat T cells were transfected with an NFAT luciferase reporter, along with expression constructs for WT LAX and all of the LAX truncations. NFAT activation was inhibited by WT LAX, as well as LAX D1, D2, and D3. However LAX D4, as well as the further truncated constructs, lacked all ability to inhibit NFAT. Since LAX D3 contains only one of the four tyrosines found to be critical for inhibition of NFAT, Y193, we can conclude that the presence of at least this tyrosine residue is necessary for LAX-mediated inhibition of NFAT. LAX Y193 is not sufficient, however, since a mutant of LAX in which only Y193 was mutated to phenylalanine inhibited NFAT activation to a similar extent as WT LAX (data not shown), most likely due to the presence of a similar and presumably redundant motifs at Y294 and Y393.

Discussion

The mechanism by which adaptors can negatively regulate signaling pathways is not well defined at present. LAX was originally identified as a transmembrane adaptor that contained similar tyrosine-containing motifs as the transmembrane adaptor LAT (11). LAT is absolutely required for TCR signaling (6). In LAT-deficient mice, T cells do not develop, and no TCR signaling occurs in a Jurkat T cell line deficient in LAT (16). T cell activation results in the tyrosine phosphorylation of LAT and subsequent recruitment of SH2 containing proteins including Grb2 and GADS, which nucleate a complex that transmits TCR signals to downstream pathways. LAX has also been shown to undergo tyrosine phosphorylation upon TCR signaling, leading to the recruitment of Grb2 and GADS. However, while LAT localizes to lipid rafts, LAX is excluded from them (11). In the LAX 4YF mutant, substitutions at four tyrosine residues were shown to eliminate both LAX phosphorylation and the protein associations triggered by TCR signaling, as well as the inhibition of NFAT activation (11). Thus, one mechanism by which LAX may negatively regulate TCR signaling is to function as a “sink” for SH2 containing proteins that bind to its phosphoryrosines, thus sequesterating these proteins away from LAT and lipid rafts.

The results presented here illustrate a second mechanism by which LAX can function, in which it may nucleate a distinct complex that inhibits TCR signals. LAX was shown to inhibit activation of the RE/AP composite element, which integrates TCR and costimulatory signals leading to IL-2 up-regulation (4, 5). However, unlike the inhibition of NFAT, RE/AP activation was not abrogated in LAX 4YF, suggesting that a mechanism of inhibition

![FIGURE 6](https://www.jimmunol.org/DownloadedFrom/7060.png)
independent of LAX tyrosine phosphorylation must exist. The cytoplasmic adaptor ALX was previously shown to bind to LAX but not to LAT (9). ALX deficient mice display a phenotype similar to LAX-deficient mice (9, 10), and ALX also inhibits RE/AP upon overexpression (8). In this study, we show that the interaction between ALX and LAX is independent of LAX tyrosine phosphorylation as the association is observed in unstimulated cells and is not abrogated by mutations in ALX that impair phosphotyrosine binding or mutations in LAX that impair its phosphorylation. Taken together, these results illustrate how LAX can inhibit T cell signaling by a mechanism distinct from simply functioning as a molecular sink for signaling molecules that might otherwise positively regulate T cell activation.

The data presented here also clarify the structural features of ALX and LAX involved in their interaction and their inhibitory effects on signaling. The association of ALX and LAX involves two distinct sites, each interaction occurring independent of tyrosine phosphorylation: ALX ΔC associates with LAX between aa 67–111, while the C-terminal portion of ALX associates with LAX between aa 169–233. ALX contains a single SH2 domain and no other known structural motifs. Four conserved PxxP polyproline sequences, three of which are located outside of the SH2 domain, are present in ALX. However, LAX does not contain any modular binding motifs, such as SH3/WW domains, that associate directly with polyproline sequences. In fact, the polyproline sequences in ALX are not required for LAX association, as LAX associates with an ALX mutant in which these sequences are mutated (data not shown). Thus, the association of ALX and LAX must occur via mechanisms independent of SH2/phosphotyrosine as well as polyproline-based interactions, and additional motifs that could mediate an interaction are not obvious. Future characterization of the interaction will require more refined truncations or scanning mutagenesis.

Finally, the truncation analyses presented here also defines the structural features of LAX required for inhibition of RE/AP and NFAT activation. Previously, four tyrosine-containing motifs in the cytoplasmic tail of LAX were shown to be critical for its inhibition of NFAT (Y193, Y268, Y294, and Y373), as their combined mutation abrogates this inhibition (11). LAX D3, which retains Y193 but not Y268, Y294, or Y373, inhibits NFAT activation similar to WT, while LAX D4, which lacks all four critical tyrosines, does not. Hence, inhibition of NFAT by LAX depend on the presence of at least one tyrosine phosphorylation site, and Y193 can serve this function. The truncation analysis also shows that inhibition of RE/AP by LAX depends on sequences contained within aa 169–233 (between D3 and D4 truncations), correlating with the sequences required for binding to ALX. The importance of this segment of LAX for NFAT inhibition may, in fact, reflect a dependence on ALX for LAX tyrosine phosphorylation.

The results presented here lead to a model of LAX function, shown in Fig. 7. In the absence of stimulation, nonphosphorylated LAX associates with ALX via two independent interactions. LAX is able to inhibit RE/AP activation without undergoing phosphorylation. ALX, via its SH2 domain, associates with Lck and recruits it to the complex with LAX. This results in LAX tyrosine phosphorylation, enabling LAX to associate with additional signaling molecules, including Gads, Grb2, and PLCγ1. These associations allow LAX to inhibit activation of NFAT. Thus, LAX regulates T cell activation by two distinct but interconnected mechanisms.