Activation of naive T cells occurs when two signals are received. The first signal is received through the T cell antigen receptor (TCR), and a second costimulatory signal is primarily provided by CD28. We have recently identified a novel adaptor molecule, ALX, which is expressed exclusively in hematopoietic cells. ALX contains several sites for potential protein-protein interaction, including an Src homology 2 (SH2) domain, four PXXP polyproline sequences, and two likely sites of tyrosine phosphorylation. Overexpression of ALX inhibits the transcriptional activation of the interleukin 2 promoter during T cell activation, specifically affecting CD28-mediated activation of the RE/AP element of the interleukin 2 promoter. To understand how ALX functions downstream of CD28, we generated a panel of site-directed mutants as well as truncations in which potential protein-binding sites were mutated or absent. We found that the ALX SH2 domain is both necessary and sufficient to mediate inhibition of RE/AP activation. Mutation of the SH2 domain did not affect ALX expression, relative localization in the cytoplasm and nucleus, phosphorylation, or a mobility shift in response to TCR signaling alone. However, an activation-induced mobility shift triggered by CD28 was reduced in the ALX SH2 domain mutant. In addition, the isolated ALX SH2 domain was found to associate with a phosphoprotein from Jurkat T cells on TCR/CD28 stimulation. Therefore, the ALX SH2 domain plays a critical role in ALX function downstream of CD28.

The activation of T cells is critical to the generation of an immune response. Minimally, two signals are required to activate naive T cells into effecter T cells, an antigen-specific signal through the T cell receptor (TCR) and a second antigen-independent “costimulatory” signal, which is primarily provided by CD28 (reviewed in Ref. 1). A critical step in T cell activation is the induction of the interleukin 2 (IL-2) gene, which occurs through both a transcriptional up-regulation of its promoter and an initiation of CD28 costimulation.

The ALX Src Homology 2 Domain Is Both Necessary and Sufficient to Inhibit T Cell receptor/CD28-mediated Up-regulation of RE/AP*

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The activation of T cells is critical to the generation of an immune response. Minimally, two signals are required to activate naive T cells into effecter T cells, an antigen-specific signal through the T cell receptor (TCR) and a second antigen-independent “costimulatory” signal, which is primarily provided by CD28. We have recently identified a novel adaptor molecule, ALX, which is expressed exclusively in hematopoietic cells. ALX contains several sites for potential protein-protein interaction, including an Src homology 2 (SH2) domain, four PXXP polyproline sequences, and two likely sites of tyrosine phosphorylation. Overexpression of ALX inhibits the transcriptional activation of the interleukin 2 promoter during T cell activation, specifically affecting CD28-mediated activation of the RE/AP element of the interleukin 2 promoter. To understand how ALX functions downstream of CD28, we generated a panel of site-directed mutants as well as truncations in which potential protein-binding sites were mutated or absent. We found that the ALX SH2 domain is both necessary and sufficient to mediate inhibition of RE/AP activation. Mutation of the SH2 domain did not affect ALX expression, relative localization in the cytoplasm and nucleus, phosphorylation, or a mobility shift in response to TCR signaling alone. However, an activation-induced mobility shift triggered by CD28 was reduced in the ALX SH2 domain mutant. In addition, the isolated ALX SH2 domain was found to associate with a phosphoprotein from Jurkat T cells on TCR/CD28 stimulation. Therefore, the ALX SH2 domain plays a critical role in ALX function downstream of CD28.

The activation of T cells is critical to the generation of an immune response. Minimally, two signals are required to activate naive T cells into effecter T cells, an antigen-specific signal through the T cell receptor (TCR) and a second antigen-independent “costimulatory” signal, which is primarily provided by CD28. We have recently identified a novel adaptor molecule, ALX, which is expressed exclusively in hematopoietic cells (7). ALX was originally identified as a protein with considerable homology to another hematopoietic adaptor, TSAd (also known as RIBP, Lad, and VRAP, (8–11)). This adaptor was identified in separate two-hybrid screens for proteins that associate with the Tec family kinases Rlk and Itk (9), the Src family kinase Lck (10), and the mitogen-activated protein kinase 3-kinase MEKK2 (12). TSAd has been reported to localize to the cytoplasm in T cells and to translocate to the immunological synapse during T cell activation (12) but has also been reported to be primarily nuclear (13). A role for TSAd in T cell activation was demonstrated in knock-out mice (9). These mice have no gross abnormalities in T cell development, but mature T cells show a moderate defect (~70% decrease) in proliferation as well as IL-2 and interferon-γ production on TCR or TCR/CD28 stimulation. TSAd-deficient animals develop an autoimmune syndrome similar to lupus, with hypergammaglobulinemia, glomerulonephritis, and production of autoantibodies, including anti-DNA antibodies (14). TSAd and ALX share a similar overall structure containing a single SH2 domain near the N terminus, several PXXP polyproline sequences, and a potential site of tyrosine phosphorylation (7). Overexpression of ALX in either the Jurkat or CEM25 T cell lines inhibited the activation of an RE/AP but not an AP-1 reporter (7). In these cell lines, the activation of RE/AP is dependent on CD28 costimulation, whereas activation of AP-1 occurs with TCR stimulation alone, implying that the primary role of ALX is downstream of CD28 rather than TCR signaling. In support of this, ALX overexpression inhibits activation of RE/AP in response to phorbol 12-myristate 13-acetate (PMA)/CD28 stimulation but had little effect on PMA/TCR stimulation. In addition, ALX was also shown to be a target of CD28 signaling. Stimulation with TCR
alone caused an activation-induced shift in ALX mobility within 5 min, whereas CD28 costimulation further enhanced this shift at later time points (7).

To understand how ALX functions, we initiated a structure/function analysis of ALX. Several potential sites of protein-protein interaction were mutated or truncated, including the SH2 domain, each of four PXXP polyproline sequences, and two potential SH3/WW domains. Here we demonstrate that the only protein interaction site that, on mutation, abrogates the ability of ALX to inhibit RE/AP activation after TCR/CD28 stimulation is the SH2 domain. Interestingly, the ALX SH2 domain alone can mediate the inhibition of RE/AP activation and binds a phosphoprotein from TCR/CD28-stimulated but not unstimulated cells. The ALX SH2 domain mutation does not alter protein expression, relative distribution of ALX in the cytoplasm and nucleus, phosphorylation, or the activation-induced modification of ALX by TCR stimulation resulting in a mobility shift. However, the activation-induced modification in response to CD28 stimulation of the ALX SH2 domain mutant is substantially reduced. Therefore, the SH2 domain of ALX is playing a critical role in ALX function downstream of CD28.

**EXPERIMENTAL PROCEDURES**

**Generation of ALX Mutant Constructs**—All mutants of ALX as described in Figs. 1A and 2A were generated by PCR-based mutagenesis of the pCFP-C1 ALX construct (7). All products were sequenced to verify the creation of the intended mutation, without introduction of any unintended mutations.

**Reporters, Antibodies, and Cell Lines Used**—The RE/AP luciferase reporter has been described previously (5). C305 is a monoclonal antibody specific to the clonotypic TCR of Jurkat T cells and was generously provided by Art Weiss (University of California, San Francisco, CA) (15). Anti-Myc tag antibodies were from Cell Signaling Technology (catalog no. 2276). Anti-CD28 antibodies were from Caltag (catalog no. MHCDC2800). Rabbit polyclonal antisera against human ALX (1576) has been described previously (7). Anti-MEF2D and anti-Nck antibodies were from Transduction Laboratories. Jurkat T cells were provided by Art Weiss (University of California) and cultured in RPMI medium with 5% fetal calf serum. Jurkat T cells, which stably express Myc-His-Tagged ALX (wt or R/K) fused to glutathione (wt or R/K) were generated as described previously (7).

**Transfections and Luciferase Assays**—Transfections, stimulations, and luciferase assays were performed as described previously (5, 16). Briefly, 15 × 10⁶ Jurkat T cells or CEM25 T cells were washed once and resuspended in 0.4 ml of serum-free RPMI medium. 20 μg of reporter or various amounts of expression plasmid were added. Electroporation was performed using a Gene Pulser II (Bio-Rad) at 250 volts, 950 microfarads. Cells were resuspended in 10 ml of RPMI medium with 5% fetal calf serum. Jurkat T cells, which stably express Myc- His-Tagged ALX (wild-type (wt) and SH2 mutant (R/K)), were generated as described previously (7).

**Western Blotting**—To examine expression of ALX mutants, whole cell extracts from transiently transfected Jurkat T cells were lysed in 1% Nonidet P-40 lysis buffer as described previously (7). Briefly, lysates were separated by electrophoresis, transferred to an Immobilon P membrane, and then combined with reducing sample buffer and boiled. The membranes weresubjected to centrifugation, resulting in pelleting of the nuclei. Supernatants were then removed as the cytoplasmic fraction. Nuclear pellets were washed and then lysed in hypotonic buffer. Both fractions were combined with reducing sample buffer and boiled before being loaded onto gels and analyzed by Western blotting as described above.

**Protein Phosphorylation**—Jurkat T cells stably expressing Myc- His-Tagged ALX (wt or R/K) and untransfected cells were resuspended at a density of 10⁶ cells/ml in phosphate-free RPMI 1640 medium (Cell and Molecular Technologies) containing 0.1 μCi/ml [32P]H3PO4. Cells were incubated for 3 h at 37 °C and stimulated for various times with antibodies to TCR and CD28 as described above. Cells were lysed at 10⁶ cells/ml in lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% Nonidet P-40 with mammalian phosphatase, and protease inhibitor mixtures (Sigma)). Lysates were incubated at 4 °C for 20 min and then clarified by centrifugation. Anti-Myc antibodies (2 μg/ml 9B11 monoclonal) (Cell Signaling) was added to the lysates, which were then rotated for 2 h at 4 °C. Protein A-Sepharose beads (Invitrogen) were then added, and the lysates were rotated for an additional 1 h. Beads were then harvested by centrifugation, washed three times in lysis buffer, and then combined with sample buffer and boiled. Proteins were resolved by gel electrophoresis and transferred to an Immobilon P membrane. Phosphorylated proteins were visualized by autoradiography. Next, the membranes were subject to Western blotting with a polyclonal antisera to ALX as described above.

**Fusion Protein Precipitations**—Plasmids encoding the SH2 domain of ALX (wt or R/K) fused to glutathione S-transferase (GST) were generated by inserting the coding sequence for amino acids 14–133 of ALX in-frame with GST in the vector pGEX-4T-1 (Amerham Biosciences). BL21 Codon-Plus (Stratagene) was transformed with these plasmids or pGEX-4T-1 (for unfused GST), and proteins were purified on glutathione-Sepharose beads (Amerham Biosciences) according to the manufacturer’s instructions and used for precipitations without elution from the beads. Protein concentrations were estimated by subjecting beads to 10 μl of electrophoresis and Coomassie Blue staining. dsRNA precipitated was visualized by autoradiography. Next, the membranes were subject to Western blotting with an anti-GST (Amerham Biosciences) to confirm equivalent amounts of GST fusion proteins within each precipitation.

**RESULTS**

**Mutation of the SH2 Domain Abrogates ALX-mediated Inhibition of RE/AP**—Having identified a novel adaptor, ALX, which functions downstream of CD28, we were interested in the biochemical basis for ALX function. Similar to other adapters, ALX contains no domains for intrinsic catalytic activity, but it contains several domains/motifs that may be involved in mediating protein-protein interactions. ALX contains a single SH2 domain, which is predicted to bind to phosphotyrosine residues, but ALX also contains several other potential sites for protein-protein interaction, including four PXXP polyproline sequences, which may associate with SH3/WW domains as well as two potential sites of tyrosine phosphorylation, which may associate with a SH2/PTB domain containing proteins. These potential sites of tyrosine phosphorylation were identified using NetPhos, version 2.0 (18). To determine the importance of each of these potential protein binding sites, each of these sites was mutated (as outlined in Fig. 1A). To impair function of the ALX SH2 domain, a mutant (ALX R/K) in which the arginine within the highly conserved FLVR sequence was replaced with lysine was generated. This replacement at the analogous arginine within the FLVR sequences of SH2 domains in general is a well established means of eliminating phosphotyrosine binding (19). The PXXP polyproline sequences were disrupted by mutating at least one proline to alanine. The potential sites of tyrosine phosphorylation were disrupted by mutating the tyro-
sine to phenylalanine. Each mutant was transiently transfected into Jurkat T cells with an RE/AP luciferase reporter. As shown in Fig. 1B, and similar to what was demonstrated previously (7), overexpression of wild-type ALX inhibited the activation of the RE/AP composite element luciferase reporter in response to TCR/CD28 stimulation. Interestingly, ALX mutants with amino acid substitutions at any of the four PXXP sites or at the two potential sites of tyrosine phosphorylation (P-Tyr) retained their ability to inhibit RE/AP up-regulation in response to TCR/CD28 stimulation. In contrast to the other mutants, ALX R/K failed to have any inhibitory effect on RE/AP, with an activation similar to that observed with vector transfection alone. This was not because of any defect in the expression of the R/K mutant but because it was expressed at comparable levels to wt ALX and all other point mutants that inhibit RE/AP activation when overexpressed in Jurkat T cells (Fig. 1C).

The ALX SH2 Domain Alone Is Sufficient to Mediate Inhibition of RE/AP—One possible explanation for the lack of a defect in any point mutant besides ALX R/K is that effects on ALX function may be observed only when multiple sites are disrupted. To test this possibility, ALX constructs mutated at three of the PXXP polyproline sequences and both of the potential sites of tyrosine phosphorylation were generated (as shown schematically in Fig. 2A). In addition, ALX constructs lacking either the portion of ALX N-terminal or C-terminal to the SH2 domain and a construct containing only the SH2 domain were generated. Interestingly, all of these ALX mutants retained the ability to inhibit RE/AP activation in response to TCR/CD28 stimulation. Therefore, the ALX SH2 domain is both necessary (Fig. 1) and sufficient (Fig. 2) to mediate the inhibitory effects on RE/AP activation.

ALX Is Inducibly Phosphorylated on TCR/CD28 Stimulation—It had been demonstrated previously that ALX undergoes a mobility shift induced on TCR/CD28 stimulation, presumably because of phosphorylation. To verify this and to determine whether there were any defects in the induced phosphorylation of the ALX R/K mutant, Jurkat T cells stably transfected with Myc-tagged wt or ALX R/K were used. As shown in Fig. 3, both wt and mutated ALX were inducibly phosphorylated after TCR/CD28 stimulation to similar extents. Western blotting with anti-ALX antisera demonstrates that...
Similar amounts of protein were immunoprecipitated in each sample.

**Mutation of the SH2 Domain Does Not Affect Cytoplasmic/Nuclear Localization of ALX but Does Affect the ALX Mobility Shift in Response to TCR/CD28 Stimulation.** Jurkat T cells stably transfected with Myc-His-tagged wt or R/K ALX were stimulated with anti-TCR and anti-CD28 antibodies for different times as shown (minutes of stimulation are indicated). Cytoplasmic and nuclear extracts were generated and analyzed by Western blotting using a monoclonal antibody against the Myc tag. Note that different cell equivalents of the two fractions were analyzed; the volume of nuclear extract loaded was derived from four times as many cells as the volume of cytoplasmic extract. As controls for nuclear/cytoplasmic separation, extracts were also blotted with antibodies to MEF2D and Nck.

**Similarity of nuclear and cytoplasmic localization of ALX.** The indicated Jurkat cell lines were labeled with \([^{32}P]\)orthophosphate and stimulated for the indicated times with antibodies to TCR and CD28. Cells were lysed and subjected to immunoprecipitation (IP) with anti-Myc antibody. Phosphorylated ALX was visualized by autoradiography, and the total ALX protein precipitated was detected by Western blotting (WB) with polyclonal antibodies.

**Activation-induced shift in mobility of ALX.** Jurkat T cells stably transfected with Myc-His-tagged wt or R/K ALX were stimulated with anti-TCR and anti-CD28 antibodies for different times as shown (minutes of stimulation are indicated). Cytoplasmic and nuclear extracts were generated and analyzed by Western blotting using a monoclonal antibody against the Myc tag. Note that different cell equivalents of the two fractions were analyzed; the volume of nuclear extract loaded was derived from four times as many cells as the volume of cytoplasmic extract. As controls for nuclear/cytoplasmic separation, extracts were also blotted with antibodies to MEF2D and Nck.
induced modification resulting in a mobility shift in response to TCR/CD28 stimulation after 5 min, similar to wild-type ALX. However, enhancement of this mobility shift was substantially reduced in the R/K mutant at either 15 or 45 min. Because the CD28-induced shift occurs in a small percentage of the total ALX, compared with the initial TCR-induced shift, which occurs in greater proportion, it is not surprising that a difference was not observed in R/K when examining ALX phosphorylation by [32P]orthophosphate incorporation (Fig. 3). In conclusion, the failure of the ALX R/K mutant to inhibit RE/AP activation cannot be attributed to altered subcellular localization. However, the mutant does display an alteration in the mobility shift induced by TCR/CD28 stimulation, and, hence, this alteration may contribute to its defect in RE/AP inhibition.

The SH2 Domain Mutation Specifically Affects CD28- but Not TCR-mediated ALX Mobility Shift—The enhanced mobility shift in ALX after TCR/CD28 stimulation at 15 and 45 min requires CD28 costimulation and is not observed with TCR stimulation alone (7). Therefore, the R/K mutant may not be able to respond fully to CD28 costimulation, although TCR-induced modification is unaffected. Alternatively, the mobility shift could be altered in response to both TCR and CD28 stimulation at later time points. To differentiate between these possibilities, Jurkat T cells expressing either Myc-tagged wild-type ALX or the R/K mutant were stimulated with antibodies to either TCR alone or in combination with antibodies to CD28 (Fig. 5). As shown previously, stimulation with TCR alone caused an alteration in ALX mobility, and the addition of CD28 costimulation enhanced this mobility shift. Interestingly, both wild-type and R/K mutant ALX underwent a similar shift in mobility in response to TCR stimulation alone. However, the enhancement in the mobility shift resulting from CD28 costimulation in combination with TCR stimulation was substantially reduced in the R/K mutant. Therefore, mutation of the SH2 domain of ALX specifically impaired the activation-induced mobility shift caused by CD28 costimulation but not TCR stimulation. This implies that the failure of the R/K mutation to inhibit RE/AP activation after TCR/CD28 stimulation may be because of an inability to respond fully to CD28 costimulation.

ALX SH2 Domain Associates with a 66-kDa Phosphoprotein—Because SH2 domains mediate protein-protein interactions by binding to phosphorylated tyrosine residues, it was likely that the defects observed in the ALX SH2 R/K mutant were the result of an inability to associate with a tyrosine-phosphorylated protein. Recombinant GST fusion proteins were generated that contained only the ALX SH2 domain or the ALX SH2 domain with the R/K mutation. These fusion proteins were used to precipitate associated proteins from extracts from Jurkat T cells that were either unstimulated or stimulated with TCR/CD28. GST alone was used as an additional negative control. The precipitations were examined by Western blotting with a monoclonal against the Myc tag.

To further our understanding of the role of ALX downstream of CD28 leading to the activation of the RE/AP composite element from the IL-2 promoter, we generated a panel of both point mutants and truncation mutants of ALX in which potential protein-protein interactions sites were altered. Seven different potential sites of protein interaction were mutated, but only a mutation in the SH2 domain abrogated the ability of ALX to inhibit RE/AP activation when overexpressed in Jurkat T cells. The SH2 domain alone was also found to be sufficient to mediate the inhibition. Therefore, the ALX SH2 domain plays a critical role in ALX function. It should be noted that the SH2 domain of ALX contains two potential protein-protein interaction sites, the FLVR sequence, predicted to mediate phosphotyrosine binding, and a PXXP polyproline sequence near the C terminus of the SH2 domain. Furthermore, there is a precedent for a single SH2 domain to have two protein binding motifs from analysis of the adaptor SAP (20). However, in the analysis of ALX only the R/K mutation within the FLVR sequence was found to disrupt the inhibition of RE/AP by TCR/CD28 stimulation, whereas the PXXP (4PP) mutant had no defect (Fig. 1). In addition, mutation of this PXXP site within an isolated ALX SH2 domain construct also failed to cause any loss of ALX function (data not shown).

Some evidence for a critical role of the SH2 domain in TSAd, an adaptor closely related to ALX, has also been reported (13). An R/K mutation in the FLVR sequence of TSAd was shown to
diminish its ability to regulate IL-2 promoter activity and to localize to the nucleus. It is perhaps not surprising that the SH2 domain plays a critical role in both of these related adaptations; however, the proteins that associate with the TSAd SH2 domain are not yet known. Although TSAd had been found to associate with several kinases, including Lck, Itk, Rlk, and MEKK2, none of these proteins binds to the TSAd SH2 domain (13, 21). A GST TSAd SH2 fusion protein was found to bind numerous phosphotyrosine-containing proteins from PMA plus pervanadate-stimulated Jurkat cells, whereas an R/K mutant lacked these associations (13). We have also observed numerous proteins that associate with the ALX SH2 GST fusion protein from pervanadate-stimulated Jurkat cells (data not shown), although the relevance of these associations to TCR/CD28 signaling is hard to assess. We have identified an ~66-kDa protein that associates with the wt but not mutated SH2 domain of ALX, dependent on TCR/CD28 stimulation in Jurkat T cells. The identification of this protein will likely substantially advance our understanding of the signaling pathways regulated by ALX, and this work is currently being pursued.

We present here the first analysis of the subcellular localization of ALX and the direct demonstration that TCR/CD28 signaling results in ALX phosphorylation by orthophosphate incorporation. The ALX R/K SH2 mutant was not found to have altered the relative distribution of ALX in the cytoplasm and nucleus in unstimulated cells or after TCR/CD28 stimulation; nor did it display a defect in the TCR activation-induced modification resulting in an altered electrophoretic mobility. Rather, this mutation only affected the ability of ALX to undergo an enhanced activation-induced mobility shift in response to CD28 costimulation (Fig. 5). Therefore, the ALX SH2 domain appears to play a critical role in ALX signaling downstream of CD28 but not TCR stimulation. One possibility is that the inability of this mutant to inhibit RE/AP activation is directly because of a reduction in phosphorylation at a regulatory site in response to CD28. Alternatively, a decrease in phosphorylation may be indicative of a failure to associate and possibly regulate a kinase. It is difficult to discern whether a particular adaptor molecule is a positive or negative regulator of signaling based on overexpression studies alone. If we make the assumption that the function of an adaptor is to bring together two or more proteins within a single macromolecular complex, it is possible that on overexpression, a normally positive regulator may disrupt the stoichiometry of this complex and thus appear to be a negative regulator. For example, Jip-1 was originally identified as a negative regulator of Jun kinase signaling based on overexpression studies (22), although the Jip-1 knockout revealed that it played a critical role in Jun kinase activation (23).

Therefore, the inhibition of RE/AP observed on overexpression of ALX does not by itself definitively show that ALX is a negative regulator of IL2 expression. Specifically, if the function of an adaptor is to bring together two or more proteins into a single complex, then it would be predicted that at least two different protein-binding sites would be required for its normal function. If ALX is a negative regulator of CD28 signaling, then at least two protein-protein interaction sites should be required to mediate the inhibition of RE/AP activation in response to TCR/CD28 stimulation. In contrast, our mutagenesis data have only identified one site, the SH2 domain, as being necessary for ALX to mediate inhibition of RE/AP activation by CD28. Because ALX-mediated inhibition of RE/AP activation in response to TCR/CD28 is dependent on a single protein-protein interaction site rather than two, this would imply that the normal function of ALX may not be as a negative regulator of CD28 signaling. Rather, an alternative model is that ALX is a positive regulator of CD28 signaling but that only the binding partner that associated with the SH2 domain is sensitive to overexpression of ALX because of differences in the relative concentration or the affinity for ALX between the two binding partners. In this case, overexpression of ALX (or the site within ALX to which it associates) disrupts the normal stoichiometry of a critical signaling complex, giving the appearance of a negative regulator (such as in the case of Jip-1). Ultimately, analysis of ALX-deficient mice will reveal exactly how ALX contributes to CD28 signaling in T cell activation.

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