Cloning and Characterization of ALX, an Adaptor Downstream of CD28

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T cell activation requires two signals: specific recognition of antigen through the T cell receptor (TCR) and a costimulatory signal provided primarily by CD28 in naïve T cells. We cloned a novel gene with considerable homology to RIBP/TSAd/Lad, an adaptor involved in T cell activation and interleukin-2 (IL-2) promoter activation. Expression of this gene is limited to the spleen and thymus. We have named this gene ALX, adaptor in lymphocytes of unknown function X. Because the related adaptor RIBP is involved in IL-2 regulation, we investigated whether ALX had a similar function. ALX overexpression in Jurkat T cells results in inhibition of IL-2 promoter activation after stimulation with superantigen. The IL-2 promoter contains several binding sites for transcription factors including the composite element RE/AP, which is the primary site of CD28 transcriptional activation. ALX overexpression had the greatest effect on the activation of a RE/AP reporter as opposed to an AP-1 reporter. Interestingly, ALX overexpression strongly inhibited RE/AP activation in response to anti-CD28/phorbol 12-myristate 13-acetate (PMA) stimulation but had minimal effect when anti-TCR/PMA was used. Therefore, it appears that ALX may function downstream of CD28 costimulation during T cell activation. In addition, the mobility of ALX shifts upon TCR/CD28 costimulation to a greater extent than what is observed with either stimulus alone demonstrating that ALX is a target of both TCR and CD28 costimulatory signaling pathways.

The activation of T cells is critical to the generation of an immune response. Minimally, two signals are required to activate resting T cells into effector 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 in naïve T cells (reviewed in Ref. 1). The biochemical events immediately downstream of TCR engagement are well characterized, initiating with activation of Src family kinases Lck and Fyn, phosphorylation of tyrosines within ITAM motifs within CD3, and recruitment of Syk family kinases to the ITAM motifs. Their subsequent activation by Src family kinases leads to the recruitment of many signaling proteins to the site of T cell/APC interaction (reviewed in Ref. 2). The biochemical events downstream of CD28 and how they synergize with TCR signals to result in T cell activation are much less understood. A critical step in T cell activation is the induction of the IL-2 gene, which occurs through both a transcriptional up-regulation of its promoter and increased stability of its mRNA (3, 4).

One molecule involved in TCR signaling is the adaptor molecule TSAd (also designated Lad, and for the mouse ortholog RIBP) (5). This adaptor was identified in separate two-hybrid screens for proteins that associated with the Tec family kinases Rlk and Itk (6), the Src family kinase Lck (7), and the mitogen-activated protein kinase kinase kinase MEKK2 (8). Although the mechanism of TSAd action is not well characterized, a role for TSAd in the regulation of IL-2 was demonstrated in the RIBP knock-out mouse (6). These mice have no gross abnormalities in T cell development, but mature T cells show a moderate defect (70% decrease) in proliferation and IL-2 production upon TCR or TCR/CD28 stimulation. TSAd has been reported to localize to the cytoplasm in T cells and to translocate to the immunological synapse during T cell activation (8), although it also has been reported to be primarily nuclear (9). Although TSAd is reported to bind to and modulate the activity of several kinases (Lck, Itk, Rlk, and MEKK2) involved in the proximal events in T cell activation, TSAd is not expressed in resting, naïve T cells but rather is rapidly induced within hours of T cell activation, suggesting that it may not play a role in proximal events in T cell activation. Therefore, it is possible that another adaptor, belonging to the same family as TSAd, is present in naïve T cells and can mediate effects similar to those of TSAd.

Redundancy is observed in many of the signaling pathways downstream of the TCR, and strong phenotypes or defects in TCR signaling may not be observed until multiple protein family members are deleted. Mice deficient in both Src family kinases Lck and Fyn have a more severe defect in T cell development than mice singly deficient in either Lck or Fyn (10). Mice deficient in the Tec family kinases Itk and Tsk/Rlk have mild defects in T cell function, whereas the doubly deficient animals reveal a critical role for Tec family kinases in T cell activation of TSAd.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY319652 and AY319653.

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The abbreviations used are: TCR, T cell receptor; IL-2, interleukin-2; PMA, phorbol 12-myristate 13-acetate; YFP, yellow fluorescent protein; GST, glutathione S-transferase; SEC2, staphylococcal enterotoxin D; SEC21, staphylococcal enterotoxin C2; ERK, extracellular signal-regulated kinase; SH, Src homology; EST, expressed sequence tag; RIBP, Rlk- and Itk-binding protein; MEKK2, mitogen-activated protein kinase/extracellular signal-regulated kinase 2; RIBP, Rlk- and Itk-binding protein; TSAd, T cell costimulatory protein; Lad, Lck-binding adaptor.
**Northern Analysis**—A murine multiple-tissue Northern blot (FirstChoice mouse blot 1, Amhion) was probed with a 315-bp PstI fragment from the murine ALX cDNA, using UltraHyb (Ambion) as per the manufacturer’s instructions. The blot was visualized by autoradiography using Biomax MS film (Kodak) overnight at ~80 °C using Trans- Screen HE intensifying screens (Kodak). The blot was subsequently stripped and reprobed for glyceroldehyde-3-phosphate dehydrogenase to verify the presence of RNA in samples that did not express ALX.

**Generation of Rabbit Polyclonal Antisera against ALX**—An Xmal fragment of ALX, corresponding to the C terminus that is least conserved between ALX and TSAd, was subcloned into pGEX-3X (Amersham Biosciences). GST-ALX fusion protein was expressed in BL21 Codon- Plus (DE3)-RP (Stratagene), purified using glutathione-Sepharose (Amersham Biosciences), and cleaved from glutathione S-transferase with Factor Xa (New England Biolabs) as per the manufacturers’ instructions. Recombinant ALX was sent to Cocalico Biologicals for generation of rabbit polyclonal antisera using a standard 90-day protocol. Three rabbits were each injected with a total of 500 μg of purified recombinant ALX. To verify specificity of the antisera, 280T cells were transfected with 1 μg of pGEXF-C1 (BD Biosciences), YFP-ALX, or YFP-TSAd with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Twenty-four hours later, Nonidet P-40 lysates were generated and examined by Western blotting with anti-YFP (to verify expression of all three constructs) and polyclonal antisera from rabbit 1576T (to demonstrate specificity of the antisera for ALX). This anti-ALX antisera (1576T) was used in all experiments.

**Transfections and Luciferase Assays**—Transfections, stimulations, and luciferase assays were performed as described previously (14, 17). Briefly, 15 × 10⁴ Jurkat T cells or CEM25 T cells were washed once and resuspended in 0.4 ml of serum-free RPMI. 20 μg of reporter or various amounts of expression plasmid (see legend for Fig. 4) were added. Electroporation was performed using a Gene Pulser II (Bio-Rad) at 250 V and 950 microfarads. Cells were resuspended in 10 ml of RPMI with 5% fetal calf serum (Invitrogen). The following day, live cells were counted by trypan blue exclusion (Bio-Whittaker), and 10⁵ cells/sample were stimulated as denoted in Figs. 4, 5, and 6. Cells were left either unstimulated or stimulated with antibodies to TCR (C305, 1:1000 final dilution), CD28 (1:200), or CD40 (1:1000). Jurkat T cells were stimulated with 300 ng/ml SED (staphylococcal enterotoxin D, Toxin Technology). CEM25 cells were stimulated with 300 ng/ml SEC2 (staphylococcal enterotoxin C2, Toxin Technology). Luciferase assays were performed as described previously (14).

**Western Blotting**—To examine ALX expression, whole cell extracts from various human hematopoietic cell lines were lysed in Nonidet P-40 lysis buffer as described previously. Total peripheral blood monoclonal cell lysate and cytoplasmic lysate from purified CD4+ T cells were gifts from Randy Hon (Children’s Hospital of Philadelphia) and Jim Riley and Gary Koretsky (University of Pennsylvania). Briefly, lysates were separated by SDS-PAGE, transferred to an Immobilon-Membrane (Millipore), and examined using rabbit 1576 polyclonal antisera against ALX at a final dilution of 1:1000. The blot was stripped and reprobed with anti-ERK1/2 as a control (p44/42 mitogen-activated protein kinase, Cell Signaling Technologies, catalog no. 9102).

**Results**

**Identification of ALX, an Adaptor Related to TSAd**—While examining Jurkat T cell microarray data, we found an EST (IMAGE no. 711563) that demonstrated considerable homology when translated and compared with the SH2 domain of TSAd, and we hypothesized that this gene could be related to TSAd and also play a role in T cell activation. The large open reading frame extended through to the 5’ end of this clone. Consequently, we used 5’ rapid amplification of cDNA ends to isolate further upstream sequences of the cDNA until a stop codon was reached in-frame with the large open reading frame. Translation of this large open reading frame predicted a protein containing 352 amino acids with considerable homology to TSAd (Fig. 1). We have designated this gene ALX, for adaptor in lymphocytes of unknown function X. Performing a BLAST search, we identified an EST that contained the complete cDNA for the murine homolog of ALX. Comparison of the mouse and human ALX protein sequences demonstrated that they share a 52% identity (Fig. 1). Like TSAd, the ALX protein
contains a single SH2 domain and several potential sites for protein-protein interactions including several polyproline PXXP (SH3-binding) motifs and potential sites of tyrosine phosphorylation (a tyrosine closely preceded by an acidic residue). The similar size, relative placement of the SH2 domain, and potential sites of protein-protein interaction, as well as the degree of homology (35%), all indicate that ALX might be functionally related to TSAd and represent the second member of a new family of adaptor molecules (Fig. 1).

**ALX Expression Is Limited to Hematopoietic Cells, Primarily Lymphocytes**

To determine the tissue distribution of ALX expression, a murine multiple-tissue Northern blot was examined (Ambion FirstChoice Mouse Blot 1). Because there is substantial homology between mouse RIBP and ALX in the SH2 do-

**Fig. 1.** Amino acid sequence of ALX. a, schematic representation of the adaptors TSAd and ALX showing similar placement of protein-protein interaction sites including a single N-terminal SH2 domain, potential sites of tyrosine phosphorylation, and polyproline PXXP sites. b, alignment of protein sequences of TSAd, RIBP, and human and mouse ALX (hALX and mALX, respectively). A ClustalW alignment was performed using MacVector (Genetics Computer Group). Positions are highlighted in the alignment where an identical or similar amino acid is present in at least three of the four proteins. The GenBank™ accession numbers for the human and mouse ALX nucleotide/protein sequences are AY319652 and AY319653, respectively.
molecular weight of the endogenous ALX protein matched our untagged ALX expression construct, confirming our conclusion from 5' rapid amplification of cDNA ends that we had cloned the entire ALX coding sequence. In the course of the work presented here, the predicted protein sequence of a gene designated HSH2 was reported (18). In this report, the initiating methionine was not conclusively identified, as the open reading frame extended through to the 5' end of the clone, and protein expression was not determined. However, it should be noted that the sequence of ALX matches the predicted protein sequence of HSH2.

**ALX Overexpression in Jurkat T Cells Inhibits IL-2 Promoter Activation**—T cells from RIBP-deficient mice (the murine ortholog of human TSAd) produce decreased IL-2 upon T cell stimulation demonstrating that TSAd/RIBP functions as a positive regulator of T cell activation. However, overexpression of TSAd in Jurkat T cells led to an inhibition of IL-2 promoter activation (6, 19). This is not unusual for an adaptor/scaffolding protein, because overexpression may disrupt the stoichiometry of a complex, giving the appearance of being a negative regulator of signaling. To determine whether ALX and TSAd may have similar functions, we compared the effects of ALX and TSAd overexpression on IL-2 promoter activation using YFP-tagged proteins. As shown in Fig. 4, and as has been demon-

![Fig. 2. ALX mRNA expression is limited to hematopoietic tissues. A mouse multiple-tissue Northern blot (Ambion) was probed with mouse ALX cDNA (upper blot) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (lower blot). RNA size markers are shown on the left. The tissues used in each lane are labeled at the top of the blot.

![Fig. 3. Expression of ALX protein in T and B cells. a, specificity of the anti-ALX antisera. 293T cells were transfected with 1 μg of pEYFP-C1 vector, YFP-ALX, or YFP-TSAd as denoted in the figure. Nonidet P-40 whole cell extracts were prepared ~24 h after transfection and examined by Western blotting (WB) for expression of either the YFP constructs using anti-YFP antisera (Clontech) or 1576T anti-ALX antisera. Arrows denote the mobility of the YFP constructs. b, Nonidet P-40 whole cell extracts were prepared from several hematopoietic cell lines and analyzed for expression of ALX protein using anti-ALX antisera. As a control, 293T cells were transfected with either vector control or untagged ALX expression construct to define the specific ALX band in the Western blot. Arrows denote the specific ALX band or a nonspecific (NS) band. Cell extracts from normal human peripheral blood mononuclear cells and purified (95%) CD4+ T cells as well as from the non-hematopoietic cell line 293T (human embryonic kidney) were also examined. The blot was stripped and reprobed with antibodies to ERK1/2 as a control for total protein level. Note that in lanes 1 and 2 significantly lower total protein was loaded, resulting in a greatly decreased signal for ERK1/2 as compared with the other samples.**
strated previously, overexpression of TSAd in Jurkat T cells inhibited IL-2 promoter activity (19). Overexpression of ALX had an even greater effect on IL-2 promoter activity, whether the cells were stimulated with anti-TCR/CD28 antibodies (data not shown) or with SED presented by Raji B cells (Fig. 4a). Co-transfection of ALX and TSAd did not dramatically change the inhibition that is seen with ALX alone (data now shown). Therefore, ALX and TSAd may be similar in function as well as structure. An additional experiment demonstrated that the addition of the YFP tag did not alter ALX function. When either untagged ALX or YFP-tagged ALX were expressed at similar levels in Jurkat T cells, both showed the same inhibition of IL-2 luciferase activation (Fig. 4b).

ALX Overexpression Inhibits Activation of RE/AP but Not AP-1—TSAd has been demonstrated to associate with Lck and Itk, two kinases involved in T cell activation (6, 7). Both Lck and Itk can be activated by stimulation through the TCR, and both have been demonstrated to bind to the cytoplasmic tail of CD28 (20, 21). Therefore, we were interested in whether ALX overexpression had an effect on RE/AP transcriptional activation, which depends upon both TCR and CD28 signals. For comparison, we examined the effect of ALX overexpression on a consensus AP-1 reporter, which in Jurkat T cells is activated by TCR engagement alone with no up-regulation by CD28 (17). Jurkat T cells were transfected with either RE/AP or AP-1 reporter constructs, along with vector control or expression plasmids for ALX or TSAd. Transfectants were left unstimulated or stimulated with SED superantigen presented by Raji B cells. In this superantigen system, activation of RE/AP requires both TCR and CD28 signaling, whereas AP-1 is dependent solely on TCR signaling (17). As shown in Fig. 5, overexpression of ALX had a significant effect on the activation of the RE/AP reporter but had a minimal effect on the activation of an AP-1 reporter. Similar results were observed when CEM25 (a high TCR-expressing subline of CEM, data not shown) cells were stimulated with superantigen SEC2 presented by Raji B cells (see Fig. 7). (Different superantigens were used to stimulate Jurkat and CEM25 cell lines based on their differential Vβ usage, CEM25 expresses Vβ13 and can only be stimulated by SEC2 superantigen, whereas Jurkat T cells express Vβ8 and can be stimulated by SED.) Because the effect of ALX overexpression is on RE/AP activation and not AP-1 activation, these data suggest that the primary effect of ALX overexpression is on CD28 costimulation rather than TCR signaling.

The Primary Effect of ALX Overexpression Is on CD28, Rather Than TCR, Stimulation Leading to RE/AP Activation—RE/AP stimulation in Jurkat T cells requires two signals, through both TCR and CD28 or through either TCR or CD28 in combination with PMA. We have taken advantage of this system previously to confirm differential signaling requirements for calcineurin downstream of TCR and CD28 (14). We demonstrated that RE/AP stimulation by anti-TCR/PMA is sensitive to FK506 (an inhibitor of calcineurin), whereas stimulation by
PM/anti-CD28 activation is not. To determine whether the effect of ALX overexpression on RE/AP activation is on TCR signaling, CD28 signaling, or both, Jurkat T cells were transfected with RE/AP luciferase reporter with or without ALX and stimulated with anti-TCR/CD28, PM/anti-TCR, or PM/anti-CD28 (Fig. 6). As expected, ALX overexpression inhibited RE/AP activation by anti-TCR/CD28. Interestingly, PM/anti-TCR stimulation of RE/AP was largely unaffected by ALX overexpression, whereas PM/anti-CD28 was inhibited to an extent similar to that seen with anti-TCR/CD28. Therefore, we conclude that ALX overexpression preferentially targets the CD28 signaling pathway leading to RE/AP transcriptional activation.

ALX Is a Downstream Target of TCR/CD28 Signaling—To confirm that ALX is part of the pathway downstream of CD28, Jurkat stable cell lines expressing myc-tagged ALX at levels that do not interfere with RE/AP activation were generated (data not shown). These cell lines were stimulated with either anti-TCR, anti-CD28, or both over a 45-min time course, and cell extracts were subject to electrophoresis and immunoblotting with antibodies to the myc tag (Fig. 7). Stimulation with anti-CD28 alone had minimal effect on the mobility of ALX, whereas anti-TCR stimulation alone caused a shift in the mobility of ALX. Interestingly, when cells were stimulated with both anti-TCR and anti-CD28, a further shift in ALX mobility is observed compared with either stimulants alone. Therefore, ALX is a target of TCR and CD28 costimulation during T cell activation.

**DISCUSSION**

We have isolated an adaptor, ALX, which appears to be the second member of a family of adaptors which includes RIBP/TSAd/Lad. ALX and TSAd are similar in size and sequence, and both contain a single SH2 domain in the N terminus, as well as additional sites for protein-protein association (potential sites of tyrosine phosphorylation and polyproline PXXP sequences). Both have a similar function in that overexpression of either TSAd (19) or ALX (data herein) inhibits the activation of the IL-2 promoter during T cell activation in Jurkat T cells. Specifically, overexpression of ALX had a substantial inhibitory effect on the RE/AP composite element from the IL-2 promoter, which is the major site of CD28 costimulation (17), while having minimal effects on TCR-mediated activation of an AP-1 reporter. The effect of ALX overexpression appears to be primarily along the CD28, rather than TCR, pathway leading to RE/AP activation, because ALX overexpression had minimal effect on TCR/PMA-induced activation of RE/AP while having a strong effect on PMA/CD28-mediated RE/AP activation. The electrophoretic mobility of ALX was synergistically shifted in response to stimulation with anti-TCR and anti-CD28, as compared with either stimuli alone. Therefore, the adaptor ALX may function downstream of CD28 during T cell activation leading to RE/AP and IL-2 promoter activation.

Based on the overexpression studies presented here, it cannot be determined conclusively whether ALX is a positive or negative regulator of CD28 signaling. If an adaptor serves as a bridge that assembles two or more proteins into a complex, the stoichiometry of this complex could be disrupted upon transient overexpression of the adaptor, and hence, a positive regulator could appear to act as a negative regulator. For example, RIBP-deficient mice (TSA ortholog) exhibited defects in IL-2 production during T cell activation, indicating that TSA functions as a positive regulator of TCR/CD28 signaling (6). However, TSAd overexpression in Jurkat T cells inhibited IL-2 promoter activation, giving the appearance of a negative regulator (19). Further elucidation of the role of ALX awaits the generation and examination of ALX-deficient animals. Although the exact role of ALX in normal T cell function has yet to be determined, the effects observed with ALX overexpression on RE/AP and IL-2 promoter activation provide a new tool with which we can probe the signaling pathways that are required downstream of CD28 costimulation. By analysis of the signaling pathways that are affected by ALX overexpression additional insight into the mechanism of CD28 signaling may be gained.

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