The Carboxyl-terminal Segment of the Adaptor Protein ALX Directs Its Nuclear Export during T Cell Activation*

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The adaptor protein ALX acts downstream of CD28 to regulate the interleukin-2 (IL-2) promoter during T cell activation. Whereas ALX is predominantly localized to the cytoplasm, ALX partially resides in the nucleus, and the nuclear pool is rapidly depleted in response to T cell receptor (TCR)/CD28 signaling. Here it is shown that this depletion occurs via nuclear export of ALX, which depends on a leucine-rich nuclear export signal (NES) in its carboxyl segment and on the CRM-1 transport protein. Nuclear import of ALX also depends on its carboxyl-terminal segment. Blocking nuclear export of ALX, either pharmacologically, by leptomycin B, or by site-directed mutation of the ALX NES, impairs CD28-mediated phosphorylation of ALX. Additionally, upon overexpression, the ALX NES mutant was found to be impaired in inhibiting TCR/CD28-induced transcriptional up-regulation of the RE/AP composite element from the IL-2 promoter, whereas a truncated form of ALX that is a potent inhibitor of RE/AP activation was found to reside entirely in the cytoplasm. Together, these results show that ALX exerts its effect on IL-2 up-regulation in the cytoplasm and suggest an intricate relationship between the nuclear localization/export, phosphorylation, and activity of ALX in response to TCR and CD28 signaling.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies Used—Myc epitope-tagged ALX and yellow fluorescent protein (YFP)-ALX fusion proteins (wild type and truncations) were described previously (3, 9). The nuclear export signal in ALX was mutated by substituting alanines for the leucines at positions 203, 207, and 210 (numbers refer to human ALX amino acid sequence) by PCR mutagenesis, and the mutant was subcloned into the same vectors as wild type ALX for expression of Myc and YFP-tagged versions. The RE/AP luciferase reporter was described previously (2). Monoclonal antibodies to the Myc epitope (clone 9B11; Cell Signaling), YFP (clone 11E5; Molecular Probes, Inc., Eugene, OR), Nck (clone 108; BD Biosciences), MEF2D (clone 9; BD Biosciences), and CD28 (clone 15E8; Caltag) were purchased from the indicated suppliers. 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) (10).

Transfections and Luciferase Assays—Jurkat transfections, stimulations, and luciferase assays were performed as previously described (2, 11). Briefly, 15 × 10⁶ Jurkat T cells were washed once and resuspended in 0.4 ml of serum-free RPMI. 20 μg of reporter with 1 μg of YFP expression plasmid (as described in the legend to Fig. 5) were added. Electroporation was performed using a Gene Pulser II (Bio-Rad) at 250 V, 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 1 × 10⁶ cells/sample were stimulated as denoted in the figures. Cells were left unstimulated or stimulated with antibodies to TCR (C305; 1:1000 final dilution) and antibodies to CD28 (1 μg/ml) for 7 h. Alternatively, they were incubated undergoing phosphorylation in response to TCR activation as well as CD28 costimulation (3). ALX is closely related to another hematopoietic adaptor protein, T cell-specific adaptor (TSAd), the murine homolog of which is known as RLK/TK-binding protein (4, 5). Similar to ALX, overexpression of TSAd affects up-regulation of the IL-2 promoter in Jurkat T cells (6, 7). T cells from mice with a targeted deletion of TSAd display a defect in IL-2 production (4), and these mice develop autoimmune disease as they age, including the development of autoantibodies (8).

ALX contains a single Shc homology 2 (SH2) domain followed by a longer carboxyl-terminal segment. The SH2 domain of ALX has been shown previously to be required for the inhibition of RE/AP and recognition of a tyrosine-phosphorylated binding partner (9). Here, it is shown that the carboxyl-terminal segment of ALX is critical for the trafficking of ALX between the cytoplasm and nucleus, containing both sequences required for import into the nucleus and for TCR signal dependent nuclear export. It is also shown that nuclear export is a CRM-1-dependent process. Further, we are able to conclude that the inhibition of RE/AP by ALX involves an effect exerted in the cytoplasm and that this inhibition is enhanced by the exit of ALX from the nucleus.

T cell activation, a critical step in the immune response, is triggered by two signals: activation of the T cell receptor (TCR)2 by a specific antigen and a costimulatory signal, generally provided by the cell surface receptor CD28 (reviewed in Ref. 1). Up-regulation of the cytokine interleukin-2 (IL-2) is a key event in T cell activation that results, at least partly, from the convergence of TCR and CD28 signaling pathways, leading to an increase in transcription of the IL-2 promoter through the RE/AP composite element (2).

We recently identified an adaptor protein designated ALX (for adaptor from lymphocytes of unknown function (“X”), which has a hematopoietic specific expression pattern and may play a role in CD28 mediated up-regulation of the IL-2 promoter (3). Upon overexpression in Jurkat T cells, ALX inhibited up-regulation of an RE/AP luciferase reporter in response to TCR and CD28 activation, whereas activation of an AP-1 reporter, responsive to TCR signaling alone, was unaffected. Hence, ALX is believed to act predominantly in the signaling pathway triggered by CD28, which leads to RE/AP transcriptional up-regulation. ALX was also shown to be a downstream target of TCR/CD28 signaling.

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2 The abbreviations used are: TCR, T cell receptor; IL-2, interleukin 2; TSAd, T cell-specific adaptor; SH2, Src homology 2; YFP, yellow fluorescent protein; LMB, leptomycin B; NES, nuclear export signal.

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with 1 μg/ml staphylococcal enterotoxin D and 1 × 10^6 Raji B cells for 16 h. Luciferase assays were performed as previously described (11).

293T cells were transfected using Fugene6 reagent (Roche Applied Science). Briefly, 1 μg of DNA and 3 μl of Fugene6 were diluted in 100 μl of serum-free medium. After a 20-min incubation at room temperature, this mixture was added to one well in a 6-well dish containing a monolayer of cells in 1 ml of complete medium. The cells were incubated overnight, washed in phosphate-buffered saline, and then harvested and subject to fractionation.

Stimulations and Subcellular Fractionation—Jurkat cell lines stably expressing wild type and mutant ALX proteins were generated as described previously (3). For stimulations, Jurkat cells were washed and resuspended in phosphate-buffered saline at a concentration of 10 million/ml. If indicated, leptomycin B (Calbiochem) was added at a concentration of 25 ng/ml, and the cells were incubated for 2 h at 37 °C. At various times, antibodies to TCR and CD28 were added (see Ref. 9), and the cells were returned to 37 °C. Nuclear and cytoplasmic fractionation was performed according to a published protocol (9, 12). Briefly, cell pellets were resuspended in a hypotonic buffer and incubated on ice. Nonidet P-40 was then added, and the lysates were vortexed briefly and then subject to centrifugation, resulting in pelleting of the nuclei. Supernatants were then removed as the cytoplasmic fraction. Nuclear pellets were washed, lysed in hypertonic buffer, and subject to centrifugation. Supernatants were then removed as the nuclear fraction. Both fractions were combined with reducing sample buffer and boiled before loading on gels and analyzed by Western blotting.

RESULTS

In previous work on the regulation of the IL-2 promoter in Jurkat T cells by the adaptor protein ALX, it was noted that a fraction of ALX is located in the nucleus and that this nuclear pool is rapidly depleted in response to TCR and CD28 signaling (9). To further investigate the function of ALX, experiments were performed to characterize the mechanism responsible for the disappearance of ALX from the nucleus. As shown in Fig. 1, ALX was found in both cytoplasmic and nuclear pools as determined by anti-Myc immunoblotting of subcellular fractions of a Jurkat T cell line expressing Myc-tagged wild-type ALX. To allow for comparison of nuclear and cytoplasmic protein in the same exposure, 4 times more cell equivalents of nuclear extracts were loaded per lane relative to cytoplasmic extracts. Since similar levels of ALX protein were apparent when nuclear and cytoplasmic extracts from unstimulated cells were analyzed, we can conclude that roughly 20% of ALX resides in the nucleus (Fig. 1; see Ref. 9). A similar subcellular distribution was also observed in 293T cells (see below). Upon stimulation of the cells with antibodies to the TCR and CD28, significant depletion of ALX from the nuclear pool was observed within 5 min, and nearly all of the ALX protein was depleted by 15 min. Stimulation with antibodies to the TCR alone was sufficient to drive ALX nuclear depletion (data not shown). As a control for effective fractionation, the extracts were also blotted with antibodies to MEF2D and Nck. MEF2D was detected only in nuclear extracts, and Nck was predominantly cytoplasmic. Equivalent amounts of MEF2D and Nck were also observed in samples from each time of stimulation, demonstrating equal protein loading between lanes.

The nuclear export of many molecules has been shown to be mediated by the protein CRM-1 (reviewed in Ref. 13), a process that can be inhibited by the fatty acid leptomycin B (LMB). To determine whether the disappearance of ALX from the nucleus is due to CRM-1-mediated nuclear export, cells were treated with LMB for 2 h prior to stimulation with antibodies to the TCR and CD28. As shown in Fig. 1, this treatment did not alter the subcellular distribution of ALX in unstimulated cells. However, LMB treatment nearly eliminated TCR/CD28-mediated nuclear export of ALX.

Nuclear export of proteins by CRM-1 has been found to depend on the presence of a leucine-rich nuclear export signal (NES) within these proteins. The consensus NES consists of three leucine residues separated by two or three intervening residues, followed by a fourth leucine one residue carboxyl to the third (LX^2,LX^2,LX^2). However, substitutions of different hydrophobic residues for the leucines seem to be tolerated (see NESBase 1.0 (14)). NES sequences lacking the fourth leucine have also been reported, for instance in NFAT and cellular inhibitor of apoptosis-1 proteins (15, 16). The sequence LXXLXXLX, similar to the consensus NES, was found in human ALX carboxyl to its SH2 domain (Fig. 2). To determine whether this sequence is conserved in ALX from different species, the sequence of human ALX was aligned with that of the previously identified mouse ALX (3) and of ALX orthologues from rat, horse, pig, cow, and dog, which were obtained by a Blast search using human ALX protein sequence against the translated EST data base through NCBI (rat/Rattus norvegicus Unigene Rn.126627, horse/Equus caballus GenBank™ accession CD66966, pig/Sus scrofa Unigene Ssc. 9327, cow/Bos taurus Unigene Bt. 26963, and dog/Canis familiaris GenBank™ accession XM541968). Strikingly, the leucines in the putative NES sequence in human ALX are completely conserved in
Nuclear Export of ALX

To begin to identify the region of ALX responsible for import into the nucleus, we examined the localization of a set of ALX truncation mutants fused to YFP. As shown in Fig. 4, 293T human embryonic kidney cells were transiently transfected with an expression construct for YFP or for various YFP-ALX fusion proteins. Immunoblotting of subcellular fractions with antibodies to YFP showed that fused YFP was almost entirely cytoplasmic. A fusion protein containing full-length, wild type ALX (YFP-ALX(wt)) was found in both cytoplasmic and nuclear pools in proportions closely matching those observed in Jurkat cells (about 20% nuclear). A full-length fusion in which the putative NES was mutated (YFP-ALX(NES)) also was distributed in the same proportions in 293T cells as in Jurkat cells. ALX can be divided into three segments: a short amino-terminal segment followed by an SH2 domain and a larger carboxyl-terminal segment after the SH2 domain (9). A fusion protein in which the amino-terminal segment of ALX was deleted localized to both the cytoplasm and nucleus, as did full-length ALX, whereas a fusion protein in which the carboxyl-terminal segment was deleted was found to be entirely cytoplasmic. Similarly, a fusion containing only the SH2 domain of ALX was entirely cytoplasmic. This analysis was also performed by transiently transfecting Jurkat cells with the same set of YFP-ALX constructs. Their distributions between the cytoplasm and nucleus, assessed with polyclonal antibodies to ALX, were found to be identical to the distributions observed in 293T cells (data not shown). These results demonstrate that the carboxyl-terminal segment of ALX, but not the SH2 domain or amino-terminal segment, contains a sequence that can confer nuclear localization to a cytoplasmic protein (YFP) and presumably mediates the nuclear import of ALX. Because analysis of the sequence of ALX did not reveal an obvious nuclear localization signal (reviewed in Ref. 17), it is possible that nuclear import of ALX depends on an interaction of its carboxyl-terminal segment with another protein.
The results of Figs. 1 and 3 also yield insight into the mechanism of TCR-mediated nuclear export of ALX. This export can be explained by either a direct or indirect effect of signaling. First, ALX might cycle constitutively between the cytoplasm and nucleus, resulting in a steady state level in each pool. In this case, TCR signaling could indirectly trigger a depletion of the nuclear pool by causing ALX to be trapped in the cytoplasm, for example, promoting the formation of a complex of ALX with a tyrosine-phosphorylated protein. This model would imply that blocking nuclear export, even in the absence of TCR stimulation, should result in the rapid accumulation of ALX in the nucleus due to constitutive import. However, neither the NES mutation nor the inhibitor LMB caused a substantial increase in the proportion of ALX in the nucleus in unstimulated cells. Hence, the data support a model in which TCR signaling directly results in the export of ALX from the nucleus. Perhaps the NES sequence in ALX is exposed to the nuclear export machinery after TCR signaling due to changes in the phosphorylation state of nuclear ALX or changes in protein-protein interactions.

Another conclusion that can be drawn from the results presented here is that the inhibition of RE/AP by ALX depends on the activity of ALX residing in the cytoplasm. Importantly, an ALX mutant lacking the carboxyl-terminal segment, as well as a mutant composed of the SH2 domain in isolation, was found to be entirely cytoplasmic. Both of these mutants were unimpaired in their ability to inhibit activation of RE/AP (9). Hence, it is likely that ALX exerts its inhibitory effect in the cytoplasm by binding to a target protein involved in the signaling pathway leading to RE/AP up-regulation and IL-2 promoter activation. These findings might suggest that movement of ALX between the nucleus and cytoplasm directed by its carboxyl-terminal segment is dispensable for its function. However, it is unlikely that overexpression studies fully reveal the function and regulation of ALX. For instance, ALX may have some activity involving interactions with nuclear proteins that is entirely separable from the inhibition of RE/AP but that is influenced by TCR and CD28 signaling. Further, movement of ALX out of the nucleus triggered by TCR signaling may have a crucial influence on the amount of ALX available to act on proteins in the cytoplasm.

The ALX(NES) mutant was found to be defective in the inhibition of RE/AP. One explanation for this phenotype is that the defect in TCR-mediated nuclear export reduces the amount of ALX in the cytoplasm available to act on the pathway upstream of RE/AP. In addition, the NES mutant also displayed a defect in CD28-mediated phosphorylation, which might be important for ALX to function downstream of CD28. In any case, it is interesting that impairing nuclear export of ALX affects both CD28-mediated phosphorylation and the function of ALX in inhibiting RE/AP. Hence, the data suggest some intricate connection between the subcellular localization, phosphorylation, and function of ALX downstream of TCR and CD28.

Adaptor proteins, which propagate signals from cell surface receptors through protein-protein interactions, are generally thought to act in the cytoplasm. However, there are other proteins that, like ALX, have a signaling function in the cytoplasm but also have a nuclear pool. For example, the Fas-associated death domain and cellular inhibitor of apoptosis-1 proteins are both recruited by plasma membrane receptors that trigger cell death. However, each also localizes to the nucleus and has a leucine-rich NES necessary for export (16, 18). Mutation of the NES in the Fas-associated death domain impairs its ability to promote cell death (18). Further, the neural Wiskott-Aldrich syndrome protein regulates actin nucleation at the cell periphery and also localizes to the nucleus (19). Nuclear export of neural Wiskott-Aldrich syndrome protein was found to be promoted by a tyrosine kinase. A phosphorylation site mutant of neural Wiskott-Aldrich syndrome protein was found to

To determine how the nuclear export of ALX influences its ability to inhibit RE/AP up-regulation, Jurkat cells were transiently transfected with an RE/AP luciferase reporter (2) along with expression constructs for either YFP, YFP-ALX(wt), or YFP-ALX(NES). Immunoblotting showed that the wild type and NES mutant YFP-ALX proteins were expressed at similar levels (data not shown). Cells were then stimulated using antibodies to TCR/CD28 or staphylococcal enterotoxin D (SED) presented by Raji B cells for 16 h (Fig. 5A) or with a combination of antibodies against TCR and CD28 for 7 h (Fig. 5B), prior to measurement of luciferase activity. Results shown are -fold activation (as compared with unstimulated), and the mean and S.D. are derived from three independent transfections per experimental condition. wt, wild type.

**DISCUSSION**

In summary, it has been shown that the nuclear export of ALX, triggered by TCR stimulation, is inhibited by LMB, an inhibitor of CRM-1 activity, and that this export depends on a leucine-rich NES sequence similar to the consensus sequence for CRM-1-mediated export. In addition to containing an NES sequence, the carboxyl-terminal segment of ALX has also been shown to contain a sequence necessary for its nuclear import. The results of Figs. 1 and 3 also yield insight into the mechanism of nuclear import of ALX. The import of ALX is promoted by a tyrosine kinase. A phosphorylation site mutant of neural Wiskott-Aldrich syndrome protein was found to reveal the function and regulation of ALX. For instance, ALX may have some activity involving interactions with nuclear proteins that is entirely separable from the inhibition of RE/AP but that is influenced by TCR and CD28 signaling. Further, movement of ALX out of the nucleus triggered by TCR signaling may have a crucial influence on the amount of ALX available to act on proteins in the cytoplasm.

**FIGURE 5.** Mutation of the NES sequence impairs the function of ALX. Jurkat cells were transiently transfected with an RE/AP luciferase reporter plasmid along with plasmids encoding the indicated proteins (see Fig. 4). 24 h after transfection, the cells were divided and then either left unstimulated or stimulated by staphylococcal enterotoxin D (SED) presented by Raji B cells for 16 h (Fig. 5A) or with a combination of antibodies against TCR and CD28 for 7 h (Fig. 5B), prior to measurement of luciferase activity. Results shown are -fold activation (as compared with unstimulated), and the mean and S.D. are derived from three independent transfections per experimental condition. wt, wild type.
accumulate in the nucleus and to block focus formation caused by over-expression of this kinase. The subcellular localization of TSAd, a lymphocyte-specific adaptor closely related to ALX, has been described in a number of reports. In one, TSAd was shown to be localized to the cytoplasm and to accumulate at immune synapses after T cell activation (20). However, in another report, TSAd was shown to localize predominantly to the nucleus (21). Both the function of TSAd and its nuclear localization have been shown to depend on its SH2 domain (21). It is plausible that both ALX and TSAd have functions in the cytoplasm and also localize to the nucleus, which might serve as a means to regulate the concentration of cytoplasmic protein or to allow them to serve a separate function. In any case, it is clear that further investigation of the subcellular localization of these two related proteins and how TCR and CD28 signals influence this localization will be critical to understanding their role in T cell activation.

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