Membrane-associated adaptors play an important role in coupling antigen receptor engagement to downstream signaling events, such as Ras-MAPK activation, Ca$^{2+}$ flux, and nuclear factor of activated T cells (NFAT) activation. Here we identified a novel membrane-associated adaptor protein, LAX. LAX is mainly expressed in B cells, T cells, and other lymphoid-specific cell types. It shares no overall sequence homology with LAT and is not localized to lipid rafts. However, like LAT, LAX has tyrosine motifs for binding Grb2, Gads, and the p85 subunit of phosphatidylinositol 3-kinase. Upon stimulation via the B or T cell receptors, LAX is rapidly phosphorylated by Src and Syk family tyrosine kinases and interacts with Grb2, Gads, and p85. Overexpression of LAX in Jurkat cells specifically inhibits T cell receptor-mediated p38 MAPK activation and NFAT/AP-1 transcriptional activation. Our data suggested that LAX functions to negatively regulate signaling in lymphocytes.

Recognition of antigens by antigen receptors, the B cell receptor (BCR) and the T cell receptor (TCR), initiates a series of biochemical events involving a variety of distinct signaling pathways that eventually lead to gene transcription, clonal expansion, and cellular differentiation. Although the BCR and TCR have different structures and recognize different forms of antigens, signaling pathways activated via these two receptors are strikingly similar. Both the BCR and TCR utilize receptor-encoded signaling motifs termed ITAMs (immunoreceptor tyrosine-based activation motifs) to activate non-receptor tyrosine kinases (1). Following receptor engagement, Src family tyrosine kinases, activated by the CD45 phosphatase, phosphorylate the paired tyrosine residues within ITAMs. Syk family tyrosine kinases, activated by the CD45 phosphatase, further phosphorylate multiple cellular proteins, including enzymes and adaptor proteins (3–8), leading to activation of the Ras-MAPK pathway and Ca$^{2+}$ flux.

Several studies using deficient cell lines have shown that adaptor proteins are essential for lymphocyte activation by coupling receptor engagement to activation of the Ras-MAPK pathway, Ca$^{2+}$ mobilization, and cytokine production (9–11). In T cells, LAT and SLP-76 have been intensively studied in recent years (6, 12–15). LAT is a membrane-associated adaptor protein. Upon phosphorylation, LAT interacts with Grb2, Gads, and PLC-γ1 (16). The binding of Grb2 to LAT is postulated to recruit Sos to the membrane to activate Ras. The association of LAT with PLC-γ1 recruits PLC-γ1 to the membrane so it can be phosphorylated and activated. Activation of PLC-γ1 is essential for TCR-mediated Ca$^{2+}$ flux and activation of Ras-GRP, a molecule that functions to activate Ras (17, 18). TCR-mediated Ras-MAPK activation and Ca$^{2+}$ flux are defective in LAT-deficient Jurkat cells, indicating that LAT is essential for TCR-mediated signaling (9, 19). Binding of Gads to LAT recruits SLP-76 indirectly to the membrane (20, 21). SLP-76 is also essential in TCR-mediated signaling as indicated in SLP-76-deficient cells (11, 22). TCR-mediated MAPK activation and Ca$^{2+}$ flux are severely compromised in these cells.

As a membrane-associated adaptor protein, LAT plays a critical role in signaling in T cells. Similar molecules might also exist in other cell types with immune receptors. A similar molecule has not been found in B cells. It has been proposed that BLNK functions as both LAT and SLP-76 to link the BCR engagement to MAPK activation and Ca$^{2+}$ flux (23). BCR-induced PLC-γ2 activation and Ca$^{2+}$ flux are defective in BLNK-deficient cells (24). BCR-mediated JNK and Erk activation are also compromised. However, in contrast to LAT, BLNK is not constitutively localized to the membrane. Furthermore, LAT deficiency in Jurkat cells cannot be complemented by BLNK (25). Therefore, it is less likely that BLNK functions as both LAT and SLP-76 in B cells. It is possible that B cells or other lymphoid cells use a LAT-like molecule to link the receptor engagement to Ras-MAPK activation and Ca$^{2+}$ flux. Due to the near completion of the human genome sequencing, it might be possible to find a LAT homolog in the human genome database.

To look for a LAT-like molecule in B cells and other cell types, we searched the human genome database with the tyrosine motifs in LAT and identified a novel gene. We named it LAX. Like LAT, LAX is a membrane-associated adaptor protein. It is expressed in T cells, B cells, and other cell types of lymphoid origin. It associates with Grb2, Gads, and the p85 subunit of PI-3 kinase. However, it is unlikely that LAX functions as a B cell LAT-like molecule. Our data show that as opposed to LAT, LAX functions to negatively regulate antigen-
LAX in Lymphocyte Signaling

Transfection and Luciferase Assay—For luciferase assays, 1 × 10^6 Jurkat cells (26.1 or LAT deficient ANJ3) were transfected with 5 μg of pNFAT/AP-1-luciferase or AP-1-luciferase plasmids, 20 ng of Renilla-TK luciferase plasmid, and LAT or different amounts of LAX plasmids by electroporation using a BTX electroporator (310 V, 10 ms). Sixteen to twenty-four hours after transfection, cells were stimulated with OKT3 (1:500 ascites), FMA (10 ng/ml) plus ionomycin (1.5 μM), or left untreated for 6 h. Dual luciferase activity was assayed according manufacturer’s protocol (Promega). NFAT-luciferase activity was normalized by Renilla-TK activity.

RESULTS

Cloning of LAX—To look for proteins that are homologous to LAT, we performed an extensive search of the NCBI data base using the entire coding sequence of LAT, and we failed to find any LAT homolog in the data base. Next we used the tyrosine motifs in LAT to search the data base. A LAT-like molecule might have no overall sequence homology to LAT but may contain similar tyrosine motifs that are responsible for binding important signaling proteins and are essential in LAT function. Of the nine conserved tyrosines in the cytoplasmic domain of LAT, Tyr-171 and Tyr-191 are particularly important. Mutations of those tyrosines abolished the association of LAT with Grb2, Gads, and PLC-γ1 and consequently LAT function (16). Tyr-171 and Tyr-191 are both within a YVNV sequence context. In addition to these tyrosine motifs, we also searched for novel proteins containing a transmembrane domain. The peptide sequences from residues 160–180 and 181–200 of LAT were used to search the human genome data base. Of the many candidate proteins that have a YVNV motif, we identified one candidate for a LAT-like molecule in the human genome. It is a hypothetical protein encoded by one large exon in human chromosome 1 (FLJ20340, GenBankTM accession number, XP_001752). This protein contains a YVNV motif like Tyr-171 and Tyr-191 in LAT and a YVNN motif that is a potential binding site for Grb2, Gads, and the p85 subunit of PI-3 kinase. Furthermore, it also contains one YENV and one YENL motif similar to Tyr-226 (226YENV) in LAT. However, there is no potential transmembrane domain present in this hypothetical protein. Because the sequence of this protein was translated from a single exon in the genome, it is likely to be partial.

To identify the full-length sequence of this protein, we used the nucleotide sequence of this hypothetical protein to search the human EST data base, and we found three overlapping EST clones in the data base. These EST clones were from germinal center B cells, pre-B cells, and Jurkat T cells. Based on the nucleotide sequence from these EST clones and the genomic sequence, we designed primers to amplify a cDNA fragment by Pfu DNA polymerase using cDNAs from Jurkat cells as template. The amplified cDNA fragment was cloned into an mammalian expression vector and sequenced. We named this gene, LAX (Linker for Activation of X cells, X indicates “to be defined”). Translation of the human LAX cDNA revealed that the LAX gene encodes a putative protein of 398 residues (Fig. 1A). We also used the human LAX sequence to search a mouse EST data base, and we identified several EST clones from mouse lymph nodes. Based on the merged sequence of these EST clones, we designed PCR primers and amplified mouse LAX using cDNA from mouse spleen. Translation of the mouse LAX cDNA revealed that mouse LAX has 407 residues (Fig. 1A). Comparison of mouse and human LAX proteins showed that they share 52% identity.

Tyrosine Motifs in LAX—A pairwise comparison of LAX with LAT revealed no significant sequence homology. Despite the lack of homology to LAT in protein sequence, the overall domain organization of LAX was very similar to that of LAT.

Molecular Cloning of LAX—The LAT peptide sequence from residues 160–180, which contains an important motif (Tyr-171) in LAT function, was used to search the human genome data base in NCBI using the BLAST program to identify molecules similar to LAT. We looked for novel transmembrane proteins that contain tyrosine motifs similar to those in LAT. A hypothetical protein FLJ20340 (GenBankTM accession number XP_001752) was identified as a potential candidate. The nucleotide sequence of this protein was then used to search the human EST data bases to find corresponding EST clones. Based on the nucleotide sequence of merged EST clones, we designed two primers (5'-CAGGAGATAGGGAGTTTGTTGCGGG-3' and 5'-GGGCGGTAGGCA-CTTTTCTCAGTCAC-3') to amplify cDNAs from Jurkat cells with Pfu DNA polymerase. The PCR product was cloned into pBluescript (KS-) and sequenced by automated sequencing. The mouse LAX sequence was obtained by BLAST searching a mouse EST data base using the human LAX sequence. The mouse LAX cDNA was cloned using primers (5'-CTTCCGATTGCCGCGAAGCTAACAGC-3' and 5'-CCCCCTCAGGAGGCCAGTGTACAC-3') to amplify the cDNA from the mouse spleen. The GenBankTM accession number for human LAX is H11001 and the accession number for mouse LAX is H110075.

Tissue Expression of LAX—Detection of LAX expression in different human tissues was done by RT-PCR using primers (5'-TTTCCGATCTGAGAGCCTTTTCAC3'- and 5'-GGGCGGATCCAGGAGGCCAGTGTACAC-3') cDNAs from different human tissues were purchased from Clontech and used in PCRs. cDNAs from different human cell lines were obtained by reverse transcription with total RNAs. GSPDH primers (5'-TGAAGGTTGCGGTTACAAATATT-3' and 5'-CATGAGGGCCATGAGGTCCACC-3') were included in the PCR as a control. The PCR for amplification of LAX was done using the following condition: 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min for 35 cycles. PCR products were resolved on a 1.5% agarose gel.

Antibodies—Rabbit anti-LAX antisera were obtained by immunization of rabbits with GST-LAX fusion proteins. Anti-human LAX monoclonal antibodies were made by fusion of splenocytes from mice immunized with GST-LAX with NSO cells. Other antibodies used in this study are as follows: rabbit anti-LAT (28), anti-Grb2 antisera from Santa Cruz Biotechnology, rabbit anti-p85 antisera, rabbit anti-Gads antisera, anti-phosphotyrosine, and anti-PLCγ1 monoclonal antibodies from Upstate Biotechnology, Inc; anti-phospho-JNK MAPK, anti-phospho-p44/p42 MAPK, anti-phospho-p38 MAPK, and anti-p38 MAPK antisera from Cell Signaling Technology, Inc.

Immunoprecipitation and Cell Fractionation—Jurkat and Daudi cells were cultured in RPMI 1640 with 10% fetal bovine serum. Before stimulation, cells were removed from the culture, washed, and resuspended at 10^6 cells/ml in RPMI 1640. Cells were stimulated with anti-CD3 (C305, 1:50 dilution) for Jurkat cells or goat anti-human IgM Fab'2 for Daudi cells for 1.5 min, left untreated, or, as indicated in the figures, lysed in 1% Brij lysis buffer with different protease inhibitors and phosphatase inhibitors. Postnuclear supernatants were used in immunoprecipitation with different antibodies as indicated in each figure. Separation of the cytosolic fraction and membrane fraction was performed using Dounce homogenization and ultracentrifugation. Purification of lipid rafts was done using a sucrose gradient (27).

Stable Transfection, Ca2+ Flux, and MAPK Activation—Jurkat cells were transfected with 5–10 μg of LAX-WT or LAX-4YF cDNA plasmid by electroporation using a BTX electroporator (310 V, 10 ms). Stable transfectants were selected in the media containing G418 and further subcloned by limiting dilution. Clones expressing similar levels of CD3, CD28, and LAX were selected and used for further experiments. Intraacellular free Ca2+ measurement was performed as described previously (25). Jurkat cells and three transfectant clones, which expressed either LAX-WT or LAX-4YF, were stimulated with C305 plus anti-CD28 for 0, 5, 10, and 15 min. Equal volume of 2× SDS sample buffer were added to stop the stimulation, and samples were resolved on SDS-PAGE. Activation of p38, Erk, and JNK were detected by blotting the membranes with different antibodies.
LAX in Lymphocyte Signaling

Human gene MOC17377 encodes LAX. A comparison of human and mouse LAX proteins shows that LAX is predominantly expressed in cells of hematopoietic origin. In contrast to LAT, LAX is expressed in B cell as well as T cells. LAX was not present in HeLa (fibroblastoid) and K562 (erythroid) cells. LAX was protected in several B cell lines (BJAB, Daudi, Raji, and Jiyoye), YT (NK-like cells), THP1 (monocytes), and Jurkat cells (T cells). LAX was not present in HeLa (fibroblastoid) and K562 (erythroid) cells. These results indicated that LAX is predominantly expressed in cells of hematopoietic origin. In contrast to LAT, LAX is expressed in B cell as well as T cells and other cell types.

Subcellular Localization of LAX—To study the function of LAX, we made a GST-LAX fusion protein and used it as an antigen to raise polyclonal antisera against human LAX. First, we examined the subcellular localization of LAX by fractionating Jurkat cells into cytosolic and membrane fractions. The membrane fraction was further solubilized with 1% Brij97 lysis buffer. Because it was difficult to detect LAX by blotting total lysates directly with our antisera, LAX protein was immunoprecipitated and resolved on SDS-PAGE. LAX was then detected by anti-LAX immunoblotting.

As shown in Fig. 2, a PCR fragment corresponding to the predicted size was clearly seen when cDNAs from the spleen, thymus, and peripheral blood leukocytes were used in the PCR amplification, suggesting that LAX is predominantly expressed in these tissues. We also used cDNAs from nine different cell lines in PCRs to detect LAX expression. LAX cDNA was detected in several B cell lines (BjAB, Daudi, Raji, and Jiyoye), YT (NK-like cells), THP1 (monocytes), and Jurkat cells (T cells). LAX was not present in HeLa (fibroblastoid) and K562 (erythroid) cells. These results indicated that LAX is predominantly expressed in cells of hematopoietic origin. In contrast to LAT, LAX is expressed in B cell as well as T cells and other cell types.

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further analyzed. Most proteins in the lipid rafts were in fraction 3, and Triton-soluble proteins were in fractions 8–12. As shown in Fig. 3B, LAX was primarily detected in fractions 8–12 and not in fraction 3. Conversely, a large portion of LAT was found in lipid rafts (Fig. 3B, fraction 3) as reported previously (27). A similar result was obtained when Daudi B cells were used (not shown). In addition, LAX was not localized to the lipid rafts further solubilized with 1% Brij and 30 mM octyl-β-D-glucoside before being subjected to anti-LAX immunoprecipitation. The presence of LAX was detected by anti-LAX immunoblotting.

**LAX Is Phosphorylated Upon Antigen Receptor Stimulation**—Because LAX is expressed in both B and T cells and contains multiple tyrosine motifs that could potentially bind Grb2 and p85, we examined whether LAX can be phosphorylated upon stimulation via the BCR or TCR. Jurkat cells were stimulated with an anti-TCRβ antibody (C305) and Daudi cells were stimulated with goat anti-human IgM F(ab’)2, for 0, 1.5, 5, 10, and 20 min before lysis. These lysates were then subjected to immunoprecipitation with rabbit anti-LAX antiserum. Anti-LAX immunoprecipitates were resolved on SDS-PAGE and blotted with anti-LAX monoclonal antibody. HC indicates heavy chain from the antibody for immunoprecipitation (IP). B, lipid rafts and Triton-soluble fractions were isolated using sucrose gradients as described previously (27). Most proteins in lipid rafts were present in fraction 3. Fractions 8–12 were Triton-soluble. Lipid rafts were further solubilized with 1% Brij and 30 mM octyl-β-D-glucoside before being subjected to anti-LAX immunoprecipitation. The presence of LAX was detected by anti-LAX immunoblotting.

**LAX Is a Substrate of Src and Syk Family Tyrosine Kinases**—The cytoplasmic domain of LAX contains multiple tyrosine motifs that may interact with Grb2, Gads, and p85. We tested whether LAX can associate with these proteins after T cell activation. Myc-tagged wild-type LAX and a mutant LAX coexpressed with Syk, Lck, or Lck and ZAP-70, LAX became tyrosine-phosphorylated, suggesting that LAX is likely a substrate of these tyrosine kinases. LAX was phosphorylated similarly by these tyrosine kinases as reported previously (26).

**LAX Interacts with Grb2, Gads, and the p85 Subunit of PI-3 Kinase**—The cytoplasmic domain of LAX contains multiple tyrosine motifs that may interact with Grb2, Gads, and p85. We tested whether LAX can associate with these proteins after T cell activation. Myc-tagged wild-type LAX and a mutant LAX with mutation of four tyrosines (Tyr-193, Tyr-268, Tyr-294, and Tyr-373) were transfected into Jurkat cells. As a control, preimmune serum was used (not shown). In addition, LAX was not localized to the raft fractions after T cell or B cell activation (not shown). These data indicated that different from LAT, LAX is not localized in lipid rafts although it is present in the membrane.

**LAX Is Phosphorylated Upon Antigen Receptor Stimulation**—Because LAX is expressed in both B and T cells and contains multiple tyrosine motifs that could potentially bind Grb2 and p85, we examined whether LAX can be phosphorylated upon stimulation via the BCR or TCR. Jurkat cells were stimulated with an anti-TCRβ antibody (C305) and Daudi cells were stimulated with goat anti-human IgM F(ab’)2, for 0, 1.5, 5, 10, and 20 min before lysis. These lysates were then subjected to immunoprecipitation with rabbit anti-LAX antiserum. Anti-LAX immunoprecipitates were resolved on SDS-PAGE and blotted with anti-LAX monoclonal antibody. HC indicates heavy chain from the antibody for immunoprecipitation (IP). B, lipid rafts and Triton-soluble fractions were isolated using sucrose gradients as described previously (27). Most proteins in lipid rafts were present in fraction 3. Fractions 8–12 were Triton-soluble. Lipid rafts were further solubilized with 1% Brij and 30 mM octyl-β-D-glucoside before being subjected to anti-LAX immunoprecipitation. The presence of LAX was detected by anti-LAX immunoblotting.
from lysates and resolved on SDS-PAGE, immunoblotted with anti-Tyr(P), anti-p85, anti-Grb2, and anti-LAX antibodies. As shown in Fig. 6A, Myc-tagged LAX-WT was phosphorylated upon TCR stimulation. Mutation of these four tyrosines completely abolished LAX phosphorylation. Upon stimulation, LAX-WT, but not LAX-4YF, associated with p85 and Grb2 upon TCR cross-linking. We also immunoprecipitated p85, Gads, and Grb2 from these lysates. The association of LAX with these proteins was detected by blotting with an anti-LAX monoclonal antibody. C, Grb2 was immunoprecipitated from Jurkat and Daudi lysates followed by blotting with an anti-LAX monoclonal antibody. WCL, whole-cell lysate; PY, phosphotyrosine.

**Fig. 6.** LAX interacts with Grb2, the p85 subunit of PI-3 kinase, and Gads upon antigen receptor stimulation. Myc-tagged wild-type LAX and mutant LAX with four mutations at Tyr-193, Tyr-268, Tyr-294, and Tyr-373 were stably expressed in Jurkat cells. LAX (A), p85, Grb2, and Gads (B) were immunoprecipitated (IP) and blotted with antibodies against LAX, p85, and Grb2 to detect specific interactions. C, Grb2 was immunoprecipitated from Jurkat and Daudi lysates followed by blotting with an anti-LAX monoclonal antibody. WCL, whole-cell lysate; PY, phosphotyrosine.

examined whether LAX interacts with other SH2 domain-containing proteins, such as PLCγ1, PLCγ2, Vav, SHP1, and SHP2. We failed to detect any significant interaction between LAX and these proteins (data not shown). Our data indicated that upon antigen receptor stimulation, LAX could interact with Grb2, Gads, and p85 and recruit these proteins to the membrane to activate downstream signaling pathways.

**LAX Functions Differently from LAT—**TCR cross-linking leads to activation of NFAT and AP-1, two critical transcription factors in TCR-induced IL-2 production. Previous studies showed that LAT is required for TCR-mediated activation of NFAT and AP-1 (9, 19). Because LAX shares some similar features as LAT, such as binding of Grb2, Gads, and p85, we next examined whether LAX could play a similar role in T cell activation as LAT. LAT-deficient Jurkat cells (ANJ3) were transiently transfected with a plasmid with LAT-WT, a mutant LAT with mutation of 10 tyrosines, or a plasmid with LAX-WT together with a luciferase reporter construct driven by a synthetic promoter containing three copies of NFAT/AP-1-binding sites and the IL-2 minimal promoter. Sixteen to twenty four hours after transfection, these transfected cells were activated with anti-CD3/OKT3 or PMA/ionomycin for 6 h and lysed. Cell lysates were used to determine luciferase activity. As shown in Fig. 7A, the defect in NFAT/AP-1 activation in LAT-deficient cells could be corrected by introducing wild-type LAT into these cells and not the LAT-10YF mutant. Transfection of these cells with LAX failed to restore NFAT/AP-1 activation, suggesting that LAX might play a different role in T cell activation.

**Overexpression of LAX Inhibits TCR-mediated T Cell Activation—**To examine the role of LAX in TCR-mediated signaling, we transiently transfected wild-type Jurkat cells with an empty plasmid, a plasmid with LAT-WT, or a plasmid with LAX-WT together with an NFAT/AP-1 luciferase reporter plasmid. As shown in Fig. 7B, overexpression of LAX-WT inhibited NFAT-mediated transcription in a dose-dependent manner. Maximal inhibition was achieved when 10 μg of plasmid was used. However, overexpression of the LAX-4YF mutant had no inhibitory effect on NFAT/AP-1 activation, suggesting that these tyrosine residues are required in LAX-mediated
inhibition. We also transfected Jurkat cells with these plasmids and an AP-1 luciferase construct. Overexpression of LAX-WT also inhibited AP-1-mediated transcription in a dose-dependent manner, and the LAT-4YF mutant failed to inhibit AP-1 activation (Fig. 7C).

To determine the biochemical basis for LAX-mediated inhibition, we transfected Jurkat cells with plasmids expressing LAX-WT and LAX-4YF to establish stable cell lines. Total lysates from these cells were analyzed by an anti-LAX Western blot. As shown in Fig. 8C, LAX-WT and LAX-4YF proteins were overexpressed in these stable cell lines in comparison with LAX in untransfected Jurkat cells. Similar amounts of protein were loaded on SDS-PAGE as indicated by an anti-PLC-1 blot (Fig. 8C). Overexpression of either WT or mutant LAX had no significant effect on TCR-mediated tyrosine phosphorylation of cellular proteins (Fig. 8A) and TCR-mediated Ca\(^{2+}\) Flux (Fig. 8B). We also determined the effect of overexpression of LAX on TCR-mediated Ca\(^{2+}\) flux by cotransfection with a plasmid expressing green fluorescent protein (GFP) in a transient transfection assay. We did not observe any difference of Ca\(^{2+}\) flux in GFP+ cells that likely overexpressed LAX protein (data not shown). These data suggested that LAX is not likely involved in TCR-mediated Ca\(^{2+}\) mobilization.

Next, we determined whether overexpression of LAX affects TCR-mediated MAPK activation. These stable transfectants and non-transfected Jurkat cells were stimulated with antibodies against TCR and CD28 for 5, 10, and 15 min or left untreated. Total lysates from these cells were resolved on SDS-PAGE and analyzed by Western blotting with antibodies against the active form of Erk, Jnk, or p38, respectively. As shown in Fig. 8C, TCR-mediated Erk and Jnk activations were not affected by overexpression of LAX-WT or LAX-4YF. However, in contrast to Erk and Jnk, TCR-mediated p38 MAPK activation was strongly suppressed in Jurkat cells overexpressing LAX-WT and not LAX-4YF. Although how LAX functions in

**Fig. 7.** Overexpression of LAX inhibits TCR-mediated NFAT/AP-1 activation. A, LAX functions differently from LAT. 5 \(\mu\)g of pNFAT-luciferase plasmid, 20 ng of Renilla-TK luciferase plasmid, and 5 \(\mu\)g of LAT-WT, LAT-10YF, or LAX-WT plasmid were used to transfect LAT-deficient Jurkat cells by electroporation. Sixteen to twenty hours after transfection, transfectants were left untreated and stimulated with OKT3 or PMA plus ionomycin for maximal activity for 6 h. Dual luciferase assay was assayed, and NFAT-luciferase activity was normalized by Renilla luciferase activity and represented as the percentage of maximal activity. B, LAX-mediated inhibition is dose-dependent. 5 \(\mu\)g of pNFAT/AP-1-Luciferase plasmid, 20 ng of Renilla-TK luciferase plasmid, and 2.5, 5, or 10 \(\mu\)g of WT-LAX plasmid, 10 \(\mu\)g of an empty vector, or 10 \(\mu\)g of LAX-4YF mutant with mutations at Tyr-193, Tyr-268, Tyr-294, and Tyr-373 were used to transfect wild-type Jurkat cells by electroporation. The assay of luciferase activity was done similarly as in A. C, AP-1-mediated transcription is inhibited by overexpression of LAX. This experiment was performed similarly as in B except that an AP-1-luciferase reporter construct was used. All experiments were performed in triplicate and are presented as the means ± S.D.

**Fig. 8.** Overexpression of LAX selectively inhibits TCR-mediated p38 MAPK activation. A, Jurkat cells and transfectants that expressed either LAX-WT or LAX-4YF were stimulated with an anti-TCR antibody (C305) for 1.5 min before lysis. Post-nuclear lysates were analyzed by Western blotting with an anti-Tyr(P) antibody. B, Ca\(^{2+}\) flux in Jurkat cells and stable transfectants that expressed either LAX-WT or LAX-4YF. C, Jurkat cells and stable transfectants were stimulated with anti-TCR plus anti-CD28 antibodies for 0, 5, 10, and 15 min. Activation of p38, JNK, and Erk MAPK was detected by Western blotting with anti-phospho-p38 MAPK, anti-phospho-JNK, and anti-phospho-Erk antibodies. The expression level of LAX was detected by blotting with an anti-LAX antibody. The same membrane was blotted with an anti-PLC-1 antibody for equal protein loading. The stable transfectant clones shown in A–C are representative of three independent clones that expressed either LAX-WT or LAX-4YF.
T cell activation remains to be determined, our data clearly indicated that in contrast to LAT, LAX functions to negatively regulate T cell activation by inhibiting TCR-mediated p38 MAPK activation. Our data also suggested that p38 MAPK activation is required for TCR-mediated AP-1/NFAT transcriptional activation in agreement with the previous finding (29, 30) that the p38 MAPK inhibitor, SB203580, inhibits the transcriptional activation of the IL-2 promoter.

**DISCUSSION**

Accumulating evidence indicates that adaptor proteins are important in antigen receptor-mediated signaling pathways. In this paper, we report identification of a novel membrane-associated adaptor molecule, LAX. LAX was exclusively expressed in lymphoid tissues. Of the several cell lines we tested, LAX was found in B, T, NK, and monocyte cell lines. In the cytoplasmic domain of LAX, there are multiple tyrosines. These tyrosines are within the Grb2- or p85-binding motifs. Upon stimulation via the TCR or BCR, LAX was tyrosine-phosphorylated and interacted with Grb2, Gads, and p85. By coexpressing LAX with Src and/or Syk tyrosine kinases, we showed that LAX could be phosphorylated by Lck, Syk, and ZAP-70. Phosphorylation of LAX was reduced in ZAP-70-deficient cells and was abolished in Lck-deficient cells. By overexpressing wild-type LAX and a mutant LAX with mutations at four critical tyrosines, we showed that overexpression of wild-type LAX inhibited p38 MAPK activation and NFAT/AP-1-mediated transcription in Jurkat cells, whereas overexpression of the mutant LAX had no effect. Our data indicated that LAX is an adaptor molecule that potentially functions to negatively regulate TCR signaling.

LAX and LAT are membrane-associated adaptor proteins. Both of them have a short extracellular domain, a transmembrane domain, and a cytoplasmic domain. Whereas LAT is expressed in T cells, NK cells, mast cells, and platelets (31), LAX is expressed in T cells, B cells, NK cells, and monocytes. We have not tested whether LAX is present in mast cells, platelets, or other cell types. Although LAT and LAX have no overall homology in amino acid sequences, the tyrosine motifs in their cytoplasmic domains are very similar (Fig. 1B). These motifs are responsible for binding the SH2 domain-containing proteins. LAX has five Grb2-binding motifs (YXXL) in its cytoplasmic tail. It also has a Gads motif (YVNV) identical to those in LAT. In addition, LAX has a p85-binding motif (YXXM), which is not present in LAT, although LAT is able to associate with p85. Upon antigen receptor stimulation, LAX also interacted with Grb2, Gads, and p85 like LAT. However, we have not been able to detect any significant interaction between LAX and PLC-γ1/2. Overexpression of LAX-WT inhibited NFAT activation, whereas overexpression of a LAX mutant with mutations at four tyrosines had no effect. It is possible that overexpression of LAX could sequester other signaling proteins from LAT and further inhibit TCR-mediated signaling. Biochemical analysis of Jurkat cells stably transfected with WT-LAX and LAX-4YF showed that overexpression of LAX had no significant effect on tyrosine phosphorylation of proteins, Ca++, Erk, or Jnk activation, suggesting that overexpression of LAX did not inhibit LAT-mediated signaling by sequestering Grb2, Gads, and p85 from LAT. Interestingly, overexpression of LAX specifically inhibited TCR-mediated p38 MAPK activation. This suggested that LAX likely functions in the pathway of p38 MAPK.

Erk, Jnk, and p38 MAPks are three subgroups of the MAPK superfamily. These three kinases are all activated following T cell activation. These MAPks phosphorylate different subsets of substrates (32). The substrates for p38 MAPK include transcription factors (Elk-1, ATF2, CHOP, MEF2C, and SAP-1) and downstream protein kinases (Mnk1, Mnk2, PRAK, MSK1, etc). It is not clear how p38 MAPK activation is coupled to TCR engagement and how p38 contributes to IL-2 production in T cells. Pretreatment of Jurkat cells with a specific p38 MAPK inhibitor, SB203580, or expression of a dominant negative form of MKK6, one of the upstream kinase of p38, can suppress the transcriptional activation of the IL-2 promoter (29). It has been shown in mice that p38 MAPK activation can modulate T cell development and is required for the activation of Th1 cells but not for activation of Th2 cells (32, 33). Our data placed LAX in the p38 pathway. However, how LAX suppresses p38 activation remains to be determined. Because the LAX mutant with mutations of four tyrosine residues failed to inhibit p38 activation, the interaction between LAX with Grb2, Gads, or p85 might be required for LAX function. It is likely that LAX might recruit a negative regulator, such as phosphatase, to the membrane to turn off p38 MAPK activation. However, we have not been able to detect any interaction between LAX and phosphatases.

Previous studies (9, 19) showed that LAT is critical in T cell activation. LAT-deficient cells are defective in TCR-mediated Ras-MAPK activation and Ca+2 flux. Our data here suggested that LAX functions differently from LAT as follows. 1) In contrast to LAT, LAX is not localized in lipid rafts. 2) Defective signaling in LAT-deficient Jurkat cells could not be rescued by expression of LAX. 3) LAX does not interact with PLC-γ1 or -γ2. Thus, LAX is less likely to function in linking receptor engagement to Ca++ flux.

Signaling via the BCR shares many similar features as signaling via the TCR. BLNK associates with Grb2, Vav, PLC-γ1, and Nck in B cells similar to LAT and SLP-76 in T cells. BLNK is not constitutively localized in the membrane, which is different from LAT. Therefore, there might exist a LAT-like molecule in B cells. This molecule functions to recruit BLNK and its associated proteins to the membrane. As opposed to LAT, LAX is expressed in B cells. Upon activation via the BCR, LAX became tyrosine-phosphorylated. We have attempted to perform similar experiments to determine whether overexpression of LAX affects BCR-mediated NFAT activation. We failed to obtain any conclusive results due to a low efficiency of transfection with these B cells. The function of LAX in B cells needs to be further studied. Because LAX is not localized in lipid rafts and does not associate with PLC-γ1/2, it is less likely that LAX functions like LAT in B cells.

In summary, we identified a novel membrane-associated adaptor protein, and we showed that it functions to negatively regulate TCR signaling. This inhibitory signal delivered by LAX may be critical for terminating IL-2 production in the late stage of immune responses. Interestingly, consistent with this notion, the amount of LAX protein extracted by Brij detergent was increased dramatically upon stimulation of Jurkat cells with anti-TCR antibody or PMA. Increased LAX protein at the membrane might bring more inhibitory molecules to the membrane to turn off a T cell response. The mechanism by which LAX inhibited p38 MAPK and NFAT/AP-1 transcriptional activation remains to be determined. The precise function of LAX in lymphocyte signaling and immune response will be revealed by analysis of LAX-deficient mice.

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