Regulation of Lck and Fyn Tyrosine Kinase Activities by Transmembrane Protein Tyrosine Phosphatase Leukocyte Common Antigen-Related Molecule

Kazutake Tsujikawa,[1] Tomoko Ichijo,[1] Kazuki Moriyama,[1] Noriko Tadotsu,[1] Kazuhiro Sakamoto,[1] Naoki Sakane,[1] So-ichiro Fukada,[1] Tatsuhiko Furukawa,[2] Haruo Saito,[3] and Hiroshi Yamamoto[1]

[1]Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Yamadaoka, Suita, Osaka, Japan; [2]Faculty of Medicine, Department of Cancer Chemotherapy, Institute for Cancer Research, Kagoshima University, Sakuragaoka, Kagoshima, Japan; and [3]Dana-Farber Cancer Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

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Abstract

Leukocyte common antigen-related molecule (LAR) is a receptor-like protein tyrosine phosphatase (PTPase) with two PTPase domains. In the present study, we detected the expression of LAR in the brain, kidney, and thymus of mice using anti-LAR PTPase domain subunit monoclonal antibody (mAb) YU1. In the thymus, LAR was expressed on $CD4^-CD8^-$ and $CD4^-CD8^{low}$ thymocytes. The development of thymocytes in CD45 knockout mice is blocked partially in the maturation of $CD4^-CD8^-$ to $CD4^+CD8^+$. We postulated that LAR regulates Lck and Fyn in the immature thymocytes. Transfection of wild-type LAR activated extracellular signal-regulated kinase signal transduction pathway in CD45-deficient Jurkat cells stimulated with anti-CD3 mAb. LAR mutants, with Cys to Ser mutation in the catalytic center of PTPase D1, bound to tyrosine-phosphorylated Lck and Fyn, and LAR PTPase domain 2 was tyrosine phosphorylated by Fyn tyrosine kinase. The phosphorylated LAR was associated with Fyn Src homology 2 domain. Moreover, LAR dephosphorylated phosphorylated tyrosine residues in both the COOH terminus and kinase domain of Fyn *in vitro*. Our results indicate that Lck and Fyn would be substrates of LAR in immature thymocytes and that each LAR PTPase domain plays distinct functional roles in phosphorylation and dephosphorylation.

Introduction

Protein tyrosine kinases (PTKs) transduce cellular signaling by phosphorylating the tyrosine residues in their substrates. On the other hand, protein tyrosine phosphatases (PTPases) are involved in the reverse reaction, dephosphorylation of phosphorylated tyrosine residues. Orderly and tightly regulated activation of PTKs and PTPases is necessary for normal signal transduction in development, proliferation, differentiation, and functions of cells (1, 2). For example, PTKs and PTPases play key functions in thymocyte development (3, 4). Thymocytes develop from $CD4^-CD8^-$ (DN) to $CD4^+CD8^+$ (DP) cells, followed by maturation to $CD4^+CD8^-$ and $CD4^-CD8^+$ (SP) cells. The initial signaling of each development stage is triggered by tyrosine phosphorylation of immunotyrosine activating motif (ITAM) in CD3 and TCRζ (5, 6), leading to recognition of antigen/MHC through pre-T-cell receptors (pre-TCR) or T-cell receptors (TCR). The tyrosine phosphorylation of ITAM is induced by the Src family PTKs, Lck and Fyn (7, 8). Analyses of thymic development in Lck knockout mice (9) and dominant-negative Lck transgenic mice (10, 11) indicate defective development of DN to DP thymocytes. Moreover, DN-to-DP transition in Lck/Fyn double knockout mice is more severely affected than that in Lck knockout mice (12), supporting the critical role of these kinases in the development of immature thymocytes. Activation of Lck and Fyn involves tyrosine dephosphorylation of the COOH-terminal regulatory domain of kinases, followed by autophosphorylation of the kinase domain. A PTPase that catalyzes the tyrosine dephosphorylation of kinases is CD45 (13).

Mammalian PTPases that have been cloned to date are structurally divided into two subgroups: intracellular PTPases and receptor-like PTPases (1, 2). PTPases are characterized by PTPase domain composed of ∼250 amino acids with a conserved signature motif ([I/V]HCXAGXXR[S/T]G) around the active site. All known intracellular PTPases have one PTPase domain. Many of them, however, also have accessory domains such as the Src homology 2 (SH2) domain, the ezrin-like domain, or the PEST domain. These accessory domains may regulate the activity, substrate specificity, or subcellular localization of the intracellular PTPases. In contrast, most receptor-like PTPases possess two tandemly repeated PTPase domains in the cytoplasm: The membrane-proximal and membrane-distal PTPase domains are called PTPase D1 (domain 1) and PTPase D2 (domain 2), respectively. The cysteine residue in the signature motif of PTPase D1 is essential for the PTPase activity, whereas PTPase D2 has no or less catalytic activity. Instead, the PTPase D2 is thought to provide the substrate specificity (14, 15). On the other hand, the extracellular domains of receptor-like PTPases are composed of various

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Requests for reprints: Kazutake Tsujikawa, Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8192; Fax: 81-6-6879-8194. E-mail: tujikawa@phs.osaka-u.ac.jp
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combinations of functional motifs such as the immunoglobulin-like (Ig) repeat, the fibronectin type III (FnIII) repeat, and the carbonic anhydrase-like domain, suggesting that the extracellular domain may modulate PTPase activity by binding to specific ligands (1, 2). CD45 and leukocyte common antigen-related molecule (LAR) are prototypes of the receptor-like PTPases.

LAR cDNA was originally cloned from human tonsil lymphocyte cDNA library by using mouse CD45 cDNA as a probe (16). LAR consists of noncovalently bound subunits, designated the E (extracellular) and P (phosphatase) subunits, generated by proteolytic cleavage of a single precursor protein between the eighth FnIII domain and transmembrane segment (17). The M_r 150,000 E-subunit is composed of three immunoglobulin-like domains and eight FnIII domains, whereas the M_r 85,000 P-subunit has a short extracellular domain, a transmembrane domain, and two tandemly repeated PTPase domains in the cytoplasm. The LAR mRNA was detected in a variety of cells, including T-cell lines, kidney cell lines, and prostate cell lines. Establishment of antihuman LAR E-subunit monoclonal antibodies (mAbs) and their use in immunohistochemical studies have confirmed that human LAR is expressed in various cell lineages such as epithelial cells, smooth muscle cells, and cardiac myocytes, but not in the spleen, brain, or neurons (17). Subsequently, mouse and rat LAR cDNAs were cloned and Northern blot and reverse transcription-PCR analyses identified the distribution of mRNA not only in the organs in which human LAR mRNA was detected, but also in the thymus, brain, and neurons (18–23). Further studies showed that LAR extracellular region is released from the cell surface by proteolytic cleavage (24) and the exons encoding extracellular FN-III domains of LAR are alternatively spliced to form various isoforms (25). These results indicate that LAR or shed LAR could play an important role in signal transduction of these tissues. In fact, various disorder phenotypes are recognized in LAR-deficient mice: reduction of the size of basal forebrain cholinergic neurons and decrease in cholinergic innervation of the dentate gyrus (26, 27); differentiation of mammary gland epithelial cells (28); regulation of regenerative neurite outgrowth (29); and low plasma insulin and glucose levels and reduced rate of hepatic glucose production (30). Moreover, insulin receptor (31, 32), EGF receptor, HGF receptor (33), and RET (34) have been reported as substrates of LAR.

In the present study, we examined the expression of LAR using YU1, an anti-LAR P-subunit mAb, as well as the functions of its two PTPase domains *in vivo*. The expression of LAR was detected not only in the brain and kidney but also in the thymus. In thymocytes, LAR was detected on parts of $CD4^-CD8^-$ cells and $CD4^-CD8^{low}$ cells. LAR transduced the signaling through TCR downstream by regulation of Lck and/or Fyn tyrosine kinase activities. In the transient transfection assay, we confirmed that LAR dephosphorylated the phosphorylated tyrosine residues of Lck and Fyn, and tyrosine residue(s) in LAR PTPase D2 was phosphorylated by Fyn to supply Fyn SH2 binding site. Our results indicate that LAR PTPases D1 and D2 are essential for the expression of PTPase activity and the recognition of Fyn and Lck, respectively.

Results

Expression of LAR in Mouse Tissues

We first examined the expression of LAR protein in various mouse tissues using YU1 mAb, which was recently raised in our laboratory. This mAb was produced by immunizing the bacterially expressed glutathione *S*-transferase (GST)-LAR intracellular domain fusion proteins (35). We confirmed that YU1 recognized LAR but not CD45, SH-PTP1, or SH-PTP2 and that its epitope region was located between downstream of transmembrane domain and upstream of PTPase D1 of LAR. By using this mAb, it was expected that more precise analysis of LAR expression in the tissues became possible irrespective of the shedding or alternative splicing of the LAR E-subunit. Aliquots (0.2 mg protein) of lysates of body organs of a 7-week-old mouse were resolved on SDS-PAGE and immunoblotted with YU1 mAb. As shown in Fig. 1, YU1 detected an approximately M_r 85,000 protein in the brain, and approximately M_r 82,000 proteins in the thymus and kidney. Moreover, approximately M_r 82,000 proteins were also detected in the lung and liver by long exposure (data not shown). The immunoblot with anti-β-tubulin antibody was performed to control for protein loading.

The intracellular domain of LAR P-subunit is alternatively spliced between the transmembrane region and the PTPase D1 in humans (36) and rats (19). We, therefore, postulated that the difference in molecular masses of the bands detected with YU1 in the thymus and brain is derived from alternative splicing of LAR P-subunit. To confirm this, reverse transcription-PCR of mouse brain and thymus cDNAs was performed using a specific LAR primer pair located in exons 20 and 22. Sequence analysis indicated that the 33-bp nucleotides (LASE-b) (19) in the main product of the brain were missing in the PCR product

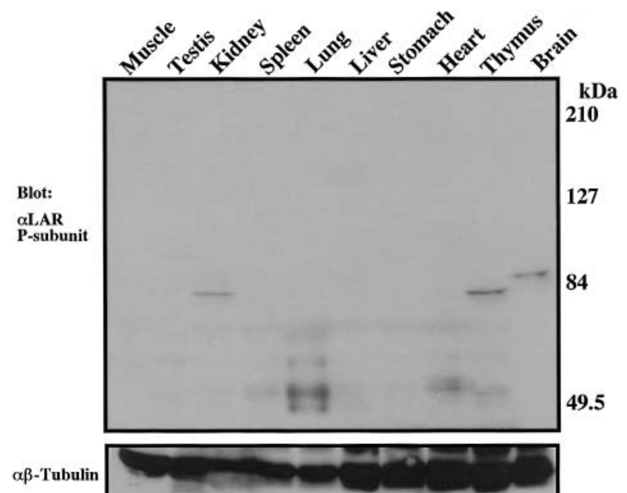

FIGURE 1. Immunoblot analysis of mouse tissues with anti-LAR P-subunit mAb YU1. Aliquots (0.2 mg protein) of mouse tissue homogenates were resolved on 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immunoblotted with anti-LAR P-subunit mAb YU1.

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of the thymus (data not shown). These results indicate that LAR is mainly expressed in the brain as well as in the thymus and could play an important physiological function in these organs.

Rescue of TCR Signal Transduction by LAR PTPase Activity in CD45-Deficient Jurkat Cells

Recently, Terszowski *et al*. (37) reported that LAR expresses on human $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes using an anti-LAR E-subunit mAb. We also detected the expression of LAR in mouse immature $CD4^-CD8^-$ and $CD4^-CD8^{low}$ thymocytes by using YU1 (data not shown). We, therefore, postulated that LAR could regulate signal transduction by dephosphorylation of tyrosine-phosphorylated protein(s) during the development stage of immature $CD4^-CD8^-$ to $CD4^+CD8^+$ thymocytes.

To clarify the physiological function as well as the roles of two PTPase domains of LAR in T cells, we constructed various expression vectors of LAR mutants with deletion of PTPase domain(s) and/or Cys to Ser mutation in the active center of each PTPase domain as follows: LAR D1CSD2 (Cys-1522 to Ser mutation in PTPase D1), LAR D1D2CS (Cys-1813 to Ser mutation in PTPase D2), LAR DCS (both Cys-1522 in PTPase D1 and Cys-1813 in PTPase D2 to Ser mutation), LAR ΔD1D2 and LAR D1ΔD2 (mutants lacking PTPase D1 and PTPase D2, respectively), and their Cys to Ser mutants (LAR ΔD1D2CS and LAR D1CSΔD2) (Fig. 2A). In an *in vitro* PTPase assay using *p*-nitrophenylphosphate as a substrate, LAR WT, D1D2CS, and D1ΔD2 expressed phosphatase activity, but the other mutants showed no enzymatic activity (data not shown). The PTPase D1 of these three LAR constructs is wild type, indicating that the PTPase activity of LAR is derived from PTPase domain 1 but not domain 2, at least *in vitro*.

CD45 is a key molecule in TCR-mediated signal transduction, which up-regulates tyrosine kinase activities of Lck and Fyn by tyrosine dephosphorylation. We hypothesized that LAR expressed on immature thymocytes could also regulate the tyrosine kinase activity by tyrosine dephosphorylation of Lck and Fyn as its substrates. To evaluate this hypothesis, LAR or its mutants were transfected into CD45-deficient Jurkat cells concomitant with both pFA2-Elk1 and pFR-Luc. If LAR up-regulates the tyrosine kinase activity of Lck and Fyn like CD45, anti-CD3 mAb stimulation should increase Elk transcription activity through extracellular signal-regulated kinase (ERK) pathway in this cell line, resulting in enhancement of Luc activity. CD45-deficient Jurkat cells transfected with LAR WT or its mutants were stimulated with plate-bound anti-CD3 mAb OKT3 for 24 h and the relative Luc activities of the lysates were determined. The activity was normalized using an internal control vector pRL-TK and expressed as relative Luc activity. As shown in Fig. 2B, the signal transduction to ERK pathway through CD3 in CD45-deficient Jurkat cells was restored by transfection of CD45 as well as LAR WT. Interestingly, LAR D1ΔD2, a mutant with wild-type PTPase D1 but without PTPase D2, expressed approximately 50% activity of LAR WT. In contrast, the other LAR mutants (LAR DCS and ΔD1D2)

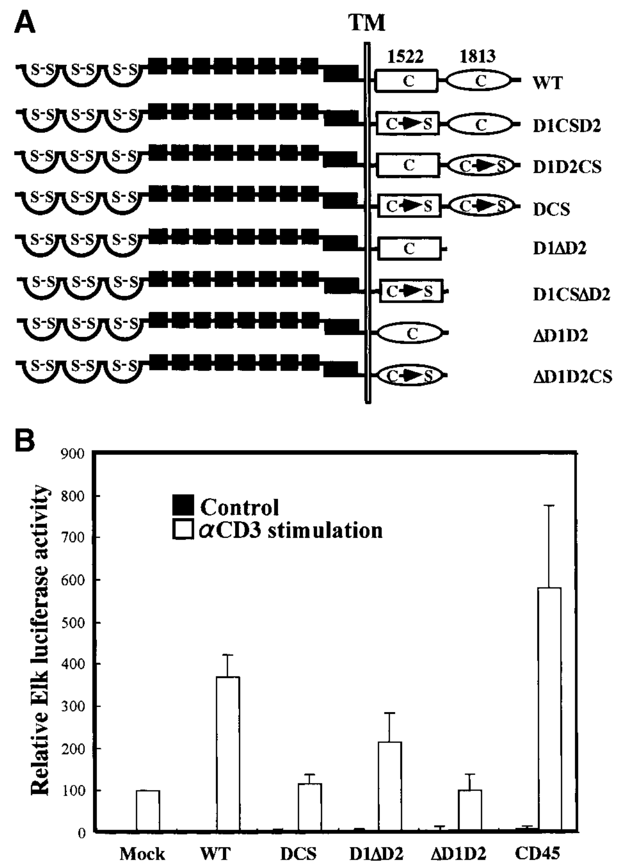

FIGURE 2. **A.** Schematic representation of LAR constructions. Wild-type LAR (*WT*) and its various mutants (*D1CSD2*, *D1D2CS*, *DCS*, *D1ΔD2*, *D1CSΔD2*, *ΔD1D2*, and *ΔD1D2CS*) were constructed and ligated into pSP65-SRα2 expression vectors. *TM,* transmembrane domain; *D1,* NH_2-terminal PTPase domain; *D2,* COOH-terminal PTPase domain; *C→S,* a point mutation of cysteine to serine in each PTPase domain. Numbers above LAR WT construct represent positions of the cysteine residue in each PTPase signature motif. **B.** LAR rescues ERK signal transduction via CD3 in CD45-deficient Jurkat cells. CD45-deficient Jurkat cells were transfected with indicated LAR expression vectors with ERK *trans*-reporter plasmids and pRL-TK by electroporation at 250 V and 960 μF capacitance. After 24 h of transfection, 10^6 cells were stimulated for 16 h with plate-bound anti-CD3 mAb OKT3 (3 μg/ml) and then lysed in lysis buffer. Luciferase (*Luc*) activity in cell lysates was measured by a luminometer using a dual-luciferase reporter assay system. *Columns,* means of three independent experiments performed in triplicate; *bars,* SE. Data are shown as a percentage of Luc activity induced by anti-CD3 stimulation in cells transfected with the empty vector.

showed no activity. These results resembled those noted in the *in vitro* phosphatase assay using *p*-nitrophenylphosphate as described above, indicating that LAR PTPase D1 has enzymatic activity, the cysteine residue in PTPase D1 is necessary for the catalytic activity, and both PTPase domains 1 and 2 are essential for the expression of the full activity of LAR PTPase *in vivo*.

LAR Is Associated with and Tyrosine Phosphorylated by Fyn and Lck

To clarify whether Fyn and Lck are substrates of LAR, we examined the association between LAR and Fyn or Lck in a

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transient transfection assay. For this purpose, we used a LAR D1CSD2 expression vector, because the mutant was expected to have stable binding affinity for its substrate due to the lack of enzymatic activity (38). NP40 lysates were prepared from COS-7 cells transfected with LAR D1CSD2, together with FynYF or LckYF, and immunoprecipitated with anti-LAR E-subunit mAbs. Immunoblot analysis with anti-phosphotyrosine mAb 4G10 indicated that tyrosine-phosphorylated proteins were coimmunoprecipitated with LAR D1CSD2 (Fig. 3A). The bands detected by reprobing with anti-Fyn and anti-Lck Abs corresponded precisely to tyrosine-phosphorylated M_r 59,000 and 56,000 proteins, respectively, indicated as Src family. Interestingly, the M_r 85,000 tyrosine-phosphorylated bands were detected in both the immunoprecipitates from lysates of cotransfectants of LAR and Fyn or Lck. The bands detected by reprobing with anti-LAR P-subunit mAb YU1 corresponded precisely to the tyrosine-phosphorylated M_r 85,000 proteins (Fig. 3B). To examine that LAR was tyrosine phosphorylated by Fyn tyrosine kinase activity, we cotransfected LAR D1CSD2 with FynWT, FynYF, or FynKM into COS-7 cells. The lysates were immunoprecipitated with anti-LAR E-subunit mAbs and then immunoblotted with anti-phosphotyrosine 4G10. Tyrosine-phosphorylated LAR was clearly detected in cotransfectants with FynWT and FynYF, but not FynKM (data not shown). Moreover, we also confirmed that bacterially expressed GST-LAR was tyrosine phosphorylated by immunoprecipitates with anti-Fyn mAb from the lysates of COS-7 cells transfected with FynWT and FynYF, but not FynKM in an *in vitro* kinase assay (data not shown). These results indicate that LAR P-subunit is a substrate of Fyn and Lck tyrosine kinases.

Tyrosine Dephosphorylation of Fyn by LAR in Vitro

The expression vectors of Lck and Fyn used in the experiments described above were constitutively active mutants in which the COOH-terminal tyrosine residue of each kinase was changed to phenylalanine. Next, we examined whether LAR could dephosphorylate phosphotyrosine residues not only in the tyrosine kinase domains, but also in COOH-terminal regulatory sites of the kinases in an *in vitro* PTPase assay. In the following experiments, we used Fyn as a target, because Fyn is known to be expressed in both thymocytes and the brain like LAR (39). Like the other Src kinase family molecules, Fyn contains two tyrosine phosphorylation sites, these being the autophosphorylation site (Tyr-419) in the kinase domain and the negative regulating tyrosine phosphorylation site (Tyr-530) within the COOH-terminal tail. We examined whether LAR could dephosphorylate these two phosphorylated tyrosine residues in Fyn. GST-LAR WT, GST-LAR D1CSD2, and GST were bacterially expressed and purified by reduced glutathione (GSH)-Sepharose beads. The expression vectors of FynWT, FynYF, or FynKM were transfected in COS-7 cells and the cell lysates were immunoprecipitated with anti-Fyn mAb. The immunoprecipitates were incubated with the purified GST-LAR fusion proteins in PTPase assay buffer. FynWT has two tyrosine residues that receive phosphorylation in both the kinase domain and the COOH-terminal tail. FynKM is a kinase-inactive mutant in which Lys-299 at the ATP binding site is mutated to Met, resulting in phosphorylation of only the COOH-terminal tyrosine by Csk. On the other hand, because tyrosine 531 in Fyn is mutated to phenylalanine in FynYF, phosphorylation is induced in only the tyrosine residue in the kinase domain. After 1 h incubation with GST-LAR fusion proteins, the immunoprecipitates were resolved on SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb 4G10 (αpTyr). As shown in Fig. 4, GST-LAR WT markedly reduced the tyrosine phosphorylation levels of FynWT as well as FynYF and FynKM, but not GST-LAR D1CSD2 or GST. In these experiments, expression levels of Fyn were almost identical (αFyn). These results indicate that LAR could dephosphorylate the phosphorylated tyrosine residues not only in the COOH-terminal domain but also in the kinase domain of Fyn by the catalytic activity of the PTPase D1.

LAR D1 Dephosphorylates Tyrosine-Phosphorylated Fyn and LAR D2 Is Phosphorylated by Fyn

Next, we further examined the functions of two PTPase domains of LAR on tyrosine dephosphorylation of Fyn. Various forms of LAR D1 and/or D2 mutants were cotransfected with FynYF in COS-7 cells and the lysates were immunoprecipitated with anti-LAR E-subunit mAbs. The immunoprecipitates were resolved on SDS-PAGE, and then immunoblotted with anti-phosphotyrosine mAb 4G10. As shown in Fig. 5A, tyrosine-phosphorylated Fyn were efficiently coimmunoprecipitated with LAR D1CSD2 and DCS, but weakly with LAR WT and D1D2CS, even though the levels of immunoprecipitated LAR detected with YU1 (Fig. 5B) and the expression levels of tyrosine-phosphorylated Fyn (Fig. 5C) in these transfected cells were very similar. Moreover, the clear M_r 85,000 tyrosine-

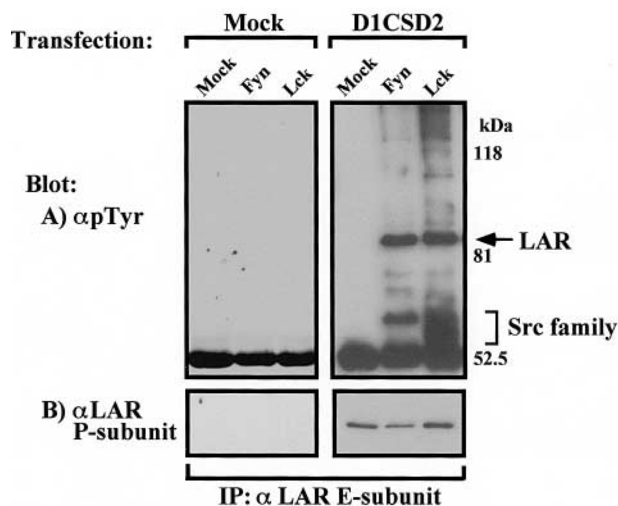

FIGURE 3. LAR D1CSD2 is associated with and phosphorylated by Lck and Fyn. COS-7 cells were cotransfected with 5 μg of pSP65-SRα2-LARD1CSD2 and 1 μg of either pME18S vector (*Mock*), pME18S-FynYF (*Fyn*), or pME18S-LckYF (*Lck*), using the DEAE-dextran method. After incubation for 48 h, cell lysates were prepared and immunoprecipitated (*IP*) with anti-LAR E-subunit mAbs (α*LAR E-subunit*), separated on 12% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was immunoblotted (*Blot*) with antiphosphotyrosine mAb 4G10 (α*pTyr*) (**A**). The filter was stripped and reprobed with anti-LAR P-subunit mAb YU1 (α*LAR P-subunit*) (**B**).

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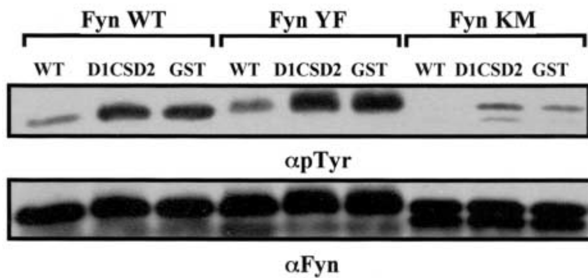

FIGURE 4. Tyrosine dephosphorylation of Fyn by LAR *in vitro*. Immunoprecipitates with anti-Fyn mAb from COS-7 cells transfected with pME18S-FynWT, pME18S-FynYF, or pME18S-FynKM were incubated for 1 h at 37°C with 5 μg of GST-LAR WT (*WT*), GST-LAR D1CSD2 (*D1CSD2*), and GST in PTPase assay buffer. The immunoprecipitates were then washed with TBS-T, separated on 10% SDS-PAGE, and immunoblotted with antiphosphotyrosine mAb 4G10 (α*pTyr*) and anti-Fyn mAb (α*Fyn*).

phosphorylated bands were also detected in transfectants with LAR D1CSD2 and DCS (indicated as D1D2 in Fig. 5, A and B). The bands detected by reprobing with YU1 corresponded precisely to tyrosine-phosphorylated M_r 85,000 proteins (Fig. 5B). These results indicate that the LAR PTPase D1 mainly functions for dephosphorylation of tyrosine-phosphorylated Fyn and LAR. We performed further transfection assays using LAR PTPase domain deletion mutants and their Cys to Ser mutants. A clear association between LAR and tyrosine-phosphorylated Fyn was detected in LAR D1CSΔD2, ΔD1D2, and ΔD1D2CS, but not in LAR D1ΔD2, which has a wild-type PTPase D1 (Fig. 5D). Moreover, M_r 50,000 tyrosine-phosphorylated bands were detected in LAR ΔD1D2 and ΔD1D2CS transfectants (indicated as D2 in Fig. 5D), but not in LAR D1ΔD2 or D1CSΔD2. Reprobing the filter with anti-LAR P-subunit mAb YU1 indicated that these tyrosine-phosphorylated bands were LAR PTPase D2 (indicated as D2 in Fig. 5E). The expression levels of tyrosine-phosphorylated Fyn in these transfected cells were again very similar (Fig. 5F). These results suggest that tyrosine-phosphorylated Fyn is recognized by LAR PTPase D1 and then dephosphorylated; activated Fyn tyrosine kinase phosphorylates tyrosine residue(s) in LAR PTPase D2.

GST-LAR Pull-Down Assay

Src family tyrosine kinases contain the unique domain, the Src homology 3 (SH3) domain, the SH2 domain, and the kinase domain in their molecule (8). To determine the domain of Fyn that is related to the association with LAR, bacterially expressed GST-Fyn unique, GST-Fyn SH2, and GST-Fyn SH3 fusion proteins were purified by GSH-Sepharose beads (Fig. 6A), incubated with lysates of COS-7 cells transfected with LAR DCS or with both LAR DCS and Fyn YF, and immunoblotted with anti-LAR E-subunit mAbs. As shown in Fig. 6B, the LAR E-subunit was detected only when GST-Fyn SH2 fusion proteins were incubated with lysates of cells transfected with both LAR DCS and Fyn YF. In contrast, incubation with lysates from transfectants of LAR DCS alone did not provide obvious association of LAR with Fyn SH2 domain. LAR PTPase D2 was tyrosine phosphorylated by FynYF (Fig. 5) and the SH2 domain is known to recognize a phosphorylated tyrosine residue. Therefore, Fyn SH2 domain would be essential for the association with tyrosine-phosphorylated LAR PTPase D2. In this experiment, two bands were detected in immunoblots with anti-LAR E-subunit antibodies. The lower band was of almost the same molecular mass as the E-subunit of LAR applied as a positive control (LAR WT), whereas the upper one was of a little larger molecular weight. The precise reason is unknown, but it could be due to modification or aggregation of LAR E-subunit.

Discussion

In the present study, we have clarified the abundant expression of LAR in the kidney, brain, and thymus of mice using anti-LAR P-subunit mAb. Moreover, it was confirmed that LAR was expressed in immature $CD4^-CD8^-$ and $CD4^-CD8^{low}$ populations in thymocytes. During the early development of thymocytes, immature $CD4^-CD8^-$ DN thymocytes initially express CD8 on the cell surface, progressively acquire the expression of CD4, and then become $CD4^+CD8^+$ DP thymocytes. Signaling via pre-TCR/CD3 complex expressed during these cell development stages activates DN-to-DP transition by regulating the Lck and Fyn tyrosine kinase activity (7, 8). A critical molecule in the regulation of these kinases in T cells is CD45 PTPase. CD45 can activate Lck and Fyn by dephosphorylating a phosphorylated tyrosine residue in their COOH-terminal regulatory domain (40). Recently, CD45 knockout mice were generated and analysis of thymocytes indicated a severe disruption of the transition from $CD4^+8^+$ DP to $CD4^+CD8^-$ or $CD4^-CD8^+$ SP thymocytes in these mice, but only a partial disruption of the

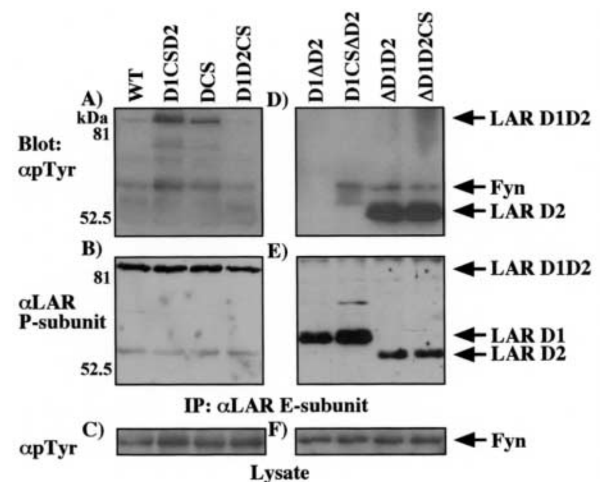

FIGURE 5. Roles of LAR PTPase D1 and D2 in Fyn recognition. pSP65-SRα2-LAR expression vectors (*WT*, *D1CSD2*, *DCS*, *D1D2CS*, *D1ΔD2*, *D1CSΔD2*, *ΔD1D2*, and *ΔD1D2CS*) were cotransfected with pME18S-FynYF to COS-7 cells as described in the figure. After incubation for 48 h, cell lysates were immunoprecipitated (*IP*) with anti-LAR E-subunit mAbs (α*LAR E-subunit*), and immunoblotted with antiphosphotyrosine mAb 4G10 (**A** and **D**). The filter was stripped and reprobed with anti-LAR P-subunit mAb YU1 (**B** and **E**). Aliquots of cell lysates were immunoblotted with antiphosphotyrosine mAb 4G10 for detecting tyrosine-phosphorylated Fyn (**C** and **F**).

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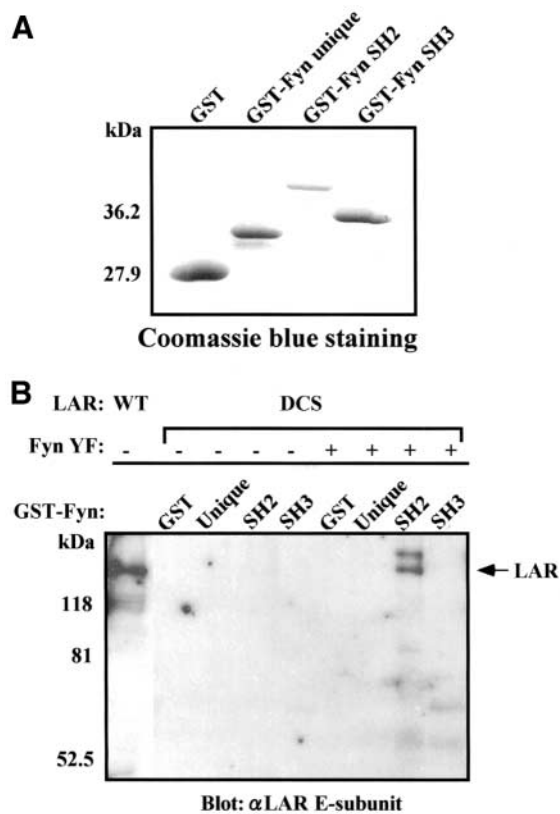

FIGURE 6. Association of Fyn SH2 domain with tyrosine-phosphorylated LAR. GST pull-down assay was performed with recombinant GST, GST-Fyn unique domain (*Unique*), GST-Fyn SH2 domain (*SH2*), or GST-Fyn SH3 domain (*SH3*) fusion proteins, and LAR DCS expressed with and without FynYF in COS-7 cells. **A.** Coomassie blue staining of the input GST fusion proteins. **B.** Immunoblotting (*Blot*) with anti-LAR E-subunit mAbs (α*LAR E-subunit*).

transition from $CD4^-8^-$ DN to $CD4^+8^+$ DP thymocytes (41, 42). On the other hand, DN-to-DP transition in Lck/Fyn double knockout mice is almost completely blocked (12). These results suggest that there exist other PTPase(s) that could regulate Lck and Fyn tyrosine kinase activities in $CD4^-CD8^-$ DN thymocytes. Therefore, we postulated that LAR might regulate Lck and/or Fyn in the thymocyte population. In CD45-deficient Jurkat cells, LAR transfection could rescue the TCR signal transduction by anti-CD3 mAb stimulation. Moreover, LAR dephosphorylated phosphorylated tyrosine residues of the COOH terminus as well as in the kinase domain of Fyn *in vitro*, indicating that LAR expressed on immature thymocytes could be a regulator of Src-family kinase.

LAR could also function as an adhesion molecule in thymocyte development. The extracellular domain of LAR is closely similar to the neural cell adhesion molecule (N-CAM) family of cell-adhesion molecules (16). The thymus can be regarded as a mesh of epithelial cells surrounding maturing T cells; thus, binding of adhesion molecules such as integrins displayed on thymocytes to laminin, one of the extracellular matrix components, strongly influences T-cell differentiation/ maturation (43, 44). Recently, the extracellular matrix component laminin-nidogen complex was reported to be a ligand for LAR extracellular domain (36). Therefore, adhesion of immature thymocytes to thymic stromal cells via LAR extracellular domain might be involved in their differentiation and/or proliferation mechanisms.

LAR has two tandemly repeated PTPase domains in the cytoplasm, although the precise function and the regulatory mechanism of each PTPase domain have not been fully clarified. Previous studies indicated that the membrane-proximal PTPase D1 of LAR has catalytic activity by itself, because the cysteine to serine-substituted mutation at the catalytic center of PTPase D1 completely eliminated the PTPase activity *in vitro* (15). In contrast, the membrane-distal PTPase D2 has no detectable enzymatic activity, but plays a role in recognition of substrates. In our study, PTPase D2-deficient LAR exhibited only half activity of full-length wild-type LAR in downstream signal transduction of CD45-deficient Jurkat cells stimulated with anti-CD3 mAb. Moreover, LAR DCS and LAR ΔD1D2, which show no PTPase activity *in vitro*, did not transduce the signaling of CD3 downstream. These results strongly support the suggestion that LAR PTPase D1 has enzymatic activity and that PTPase D2 is essential for full activation of PTPase D1 *in vivo*.

In the transient expression system of COS-7 cells using various LAR mutants, the tyrosine-phosphorylated Fyn was coprecipitated with LAR mutants except LAR WT, D1D2CS, and D1ΔD2. Because PTPase D1 in these three LAR constructions is wild type, they would not be able to trap Fyn by rapid dephosphorylation *in vivo*. In contrast, the association of tyrosine-phosphorylated Fyn with LAR D1CSD2, DCS, and D1CSΔD2 would result in the substrate trapping mechanism, indicating that Cys-1522, which is located at the catalytic center of the PTPase D1, is critical for its catalytic activity *in vitro* as well as *in vivo*. The association mechanism of Fyn with LAR ΔD1D2 and ΔD1D2CS might be different from that with the substrate trapping mutants. We previously reported that the association of insulin receptor to LAR WT and LAR D1CSD2, which have no mutation in PTPase D2, is significantly weakened by the introduction of Cys-1813 to Ser-substituted mutation in LAR PTPase D2 (LAR D1D2CS and DCS) (35). These results suggest that signature motif of LAR PTPase D2 is essential for substrate recognition. In the present study, however, LAR D1CSD2, DCS, ΔD1D2, and ΔD1D2CS were associated with phosphorylated Fyn. In addition, these mutants were tyrosine phosphorylated by Fyn tyrosine kinase activity. Moreover, tyrosine-phosphorylated LAR could bind Fyn SH2 domain, indicating that the function of LAR PTPase D2 could be the supply of a binding site to SH2 domain of the Src family kinases by tyrosine phosphorylation.

Tyrosine phosphorylation of PTPases by PTKs has already been reported in some PTPases. PTP1C (45) and PTP 1B (46) are tyrosine phosphorylated by the insulin receptor tyrosine kinase. The PTPase activity of PTP1C is enhanced by tyrosine phosphorylation, and the phosphorylation of PTPase 1B is involved in the interaction with insulin receptor. SHP-1 (47) and SHP-2 (48) are phosphorylated by Src-family tyrosine kinases, and Fyn binds directly to the phosphorylated SHP-2 via its SH2 domain. CD45 is also phosphorylated by Csk tyrosine kinase (49). Phosphorylation causes a marked increase in CD45 PTPase activity and binding to Lck via its SH2 domain. Tyrosine phosphorylation sites in LAR PTPase D2 are

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not clear; however, the phosphorylation is at least necessary for the association with Fyn via its SH2 domain. LAR PTPase D2 contains several potential tyrosine phosphorylation sites. Of these, Y^{1835}EGV is a potential binding site for the SH2 domains of Fyn and Lck (50). Further studies using tyrosine to phenylalanine-substituted mutants of the phosphorylation sites are necessary for elucidation of the precise SH2 binding site in LAR molecule.

Recently, Wills *et al*. (51) reported the molecular mechanisms of involvement of *Drosophila* LAR (Dlar) in the control of motor axon guidance. Dlar PTPase D2 is involved in the direct binding of *Drosophila Abl tyrosine kinase* and is tyrosine-phosphorylated by the kinase. The Abl substrate Enabled (Ena), which is required for choice point navigation, also binds Dlar PTPase D2 and is then tyrosine phosphorylated by Abl. The phosphorylated Abl and Ena seem to be tyrosine dephosphorylated by the catalytic activity of Dlar PTPase D1. These regulation mechanisms are similar to those of the Src family kinases by each PTPase domain of mammalian LAR clarified in the present study.

In conclusion, we have demonstrated for the first time that LAR dephosphorylates phosphotyrosine residues of Lck and Fyn; regulation of the Src family kinase activity by LAR transduces CD3 signaling downstream; LAR PTPase D2 is a target of Fyn and Lck tyrosine kinases and its tyrosine phosphorylation leads to the recognition by Fyn SH2 domain. However, the most straightforward examination to clarify the function of LAR in the thymocyte development will be a genetic cross of LAR-mutant mice with CD45-deficient mice. We recently developed LAR D1CSD2 and LAR DCS transgenic mice. These mice should allow us to explore the above missing issues and provide detailed information about the regulation of Lck and Fyn by LAR as well as the functions of two LAR PTPase domains *in vivo*.

Materials and Methods

Plasmid Constructions

The LAR expression vectors used in the transient transfection assay were constructed by inserting full-length LAR (WT), its PTPase domain deletion mutants (ΔD1D2 and D1ΔD2), and its Cys to Ser mutants (D1CS, D2CS, DCS, ΔD1D2CS, and D1CSΔD2) into a modified version of the pcDL-SRα296 expression plasmid termed pSP65-SRα2 (44) using standard cloning procedure. The numbering of LAR amino acid residues was in accordance with Streuli *et al*. (16). Human Fyn and Lck expression vectors, pME18S-Fyn (FynWT, a wild-type Fyn), pME18S-FynY531F (FynYF, a constitutive active form of Fyn) (52) or pME18S-FynK299M (FynKM, a kinase negative mutant), pME18S-Lck (LckWT, a wild-type Lck), and pME18S-LckY505F (LckYF, a constitutive active form of Lck) and vectors for bacterial expression of GST-Fyn SH2 domain, GST-Fyn SH3 domain, and GST-Fyn unique domain were kindly provided by Dr. Yamamoto (University of Tokyo).

Expression of LAR in Mouse Tissues

Various tissues from specific pathogen-free C57BL/6 mice (Charles River, Japan) were homogenized in ice-cold lysis buffer [1% NP40, 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 10 mM iodoacetamide, 10 mM NaF, 10 mM sodium PP_i, 0.4 mM sodium vanadate, 0.1 mM phenylarsine oxide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine]. The supernatants were collected after centrifugation and the resultant protein (0.2 mg) was resolved on 7.5% SDS-PAGE and immunoblotted with anti-LAR mAb YU1 raised against bacterially expressed GST-LAR intracellular domain as an antigen (35). The experiments were conducted according to the institutional ethical guidelines for animal experiments.

Cell Culture and Transfections

COS-7 cells, obtained from the Human Science Research Resources Bank (Osaka, Japan), were grown at 37°C in 5% CO_2 atmosphere in DMEM (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Nichirei, Japan) and 10 mg/ml kanamycin. Cells were cotransfected with 5 μg of pSP65-SRα-LAR expression vector and 1 μg of pME18S-Fyn or pME18S-Lck expression vectors by the DEAE-dextran method. After incubation for 48 h, the transfected cells were washed with ice-cold PBS containing 1 mM sodium vanadate, 5 mM NaF, 5 mM sodium PP_i, and 5 mM EDTA, and then lysed on ice in 1 ml of ice-cold lysis buffer [1% NP40, 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 10 mM iodoacetamide, 10 mM NaF, 10 mM sodium PP_i, 0.4 mM sodium vanadate, 0.1 mM phenylarsine oxide, 1 mM PMSF, and 1 mM benzamidine]. The cell lysates were centrifuged to remove insoluble materials before immunoprecipitation and immunoblotting.

Immunoprecipitation and Immunoblotting

Cell lysates were precleared with 20 μl protein G-Sepharose (Amersham Pharmacia Biotech, Arlington Heights, IL) for over 2 h at 4°C. For immunoprecipitation, cell lysates were incubated with 5 μg anti-LAR mAbs (1:1:1 mixture of 11.1A, 75.3A, and 71.2E) (17) for over 1 h at 4°C, and then with 20 μl protein G-Sepharose for 1 h at 4°C. After washing twice with 1 ml lysis buffer as described above and twice with 1 ml TBS-T [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 0.1% Tween 20], immunoprecipitates were resolved by SDS-PAGE. Proteins in immunoprecipitates and cell lysates were transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), blocked with 3% BSA in TBS-T and incubated with primary antibodies at room temperature for 1 h, followed by washing three times with TBS-T. To detect antibody binding, horseradish peroxidase (HRP)-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-T was incubated at room temperature for 1 h. After washing in TBS-T three times, bound HRP conjugates were visualized with enhanced chemiluminescent reagent (Wako, Japan). The primary antibodies used were anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-Fyn mAb (Santa Cruz Biotechnology), anti-LAR E-subunit mAbs (1:1:1: mixture of 75.3A, 11.1A and 71.2E), and YU1, the anti-LAR P-subunit mAb.

Luciferase Assay

The CD45-deficient Jurkat cell line J45.01 (American Type Culture Collection, Manassas, VA) was transfected with pSP65-

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SRα-LAR expression vectors concomitant with pFR-Luc, pFA-Elk1 (PathDetect Elk *trans*-reporting system) (Stratagene, La Jolla, CA), and pRL-TK (Promega, Madison, WI) by electroporation using the Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) at 250 V and 960 μF capacitance. After 24 h of transfection, 10^6 cells were stimulated for 16 h with plate-bound anti-CD3 mAb OKT3 (3 μg/ml) (American Type Culture Collection). Luc in cell lysates was measured by a luminometer Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) using a dual-luciferase reporter assay system (Promega).

In Vitro Dephosphorylation Assay of Tyrosine-Phosphorylated Fyn

Lysates of COS-7 cells transfected with pME18S-Fyn, pME18S-FynYF, and pME18S-FynKM were immunoprecipitated with anti-Fyn mAb. The immunoprecipitates were washed with PBS twice and then with PTPase assay buffer [25 mM HEPES (pH 7.4), 5 mM EDTA, 100 mM DTT, 50 mM NaCl, and 50 μg/ml BSA]. GST-LAR fusion proteins were generated by subcloning the following human cDNAs into a pGEX-2T vector (Amersham Pharmacia Biotech): LAR PTPase domain cDNA (GST-LAR) and LAR PTPase domain cDNA containing a Cys-1522 to Ser mutation (GST-LAR D1CSD2).

GST Pull-Down Assay

GST-Fyn unique domain, GST-Fyn SH2 domain, and the GST-Fyn SH3 domain were expressed in *Escherichia coli* NM522 according to standard procedures. Briefly, bacteria were grown to mid-log phase at 37°C and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Bacteria were then pelleted, washed, resuspended in PBS containing 1 mM PMSF, and sonicated. Insoluble material was removed by centrifugation. GST fusion proteins were bound to glutathione-*S*-Sepharose 4B beads (Amersham Pharmacia Biotech). After washing three times with PBS, the fusion proteins were eluted twice in 50 mM Tris-HCl (pH 8.0) containing 10 mM GSH (Sigma). Five micrograms of the purified GST fusion proteins were incubated at 4°C for 1 h with immunoprecipitates with anti-LAR E-subunit mAbs from lysates of COS-7 cells cotransfected with pSP65-SRα2-LAR WT or pSP65-SRα-LAR D1CSD2 concomitant with pME18S-FynYF. The complexes were then washed with TBS-T three times, and resuspended in SDS-sample buffer, separated by SDS-PAGE, and immunoblotted with anti-LAR E-subunit mAbs and anti-Fyn mAb.

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