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Direct Interaction of the CD38 Cytoplasmic Tail and the Lck SH2 Domain

CD38 TRANSDUCES T CELL ACTIVATION SIGNALS THROUGH ASSOCIATED Lck*

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CD38 ligation has been shown to induce activation of intracellular signaling cascade in T lymphocytes through a Lck-dependent pathway. However, it is not clear how Lck initiates the CD38-mediated signaling process. In the present study, we showed that CD38 and Lck were physically associated through the cytoplasmic tail and the Src homology 2 domain, respectively. This was evidenced by coimmunoprecipitation of Lck with CD38 and Lck with isolated CD38 cytoplasmic domain from T cell lysate, cell lysate of COS-7 cells cotransfected with cDNAs of Lck and CD38, or a mixture of *in vitro* translated CD38 and Lck. Because the CD38 cytoplasmic domain does not contain any tyrosine residue, the interaction should be independent of phosphotyrosine. The interaction was further confirmed by *in vitro* interaction between a purified Lck Src homology 2 domain and a nonphosphosynthetic peptide corresponding to the membrane proximal region of the CD38 cytoplasmic domain. In addition, CD38 ligation resulted in an elevated tyrosine kinase activity of the CD38-associated Lck and ultimate activation of interleukin-2 gene transcription. Furthermore, expression of a kinase-deficient Lck mutant suppressed interleukin-2 gene activation in a dose-dependent manner. These results strongly suggested that CD38 ligation indeed tranduced signals for T cell activation using its associated Lck.

CD38 is a type II transmembrane glycoprotein with a long C-terminal extracellular domain and a short N-terminal cytoplasmic tail (1). The extracellular domain of CD38 has bifunctional enzyme activities that catalyze synthesis of cyclic ADP ribose from nicotinamide adenine dinucleotide (NAD) and hydrolysis of cyclic ADP ribose to adenosine diphosphoribose (1–3). Cyclic ADP ribose is a novel Ca^{2+} mobilizer in different eukaryotic cells where it can modulate the intracellular calcium levels by releasing it from ryanodine-sensitive intracellular stores (4–10).

In addition to the catalytic activities of the extracellular domain, the CD38 cytoplasmic domain seems to independently transduce activation signals. CD38 ligation with an agonistic anti-CD38 monoclonal antibody (mAb)[1] stimulates proliferation of B and T lymphocytes (11–13) and increases tyrosine phosphorylations of a set of intracellular proteins (14–17). Targets for the CD38-mediated tyrosine phosphorylation and activation of downstream signaling cascade in T cells include the TCR/CD3ζ chain, ZAP-70, components of Ras-mitogen-activated protein kinase pathway, and c-Cbl (17). However, CD38 itself lacks intrinsic protein-tyrosine kinase activity, and such molecular modifications do not occur in a Lck-deficient Jurkat cell line, J.Cam1 (17). These results indicate that Lck likely plays an important role in signal transduction through the cytoplasmic domain of CD38. However, it is unknown how CD38 recruits and uses Lck in transducing signals further downstream in the cell.

In the present study, it is demonstrated that CD38 and Lck molecules are directly associated through a phosphotyrosine-independent interaction between the cytoplasmic domain of the former and the SH2 domain of the latter. Upon CD38 ligation, tyrosine kinase activity of the associated Lck is up-regulated and relays signals to induce the ultimate activation of IL-2 gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—Adherent (COS-7) and suspension (Jurkat, J.Cam1) cells were maintained in Dulbecco's modified Eagle's medium and RPMI (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. T lymphocytes were isolated from spleens of 4-week-old BALB/c mice by using a nylon wool column as described (18). Antibodies were purchased from Pharmingen (murine monoclonal anti-CD38), Immunotech (T16, human monoclonal anti-CD38), Serotec ($F(ab')_2$ goat anti-rat IgG), UBI (monoclonal 4G10 anti-phosphotyrosine, polyclonal anti-Lck), Sigma (anti-rat and mouse IgG-agarose), IBI-Kodak (monoclonal anti-FLAG epitope), and Amersham Pharmacia Biotech (anti-GST). For cross-linking of CD38, T cells were incubated with anti-CD38 mAb (10 $\mu g/10^7$ cells) or control rat IgG for 30 min on ice. After removal of unbound antibodies, cells were resuspended in serum-free RPMI and cross-linked for 5 min at 37 °C with soluble $F(ab')_2$ goat anti-rat IgG (20 $\mu g/10^7$ cells). Cells were immediately lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% digitonin, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 100 μM Na_3VO_4, 50 mM NaF) and centrifuged at 20,000 × *g* for 30 min at 4 °C, and resulting supernatant was used for further analysis.

Cloning and Expression of Recombinant CD38 and Lck—cDNAs encoding human CD38 and Lck were prepared by reverse transcriptase-polymerase chain reaction and cloned into pFLAG-CMV2 (IBI, Kodak),

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[1] The abbreviations used are: mAb, monoclonal antibody; IL, interleukin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; Ab, antibody; wt, wild type; cyt, cytoplasmic; MAPK, mitogen-activated protein kinase; SH, Src homology; TCR, T cell receptor.

pRc/CMV (Invitrogen), and pCDNAI vectors. A kinase-defective mutant Lck (Lck.A273) was generated by replacing a coding sequence of the lysine 273 (AAG) with that of alanine (GCG) using the QuickChange™ site-directed mutagenesis system (Stratagene). A sequence of mutated nucleotide was confirmed by DNA sequencing. The IL-2-Luc reporter contains the luciferase gene downstream from the IL-2 promoter region, including 548 base pairs 5′ of the transcription start site and was generated by subcloning a *Hin*dIII fragment from the IL-2 promoter into the *Hin*dIII site of pGL3-basic vector (Promega) (19). An expression vector for the CD38 cytoplasmic domain alone was constructed by inserting a polymerase chain reaction fragment encoding the specific region into a pFLAG-CMV2 vector. Construction and preparation of GST-fused Lck subdomains were described elsewhere (20). GST-fused SH2 domains of Src and Crk and N-terminal SH2 domains of GTPase-activating protein, phospholipase C-γ1, and p85 phosphatidylinositol 3-kinase were generous gifts of Dr. Steven E. Shoelson at the Harvard Medical School. Expressions of CD38 and Lck were achieved by transient transfection of corresponding plasmids (5 μg) into COS-7 or T cell lines using DEAE-dextran or Superfect™ (Quiagen Inc.). Transfected cells were analyzed after 48 h posttransfection. *In vitro* transcription and translation of CD38 and Lck were performed according to manufacturer's instructions using RiboMAX™ and rabbit reticulocyte lysate systems (Promega).

Immunoprecipitation and Immunoblotting—For immunoprecipitation, cell lysates were incubated for 1 h at 4 °C with anti-CD38 mAb or 4G10 anti-phosphotyrosine antibody and further incubated for 30 min with anti-rat or anti-mouse IgG-agarose. Immunoprecipitates were washed three times with the lysis buffer, and proteins were separated on a 10% SDS-PAGE. After transfer to a nitrocellulose membrane, protein bands were visualized by immunoblotting using specific antibodies, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Advanced Biochemicals Inc., Korea), and an ECL substrate kit (Amersham Pharmacia Biotech). Similarly, Lck subdomain binding proteins were analyzed; after preclearing with GST-glutathione Sepharose beads or anti-FLAG (M2) affinity gel, cell lysates were incubated with purified GST fusion proteins (~5 μg), and bound proteins were precipitated with glutathione-Sepharose beads and analyzed by immunoblotting.

Real Time Binding Analysis—Three peptides corresponding to amino acids 1–10 (ANCEFSPVSG), 5–15 (SPVSGDKPCC), and 11–20 (DKPCCRLSRR) of CD38 were synthesized at Korea Basic Science Center and subsequently purified on C18 reversed phase high pressure liquid chromatography. Purified GST-Lck.SH2 domain fusion protein was covalently coupled to the dextran surface of a BIAcore sensorchip. The synthetic peptide solution (0.25 mg/ml) was injected onto immobilized Lck SH2 surface, and binding was measured as responses from 25 s postinjection relative to a baseline determined immediately before injection.

In Vitro Transcription and Translation—Recombinant plasmids, pRc/CMV containing human CD38 cDNA and pCDNAI containing human LCK cDNA, were linearized with restriction enzymes, *Xho*I and *Xba*I, respectively. *In vitro* transcription was performed using RiboMAXTM Large Scale RNA production system, and *in vitro* translation was performed with resulting RNA products using a rabbit reticulocyte lysate system according to the manufacturer's instructions (Promega).

RESULTS

Direct Interaction of CD38 and Lck—CD38 ligation induces a rapid increase in Lck-dependent tyrosine phosphorylation of a set of cellular proteins; however, a mechanism through Lck is recruited to the CD38-signaling complex is not known (17). One possibility is that Lck directly associates with CD38, and upon CD38 ligation, the associated Lck is activated to phosphorylate its cellular substrates. To examine the possibility, coimmunoprecipitation of CD38 with Lck was analyzed in cell lysates of purified murine T cells. Interestingly, Lck was found in an immune complex precipitated by a specific anti-murine CD38 mAb (clone 90, Pharmingen) but not by an immunoprecipitate of an isotype matched control Ab (Fig. 1*A*, *lanes 2* and *1,* respectively). Furthermore, when Lck was precleared from the cell lysates using anti-Lck antiserum, CD38 no longer coprecipitated with Lck (Fig. 1*A*, *lane 3*). These results indicate that Lck is associated, either directly or indirectly, with CD38 in T cells.

When mixed together, *in vitro* translation products of

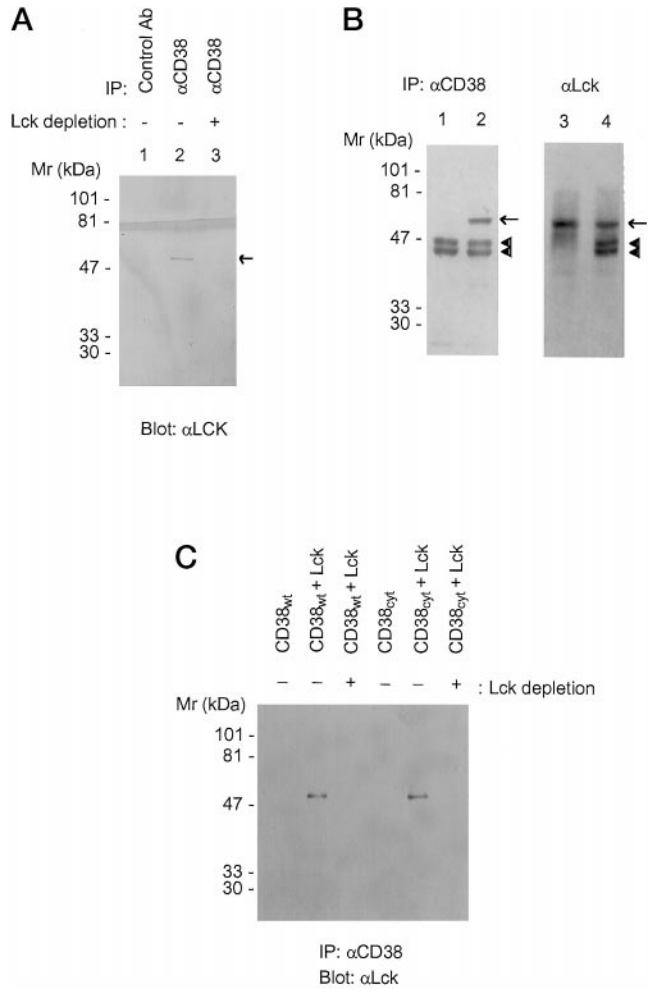

FIG. 1. **Direct interaction of CD38 and Lck.** *A,* coimmunoprecipitation of CD38 and Lck. Murine T cells were prepared, lysed, and immunoprecipitated with a normal mouse IgG (*lane 1*) and anti-CD38 monoclonal antibody (*lanes 2* and *3*), before (*lanes 1* and *2*) and after (*lane 3*) depletion of Lck from the lysate using anti-Lck antibody. Immunoprecipitates were then analyzed by Western blot using anti-Lck antibody. The *arrow* points to the Lck molecule. *B,* association of *in vitro* translated CD38 and Lck. *In vitro* translation products of [S^{35}]methionine-labeled CD38 and Lck (see "Experimental Procedures") were incubated separately (*lanes 1* and *3*) or mixed and incubated (*lanes 2* and *4*) for 4 h at 4 °C. CD38 (*lanes 1* and *2*) and Lck (*lanes 3* and *4*) were immunoprecipitated, and the immune complexes were analyzed on a 10% SDS-PAGE followed by autoradiography. *Arrows* point to Lck, and *arrowheads* to CD38 molecules. *C,* interaction of CD38 and its cytoplasmic tail with Lck in COS-7 cells. COS-7 cells were cotransfected with cDNAs encoding Lck and wild type CD38 (CD38wt) or cytoplasmic domain of CD38 (CD38cyt). CD38 was immunoprecipitated from cell lysates before and after Lck depletion. Lck content in the immunoprecipitates was measured by immunoblot analysis using ant-Lck Ab. *IP*, immunoprecipitate.

[^{35}S]methionine labeled Lck and CD38 were coimmunoprecipitated by either anti-CD38 mAb or anti-Lck antiserum (Fig. 1*B, lanes 2* and *4*). In addition, Lck and CD38 were also coimmunoprecipitated from lysates of COS-7 cells ectopically expressing both CD38 and Lck (Fig. 1*C*). Interestingly, expression of only the cytoplasmic domain of CD38 was sufficient to precipitate the cotransfected Lck (Fig. 1*C*). These results strongly indicate that the cytoplasmic domain of CD38 interacts directly with Lck and that the interaction does not require any other T cell-specific component.

Interaction of the Cytoplasmic Domain of CD38 and the SH2 Domain of Lck—To delineate subregion(s) of Lck required for association with CD38, GST fusion proteins containing the N-terminal unique region, SH3 domain, SH2 domain, or a

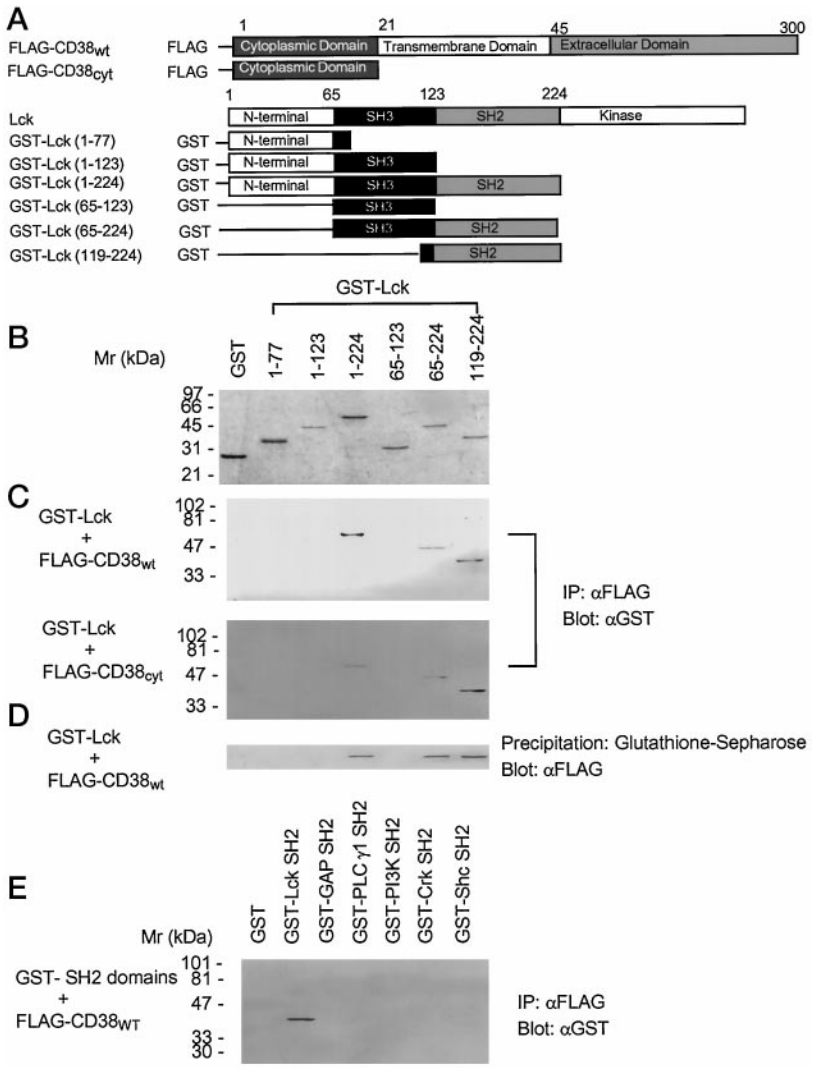

FIG. 2. **Association of the CD38 cytoplasmic tail and the Lck SH2 domain.** *A,* schematic presentation of FLAG-tagged CD38 (FLAG-CD38wt), isolated CD38 cytoplasmic tail (FLAG-CD38cyt), and GST fusion proteins of Lck subdomains. *B*, bacterially expressed and purified GST fusion proteins. Fusion proteins were expressed and purified as described under "Experimental Procedures," and analyzed on a SDS-PAGE by Coomassie Blue staining. *C,* interaction of the Lck SH2 domain with CD38. Lysates of COS-7 cells transfected with cDNAs of FLAG-CD38wt or FLAG-CD38cyt were incubated with purified GST fusion proteins at 4 °C. FLAG-tagged proteins were immunoprecipitated by anti-FLAG (M2) antibody, and coprecipitated GST-fused Lck subdomains were measured by immunoblot analysis using anti-GST Ab. *D,* GST-fused Lck subdomains were precipitated by glutathione-Sepharose beads from the same incubation mixture as in *C*, and coprecipitated CD38 was measured by immunoblot analysis using anti-FLAG Ab. *E,* only the Lck SH2 domain interacts with CD38. Interaction of CD38 with GST-fused SH2 domains of various proteins was analyzed as in *C*. *IP*, immunoprecipitate.

combination of these subregions of Lck were examined for their ability to associate with CD38 (Fig. 2). To this end, cell lysates of COS-7 cells expressing either FLAG epitope-tagged wild type CD38 (FLAG-CD38wt) or the cytoplasmic domain of CD38 (FLAG-CD38cyt) were incubated with purified GST fusion proteins. FLAG-CD38wt was immunoprecipatated using anti-FLAG mAb, and the associated GST-fused Lck subdomain(s) was examined by immunoblot analysis using an anti-GST antiserum (Fig. 2*C*). Interestingly, both FLAG-CD38wt and FLAG-CD38cyt interacted well with GST fusion proteins containing the Lck SH2 domain (1–224, 65–224, and 119–224) but not with GST fusion proteins of other subdomains. Furthermore, when GST fusion proteins were precipitated by glutathione-Sepharose beads, CD38 was clearly detected only in the precipitates of GST fusion proteins containing the Lck SH2 domain (Fig. 2*D*). The same experiments using GST fusion proteins containing SH2 domains of other proteins failed to show any interaction with CD38 (Fig. 2*E*). Taken together, these data showed that the cytoplasmic domain of CD38 specifically associated with the Lck SH2 domain.

Interaction of the CD38 cytoplasmic domain and the Lck SH2 domain was further confirmed by measuring real time binding between purified GST-fused Lck SH2 domain and synthetic peptides derived from the CD38 cytoplasmic domain (Fig. 3*A*). Interestingly, a peptide corresponding to amino acid residues 11–20 of CD38 showed clear interaction with immobilized GST-Lck SH2 domain, whereas N-terminal peptides did not. Furthermore, the interaction of CD38 with the purified GST-fused Lck SH2 domain was inhibited by a peptide derived from amino acid residues 11–20 of CD38 dose dependently, but not by N-terminal peptides or a random amino acid sequence peptide (Fig. 3*B*). Thus, it is likely that CD38 and Lck form a complex through the membrane proximal region of the CD38 cytoplasmic domain and the Lck SH2 domain.

Lck Is Essential in CD38-mediated T Cell Activation—Lck has an essential role in TCR/CD3-mediated activation of the Ras-MAPK pathway, which eventually influences IL-2 gene activation in a subset of T cells (21, 22). CD38 ligation also has been suggested to activate MAPK and thereby induce slower migration of Lck on SDS-PAGE, probably because of MAPK-mediated serine phosphorylation of Lck (17). Thus, the physical interaction of CD38 and Lck may be a mechanism resembling the mechanism of TCR/CD3-mediated signaling. Indeed, ligation of CD38 substantially increased the tyrosine kinase activity of Lck (Fig. 4*A*). Furthermore, ligation of both CD38 and CD3 induced a similar mobility change of CD38-associated Lck (Fig. 4*B*).

Finally, to examine the requirement of the kinase activity of Lck in CD38-mediated signaling, we investigated the effects of a kinase inactive mutant Lck, Lck.A273, which is defective in a part of the ATP binding site (23, 24). Jurkat T cells were cotransfected with IL-2-Luc reporter and various amounts of Lck.A273, and the CD38 ligation-mediated IL-2 promoter activation was analyzed. As shown in Fig. 4*C*, CD38 cross-linking

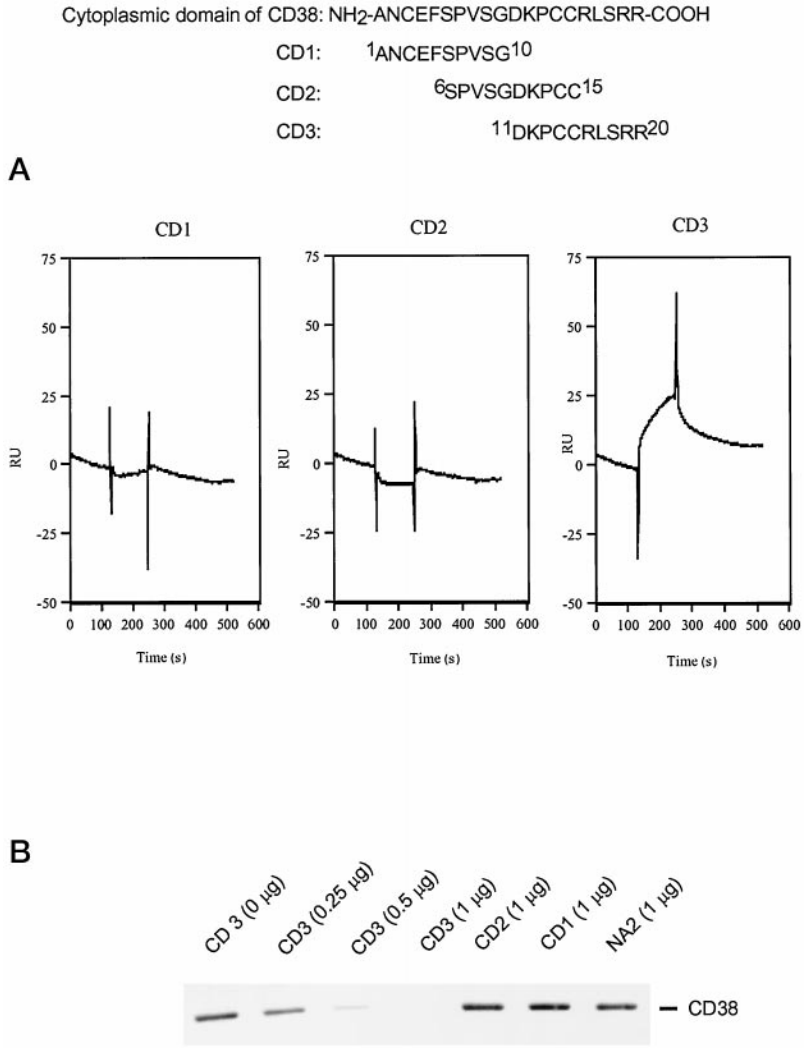

FIG. 3. **Interaction of CD38 cytoplasmic tail peptides and Lck SH2 domain.** Three peptides corresponding to amino acid residues 1–10 (CD1, ANCEFSPVSG), 6–15 (CD2, SPVSGDKPCC), and 11–20 (CD3, DKPCCRLSRR) of CD38 are shown in the *upper panel*. *A,* peptide solutions (0.25 mg/ml) were injected onto the immobilized Lck SH2 surface of a BIAcore sensorchip, and binding was measured as responses from 25 s postinjection relative to a baseline determined immediately before injection. *RU*, relative units. *B,* inhibition of CD3 peptide on the interaction of Lck SH2 with CD38. Each peptide was incubated with GST- Lck SH2 for 30 min and further incubated with FLAG-CD38wt for 1 h. GST-Lck SH2 was precipitated with glutathione-Sepharose beads, and coprecipitated CD38 was detected by anti-FLAG Ab. NA2 is a control peptide of random amino acid sequence (DAQLDMALDS).

enhanced IL-2 promoter activity more than 3-fold over the basal level in Jurkat cells, and this enhancement was abolished upon coexpression of Lck.A273 in a dose-dependent manner. However, this mutation did not affect the interaction with CD38 (data not shown), and the basal IL-2 promoter activity was not affected by coexpression of Lck.A273. These results suggest that CD38 transduces a signal for IL-2 gene activation through the associated Lck activation in a manner similar to the Lck-mediated TCR/CD3 signaling.

DISCUSSION

In a previous study, it was shown that CD38 ligation induced Lck-regulated phosphorylation and/or activation of CD3ζ, ZAP-70, Ras-mitogen-activated protein kinase pathway, and other signaling molecules in Jurkat T cells (17). However, it was unclear how CD38 ligation recruited Lck to its signaling of downstream cascades. The present study demonstrated that the cytoplasmic domain of CD38 directly associated with Lck via its SH2 domain (Figs. 1 and 2) and that ligation of CD38 up-regulates Lck tyrosine kinase, thereby inducing the ultimate activation of IL-2 gene transcription (Fig. 4). Thus, the present data confirm the CD38-induced Lck-dependent signaling and provide further insight of molecular mechanisms involved in this signaling pathway.

The SH2 domain is a highly conserved structural module of about 100 amino acid residues and mediates protein-protein interactions (25). In general, SH2 domains interact with phosphotyrosine residues of a specific ligand using a well conserved phosphotyrosine binding pocket (26). However, the CD38 cytoplasmic domain does not have any tyrosine residues (1), and it binds well to an Arg-154 mutant Lck, which is defective in the phosphotyrosine binding pocket (Ref. 26 and data not shown). Thus, the CD38 cytoplasmic domain interacts with the Lck SH2 domain in a phosphotyrosine-independent manner. There are several reports showing that SH2 domains can also serve as phosphotyrosine-independent protein binding sites (20, 27–30). The interaction of some phosphotyrosine-independent ligands with SH2 domain seemed to depend on extensive phosphorylation on serine/threonine residues (31–33). However, three serine residues present in the CD38 cytoplasmic domain were not efficiently phosphorylated before and after CD38 ligation (data not shown). Furthermore, a decapeptide derived from the membrane proximal region of the CD38 cytoplasmic domain having only one nonphosphorylated serine residue bound well to the Lck SH2 domain (Fig. 3). Thus, the CD38 cytoplasmic domain requires neither tyrosine nor serine phosphorylation for its interaction with the Lck SH2 domain. At present, it is not clear whether or not the 10 amino acids are the minimal length required for SH2 domain binding and how the interaction occurs between such a short non-phosphopeptide and the Lck SH2 domain. Further analysis is needed to better understand this novel interaction mode, which provides a potential way to modulate SH2 domain-mediated protein-protein interaction.

Lck has been known to interact with a few T cell surface molecules including CD4, CD8, TCR/CD3ζ chain, and IL-2Rβ

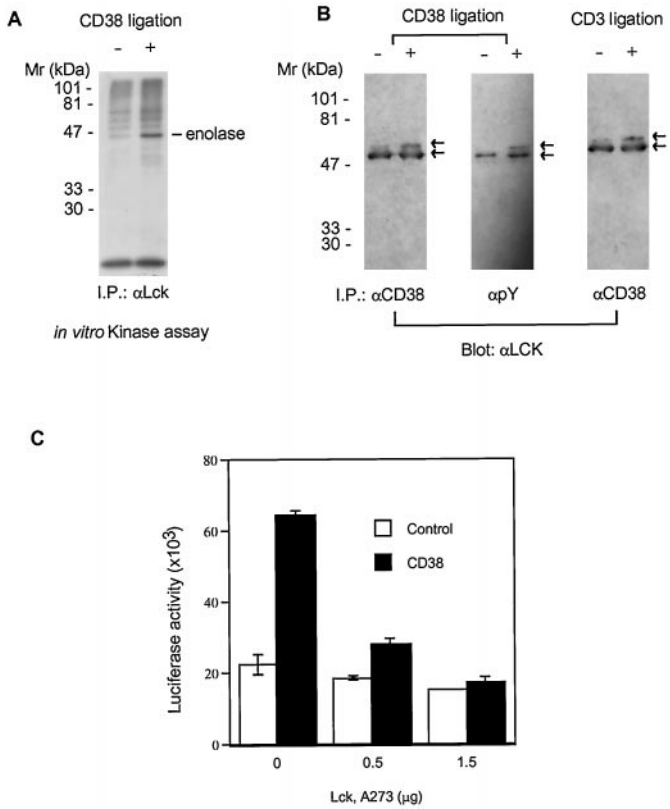

FIG. 4. **Lck-mediated CD38 signaling for T cell activation.** *A,* activation of Lck catalytic activity by CD38 ligation. 5×10^6 Jurkat cells were ligated by an agonistic anti-CD38 antibody (+) or a control antibody (−) for 10 min and lysed. Lck was immunoprecipitated from the cell lysates and subjected to *in vitro* kinase assay in the presence of 100 ng of enolase in 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, and 10 μCi of [^{32}P]ATP. After a 20-min incubation at room temperature, reaction was stopped by adding same volume of 2X SDS-sample buffer. Samples were boiled for 3 min, separated on a 10% SDS-PAGE, and visualized by autoradiography. *B,* Lck gel shift induced by CD38 cross-linking. Partially purified murine T cells were treated on ice with anti-CD38, anti-CD3, or mouse IgG for 30 min and then secondary antibody for 5 min at 37 °C as described under "Experimental Procedures." Lck was immunoprecipitated from the cell lysates using anti-CD38 or anti-phosphotyrosine Ab, and its mobility was measured on a 9% SDS-PAGE, followed by immunoblot analysis using anti-Lck Ab. *Arrows* indicate two species of Lck molecules with different mobilities at the 56 and 60 kDa regions. *C,* Lck is required for the CD38 ligation-mediated activation of IL-2 promoter. 10^6 Jurkat T cells were cotransfected with 1.0 μg of IL-2-Luc reporter plasmids and indicated amounts of expression plasmids encoding kinase-defective Lck (Lck.A273). At 24 h posttransfection, cells were ligated with anti-CD38 antibody and incubated for an additional 14 h. The experiments were performed in duplicate three times, and the standard deviations are shown on top of each bar. *I.P.*, immunoprecipitates.

(34–36), and distinct subregions of Lck are used for interaction with these molecules (a unique N-terminal region for CD4 and CD8, the SH2 domain for the tyrosyl-phosphorylated CD3ζ chain, and the SH1 or SH3 domain for IL-2Rβ). Through these interactions, Lck plays important roles in signaling at different T cell environments. The unique interaction of CD38 with Lck, therefore, represents another addition to the divergent roles of Lck in T cell regulation.

Because both CD38 and CD3 activate Lck and utilize it to initiate downstream signaling cascades, there may be a cross-talk between signaling pathways transduced by these two distinct T cell surface receptors. Indeed, CD38 ligation resulted in a sequence of events that occur during CD3-mediated T cell activation: tyrosyl phosphorylation of the CD3ζ chain and ZAP-70, activation of the Ras-Raf-MAPK pathway, and mobility shift of Lck, likely due to serine phosphorylation by the activated MAPK (17). In a reverse direction, CD3 cross-linking induced the same mobility shift of CD38-associated Lck (Fig. 4*B*). Interestingly, proper CD38 signaling seemed to require expression of TCR/CD3 on human T cell surface (37). Thus, some of the CD38-mediated signaling events might be elicited through signaling machineries shared with TCR/CD3 and vice versa. However, there are also apparent differences between outcomes of signaling through CD38 and CD3. For example, the extents of cytokine production by individual ligation of these two cell surface receptors are significantly different from one cytokine to the other (38). Distinct kinetics of Ca^{2+} mobilization induced by cross-linking of CD38 and CD3 would be another example (37). Thus, although CD38 and CD3 may share common signaling components in some part, these two receptors likely use their own unique mechanisms to transduce distinct signals. The presence of a signaling pathway unique for CD38 is supported by observations of CD3 expression-independent phosphorylation of c-Cbl (17), the CD38 cytoplasmic domain-independent intracellular tyrosine phosphorylation, and IL-2 production in myeloid and B cell lines (39, 40). Nevertheless, CD38 provides an alternative signaling pathway for T cell activation via its associated tyrosine kinase, Lck. Further investigations of interaction between the CD38 cytoplasmic domain and the Lck SH2 domain will shed more light to better understanding of CD38-mediated T cell signaling.

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Direct Interaction of the CD38 Cytoplasmic Tail and the Lck SH2 Domain: CD38 TRANSDUCES T CELL ACTIVATION SIGNALS THROUGH ASSOCIATED Lck

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