MOLECULAR AND CELLULAR BIOLOGY, Dec. 1996, p. 7151–7160 Vol. 16, No. 12
0270-7306/96/$04.00+0

The Kinase, SH3, and SH2 Domains of Lck Play Critical Roles in T-Cell Activation after ZAP-70 Membrane Localization

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Received 17 June 1996/Returned for modification 19 August 1996/Accepted 18 September 1996

Antigenic stimulation of the T-cell antigen receptor initiates signal transduction through the immunoreceptor tyrosine-based activation motifs (ITAMs). When its two tyrosines are phosphorylated, ITAM forms a binding site for ZAP-70, one of the cytoplasmic protein tyrosine kinases essential for T-cell activation. The signaling process that follows ZAP-70 binding to ITAM has been analyzed by the construction of fusion proteins that localize ZAP-70 to the plasma membrane. We found that membrane-localized forms of ZAP-70 induce late signaling events such as activation of nuclear factor of activated T cells without any stimulation. This activity was observed only when Lck was expressed and functional. In addition, each mutation that affects the function of Lck in the kinase, Src homology 2 (SH2), and SH3 domains greatly impaired the signaling ability of the chimeric protein. Therefore, Lck functions in multiple manners in T-cell activation for the steps following ZAP-70 binding to ITAM.

Antigenic stimulation of the T-cell antigen receptor (TCR) initiates signal transduction through a motif called ITAM (immunoreceptor tyrosine-based activation motif), which is composed of the consensus sequence (D/E)xxYxx(I/L)x_{6-8}Yxx(I/L) (12, 57, 75). Three repeats of ITAMs are contained within the ζ chain of TCR, and a single motif for each of the three CD3 molecules (γ, δ, and ε) is present. Motifs in both CD3ε and ζ have sufficient structure to convey the activation signals (for a review, see reference 75).

A key role of ITAM in TCR signal transduction is to initiate sequential interactions of protein tyrosine kinases (PTKs). At least three classes of PTKs are involved in TCR signal transduction, namely, (i) Lck and Fyn, (ii) ZAP-70 and Syk, and (iii) Itk (for reviews, see references 53, 58, and 75).

Lck, a Src family PTK, appears to play a major role in phosphorylating both tyrosines in ITAM when antigenic stimulation is presented to TCR (15, 36). Fyn may play a role equivalent to that of Lck (19, 31); however, the phenotype of T cells from Fyn knockout mice is much less dramatic than that of cells from Lck knockout mice (6, 63).

Phosphorylation of both tyrosines in ITAM creates a binding site for ZAP-70 (36, 73). The crystal structure of the complex consisting of ZAP-70 Src homology 2 (SH2) domains and ITAM clearly demonstrated that two phosphotyrosines of ITAM bind the two SH2 domains (34). Following ITAM binding, ZAP-70 appears to become activated and thereafter initiate the downstream signaling cascade (49). Syk appears to play roles equivalent to those of ZAP-70 in certain types of cells (19, 32).

Itk, a cytoplasmic PTK and a member of the Itk-Btk family, is expressed preferentially in T lymphocyte lineages (reviewed in reference 53). Loss of Itk expression by homologous recombination results in the impaired signaling ability of TCR (42). It should be noted that Btk is activated upon stimulation and subsequent tyrosine phosphorylation of B-cell antigen receptor (5, 22, 59).

Expression of Lck and ZAP-70 is required for the signal transduction of TCR. Loss of Lck expression in mice, via homologous recombination, severely disrupts T-cell development and abrogates the TCR signal (46). Neither ZAP-70 recruitment nor late signaling events are observed when TCR is stimulated in T cells lacking Lck (37, 64). Loss or altered expression of ZAP-70 was found in patients with a rare immune deficiency syndrome (7, 16, 25). Their peripheral T cells do not show intracellular responses upon antigenic stimulation. Furthermore, thymocytes from mice lacking in ZAP-70 expression were not able to respond to TCR stimulation and did not go through either positive or negative selection to form a normal peripheral T-cell repertoire (48). In addition, when T-cell lines were transfected to express the chimeric molecules of CD16 (extracellular domain)–CD7 (transmembrane domain)–ZAP-70 and CD16-CD7-Lck, cross-linking of chimeras via anti-CD16 antibody induced T-cell activation (39).

To understand the molecular events that follow ZAP-70 membrane recruitment, we have constructed several chimeric molecules that localize ZAP-70 to the plasma membrane. Surprisingly, the membrane localization of ZAP-70 was sufficient to trigger late signaling events, such as the activation of nuclear factor of activated T cells (NF-AT). This activation was not observed in T cells that had lost the expression of Lck. Introduction of Lck into these Lck-less T cells recovered the signaling ability of the chimera. Using this reconstitution system, we found that SH3 and SH2 domains of Lck, as well as the kinase domain, play critical roles in inducing NF-AT activation. These data revealed that Lck functions in multiple manners for signal transduction after ZAP-70 is recruited to the plasma membrane.

MATERIALS AND METHODS

Cell lines and culture conditions. Jurkat, J.CaM1.6, and J.RT3.T3.5 cells (gifts from A. Weiss, University of California, San Francisco) and simian virus 40 large T-antigen (TAg)-transfected Jurkat cells (Jurkat TAg cells) (51) (a gift from G. Crabtree, Stanford University) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml. COS7 cells, a gift from M. Tanaka (Mitsubishi Kasei

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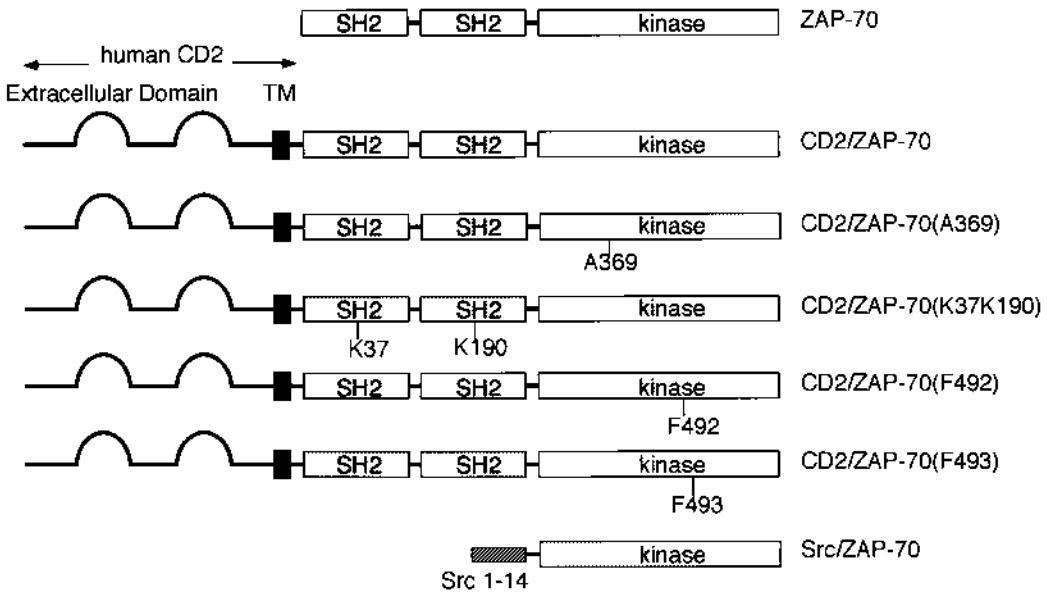


FIG. 1. Schematic representations of the chimeric CD2/ZAP-70 and Src/ZAP-70 molecules used in this study. CD2/ZAP-70 chimeras were constructed by attaching the entire coding region of human ZAP-70 to the extracellular and transmembrane (TM) domains (including 3 amino acids from the cytoplasmic region) of human CD2. Mutated amino acids are shown for each construct of the kinase domain mutants (A369, F492, and F493) and the SH2 domain mutant (K37K190). For Src/ZAP-70, residues 1 to 14 of chicken Src and a spacer glycine were attached to the C-terminal fragment of human ZAP-70 (from residue 263 to the C terminus).

Institute of Life Sciences), and BW5147.hCD4 cells, a gift from N. Shinohara (Mitsubishi Kasei Institute of Life Sciences), were maintained in Dulbecco's modified minimal essential medium with 5% fetal calf serum, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml. Stable transfectants were established by selection in the culture medium with 250 U of hygromycin B (Wako Chemicals, Tokyo, Japan) per ml.

Construction of the expression and reporter plasmids. CD2/ZAP-70 chimeras were constructed by attaching the entire coding regions of human ZAP-70 to the transmembrane and extracellular domain of human CD2 (amino acid residues 1 to 218) (60). CD2/ZAP-70 mutants were constructed by a method based on PCR as previously described (36). For mutant CD2/ZAP-20(A369) (mutant A369), K-369 (AAG) of human ZAP-70 was mutated to A (GCG). For mutant F492, Y-492 (TAC) was mutated to F (TTC). For mutant F493, Y-493 (TAC) was mutated to F (TTC). For mutant K37K190, R-37 and R-190 were mutated to K. For the Src/ZAP-70 construct, an oligonucleotide containing the 5′ untranslated region (positions −1 to −30) of ZAP-70 (15), amino acid residues 1 to 14 of chicken Src (67), and a spacer amino acid of glycine (nucleotide sequence, 5′-ACGTCCCCAGGTTTCGGGAGGCCCAGGGGCGATGGGGAGTAGC AAGAGCAGCCTAAGGACCCCAGCCAGCGCGGG-3′) was ligated to a DNA fragment coding for the amino acids from position 263 to the C terminus of human ZAP-70 (15). All CD2/ZAP-70 chimeras and Src/ZAP-70 were cloned into pME18S for transient transfections and into pMIKHygB for stable transfections (gifts from K. Maruyama, Tokyo Medical and Dental University). The cDNA of murine Lck, a gift from D. Littman, was also cloned into pME18S. The Lck mutants with alterations in the SH2 domain (K154 mutants) and the kinase domain (A273 mutants) were previously described (36). For the mutation at the autophosphorylation site (F394 mutation), Y-394 (TAC) was changed to F (TTC). For the SH3 domain mutation, P-112 (CCC) was changed to L (CTC). Both mutations were introduced by replacing the wild-type DNA fragments with oligonucleotides containing the mutations. The NF-AT luciferase reporter construct (NF-AT-luc) (50) was a gift from G. Crabtree (Stanford University).

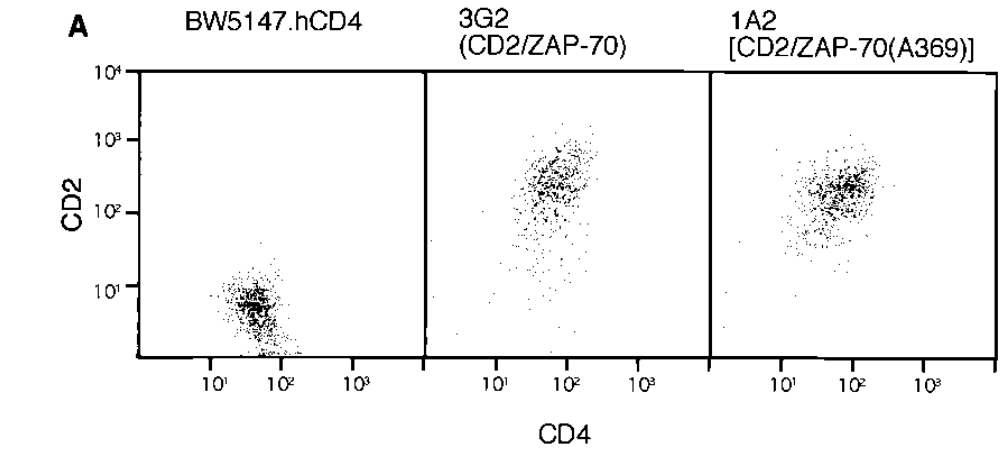
Cell transfections, stimulations, and luciferase assay. For stable transfectants, cells (10^6) were electroporated with 40 μg of DNA at 800 μF and 400 V (Cell porator; GIBCO BRL). For the luciferase assay, cells (5×10^6) were transfected by electroporation with 15 μg of NF-AT-luc and the expression constructs at 800 μF and 350 to 400 V. At 24 to 36 h after transfection, the cells were divided and cultured in a final volume of 5 ml of the culture medium with or without 1.0 μM ionomycin and 10 ng of phorbol myristate acetate (PMA) (Calbiochem, San Diego, Calif.) per ml. After 8 to 12 h of stimulation, cells were harvested and luciferase activity was measured with a PicaGene kit (Wako Chemicals) and a luminometer (Lumat-LB9501; Berthold). For each transfection condition, the percent activity against the maximal stimulation (induced by treatment with PMA and ionomycin) was calculated. With this percent activity, the average fold induction for each transfection (triplicate) over that of the cells transfected with the empty vector was determined. COS7 cells were transfected with DEAE-dextran and analyzed as described elsewhere (15).

Immunoprecipitation and Western blot (immunoblot) analysis. CD2/ZAP-70-transfected Jurkat TAg and Lck-transfected BW5147.hCD4 cells were harvested at 10 to 24 h after transfection. The cells were lysed in lysis buffer containing 1% Nonidet P-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors as previously described (35). Western blots were carried out with an enhanced chemiluminescence assay according to the manufacturer's recommendation (ECL kit; Amersham). To detect the expression of the CD2/ZAP-70 chimera, cell lysates were immunoprecipitated with biotinylated anti-CD2 monoclonal antibody (MAb) (Pharmingen) and streptavidin-agarose (Sigma). 2F3.2, an anti-ZAP-70 MAb, was described previously (36). Mol171 (47), an anti-Lck MAb, was a gift from Y. Koga (Tokai University), and anti-HS1 rabbit polyclonal antiserum (38) was a gift from T. Watanabe (Kyusyu University). Anti-Fyn, anti-phospholipase C γ1 (anti-PLC-γ1), anti-Vav, and antiphosphotyrosine (4G10) antibodies were purchased from Upstate Biotechnology Inc. Anti-p85 rabbit antiserum was purchased from Signal Transduction Lab. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G was from Zymed, and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G was purchased from Jackson Immuno Research.

Flow cytometric analysis. Cells were stained with biotin-conjugated anti-human CD2 (Pharmingen) and fluorescein isothiocyanate-conjugated anti-human CD4 (Becton Dickinson) antibodies in Hanks balanced salt solution containing 5% calf serum and 0.02% sodium azide. After being extensively washed, the cells were stained with TRICOLOR-labeled streptavidin (Caltag, South San Francisco, Calif.). Samples were analyzed by flow cytometry with a FACScan (Becton Dickinson).

RESULTS

Activation of NF-AT by CD2/ZAP-70. To study the functional consequences of ZAP-70 membrane localization, we have created chimeric molecules between human ZAP-70 and human CD2, a surface antigen of T-lymphocyte lineage (reviewed in reference 45). The extracellular and transmembrane regions with three cytoplasmic amino acids of CD2 were fused to the entire sequence of human ZAP-70 (Fig. 1). Several stable transfectants were established by using BW5147.hCD4, a BW5147 murine thymoma expressing exogenous human CD4 (Fig. 2A). Cross-linking of CD4 and CD2 with antibodies failed to activate these transfectants (data not shown). Surprisingly, two cell lines [2G6(WT) and 3G2(WT)] expressing the wild-type ZAP-70 chimera lost more than 90% of the expression of Lck (Fig. 2B). No significant change in expression was observed for other signaling molecules, such as Fyn, PLC-γ1, Grb2 (the p85 subunit of phosphatidylinositol 3-kinase), HS1,



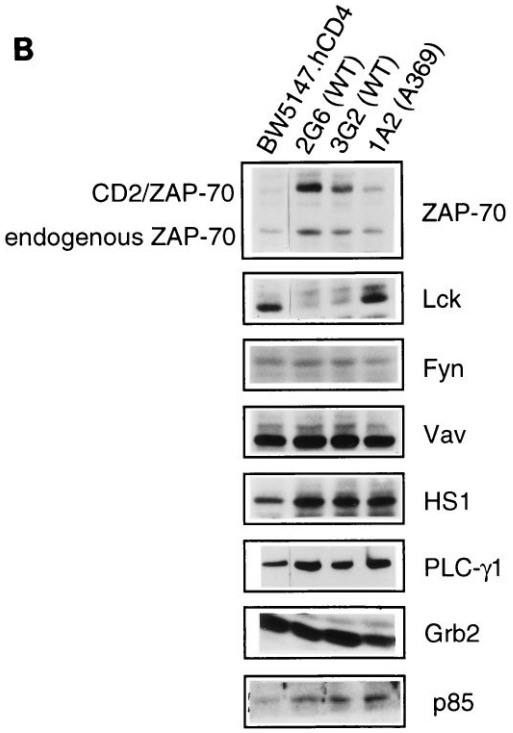


FIG. 2. Loss of Lck expression in the stable transfectants of CD2/ZAP-70. (A) Fluorescence-activated cell sorter analysis of CD2/ZAP-70 stable transfectants for CD4 and CD2 expression. The transfection host BW5147.hCD4; 3G2, a stable transfectant line with the wild-type CD2/ZAP-70; and 1A2, a stable transfectant line with the kinase-inactive CD2/ZAP-70 A369 mutation, were analyzed with anti-CD4 and anti-CD2. (B) Western blot analysis of the expression of CD2/ZAP-70 and endogenous proteins in CD2/ZAP-70 transfectants. Samples (10 μg) of total cellular lysates from BW5147.hCD4, the wild-type CD2/ZAP-70 transfectant lines (2G6 and 3G2), and the kinase-inactive CD2/ZAP-70 transfectant line (1A2) were analyzed by Western blotting for the expression of endogenous proteins. Each membrane was hybridized with the antibody shown on the right. CD2/ZAP-70 and endogenous ZAP-70 are indicated on the left for the membrane hybridized with anti-ZAP-70 antibody.

and Vav (Fig. 2B). Loss of Lck expression was not observed with cell lines expressing kinase-inactive CD2/ZAP-70 mutant A369 [an example of 1A2(A369) is presented]. Since T-cell tumors undergo growth arrest and apoptotic cell death when they are activated by TCR stimulation (8), we speculated that CD2/ZAP-70 induces activation signals that cause apoptotic cell death without any treatment and that cell lines established were selected for the loss of function in the downstream signaling events of ZAP-70 membrane recruitment.

To test this, CD2/ZAP-70 was expressed transiently in the Jurkat T-cell leukemia line and the activity of NF-AT, a transcription complex involved in early gene activation by TCR stimulation (reviewed in reference 21), was measured. When CD2/ZAP-70 was cotransfected with the reporter construct NF-AT-luc (50) in Jurkat cells, a significant increase of NF-AT-driven transcription activity was observed (Fig. 3A, left panel). The level of induction was comparable to that induced by the anti-TCR antibody (Fig. 3A, right panel). The kinase-inactive CD2/ZAP-70 chimera (A369) and nonchimeric wild-type ZAP-70 did not induce NF-AT activity.

Next, we investigated how ZAP-70 kinase activity contributes to the activation of NF-AT. Previous studies showed that kinase activity of ZAP-70 is greatly reduced when Y is changed to F at position 493. On the other hand, kinase activity is enhanced when Y is changed to F at 492 (14, 40, 72). Jurkat TAg cells were used as the host to enhance the expression of transfected genes. Figure 3B shows representative data from this transfection. Y-to-F mutation at Y-493 (F493 mutation) abolished the induction of NF-AT by CD2/ZAP-70, and Y-to-F mutation at Y-492 (F492 mutation) enhanced NF-AT induction by three- to fivefold over the wild type. The equivalent amount of chimeric protein was expressed in each transfectant (Fig. 3C). These results indicate that the level of NF-AT activation by CD2/ZAP-70 correlates with the kinase activity of ZAP-70.

In addition, FK-506 completely inhibited the NF-AT activation by CD2/ZAP-70 (data not shown). The effect was observed with as little as 0.1 ng of FK-506 per ml, the concentration required to block NF-AT activation in stimulated T cells (28, 43).

CD2/ZAP-70 functions independently from TCR expression. The association of ZAP-70 with phosphotyrosines of ITAMs is an essential step for T-cell activation via TCR stimulation. Binding of ITAMs and ZAP-70 induces autophosphorylation of ZAP-70 and facilitates binding sites for the SH2 domain of Src family kinases (49). For Syk, the kinase activity is induced by the association with ITAMs (57, 61). We investigated whether the association of CD2/ZAP-70 with ITAMs is required to induce NF-AT activity. A mutant cell line derived from Jurkat, J.RT.T3.5, lost surface expression of TCR because of a defect in

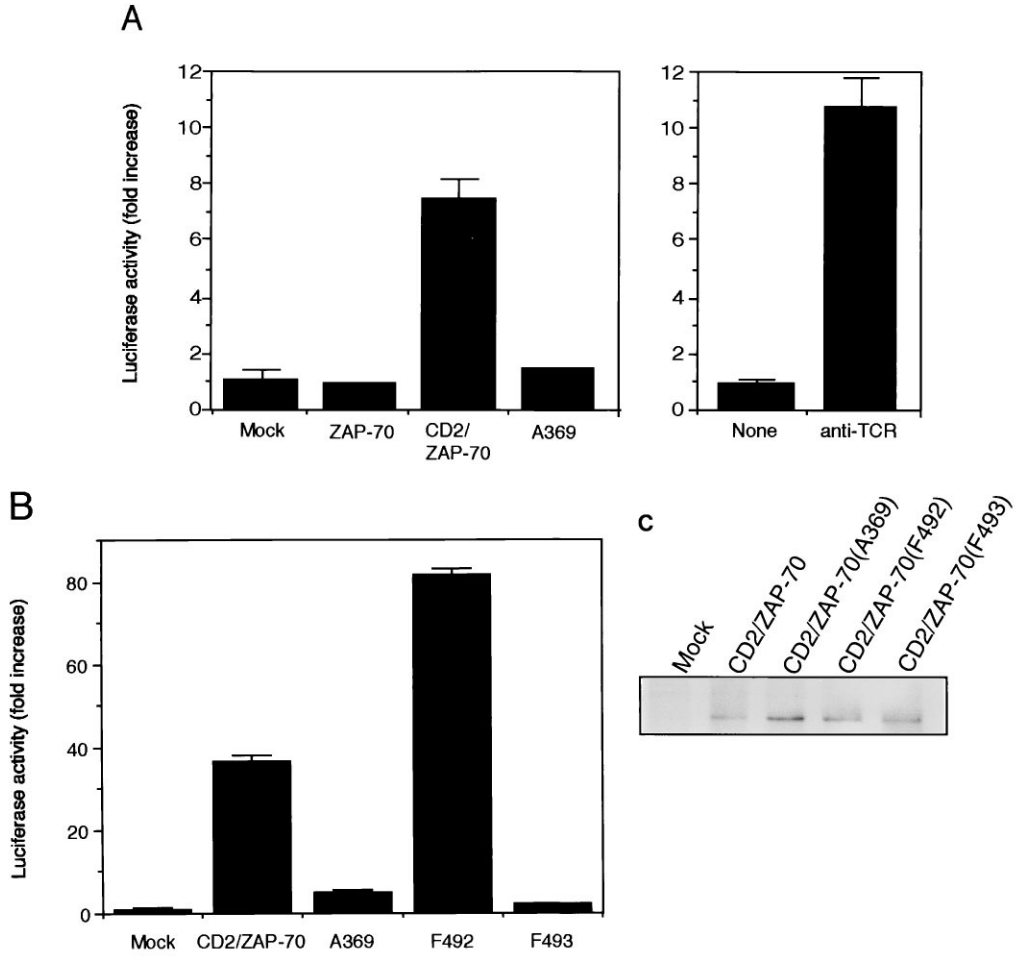


FIG. 3. Transient expression of CD2/ZAP-70 in Jurkat cells activates NF-AT-dependent transcription. (A) (Left panel) Jurkat cells were transiently transfected with NF-AT-luc and with expression vectors for no insert (Mock), wild-type ZAP-70, CD2/ZAP-70, and kinase-inactive CD2/ZAP-70 (A369). (Right panel) Jurkat cells were transiently transfected with NF-AT-luc and treated with medium alone (None) or anti-TCR MAb C305 (1:1,000 dilution of ascites) (anti-TCR). For each transfection condition, the percent activity against the maximal stimulation (induced by treatment with PMA and ionomycin) was calculated. The results are shown as fold induction of NF-AT response over the response in cells transfected with the empty vector. Each experiment was performed in triplicate more than once, and representative data are presented. (B) Jurkat TAg cells were transfected with NF-AT-luc and the expression vectors for no insert (Mock), CD2/ZAP-70, and three kinase domain mutants of CD2/ZAP-70 [(A369), (F492), and (F493)]. Induced luciferase activity was determined for each transfectant as for panel A. (C) Expression constructs for CD2/ZAP-70 and its mutants produce equivalent amounts of chimera proteins. Jurkat TAg cells were transiently transfected as for panel B with the expression constructs shown above each lane. CD2/ZAP-70 chimeras were immunoprecipitated with anti-CD2 and analyzed by Western blotting with anti-ZAP-70 MAb 2F3.2.

the TCR β chain (76). When this cell line was transfected with CD2/ZAP-70, we observed the induction of NF-AT activity as well as in the wild-type Jurkat cells (Fig. 4A).

Since the ζ chain can be expressed on the cell surface without forming a complex with TCR (4, 41), it was possible that CD2/ZAP-70 interacts with ζ to initiate signal transduction. To test this, we introduced mutations that abolish the ability of ZAP-70 to bind to phosphotyrosines of ITAMs and investigated whether these mutations would impair the level of activation (R-37 and R-190, which are critical for binding to phosphotyrosine, were mutated to K [K37K190 mutation]). As shown in Fig. 4B, mutant K37K190 induced NF-AT activity as well as the wild-type chimera. These results demonstrate that CD2/ZAP-70 does not require TCR expression or ITAM binding for the NF-AT activation.

Since CD2 is known as an activation molecule for T cells (reviewed in references 10 and 45), we tested whether the CD2 part of this chimeric molecule is contributing to the activation process. The N-terminal 14-amino-acid sequence from Src, which includes the sufficient signal for myristylation and membrane localization (reviewed in reference 55), was used as the fusion partner. To avoid further complexity by the function of SH2 domains, the N-terminal region of ZAP-70 containing both SH2 domains (residues 1 to 263 of ZAP-70) was deleted (Fig. 1, Src/ZAP-70). When expressed in Jurkat cells, Src/ZAP-70 induced NF-AT activity at a level comparable to that of CD2/ZAP-70 (Fig. 4C). Thus, neither the CD2 portion nor the SH2 domains of ZAP-70 are required for NF-AT activation.

Lck is essential for the induction of NF-AT activity by CD2/ZAP-70. To understand how CD2/ZAP-70 initiates the signaling pathway that activates NF-AT, we investigated whether Lck plays a role in the process. Involvement of Lck was suggested by the observation that two CD2/ZAP-70 stable transfectant lines of BW5147.hCD4 expressed greatly reduced levels of Lck (Fig. 2B). A mutant Jurkat line that had lost the expression of Lck, J.CaM1.6 (33, 64), was transfected with CD2/ZAP-70. As shown in Fig. 5A (left panel), no increase of NF-AT activity was observed when J.CaM1.6 was transfected separately with the expression construct for CD2/ZAP-70 or Lck. However, when cells were transfected with a mixture of

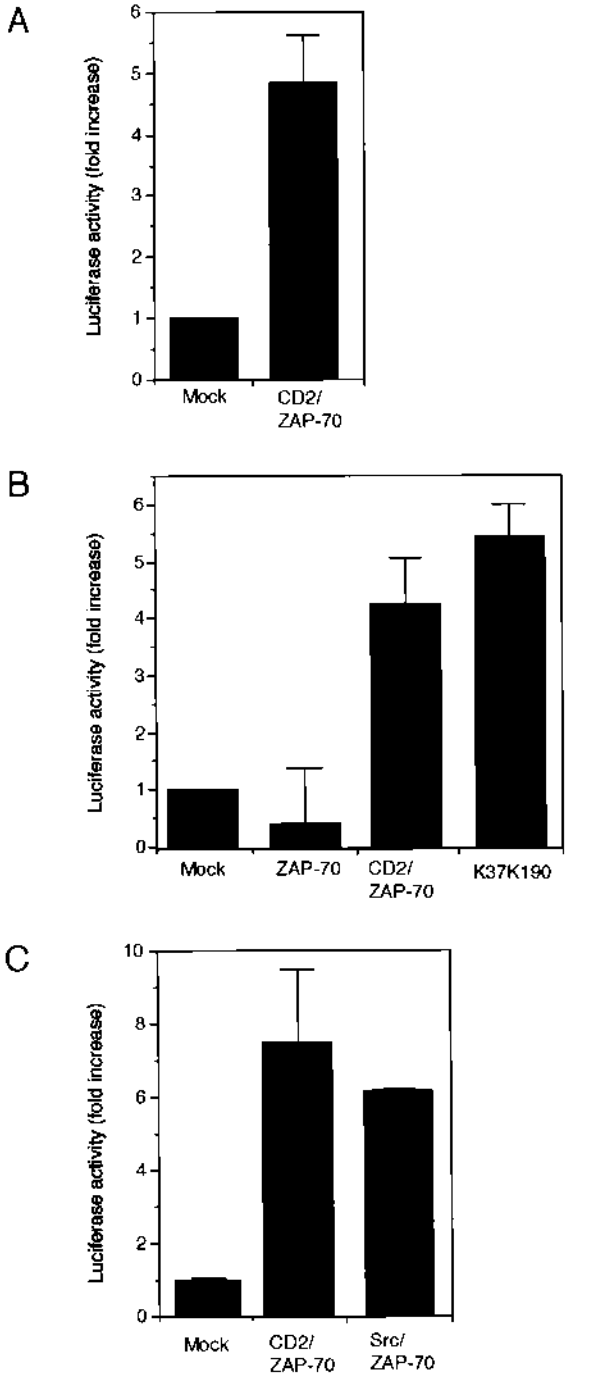


FIG. 4. Membrane-localized forms of ZAP-70 activate NF-AT without TCR expression and without SH2 domains. (A) J.RT3.T3.5, a Jurkat mutant line that lost the expression of the β chain of TCR, was transiently transfected with constructs CD2/ZAP-70 and NF-AT-luc. Induced luciferase activity was measured as described for Fig. 3. (B) Jurkat cells were transfected with NF-AT-luc and the expression vectors for no insert (Mock), ZAP-70, CD2/ZAP-70, and K37K190. Induced luciferase activity was determined for each transfectant as for panel A. (C) Jurkat cells were transfected with NF-AT-luc and the expression vectors for no insert, CD2/ZAP-70, and Src/ZAP-70. Induced luciferase activity was determined as described above.

these expression constructs, a significant level of NF-AT activity was induced. The same requirement for Lck was observed for Src/ZAP-70 (Fig. 5A, right panel). This shows that Lck plays an essential role in the signaling process initiated by membrane-localized ZAP-70.

We also tested whether the NF-AT activity in the CD2/ZAP-70 stable transfectants that had lost the expression of endogenous Lck can be induced by expressing exogenous Lck. 2G6(WT), a CD2/ZAP-70 stable transfectant that expresses a minimum level of endogenous Lck (Fig. 2B), and 2B2(A369), a stable transfectant line expressing kinase-inactive CD2/ZAP-70 mutant A369, were cotransfected with NF-AT-luc and the expression construct for Lck. When exogenous Lck was expressed in 2G6(WT), a marked increase of NF-AT activity was observed (Fig. 5B, left panel). A similar increase was observed with 3G2, another stable transfectant of CD2/ZAP-70 (data not shown). In contrast, Lck transfection did not induce any NF-AT activity in 2B2(A369) (Fig. 5B, right panel).

These results clearly demonstrate that Lck is essential for induction of NF-AT-dependent transcription by CD2/ZAP-70. Thus, Lck functions not only for the recruitment of ZAP-70 to ITAMs, but also for the signaling process following the recruitment.

Requirement for the kinase, SH2, and SH3 domains of Lck. We investigated what function of Lck is required for NF-AT activation by CD2/ZAP-70. For this purpose, loss-of-function mutations were introduced into the kinase (K-273 to A [A273] and Y-394 to F [F394]), SH2 (R-154 to K [K154]), and SH3 (P-112 to L [L112]) domains of Lck (Fig. 6A). Mutations at the site critical for phosphotransferase activity (A273 mutation) and at the site for autophosphorylation (F394 mutation) abolished the kinase activity of Lck, as previously reported (1, 36). R-154 is a critical residue for the SH2 domain to facilitate its binding to the phosphotyrosine-containing ligand. Mutation of this R to K abrogates this interaction (for a review, see reference 51). The L112 mutation corresponds to the *sem-5* mutation (*n1619* allele) of *Caenorhabditis elegans*, a loss-of-function mutation of an SH3 domain of Sem5 (18).

The effect of each mutation was tested by transiently transfecting 2G6(WT) with the expression plasmid for mutated Lck and NF-AT-luc (Fig. 6B). Expression of the kinase-inactive forms of Lck, A273 and F394, failed to induce NF-AT-dependent transcription although equivalent amounts of protein were expressed (Fig. 6C). This shows that kinase activity of Lck is essential for the signaling pathway which is initiated by CD2/ZAP-70. In addition, an increase of tyrosine phosphorylation of cellular proteins as well as CD2/ZAP-70 was observed with the wild-type Lck transfectants but not with the kinase-inactive Lck transfectants (Fig. 6D).

A remarkable effect of mutation was observed with the SH3 domain mutation, L112. Transfection of L112 cells resulted in induction of a minimum amount of NF-AT activity. The SH2 domain mutation, K154, also reduced the signal significantly. These observations were constant throughout a number of experiments. Both K154 and L112 Lck proteins were expressed at a level equivalent to that of the wild-type Lck (Fig. 6C). In addition, we observed no significant alteration of kinase activity with K154 and L112 (data not shown). These results show that SH2 and SH3 domains of Lck function independently from the kinase domain in this signaling process.

DISCUSSION

We have demonstrated here that Lck is essential for NF-AT activation initiated by membrane-localized forms of ZAP-70. To analyze how ZAP-70 facilitates the signaling process when

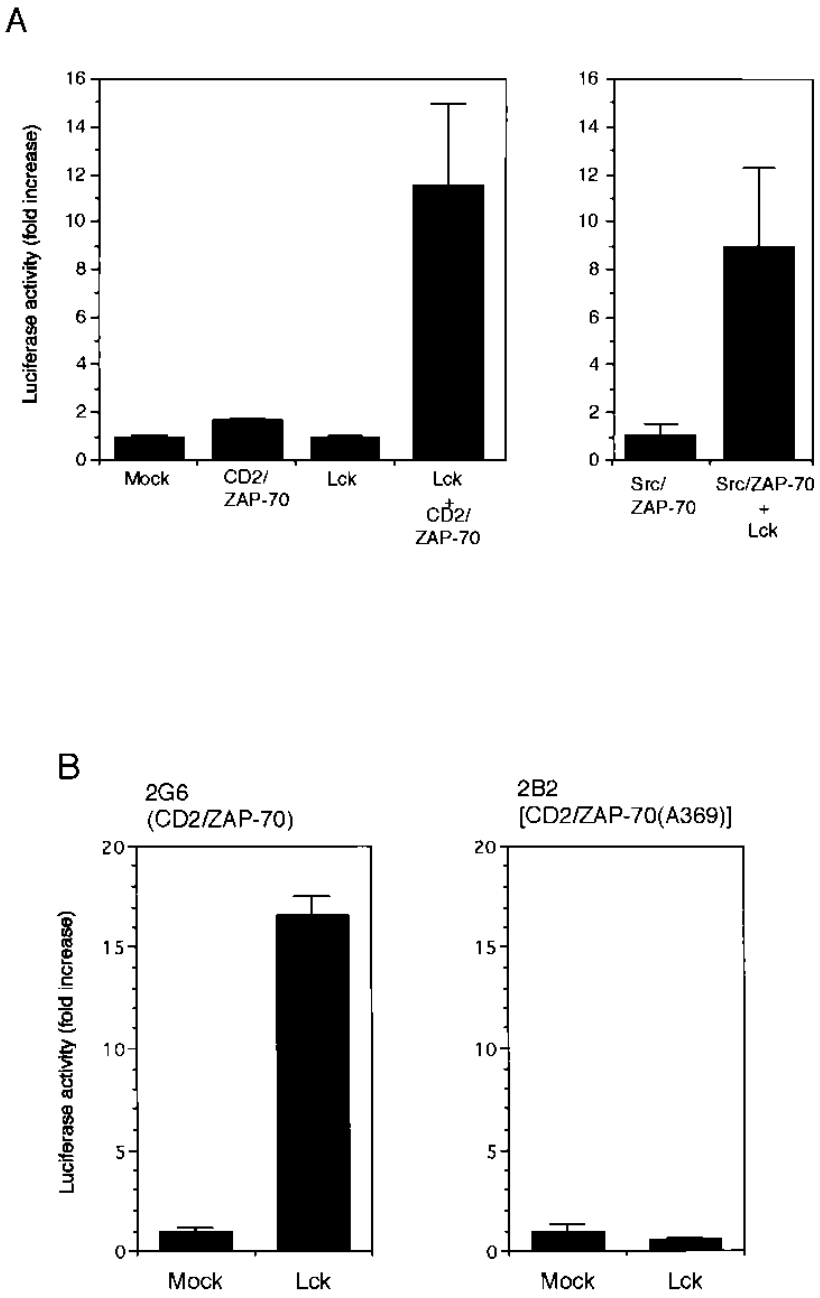


FIG. 5. CD2/ZAP-70 requires Lck for the activation of NF-AT-dependent transcription. (A) J.Cam1.6, a Lck-less mutant line of Jurkat, was transiently transfected with expression vectors for CD2/ZAP-70 alone, Lck alone, and the mixture of the two (Lck + CD2/ZAP-70) or with vectors for Src/ZAP-70 alone and the mixture of Src/ZAP-70 and Lck (Src/ZAP-70 + Lck), along with NF-AT-luc. Induced luciferase activity was determined as described for Fig. 3. (B) A stable transfectant line of BW5147.hCD4 with CD2/ZAP-70, 2G6, and a stable transfectant line with kinase-inactive CD2/ZAP-70 mutant A369, 2B2, were transfected with the expression construct for Lck and NF-AT-luc. Induced luciferase activity was determined as described above.

recruited to the plasma membrane by TCR stimulation, a chimeric molecule consisting of ZAP-70 and a cell surface antigen, CD2, was constructed and expressed in a murine thymoma line, BW5147. Unexpectedly, stable transfectants that express high levels of CD2/ZAP-70 lost the majority of the expression of endogenous Lck. We found that transient expression of CD2/ZAP-70 induced a significant level of NF-AT-driven transcription without any stimulation. Neither TCR expression nor association with ITAMs was necessary for this process, whereas expression of Lck was essential. Introduction of exogenous Lck into cell lines that had lost the expression of endogenous Lck functionally reconstituted the signal for NF-AT activation by CD2/ZAP-70. In this reconstitution system, each mutation in the kinase, SH2, and SH3 domains of Lck impaired the signal significantly. These results provide the first in vivo evidence that Lck is an essential element for signal transduction that follows the membrane recruitment of ZAP-70 and suggest insight into the role Lck plays.

The data presented here clearly demonstrate that the function of Lck as a kinase is essential for signal transduction

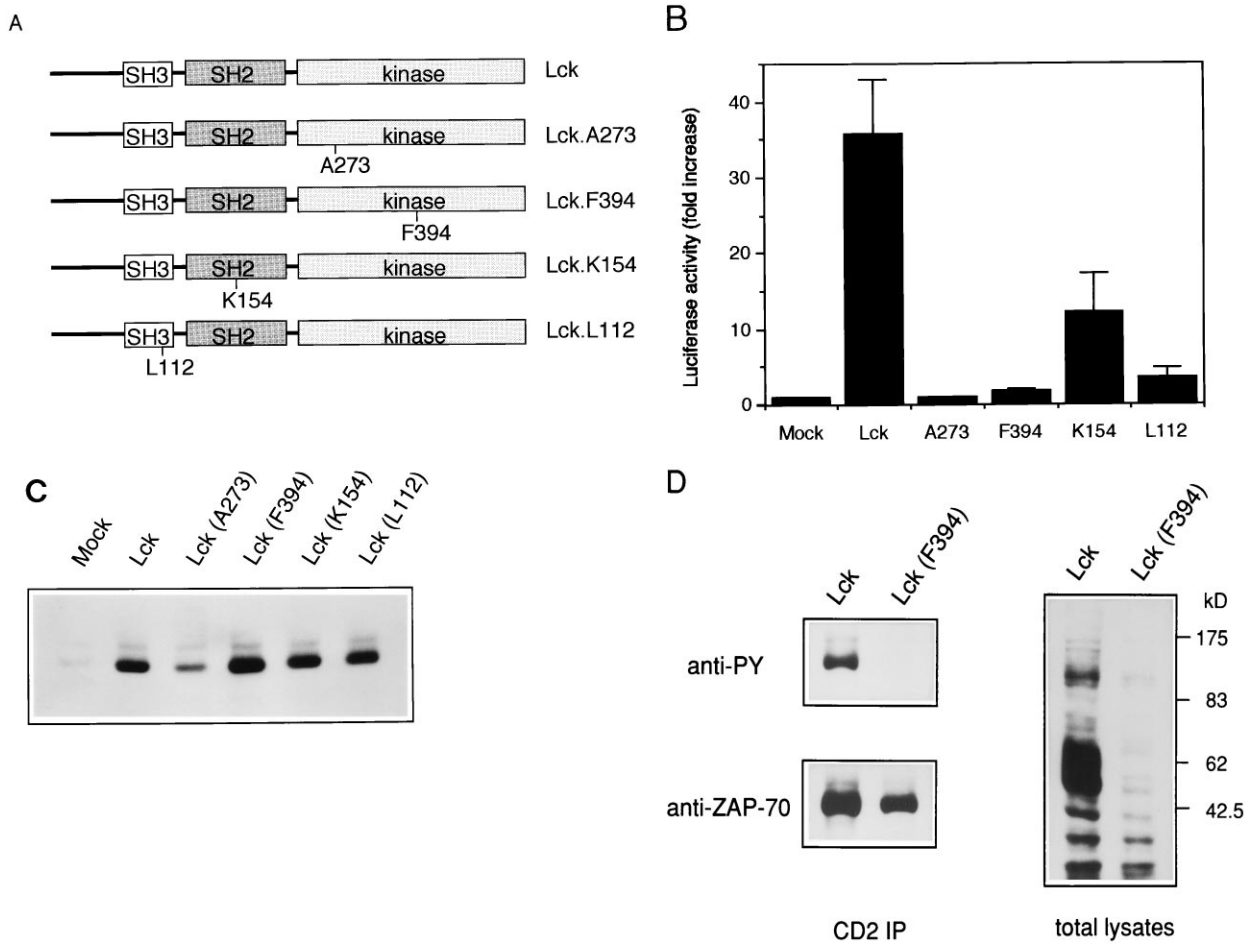


FIG. 6. The kinase, SH2, and SH3 domains of Lck play critical roles for NF-AT activation by CD2/ZAP-70. (A) Schematic representations of wild-type and mutated forms of Lck used in this study. Functionally critical amino acids for the kinase, SH2, and SH3 domains are changed as indicated. (B) 2G6, the stable transfectant of BW5147.hCD4 with CD2/ZAP-70, was transiently transfected with expression constructs for wild-type Lck (Lck), kinase-inactive forms of Lck (A273 and F394), SH2-mutated Lck (K154), and SH3-mutated Lck (L112). Each construct was cotransfected with NF-AT-luc, and the induction of NF-AT activity was determined as described above. (C) Aliquots of 2G6 total cell lysates transfected in panel B were analyzed by Western blotting to confirm that equivalent levels of Lck are expressed by the constructs. (D) 2G6 was transiently transfected with wild-type Lck or a kinase-inactive form of Lck (F394). Each transfectant was harvested 10 h after transfection and analyzed by Western blotting. (Left panels) CD2/ZAP-70 was immunoprecipitated, and its tyrosine phosphorylation was determined by Western blotting with antiphosphotyrosine MAb 4G10 (anti-PY). The same precipitates were analyzed with anti-ZAP-70 MAb 2F3.2. (Right panel) Total cell lysates were analyzed by Western blotting with 4G10. IP, immunoprecipitate.

initiated by CD2/ZAP-70. Y-493 of ZAP-70 is a likely site of phosphorylation to activate this kinase. As shown previously, this Y is one of the major phosphorylation sites induced by TCR stimulation (14, 74). Coexpression of Lck and ZAP-70 in either SF9 cells or COS cells induced heavy phosphorylation at this residue (14, 72). Finally, mutation of Y to F greatly reduces ZAP-70 kinase activity and the signaling function of CD2/ZAP-70 (references 14 and 72 and this study).

The roles of the SH2 and SH3 domains of Lck are less well characterized than the function of the kinase domain. Deletion of either the SH2 or the SH3 domain blocked Lck.F505 (an activated form of Lck with a Y-to-F mutation at the negative-regulatory Y-505) from enhancing TCR-induced interleukin 2 production (13). A recent study also showed that the SH2 domain of Lck plays an essential role for TCR signal transduction (65). A study using a CD4-Lck chimera showed that the K154 mutation of the SH2 domain partially impaired the ability of the chimera to potentiate the antigen-induced activation of a T-cell line (78). In the case of the latter, since the SH2 domain of Lck can bind tyrosine-phosphorylated ZAP-70, it was illustrated that Lck might bring in the coreceptor molecule CD4 or CD8 into the TCR complex by associating with tyrosine-phosphorylated ZAP-70 (78). Clustering of CD4 with TCR in a manner dependent on Lck was also reported for Jurkat cells (69). However, in our study, there is no requirement for the clustering of CD4 or CD8 with TCR. Thus, the SH2 domain of Lck must play another role in signal transduction.

The L112 mutation of Lck introduced in this study may impair the ability of the SH3 domain to bind to its target. This amino acid change corresponds to the mutation found in the *n1619* allele of *sem-5* (18). Structural analysis of the SH3 domain revealed that the residue corresponding to P-112 of Lck exists in the pocket which makes contact with the ligand (27). The same mutation at this residue of the SH3 domain abrogated association of Src with various molecules (26). A number of molecules have been shown to bind the SH3 domain of Lck, such as HS1 (9, 30, 31, 64, 79), p120 Cbl (23, 29, 58), p120 Ras-GAP (2, 3, 11), Ras-GAP-associated molecules p62 and p190 (3), and phosphatidylinositol 3-kinase (53, 71). It should

be noted that Btk, a member of the Itk-Btk family, binds the SH3 domains of Src family kinases (17, 80). With this mutation, the SH3 domain of Lck fused to glutathione transferase lost its ability to bind to a number of molecules (not shown). Thus, we speculate that the L112 mutation affected NF-AT activation by abrogating the association of the SH3 domain of Lck and its ligands.

One possibility that explains our observation is that Lck functions as an adapter molecule and also as a kinase. Association between Lck and ZAP-70 was detected in T cells activated by anti-CD3 antibody (24, 69). Thus, one of the functions of Lck may be to bind tyrosine-phosphorylated ZAP-70 via the SH2 domain and bring SH3-binding proteins into the TCR complex. Association between Lck and ZAP-70 could enhance the kinase activity of Lck (20). Thereafter, the complex formation, along with ZAP-70 activation, could lead to tyrosine phosphorylation of SH3-binding proteins such as Cbl and initiation of the following process. Indeed, many of these SH3-binding proteins are heavily tyrosine phosphorylated after TCR stimulation. This possibility is also in agreement with data presented previously (65, 78).

Interestingly, no T-cell activation was observed even when ZAP-70 was localized to the plasma membrane in thymic and peripheral T cells (70) and in T-cell lines stimulated with antagonistic peptides (44, 62). The difference between the data of the previous studies and our data may be derived from the difference in the amount of ZAP-70 recruited to the membrane. In our system, all chimeric proteins expressed on the cell surface localize ZAP-70 to the plasma membrane, whereas only a minor portion of ZAP-70 may be in association with the surface TCR under the conditions in the previous studies. Alternatively, but not exclusively, the expression and activity of Lck in the tumor lines used here may be higher than those of the T cells used in the earlier studies. Indeed, it was shown recently that the availability of Lck in the thymus for TCR signal transduction is limited (77).

Another approach using a chimeric molecule of ZAP-70 showed that T-cell activation was observed only when the ZAP-70 chimera was clustered with another chimera of CD16-CD7-Lck (39). Methods used for the experimental system could contribute to the difference between those data and ours. In this study, we introduced CD2/ZAP-70 into lymphoma lines by electroporation along with the reporter construct which contains the triplicated NF-AT sites to enhance the sensitivity (50). Kolanus et al. (39) transfected T-cell lines with viral vectors and analyzed the function of their chimeras by methods such as antibody-mediated cytotoxicity and the change of intracellular Ca^{2+} in a cytotoxic T-cell line. These assays are more standard for measuring T-cell activation but less sensitive than the transcriptional assay we used.

Although the requirement for clustering is different, all data agree on the point that steps other than the membrane localization of ZAP-70 are required to activate T cells. The data presented here illustrate that Lck is one of the key elements involved in the process that follows the recruitment of ZAP-70 by phosphorylated ITAMs. One of the functional roles of Lck appears to be tyrosine phosphorylation of ZAP-70 to activate the kinase. In addition, both SH2 and SH3 domains of Lck are necessary for efficient signaling. This indicates that Lck may play a role as an adapter molecule to recruit downstream signaling molecules to the TCR complex and induce their tyrosine phosphorylation. Whether these proteins are direct substrates for ZAP-70 is now under investigation.

ACKNOWLEDGMENTS

We are very grateful to Arthur Weiss, Dan Littman, Gerald Crabtree, Takeshi Watanabe, Yoshihiro Takemoto, Kazuo Maruyama, Yasuhiro Koga, and Nobukata Shinohara for reagents; to Kaoru Matsuzaki, Kaoru Nasu, Sachiko Ohta, and Yuko Ikegami for excellent technical assistance; to Fumie Sahira for secretarial assistance; to Noemi Fusaki for helpful suggestions; and to Shintaro Iwashita, Hidetaka Yakura, John Gill, and Kathy Adams-Iwashima for critical reading of the manuscript. We are also grateful to Nobukata Shinohara and Minoru Muramatsu for continuous encouragement.

We thank the Mitsubishi Kasei Institute for Life Sciences and the Mitsubishi Chemical Corporation Yokohama Research Center for generous support.

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