The Src-like protein-tyrosine kinase Fyn is associated with T-cell antigen receptor. Transient expression of actively mutated Fyn, having Phe-528 instead of Tyr-528 or Thr-338 instead of Ile-338, in Jurkat T-cells stimulated the serum response element (SRE), 12-O-tetradecanoylphorbol-13-acetate response element, cyclic AMP response element, and c-fos promoter. The stimulation of SRE was particularly prominent not only with active Fyn but also with normal (wild-type) Fyn. SRE was also stimulated by both normal and active Lck. Furthermore, normal and active Fyn stimulated transcription from the IL-2 gene promoter when transfected cells were stimulated by concanavalin A plus 12-O-tetradecanoylphorbol-13-acetate. Under the same conditions, Lck did not stimulate IL-2 promoter unless it was activated by mutation. Interestingly, a mutant Fyn, which has deletions within the SH2 region and so is able to transform chicken embryo fibroblasts, did not stimulate either the c-fos or IL-2 promoter, suggesting the importance of this region in T-cell signaling. Csk, which phosphorylates tyrosine residues in the negative regulatory sites of Src family kinases, down-regulated Fyn- and Lck-mediated stimulation of the serum response element and Fyn-mediated enhancement of IL-2 promoter activity. These data suggest that Fyn and Lck, whose activities are regulated by Csk, are involved in different phases of T-cell activation.

The Src-like kinases are generally associated with the internal portion of the plasma membrane and are suggested to play crucial roles in surface receptor-mediated signaling (1-8). Since the enzymatic activities of Src-like kinases are regulated by phosphorylation and dephosphorylation of tyrosine residues in their carboxyl-terminal regions (1, 2), it is important to identify the enzymes responsible for these activities. Recently an enzyme that phosphorylates a tyrosine residue close to the carboxyl terminus of c-Src, tyrosine 527, was purified from the membrane fraction of neonatal rat brain and named Csk (9). Csk can also phosphorylate other members of the Src family such as Fyn and Lyn at the conserved tyrosine residue within the carboxyl-terminal region. Consequently, Csk down-regulates the kinase activities of Src, Fyn, and Lyn in vitro (10) and suppresses cell transformation by a combination of c-Src and Crk (11). Recent evidence also shows that Csk is able to rescue the c-Src-induced lethal phenotype of Saccharomyces pombe, presumably by regulating kinase activity of the c-Src protein (12). Csk is expressed in lymphoid tissues and in neural brain (10), which is rich in Src family kinases (1, 2, 13). Therefore, Csk may negatively regulate the functions of these Src-like kinases in lymphoid and neural cells.

The response of T-cells to antigen bound to the antigen receptor (T-cell receptor (TCR))-major histocompatibility complex consists of a series of cellular events that result in expression of a number of genes including the c-fos and IL-2 genes (14). There are reports that activation of protein-tyrosine kinases following TCR stimulation is required and precede these intracellular events (15). In most cases TCR consists of an a/b heterodimer and CD3 subunits that are g, 6, and (and/or y) chains. However, none of these molecules possesses intrinsic protein-tyrosine kinase activity. Recent evidence suggests that Src-like protein-tyrosine kinases couple the TCR for the signaling events (15). In particular, the Fyn protein, which is expressed in mature T-cells, physically associates with the CD3 complex (16). The importance of Fyn in TCR-mediated signal transduction is further suggested by the following studies. By use of a transgenic mouse system, thymocytes expressing a high level of the Fyn transgene were shown to be hyperstimulatable (17). Studies using fn+ mice revealed that TCR-mediated signaling was defective in mature single positive (CD4+CD8- or CD4+CD8+) thymocytes, although the signaling was still present in immature thymocytes and peripheral T-cells (18, 20). The latter data suggest that fn is critically important in TCR-mediated signaling, at least in a developmentally restricted subpopulation of thymocytes. In addition, our unpublished data suggest that IL-2 production is elevated in T-cell hybridomas expressing high levels of wild-type and actively mutated Fyn upon mitogenic stimulation. To obtain further details of the molecular mechanisms of T-cell signaling, we have examined the effects of Fyn and Csk expression on the activities of the c-fos and IL-2 promoters.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Human Jurkat T-cells, donated from T. Taniguchi (Osaka University, Japan) and Class II-restricted mouse helper T-cell hybridoma HBC21.7.31 and its derivatives were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The monkey

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1 The abbreviations used are: TCR, T-cell receptor; CAT, chloramphenicol acetyltransferase; ConA, concanavalin A; TPA, 12-O-tetradecanoylphorbol-13 acetate; SRE, serum response element; TRE, TPA response element; CRE, cyclic AMP response element; SH2, Src homology-2.

2 N. Fusuki, S. Matsuda, G. Suzuki, K. Semba, T. Katagiri, and T. Yamamoto, manuscript in preparation.
Regulation of T-cell Signaling by Protein-tyrosine Kinases

A

![Diagram of protein kinase activity](image)

B

Brain-type: RAAGLCRLVYVFCHKMPRLTDLYVKTQWEPLSIQLRGLNGQEGYVW

Thymus-type: K+D++F NT-T-1A5S CT-Qt-+GLA+VA-+B+C K+Q+CA+L

![Image of protein kinase activity](image)

Kidney fibroblast cell line CV-1 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

Construction of Expression Plasmids—The expression vector pmE185M containing Srα promoter (26) was a gift from K. Maruyama (Tokyo University). The complementary DNAs encoding T-Fyn were obtained by following the modifications of pSRD-FN (21), which contained 1.8-kilobase pair B-fyn cDNA. A sequence corresponding to exon 7A (22) specific to brain type Fyn was replaced by that corresponding to exon 7B (22), which was synthesized by reverse transcription of mRNA from Jurkat T-cells followed by polymerase chain reaction (23). The primers used for polymerase chain reaction were 5'-CCACCCGcGc-

Phylogenetic analysis of amino acid sequences is shown in Fig. 1. B-fyn, 1-fyn, and f-14/fyn cDNAs were excised from pSRD vector (21) and recloned into pME185M at the MluI site. The DNA fragments of the lck cDNA (25) obtained from T. Shimotohno (National Cancer Center, Tokyo), the lyn cDNA (13), and the csk cDNA (26) were converted to the MluI fragments by attaching the MluI linker (Life Technologies, Inc.), which were then recloned into the MluI site of pME185M. Tyrosine to phenylalanine mutation at amino acid positions 528 and 531 in the authentic sequence of exon 7B was determined by the standard dideoxy chain termination method. The deduced amino acid sequence of exon 7B was compared with that of normal T-Fyn.

Fyn-The cDNA of active T-fin (T-FynF, Fig. 2) was replaced by that corresponding to exon 7B of human B-fyn and T-Fyn, respectively, and compared. Only amino acid residues of T-Fyn that differ from the corresponding residues of B-Fyn are indicated. Dots show the identical residues between the two Fyns. Dashes show the residues missing in T-Fyn.

Fig. 1. Schematic representations of wild-type and mutant fyn gene products. A, the structures of B-Fyn and its derivatives (f-14 and f-1) are according to Sema et al. (21), and that of T-Fyn was obtained by determining the nucleotide sequence of exon 7B (22) of the human fyn gene. The transforming activity of each Fyn is as determined on chick embryo fibroblasts (22). Relative kinase activity was determined by measuring the level of in vitro autophosphorylation of each Fyn immunoprecipitated from CV-1 cells that had been transfected with the expression plasmid containing corresponding fyn cDNA. Tyrosine to phenylalanine mutation at amino acid positions 528 and 531 is indicated as Y528F and Y531F, respectively, and isoeucine to threonine mutation at position 538 as I338T.

B-Fyn (Brain-type) 
B-FynF (Y552F) 
T-Fyn (Thymus-type) 
T-FynF (Y552F) 

Transforming Activity 

Kinesin Activity 

normal resting T-cells (34). Transfection of T-FynF significantly increased the c-fos promoter activity (Table I). Since the c-fos promoter contains the serum response element (SRE), the TPA response element (CRE), and the cyclic AMP response element (CRE) (35), we examined which element was responsible for the T-FynF-mediated stimulation of the c-fos promoter. For this, we transfected the T-FynF construct into Jurkat cells together with the SREC, TREC, or CRECAT reporter plasmid (28). Results showed that T-FynF stimulated these elements and the stimulation of SRE was more than that of TRE and CRE (Fig. 2).

RESULTS

Stimulation of c-fos Promoter and IL-2 Promoter Activities by Kinase-active Fyn—The cDNA of active T-Fyn (T-FynF, Fig. 1) that encodes thymus-type Fyn (22) with Phe-528 instead of Tyr-528 was cloned into the expression plasmid pME185M containing a chimeric promoter (SRα) of the SV40 and human T lymphotropic virus-I promoters (20). Reporter plasmid c-fos CAT (28) was cotransfected with the T-FynF expression plasmids into Jurkat T-cells, which were chosen because of the similarity of their response to TCR stimulation to that of normal resting T-cells (34). Transfection of T-FynF significantly increased the c-fos promoter activity (Table I). Since the c-fos promoter contains the serum response element (SRE), the TPA response element (TRE), and the cyclic AMP response element (CRE) (35), we examined which element was responsible for the T-FynF-mediated stimulation of the c-fos promoter. For this, we transfected the T-FynF construct into Jurkat cells together with the SREC, TREC, or CRECAT reporter plasmid (28). Results showed that T-FynF stimulated these elements and the stimulation of SRE was more than that of TRE and CRE (Fig. 2).
were subjected to CAT assay as described (30, 31). The transfectants of IL-2CAT were treated with ConA (30 pg/ml) and TPA (50 ng/ml) for (Fos), SRECAT (SRE), TRECAT (TRE), CRECAT (CRE), (Cod
ZL-2 promoter (Table I). In this system, the phorbol ester TPA
kat cells transfected with T-fynF showed little activation of
vector plasmid (Table I). This result indicates that elevated
in independent experiments:
TPA treatment, CD3 cross-linking induced significant transac-
by TPA and a mitogen such as ConA
stimulation of the ZL-2 promoter only when the transfectants
transfected into Jurkat cells stimulated SRECAT and caused
increased kinase activity of T-FynF markedly stimulated the
ZL-2 promoter. For comparison, the effect of the
in T-cells and can contribute to T-cell signaling
important to clarify whether Fyn and Lck regulate the signal-
into Jurkat T-cells (2 x 10^6) together with reporter plasmids, c-fosCAT (Fos), SRECAT (SRE), TRECAT (TRE), CRECAT (CRE), or IL-2CAT (5 μg) as described (28, 29). The lysates (80 μg of protein) of the transfectants were subjected to CAT assay as described (30, 31). The transfectants of IL-2CAT were treated with ConA (30 μg/ml) and TPA (50 ng/ml) for 8 h (ConA + TPA) before cell harvest. Relative CAT activities of Fyn transfectants in comparison with mock (vector) transfectants are presented.

| Enhancer/promoter | Fos | SRE | TRE | CRE | IL-2         |
|-------------------|-----|-----|-----|-----|-------------|
| None              | 1.0 | 0.7%| 1.0 | 0.5%| 1.0 (0.5%)  |
| ConA + TPA        | 1.0 | 4.2 | 1.1 | 4.0 | 1.1 (1.1%)  |

| Vector | 1.0 (4.1%)^a | 1.0 (0.7%) | 1.0 (3.2%) | 1.0 (0.5%) | 1.0 (0.3%) |
|--------|--------------|------------|------------|------------|------------|
| T-Fyn  | 2.2          | 9.6        | 1.8        | 1.3        | 1.0        |
| T-FynF | 5.7          | 20.4       | 4.0        | 2.7        | 1.1        |
| B-Fyn  | NT^a         | 8.2        | NT         | NT         | 1.1        |
| B-FynF | NT           | 13.2       | NT         | NT         | 1.1        |
| f-14   | NT           | 12.7       | NT         | NT         | 1.1        |
| t-1    | NT           | 1.0        | NT         | NT         | 1.0        |

^a Numbers in percentage indicate values of percent conversion of [14C]chloramphenicol to its acetylated form. All values were average of three independent experiments.

^ NT, not tested.

Table 1

Regulation of T-cell Signaling by Protein-tyrosine Kinases

Various Fyn cDNA constructs (5 μg) in pME18SM vector were transfected into Jurkat T-cells (2 x 10^6) together with reporter plasmids, c-fosCAT (Fos), SRECAT (SRE), TRECAT (TRE), CRECAT (CRE), or IL-2CAT (5 μg) as described (28, 29). The lysates (80 μg of protein) of the transfectants were subjected to CAT assay (Table I). These data suggested that the c-fos promoter was activated by T-FynF primarily through SRE. In contrast, Jurkat cells transfected with T-fynF showed little activation of CAT activity of IL-2 CAT over the cells cotransfected with the vector plasmid (Table I). This result indicates that elevated tyrosine kinase activity alone is not sufficient for induction of the IL-2 gene. However, when T-cells cotransfected with IL-2 CAT and the T-fynF constructs were stimulated simultaneously by TPA and a mitogen such as ConA or phorbol ester, enhanced kinase activity of T-FynF markedly stimulated the IL-2 promoter (Table I). In this system, the phorbol ester TPA provided a second signal that is required for TCR-mediated mitogenesis. Next, we cross-linked the CD3 molecule with anti-CD3ε antibodies on the surface of the Jurkat T-cells that had been transfected with the IL-2 CAT and T-fynF constructs. On TPA treatment, CD3 cross-linking induced significant transactivation of the IL-2 promoter (Fig. 3). Treatment with TPA and anti-CD3ε without exogenous T-fynF had little effect. Thus we concluded that increased kinase activity of Fyn augments transcription of the IL2 gene upon T-cell activation.

Effects of Normal Fyn, Lck, and Lyn on SRE and IL-2 Promoter—Previous reports show that active forms of other Src family members, namely F505 p56^Lck (36) and v-Src (37), enhance T-cell responsiveness. As p56^Lck is abundantly expressed in T-cells and can contribute to T-cell signaling (3–5, 38), it is important to clarify whether Fyn and Lck regulate the signaling through similar or different mechanisms. Then we examined the effects of normal Fyn and normal Lck on SRE and the IL-2 promoter. For comparison, the effect of the lyn gene product, which is not expressed in normal T-cells, was also tested. Two forms of normal lyn cDNAs, T-fyn and B-fyn (Fig. 1) (22), transfected into Jurkat cells stimulated SRECAT and caused stimulation of the IL-2 promoter only when the transfectants were stimulated with TPA plus ConA (Table I). No significant difference was observed between T-fyn and B-fyn. SRE was also stimulated by normal and active F505Lck (Table II). Furthermore, as c-fos CAT was transactivated by Lck to a similar extent as by Fyn (Fig. 2B), we tentatively concluded that the c-fos gene is also regulated by Lck most likely through SRE on T-cell activation. Active Lyn (F508Lyn) also stimulated SRE, while normal Lyn had only a slight effect (Table II). These findings were substantiated by the observation that introduction of increasing amounts of the lyn and lck constructs, but not the lyn construct, stimulated SRE dose dependently (Fig. 4A).

In contrast, we found that neither normal Lck nor Lyn activated the IL-2 promoter in the presence or absence of ConA plus TPA stimulation (Table I). When the amounts of the expression constructs were increased, the level of transactivation of the IL2 promoter by normal Lck or Lyn kinase remained low, while that by normal Fyn increased with an increase in the amount of lyn construct transfected (Fig. 4B). From these data, we conclude that Lyn is a key protein-tyrosine kinase involved in the process of T-cell activation. It should be noted, however, that both active F505Lck (LckF) and F508Lyn (LynF) together with ConA plus TPA stimulated the IL-2 promoter (Table II), though less significantly than Fyn.

Importance of Fyn SH2 Domain in Signaling—In order to
Regulation of T-cell Signaling by Protein-tyrosine Kinases

To dissect further the molecular mechanisms of T-cell responsiveness by Fyn, we examined the effects of Fyn mutants on SRE and IL-2 promoters. Two mutants of B-Fyn, f-14 and t-1fyns, which we isolated before (Fig. 1), transform chicken embryo fibroblasts while normal Fyn does not (21). The products of these mutant fyn genes were shown to have elevated protein-tyrosine kinase activities in vitro ((21) summarized in Fig. 1). The mechanisms of activation seem to be different because the nature of the mutations in the genes are different from each other. The f-14 mutant carries a point mutation resulting in Thr-338 instead of Ile-338 in the kinase domain. The t-1 mutant came a point mutation resulting in a significant amount of the exogenous Fyn protein as determined by immune complex kinase assay (Fig. 5B). Third, even when the increased amounts of the t-1fyn construct were cotransfected, the CAT activity of SRECAT remained at low levels (Fig. 4A). Thus, the SH2 region seems important in Fyn-mediated transactivation of SRE. Similarly the CAT activity of IL-2 CAT was stimulated by f-14 Fyn but not by t-1Fyn (Table I) in Jurkat cells treated by ConA plus TPA. These findings suggest that Fyn kinase enhances signaling into the nucleus and further that the SH2 domain of Fyn is involved in this enhancement.

**Functional and Physical Interactions of Fyn with Csk in TCR-mediated Signaling**—To determine the effects of Csk on transactivation of c-fos and IL-2 promoters by Src-like kinases, we inserted csk cDNA (26) in the correct and reverse orientations into the corresponding vector. The expression plasmids pME18SM (5 μg/reaction) with (shaded bars) or without (open bars) T-fynF cDNA were transfected into Jurkat T-cells (2 × 10⁶) together with IL-2 CAT constructs (5 μg each). The transfectants were treated with TPA (50 ng/ml) and anti-CD3e antibodies (50 ng/ml; provided by T. Uchiyama, Kyoto University) for 6 h before each harvest as described (55). The lysates of these transfectants were subjected to CAT assay.

**TABLE II**

Stimulation of SRE and IL-2 promoter by Lck and Lyn

Both normal and active lck or lyn cDNAs in pME18SM vector (5 μg) were transfected into Jurkat T-cells (2 × 10⁶) together with reporter plasmids, SRECAT (SRE), or IL-2CAT (5 μg) as described in Table I. The lysates (50 μg of protein) of the transfectants were subjected to CAT assay, and the enhancer/promoter activities were shown as described in Table I.

| Enhancer/promoter | SRE | IL-2 |
|-------------------|-----|------|
|                   |     | None | ConA + TPA |
| Vector            | 1.0 (0.8%) | 1.0 (0.5%) | 1.0 (1.2%) |
| Lck               | 5.9 | 1.1  | 1.0 |
| LckF              | 8.8 | 1.0  | 3.4 |
| Lyn               | 2.2 | 1.0  | 0.9 |
| LynF              | 3.7 | 1.0  | 2.8 |

**Fig. 3.** Stimulation of IL-2 promoter upon cross-linking of CD3e. The expression plasmids pME18SM (5 μg/reaction) with (shaded bars) or without (open bars) T-fynF cDNA were transfected into Jurkat T-cells (2 × 10⁶) together with IL-2 CAT constructs (5 μg each). The transfectants were treated with TPA (50 ng/ml) and anti-CD3e antibodies (50 ng/ml; provided by T. Uchiyama, Kyoto University) for 6 h before each harvest as described (55). The lysates of these transfectants were subjected to CAT assay.

**Fig. 4.** Differential effects of Src-like kinases on SRE and IL-2 promoter. Increasing amounts of the cDNAs for T-Fyn, Lck (25), and Lyn (13) cloned in pME18SM (1–9 μg) were transfected into Jurkat cells (5 × 10⁶ cells) together with SRECAT (3 μg) (A) or with IL-2 CAT (3 μg) (B). In B, transfectants were treated with ConA and TPA as described in Table I. The lysates of these transfectants were subjected to CAT assay. In A, the effect of t-1Fyn was also analyzed by transfecting its cDNA in pME18SM. Three independent experiments gave virtually identical results. The percentage conversions of [14C]chloramphenicol to its acetylated form with 1 μg of expression plasmids of fyn, lck, and lyn, respectively, in a particular experiment were, respectively, 0.7, 0.6, and 0.9 in A and 0.45, 0.2, and 0.3 in B. The data with t-1fyn (A) were obtained with a different set of experiments, and the percentage conversion with 1 μg of DNA was 0.66.

**Fig. 5.** Transient expression of t-1fyn in fibroblasts and T-cells. Expression plasmids pME18SM containing cDNAs for normal B-fyn (lanes A and B4), f-14fyn (lanes A3 and B3), and t-1fyn (lanes A2 and B2) or no insert (lanes A1 and B1) were transfected into CV-1 cells (A) and Jurkat T-cells (B). The transfectants were harvested 48 h after transfection and lysed with RIPA buffer, and the lysates were subjected to Western blot analysis (A) or to immune complex kinase assay (B) using anti-Fyn antibodies. The position of Fyn is indicated on the right. Only a slight difference of migration between t-1Fyn and normal or f-14Fyn was acknowledged in B, since the kinase reaction products were analyzed in SDS-polyacrylamide gel containing a relatively higher concentration (12%) of polyacrylamide.
Regulation of T-cell Signaling by Protein-tyrosine Kinases

Using a transient expression system we have shown that Fyn kinase activated by a point mutation, Tyr-528 → Phe, confers hyperresponsiveness on T-cells. The T-cell responsiveness was determined by measuring stimulation of the promoter activities of both the c-fos and IL-2 genes. Our evidence suggests that the c-fos promoter is stimulated by T-FynF through enhancement of SRE and TRE within the promoter (Fig. 2A, Table I). The IL-2 promoter activity was augmented by T-FynF only when the cells received mitogenic stimulation that mimics TCR-mediated activation (Table I). This suggests that the elevated kinase activity alone is not sufficient to deliver the signal toward the nucleus. It is likely that T-cell specific signaling through Fyn may require its dimerization, as the signaling of the receptor-type protein-tyrosine kinase occurs via

![Diagram](image-url)

**Fig. 6. Interaction of Csk with Fyn and Lck.** Samples of 3 μg of the expression plasmid pMECsk (sense; +, shaded bars) or pMEKsc (antisense; −, solid bars) were transfected into Jurkat T-cells (5 × 10⁶) together with the expression plasmids (3 μg) for normal T-Fyn (lanes 3 and 4), normal Lck (lanes 7 and 8), activated T-FynF (lanes 5 and 6), or activated LckF (lanes 9 and 10) and SRECAT (A) or IL-2 CAT (B) reporter plasmid (3 μg). When the IL-2 CAT construct was used as a reporter plasmid, the transfectants were treated with ConA and TPA as described in Table I. The pMECsk (lane 1) or pMEKsc (lane 2) plasmid (3 μg) was transfected with the reporter plasmids (3 μg) and pME18SM vector plasmid without fyn cDNA (3 μg). At least two independent experiments gave essentially identical results. It should be noted that the data with antisense csk cDNA in this experiment were consistent with those given in Tables I and II except that the level of stimulation of SRE by LckF was relatively lower than that by Lck. Probably this could be attributed to the poorer quality of the LckF plasmid that we prepared for this particular experiment.

**Fig. 7. Co-immunoprecipitation of Fyn with Csk.** The HBC21.7.31 cells and their derivatives (10⁶) that express normal B-Fyn (lanes 2 and 5) and F-14Fyn (lanes 3 and 6) exogenously were lysed with TNE buffer. The lysates were subjected to immune complex kinase assay using normal rabbit serum (lanes 1-3) or anti-Csk antibodies (lanes 4-6). The Fyn protein was then re-immunoprecipitated from the reaction products (A). Aliquots of the lysates were subjected to Western blotting to examine the amount of Fyn (B) and Csk (C) expressed in each cell line. The positions of Fyn and Csk are indicated on the right by arrows.

amined using the T-cell hybridomas HBC21.7.31 in which normal or f-14fyn was exogenously introduced.² From lysates of both normal fyn and f-14fyn overexpressed cells, we could co-immunoprecipitate Fyn with Csk using anti-Csk antibodies, which was demonstrated by in vitro kinase reaction (Fig. 7). The amount of Fyn co-immunoprecipitated with Csk was apparently dependent on the level of Fyn expression (lanes 4 and 5). More phosphorylation of F-14Fyn as compared with normal Fyn was detected in the Csk immunoprecipitates (lanes 5 and 6). This may suggest that F-14Fyn that is supposed to be highly phosphorylated in vivo is only partially phosphorylated in vitro. It is also possible that both forms of Fyn equally associate with Fyn and the levels of phosphorylation merely reflect the in vivo kinase activities of F-14Fyn and normal Fyn. Our experimental data do not discriminate these two possibilities. It is important to determine whether phosphorylation in vitro of Fyn in vivo. It is also possible that both forms of Fyn equally associate with Fyn and the levels of phosphorylation merely reflect the in vitro kinase activities of F-14Fyn and normal Fyn. Our experimental data do not discriminate these two possibilities. It is important to determine whether phosphorylation in vitro of Fyn in vivo. It is also possible that both forms of Fyn equally associate with Fyn and the levels of phosphorylation merely reflect the in vitro kinase activities of F-14Fyn and normal Fyn.
dimerization of the receptor (39). Since Fyn interacts with TCR complex, ligand-dependent clustering of TCR would result in Fyn dimerization. Similar, though less, stimulation of these promoters was observed by overexpressing normal Fyn (Table 1). In addition, kinase-active Fyn having a deletion in the SH2 region could hardly stimulate SRE or affect the promoter activity of the IL-2 gene in response to activating signal (Table 1, see below). From these data we have concluded that Fyn plays an important role in delivering signal from TCR to nucleus. In addition we would like to point out that the experimental system employed in this study is useful to analyze the molecular basis of Fyn function in T-cell activation. Namely, it is possible to coexpress possible regulatory proteins such as protein tyrosine kinase Csk and protein tyrosine phosphatases together with Fyn in Jurkat cells and examine the activities of c-fos and IL-2 promoters in T-cell signaling (see below).

The suggestion of functional involvement of Fyn in TCR signal transduction is provided by previous studies. First, a rapid but transient increase in Fyn kinase activity followed TCR stimulation (40). Second, overexpression of Fyn in T-cell hybridomas (Ref. 41 and our unpublished data) or in thymocytes of transgenic mice (17) resulted in augmented responses to TCR stimulation. Finally, TCR-mediated signaling was defective in immature thymocytes of Fyn-deficient mice, although the signaling was unaffected in immature thymocytes and peripheral T-cells (18, 19). The conclusion of this manuscript is consistent with these observations.

Previous studies have shown that another Src-like kinase Lck that is primarily expressed in T-cells is also important in the process of T-cell activation. For example, a failure to express functional Lck was unable to activate the TCR-regulated pathway of the protein tyrosine kinase (42). Consistently SRE and the c-fos promoter were stimulated by Lck as well as T-Fyn (Table II, Fig. 2). However, we also demonstrated that normal Lck, unlike normal Fyn, was unable to stimulate transcription of the IL-2 gene upon T-cell activation (Table II, Fig. 4B), suggesting that Lck and Fyn play roles in different phases of signaling. We propose that Fyn functions more proximal to the IL-2 gene than Lck.

The experimental data in this study also show that at least part of the SH2 region of Fyn is important in Fyn-mediated signaling in T-cells (Table I, Fig. 4A). Many lines of evidence show that the SH2-containing proteins are important in signal transduction (43-45). These proteins include a phosphatidylinositol-specific phospholipase C-γ, the ras GTPase-activating protein, the p85 subunit of phosphatidylinositol 3-kinase, and Grb-2/Sem5, which have been demonstrated to be phosphorylated at tyrosine residues and/or associated with receptor protein-tyrosine kinases upon ligand stimulation. In a preliminary study, we found that the CD3-ζ chain was associated with the Fyn protein when these proteins were expressed together in CV-1 cells (data not shown). Others also showed physical interaction between Fyn and the ζ chain and demonstrated that the 10-amino acid sequence in the amino-terminal unique domain was responsible for the association (46). The critical role of the ζ chain, which has multiple tyrosine phosphorylation sites (47), in signal transduction was clearly demonstrated by analyzing a chimeric protein consisting of the extracellular domain of CD4 or CD8 and the cytoplasmic domain of ζ (48-50). Taken together we assume that the SH2 sequence of Fyn interacts with the partly phosphorylated ζ chain and that this interaction increases the stability of Fyn-ζ association and is a prerequisite for further tyrosine phosphorylation of ζ by the Fyn kinase. The resulting, heavily phosphorylated ζ chain would in turn recruit SH2-containing signaling molecules such as phospholipase C-γ, GTPase-activating protein, and phosphatidylinositol 3-kinase, as insulin receptor-associated p185, which becomes phosphorylated at multiple tyrosine residues by the insulin receptor upon insulin stimulation, interacts with phosphatidylinositol 3-kinase (51). It should be noted that ZAP-70 protein-tyrosine kinase, which was identified as ζ-associated kinase, contains two SH2 motives and is rapidly tyrosine-phosphorylated after stimulation of T-cells (52, 53). In addition, our present data suggest that at least some fraction of Csk is associated with TCR. Csk might be recruited by phosphorylated ζ to complete negative regulation of Fyn and/or Lck kinases, resulting in down-regulation of signaling.

The kinase activity of the Src family is regulated by phosphorylation and dephosphorylation of a tyrosine residue that corresponds to Tyr-527 of c-Src and is conserved among the family members (1, 2). Phosphorylation of this regulatory tyrosine is catalyzed by a cytoplasmic protein-tyrosine kinase Csk (9). Regulation of the Src kinase activity by Csk has been demonstrated in vitro and in yeast (10, 12, 26). Although the kinase activity of Fyn has been shown to be down-regulated in vitro by Csk (10), regulation of Fyn kinase by Csk in intact cells has not been demonstrated. Our present data clearly show that Csk suppresses activities of Fyn and Lck to stimulate the c-fos and IL-2 promoters in T-cells (Fig. 6), which suggests that Csk is involved in TCR-mediated signaling. Furthermore we demonstrated co-immunoprecipitation of Csk and Fyn from the lysates of Fyn-overexpressed cells by means of in vitro kinase assay (Fig. 7). The efficiency of co-immunoprecipitation was low, and preliminary data indicated that no more than 1% of Fyn was physically associated in unstimulated cells. The molecular basis of this interaction remains to be elucidated. It should be noted, however, that no association of Csk with Src was observed in fibroblasts (54). Nevertheless recent studies in this laboratory suggested physical interaction of Lyn and Fyn with Csk when both kinases were overexpressed in CV-1 monkey kidney cells.3 Further studies are needed to explain the apparent discrepancy. Both Fyn and Lck are associated with the antigen-major histocompatibility complex receptor system (3, 4, 6). Moreover Csk is expressed ubiquitously but is expressed at highest levels in certain tissues that include thymus (10) and has been isolated from the cellular membrane fraction (9). These data together suggest that Csk functions in the TCR-CDS3-mediated signaling pathway.

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