A general strategy for producing conditional alleles of Src-like tyrosine kinases

DAVID M. SPENCER*, ISABELLA GRAEF*, DAVID J. AUSTIN†, STUART L. SCHREIBER†‡, AND GERALD R. CRABTREE*

*Howard Hughes Medical Institute, Departments of Developmental Biology and Pathology, Stanford University Medical School, Stanford, CA 94062; and †Howard Hughes Medical Institute, Department of Chemistry, Harvard University, Cambridge, MA 02138

ABSTRACT   The Src-like tyrosine kinases require membrane localization for transformation and probably for their normal role in signal transduction. We utilized this characteristic to prepare Src-like tyrosine kinases that can be readily activated with the rationally designed chemical inducer of dimerization FK1012. Dimerization of cytoplasmic Src-like tyrosine kinases was not sufficient for signaling, but their recruitment to the plasma membrane led to the rapid activation of transcription factors identical to those regulated by crosslinking the antigen receptor. Moreover, recruitment of activated Src-like kinases to the membrane replaced signaling by the T-lymphocyte antigen receptor complex, leading to the activation of both the Ras/protein kinase C and Ca^{2+}/calcineurin pathways normally activated by antigen receptor signaling. Since these chemical inducers of dimerization are cell permeable, this approach permits the production of conditional alleles of any of the Src-like tyrosine kinases, thereby allowing a delineation of their developmental roles.

Conditional alleles have proven essential for understanding the role of many genes because they add the dimension of time to genetic analyses and thereby avoid the myriad secondary effects and compensatory mechanisms brought into play when a gene is constitutively absent or active. For example, isolation of the temperature-sensitive (ts) cdc alleles of the cell cycle control genes was essential to understanding their function (1, 2), since it permitted the definition of execution points and a clarification of the complex feedback relationships that are fundamental to cell cycle control. While ts alleles of mammalian genes, including those encoding Src-like kinases (3), have been engineered to elucidate their roles in signaling, they can only be utilized in tissue culture and are typically inactivated at temperatures that induce the heat-shock response, which activates numerous genes that can potentially obfuscate the physiologic role(s) of the gene being studied. Finally, most ts alleles require several hours for the inactive protein to accumulate, since ts alleles are almost always ts for posttranslational folding and not stability (4). Recently, we have developed an alternative to ts alleles using the cell-permeable chemical inducer of dimerization (CID) FK1012 (5). This molecule is a dimer of FK506 and induces dimerization or, more simply, proximity between two chimeric proteins containing the FK506-binding protein FKBP12 (K_d = 0.4 nM).

Mounting evidence indicates that the recruitment of signaling molecules to the proximity of their substrates is a major mechanism of regulatory control. Enzymes such as protein kinase C, phospholipase C, and c-Raf are localized to the plasma membrane after receptor activation (for a review, see ref. 6). The importance of proximity would be simplistically predicted to arise because the probability of effective enzyme–substrate interactions is inversely proportional to the cube of the distance between them. Therefore it should be possible to activate certain signaling molecules by artificially inducing proximity to their effector targets. For the Src-like tyrosine kinases, we have relied on the fact that their activity requires membrane attachment by an N-terminal myristoyl group (7, 8). Additionally, all members of this family have a conserved regulatory tyrosine residue at their C terminus, which must be dephosphorylated for maximum activity (9, 10); removal of this tyrosine residue constitutively activates these enzymes. The tyrosine kinase Csk and the phosphatase CD45 are apparently involved in this regulation in vivo (11–14). By regulating the membrane localization of activated protein-tyrosine kinases (PTKs), we can recapitulate the "downstream" or activation-independent steps of Lck or Fyn signaling by the T-lymphocyte antigen receptor (TCR), allowing a delineation of catalytic activities without contribution of the activation functions. Surprisingly, we find that this activation occurs in the absence of a functional TCR, indicating that there may be a role for Lck or Fyn downstream of the antigen receptor, a result that is consistent with several studies on the development of anergy (15).

MATERIALS AND METHODS

Plasmid Clones. The expression plasmids used in this study are described in Fig. 1A or below. All of the constructs made by PCR were sequenced. Protein expression was verified by Western blot analysis using the influenza hemagglutinin epitope tag (12CA5) (7). The murine Lck, Fyn, and Lyn templates are from m-lck, pmTF, and lynAF, respectively (18, 19). Primers were flanked by Xho I (5' primer) or Sal I (3' primer) sites, and the resulting fragments were subcloned into pKS (Stratagene), sequenced, and subcloned into the Sal I site of SF1E (SF1 series) or MF1E (MF1 series) described previously (5). RSV-N17Ras (20) is a dominant-negative Harvey Ras mutant.

The reporter plasmids NF-AT-SX, IL-2-SX, AP-1-SX, NFκB-SX, and Oct/OAP-SX have been described (5). Briefly, they contain multiple binding sites for the various transcription factors cloned upstream of a minimal interleukin 2 (IL-2) promoter [−70 to +47 (21)] driving secreted alkaline phosphatase (SEAP) expression.

Cell Lines and Tissue Culture. Jurkat-TAg cells, J.RT-T3.5 cells (22), and COS (monkey kidney) cells were used for these studies. Jurkat-TAg cells and COS cells contain the simian virus 40 large tumor antigen, which permits replication of plasmids containing the simian virus 40 origin. J.RT-T3.5 is a TCR β-chain-deficient subclone of Jurkat cells (20). All cells were grown and electroporated in RPMI 1640 medium, 10%

Abbreviations: ts, temperature sensitive; FKBP12, FK506-binding protein; TCR, T-lymphocyte antigen receptor; IL-2, interleukin 2; SEAP, secreted alkaline phosphatase; mAb, monoclonal antibody; SH, Src homology; PTK, protein-tyrosine kinase; CID, chemical inducer of dimerization.
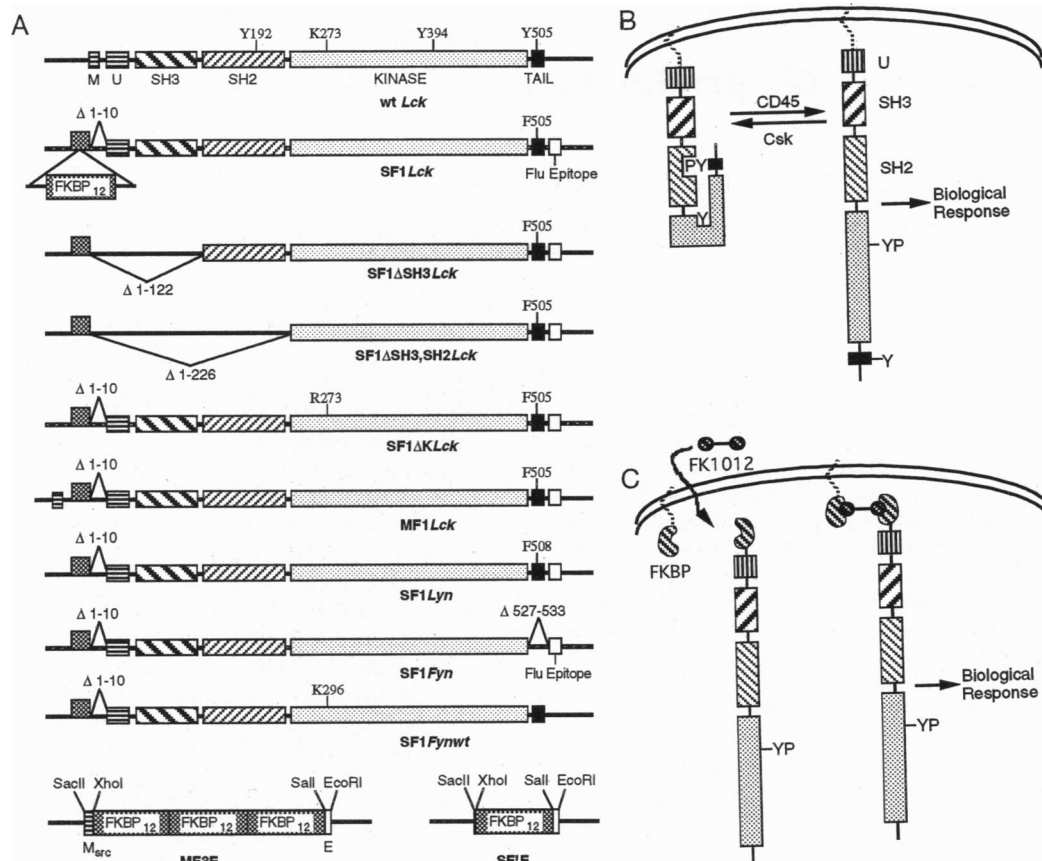‡To whom reprint requests should be addressed.

FIG. 1.   (*A*) Construction of chimeric intracellular signaling molecules. Schematic of Src-family kinase–FKBP12 chimeras used for inducible membrane targeting. Src-family kinases are deregulated by mutation of the C-terminal tyrosine residue as shown and inactivated by truncation of the N-terminal myristoylation targeting peptide (residues 1–10). SF1ΔSH3Fyn lacks residues 1–144, SF1ΔSH3,SH2Fyn lacks residues 1–254, and SF1ΔKFyn has substitution K296E (not shown). Membrane targeting is achieved by subcloning these modified kinases into the FKBP12-tagging vector MF1E (myristoylated) or SF1E (cytosolic). M, myristoylation targeting sequence from v-src (residues 1–14) (5, 7); S, soluble, nonmyristoylated; U, unique domain; E, influenza hemagglutinin epitope tag (16); TAIL, C-terminal regulatory peptide. (*B*) Model of the regulation of Lck/Fyn by the CD45 protein-tyrosine phosphatase and Csk PTK. Removal of the C-terminal phosphate by the CD45 protein tyrosine phosphatase relieves Lck/Fyn tyrosine kinase inhibition, permitting downstream signaling (modified from ref. 17). (*C*) Model of inducible membrane targeting with synthetic ligands. (*Left*) Src-family kinases have been inactivated by the replacement of their membrane targeting motifs by FKBP12. (*Right*) With the addition of membrane-permeable FK1012, the tyrosine kinases are recruited to the plasma membrane docking protein MF1, eliciting a biological response. Y, tyrosine; YP, phosphotyrosine.

(vol/vol) fetal calf serum, 10 mM Hepes (pH 7.4), and penicillin/streptomycin.

Electroporations and SEAP Assays. Jurkat-TAg cells were electroporated (Bio-Rad Gene Pulser; 960 μF and 250 V in a 0.4-cm-wide cuvette) with 2–3 μg of the reporter plasmid NF-AT-SX or one of its derivatives (5, 23), 1 μg of the pBJ5 expression vector containing the "docking protein" MF1E or MF3E, and 2 μg of pBJ5 containing one of the Src-family kinases or the control construct SF1. Alternatively, 1 μg of MF1Lck was cotransfected with 2 μg of reporter. For COS cells, one-half of the above amounts were used. For J.RT-T3.5 cells, 0.2 μg of expression plasmid RSV-TAg (24) was cotransfected to increase expression. After 24 hr, aliquots of cells were stimulated with dilutions of FK1012A or mitogen (1 μm ionomycin plus phorbol 12-myristate 13-acetate at 25 ng/ml) plus 1 μM nonreactive, monomeric control ligand FK506-M (5). After 20 hr, supernatants were assayed for SEAP activity as reported (5), and the data are presented relative to mitogen stimulation. Each data point was performed in duplicate, and the data are the average of two experiments. All reagents were dissolved in ethanol, and the maximum concentration of solvent in culture never exceeded 0.1%.

Kinetics of Activation. Anti-TCR monoclonal antibody (mAb) UCHT1 (Sigma)-coated microtiter wells were incubated for 1 hr at 37°C with 10 μg of mAb per ml of

phosphate-buffered saline (PBS) and blocked for 1 hr 37°C in PBS containing 1% fetal calf serum. Cells were divided into these wells or untreated wells containing mitogen or FK1012. Aliquots of cells were removed from the microtiter wells at each time point and frozen until the last time point. The data are presented as the average of two experiments performed in duplicate.

RESULTS

To be able to reversibly recruit the Src kinases to the membrane, we removed the myristoylation-targeting peptides from the N termini of Fyn, Lck, and Lyn and replaced them with FKBP12 (Fig. 1*A*) (8, 25). We also designed a membrane-bound docking protein, MF*n*E, composed of the N-terminal myristoylation targeting peptide from v-src followed by *n* = 1–3 copies of FKBP12 (5). To focus on downstream signaling events, we reasoned that we needed to eliminate the influence of upstream regulatory events [e.g., Csk phosphorylation and CD45 dephosphorylation (Fig. 1*B*)] in kinase activation. Therefore, chimeric Lck (SF1Lck), Fyn (SF1Fyn), and Lyn (SF1Lyn) were deregulated by eliminating their regulatory, C-terminal tyrosine residues (Fig. 1*A*). *A priori*, the administration of the CID FK1012 (5; 26) should lead to the formation of hetero- and homodimers (Fig. 1*C*).

Cell Biology: Spencer *et al.*

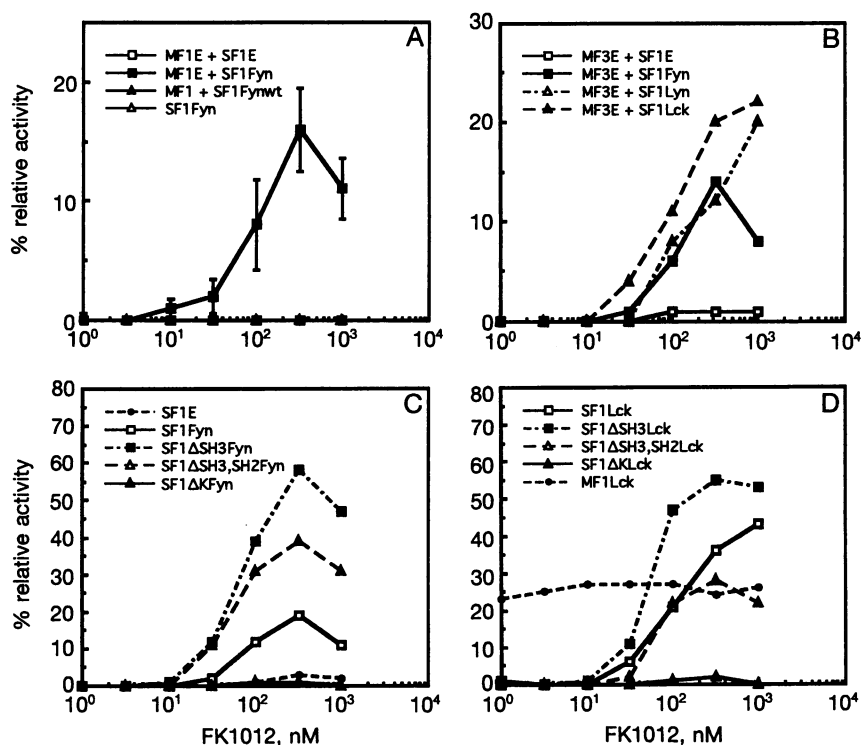*Proc. Natl. Acad. Sci. USA* 92 (1995)   9807

FIG. 2. Inducible signal transduction using synthetic dimers by membrane targeting of Src-family kinases. (*A*) The ability of dimeric ligand FK1012 to recruit SF1Fyn to the plasma membrane docking protein MF1E is assayed by the induction of an NF-AT-responsive reporter plasmid NF-AT-SX in Jurkat-TAg cells (23, 24). This is compared to the FK1012-dependent recruitment of wild-type Fyn (SF1Fynwt) or cytosolic FKBP12 (SF1E). (*B*) The ability of FK1012 to target multiple Src family members to the plasma membrane docking protein MF3E compared to control protein SF1E. (*C* and *D*) The signaling capacity of various Fyn (*C*) or Lck (*D*) mutants lacking their SH3 (ΔSH3), SH3 and SH2 (ΔSH3,2), or kinase (ΔK) domain is compared to the parent constructs SF1Fyn (*C*) or SF1Lck (*D*) by membrane targeting with FK1012. As a control, CID-independent signaling by myristoylated FKBP-Lck (MF1Lck) is shown. All of the constructs were similarly expressed as assayed by Western blot using the 12CA5 mAb against the influenza hemagglutinin epitope (D.M.S., I.G., and G.R.C., unpublished results and ref.7).

To demonstrate the feasibility of this approach, Jurkat-TAg cells (24) were cotransfected with one of several FKBP12-containing constructs and a reporter plasmid (NF-AT-SX) in which the SEAP gene is under the control of the NF-AT transcription factor (23, 27). NF-AT-dependent transcription of this reporter is elicited by the antigen receptor through a bifurcating signaling pathway (28–30) that requires both calcineurin and Ras and that is inhibited by cyclosporin A and

FK506 (31). Thus, in several respects, it faithfully mimics the activation requirements of T lymphocytes. When the cytosolic FKBP12–Fyn chimera (SF1Fyn) or the docking protein (MF1E) is transfected into Jurkat-TAg cells, NF-AT activity is undetectable at all concentrations of FK1012 (Fig. 2*A*). However, if the docking protein and the FKBP12–Fyn chimera are cotransfected into Jurkat-TAg cells, FK1012 activates signaling at concentrations as low as 1 nM. Membrane recruitment
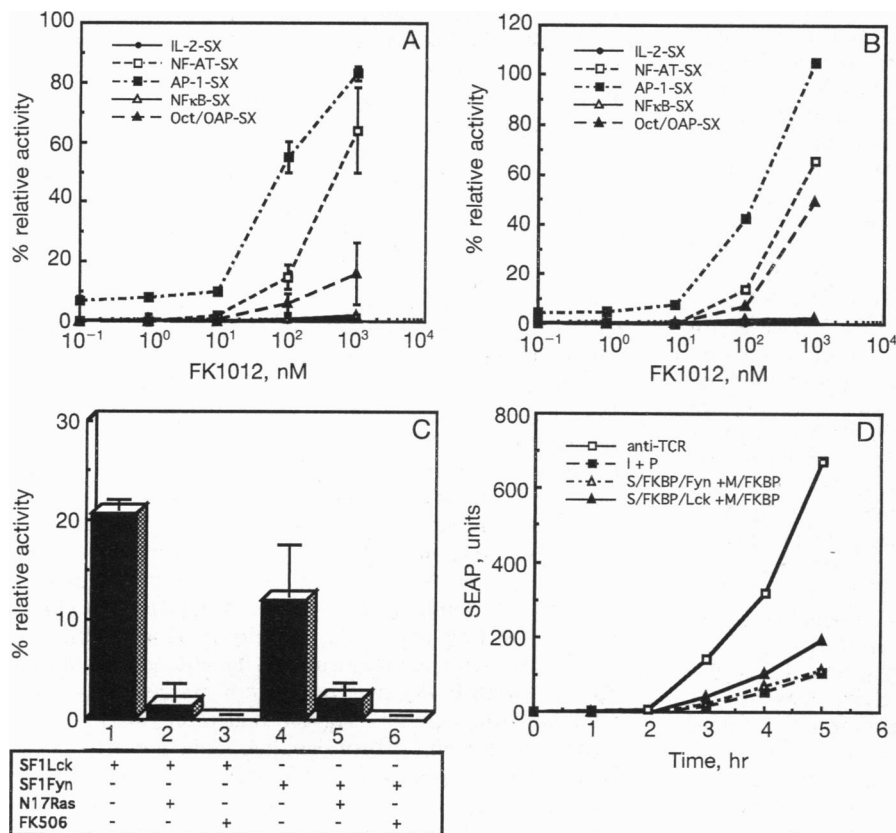

FIG. 3. Conditional activation of Src-family kinases mimics TCR signaling. Comparison of the induction of a panel of transcription factors (5) by CID-induced Fyn (*A*) or Lck (*B*). (*C*) The ability of dominant-negative Ras (RSV-N17Ras, 2 μg) or FK506 (2 ng/ml) to block NF-AT activation by membrane recruitment of Fyn or Lck by 300 nM FK1012. (*D*) An examination of the kinetics of activation by Fyn and Lck using 1 μM FK1012 relative to that by mitogen or direct TCR crosslinking (see *Materials and Methods*).

of "wild-type" Fyn, SF1Fynwt, is insufficient for signaling (Fig. 2*A*). As expected, FK1012-mediated membrane recruitment and crosslinking of SF3Fynwt, which contains three FKBP12s, signaled effectively. Also, membrane-tethered, deregulated Fyn (MF1Fyn) activated signaling in a FK1012-independent fashion ($\approx$40% relative to ionomycin and phorbol 12-myristate 13-acetate activation (refs. 7 and 9; D.M.S. and G.R.C., unpublished results).

To investigate potential specialization among several Src family members, we compared the ability of Fyn, Lck, and Lyn to activate signaling. Lck activates slightly, but reproducibly, better in this system at lower FK1012 concentrations than Fyn or Lyn, whereas there is no significant difference in the efficiency by which membrane recruitment of deregulated Fyn or Lyn activates signaling (Fig. 2*B*). Although Lyn is predominately B-cell specific (32), it can apparently signal in T cells when artificially targeted to the plasma membrane. These results suggest that the kinase domains of Src family members may not contribute significantly to their specific roles but that specific mechanisms of activating these kinase are important for their biologic specificity.

We investigated the role of the Src homology (SH) 2 and 3 domains in signaling induced by FK1012 by constructing a series of Fyn and Lck mutants lacking one or both SH3 and SH2 domains. MF3E was cotransfected into Jurkat cells alone with one of the various Fyn or Lck constructs or kinase-deficient mutants (Figs. 1*A* and 2 *C* and *D*). These results indicate that the kinase domains are not only necessary but also sufficient for signaling, whereas neither the SH3 nor the SH2 domain is required for the induction of signaling and, in fact, may be inhibitory. The dispensability of these N-terminal domains rules out the formal possibility that membrane recruitment of SH3- or SH2-associated signaling molecules is responsible for signaling.

As previously shown, crosslinking the intact TCR or the ζ chain of the TCR complex activates a subset of transcription factors necessary for the transcriptional induction of the IL-2 gene, including NF-AT, Oct/OAP, and AP-1, whereas activation of NF-κB factors requires costimulatory signals (5, 33). To assay whether inducible Fyn or Lck can activate a similar panel of factors, we cotransfected various reporter plasmids into Jurkat-TAg cells along with MF3E and SF1Fyn or SF1Lck (Fig. 3 *A* and *B*, respectively). Upon addition of FK1012, only the NF-AT-, Oct/OAP-, and AP-1-responsive reporters were activated, whereas the NF-κB-responsive and the IL-2 enhancer-containing reporter were completely inactive. Additionally, NF-AT-dependent transcription induced by Fyn or Lck is blocked by FK506, a potent inhibitor of the Ca^{2+}-regulated phosphatase calcineurin (23, 34, 35) and requires functional Ras activity, because a dominant-negative Ras, RSV-N17Ras, completely blocks induction (Fig. 3*C*) (20). Finally, we examined the kinetics of activation by Fyn and Lck and compared them to that of direct TCR stimulation or mitogen stimulation. Although the total levels of reporter enzyme secretion are severalfold higher by TCR-stimulated cells than by Fyn or Lck-stimulated cells, in either case, reporter activity is first measurable at 3 hr, demonstrating that FK1012-activated signaling is comparable to that of the receptor (Fig. 3*D*). Therefore, by multiple criteria the induction of Fyn or Lck by FK1012 seems to reproduce TCR-mediated signaling faithfully.

To investigate the dependence of Fyn or Lck signaling on the TCR complex, SF1Fyn or SF1Lck plus MF3E were transiently transfected into the TCR$^-$ subclone of Jurkat cells, J.RT-T3.5 (22). Since these cells lack the TCR β chain, they do not assemble a TCR complex at the plasma membrane, resulting in the enhanced degradation or retention in the endoplasmic reticulum of the unassembled TCR complex subunits (36). Surprisingly, membrane recruitment of Fyn (Fig. 4*A*) or Lck (D.M.S., I.G., and G.R.C., unpublished results) initiated sig-
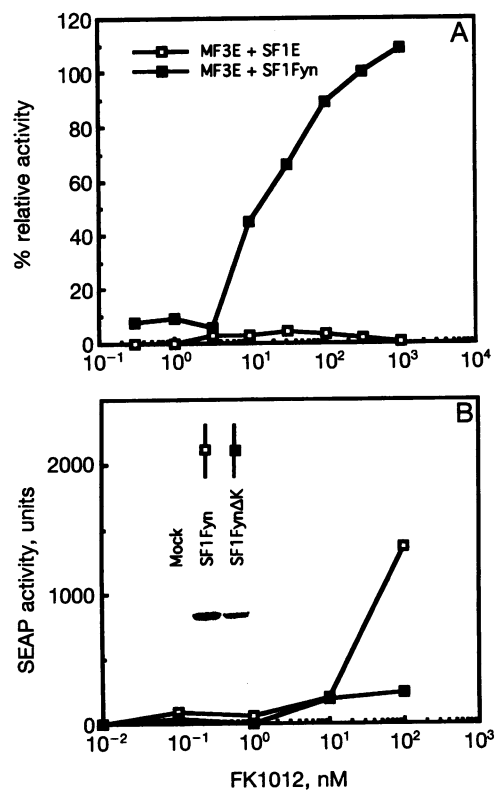

FIG. 4. Membrane recruitment of SF1Fyn activates signaling independent of the TCR complex. (*A*) The signaling capacity of Fyn was examined in the TCR$^-$ variant of Jurkat cells, J.RT-T3.5 (22), by assaying the induction of NF-AT. (*B*) The signaling capacity of Fyn or kinase-deficient Fyn (SF1FynΔK) was examined in non-T COS cells by assaying the induction of AP-1. (*Inset*) Western blot of samples from the above transfections using 12CA5 anti-hemagglutinin mAb.

naling in the absence of a functional TCR. To investigate this, we transfected SF1Fyn or kinase-deficient SF1FynΔK plus MF3E into nonlymphocyte COS cells (Fig. 4*B*). Again, membrane recruitment of Fyn was sufficient for signaling, whereas the kinase-deficient SF1FynΔK was defective in its ability to signal. These results indicate that signaling by Src family kinases may be able to bypass the antigen receptor complex.

CONCLUSIONS

Our results demonstrate that FK1012-mediated membrane recruitment of deregulated Src-like kinases is sufficient to initiate T-cell signaling. In contrast, studies by Kolanus *et al.* (37) indicated that signaling required antibody-induced coaggregation of both membrane-tethered chimeric Fyn and a distinct PTK, ZAP-70 or Syk. However, we have employed an activated version of Lck or Fyn in our studies, implying that the need for colocalization of Src-like tyrosine kinases and ZAP-70 may be related to the ability of aggregation to activate the Src-like tyrosine kinase. Another possibility is that the FK1012-localized Src family kinases used in this study may be less conformationally restricted than the CD16-PTK chimeras used in the previous study, permitting phosphorylation of downstream signaling molecules in the absence of colocalization of putative docking proteins or targets, like Syk or ZAP-70. Other studies suggest that ZAP-70 may not be required for certain types of signaling. Constitutively active Src can activate IL-2 production without ZAP-70 phosphorylation (8), Fyn and TCR ζ can initiate signaling in COS cells in an apparently ZAP-70-independent manner (38), and CD4$^+$ thy-

Cell Biology: Spencer *et al.*

Proc. Natl. Acad. Sci. USA 92 (1995) 9809

mocytes can develop and emigrate from the thymus in ZAP-70-deficient humans (39–41).

We find that when artificially recruiting an activated Src kinase to their site of action, the SH3, SH2, and N-terminal "unique" domains are not essential for the activation of signaling. This is consistent with the putative normal role for these domains in membrane targeting or activation of Lck or Fyn. Moreover, we find that targeting these Src-family kinases to an N-myristoylated FKBP12 eliminates potential subtype specificity. This finding is consistent with previous reports that Src family members can in several circumstances compensate for the loss of other family members (42, 43) and suggests that the specificity of the actions of these kinases arises from specific means of activation rather than kinase function. Furthermore, the crystal structure of the SH2–SH3 region of Lck, which shows that both of these domains are involved in the reversible repression of Src-family kinases by a tripartite interaction with the C-terminal regulatory tyrosine residue (44), is consistent with the inhibitory effects these regions showed in our assays. While these domains have been implicated in interactions with downstream effector molecules, like phospholipase C-γ2, mitogen-activated protein kinase, Ras GTPase-activating protein, and phosphatidylinositol 3-kinase (45), other studies show that SH3, SH2, or both domains may be dispensable. The SH3 domain of Lck^{F505} is not necessary for transformation, and the dispensability of the SH2 and SH3 domains for tyrosine kinase activity has been well documented (46). While our results contrast with reports that found that the SH2 domain of Lck^{F505} is necessary for IL-2 production (47) or proliferation (46, 48), we assayed the ability of relatively short-term activation of Lck or Fyn (more similar to the physiologic regulation) to initiate transcription. Our results cannot rule out that the posttranscriptional regulation of IL-2 or mitogenesis may require the SH2 domain of Lck or Fyn.

Last, the results here suggest that bringing a constitutively active Src-family kinase to the plasma membrane can bypass signaling by the TCR complex and associated molecules, like the Syk-family tyrosine kinase ZAP-70. This is consistent with at least two observations. First, p56^{lck} coprecipitates with phospholipase C-γ1 after TCR stimulation (49), and, second, the induction of anergy requires calcineurin activation, as reflected by the fact that induction of anergy is inhibited by cyclosporin A, without activation of ZAP-70 (15). We cannot yet exclude the formal possibility that endoplasmic reticulum-retained ζ chains or CD3 subunits can functionally substitute for plasma membrane-bound TCR complexes (50, 51) or that other cell surface receptors might substitute for the antigen receptor in this context.

The approach described above may be generalizable to any situation in which a protein becomes active by virtue of its localization or proximity to its substrate. In the case of the tyrosine kinases, we have taken an unregulated requirement for activity (i.e., myristoylation) and made it the basis of regulation. Such an approach has also been applied to the CID-mediated activation of Ras (52). These reagents should also be useful for reconstitution of genetically ablated genes with chimeric genes that can be regulated on physiologic time scales.

1. Hartwell, L. H. & Weinert, T. A. (1989) *Science* **246**, 629–634.
2. Norbury, C. & Nurse, P. (1992) *Annu. Rev. Biochem.* **61**, 441–470.
3. Hurley, T. R., Amrein, K. E. & Sefton, B. M. (1992) *J. Virol.* **66**, 7406–7413.
4. Alber, T. (1989) *Annu. Rev. Biochem.* **58**, 765–798.
5. Spencer, D. M., Wandless, T. J., Schreiber, S. L. & Crabtree, G. R. (1993) *Science* **262**, 1019–1024.
6. Mochly-Rosen, D. (1995) *Science* **268**, 247–251.
7. Cross, F. R., Garber, E. A., Pellman, D. & Hanafusa, H. (1984) *Mol. Cell. Biol.* **4**, 1834–1842.
8. Resh, M. D. (1994) *Cell* **76**, 411–413.
9. Marth, J. D., Cooper, J. A., King, C. S., Siegler, S. F., Tinker, D. A., Krebs, E. G. & Perlmutter, R. M. (1988) *Mol. Cell. Biol.* **8**, 540–550.
10. Amrein, K. E. & Sefton, B. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4247–4251.
11. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P. & Alitalo, K. (1992) *EMBO J.* **11**, 2919–2924.
12. Mustelin, T., Pessa-Morikawa, T., Autero, M., Gassmann, M., Andersson, L. C., Gahmberg, C. G. & Burn, P. (1992) *Eur. J. Immunol.* **22**, 1173–1178.
13. Chow, L. M., Fournel, M., Davidson, D. & Veillette, A. (1993) *Nature (London)* **365**, 156–160.
14. Mustelin, T., Coggeshall, K. M. & Altman, A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6302–6306.
15. Sloan-Lancaster, J., Shaw, A. S., Rothbard, J. B. & Allen, P. M. (1994) *Cell* **79**, 913–922.
16. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. & Wigler, M. (1988) *Mol. Cell. Biol.* **8**, 2159–2165.
17. Weiss, A. & Littman, D. R. (1994) *Cell* **76**, 263–274.
18. Appleby, M. W., Gross, J. A., Cooke, M. P., Levin, S. D., Qian, X. & Perlmutter, R. M. (1992) *Cell* **70**, 751–763.
19. Yi, T. L., Bolen, J. B. & Ihle, J. N. (1991) *Mol. Cell. Biol.* **11**, 2391–2398.
20. Rayter, S. I., Woodrow, M., Lucas, S. C., Cantrell, D. A. & Downward, J. (1992) *EMBO J.* **11**, 4549–4556.
21. Fiering, S., Northrop, J. P., Nolan, G. P., Mattila, P. S., Crabtree, G. R. & Herzenberg, L. A. (1990) *Genes Dev.* **4**, 1823–1834.
22. Weiss, A. & Stobo, J. (1984) *J. Exp. Med.* **160**, 1284–1299.
23. Clipstone, N. A. & Crabtree, G. R. (1992) *Nature (London)* **357**, 695–697.
24. Northrop, J. P., Ullman, K. S. & Crabtree, G. R. (1993) *J. Biol. Chem.* **268**, 2917–2923.
25. Standaert, R. F., Galat, A., Verdine, G. L. & Schreiber, S. L. (1990) *Nature (London)* **346**, 671–674.
26. Pruschy, M. N., Spencer, D. M., Kapoor, T. M., Miyake, H., Crabtree, G. R. & Schreiber, S. L. (1994) *Chem. Biol.* **1**, 163–172.
27. Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Nolan, G. P., Admon, A. & Crabtree, G. R. (1994) *Nature (London)* **369**, 497–502.
28. Shaw, J.-P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A. & Crabtree, G. R. (1988) *Science* **241**, 202–205.
29. Flanagan, W. F., Corthesy, B., Bram, R. J. & Crabtree, G. R. (1991) *Nature (London)* **352**, 803–807.
30. Karttunen, J. & Shastri, N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3972–3976.
31. Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E. & Crabtree, G. R. (1989) *Science* **246**, 1617–1620.
32. Hutchcroft, J. E., Harrison, M. L. & Geahlen, R. L. (1992) *J. Biol. Chem.* **267**, 8613–8619.
33. Verweij, C. L., Geerts, M. & Aarden, L. A. (1991) *J. Biol. Chem.* **266**, 14179–14182.
34. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. & O'Neill, E. A. (1992) *Nature (London)* **357**, 692–695.
35. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I. & Schreiber, S. L. (1991) *Cell* **66**, 807–815.
36. Klausner, R. D., Lippincourt-Schwartz, J. & Bonifacino, J. S. (1990) *Annu. Rev. Cell Biol.* **6**, 403–431.
37. Kolanus, W., Romeo, C. & Seed, B. (1993) *Cell* **74**, 171–183.
38. Hall, C. G., Sancho, J. & Terhorst, C. (1993) *Science* **261**, 915–918.
39. Chan, A. C., Kadlecek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L., Iwashima, M., Parslow, T. G. & Weiss, A. (1994) *Science* **264**, 1599–1601.
40. Arpaia, E., Shahar, M., Dadi, H., Cohen, A. & Roifman, C. M. (1994) *Cell* **76**, 947–958.
41. Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A. & Parslow, T. G. (1994) *Science* **264**, 1596–1599.
42. Lowell, C. A., Soriano, P. & Varmus, H. E. (1994) *Genes Dev.* **8**, 387–398.
43. Stein, P. L., Vogel, H. & Soriano, P. (1994) *Genes Dev.* **8**, 1999–2007.
44. Eck, M. J., Atwell, S. K., Shoelson, S. E. & Harrison, S. C. (1994) *Nature (London)* **368**, 764–769.
45. Plaiman, C. M., Clark, M. R., Gauen, L. K., Winitz, S., Coggeshall, K. M., Johnson, G. L., Shaw, A. S. & Cambier, J. C. (1993) *Mol. Cell. Biol.* **13**, 5877–5887.
46. Reynolds, P. J., Hurley, T. R. & Sefton, B. M. (1992) *Oncogene* **7**, 1949–1955.
47. Luo, K. & Sefton, B. M. (1992) *Mol. Cell. Biol.* **12**, 4724–4732.
48. Veillette, A., Caron, L., Fournel, M. & Pawson, T. (1992) *Oncogene* **7**, 971–980.
49. Weber, J. R., Bell, G. M., Han, M. Y., Pawson, T. & Imboden, J. B. (1992) *J. Exp. Med.* **176**, 373–379.
50. Chen, C., Bonifacino, J. S., Yuan, L. C. & Klausner, R. D. (1988) *J. Cell Biol.* **107**, 2149–2161.
51. Bonifacino, J. S., Suzuki, C. K., Lippincott-Schwartz, J., Weissman, A. M. & Klausner, R. D. (1989) *J. Cell Biol.* **109**, 73–83.
52. Holsinger, L. J., Spencer, D. M., Austin, D. J., Schreiber, S. L. & Crabtree, G. R. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9810–9814.