Direct T Cell Activation by Chimeric Single Chain Fv-Syk Promotes Syk-Cbl Association and Cbl Phosphorylation*

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The protein tyrosine kinase Syk is activated upon engagement of immune recognition receptors. We have focused on the identification of signaling elements immediately downstream to Syk in the pathway leading to T cell activation. To circumvent T cell receptor (TCR)-CD3 activation of Src family kinases, we constructed a signaling molecule with an extracellular single chain Fv of an anti-TNP antibody, attached via a transmembrane region to Syk (scFv-Syk). In a murine T cell hybridoma, direct aggregation of chimeric Syk with antigen culminates in interleukin-2 production and target cell lysis. Initially, it causes an increase in the association between scFv-Syk and the cytosolic protein Cbl and subsequently promotes tyrosine phosphorylation of Cbl. Interestingly, although both Cbl and phospholipase C-γ (PLC-γ) are phosphorylated in this hybridoma upon TCR CD3 cross-linking, these two events are uncoupled in scFv-Syk-transfected cells, in which we were unable to detect antigen-driven PLC-γ phosphorylation. These results support a model in which Syk can initiate and directly activate the T cell's signaling machinery and position Cbl as a primary tyrosine kinase substrate in this pathway. Furthermore, for efficient PLC-γ phosphorylation to occur in these cells, the combined actions of different tyrosine kinase families may be required.

A variety of biochemical changes occur in T lymphocytes after antigen stimulation including increases in protein phosphorylation, lipid turnover, and intracellular Ca²⁺ levels, activation of specific enzymes, and induction of gene expression (1, 2). The earliest of these T cell receptor (TCR)1-triggered events is activation of protein tyrosine kinases (3, 4), although none of the subunits of the TCR-CD3 complex possess an intrinsic tyrosine kinase activity. The ability of the ligated receptors to induce increased tyrosine phosphorylation lies in their ability to interact with cytoplasmic non-receptor protein tyrosine kinases (5), including members of the Src, Csk, and Syk kinase families (1, 2).

Syk, a 72-kDa protein tyrosine kinase, is abundant in several hematopoietic lineages, such as B cells, myeloid cells, and thymocytes (6). Syk has been shown to associate with the cytoplasmic domains of many immune recognition receptors including the Igα and Igβ chains of the B cell antigen receptor (7), the Fce receptor of mast cells (8), and the Fcy receptors of monocytes and macrophages (9–12). Upon engagement of these receptors, Syk becomes phosphorylated and is thereby activated to phosphorylate itself and additional cellular proteins. Participation of Syk in T cell activation was first suggested by the finding that this kinase, when fused to the transmembrane and extracellular domains of CD7 and CD16, respectively, could induce complete T cell activation (13). More recently, Syk was found to be constitutively associated with the TCR-CD3 complex in a basal state, then rapidly autophosphorylated and enzymatically activated up to 20-fold after T cell stimulation (14). Importantly, the activation of Syk does not depend on the presence of Lck in T cells, although both Lck and Fyn can act as downstream amplifiers of Syk’s initial signal (14).

A model of sequential activation of Src and Syk family kinases has been proposed (15, 16) whereby receptor clustering stimulates one or more of the membrane-associated Src kinases, resulting in phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosines within receptor chains. The tandem SH2 domains of Syk (and Zap-70 in T cells) can then bind to these newly created docking sites, leading to the phosphorylation of Syk and an increase in its intrinsic kinase activity (17). The molecular events leading to downstream processes such as phosphatidylinositol 4,5-bisphosphate breakdown, PI3-kinase activation, and stimulation of the Ras pathway is likely attributed to phosphorylation of target proteins by both Src and Syk families of kinases. Since Src kinases are localized within particulate fractions, whereas Syk kinase is present mostly in the cytosolic fraction of cell lysates, it has been proposed that each type of kinase is positioned within the cell to interact with and phosphorylate a distinct subset of proteins (18). Recently a number of reports have emerged implicating the proteins HS1, cortactin, α-tubulin, and phospholipase C-γ (PLC-γ) as specific substrates for Syk (18–21).

In this study, we focused on the identification of signaling elements immediately downstream to Syk in the pathway leading to complete T cell activation. Most studies on T cell signaling are carried out on cells stimulated with a cross-linking antibody to the TCR-CD3 complex. In this manner, both Src and Syk kinase families are rapidly activated, and intricate signaling complexes are formed (22), making it difficult to assign a particular T cell substrate to one kinase. To circumvent this problem, we have constructed a signaling molecule with an extracellular single chain Fv (scFv) motif attached via a transmembrane region to Syk. This design, similar to one devised by Kolanus et al. (13), allows for simple and direct clustering of Syk molecules with antigen, thus bypassing the...

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The abbreviations used are: TCR, T cell receptor; PLC, phospholipase C; IL, interleukin; TNP, trinitrophenyl; TNP-Fγ, trinitrophenyl-fowl γ-globulin; sc, single chain; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; ITAM, immunoreceptor tyrosine-based activation motif.
initial activation of Src family kinases and their phosphorylation of receptor ITAM sequences. We show here that direct aggregation of Syk molecules in a T cell hybridoma transmits a signal culminating in the effective production of IL-2 and target cell lysis. Initially, Syk aggregation causes an increase in the association between Syk and Cbl and promotes the tyrosine phosphorylation of Cbl, a cytosolic multidomain protein. Interestingly, in this system we were unable to detect significant phosphorylation of PLC-γ, a proposed substrate of T cell tyrosine kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following antibodies were used in these experiments: GK20.5 (anti-Sp6 monoclonal antibody), anti-Cbl and anti-Syk (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PLC-γ (for Western blotting from Transduction Laboratories, Lexington, KY) and for immunoprecipitation an anti-peptide polyclonal antibody kindly provided by Yosef Yarden at the Weizmann Institute (23), and anti-phosphotyrosine 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Protein A- and protein G-Sepharose were from Pharmacia (Uppsala, Sweden). Oligonucleotide synthesis and DNA sequencing were performed by the Biological Services at the Weizmann Institute of Science. Trinitrophenyl-fowl γ-globulin (TNF-FG) or TNF-A.20 were made as described previously (24, 25).

**Construction of Chimeric Syk Molecules**

Constitution of the anti-TNP single chain Fv genes from the Sp6 anti-TNF monoclonal antibody has been described (25). For isolation of the Syk tyrosine kinase cDNA, reverse transcription-polymerase chain reaction was carried out on RNA from both Jurkat human leukemia and anti-CD3-stimulated human T cells. The polymerase chain reaction was performed in two stages, and DNA pieces were later joined at a native HindIII site. Primers were designed based on the porcine sequence with comparison to the predicted human amino acid sequence (13). Similarly, the CD8 transmembrane and hinge regions were amplified by reverse transcription-polymerase chain reaction from Jurkat RNA. The construct (scFv-Syk) was sequenced in its entirety before insertion into the pBSV expression vector. Oligonucleotides for construction of chimeric genes were synthesized as follows.

**Syk—Amino-terminal fragment 5′ primer: 5′-CTCCTGAGAATG-3′; amino-terminal fragment 3′ primer: 5′-TCTTCCCTCTGGGAGTGAAGGTCC-3′; carboxy-terminal fragment 5′ primer: 5′-AAGGGACAAAACTGGG-3′; carboxy-terminal fragment 3′ primer: 5′-GGCTGCAGGATTACCAACATCTGATGATA-3′.

**CD8 Transmembrane + Hinge (Including J Region of Heavy Chain)—5′ primer: 5′-CCGGTACGGCTTCTTCCGCGTGACACATGCTGATC-3′; 3′ primer: 5′-CTGCTAGGTTGGCGATTAAGGGGATACAC-3′.

**Expression of Chimeric Syk Molecules**

Twenty µg of pHSV-scFv-Syk DNA was transfected into 273 murine hybridoma cells by electroporation as described elsewhere (25). Transfectants were selected in G418 at 2 mg/ml. Expression of chimeric protein on the surface of transfected cells was evaluated by immunofluorescence staining using the GK20.5 anti-Sp6 idotype and fluorescein isothiocyanate-labeled anti-mouse Fab′ antibody. For Western blotting, cells were washed in phosphate-buffered saline and solubilized in lysis buffer (50 mM Hapes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.4% aprotonin (24.4 trypsin inhibitory units/ml), 5 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor) for 1 h at 4 °C. After centrifugation at 17,000 g, supernatants were collected at 3 x 10⁷/ml in the same medium. 1.5 ml of cell suspension were placed on each well for the indicated time periods at 37 °C, after which cells were collected, quickly spun, and solubilized as above in lysis buffer without NaF but containing 10 mM sodium pyrophosphate and 80 µM β-glycerophosphate disodium salt. The TCR-expressing hybridoma, MD45, was stimulated by incubating 1 x 10⁶ cells with 0.5 µl of supernatant from the anti-CD3 hybridoma 2C11. After indicated time periods, cells were quickly spun and solubilized as above. Upon separation by SDS-PAGE under reducing conditions and transfer of proteins to nitrocellulose, immunoblotting was performed with the monoclonal anti-phosphotyrosine antibody 4G10 followed by detection with peroxidase-labeled goat anti-mouse antibody and ECL.

For immunoprecipitation experiments, 10 µg of anti-Cbl or 7 µl of PLC-γ, antisera were added to equivalent amounts of lysate protein for 2 h at 4 °C, after which 30–40 µl of protein A- or protein G-Sepharose were added for another 2 h with constant agitation. Anti-Sp6 antibody GK20.5, first bound to protein G-Sepharose, was used for immunoprecipitation of chimeric Syk through the scFv portion. Immunoprecipitates were washed three times in wash buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 10 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.4% aprotonin (24.4 trypsin inhibitory units/ml), 5 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor), resuspended in 1 x reducing SDS sample buffer, and separated by SDS-PAGE. Immunoblots were developed by ECL detection after incubation with primary antibodies. Stripping was performed according to the instructions provided by the supplier.

**Kinase assays**

Kinase assays were performed on anti-Sp6 immunoprecipitates, which were washed three times in a buffer containing 50 mM Hapes, pH 7, 150 mM NaCl, 0.1% Triton, 10% glycerol, 1 mM Na3VO4, and 5 mM NaF. Sepharose-bound proteins were then incubated in the same buffer containing 10 mM magnesium acetate, 10 mM MnCl2, and 20 µCi of γ-32P[ATP (3000 Ci/mmol, DuPont NEN) for 10 min at room temperature and washed three more times in wash buffer before resuspension in reducing sample buffer. After separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride (MSI, Westboro, MA), and the membrane was exposed to x-ray film both before and after treatment with 1 N KOH for 1 h at 55 °C.

**Functional Assays**

To measure specific IL-2 production, 10⁵ transfectants expressing the scFv-Syk chimera were incubated with 3 x 10⁵ TNF-modified A.20 or L1210 B lymphoma cells in DMEM containing 10% fetal calf serum for 18–24 h. Alternatively, transfectants were reacted with plastic-immobilized TNP-FG, at a molar ratio of TNP 11-FG to scFv moiety of 20:1, and then assayed for chimericSyk expression on the cell surface using the GK20.5 anti-Sp6 monoclonal antibody. Binding was measured by flow cytometry and was expressed as the percentage of cells reactive with GK20.5 antibody.

**RESULTS**

**Expression of Chimeric ScFv-Syk Molecules**—To achieve an effective and direct means of clustering Syk molecules, we constructed a chimeric molecule in which the scFv of an anti-TNP antibody, Sp6, is attached via the hinge and transmembrane domains of CD8α to the cytoplasmic tyrosine kinase. The scFv moiety serves as an efficient method for aggregating Syk molecules using polyvalent TNF antigen and an experimental handle to detect and isolate chimeric Syk molecules. The CD8α hinge region has been shown to be important in the expression and extension of Ig-like domains (27). Our approach, as described under “Experimental Procedures,” the chimeric signal transduction molecule was cloned into an expression vector that contains the Rous sarcoma virus-long terminal repeat promoter and the neomycin resistance gene (28). DNA was transfected into a mutant of the murine T cell hybridoma MD45, named 27J, which lacks the T cell receptor as a result of defective production of its α chain (29). Stable transfectants were selected in medium containing G418 and assayed for chimeric Syk expression in the supernatants of transfected cells. Immunoprecipitated proteins were then analyzed by SDS-PAGE of proteins, Western blotting, and immunoblotting was performed with the monoclonal anti-phosphotyrosine antibody 4G10 followed by detection with peroxidase-labeled goat anti-mouse antibody and ECL. The immunoprecipitated proteins were then analyzed by Western blotting experiments.
protein expression. When total cell lysates were separated by SDS-PAGE and then immunoblotted with an anti-idiotype antibody (GK20.5), chimeric Syk molecules are detected at the expected size of 112 kDa (Fig. 1A). The larger protein band at approximately 200 kDa seen in Fig. 1A is the dimeric form of the chimera (since recognition of the Sp6 antigen by GK20.5 is destroyed under completely reducing conditions, boiling of lysates was avoided).

To assure that the Syk molecules in this configuration retained protein tyrosine kinase activity, chimeras were immunoprecipitated from scFv-Syk-transfected cells (clone S8-1.9) with anti-idiotype antibodies, and washed immunoprecipitates were subjected to an in vitro kinase assay. As seen in Fig. 1B, there was effective incorporation of [γ-32P]ATP into several molecules, the major one corresponding in size to that of the scFv-Syk chimera itself. The gel in Fig. 1B was treated with 1 N KOH before exposure to film, yet the same pattern of phosphorylated protein bands was seen before treatment, confirming that the incorporation of [γ-32P]ATP was for the most part on tyrosine residues. The same antibody did not precipitate any kinase activity from nontransfected 27J cells.

Fig. 1C shows fluorescence-activated cell sorter analysis of three representative transfectants and nontransfected 27J cells after staining with antibody to the extracellular scFv, and then with a secondary fluorescein isothiocyanate-conjugated antibody. These results confirm the surface expression of the chimeric scFv-Syk protein.

Cross-linking of Chimeric scFv-Syk Initiates Production of IL-2 and Target Cell Cytolysis—To assess the ability of scFv-Syk to initiate IL-2 production in the T cell hybridoma, transfectants were incubated with antigen either as TNP-modified carrier protein (TNP-FgG) or TNP-modified A.20 or L1210 cells. Supernatants were then tested for their ability to support the growth of an IL-2-dependent CTL-L line. The results in Fig. 2A show the significant secretion of IL-2 from chimeric Syk-transfected cells upon stimulation with either type of antigen. The homologous kinase Zap-70 in chimeric form, however, was not able to trigger IL-2 production upon antigen triggering, despite being present on the cell surface (not shown). The scFv-Syk chimeras can also mediate specific, non-major histocompatibility complex-restricted cytolytic activity toward TNP-modified A.20 target cells as shown in Fig. 2B.
Triggering of Chimeric Syk Molecules Results in Cbl Phosphorylation—To reveal the major proteins that undergo tyrosine phosphorylation due to antigen-induced clustering of Syk molecules, nontransfected 27J and scFv-Syk bearing cells (clone S8-1.9) were stimulated with TNP-F-G, lysed, and analyzed by anti-phosphotyrosine immunoblotting (Fig. 3A). In transfected cells only, a protein with a molecular mass of ~112 kDa is detected at all time points tested, corresponding in size to that of the chimeric scFv-Syk molecule. Immunoprecipitation experiments with anti-idiotype antibodies (GK20.5), followed by anti-phosphotyrosine immunoblotting, confirmed that scFv-Syk chimeras are indeed constitutively phosphorylated (Fig. 3C). In many experiments we have noted a slight decline in chimera phosphorylation upon longer stimulation periods (Fig. 3C, 30-min stimulation). However, we were unable to observe any reproducible change in Syk's kinase activity upon antigen stimulation when tested by in vitro phosphorylation of immunoprecipitated molecules (data not shown).

After 10-min stimulation with antigen, the first tyrosine-phosphorylated protein detected in S8-1.9 transfecants has a molecular mass of ~120 kDa (Fig. 3A). As expected, nontransfected 27J cells show no phosphotyrosine response upon incubation with TNP-F-G. Since the TCR tyrosine kinase substrate Cbl is of the same size, we tested whether Cbl is identical to the protein phosphorylated upon stimulation of chimeric Syk. This 120-kDa cytoplasmic protein contains potential nuclear localization and zinc finger domains, proline-rich, and leucine zipper regions (30). Cbl is the cellular homolog of \( v\)-cbl, first discovered as the transforming gene of the CaS NS-1 murine retrovirus which causes pre-B lymphomas and myelogenous leukemias (31).

When the blot in Fig. 3A was stripped and reprobed with anti-Cbl antibodies, Cbl protein exactly aligned with the upper tyrosine-phosphorylated band (Fig. 3B). To confirm this finding, immunoprecipitation experiments were performed with anti-Cbl antibodies as shown in Fig. 4. Antigen stimulation of scFv-Syk molecules for 10 min results in the specific tyrosine phosphorylation of Cbl, which peaks at 20 min (Fig. 4, left panel). Importantly, the amounts of Cbl immunoprecipitated from the various lysates were approximately equal (Fig. 4, middle panel). The lower band in the anti-phosphotyrosine immunoblot corresponds to the scFv-Syk chimera (see below). Interestingly, we also see a transient increase in phosphorylation of this Cbl-bound chimera, which was undetectable in anti-idiotype immunoprecipitates (see Fig. 3C).

An Increase in Syk-Cbl Association Precedes the Syk-mediated Phosphorylation of Cbl—The finding that aggregation of chimeric Syk triggers phosphorylation of Cbl suggested a possible association between these two molecules. Indeed, we do detect a constitutive association between chimeric Syk and Cbl, as seen by immunoblotting Cbl immunoprecipitates with anti-Syk antibodies (Fig. 4, right panel, 0 min). This basal association is most likely not mediated by tyrosine phosphorylation since addition of up to 50 mM of \( p\)-nitrophenyl phosphate to immunoprecipitation reactions does not disrupt it (Fig. 5A). This compound has been shown to inhibit binding of antiphosphotyrosine antibodies (32) and to disrupt protein interactions that depend on the presence of phosphotyrosine (33, 34).

Interestingly, when chimeric Syk molecules were immunoprecipitated with the GK20.5 anti-idiotype antibody, we detected a significant increase in their association with Cbl upon antigen stimulation (Fig. 5B). As a result of this switch in experimental protocol, the coprecipitation of Cbl through anti-scFv idiotype antibodies is often much lower than in the reciprocal configuration, and the detectable increase in Syk-Cbl association is more pronounced (compare Fig. 4, right panel, and Fig. 5B, left panel). This is not surprising considering that scFv-Syk chimeras are greatly overexpressed in S8-1.9 cells, in excess of possible Cbl molecules to bind, whereas a large proportion of endogenous Cbl protein does bind the Syk chimera in the basal state. Fig. 5B shows a peak in Syk-Cbl association after 10-min antigen stimulation, just preceding that seen for Cbl phosphorylation (Fig. 4). This heightened association between Syk and Cbl molecules persisted for at least 40 min. Although the amount of basal and induced Syk-Cbl association can vary between experiments, densitometer scanning of autoradiograms from several experiments confirmed a 2–7-fold increase upon stimulation of chimeric Syk molecules with immobilized antigen.

![Fig. 3. Tyrosine phosphorylation in total cell lysates after antigen stimulation. A, cell lysates from TNP-F-G-stimulated 27J or scFv-Syk-transfected cells (clone S8-1.9) were separated by SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody. The blot was then stripped and reprobed with anti-Cbl antibodies (B), anti-idiotype (GK20.5) immunoprecipitates from S8-1.9 cells were immunoblotted with either anti-phosphotyrosine (pTyr) or anti-Syk antibodies.](image1)

![Fig. 4. Tyrosine phosphorylation of Cbl after antigen stimulation. scFv-Syk-transfected cells (clone S8-1.9) were stimulated on TNP-F-G-coated wells for the indicated times. Cells were sedimented by brief centrifugation and lysed, and equivalent amounts of protein were reacted with anti-Cbl antibodies. Washed immunoprecipitates were immunoblotted with anti-phosphotyrosine (pTyr) antibodies (left panel). The same blot was stripped and reprobed with anti-Cbl antibodies (middle panel), stripped again, and reprobed with anti-Syk antibodies (right panel).](image2)
Activation of Chimeric Syk Molecules Does Not Cause Significant Phosphorylation of PLC-γ—Many studies on T cell activation have shown the importance of PLC-γ as a substrate for T cell tyrosine kinases (35–38). In fact, a number of reports have suggested this enzyme is a direct substrate for the Syk kinase (13, 21, 39–41). To assess the state of tyrosine phosphorylation of PLC-γ in our system, cells containing chimeric Syk molecules were stimulated with antigen as above, and cell lysates were probed with anti-PLC-γ antibodies. When PLC-γ1 immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies, we could not detect significant PLC-γ1 phosphorylation upon cross-linking of chimeric Syk, despite notable levels of PLC-γ in these cells (Fig. 6, panels A and B). This is in marked contrast to the pronounced phosphorylation of Cbl in stimulated cells of the same experiment (Fig. 6, panels C and D). Interestingly, despite the lack of PLC-γ1 phosphorylation in antigen-stimulated S8-1.9 cells, there is a constitutive association between PLC-γ1 and the scFv-Syk chimera (Fig. 7). This is likely due to an interaction between the SH2 domains of PLC-γ1 and tyrosine phosphorylated scFv-Syk (see Fig. 3C), as has been shown to occur in antigen receptor-stimulated B cells (41).

To verify that PLC-γ1 could serve as a substrate for activated tyrosine kinases in these cells, we tested whether it underwent phosphorylation upon stimulation of the endogenous TCR complex in the TCR-expressing hybridoma, MD45. These cells indeed display a pronounced increase in both PLC-γ1 and Cbl phosphorylation in response to anti-CD3 triggering (Fig. 8). Thus, although the activation of PLC-γ1 may be an important step in the pathway emanating from the TCR-CD3 complex, it appears to be much less crucial in the signal propagated through chimeric Syk molecules.

**DISCUSSION**

Given the importance of Syk kinase activity in lymphocyte activation (2, 16), identification of its early targets of phosphorylation and association in T cells is an immediate goal for understanding propagation of the signal leading to T cell activation. In the direct triggering of the chimeric scFv-Syk molecules by an antigen stimulus, we found that the first detectable tyrosine kinase event was phosphorylation of the proto-oncogene c-cbl (Figs. 3 and 4). Cbl is strongly phosphorylated on tyrosine residues upon stimulation of a variety of growth factor and antigen receptors (42–47). As a multidomain protein, Cbl...
is capable of interacting in vivo with a variety of molecules involved in cellular signaling, including protein tyrosine (43, 48–52) and lipid (33, 53) kinases, and adaptor proteins (44, 51, 53–57). Although its biological role is at present unclear, its participation in cellular transformation (58–60) suggests an important function for Cbl in cellular proliferation.

Cbl is also one of the earliest and most prominent protein tyrosine kinase substrates in this T cell hybridoma when stimulated through the TCR-CD3 receptor complex (Fig. 8, C and D). It has recently been suggested that by interacting with adaptor proteins, such as Grb2, Crk, and Crk-L, Cbl may affect the function of nucleotide exchange factors including sos, vav, and C3G (57, 61). Since direct triggering of Syk allows for Cbl phosphorylation, we speculate that this kinase may be an important initiator of the pathway that regulates guanine nucleotide exchange on small G proteins in T cells.

We observed a basal level of association between scFv-Syk chimeras and Cbl (Figs. 4 and 5), which is increased in a time-dependent fashion upon antigen stimulation (Fig. 5). Thus our experiments demonstrate that direct aggregation of Syk kinase molecules in T cells augments their association with Cbl, while the kinetics of this event just precedes that of aggregation-induced Cbl phosphorylation. A similar activation-dependent complex was shown to form between Syk and Cbl upon stimulation of the B cell antigen receptor (51). Furthermore, we could only detect antigen-induced phosphorylation of scFv-Syk chimeras which coimmunoprecipitate with Cbl (Fig. 4), and not in the larger cellular pool of chimeric molecules (Fig. 3). These data suggest a direct phosphorylation of Cbl by the chimeric Syk kinase, although the indirect involvement of additional kinases cannot be ruled out (51). The Src family member Fyn is one candidate kinase, as several studies have shown it to exist in preformed complexes with Cbl in unstimulated T cells (45, 62). We have also noted a constitutive association between Fyn and Cbl in both scFv-Syk-transfected and TCR-expressing MD45 cells (not shown). However, although antigen-CD3 stimulation results in an increase in Fyn-associated phospho-Cbl in MD45 cells, the same is not true for antigen stimulation of scFv-Syk receptors (not shown). Another Src family member, Lck, although expressed in the hybridoma (not shown), is likely not involved in the phosphorylation of Cbl, since this event can occur efficiently in the mutant Jurkat cell line, JCaM1.6, which lacks Lck expression (63).

The nature of the Syk-Cbl association in scFv-Syk transfected cells is not clear. Band and colleagues (64) just recently described a novel phosphotyrosine-binding domain in the NH2-terminal region of Cbl that can directly interact with Zap-70 in membrane, and Syk intracellular regions) in Jurkat leukemia cells is in fact shown to result in PLC-γ1 phosphorylation (13). Differences in the cell type studied may be an important differentiating factor since we have noticed that, in MD45 cells (although there is clear phosphorylation of this protein upon anti-CD3 stimulation (Fig. 8)), the ratio of phosphorylated to total PLC-γ1 immunoprecipitated is much less than that in Jurkat cells (not shown), in which most of the studies on PLC-γ phosphorylation have been performed.

Earlier reports have suggested a role for Src family kinases in coupling the TCR to the activation of PLC-γ1 (66, 67). In fact, five different Src family kinases were shown to efficiently phosphorylate purified PLC-γ1 and PLC-γ2 in vitro (68). Furthermore, a recent study of chicken B cells deficient in Bruton’s tyrosine kinase revealed that PLC-γ2 phosphorylation and activity are regulated by this kinase as well (69). Homologous Bruton’s tyrosine kinase family members, Emt and Itk, are present in T cells (70, 71) where they may play a role, along with Src and Syk family kinases, in the activation of PLC-γ. Interestingly, in a heterologous system in which B cell receptor-induced signaling was reconstituted in a nonlymphoid cell line (72), Syk activity was necessary but not sufficient to couple the B cell receptor to activation of PLC-γ2. Collectively, these data suggest that for efficient PLC-γ2 phosphorylation and/or activation, the combined actions of different tyrosine kinase families are required, a situation which may be unattainable by direct triggering of chimeric Syk molecules.

While several cytoplasmic protein tyrosine kinases have been implicated in the development and activation of T lymphocytes (1, 2), and a number of models proposed to describe the sequence of events following engagement of the TCR, discrepancies still remain. One open question concerns the requirement for Src kinases in the activation of Syk (14, 73). The construction of a chimeric protein based on this key intracellular tyrosine kinase, linked to the extracellular scFv, has allowed for direct triggering of the T cell-signaling machinery. In this T cell hybridoma, clustering of Syk is sufficient to achieve complete T cell activation, leading to antigen-specific IL-2 production and target cell lysis (Fig. 2). Similar conclusions were drawn from studies in Jurkat T cells transduced with CD16-
CD7-Syk chimeric molecules (13), as discussed above. In these studies, aggregation of Syk chimeras alone, but coaggregation of chimeras bearing Fyn and Zap-70 kinases, allowed for initiation of B cell receptor signaling a requirement for Lyn to induce tyrosine phosphorylation and activation of Syk was demonstrated (75). Recent studies with Syk chimeric molecules support a model in which Syk, as an integral part of the TCR complex, possesses initiating capabilities once aggregated. This aggregate can result from engagement of the TCR-CD3 complex and may be Src family kinase-independent. The situation with Syk is more complex. In B cell receptor signaling a requirement for Lyn to induce tyrosine phosphorylation and activation of Syk was demonstrated (75). In trans-signaling a requirement for Lyn to induce tyrosine phosphorylation and activity of Src kinases Lyn or Fyn. In the MD45 T cell hybridoma and activation of Syk, as an integral part of the TCR complex, possibly explaining its functional autonomy. The results we present herein with chimeric Syk molecules support a model in which Syk, as an integral part of the TCR-CD3 complex and rapidly activated upon stimulation, even in the absence of Lck (14). Furthermore, a recent report showed the intrinsic kinase activity of Syk to be significantly superior to that of Zap-70 (77), possibly explaining its functional autonomy. The results we present herein with chimeric Syk molecules support a model in which Syk, as an integral part of the TCR-CD3 complex, possesses initiating capabilities once aggregated. This aggregate can result from engagement of the TCR-CD3 complex and may be Src family kinase-independent.

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