Differential Regulation of T Cell Antigen Responsiveness by Isoforms of the src-related Tyrosine Protein Kinase p59sYN
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Summary
Recent observations suggest that the src-related tyrosine protein kinase p59sYN may be involved in antigen-induced T lymphocyte activation. As a result of alternative splicing, p59sYN exists as two isoforms that differ exclusively within a short sequence spanning the end of the Src Homology 2 (SH2) region and the beginning of the tyrosine protein kinase domain. While one p59sYN isoform (fynB) is highly expressed in brain, the alternative product (fynT) is principally found in T lymphocytes. To further understand the role of p59sYN in T cell activation and to test the hypothesis that p59sYN serves a tissue-specific function in T lymphocytes, we have examined the effects of expression of activated versions (tyrosine 528 to phenylalanine 528 mutants) of either form of p59sYN on the physiology of an antigen-specific mouse T cell hybridoma. Our results demonstrated that the two forms of fyn, expressed in equivalent amounts, efficiently enhance antibody-induced T cell receptor (TCR)-mediated signals. In contrast, only p59sYN increased interleukin 2 production in response to antigen stimulation. This finding implies that the distinct p59sYN isoform expressed in T lymphocytes regulates the coupling of TCR stimulation by antigen/major histocompatibility complex to lymphokine production.

Materials and Methods

Cells. The antigen-specific murine T cell hybridoma BI-141 (10) and BI-141-derived cell lines were grown in RPMI 1640 medium.
supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), penicillin, and streptomycin. Note that specific ribonuclease protection assays showed that parental BI-141 cells exclusively express fynT transcripts (L. M. Chow, A. Veillette, and D. Davidson, unpublished observations). The IL-2-dependent HT-2 indicator cells (11) were cultured in RPMI 1640 medium supplemented as mentioned above, and also containing 50 U/ml rIL-2 and 55 μM β-ME (Gibco Laboratories). FT 5.7 cells are L fibroblasts expressing the hybrid class II MHC molecule A2A3 (kindly provided by Ron Germain, National Institutes of Health, Bethesda, MD). They were grown in HAT-supplemented alpha MEM containing 10% FCS and antibiotics. All cells were maintained at 37°C in a humidified CO2 incubator.

Site-directed Mutagenesis. Wild-type murine fynT (MM23) and fynB (MBI) complementary DNAs (cDNAs) were provided by M. P. Cooke and R. M. Perlmutter (University of Washington, Seattle, WA) (7). All mutations were introduced by oligonucleotide-directed mutagenesis of uracil-enriched double-stranded plasmid DNA, as described by Siliaty et al. (12). Initially, an EcoRI site was introduced at position 210 of the 5' untranslated (UT) region of the fynT cDNA MM23 (using the oligonucleotide 5'AAGCTTCCGAATTCCCCACCAACTG3'). This allowed subsequent removal of most of the 5' UT sequences of MM23 through standard rDNA technology. As this portion of the cDNA contains multiple AUG codons, it may repress the translation of fyn mRNAs (13). Then, to generate activated versions of p59Fσ, tyrosine 528 was mutated to a phenylalanine using the oligonucleotide 5'CGGGCTGAACTGGGCG3'. The mutant fynB cDNA was created by exchanging the Apal-BamHI fragment of MM23 (which contains the seventh exon) with that of the fyn CDNA MBI. All exchanged fragments were fully resequenced and found to contain no additional mutations (data not shown). Similarly kinase-negative variants of F528 p59Fσ and F528 p59Fσ were engineered by introducing a lysine to methionine substitution at position 210 of the ATP-binding site of these polypeptides using oligonucleotide-directed mutagenesis (mutagenic oligonucleotide: 5'TAAGGGTTGAGCAACTG C3'). An identical mutation in other tyrosine protein kinase genes has been shown to abolish catalytic activity (14). After fully resequencing to ensure that no additional mutations were introduced, the smallest restriction fragments containing the mutated nucleotide were substituted for the equivalent fragments in the activated fyn cDNAs.

Retroviral Expression Vectors. The wild-type or mutant fyn cDNAs were inserted in the EcoRI site of the retroviral expression vector pLXSN (provided by D. Miller, Fred Hutchinson Cancer Center, Seattle, WA) (15). Generation of retrovirus packaging cell lines and retrovirus stocks, as well as retroviral infection of BI-141 cells, were performed as described previously (16). Cells were selected for growth in media containing 750 μg/ml G418 and monoclonal vector pLXSN (provided by D. Miller, Fred Hutchinson Cancer Seattle, WA) (7). All mutations were introduced by oligonucleotide-directed mutagenesis (mutagenic oligonucleotide: 5'TAAGGGTTGAGCAACTGC 3'). Equivalent numbers of ceils were plated in triplicate with serial dilutions of mAb F23.1 at 37°C overnight. After removal of the unbound antibody, 2 × 10^6 BI-141 derivatives were added in 200 μl tissue culture medium and incubated at 37°C for 24 h. 50 μl of supernatant were removed, frozen for 1 h at -70°C to destroy carry-over cells, and then tested for IL-2 content. This was performed by measuring [3H]thymidine incorporation in 10^4 IL-2-dependent HT-2 indicator cells (11). HT-2 cells did not respond to mAb F23.1 alone (our unpublished data). Controls were without addition.

To test antigen-induced IL-2 production, BI-141 cells (10^4 cells/well) were plated in triplicate with 5 × 10^6 irradiated MHC class II A2A3-transfected L cells (FT 5.7) in 96-well flat-bottomed tissue culture plates containing serial dilutions of beef insulin in a final volume of 200 μl tissue culture medium (25). After incubation at 37°C for 24 h, supernatants were collected and assayed for IL-2 content as described above. Controls were without any addition.

Results

To better understand the role of p59FσT in mature T cell physiology, we have introduced constitutively activated versions of murine p59FσT and p59FσB in the CD4-negative, class II MHC-restricted beef insulin-specific murine helper
T cell hybridoma BL-141 (10). These activated fyn polypeptides were generated by mutating a COOH-terminal site of tyrosine phosphorylation (tyrosine 528) to a phenylalanine residue, which cannot be phosphorylated (F528 p59\textsuperscript{mu} mutants; Fig. 1 A). Phosphorylation of this conserved tyrosine residue represses the enzymatic function of src-related tyrosine protein kinases (26; for review, see reference 27). Cells were selected for resistance to the aminoglycoside G418 and individual clones isolated by limiting dilution. Clones were first assayed for expression of the appropriate retroviral transcript by Northern blot analysis (Fig. 1 B). Four cell lines expressing comparable levels of either of the two mutant fyn RNAs (Fig. 1 B, lanes 3–6 and 7–10) were randomly selected for further studies. BI-141 cell lines expressing the neomycin marker alone (Neo; Fig. 1 B, lane 2) or comparable amounts of transcripts encoding an activated version of p56\textsuperscript{kk} (F505 p56\textsuperscript{kk}; Fig. 1 B, lanes 11–13) (16) were also included in these analyses. A subsequent fyn-specific immunoblot assay revealed that F528 p59\textsuperscript{mu} expressing cells (Fig. 1 C, lanes 4–11) contained 1.5–3 times more p59\textsuperscript{mu} than parental BI-141 cells (Fig. 1 C, lane 1) controls (Fig. 1 C, lanes 2 and 3). This level of overexpression most likely reflects the high levels of endogenous p59\textsuperscript{AT} in BI-141 cells. All cell lines expressed levels of TCR, CD3, Thyl.2, CD45, and p56\textsuperscript{kk} that were identical to those of parent BI-141 cells. Moreover, all clones remained CD4-negative (data not shown).

Tyrosine protein phosphorylation is the earliest biochemical alteration detected after antigen receptor stimulation (1). It is important to note that, as shown by studies using tyrosine-specific protein kinase inhibitors, this signal is a prerequisite for the subsequent increase in intracellular calcium and IL-2 production (28, 29). To investigate the ability of the two fyn isoforms to contribute to antigen receptor signaling, the effects of expression of these polypeptides on TCR-induced tyrosine protein phosphorylation were examined using anti-TCR mAb H57.59.7 or anti-CD3 epsilon mAb 145-2C11 (data not shown). Although the pattern and duration of substrate tyrosine phosphorylation were generally the same for cells expressing either fyn mutant, quantitative analyses suggested that tyrosine phosphorylation of certain substrates (p70 and p36) was more prolonged in p59\textsuperscript{mu} than p59\textsuperscript{mu} expressing cells. Both mutants also augmented baseline tyrosine phosphorylation of polypeptides migrating at 100, 85, 59, and 54 KD. The substrates showing augmented TCR-induced tyrosine phosphorylation in F528 p59\textsuperscript{mu} expressing cells (Fig. 2 B, lanes 5–12) had the same apparent molecular weights as those previously shown to be regulated by F505 p56\textsuperscript{kk} (Fig. 2 B, lanes 13–16) (16). However, the enhancement by F528 p59\textsuperscript{mu} was generally less than that conferred by the activated kk polypeptide.

Previous studies have shown that the CD45 tyrosine phosphatase can significantly modulate antigen receptor-induced signals (1, 2). This implies that such an enzyme may physiologically regulate the tyrosine protein kinases mediating the TCR-induced signals. Thus, in an attempt to further characterize the mechanisms by which the two fyn isoforms regulate TCR-induced tyrosine protein phosphorylation, the effects of coaggregation of TCR with CD45 were evaluated (Fig. 2 C). As is the case for Neo expressing cells (Fig. 2 C, lane 4), or cells containing F505 p56\textsuperscript{kk} (Fig. 2 C, lane 16), coaggregation of TCR with CD45 on F528 fyn\textsuperscript{AT} (Fig. 2 C, lane 8) or F528 fynB (Fig. 2 C, lane 12) expressing cells abrogated anti-TCR antibody-induced tyrosine protein phosphorylation (Fig. 2 C, lanes 3, 7, 11, and 15). Coupled with the findings reported above, this result suggests that the activated p59\textsuperscript{mu} variants, as well as activated p56\textsuperscript{kk}, regulate antigen receptor-mediated signals through overlapping and possibly similar mechanisms.

We next wanted to ascertain whether the enhancement of TCR-induced signals by fyn resulted in improved lymphokine production (Fig. 3). To this end, cells were stimulated with anti-TCR mAb F23.1 immobilized on plastic and IL-2 production was assayed using the IL-2-sensitive cell line HT-2 (Fig. 3 A). When compared with Neo cells, F528 p59\textsuperscript{mu} and F528 p59\textsuperscript{mu} expressing cells demonstrated a comparable and marked enhancement of antibody-induced IL-2 production. This was most obvious at low concentrations of antibody (30 and 100 ng/ml), at which no response was noted in control cells. In fact, IL-2 release after stimulation with 30 nl/ml of mAb F23.1 tended to be more substantial for cells expressing F528 p59\textsuperscript{mu}.

Cell lines were also tested for reactivity to beef insulin presented in association with the appropriate class II MHC molecules (A\textsubscript{b}A\textsubscript{b}). Two assays, representative of a total of eight independent experiments, are shown in Fig. 3, B and C. These studies revealed that cells expressing F528 p59\textsuperscript{mu} had significantly improved antigen/MHC-induced IL-2 responses when compared with control Neo cells. Surprisingly, this was not the case for cells expressing equivalent amounts of F528 p59\textsuperscript{mu}, which had essentially unaltered responses to antigen (Fig. 3, B and C). Only one F528 p59\textsuperscript{mu} clone (FynB F18, Fig. 3 C) showed a small but consistent increase in antigen-induced lymphokine production. The improvement of antigen responsiveness by F528 p59\textsuperscript{mu} was less than that conferred by expression of CD4 or F505 p56\textsuperscript{kk} in BI-141 cells. A comparison with representative clones is included in the experiment depicted in Fig. 3 C.

We finally verified that the enhancement of T cell responsiveness by F528 p59\textsuperscript{mu} was related to the elevated tyrosine protein kinase activity of this mutant. To this effect, F528 fyn\textsuperscript{AT} and F528 fynB polypeptides with abolished catalytic activity (M296F528 p59\textsuperscript{mu}) (Fig. 4) or wild-type fyn proteins (Fig. 5) were expressed in BI-141 cells. Cell lines containing equivalent amounts of retroviral fyn transcripts were selected for comparison with F528 p59\textsuperscript{mu} expressing cells (Fig. 4 A, and data not shown). The M296F528 p59\textsuperscript{mu} expressing cells contained 1.5–2.5-fold higher levels of fyn protein than con-
Figure 1. Expression of F528 p59\textsuperscript{yn} in BI-141 cells. (A) Retroviral constructs. The activated fyn\textsuperscript{T} and fyn\textsuperscript{B} mutants were inserted into the retroviral vector pLXSN at a convenient multiple cloning site flanked by the 5' Moloney Murine Sarcoma Virus (MoMSV) LTR and the 3' Moloney Murine Leukemia Virus (MoMLV) LTR. The neomycin (neo) phosphotransferase gene (Tn5) is driven by the SV40 early promoter. The position of \( \psi^+ \) sequences is shown. (Arrow) Start sites and direction of transcription. (pA) Position of the polyadenylation signal. K296 is the lysine residue at position 296 of p59\textsuperscript{yn} and is part of the ATP-binding site. Y528, which represents the major site of in vivo tyrosine phosphorylation of p59\textsuperscript{yn}, tyrosine 528, was replaced through site-directed mutagenesis by phenylalanine (F528), as described in Materials and Methods. Exon 7 is designated 7A in fyn\textsuperscript{B} and 7B in fyn\textsuperscript{T}. (B) Northern blot analysis. Expression of retroviral construct-encoded transcripts was assayed in clonal BI-141 cell lines infected with retroviruses encoding the neomycin phosphotransferase alone (lane 2), F528 p59\textsuperscript{yn} (lanes 3–6), F528 p59\textsuperscript{yn} (lanes 7–10), and, for comparison, F505 p56\textsuperscript{ck} (lanes 11–13). RNA from parental BI-141 cells was also assayed as negative control (lane 1). Lanes 1: BI-141 parent; 2: Neo 2; 3: FynT F2; 4: Fyn\textsuperscript{T} F7; 5: Fyn\textsuperscript{T} F12; 6: Fyn\textsuperscript{T} F19; 7: FynB F13; 8: Fyn\textsuperscript{B} F15; 9: FynB F17; 10: FynB F18; 11: Lck F7; 12: Lck F9, and L3: Lck F13. The probe used for hybridization was a 687-bp HindIII-NaeI fragment from pLXSN that encompasses the majority of the neomycin resistance gene (neo) (Fig. 1 A). This probe recognized the chimeric retroviral transcript initiating from the 5' MoMLV LTR, incorporating the sequences inserted in the multiple cloning site and terminating at the polyadenylation signal downstream of the neo gene. This was done to allow adequate comparison of the abundance of retroviral construct-encoded fyn and lck transcripts in BI-141 derivatives. (\( \bullet \)) designates hybrid fyn and lck messages (6.0 kb) produced by the retroviral vectors. The other RNA species detected at 3.1 and 1.9 kb are retroviral vector-encoded transcripts. (Right) Positions of the 28S and 18S ribosomal RNAs. Exposure: 6 h. (C) fyn immunoblot. Levels of p59\textsuperscript{yn} were measured in parental BI-141 cells (lane 1) and in cell lines expressing the neomycin phosphotransferase alone (lanes 2 and 3), F528 p59\textsuperscript{yn} (lanes 4–7) and F528 p59\textsuperscript{yn} (lanes 8–11), using a fyn-specific immunoblot assay. Lanes 1: BI-141 parent; 2: Neo 2; 3: Neo 4; 4: Fyn\textsuperscript{T} F2; 5: Fyn\textsuperscript{T} F7; 6: Fyn\textsuperscript{T} F12; 7: Fyn\textsuperscript{T} F19; 8: Fyn\textsuperscript{B} F13; 9: Fyn\textsuperscript{B} F15; 10: Fyn\textsuperscript{B} F17, and 11: Fyn\textsuperscript{B} F18. The immunoreactive species detected at 56 and 54 kD appear to be proteolytic cleavage products of p59\textsuperscript{yn} (our unpublished observations). (Left) Position of p59\textsuperscript{yn} and (right) positions of prestained molecular weight markers (in kD). Exposure: 4 h. Levels of p59\textsuperscript{yn} expression were quantified as described in Materials and Methods and, relative to the average of Neo cells, are as follows: Fyn\textsuperscript{T} F2: 1.8; Fyn\textsuperscript{T} F7: 2.1; Fyn\textsuperscript{T} F12: 1.6; Fyn\textsuperscript{T} F19: 1.5; Fyn\textsuperscript{B} F13: 2.4; Fyn\textsuperscript{B} F15: 1.9; Fyn\textsuperscript{B} F17: 2.3, and Fyn\textsuperscript{B} F18: 2.7.
Figure 2. Enhancement of TCR-induced tyrosine protein phosphorylation signal by F528 p59fn. Antiphosphotyrosine immunoblots. (A) Time course experiments. Antibody-mediated cross-linking of clonal BI-141 cell lines expressing the neomycin phosphotransferase (Neo I; lanes 1–6), F528 p59fnT (FynT F2; lanes 7–12) and F528 p59fnB (FynB F13; lanes 13–18) was performed with anti-TCR mAb F23.1 and RAM IgG for variable periods of time at 37°C as described in Materials and Methods. Lanes 1, 7, and 13: untreated controls; lanes 2, 8, and 14: mAb F23.1 + RAM IgG for 1 min; lanes 3, 9, and 15: mAb F23.1 + RAM IgG for 5 min; 4, 10, and 16: mAb F23.1 + RAM IgG for 10 min; lanes 5, 11, and 17: mAb F23.1 + RAM IgG for 15 min; lanes 6, 12, and 18: mAb F23.1 + RAM IgG for 30 min. Exposure: 12 h. Compared to Neo expressing cells, the enhancement of tyrosine phosphorylation after 1 min of cross-linking is: FynT: p120: 2.7-fold; p100: 3.4-fold; p74: 3.5-fold; p70: 3.0-fold, and p36: 4.4-fold. FynB: p120: 2.9-fold; p100: 2.4-fold; p74: 3.8-fold; p70: 2.9-fold, and p36: 3.2-fold. (B) TCR-induced tyrosine protein phosphorylation in F528 p59fn expressing cells. Cross-linking of BI-141 cell lines expressing the neomycin marker (lanes 1–4), F528 p59fnT (lanes 5–8), F528 p59fnB (lanes 9–12) and, for comparison, F505 p56fn (Lck F7; lanes 13–16) was performed with anti-TCR mAb F23.1 + RAM IgG for 2 min at 37°C. Lanes 1 and 2: Neo I; 3 and 4: Neo 2; 5 and 6: FynT F2; 7 and 8: FynT F7; 9 and 10: FynB F13; 11 and 12: FynB F7; 13 and 14: Lck F7; 15 and 16: Lck F9. Lanes 1, 3, 5, 7, 9, 11, 13, and 15: RAM IgG alone and lanes 2, 4, 6, 8, 10, 12, 14, and 16: mAb F23.1 + RAM IgG. Exposure: 10 h. Note that similar results were consistently obtained with several other randomly selected F528 p59fn expressing clones (data not shown). (C) Effects of coaggregation of TCR with CD45 on tyrosine protein phosphorylation. Cross-linking of BI-141 cell lines expressing the neomycin marker (Neo 1; lanes 1–4), F528 p59fnT (FynT F2; lanes 5–8), F528 p59fnB (FynB F13; lanes 9–12) and, for comparison, F505 p56fn (Lck F9; lanes 13–16) was performed using biotinylated primary antibodies and avidin for 5 min at 37°C as described. Lanes 1, 5, 9, and 13: avidin alone; lanes 2, 6, 10, and 14: anti-CD45 mAb 30-F11 + avidin; lanes 3, 7, 11, and 15: anti-TCR mAb F23.1 + avidin, and lanes 4, 8, 12, and 16: mAb F23.1 + mAb 30-F11 + avidin. Exposure: 12 h. (Right) Positions of molecular weight markers. (Left) Major tyrosine phosphorylation substrates.

trol Neo cells (Fig. 4 B). These levels were comparable to the amounts detected in F528 p59fn expressing cells. Further analysis by antiphosphotyrosine immunoblot (Fig. 4 C) showed that cells expressing the inactive versions of fynT (Fig. 4 C, lanes 5–8) or fynB (Fig. 4 C, lanes 9–12) had TCR-induced tyrosine phosphorylation signals that were comparable to those of Neo cells (Fig. 4 C, 1–4). Moreover, these cells failed to demonstrate enhanced IL-2 responses to stimulation.
with antigen (Fig. 4 D) or anti-TCR antibodies (data not shown). Similarly, the BI-141 derivatives overexpressing wild-type fynT or fynB proteins (Fig. 5 A) did not show improved responsiveness to antigen (Fig. 5, B). Therefore, lack of the regulatory COOH-terminal site of tyrosine phosphorylation (tyrosine 528) is critical for enhancement of antigen responsiveness by fynT.

Discussion

The results presented in this report show that activated p59frt and p59frb efficiently enhance anti-TCR antibody-induced responses in the murine T cell hybridoma BI-141. This finding is consistent with the results of others (9) and further implicates p59frb in the regulation of T cell receptor signaling. It is interesting, however, that even though the two proteins were expressed at equivalent levels, only activated p59frt efficiently increased T cell responsiveness to antigen stimulation. This observation strongly suggests that fynT serves as a tissue-specific function in T lymphocytes.

It was intriguing to note that despite the distinct effects of p59frt and p59frb on antigen-induced T cell responses, we failed to detect consistent qualitative or quantitative differences in the ability of these two polypeptides to improve T cell responses to antibody stimulation, including tyrosine protein phosphorylation and lymphokine production. Consistently, we found that both fyn isoforms had apparently similar in vitro tyrosine protein kinase activities and seemed capable of physically interacting with the TCR complex (D. Davidson and A. Veillette, unpublished observations). Although it
Figure 4. Effects of kinase-negative p59\textsuperscript{efm} polypeptides on T cell responsiveness. (A) Northern blot analysis. Expression of retroviral fyn transcripts was assayed in clonal BI-141 cell lines expressing the neomycin phosphotransferase alone (lane 1), M296F528 p59\textsuperscript{efm} (lanes 2–5), M296F528 p59\textsuperscript{efm} (lanes 6 and 7), and, for comparison, F528 p59\textsuperscript{efm} (lanes 8 and 9). Lanes 1: Neo 2; 2: Fyn\textsuperscript{T} MF4; 3: Fyn\textsuperscript{T} MF6; 4: Fyn\textsuperscript{T} MF8; 5: Fyn\textsuperscript{T} MF11; 6: FynB MF8; 7: FynB MF11; 8: Fyn\textsuperscript{T} F12, and 9: FynB F13. In this experiment, a full-length MM23 fyn\textsuperscript{T} cDNA was used as a probe. (B) Fyn immunoblot. As in Fig. 1 C. Lanes 1 and 2: Neo; 3: Fyn\textsuperscript{T} MF4; 4: Fyn\textsuperscript{T} MF6; 5: Fyn\textsuperscript{T} MF8; 6: Fyn\textsuperscript{T} MF11; 7: FynB MF8; 8: FynB MF11; 9: Fyn\textsuperscript{T} F12, and 10: FynB F13. (Left) Position of p59\textsuperscript{efm}, and (right) positions of molecular weight markers. Exposure: 8 h. The relative amounts of fyn in M296F528 p59\textsuperscript{efm} expressing cell lines (compared with the average of Neo cells) are: Fyn\textsuperscript{T} MF4: 2.1-fold; Fyn\textsuperscript{T} MF6: 2.0-fold; Fyn\textsuperscript{T} MF8: 2.3-fold; Fyn\textsuperscript{T} MF11: 1.7-fold; FynB MF8: 2.1-fold, and FynB MF11: 2.1-fold. (C) Effects of TCR aggregation on tyrosine protein phosphorylation. As in Fig. 2 B. Crosslinking was performed with mAb F23.1 and RAM IgG for 2 min at 37\degree C. Lanes 1 and 2: Neo 1; 3 and 4: Neo 2; 5 and 6: Fyn\textsuperscript{T} MF4; 7 and 8: Fyn\textsuperscript{T} MF8; 9 and 10: FynB MF8; 11 and 12: FynB MF11; 13 and 14: Fyn\textsuperscript{T} F2, and 15 and 16: FynB F13. Lanes 1, 3, 5, 7, 9, 11, 13, and 15: RAM IgG alone and lanes 2, 4, 6, 8, 10, 12, 14, and 16: mAb F23.1 + RAM IgG. (Left) Positions of molecular weight markers, and (left) major tyrosine phosphorylation substrates. The immunoreactive species detected in all lanes at 54–56 kD is the H chain of IgG. Exposure: 10 h. (D) Antigen stimulation assay. Different clonal BI-141 cell lines expressing M296F528 p59\textsuperscript{efm} were tested for beef insulin reactivity and compared with other BI-141 derivatives, as described in Fig. 3 B.
Figure 5. Effects of wild-type p59^+ polypeptides on T cell responsiveness. (A) fyn immunoblot. As in Fig. 1 C. Lanes 1: BI-141; 2: Neo 2; 3: Neo 4; 4: FynT wt6; 5: FynT wt7; 6: FynT wt12; 7: FynB wt2; 8: FynT F2, and 9: FynB F13. (Left) Position of p59^+ and (right) positions of molecular weight markers. Exposure: 10 h. The relative amounts of fyn in wild-type p59^+ expressing cell lines (compared with the average of Neo cells) are: FynT wt6: 2.5-fold; FynT wt7: 3.1-fold; FynT wt12: 2.4-fold, and FynB wt 2: 2.1-fold. (B) Antigen stimulation assay. Different clonal BI-141 cell lines overexpressing wild-type p59^+ proteins were tested for beef insulin reactivity and compared with other BI-141 derivatives, as described in Fig. 3 B.

should be noted that distinctions in substrate specificity or catalytic function may have been masked by the stimulation provided by anti-TCR antibodies, an alternative explanation for our results is that the enhancement of TCR-induced tyrosine protein phosphorylation by activated fyn is insufficient to ameliorate response to antigen/MHC stimulation. Through its unique sequences, p59^+T may regulate additional cellular pathways which further modulate T cell responsiveness to antigen/MHC. These yet unidentified targets could be other signal-transducing molecules, adhesion molecules, or cytoskeletal constituents. Although the ranges of overexpression typically allowed by retrovirus-mediated gene transfer did not permit further overexpression of the fyn isoforms in BI-141 cells, it would be of interest to test whether a marked increase in expression of activated fynB in a permissive experimental system could mimic the ability of fynT to enhance physiological antigen receptor-mediated responses.

Based on the findings reported herein, we postulate that fynT must participate in regulatory pathways involved in coupling TCR stimulation by antigen/MHC to lymphokine production. It is interesting that a related function was recently ascribed to the y chain of the TCR complex (30). Deletion of the cytoplasmic tail of y significantly decreased the ability of this molecule to link antigen/MHC-induced TCR stimulation to lymphokine production. As is the case for p59^+B, this defect could be bypassed by stimulation with high affinity anti-TCR mAbs. These provocative similarities suggest that the function provided by the cytoplasmic tail of y is mediated either directly or indirectly through p59^+T. Further support for this view is lent by the observation that a fraction of p59^+T can be found associated with TCR in mild detergent lysates of T lymphocytes (8).

Our data, as well as other published results (9, 16), show that activated versions of the two src-related tyrosine protein kinases abundantly expressed in T cells (p59^+T and p56^+B) can enhance antigen receptor-mediated functions. It is important that this is in contrast with p60^+c, a member of the src family not normally expressed in T lymphocytes. Expression of an activated version of this tyrosine protein kinase in antigen-specific T cells fails to improve antigen receptor-induced signals, despite its ability to spontaneously enhance T cell tyrosine protein phosphorylation and lymphokine production (31, and our unpublished data).

Whereas the regulatory interactions between p56^+c and CD4/CD8 are well established (2), the physiological processes allowing the activation of p59^+T in T cells are unfortunately poorly understood. As fynT can associate with TCR, it has been proposed that the enzymatic function of p59^+T is directly regulated by TCR. However, in vitro catalytic activity of p59^+T is not enhanced upon antibody stimulation of TCR (8, and our unpublished observations). While this finding does not preclude the view that p59^+T may be regulated through physical interactions with TCR, it may indicate that the function of this enzyme is controlled through
a more complex machinery. For example, \textit{fyn}\textsuperscript{T} may first be activated by CD45-mediated dephosphorylation of tyrosine 528 (1). Subsequently, as suggested by our observations, TCR stimulation may allow activated p59\textsuperscript{ck} molecules to effectively interact with and/or phosphorylate their substrates. These events may be prohibited by coaggregation of TCR with CD45, possibly as a result of a decrease in the activity of p59\textsuperscript{ck} by CD45 approximation, or as a consequence of substrate dephosphorylation. A related model has been proposed for the regulation of the function of activated p56\textsuperscript{ck} molecules in T cells (16). Despite the fact that the basis of these phenomena remains largely undefined, it is becoming increasingly clear that through both distinct, as well as overlapping, regulatory processes, p59\textsuperscript{ck} and p56\textsuperscript{ck} play important roles in the signal transduction of T cell activation.

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References

1. Klausner, R.D., and L.E. Samelson. 1991. T cell antigen receptor activation pathways: the tyrosine kinase connection. \textit{Cell}. 64:875.

2. Veillette, A., N. Abraham, L. Caron, and D. Davidson. 1991. The lymphocyte-specific tyrosine protein kinase p56\textsuperscript{ck}. \textit{Sem. Immunol.} 3:143.

3. Kawakami, T., C.Y. Pennington, and K.C. Robbins. 1986. Iso-

4. Semba, K., M. Nishizawa, N. Miyajima, M.C. Yoshida, J.

5. Kypta, R.M., A. Hemming, and S.A. Courtneidge. 1988. Isolation and oncogenic potential of a novel human src-like gene. \textit{Mol. Cell. Biol.} 6:4195.

6. Cheng, S.H., R. Harvey, P.C. Espino, K. Semba, T. Yamamoto, K. Toyoshima. 1986. yes-related protooncogene, syn, belongs to the protein-tyrosine kinase family. \textit{Proc. Natl. Acad. Sci. USA.} 83:5459.

7. Cooke, M.P., and R.M. Perlmutter. 1989. Expression of a novel form of the fyn proto-oncogene in hematopoietic cells. \textit{New Biol.} 1:66.

8. Samelson, L.E., A.F. Phillips, E.T. Luong, and R.D. Klausner. 1990. Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. \textit{Proc. Natl. Acad. Sci. USA.} 87:4358.

9. Cooke, M.P., K.M. Abraham, K.A. Forbush, and R.M. Perlmutter. 1991. Regulation of T cell receptor signaling by a src family protein-tyrosine kinase (p59\textsuperscript{ck}). \textit{Cell}. 65:281.

10. Reske-Kunz, A.B., and E. Rüde. 1985. Insulin-specific T cell hybridomas derived from (H-2\textsuperscript{b}xH-2\textsuperscript{k})F; mice preferably employ F\textsubscript{1}-unique restriction elements for antigen recognition. \textit{Eur. J. Immunol.} 15:1048.

11. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. \textit{J. Immunol. Methods.} 65:55.

12. Slilaty, S.N., M. Fung, S.-H. Shen, and S. Lebel. 1990. Site-directed mutagenesis by complementary-strand synthesis using a closing oligonucleotide and double-stranded DNA templates. \textit{Anal. Biochem.} 185:194.

13. Math, J.D., R.W. Overell, K.E. Meier, E.G. Krebs, and R.M. Perlmutter. 1988. Translational activation of the 1k proto-

14. Jove, R., S. Kornbluth, and H. Hanafusa. 1987. Enzymati-

15. Miller, A.D., and G.J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. \textit{Bio-Techniques.} 7:980.

16. Abraham, N., M.C. Miceli, J.R. Parnes, and A. Veillette. 1991. Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56\textsuperscript{ck}. \textit{Nature (Lond.).} 350:62.

17. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. \textit{Biochemistry.} 18:5294.

18. Feinberg, A.P., and B. Vogelstein. 1983. A technique for radio-

19. Abraham, N., and A. Veillette. 1990. Activation of p56\textsuperscript{ck} through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myrist-
20. Veillette, A., J.B. Bolen, and M.A. Bookman. 1989. Alterations in tyrosine protein phosphorylation induced by antibody-mediated crosslinking of the CD4 receptor of T lymphocytes. Mol. Cell. Biol. 9:4441.

21. Veillette, A., J.C. Zástiga-Pfückler, J.B. Bolen, and A.M. Kruisbeek. 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. J. Exp. Med. 170:1671.

22. Kubo, R.T., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine alpha-beta T cell receptors. J. Immunol. 142:2736.

23. Staerz, U.D., H. Rammensee, J.D. Benedetto, and M.J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. J. Immunol. 134:3994.

24. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. Proc. Natl. Acad. Sci. USA. 84:1374.

25. Miceli, M.C., P. von Hoegen, and J.R. Parnes. 1991. Adhesion versus coreceptor function of CD4 and CD8: role of the cytoplasmic tail in coreceptor activity. Proc. Natl. Acad. Sci. USA. 88:2623.

26. Cheng, S.H., P.C. Espino, J. Marshall, R. Harvey, J. Merrill, and A.E. Smith. 1991. Structural elements that regulate pp59-src catalytic activity, transforming potential, and ability to associate with polyomavirus middle-T antigen. J. Virol. 65:170.

27. Cooper, J.A. 1990. The Src family of protein-tyrosine kinases. In Peptides and Protein Phosphorylation. B.E. Kemp, editor. CRC Press, Inc., Boca Raton, FL. 85–113.

28. Mustelin, T., K.M. Coggshall, N. Isakov, and A. Altman. 1990. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. Science (Wash. DC). 247:1584.

29. June, C.H., M.C. Fletcher, J.A. Ledbetter, G.L. Schieven, J.N. Siegel, A.F. Phillips, and L.E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T cell receptor mediated signal transduction. Proc. Natl. Acad. Sci. USA. 87:7722.

30. Frank, S.J., B.B. Niklinska, D.G. Orloff, M. Mercep, J.D. Ashwell, and R.D. Klausner. 1990. Structural mutations of the T cell receptor zeta chain and its role in T cell activation. Science (Wash. DC). 249:174.

31. O'Shea, J.J., J.D. Ashwell, T.L. Bailey, S.L. Cross, L.E. Samelson, and R.D. Klausner. 1991. Expression of v-src in a murine T-cell hybridoma results in constitutive T-cell receptor phosphorylation and interleukin 2 production. Proc. Natl. Acad. Sci. USA. 88:1741.