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Unique Catalytic Properties Dictate the Enhanced Function of $p59^{fynT}$, the Hemopoietic Cell-Specific Isoform of the Fyn Tyrosine Protein Kinase, in T cells

DOMINIQUE DAVIDSON,[1] JEAN VIALLET,[2,3,4] AND ANDRÉ VEILLETTE[1,2,3,4,5]*

McGill Cancer Centre[1] and Departments of Biochemistry,[5] Medicine,[2] and Oncology,[3] McGill University, Montréal, Québec, Canada H3G 1Y6, and Departments of Medicine and Oncology, Montreal General Hospital, Montréal, Québec, Canada H3G 1A4[4]

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As a result of alternative splicing, the *fyn* gene encodes two different tyrosine protein kinase isoforms. While one protein ($p59^{fynB}$) is abundantly expressed in the brain, the alternative product ($p59^{fynT}$) is contained only in cells of hemopoietic lineages, especially T lymphocytes. Sequence analyses have revealed that these two isoforms differ exclusively within a stretch of 52 amino acids which overlaps the end of the Src homology 2 (SH2) motif and the beginning of the catalytic domain. Consistent with the idea that FynT provides a specialized function in hemopoietic cells, we have previously shown that expression of activated FynT molecules, but not that of activated FynB polypeptides, enhanced the antigen responsiveness of a mouse T-cell line (BI-141) (D. Davidson, L. M. L. Chow, M. Fournel, and A. Veillette, J. Exp. Med. 175:1483–1492, 1992). In this study, we examined the basis for the distinct signalling capabilities of the two Fyn isoforms in T lymphocytes. Our biochemical analyses revealed that FynT is more adept than FynB at promoting antigen receptor-triggered calcium fluxes. This phenomenon likely contributes to the improved biological function of FynT during antigen stimulation, as the calcium ionophore ionomycin partially rescued the inability of FynB to enhance antigen-induced lymphokine secretion. To establish the structural basis for these observations, we also created and analyzed a series of chimeras of FynT and FynB. These studies demonstrated that the distinct catalytic domain of FynT, and not its altered SH2 motif, is responsible for the improved ability to augment antigen responsiveness. Similarly, this sequence enhances the ability to mobilize cytosolic calcium in response to antigen receptor stimulation. Taken together, these data show that the distinct biological impacts of FynT and FynB in T cells are related to limited structural differences in the amino-terminal portion of their catalytic domains and that they reflect, at least in part, the greater ability of FynT to mobilize cytoplasmic calcium.

Stimulation of T lymphocytes by an antigen or anti-T-cell receptor (TCR) antibodies causes rapid phosphorylation of several cellular proteins on tyrosine residues (for reviews, see references 25, 29, 40, and 43). This tyrosine protein phosphorylation signal is required for all subsequent events of T-cell activation, including the mobilization of intracellular calcium and the secretion of lymphokines such as interleukin-2 (IL-2). While the antigen receptor and the associated CD3 and ζ chains are devoid of intrinsic catalytic properties, recent studies indicate that TCR-induced tyrosine protein phosphorylation is mediated through the recruitment of various cytoplasmic tyrosine protein kinases. There are accumulating data showing that tyrosine protein kinases $p56^{lck}$ and $p59^{fyn}$, two members of the Src family abundantly expressed in T cells, are specifically responsible for initiation of the TCR-induced signalling cascade (for reviews, see references 40 and 43).

Whereas the regulation of Lck by the CD4, CD8, and CD45 T-cell surface antigens is well established, the mechanisms controlling the activity of Fyn in T lymphocytes are less well characterized (reviewed in references 25 and 40). Intriguingly, small amounts of $p59^{fyn}$ can be found associated with elements of the TCR complex in mild detergent lysates of T lymphocytes (30). This observation has led to the proposal that the function of Fyn may be directly controlled by TCR stimulation. Some support for this view has been provided by the finding that activation of T cells by anti-TCR antibodies results in a reproducible increase in the enzymatic activity of Fyn, as measured in immune complex kinase reactions (38).

The structure of $p59^{fyn}$ is very analogous to that of other Src-related enzymes (for reviews, see references 11 and 40). It possesses, from the amino terminus to the carboxy terminus (Fig. 1A), (i) myristylation and palmitylation signals (31) which are necessary for stable association with the inner aspect of the plasma membrane; (ii) a unique domain which seems to allow specific interactions with the CD3 and ζ chains of TCR (37); (iii) a Src homology 3 (SH3) domain presumed to mediate binding to proline-rich polypeptides (7; reviewed in reference 24); (iv) an SH2 sequence responsible for association with phosphotyrosine-containing peptides (for reviews, see references 5 and 24); (v) a catalytic domain, including sites for ATP binding, phosphotransfer, and autophosphorylation; and (vi) a negative regulatory domain which contains the major site of in vivo tyrosine phosphorylation, tyrosine 528. Mounting evidence shows that phosphorylation at tyrosine 528 is not due to autophosphorylation but is mediated by members of the Csk family of inhibitory tyrosine protein kinases (8, 21, 23). As a consequence of phosphorylation at this site, the carboxy terminus of $p59^{fyn}$ is postulated to associate with the Fyn SH2 motif, thereby interfering with interactions of the kinase domain with intracellular substrates.

Because of alternative splicing of distinct exons 7, the *fyn* gene encodes two protein isoforms termed $p59^{fynT}$ and $p59^{fynB}$ (10, 35, 36). These two polypeptides differ exclusively within a

* Corresponding author. Mailing address: Room 715, McIntyre Medical Sciences Building, McGill University, 3655 Drummond St., Montréal, Québec, Canada H3G 1Y6. Phone: (514) 398-8936. Fax: (514) 398-6769. Electronic mail address: VEILLETTE@MEDCOR.MCGILL.CA.

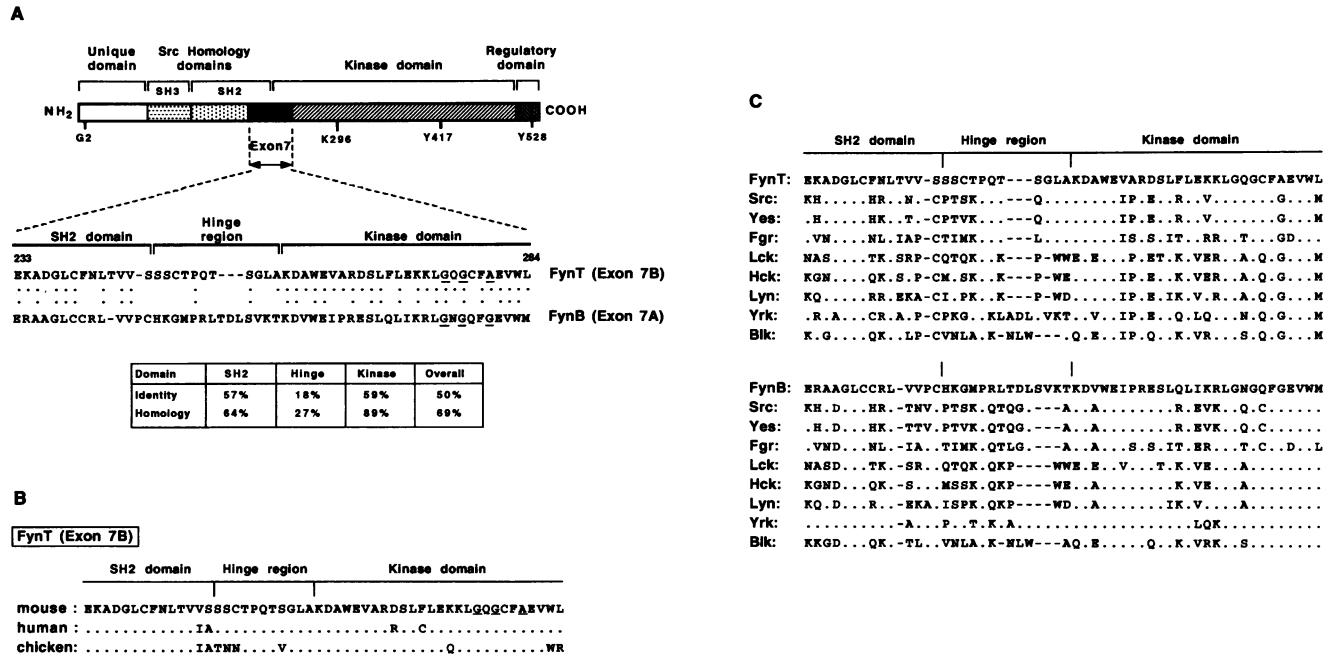

FIG. 1. The two isoforms of p59^{fyn}: FynT and FynB. (A) Schematic representation of FynT and FynB. The primary structure of p59^{fyn} is shown at the top. Salient features include a glycine residue at position 2 (G2), which is required for myristylation and association with the inner aspect of the plasma membrane; a unique domain; SH3 and SH2 domains; a kinase region, including sites for phosphotransfer (lysine 296 [K296]) and autophosphorylation (tyrosine 417 [Y417]); and a carboxy-terminal regulatory domain encompassing the major site of in vivo tyrosine phosphorylation, tyrosine 528 (Y528). The amino acids encoded by exon 7 of mouse *fynT* and *fynB* are presented below. Identical residues are indicated by two dots, while conserved amino acids are represented by a single dot. The highly conserved glycine-rich motif (G-X-G-X-X-G) in the kinase domain is underlined. The carboxy-terminal boundary of the SH2 domain was defined by alignment with other SH2 domains (14, 42). The amino-terminal border of the kinase domain was assigned to the beginning of the highly conserved sequence in the carboxy-terminal halves of Src-like enzymes (11, 35; see panel C). The percentages of identity and homology between the various subdomains encoded by exon 7 are tabulated. (B) Comparison of the exon 7-encoded sequences of mouse (10), human (36), and chicken (35) FynT. Identical residues are indicated by dots. (C) Comparison of the exon 7-encoded sequences of FynT (top) and FynB (bottom) with the corresponding region in the other Src-related tyrosine protein kinases (11, 35). Identical residues are shown as dots.

sequence of approximately 50 amino acids which overlaps the end of the SH2 domain and the beginning of the catalytic domain (Fig. 1A). While the most conventional isoform (FynB) is abundantly expressed in the brain, the alternative product (FynT) accumulates only in cells of hemopoietic lineages, especially T lymphocytes. On the basis of this expression pattern, it has been proposed that p59^{fynT} may play a specialized role in the signalling machinery of hemopoietic cells. This view was supported by our demonstration that expression of constitutively activated FynT molecules (carrying a tyrosine 528-to-phenylalanine substitution), but not that of activated FynB molecules, enhanced the responsiveness of a mouse T-cell line (BI-141) to antigenic stimulation (12).

To understand the function of p59^{fynT} better, we devised a series of experiments aimed at defining the structural and biochemical bases for its unique behavior in T cells. By creating and analyzing chimeras of p59^{fynT} and p59^{fynB}, we established that the altered catalytic sequence of FynT, and not its modified SH2 motif, is responsible for the augmented ability to enhance TCR-mediated signals. Moreover, our studies provided evidence that the distinct impacts of FynT and FynB on T-cell antigen responsiveness can be explained, at least in part, by their different abilities to regulate TCR-triggered calcium fluxes.

MATERIALS AND METHODS

Cells. BI-141, a CD4-negative, CD8-negative, class II major histocompatibility complex (MHC)-restricted, beef insulin-specific mouse T-cell hybridoma (28), was propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum. As it seemingly expresses a low-affinity TCR, BI-141 fails to respond to antigen-MHC in the absence of coexpression of CD4 (1). However, antigen responsiveness can be rescued by expression of constitutively activated Lck or Fyn molecules (see below; 1, 8, 12). Derivatives expressing the neomycin phosphotransferase alone (Neo) or in combination with constitutively activated FynT or FynB (tyrosine 528 to phenylalanine [F528] FynT or FynB) or constitutively activated Lck (tyrosine 505 to phenylalanine [F505] Lck) have been described elsewhere (1, 12). All BI-141 T-cell derivatives were maintained in growth medium supplemented with the aminoglycoside G418 (0.6 mg · ml^{-1}).

Construction of *fynT-fynB* chimeric cDNAs. *fynTTB* and *fynBBT* cDNAs were constructed as follows. Through site-directed mutagenesis (12), *Sty*I restriction endonuclease recognition sites were introduced at the junction of the hinge and kinase domains in mouse *fynT* and *fynB* by using plasmids MM23 and MB1 (10), respectively, as templates. The following oligonucleotides were used for mutagenesis: *fynT*, 5′-CAAG CATCCTTGGCCAATCC-3′; *fynB*, 5′-CAGACATCCTTG

GTTTTGAC-3′. It was important that the mutations produced by these oligonucleotides did not alter the amino acid sequence of either Fyn isoform. By using standard recombinant DNA technology, the *Apa*I-*Sty*I fragment from *fynT* was substituted for the equivalent fragment in *fynB* and vice-versa.

The other chimeric cDNAs were created by two sets of PCRs. For the first PCR, the following oligonucleotides were used: *fynTBB*, 5′-ACAGGAGTAGGATCCTTTGTGGC-3′ (2.7) and 5′-CCTTGGCATCCCTTTGTGTGAAACCACAG TTAAGTT-3′ (2.22) with the *fynT* cDNA as the template and 5′-CACAAAGGGATGCCAAGG-3′ (2.24) and 5′-CGGGCT GAAACTGGGGC-3′ (2.3) with the *fynB* cDNA as the template; *fynBTT*, 5′-ACAGGAGTAGGATCCTTTGTGGC-3′ (2.7) and 5′-TTGTGGGGTACAACTTGAACAGGGAAC TACTAGGCG-3′ (2.23) with the *fynB* cDNA as the template and 5′-TCAAGTTGTACCCCACAA-3′ (2.25) and 5′-CGG GCTGAAACTGGGGC-3′ (2.3) with the *fynT* cDNA as the template; *fynTBT*, 5′-ACAGGAGTAGGATCCTTTGTGG C-3′ (2.7) and 5′-CCTTGGCATCCCTTTGTGTGAAACCA CAGTTAAGTT-3′ (2.22) with the *fynT* cDNA as the template and 5′-CACAAAGGGATGCCAAGG-3′ (2.24) and 5′-CGGGCTGAAACTGGGGC-3′ (2.3) with the *fynBBT* cDNA as the template; *fynBTB*, 5′-ACAGGAGTAGGATCCTTT GTGGC-3′ (2.7) and 5′-TTGTGGGGTACAACTTGAA CAGGGAACTACTAGGCG-3′ (2.23) with the *fynB* cDNA as the template and 5′-TCAAGTTGTACCCCACAA-3′ (2.25) and 5′-CGGGCTGAAACTGGGGC-3′ (2.3) with the *fynTTB* cDNA as the template.

The PCR-generated products were then used as templates for a second round of PCR with common upstream and downstream oligonucleotide primers (2.7 and 2.3). The final PCR products were digested with *Apa*I and *Bgl*II, and the resulting *Apa*I-*Bgl*II fragments were exchanged into the wild-type *fynT* cDNA. All constructs were sequenced and found to contain no unwanted mutation (data not shown).

The chimeric *fyn* cDNAs also carried a point mutation which introduced a tyrosine-to-phenylalanine substitution at position 528 (F528 Fyn mutation) (12).

Creation of $fynT^{G280}$ and $fynB^{A280}$ cDNAs. Through PCR, the last residues of the glycine-rich motifs of FynT (alanine 280) and FynB (glycine 280) were mutated to glycine and alanine, respectively. The following oligonucleotides were used for the first PCR: $fynT^{G280}$, 5′-ACAGGAGTAGGATCCTT TGTGGC-3′ (2.7) and 5′-ACACTTCACCGAAACAC-3′ (AG2) with the *fynT* cDNA as the template and 5′-GTGTT TCGGTGAAGTGT-3′ (AG1) and 5′-CGGGCTGAAACT GGGGC-3′ (2.3) with the *fynT* cDNA as the template; $fynB^{A280}$, 5′-ACAGGAGTAGGATCCTTTGTGGC-3′ (2.7) and 5′-ATACTTCCGCAAACTGC-3′ (GA2) with the *fynB* cDNA as the template and 5′-GCAGTTTGCGGAAGTAT-3′ (GA1) and 5′-CGGGCTGAAACTGGGGC-3′ (2.3) with the *fynB* cDNA as the template.

The second PCR and the subsequent cloning steps were done as described above for the chimeric *fyn* cDNAs.

Retrovirus-mediated gene transfer. The *fyn* cDNAs were cloned into the multiple cloning site of retroviral vector pLXSN (20). Retroviral constructs were subsequently transfected by calcium phosphate precipitation (6) in ψ-2 packaging cells, and polyclonal virus-producing cell lines were established by growth in medium containing the aminoglycoside G418 (0.4 $mg \cdot ml^{-1}$). BI-141 T cells were infected with the appropriate retroviral stocks as described elsewhere (3). Infected cells were selected in G418 (0.75 $mg \cdot ml^{-1}$)-containing medium, and monoclonal cell lines were established by limiting dilution. Cells were screened by immunoblotting of total cell lysates with a specific anti-Fyn serum (see below). All of the cell lines used in our experiments expressed unaltered levels of TCR, CD3ε, Thy1, and CD45 and remained CD4 negative (data not shown).

Anti-Fyn immunoblots. Anti-Fyn immunoblotting was performed as previously described (2, 39, 41), with a polyclonal rabbit anti-Fyn serum directed against residues 25 to 141 of the murine $p59^{fyn}$ sequence (12). This antiserum recognizes sequences that are common to FynT and FynB. Immunoreactive products were detected by incubation of membranes with ^{125}I-labeled protein A (Amersham) and subsequent autoradiography. Radioactivity was quantitated by using a Bas2000 Phosphor-Imager (Fuji).

Antigen stimulation assays. To test antigen-induced lymphokine production (1, 12), BI-141 cells (10^5 per well) were plated with 5×10^4 irradiated class II MHC (A_α^b A_β^k)-transfected L cells (FT5.7) and serial dilutions of beef insulin (Sigma) in a final volume of 200 μl. After incubation for 24 h at 37°C, supernatants were harvested and frozen at −70°C to destroy carryover cells. IL-2 production was subsequently tested by examining [^3H]thymidine incorporation in IL-2-dependent cell line HT-2. Controls received no addition. Assays were performed in triplicate.

In some experiments, antigen stimulation was performed in the presence of the calcium ionophore ionomycin (20 $ng \cdot ml^{-1}$; Sigma).

Measurement of changes in cytosolic calcium. After extensive washing in serum-free medium, cells (5×10^6) were loaded for 30 min at 37°C with the calcium indicator dye fura-2/AM (final concentration, 5 μM; Molecular Probes, Inc., Eugene, Oreg.). Following several washes in phosphate-buffered saline, cells were incubated for 30 min on ice in various concentrations (see figure legends) of mouse anti-TCR monoclonal antibody (MAb) F23.1 (32). After washing away of the excess antibody, cells were resuspended in calcium assay buffer (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4]; 17) and introduced into a spectrophotometer cuvette. Intracellular calcium levels were then measured in a Perkin-Elmer LS50 luminescence spectrometer with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Throughout these measurements, cell suspensions were maintained at 37°C and subjected to gentle stirring with a small magnetic bar. After 60 s of incubation at 37°C, sheep anti-mouse immunoglobulin G (Organon-Teknika) was added to a final concentration of 15 $\mu g \cdot ml^{-1}$ to allow induction of the calcium flux. Cytosolic calcium was monitored for at least 5 min following the addition of sheep anti-mouse immunoglobulin G.

In some cases, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA; 2 mM) was added to the calcium assay buffer to chelate the extracellular calcium.

RESULTS

Activated FynT is more efficient than activated FynB at enhancing TCR-induced calcium mobilization. We have previously shown that FynT bearing a tyrosine 528-to-phenylalanine mutation (F528 FynT), but not F528 FynB, enhanced lymphokine production by mouse T-cell line BI-141 in response to antigen stimulation (12). Perplexingly, however, these earlier analyses revealed that the two activated Fyn isoforms caused similar enhancements of intracellular tyrosine protein phosphorylation upon antigen receptor stimulation with anti-TCR antibodies. One potential explanation for this apparent discrepancy was that subtle differences in TCR-

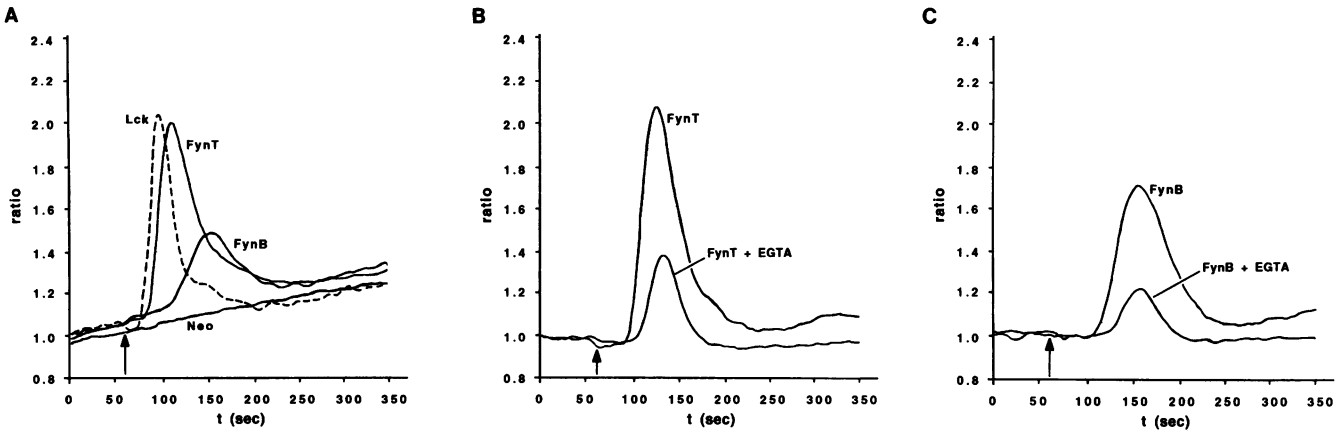

FIG. 2. Effects of F528 FynT and F528 FynB on TCR-mediated calcium mobilization. Cells were loaded with the calcium indicator dye fura-2 and stimulated with anti-TCR MAb F23.1 (8 $\mu g \cdot ml^{-1}$), as described in Materials and Methods. Changes in cytosolic calcium were monitored in a luminescence spectrometer. Sheep anti-mouse immunoglobulin G was added after 60 s of incubation at 37°C (arrow). Relative intracellular calcium concentrations are represented as the luminescence ratios at excitation wavelengths of 340 and 380 nm. All baseline values were adjusted to 1.0. Abscissa: time in seconds. Ordinate: luminescence ratio (340 nm/380 nm). (A) Comparison of cells expressing the neomycin phosphotransferase alone (Neo), F528 FynT (FynT F7), F528 FynB (FynB F15), or F505 Lck (Lck F9). (B) Effects of EGTA on TCR-mediated calcium fluxes in F528 FynT-expressing cells. Cells were stimulated as described above, with or without the calcium chelator EGTA (2 mM). The peak calcium response was decreased by 63% by addition of EGTA. (C) Effects of EGTA on TCR-mediated calcium fluxes in F528 FynB-expressing cells. Cells were stimulated as described above, with or without the calcium chelator EGTA (2 mM). The peak calcium response was decreased by 71% by addition of EGTA.

induced tyrosine protein phosphorylation caused the distinct impacts on IL-2 release.

TCR stimulation is associated with a rapid rise in the cytoplasmic calcium concentration (18; reviewed in references 26 and 43). Such a change is thought to be mediated, at least in part, by phospholipase Cγ1-mediated production of inositol trisphosphate and to reflect both release of calcium from intracellular stores and flux of the ion from the extracellular compartment. This calcium mobilization response is dependent on tyrosine protein phosphorylation and is presumed to be critical for activation of calcium-dependent enzymes and production of lymphokines such as IL-2.

To understand the biochemical basis for the differential function of FynT and FynB in T cells, we tested the impact of the two isoforms on the ability of TCR stimulation to provoke a rise in intracellular calcium (Fig. 2). Previously described BI-141 T-cell derivatives (12) expressing F528 FynT or F528 FynB were used in these experiments. Cells were loaded with the fluorescent calcium indicator dye fura-2 and activated with mouse anti-TCR MAb F23.1 plus sheep anti-mouse immunoglobulin G as described in Materials and Methods. Changes in cytoplasmic calcium were monitored in a luminescence spectrometer (Fig. 2A).

When saturating amounts of MAb F23.1 (8 $\mu g \cdot ml^{-1}$) were used for stimulation, cells expressing either F528 FynT or F528 FynB exhibited an increase in cytosolic calcium. A calcium response was also noted in BI-141 cells containing an activated version of $p56^{lck}$ (tyrosine 505-to-phenylalanine Lck mutant; F505 Lck; 1). Like F528 FynT, F505 Lck had previously been shown to enhance the antigen responsiveness of BI-141 T cells (1). In contrast to these various cell lines, no anti-TCR antibody-triggered calcium response was detected in cells expressing the neomycin phosphotransferase alone (Neo). Nevertheless, all of the cell lines showed comparable increases in cytosolic calcium in response to the calcium ionophore ionomycin (data not shown). As reported for other T-cell types (reviewed in references 26 and 43), the TCR-induced calcium flux in both F528 FynT- and F528 FynB-expressing cells was partially reduced by addition of EGTA to the assay buffer (Fig. 2B and C, respectively). Since EGTA chelated the extracellular calcium, this finding implied that the calcium mobilization was consequent to both release of the ion from intracellular stores and influx from the extracellular compartment.

In these experiments, the calcium mobilization noted in F528 FynB-expressing cells was consistently slower and of lesser magnitude than that observed in cells containing F528 $p59^{fynT}$. To evaluate this finding further, similar experiments were conducted with serial dilutions of MAb F23.1 (Fig. 3). Such a protocol was presumed to allow a closer approximation to the extent of T-cell stimulation provided by antigen-MHC. When smaller amounts of MAb F23.1 were used for activation, F528 FynB-expressing cells (Fig. 3B) clearly exhibited a weaker calcium response than F528 FynT-containing cells (Fig. 3A). This was especially obvious at the lowest concentration of anti-TCR antibody (0.9 $\mu g \cdot ml^{-1}$), which failed to trigger calcium mobilization in F528 FynB-expressing cells. The maximal increase in cytosolic calcium at the various antibody concentrations is represented graphically in Fig. 3C. Similar data were obtained with other F528 FynT- and F528 FynB-expressing cells (data not shown).

Hence, whereas we had previously found that F528 FynT and F528 FynB were equally efficient at increasing TCR-induced tyrosine protein phosphorylation (12), these data indicated that FynT had a greater propensity than FynB to enhance antigen receptor-initiated calcium fluxes.

Ionomycin can partially rescue the inability of FynB to enhance antigen responsiveness in BI-141 T cells. To evaluate whether the impacts on TCR-mediated calcium fluxes contribute to the distinct effects of F528 FynT and F528 FynB on antigen-induced IL-2 secretion, we examined the ability of a calcium ionophore (ionomycin) to rescue antigen-induced lymphokine secretion in cells expressing activated FynB. Cells were stimulated with various concentrations of the antigen beef insulin in the context of the appropriate class II MHC molecules ($A_\alpha{}^b$ $A_\beta{}^k$), with or without addition of ionomycin (Fig. 4). Lymphokine secretion was subsequently measured in

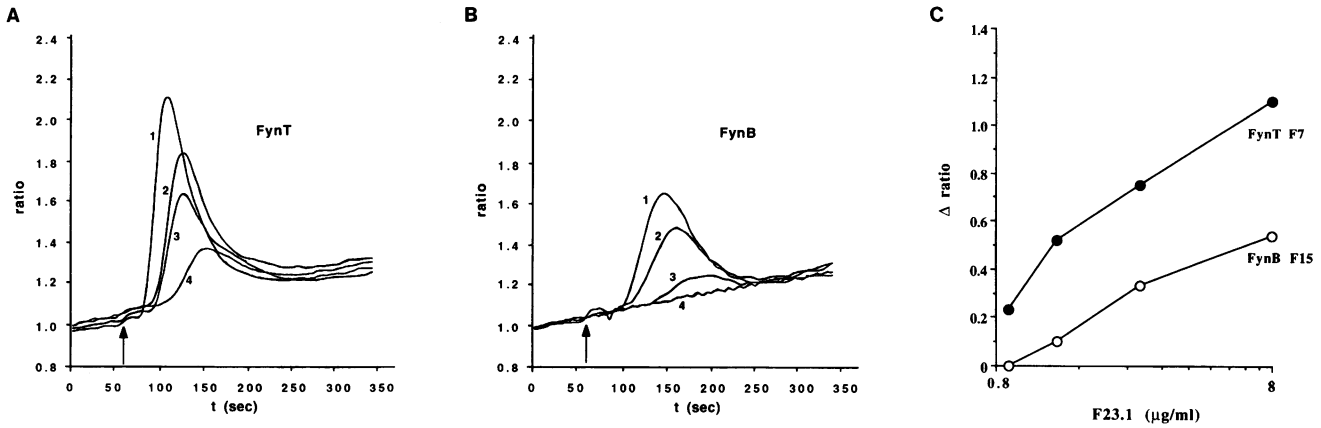

FIG. 3. Dose-response analysis of effects of F528 FynT and F528 FynB on TCR-mediated calcium fluxes. Measurements of cytosolic calcium were performed on TCR-stimulated BI-141 cells as described in the legend to Fig. 2, except that serial dilutions of MAb F23.1 were used for stimulation. Curves: 1, 8 $\mu g \cdot ml^{-1}$; 2, 2.7 $\mu g \cdot ml^{-1}$; 3, 1.5 $\mu g \cdot ml^{-1}$; 4, 0.9 $\mu g \cdot ml^{-1}$. (A) F528 FynT-expressing cells (FynT F7). (B) F528 FynB-expressing cells (FynB F15). (C) Graphic representation of the peak increase in relative intracellular calcium in F528 FynT- and F528 FynB-containing cells at various anti-TCR MAb F23.1 concentrations. Abscissa: concentration of MAb F23.1 (micrograms per milliliter), logarithmic scale. Ordinate: peak increase in luminescence ratio (Δ ratio).

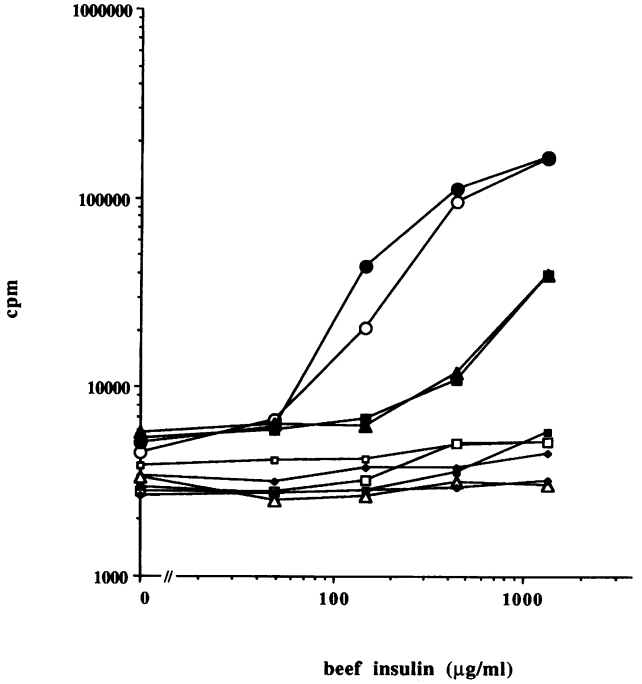

FIG. 4. Effects of the calcium ionophore ionomycin on antigen responsiveness. BI-141 T-cell derivatives expressing F528 FynT (FynT F), F528 FynB (FynB F), or the neomycin phosphotransferase alone (Neo) were stimulated with serial dilutions of the antigen beef insulin in the presence of the relevant class II MHC molecules (A_α^b A_β^k). Lymphokine release was measured in a standard IL-2 bioassay using IL-2-dependent HT-2 indicator cells. In some cases, antigen stimulation was done in the presence of ionomycin (20 $ng \cdot ml^{-1}$). Abscissa: beef insulin concentration (micrograms per milliliter), logarithmic scale. Ordinate: [^{3}H]thymidine incorporation, logarithmic scale. Controls without addition are shown as zero on the abscissa. Symbols: □, Neo 1; ◇, Neo 5; ○, FynT F7; △, FynB F15; □, FynB F13; ■, Neo 1 plus ionomycin; ◆, Neo 5 plus ionomycin; ●, FynT F7 plus ionomycin; ▲, FynB F15 plus ionomycin; ■, FynB F13 plus ionomycin.

a standard IL-2 bioassay using IL-2-dependent HT-2 indicator cells.

As previously reported (12), F528 FynT-expressing cells, but not cells containing F528 FynB or the neomycin phosphotransferase alone (Neo), promptly responded to antigen stimulation in the absence of ionomycin. However, while Neo cells remained incapable of antigen-induced lymphokine secretion in the presence of ionomycin, cells expressing F528 FynB showed a measurable improvement in antigen responsiveness. Nevertheless, this effect was partial, as the response of ionomycin-treated F528 FynB-expressing cells was intermediate between those of Neo cells and F528 FynT-containing cells.

Defining the structural basis for the different impacts of FynT and FynB on T cells: creation of FynT-FynB chimeras. As discussed earlier, the sequences of FynT and FynB differ exclusively within a short region extending over the end of the SH2 motif and the beginning of the catalytic domain (Fig. 1A; 10, 35, 36). Within the SH2 motif, this difference is limited to the second α helix (αB) and the seventh β strand (βG), which are part of the high-affinity recognition site for tyrosine-phosphorylated peptides (14, 42). This region, also termed the BG loop, presumably contributes to a pocket that binds the third residue located carboxyl to phosphotyrosine. In the catalytic region, the divergence between FynT and FynB is restricted to subdomain I (16). This portion of the kinase domain includes a highly conserved glycine-rich motif (G-X-G-X-X-G), which is involved in Mg^{2+}-ATP binding. It is provocative that FynT is the only known tyrosine protein kinase in which the last glycine of this motif is replaced by alanine (10, 35, 36; Fig. 1C). Lastly, the sequences of $p59^{fynT}$ and $p59^{fynB}$ differ in the area lying between the SH2 and kinase domains. This ill-characterized region, termed the hinge, may represent a folding point that facilitates intramolecular interactions between the amino- and carboxy-terminal halves of the enzyme.

To establish the structural basis for the distinct effects of $p59^{fynT}$ and $p59^{fynB}$ on T cells, chimeras of the two molecules were produced through standard recombinant DNA technology (Fig. 5). As explained in Materials and Methods, we generated a series of *fyn* cDNA constructs in which the sequence of the SH2, hinge, or kinase region was individually exchanged from *fynT* to *fynB* and vice versa. On the basis of the

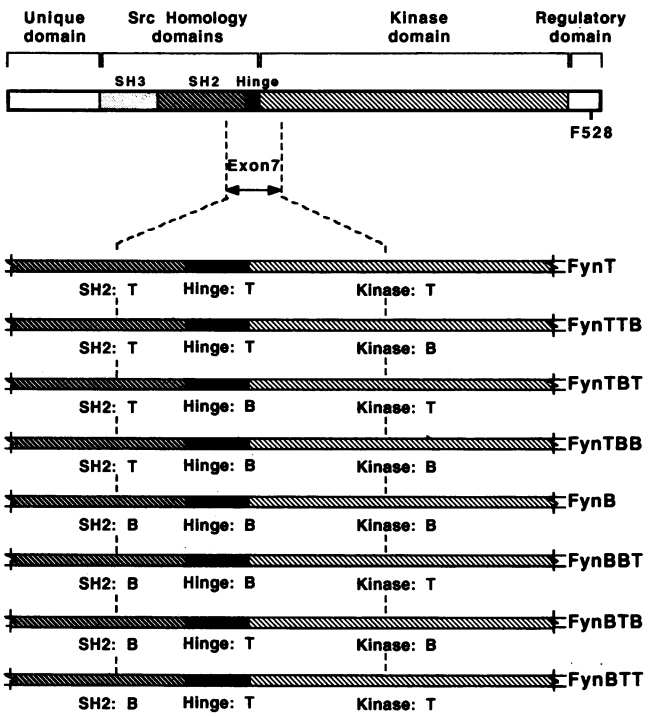

FIG. 5. Structures of FynT-FynB chimeras. Schematic representations of the various FynT-FynB chimeras are shown. All chimeric proteins contained a tyrosine-to-phenylalanine mutation at position 528 (F528).

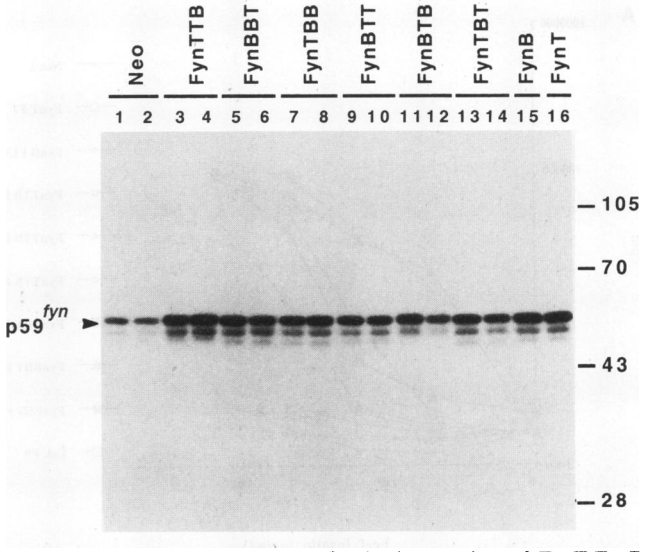

FIG. 6. Anti-Fyn immunoblot showing expression of FynT-FynB chimeras in BI-141 T cells. BI-141 T cells were infected with retroviruses encoding the various FynT-FynB chimeras and selected for growth in medium containing the aminoglycoside G418. Monoclonal cell lines were isolated by limiting dilution and screened by immunoblotting of total cell lysates with anti-Fyn antibodies. Representative cell lines were selected for further studies. Lysates corresponding to equivalent cell numbers were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto Immobilon membranes, and probed by immunoblotting with a rabbit anti-Fyn serum and ^{125}I-labeled protein A. The abundance of p59^{fyn} in these cells (relative to that of p59^{fyn} in Neo cells) is shown in brackets. Lanes: 1, Neo 1; 2, Neo 3; 3, FynTTB F15 (2.9); 4, FynTTB F16 (3.5); 5, FynBBT F1 (3.1); 6, FynBBT F9 (2.8); 7, FynTBB F6 (2.7); 8, FynTBB F10 (3.0); 9, FynBTT F4 (2.4); 10, FynBTT F22 (2.2); 11, FynBTB F14 (2.5); 12, FynBTB F15 (1.7); 13, FynTBT F12 (2.7); 14, FynTBT F21 (2.0); 15, FynB F13 (3.0); 16, FynT F7 (2.9). The positions of prestained molecular mass markers (sizes are in kilodaltons) are shown on the right, and that of p59^{fyn} is indicated on the left. The presence of equivalent amounts of cellular proteins in all of the lanes was confirmed by amido black staining of Immobilon membranes (data not shown). The exposure time was 16 h.

resulting sequence combinations, the chimeras were named FynTTB, FynBBT, FynTBT, FynBTB, FynTBB, and FynBTT (Fig. 5). All chimeric proteins also contained a tyrosine-to-phenylalanine substitution at position 528 (F528 Fyn mutation). This mutation constitutively activates the catalytic function of p59^{fyn} (12, 13) and is necessary for FynT to enhance antigen responsiveness in BI-141 T cells (12).

Expression of FynT-FynB chimeras in BI-141 T cells. The chimeric *fyn* cDNAs were inserted into the multiple cloning site of retroviral vector pLXSN (20). Because this plasmid also contains the neomycin phosphotransferase gene (*neo*), it allows selection for cell growth in medium supplemented with the aminoglycoside G418. Constructs were individually transfected in ψ-2 packaging cells, and the resulting retroviral stocks were used to infect BI-141 cells. Monoclonal G418-resistant cell lines were obtained by limiting dilution and screened for p59^{fyn} overexpression by immunoblotting of total cell lysates with a polyclonal rabbit anti-Fyn serum (data not shown). All of the cell lines selected for further studies expressed unaltered levels of TCR, CD3ε, CD45, and Thy1 and remained CD4 negative (data not shown).

An anti-Fyn immunoblot of lysates from representative clones (Fig. 6) showed that all of the Fyn chimeras were expressed at equivalent levels, resulting in a two- to threefold increase in the abundance of p59^{fyn} in BI-141 cells (compare lanes 3 to 14 with lanes 1 and 2). Such a modest degree of overexpression was similar to that achieved with F528 FynB and F528 FynT (lanes 15 and 16, respectively). It most likely reflects the high levels of endogenous p59^{fyn} present in BI-141 T cells (12). In addition to reacting with the 59-kDa Fyn protein, the antiserum recognized products migrating at 56 and 54 kDa in these gels. These polypeptides are generated by proteolysis of p59^{fyn} (our unpublished data). The appropriate expression of the various chimeric cDNAs was confirmed by Northern (RNA) blot analyses of total cellular RNA (data not shown).

The distinct catalytic domain of FynT is responsible for the enhanced ability to improve antigen responsiveness in BI-141 T cells. The responsiveness of BI-141 T cells to antigen-MHC stimulation was next evaluated (Fig. 7). By using the protocol outlined above, cell lines were stimulated with beef insulin and the appropriate class II MHC molecule and tested for lymphokine release. To simplify our analyses, chimeras bearing reciprocal modifications (i.e., FynTTB and FynBBT, FynTBT and FynBTB, and FynBTT and FynTBB) were examined in the same assay (Fig. 7A, B, and C, respectively). Like F528 FynT- and F505 Lck-bearing cells (Fig. 7), all of the cells expressing Fyn proteins with the catalytic domain from FynT (FynBBT, FynTBT, and FynBTT) showed demonstrable antigen-MHC-induced lymphokine secretion. In contrast, cells expressing chimeras with the FynB kinase domain (FynTTB, FynBTB, and FynTBB) failed to respond to the antigenic stimulus. Clearly, acquisition of the SH2 or hinge region from FynT was insufficient to allow F528 FynB to enhance antigen responsiveness.

As suggested by these and other experiments (Fig. 7; data

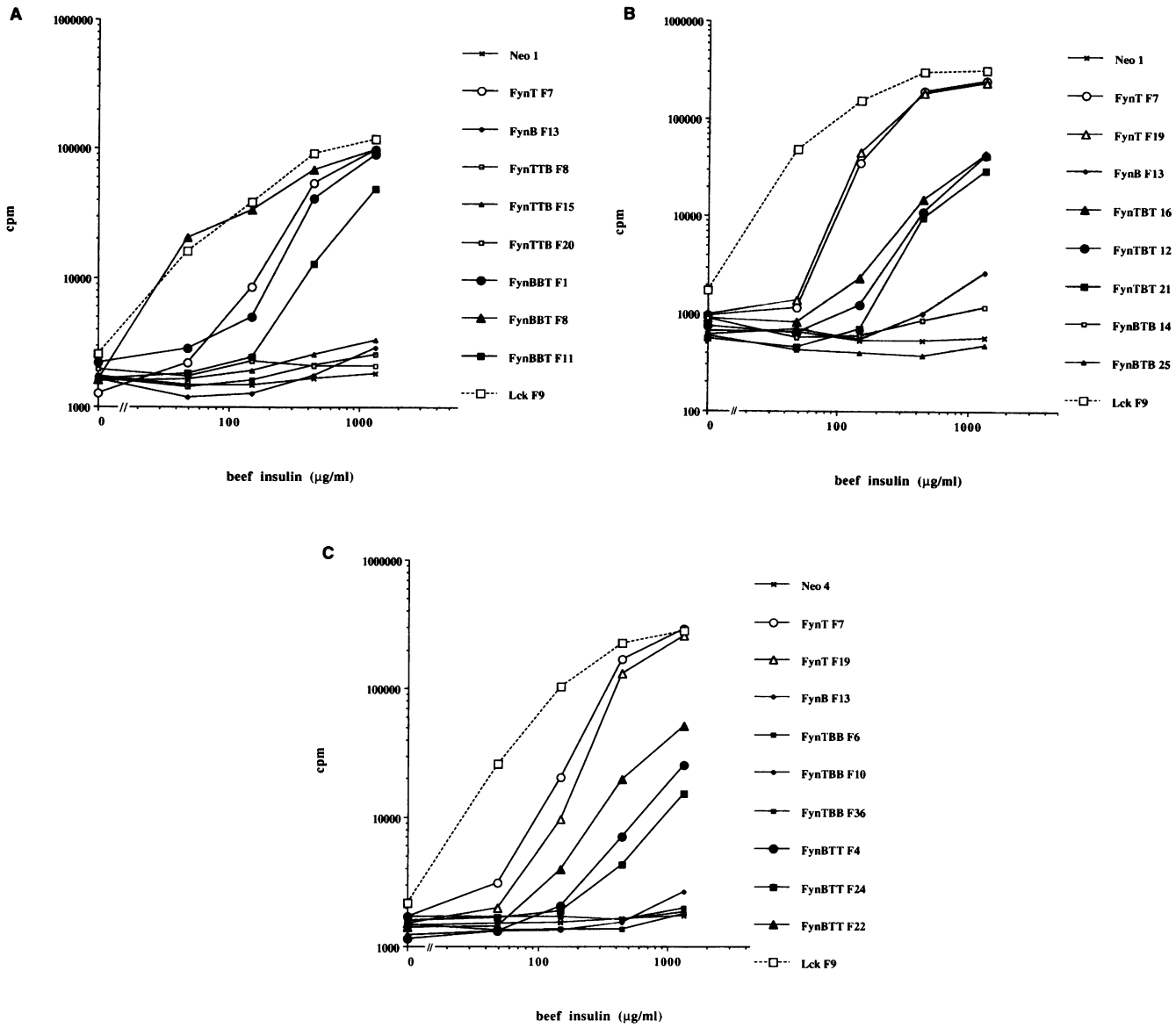

FIG. 7. Effects of FynT-FynB chimeras on antigen responsiveness. Antigen stimulation assays were performed as outlined in the legend to Fig. 4. Cells expressing Fyn chimeras bearing reciprocal modifications were analyzed in the same assay. Cells expressing F505 Lck (Lck F9) were used as a positive control. (A) FynTTB versus FynBBT. (B) FynTBT versus FynBTB. (C) FynTBB versus FynBTT.

not shown), the level of responsiveness conferred by FynTBT and FynBTT tended to be lower than that allowed by FynT or FynBBT. Whereas the basis for this finding has not been determined, it suggests that proper pairing of SH2 and hinge regions is necessary for optimal Fyn function.

The distinct catalytic domain of FynT dictates the improved ability to mobilize intracellular calcium. The influence of these chimeras on TCR-induced calcium mobilization was also examined (Fig. 8). As described earlier, cells were loaded with fura-2 and stimulated with serial dilutions of anti-TCR MAb F23.1. Calcium mobilization was then evaluated in a luminescence spectrometer. To simplify analysis of these data, the maximal increment in cytosolic calcium at the various anti-TCR antibody concentrations used was plotted for each cell line (Fig. 8). These studies revealed that all of the cells expressing chimeras with the FynT catalytic sequence had more pronounced TCR-induced calcium mobilization than did cells expressing Fyn molecules having the FynB kinase sequence. These conclusions were corroborated by examining at least two independent clones of each type (Fig. 8; also data not shown).

The alanine substitution in the glycine-rich motif of FynT does not account for its enhanced biological activity in BI-141 T cells. $p59^{fynT}$ is the only identified tyrosine protein kinase in which the G-X-G-X-X-G motif is replaced by G-X-G-X-X-A (16; Fig. 1C). Strikingly, the glycine-to-alanine modification has been documented in mouse (10), human (36) and chicken (35) FynT (Fig. 1B). To examine the role of this amino acid substitution in the function of FynT, alanine 280 of F528 FynT was replaced by glycine and the behavior of the resulting Fyn polypeptide (G280F528 FynT) was tested by expression in BI-141 cells. Similar analyses were performed on F528 FynB

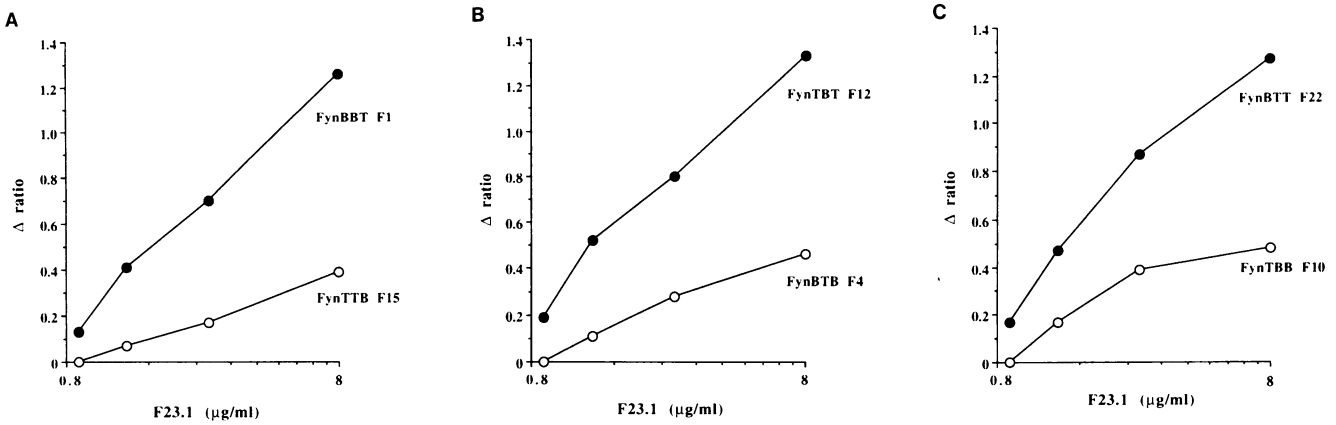

FIG. 8. TCR-induced changes in cytosolic calcium in cells expressing FynT-FynB chimeras. Graphic representation of the peak increase in relative intracellular calcium upon stimulation with various concentrations of anti-TCR MAb F23.1. Abscissa: concentration of MAb F23.1 (micrograms per milliliter), logarithmic scale. Ordinate: peak increase in luminescence ratio (Δ ratio). (A) FynTTB versus FynBBT. (B) FynTBT versus FynBTB. (C) FynTBB versus FynBTT.

polypeptides in which wild-type glycine 280 had been replaced by alanine (A280F528 FynB). As outlined earlier, monoclonal G418-resistant cell lines were screened for Fyn overexpression by anti-Fyn immunoblotting (data not shown).

Functional studies were performed on several cell lines expressing $p59^{fyn}$ in quantities analogous to those of F528 FynT- and F528 FynB-expressing cells (Fig. 9A). All of these clonal populations also exhibited unmodified levels of TCR, CD3ε, Thy1, and CD45 (data not shown). When evaluated in antigen stimulation assays, G280F528 FynT-expressing cells showed marked antigen-MHC-induced lymphokine secretion (Fig. 9B). This response was comparable to that of F528

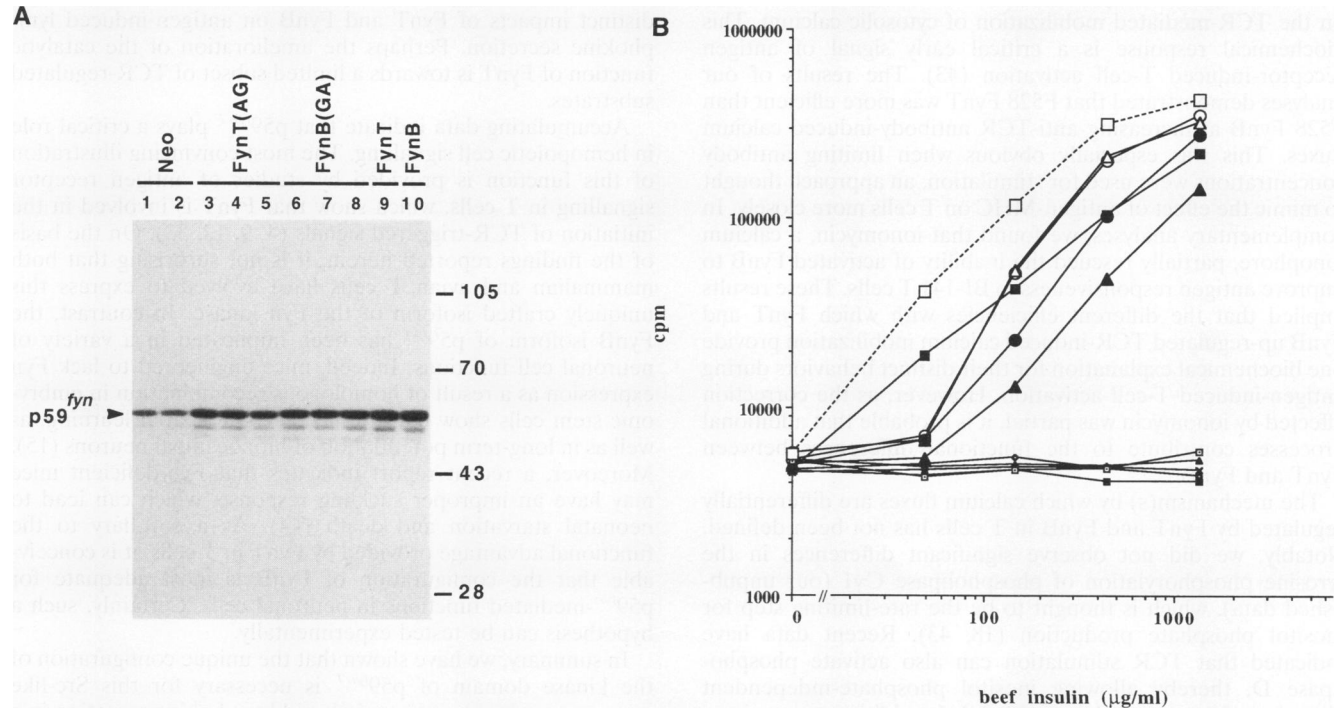

FIG. 9. Effect of the alanine substitution in the glycine-rich motif of FynT. (A) Anti-Fyn immunoblot. The abundance of $p59^{fyn}$ in cells containing G280F528 FynT [FynT(AG)] or A280F528 FynB [FynB(GA)] was determined by immunoblotting of total cell lysates with anti-Fyn antibodies. Lanes: 1, Neo 1; 2, Neo 3; 3, FynT(AG) F12 (2.1); 4, FynT(AG) F14 (2.1); 5, FynT(AG) F16 (1.9); 6, FynB(GA) F26 (2.3); 7, FynB(GA) F45 (2.2); 8, FynB(GA) F53 (2.6); 9, FynT F7 (2.9); 10, FynB F15 (2.9). The positions of prestained molecular mass markers (sizes are in kilodaltons) are shown on the right, and that of $p59^{fyn}$ is indicated on the left. The presence of equivalent amounts of cellular proteins in all of the lanes was confirmed by amido black staining of Immobilon membranes (data not shown). The exposure time was 14 h. (B) Antigen stimulation assay. Antigen responsiveness of cells expressing G280F528 FynT [FynT(AG)] or A280F528 FynB [FynB(GA)] was examined as described in the legend to Fig. 4. Symbols: ×, Neo 1; ○, FynT F7; △, FynT F19; ◇, FynB F15; ■, FynT(AG) F4; ●, FynT(AG) F14; ▲, FynT(AG) F15; ■, FynB(GA) F3; ▲, FynB(GA) F45; ⊡, FynB(GA) F53; □, Lck F9.

FynT-bearing cells. In contrast, but similar to F528 FynB-expressing cells, BI-141 derivatives containing A280F528 FynB were unresponsive to the antigenic stimulus. Thus, these results indicated that the glycine-to-alanine substitution in the glycine-rich motif did not contribute to the enhanced function of FynT in BI-141 T cells.

DISCUSSION

We have previously shown that expression of FynT molecules bearing a tyrosine-to-phenylalanine mutation at the negative regulatory site (F528 FynT) greatly augmented the ability of BI-141 T cells to release lymphokines in response to antigenic stimulation (12). This effect was distinct from that of expression of either wild-type FynT or F528 FynB, which failed to enhance antigen responsiveness in these cells. These data implied that FynT, but not FynB, was capable of coupling TCR stimulation to lymphokine production. Moreover, they showed that constitutive activation of the enzymatic function of FynT was necessary for this polypeptide to improve antigen receptor-mediated responses in these cells noticeably. Unfortunately, our previous attempts to define the biochemical basis for the functional difference between FynT and FynB were hampered by the finding that the two isoforms equally improved intracellular tyrosine protein phosphorylation in response to anti-TCR antibody stimulation. Analogous conclusions were reached in experiments evaluating the tyrosine phosphorylation of specific substrates such as Vav and ZAP-70 (our unpublished data).

To understand the mechanism underlying the distinct behaviors of FynT and FynB in T cells, we assessed their effects on the TCR-mediated mobilization of cytosolic calcium. This biochemical response is a critical early signal of antigen receptor-induced T-cell activation (43). The results of our analyses demonstrated that F528 FynT was more efficient than F528 FynB at increasing anti-TCR antibody-induced calcium fluxes. This was especially obvious when limiting antibody concentrations were used for stimulation, an approach thought to mimic the effect of antigen-MHC on T cells more closely. In complementary analyses, we found that ionomycin, a calcium ionophore, partially rescued the inability of activated FynB to improve antigen responsiveness in BI-141 T cells. These results implied that the different efficiencies with which FynT and FynB up-regulated TCR-induced calcium mobilization provide one biochemical explanation for their distinct behaviors during antigen-induced T-cell activation. However, as the correction effected by ionomycin was partial, it is probable that additional processes contribute to the functional differences between FynT and FynB.

The mechanism(s) by which calcium fluxes are differentially regulated by FynT and FynB in T cells has not been defined. Notably, we did not observe significant differences in the tyrosine phosphorylation of phospholipase Cγ1 (our unpublished data), which is thought to be the rate-limiting step for inositol phosphate production (18, 43). Recent data have indicated that TCR stimulation can also activate phospholipase D, thereby allowing inositol phosphate-independent elevation of cytosolic calcium (22, 34). In addition, others have shown that TCR stimulation causes the release of a novel messenger (termed the calcium influx factor) that stimulates calcium influx from the extracellular compartment (27). Thus, it is possible that the two Fyn isoforms regulate one or both of these pathways with different efficiencies.

Sequence analysis showed that FynT and FynB differ within a region of roughly 50 amino acids which overlaps the end of the SH2 motif and the beginning of the catalytic domain (Fig. 1; 10, 35, 36). By creation and analysis of FynT-FynB chimeras, we determined that the distinct catalytic sequence of FynT, and not its divergent SH2 or hinge region, conferred the ability to enhance the antigen responsiveness of BI-141 T cells. The same sequence allowed a greater elevation in intracellular calcium in response to TCR stimulation.

The differences in the catalytic domains of FynT and FynB extend over a very short stretch of 27 amino acids. Of these 27 residues, 11 (41%) differ between FynT and FynB (Fig. 1A). By analogy with the cyclic AMP-dependent protein kinase (the structure of which has been defined by crystallography; 19), this area presumably contains the first β strand and part of the second β strand of the Fyn kinase domain, contributing to the lobe which binds Mg^{2+}-ATP. A highly conserved glycine-rich motif (G-X-G-X-X-G) located between the two β strands appears to be particularly important for this binding. FynT is the only tyrosine protein kinase in which the last glycine of G-X-G-X-X-G (glycine 280) is replaced by alanine (16; Fig. 1C). However, our studies determined that the glycine 280-to-alanine substitution was not responsible for the ameliorated biological properties of FynT. Seemingly, one or several of the other amino acid differences confer(s) the distinct ability of FynT to improve T-cell antigen responsiveness.

In previous studies, we found that FynT and FynB display similar in vitro catalytic activities towards exogenous substrates such as rabbit muscle enolase (13). Moreover, we observed that both F528 FynT and F528 FynB are capable of oncogenic transformation of rodent fibroblasts (although activated FynT does so with somewhat higher efficiency; 13). Coupled with these results, the present study implied that rather subtle differences in catalytic activity are the basis for the strikingly distinct impacts of FynT and FynB on antigen-induced lymphokine secretion. Perhaps the amelioration of the catalytic function of FynT is towards a limited subset of TCR-regulated substrates.

Accumulating data indicate that $p59^{fynT}$ plays a critical role in hemopoietic cell signalling. The most convincing illustration of this function is provided by studies of antigen receptor signalling in T cells, which show that FynT is involved in the initiation of TCR-triggered signals (4, 9, 12, 33). On the basis of the findings reported herein, it is not surprising that both mammalian and avian T cells have evolved to express this uniquely crafted isoform of the Fyn kinase. In contrast, the FynB isoform of $p59^{fyn}$ has been implicated in a variety of neuronal cell functions. Indeed, mice engineered to lack Fyn expression as a result of homologous recombination in embryonic stem cells show consistent defects in spatial learning, as well as in long-term potentiation of hippocampal neurons (15). Moreover, a recent report indicates that Fyn-deficient mice may have an improper suckling response, which can lead to neonatal starvation and death (44). As a corollary to the functional advantage provided by FynT in T cells, it is conceivable that the configuration of FynB is most adequate for $p59^{fyn}$-mediated functions in neuronal cells. Certainly, such a hypothesis can be tested experimentally.

In summary, we have shown that the unique configuration of the kinase domain of $p59^{fynT}$ is necessary for this Src-like enzyme to enhance antigen-induced lymphokine secretion in a mouse T-cell hybridoma. Our data also indicate that the distinct impacts of FynT and FynB on T-cell physiology likely reflect subtle changes in catalytic efficiency which affect the function of selected TCR-regulated targets. Importantly, one of these substrates was shown to be involved in the regulation of cytosolic calcium upon TCR stimulation. As BI-141 is an antigen-specific mouse T-cell line, caution should be exercised in extending our findings to all T cells. Experiments with other

T cell lines or with transgenic animals are necessary to establish the universality of our findings.

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