Differential Regulation of T Cell Antigen Responsiveness by Isoforms of the *src*-related Tyrosine Protein Kinase $p59^{fyn}$

By Dominique Davidson,* Lionel M. L. Chow,*‡ Marielle Fournel,* and André Veillette*‡§‖¶

*From the *McGill Cancer Centre; the Departments of ‡Biochemistry, §Medicine, and ‖Oncology, McGill University; and the ¶Division of Oncology, Department of Medicine, Montreal General Hospital, Montréal H3G 1Y6 Canada*

Summary

Recent observations suggest that the *src*-related tyrosine protein kinase $p59^{fyn}$ may be involved in antigen-induced T lymphocyte activation. As a result of alternative splicing, $p59^{fyn}$ 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 $p59^{fyn}$ isoform (*fynB*) is highly expressed in brain, the alternative product (*fynT*) is principally found in T lymphocytes. To further understand the role of $p59^{fyn}$ in T cell activation and to test the hypothesis that $p59^{fynT}$ 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 $p59^{fyn}$ 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 enhanced antibody-induced T cell receptor (TCR)–mediated signals. In contrast, only $p59^{fynT}$ increased interleukin 2 production in response to antigen stimulation. This finding implies that the distinct $p59^{fyn}$ isoform expressed in T lymphocytes regulates the coupling of TCR stimulation by antigen/major histocompatibility complex to lymphokine production.

Antigen-induced T lymphocyte activation is primarily mediated by changes in intracellular tyrosine protein phosphorylation (for review, see reference 1). Although the cellular machinery regulating this biochemical signal remains poorly characterized, recent observations suggest that the *src*-related nonreceptor tyrosine protein kinases $p56^{lck}$ and $p59^{fyn}$ are involved in this process. $p56^{lck}$ is a lymphocyte-specific tyrosine protein kinase associated with and regulated by the CD4 and CD8 T cell surface antigens (for review, see reference 2). Increasing evidence demonstrates that a large part of the accessory function provided by CD4 or CD8 during T cell activation is mediated through $p56^{lck}$. Contrary to $p56^{lck}$, $p59^{fyn}$ is expressed in most cell types (3–6). It is interesting that as a result of alternative splicing of mutually exclusive exons 7, $p59^{fyn}$ exists as two isoforms differing solely within a stretch of 51 amino acids spanning the end of the SH2[1] region and the beginning of an otherwise classical tyrosine protein kinase domain (see Fig. 1 *A*) (7). It has been postulated that as a consequence of different substrate specificities and/or catalytic efficiencies, these two enzymes regulate distinct cellular processes.

Whereas one *fyn* isoform (*fynB*) accumulates highly in brain, the other (*fynT*) is expressed predominantly in T lymphocytes (7; L. M. L. Chow, A. Veillette, D. Davidson, unpublished data). The limited homology (53% or less) between the exon 7-encoded sequences of *fynT* and those of *fynB*, as well as of all other *src*-related tyrosine protein kinases, fostered the early view that $p59^{fynT}$ plays a specialized role in T lymphocyte physiology. The subsequent demonstration that a fraction of $p59^{fynT}$ is physically associated with elements of the TCR complex suggested that this polypeptide may in fact regulate antigen receptor-induced signals (8). Additional support for this possibility was recently provided by the observation that thymocytes of transgenic mice overexpressing either $p59^{fyn}$ isoform showed enhanced TCR-induced tyrosine protein phosphorylation, calcium response, and IL-2 production (9). However, no significant difference between the functions of $p59^{fynT}$ and $p59^{fynB}$ were noted in this study.

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

[1] *Abbreviations used in this paper:* RAM, rabbit anti-mouse; SH2, Src Homology Domain 2; UT, untranslated.

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. L. 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 $A^b_\alpha A^k_\beta$ (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 CO_2 incubator.

Site-directed Mutagenesis. Wild-type murine *fynT* (MM23) and *fynB* (MB1) 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 Slilaty 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′AAGCTCCCGAATTCCCACCAACT3′). 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 $p59^{fyn}$, tyrosine 528 was mutated to a phenylalanine using the oligonucleotide 5′CGGGCTGAAACTGGGGC3′. The mutant *fynB* cDNA was created by exchanging the ApaI-BamHI fragment of MM23 (which contains the seventh exon) with that of the *fynB* cDNA MB1. All exchanged fragments were fully resequenced and found to contain no additional mutations (data not shown). Similarly kinase-negative variants of F528 $p59^{fynT}$ and F528 $p59^{fynB}$ were engineered by introducing a lysine to methionine substitution at position 296 of the ATP-binding site of these polypeptides using oligonucleotide-directed mutagenesis (mutagenic oligonucleotide: 5′TAAGGGTCATTATGGCT3′). An identical mutation in other tyrosine protein kinases 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 cell lines established by limiting dilution. Cells expressing the neomycin resistance gene (*neo*) alone, or the activated versions of $p56^{lck}$ (F505 $p56^{lck}$) have been described elsewhere (16). BI-141 cells expressing mouse CD4 (CD4 FL3) were also generated by retroviral infection with a construct in which a full-length mouse CD4 cDNA (kindly provided by Jane Parnes, Stanford University, Palo Alto, CA) was inserted into the EcoRI site of pLXSN (L. Caron and A. Veillette, unpublished data).

RNA Isolation and Northern Blot Analysis. Total cellular RNA was prepared from frozen cell pellets by the guanidine isothiocyanate method (17). For Northern blot analysis, 10 μg of RNA was resolved on 1.1% agarose, 2.2 M formaldehyde gels, and transferred to nylon membrane by vacuum blotting. After baking the membrane at 80°C for 2 h and cross-linking the nucleic acids with UV light for 5 min, the membrane was prehybridized in a solution containing 50% formamide, 5× SSC (1× SSC is: 150 mM NaCl, 15 mM sodium citrate), 0.5% SDS, 50 mM sodium pyrophosphate, 1× Denhardt solution, and 100 μg/ml salmon sperm DNA for 15 h at 42°C. Hybridization was carried out at 42°C for 18 h in a similar solution with the addition of 10% dextran sulfate and 2× 10^6 cpm/ml of randomly primed ^{32}P-labeled probe (18). Two brief washes in 2× SSC and 0.1% SDS at 25°C and one 30-min wash in 0.1× SSC, and 0.1% SDS at 55°C were done before autoradiography.

Fyn-specific Immunoblot Assay. Equivalent numbers of cells were lysed in sample buffer and lysates resolved on 8% SDS-PAGE gels. Subsequent immunoblotting was conducted as described previously (19), using a rabbit anti-*fyn* serum generated by immunization with a trpE fusion protein containing residues 25–141 of the murine $p59^{fyn}$ sequence (7). This peptide corresponds to sequences located within the unique and Src Homology 3 (SH3) domains of $p59^{fyn}$, which are therefore common to *fynT* and *fynB*. This antiserum specifically recognized $p59^{fyn}$ in both immunoprecipitation and immunoblot assays. It did not react with $p56^{lck}$ (our unpublished data). For quantitation, bands were cut from nitrocellulose and counted in a gamma counter. The presence of equivalent amounts of proteins in each lane was subsequently confirmed by staining of nitrocellulose filters with amido black.

Antibody-mediated Cross-linking and Antiphosphotyrosine Immunoblots. These assays were done as described elsewhere (20, 21). The anti-TCR and -CD3 mAbs have been reported previously (22–24). Titration of the amount of mAb F23.1 used for cross-linking revealed that the antibody-induced tyrosine protein phosphorylation dose-responses were similar for F528 $p59^{fynT}$ and F528 $p59^{fynB}$ expressing cells (unpublished observations). Antibody-mediated cross-linking was also performed using biotinylated primary antibodies and avidin. Incubation with avidin was for 5 min. Biotinylated anti-TCR mAb F23.1 and anti-CD45 mAb 30-F11 were kindly provided by Jeff Ledbetter (Bristol-Myers-Squibb, Seattle, WA). Antiphosphotyrosine immunoblotting was performed using affinity-purified polyclonal rabbit anti–phosphotyrosine antibodies (our unpublished data). Quantitation was performed as outlined above.

IL-2 Assays. To evaluate antibody-induced IL-2 production by the different BI-141 cell lines, wells of 96-well Falcon tissue culture plates were coated in triplicate with serial dilutions of mAb F23.1 at 37°C overnight. After removal of the unbound antibody, 2 × 10^4 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 [^{3}H]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 (10^5 cells/well) were plated in triplicate with 5 × 10^4 irradiated MHC class II $A^b_\alpha A^k_\beta$-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 $p59^{fynT}$ in mature T cell physiology, we have introduced constitutively activated versions of murine $p59^{fynT}$ and $p59^{fynB}$ in the CD4-negative, class II MHC–restricted beef insulin-specific murine helper

T cell hybridoma BI-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 $p59^{fyn}$ 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 $p56^{lck}$ (F505 $p56^{lck}$; Fig. 1 *B*, lanes *11–13*) (16) were also included in these analyses. A subsequent *fyn*-specific immunoblot assay revealed that F528 $p59^{fyn}$ expressing cells (Fig. 1 *C*, lanes *4–11*) contained 1.5–3 times more $p59^{fyn}$ than parental BI-141 cells (Fig. 1 *C*, lane *1*) or control Neo clones (Fig. 1 *C*, lanes *2* and *3*). This level of overexpression most likely reflects the high levels of endogenous $p59^{fynT}$ in BI-141 cells. All cell lines expressed levels of TCR, CD3, Thy1.2, CD45, and $p56^{lck}$ 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 an antiphosphotyrosine immunoblot assay (Fig. 2). Upon stimulation with anti-TCR mAb F23.1 and rabbit anti-mouse (RAM) IgG, we found that cells expressing F528 $p59^{fynT}$ (Fig. 2 *A*, lanes *7–12*) or F528 $p59^{fynB}$ (Fig. 2 *A*, lanes *13–18*) demonstrated more rapid, robust, and sustained tyrosine protein phosphorylation signals than control cells (Fig. 2 *A*, lanes *1–6*). Similar results were noted after stimulation with anti-TCR mAb H57.597 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 $p59^{fynT}$ than $p59^{fynB}$ 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 $p59^{fyn}$ expressing cells (Fig. 2 *B*, lanes *5–12*) had the same apparent molecular weights as those previously shown to be regulated by F505 $p56^{lck}$ (Fig. 2 *B*, lanes *13–16*) (16). However, the enhancement by F528 $p59^{fyn}$ was generally less than that conferred by the activated *lck* 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 $p56^{lck}$ (Fig. 2 *C*, lane *16*), coaggregation of TCR with CD45 on F528 *fynT* (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 $p59^{fyn}$ variants, as well as activated $p56^{lck}$, 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 $p59^{fynT}$ and F528 $p59^{fynB}$ 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 ng/ml of mAb F23.1 tended to be more substantial for cells expressing F528 $p59^{fynB}$.

Cell lines were also tested for reactivity to beef insulin presented in association with the appropriate class II MHC molecules ($A^b_\alpha A^k_\beta$). 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 $p59^{fynT}$ 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 $p59^{fynB}$, which had essentially unaltered responses to antigen (Fig. 3, *B* and *C*). Only one F528 $p59^{fynB}$ clone (FynB F18, Fig. 3 *C*) showed a small but consistent increase in antigen-induced lymphokine production. The improvement of antigen responsiveness by F528 $p59^{fynT}$ was less than that conferred by expression of CD4 or F505 $p56^{lck}$ 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 $p59^{fyn}$ was related to the elevated tyrosine protein kinase activity of this mutant. To this effect, F528 *fynT* and F528 *fynB* polypeptides with abolished catalytic activity (M296F528 $p59^{fyn}$) (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 $p59^{fyn}$ expressing cells (Fig. 4 *A*, and data not shown). The M296F528 $p59^{fyn}$ expressing cells contained 1.5–2.5-fold higher levels of *fyn* protein than con-

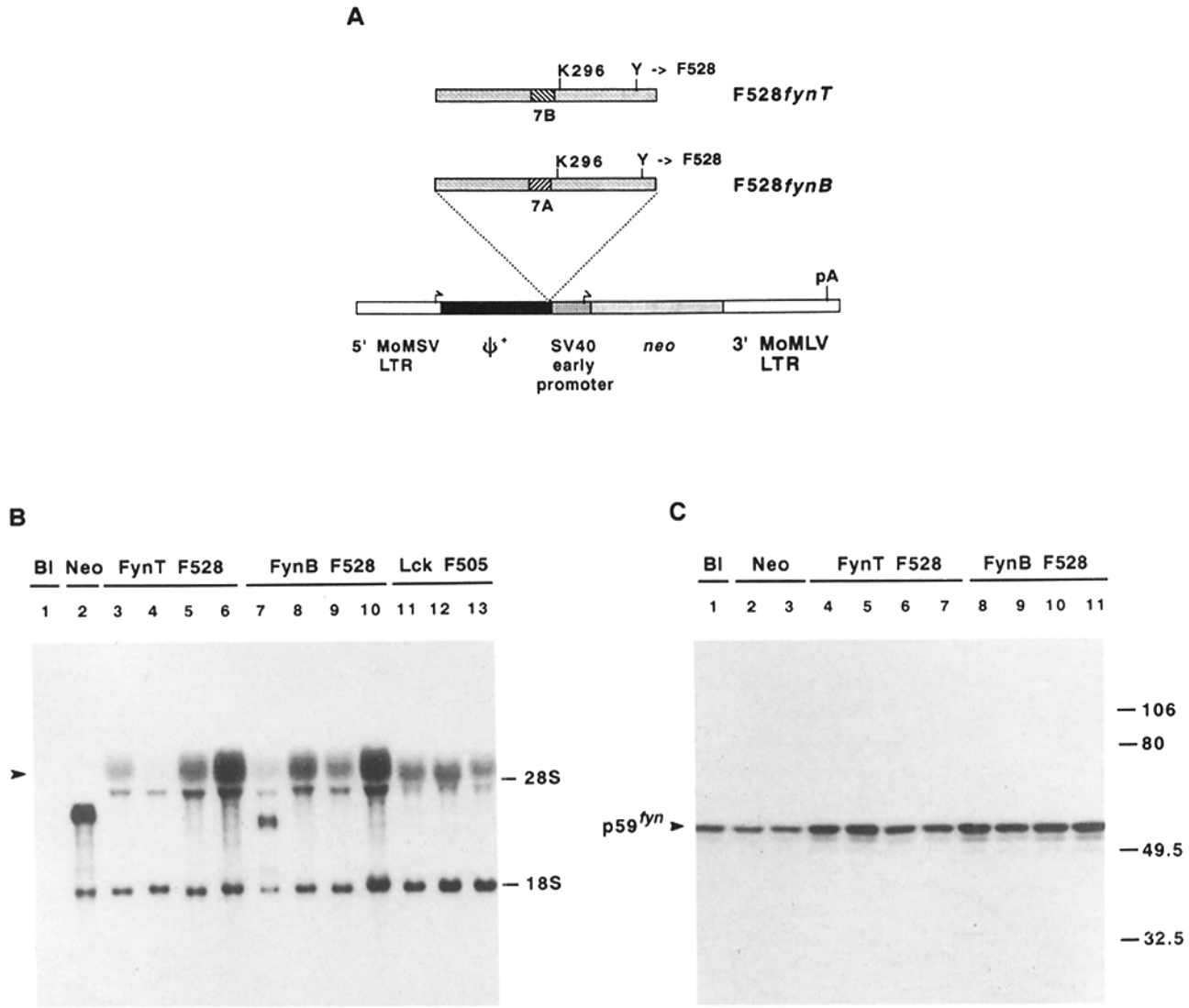

Figure 1. Expression of F528 p59^{fyn} in BI-141 cells. (*A*) Retroviral constructs. The activated *fynT* and *fynB* mutants were inserted into the retroviral vector pLXSN at a convenient multiple cloning site flanked by the 5' Moloney Murine Sarcoma Virus (MoMSV) LTR and the 3' Moloney Murine Leukemia Virus (MoMLV) LTR. The neomycin (*neo*) phosphotransferase gene (Tn5) is driven by the SV40 early promoter. The position of ψ^+ sequences is shown. (*Arrows*) Start sites and direction of transcription. (*pA*) Position of the polyadenylation signal. K296 is the lysine residue at position 296 of p59^{fyn} and is part of the ATP-binding site. Y528, which represents the major site of in vivo tyrosine phosphorylation of p59^{fyn}, tyrosine 528, was replaced through site-directed mutagenesis by phenylalanine (F528), as described in Materials and Methods. Exon 7 is designated 7A in *fynB* and 7B in *fynT*. (*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^{fynT} (lanes *3–6*), F528 p59^{fynB} (lanes 7–*10*), and, for comparison, F505 p56^{lck} (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*: FynT F7; *5*: FynT F12; *6*: FynT F19; 7: FynB F13; *8*: FynB F15; *9*: FynB F17; *10*: FynB F18; *11*: Lck F7; *12*: Lck F9, and *13*: 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. (►) 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^{fyn} were measured in parental BI-141 cells (lane *1*) and in cell lines expressing the neomycin phosphotransferase alone (lanes *2* and *3*), F528 p59^{fynT} (lanes *4–7*) and F528 p59^{fynB} (lanes *8–11*), using a *fyn*-specific immunoblot assay. Lanes *1*: BI-141 parent; *2*: Neo 2; *3*: Neo 4; *4*: FynT F2; *5*: FynT F7; *6*: FynT F12; 7: FynT F19; *8*: FynB F13; *9*: FynB F15; *10*: FynB F17, and *11*: FynB F18. The immunoreactive species detected at 56 and 54 kD appear to be proteolytic cleavage products of p59^{fyn} (our unpublished observations). (*Left*) Position of p59^{fyn} and (*right*) positions of prestained molecular weight markers (in kD). Exposure: 4 h. Levels of p59^{fyn} expression were quantified as described in Materials and Methods and, relative to the average of Neo cells, are as follows: FynT F2: 1.8; FynT F7: 2.1; FynT F12: 1.6; FynT F19: 1.5; FynB F13: 2.4; FynB F15: 1.9; FynB F17: 2.3, and FynB F18: 2.7.

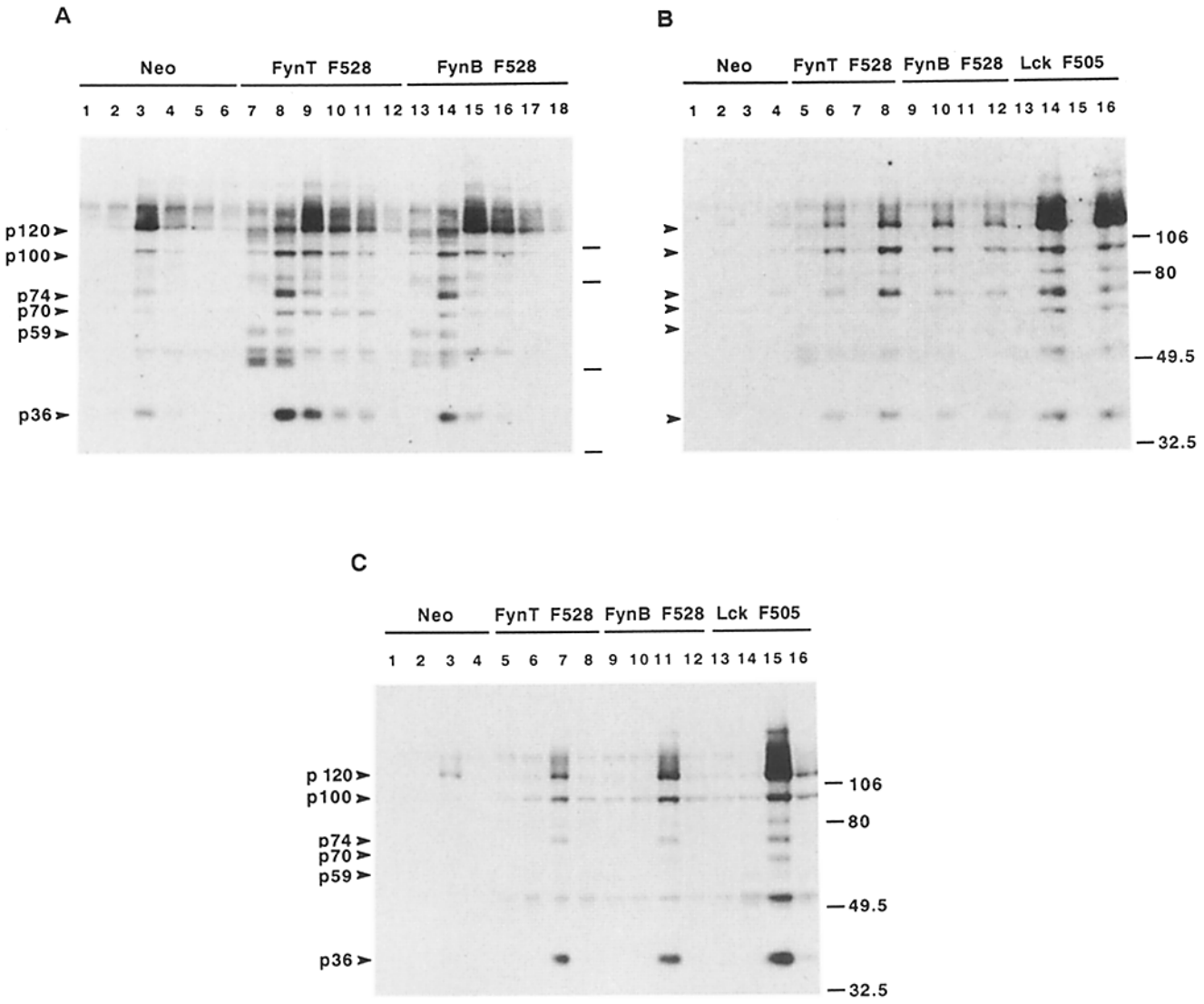

Figure 2. Enhancement of TCR-induced tyrosine protein phosphorylation signal by F528 $p59^{fyn}$. Antiphosphotyrosine immunoblots. (*A*) Time course experiments. Antibody-mediated cross-linking of clonal BI-141 cell lines expressing the neomycin phosphotransferase (Neo 1; lanes *1–6*), F528 $p59^{fynT}$ (FynT F2; lanes *7–12*) and F528 $p59^{fynB}$ (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 $p59^{fyn}$ expressing cells. Cross-linking of BI-141 cell lines expressing the neomycin marker (lanes *1–4*), F528 $p59^{fynT}$ (lanes *5–8*), F528 $p59^{fynB}$ (lanes *9–12*) and, for comparison, F505 $p56^{lck}$ (lanes *13–16*) was performed with anti-TCR mAb F23.1 + RAM IgG for 2 min at 37°C. Lanes *1* and *2*: Neo 1; *3* and *4*: Neo 2; *5* and *6*: FynT F2; *7* and *8*: FynT F7; *9* and *10*: FynB F13; *11* and *12*: FynB F15; *13* and *14*: Lck F7, and *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 $p59^{fyn}$ 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 $p59^{fynT}$ (FynT F2; lanes *5–8*), F528 $p59^{fynB}$ (FynB F13; lanes *9–12*) and, for comparison, F505 $p56^{lck}$ (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 $p59^{fyn}$ 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

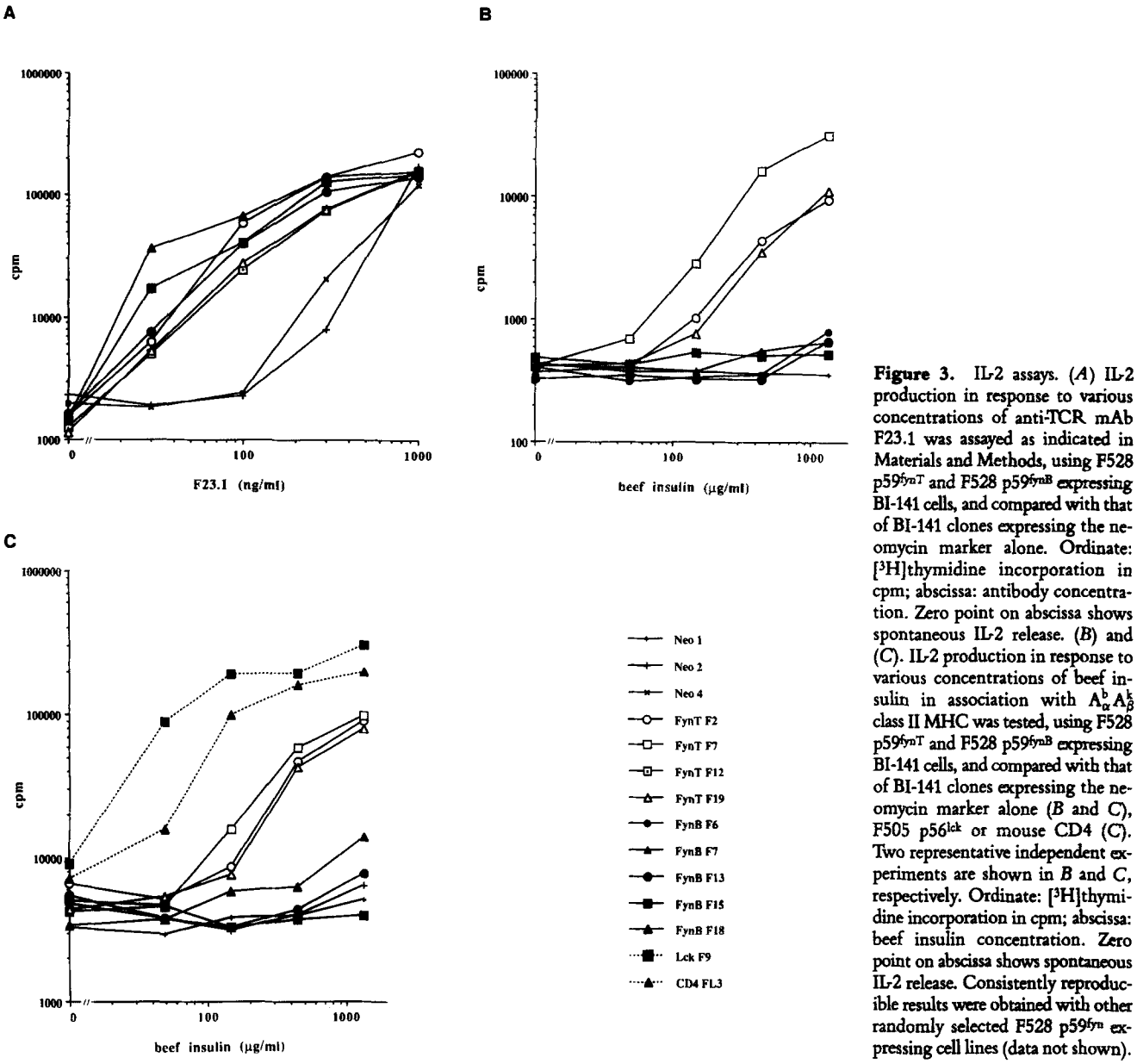

Figure 3. IL-2 assays. (*A*) IL-2 production in response to various concentrations of anti-TCR mAb F23.1 was assayed as indicated in Materials and Methods, using F528 $p59^{fynT}$ and F528 $p59^{fynB}$ expressing BI-141 cells, and compared with that of BI-141 clones expressing the neomycin marker alone. Ordinate: [^{3}H]thymidine incorporation in cpm; abscissa: antibody concentration. Zero point on abscissa shows spontaneous IL-2 release. (*B*) and (*C*). IL-2 production in response to various concentrations of beef insulin in association with $A^b_\alpha A^k_\beta$ class II MHC was tested, using F528 $p59^{fynT}$ and F528 $p59^{fynB}$ expressing BI-141 cells, and compared with that of BI-141 clones expressing the neomycin marker alone (*B* and *C*), F505 $p56^{lck}$ or mouse CD4 (*C*). Two representative independent experiments are shown in *B* and *C*, respectively. Ordinate: [^{3}H]thymidine incorporation in cpm; abscissa: beef insulin concentration. Zero point on abscissa shows spontaneous IL-2 release. Consistently reproducible results were obtained with other randomly selected F528 $p59^{fyn}$ expressing cell lines (data not shown).

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 $p59^{fynT}$ and $p59^{fynB}$ 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 $p59^{fyn}$ 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 $p59^{fynT}$ 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 $p59^{fynT}$ and $p59^{fynB}$ 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

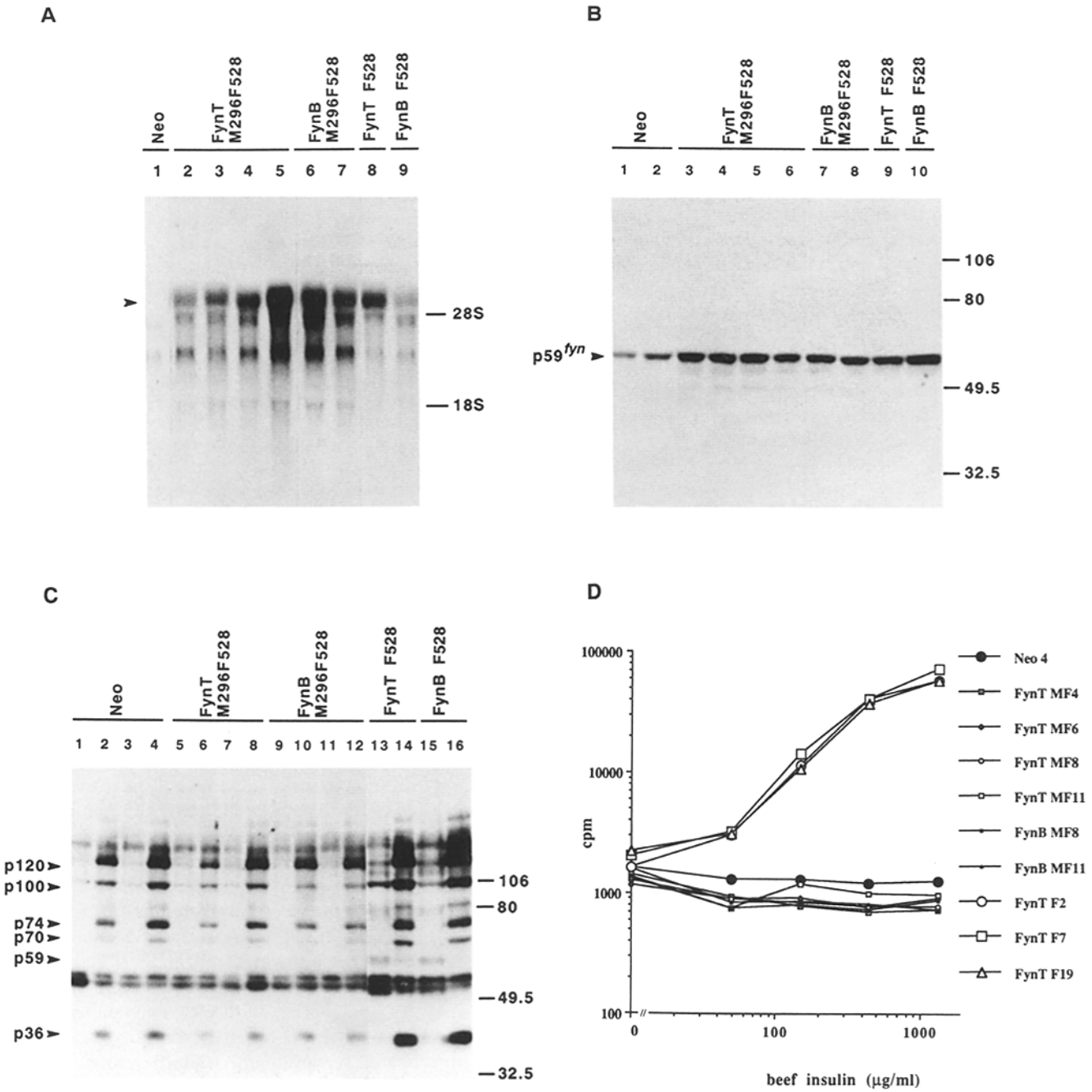

Figure 4. Effects of kinase-negative p59^{fyn} polypeptides on T cell responsiveness. (*A*) Northern blot analysis. Expression of retroviral *fyn* transcripts was assayed in clonal BI-141 cell lines expressing the neomycin phosphotransferase alone (lane *1*), M296F528 p59^{fynT} (lanes *2–5*), M296F528 p59^{fynB} (lanes *6* and *7*), and, for comparison, F528 p59^{fyn} (lanes *8* and *9*). Lanes *1*: Neo 2; *2*: FynT MF4; *3*: FynT MF6; *4*: FynT MF8; *5*: FynT MF11; *6*: FynB MF8; 7: Fyn B MF11; *8*: FynT F12, and *9*: FynB F13. In this experiment, a full-length MM23 *fynT* cDNA was used as a probe. (►) Retroviral *fyn* transcript position. (*Right*) 28S and 18S ribosomal RNA positions. The two RNA species migrating at 3.7 and 2.8 kb are the endogenous *fyn* transcripts (3–6). Exposure: 6 h. (*B*) *fyn* immunoblot. As in Fig. 1 *C*. Lanes *1* and *2*: Neo; *3*: FynT MF4; *4*: FynT MF6; *5*: FynT MF8; *6*: FynT MF11; 7: FynB MF8; *8*: FynB MF11; *9*: FynT F2, and *10*: FynB F13. (*Left*) Position of p59^{fyn}, and (*right*) positions of molecular weight markers. Exposure: 8 h. The relative amounts of *fyn* in M296F528 p59^{fyn} expressing cell lines (compared with the average of Neo cells) are: FynT MF4: 2.1-fold; FynT MF6: 2.0-fold; FynT MF8: 2.3-fold; FynT 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°C. Lanes *1* and *2*: Neo 1; *3* and *4*: Neo 2; *5* and *6*: FynT MF4; *7* and *8*: FynT MF8; *9* and *10*: FynB MF8; *11* and *12*: FynB MF11; *13* and *14*: FynT 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. (*Right*) 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^{fyn} were tested for beef insulin reactivity and compared with other BI-141 derivatives, as described in Fig. 3 *B*.

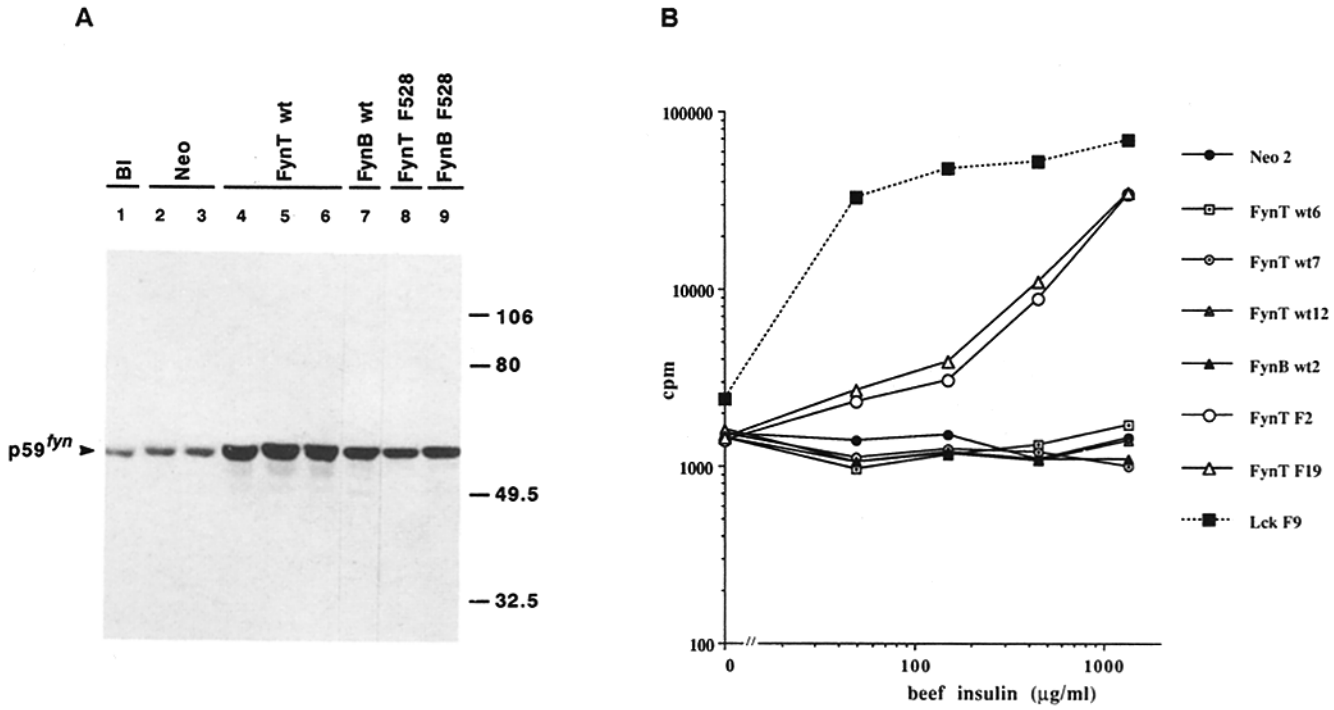

Figure 5. Effects of wild-type p59^{fyn} polypeptides on T cell responsiveness. (*A*) *fyn* immunoblot. As in Fig. 1 *C*. Lanes *1*: BI-141; *2*: Neo 2; *3*: Neo 4; *4*: FynT wt6; *5*: FynT wt7; *6*: FynT wt12; 7: FynB wt2; *8*: FynT F2, and *9*: FynB F13. (*Left*) Position of p59^{fyn}, and (*right*) positions of molecular weight markers. Exposure: 10 h. The relative amounts of *fyn* in wild-type p59^{fyn} 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^{fyn} 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^{fynT} 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 ζ chain of the TCR complex (30). Deletion of the cytoplasmic tail of ζ significantly decreased the ability of this molecule to link antigen/MHC-induced TCR stimulation to lymphokine production. As is the case for p59^{fynB}, 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 ζ is mediated either directly or indirectly through p59^{fynT}. Further support for this view is lent by the observation that a fraction of p59^{fynT} 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^{fynT} and p56^{lck}) can enhance antigen receptor-mediated functions. It is important that this is in contrast with p60^{src}, 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^{lck} and CD4/CD8 are well established (2), the physiological processes allowing the activation of p59^{fyn} in T cells are unfortunately poorly understood. As *fynT* can associate with TCR, it has been proposed that the enzymatic function of p59^{fynT} is directly regulated by TCR. However, the in vitro catalytic activity of p59^{fynT} is not enhanced upon antibody stimulation of TCR (8, and our unpublished observations). While this finding does not preclude the view that p59^{fynT} 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, *fynT* may first be activated by CD45-mediated dephosphorylation of tyrosine 528 (1). Subsequently, as suggested by our observations, TCR stimulation may allow activated $p59^{fynT}$ 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 $p59^{fynT}$ by CD45 approximation, or as a consequence of substrate dephosphorylation. A related model has been proposed for the regulation of the function of activated $p56^{lck}$ 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, $p59^{fynT}$ and $p56^{lck}$ play important roles in the signal transduction of T cell activation.

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Address correspondence to Dr. André Veillette, McGill Cancer Centre, Room 701, McIntyre Medical Sciences Building, 3655 Drummond Street, Montréal H3G 1Y6 Canada.

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