Involvement of p59*fyn*^{T} in Interleukin-5 Receptor Signaling

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Summary

Previous studies implicate the nonreceptor protein tyrosine kinase (PTK) p59*fyn* in the propagation of signals from the B cell antigen receptor. To elucidate the functions of this kinase, we examined B cell responsiveness in mice engineered to lack the hematopoietic isoform of p59*fyn*. Remarkably, antigen receptor signaling was only modestly defective in *fyn*^{Tnull} B cells. In contrast, signaling from the interleukin (IL)-5 receptor, which ordinarily provides a comitogenic stimulus with antiimmunoglobulin, was completely blocked. Our results document the importance of p59*fyn*^{T} in IL-5 responses in B cells, and they support a general model for cytokine receptor signal transduction involving the simultaneous recruitment of at least three families of PTK.

B cells interact with antigen through a multisubunit complex, the B cell antigen receptor. The receptor is composed of membrane-bound Ig noncovalently associated with one or more copies of an $\alpha\beta$ heterodimer derived from the products of the mb-1 (Igα) and b29 (Igβ) genes. Engagement of the B cell antigen receptor by antigen provokes a number of biochemical events. These culminate in the activation of resting B cells, their entry into the cell cycle, and the internalization of antigen, which is subsequently processed for presentation to T cells. An early event in this cascade is the phosphorylation of a number of cellular proteins upon tyrosine, suggesting that the activation of tyrosine kinases may be an obligate step in signaling from the antigen receptor (1–4). Indeed, both B cell proliferation (5) and the internalization of antigen are inhibited by treatment with tyrosine kinase inhibitors (6–8).

The kinases believed to mediate B cell activation include members of the *src*, *syk*, and *tec* families (reviewed in reference 9). For example, p72^{syk} is able to associate with the antigen receptor complex and becomes tyrosine phosphorylated after anti-Ig antibody stimulation of B cells (4, 10, 11). p70^{btk}, a representative of the *tec* family of protein tyrosine kinases (PTKs)[1], has also been implicated in B cell signaling as a consequence of the abnormal B cell responses observed

in lymphocytes from mice bearing a missense mutation in the *btk* gene (12, 13). This kinase also assists in regulating B cell development, as revealed by the acute block in B cell ontogeny witnessed in humans lacking functional p70^{btk} protein (14, 15).

Several members of the src family of protein tyrosine kinases, particularly p53/56^{lyn}, p59^{fyn}, p56^{lck}, and p56^{blk} have been identified in B cell receptor complexes, and the kinase activity of each of these has been reported to increase after anti-Ig stimulation (16–19). Association of these kinases with the antigen receptor appears to involve the Igα/Igβ components of the antigen receptor (20, 21) and is dependent on the activation status of the receptor complex. Thus, in the resting state, a low affinity association between the amino terminus of p59^{fyn} and a sequence within Igα appears to predominate. After B cell receptor (BCR) engagement, phosphorylation of tyrosine residues in the antigen receptor activation motif facilitates an association with p59^{fyn} via its SH2 domain, and results in an increase in kinase activity (21–23). p59^{fyn} is also capable of physical interaction with phospholipase Cγ2 (24), itself a target for tyrosine phosphorylation after receptor engagement, and therefore appears to play an important role in signaling pathways emanating from the BCR.

In addition to responding to antigenic stimulation, B cells also mature in response to numerous cytokines. These include IL-7, implicated in the early stages of B cell development (25), and IFN-γ, IL-4, and IL-5, which together par-

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[1] *Abbreviations used in this paper:* BCR, B cell receptor; PTK, protein tyrosine kinase; TPN-Ficoll, trinitrophenylated aminoethyl carboxymethyl-conjugated Ficoll; TNP-KLH, trinitrophenol-conjugated KLH.

ticipate in the regulation of isotype switching (26, 27). An evolving body of literature suggests that these molecules exert their effects in part through the activation of another family of protein tyrosine kinases, the JAK family (reviewed in references 28, 29). The *src* family protein tyrosine kinases may also be active in cytokine signaling pathways, as evidenced by the physical and functional association between p56^{lck} and the β chain of the IL-2 receptor (30), and between p59^{fyn} and the IL-7 receptor (31, 32).

We have previously described the generation of mice that lack expression of one isoform of *fyn*, that expressed within hematopoietic cells (*fyn*T), by targeted disruption (33). These *fyn*^{Tnull} animals develop normally and are indistinguishable at a gross level from wild-type siblings. However they exhibit an exquisitely specific defect: mature thymocytes from *fyn*^{Tnull} mice are acutely refractile to stimulation through the TCR (33).

Here we report that signaling from the B cell antigen receptor proceeds in a near-normal manner in the absence of p59^{fyn}. However, the absence of p59^{fyn} in the B cell lineage is not without consequence: *fyn*^{Tnull} mice show impaired B cell responses after immunization, and these *fyn*^{Tnull} B cells fail to respond to signaling through the IL-5 receptor. These observations support a general model for the concerted action of multiple tyrosine kinases in cytokine receptor signaling.

Materials and Methods

Generation of Experimental Animals and Flow Cytometric Analysis. *fyn*^{Tnull} mice were generated as previously described and were maintained by brother × sister matings. Homozygous wild-type animals derived from the intercross of *fyn*$^{T+/-}$ animals were also maintained and used as controls (33). Animals were analyzed between 8 and 12 wk of age. Single cell suspensions of splenocytes were prepared as previously described (33). Peritoneal exudate cells were harvested by lavage using PBS containing 10 μg/ml heparin (Sigma Chemical Co., St. Louis, MO). Cells were stained for surface expression of B220, IgM, IgD, and Ly1 using PE-conjugated anti-Ly5 (Caltag Laboratories, San Francisco, CA), FITC-labeled anti-IgM (Southern Biotechnology Associates, Birmingham, AL), biotinylated anti-IgD (Binding Site Inc., San Diego, CA), and biotinylated anti-Ly1 (PharMingen, San Diego, CA). Biotinylated anti-IgD and biotinylated anti-Ly1 were subsequently visualized using streptavidin-TRICOLOR® (Caltag Laboratories). Events were collected in list mode files on a FACScan® flow cytometer and analyzed using Lysys II software (Becton Dickinson & Co., Mountain View, CA).

Mitogenic Responses of fyn^{Tnull} *Splenocytes and Bone Marrow.* Purified splenic B cells were prepared by complement lysis of CD4$^+$ and CD8$^+$ T cells with the CD4- and CD8-specific mAbs GK1.5, 3.155, and 2.43. 4 × 10^5 splenic B cells were cultivated in 200 μl RPMI supplemented with 10% FCS, 2 mM glutamine, 10 mM Hepes buffer, pH 7.0, and 0.1 mM 2-ME, and 100 U/ml penicillin and 100 μg/ml streptomycin.

Cells were stimulated with goat anti–mouse IgM (Fab')2 (Organon Teknika, Durham, NC) at 50 μg/ml, bacterial LPS at 25 μg/ml (Difco Laboratories, Detroit, MI), recombinant murine IL-5 (R & D Systems, Inc., Minneapolis, MN) at the concentrations indicated in the text, dextran sulfate at 3 μg/ml (Sigma Chemical Co.) or CD40 ligand-trimer (Immunex Research and Development

Corp., Seattle, WA), as described in the text. After 48 h, cells were incubated with ^{3}[H]thymidine for 18–24 h and then harvested onto printed glass fiber filter mats using a Tomtec harvester. Incorporation of ^{3}[H]thymidine was measured using an HEB-Wallac 1205 Betaplate liquid scintillation counter (Wallace Inc., Gaithersburg, MD).

Bone marrow was harvested from femurs of control and *fyn*^{Tnull} mice by aspiration with sterile HBSS. Cells were cultured in Iscove's modification of Eagle's medium, supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and stimulated with recombinant murine IL-3 and recombinant GM-CSF (a generous gift from Immunex Research and Development Corp.). Proliferation was assessed by measuring the incorporation of ^{3}[H]thymidine as described above.

Calcium Analysis. The determination of levels of intracellular free calcium by flow cytometric analysis was performed as described previously (34). Splenocytes were stained for surface expression of B220 using PE-conjugated Ly5 (Caltag Labs) and stimulated with goat anti–mouse IgM (Fab')2 (Organon Teknika) or sheep anti–mouse IgD (Binding Site Inc.) at the indicated doses. Data were analyzed using MTIME software as described previously (35).

Responses of fyn^{Tnull} *Mice to Immunization with Thymic-dependent and -independent Antigens.* Mice were injected intraperitoneally with 1 μg of trinitrophenol-conjugated KLH (TNP-KLH) or 1 μg of trinitrophenylated aminoethyl carboxymethyl–conjugated Ficoll (TNP-Ficoll). Mice were bled 7 d after immunization and serum was prepared for ELISA analysis. Serum polyclonal IgM and IgG3 levels and anti-TNP isotype levels were determined by ELISA as previously described (36).

Immunoprecipitation and In Vitro Kinase Assay. Whole cell lysates of splenic B cells prepared by complement lysis of T cells were prepared as previously described (34). Immunoprecipitation–kinase assays were performed as described in Cooke et al. (34) or Tsukada et al. (14). Extracts were resolved by 10% SDS-PAGE and transferred to nitrocellulose for exposure to film (X-Omat AR; Eastman Kodak Co., Rochester, NY).

Association between p59^{fyn} *and p77*^{btk}. B cell blasts were derived from splenic B cells by stimulation with LPS at 50 μg/ml in RPMI supplemented as described above. After stimulation with rIL-5 at the doses described in the text, cells were lysed in a buffer containing 50 mM Tris HCl, pH 7.5, and 150 mM NaCl supplemented with either 1% Triton X-100, 1% Brij 96, or 1% 3-[(3-chola-midopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS). Kinase reactions were performed as described above. Immune complexes were then solubilized by boiling in 2% SDS, diluted 20-fold, and divided into two aliquots. p77^{btk} was immunoprecipitated from one of these as previously described (14) and p59^{fyn} immunoprecipitated from the second aliquot as a control. Phosphorylated substrates were resolved by PAGE.

Results

B Cell Development Proceeds Normally in the Absence of p59^{fyn}. B cell development from pre-B cell to mature B cell can be divided into a number of developmental steps, characterized by the ordered expression of cell surface markers and by the rearrangement status of Ig genes (for a review see reference 37). Since IL-7 assists in regulating B cell development, the reported association between the IL-7 receptor and p59^{fyn} (31, 32) suggested that B cell development in *fyn*^{Tnull} mice might be compromised. Using carefully prepared populations,

we found that p59^{fynT} is the Fyn isoform expressed in B cells and noted that fyn^{Tnull} mice lack fyn protein in both B and T cell compartments (data not shown). Hence, these animals afforded us the opportunity to assess the relevance of this interaction. Flow cytometric analysis of splenocytes from normal animals stained with mAbs to surface IgM and IgD permits the resolution of three subpopulations of cells (Fig. 1 A), including an immature population expressing high levels of IgM but low levels of IgD. All three populations are represented at equivalent levels in flow cytometric profiles from control and fyn^{Tnull} animals. Spleens from fyn^{Tnull} mice also exhibit normal cellularity (data not shown) and express appropriate numbers of B220-bearing cells (Fig. 1 B). Hence, no gross abnormalities in B lymphopoiesis result as a consequence of the absence of p59^{fyn}.

Development of the B1 B cell lineage in the absence of p59^{fynT} was also examined. B1 B cells, which represent the products of a distinct developmental lineage (38), are large granular cells that express high levels of surface IgM, low-to-moderate levels of IgD and B220 and, in most cases, CD5 (38). Although B1 B cells constitute only a small proportion of total splenocytes, such B cells represent the predominant lymphoid population in the peritoneal compartment (38). The analysis of large granular lymphocytes present in the peritoneal cavity of wild-type mice revealed the presence of two populations of IgM^{bright} cells, corresponding to B1a (CD5$^+$ IgM$^+$) and B1b (CD5$^-$ IgM$^+$) cells. Both populations were equally represented in wild-type mice and in mice carrying the fyn^{Tnull} mutation (Fig. 1 C).

Signaling through the B Cell Antigen Receptor Proceeds Normally in fyn^{Tnull} Animals. In the absence of p59^{fynT}, T lymphocyte development proceeds normally (33, 39). However, thymocytes from fyn^{Tnull} mice exhibit a substantially compromised response to stimulation through the TCR (33). We therefore evaluated B lymphocyte function in fyn^{Tnull} mice in an attempt to identify an analogous signaling defect within the B cell lineage.

The effects of antigenic stimulation on B lymphocytes can be mimicked by treatment in vitro with antibodies to surface IgM or IgD. B cell antigen receptor cross-linking promotes a number of readily measured physiologic events, including the proliferative expansion of mature B cells. This approach was used to assess the integrity of antigen receptor–mediated signaling pathways in the fyn^{Tnull} B cells. Fig. 2 illustrates the proliferative responses of splenic B cells from 12 fyn^{Tnull} animals stimulated with goat anti–mouse IgM at 50 µg/ml, represented as a percentage of the response of control animals analyzed simultaneously. The proliferative response is somewhat heterogeneous, reflecting biological variation in the animals used in these studies. However, fyn^{Tnull} B cells were clearly capable of sustaining a robust response to stimulation through surface immunoglobulin. Titration of the stimulatory dose from 50–6.25 µg/ml did not perturb this response (Fig. 2 and data not shown). Thus, p59^{fynT} is not an obligate component of the B cell receptor signaling complex. A consistent observation, but one that is difficult to resolve

satisfactorily in light of the healthy response to surface Ig stimulation, is that unstimulated splenic B cells from fyn^{Tnull} mice generally exhibited a lower basal level of proliferation as compared to control cells (see below).

Splenic B cells can also be induced to proliferate after stimulation with the nonspecific mitogen, LPS. The mechanism of LPS activation has not been fully deciphered; however, LPS stimulation does not appear to elicit the activation of src family tyrosine kinases (2, 3), leading to the suggestion that its mitogenic effect is independent of the B cell antigen receptor. fyn^{Tnull} splenocytes retain the potential to respond to LPS and also to other B cell mitogens that bypass the antigen receptor, notably ionomycin and anti-CD72 antibodies (data not shown; references 40, 41).

Early Biochemical Events after Antigen Stimulation. Although antigen receptor–induced mitogenesis appeared normal in the fyn^{Tnull} background, there remained the potential for biochemical aberrations. Engagement of the antigen receptor promotes two early and immediate events: the phosphorylation of cellular proteins on tyrosine, as well as the rapid and transient mobilization of intracellular calcium stores leading to elevation of intracellular free calcium levels. Both of these responses proceed in a normal manner in the absence of p59^{fyn} (Fig. 3 and data not shown). In particular, the stimulation of splenocytes from wild-type and fyn^{Tnull} animals with antibodies to surface IgM or IgD elicited a rapid dose-dependent mobilization of intracellular calcium stores, with the responses in wild-type and fyn^{Tnull} animals virtually identical over a range of stimulatory doses (Fig. 3 and data not shown). Thus, by the criteria evaluated here, signal transduction from the B cell antigen receptor exhibits no obligate requirement for p59^{fynT}.

Antibody Responses in fyn^{Tnull} Animals. To evaluate the immunological competence of fyn^{Tnull} animals more thoroughly, their ability to respond to immunization with both thymic-dependent and -independent antigens was assessed.

Levels of serum IgM, IgG1, and IgG3 present in nonimmunized fyn^{Tnull} mice were comparable to those present in age-matched control animals (data not shown). fyn^{Tnull} mice thus appeared capable of maintaining a normal antibody response to routinely encountered environmental agents. Moreover, although there was some heterogeneity among the responses of individual animals, fyn^{Tnull} mice proved capable of mounting a satisfactory antibody response to immunization with the thymic-dependent antigen TNP-KLH (Fig. 4 A). These normal thymic-dependent responses are consistent with our previous observation that peripheral T cell (as opposed to thymocyte) function is not severely compromised in fyn^{Tnull} mice (33).

fyn^{Tnull} mice immunized with the thymic-independent antigen TNP-Ficoll mount an IgM response that is indistinguishable from that of control animals. TNP-Ficoll immunization, however, elicits substantially less robust IgG3 responses in fyn^{Tnull} mice compared with control animals (Fig. 4 B). An inability to respond to immunization with thymic-independent antigens has previously been described in the
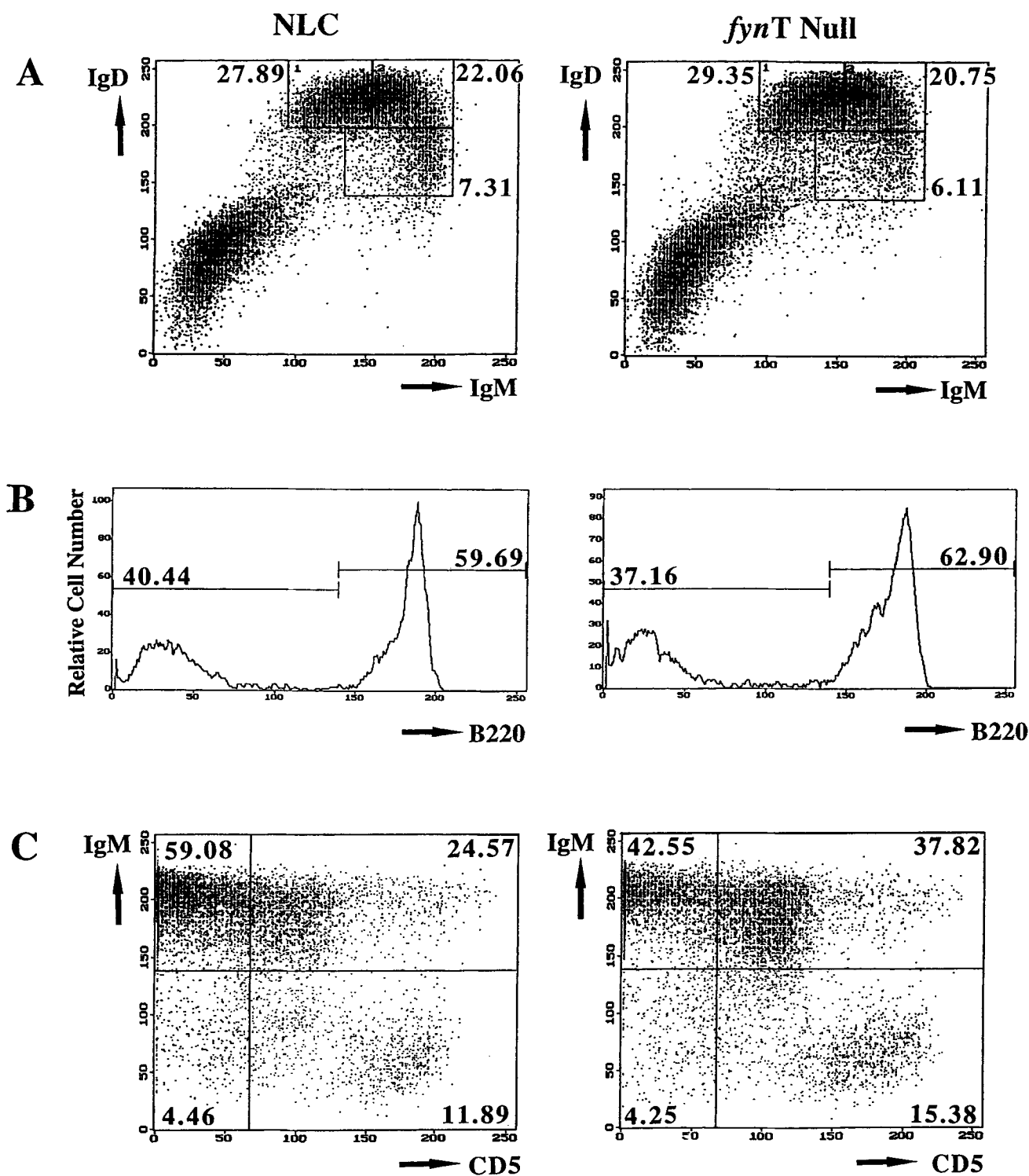
Figure 1. Flow cytometric analysis of lymphocytes from *fyn*[Tnull] mice and control animals. (*A* and *B*). Splenocytes from 8-wk-old animals were stained for the expression of the cell surface molecules IgM and IgD (*A*) or B220 (*B*) as described in Materials and Methods. 20,000 events were collected in list mode files on a FACScan® flow cytometer and analyzed using Lysys II software. The percentage of events contained in individual populations is indicated. (*C*) Peritoneal cells were harvested as described in Materials and Methods, and were stained as described above. The plot shown was generated by gating on large granular lymphocytes present in the peritoneal cavity.
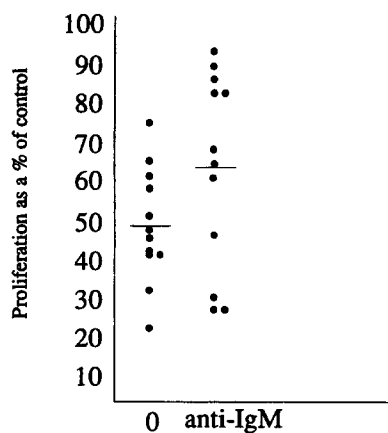
Figure 2. Mitogenic responses of *fyn*^Tbull splenic B cells. B cells purified by complement lysis were stimulated with antibody to surface IgM as described in Materials and Methods. The proliferative responses of individual animals are presented, with proliferation expressed as a percentage of the response of control animals analyzed simultaneously. The figure summarizes data obtained from six separate experiments.

CBA/N mouse strain, and is characteristic of mice bearing a mutation (XID) in the Btk PTK (12, 13, 42). Fig. 4 *B* illustrates that the XID mutation has a far more profound effect upon the IgG3 response than that observed as a consequence of the *fynT* mutation, and in addition compromises the IgM

response and reduces the levels of IgM and IgG3 in preimmune serum (Fig. 4 *B* and references 42, 43).

Impaired Cytokine Responses in fyn^Tbull *Animals.* The similarity of the B cell defect seen after immunization of *fyn*^Tbull animals with TNP-Ficoll to that observed in XID mice prompted an examination of other B cell responses known to be compromised by the XID mutation. For example, XID B cells respond poorly to the costimulatory effects of IL-5 (44). To assess the integrity of the IL-5 receptor signaling pathway, splenic B cells were cultured in vitro and stimulated with IL-5 plus CD40 ligand trimer, or IL-5 plus dextran sulfate, two mitogen combinations that support B cell growth and the generation of antibody-secreting cells. Proliferative responses were assessed after 48 h in culture (45).

Both dextran sulfate and CD40 ligand alone elicit very modest proliferative responses when supplied to B lymphocytes (Fig. 5, *A* and *B*). As previously reported, costimulation with IL-5 delivers a potent, dose-dependent, comitogenic signal. However, no IL-5–induced enhancement of B cell proliferation occurs in *fyn*^Tbull splenocytes, which mount little response over that seen after treatment with dextran sulfate or CD40 ligand trimer alone (Fig. 5, *A* and *B*).

The IL-5 receptor shares a common subunit (β_c) with two other cytokine receptors, the GM-CSF receptor and, in humans, the IL-3 receptor (reviewed in reference 46). The signaling potential of all three receptors is believed to reside in part in this β_c chain, as shown by the detrimental conse-
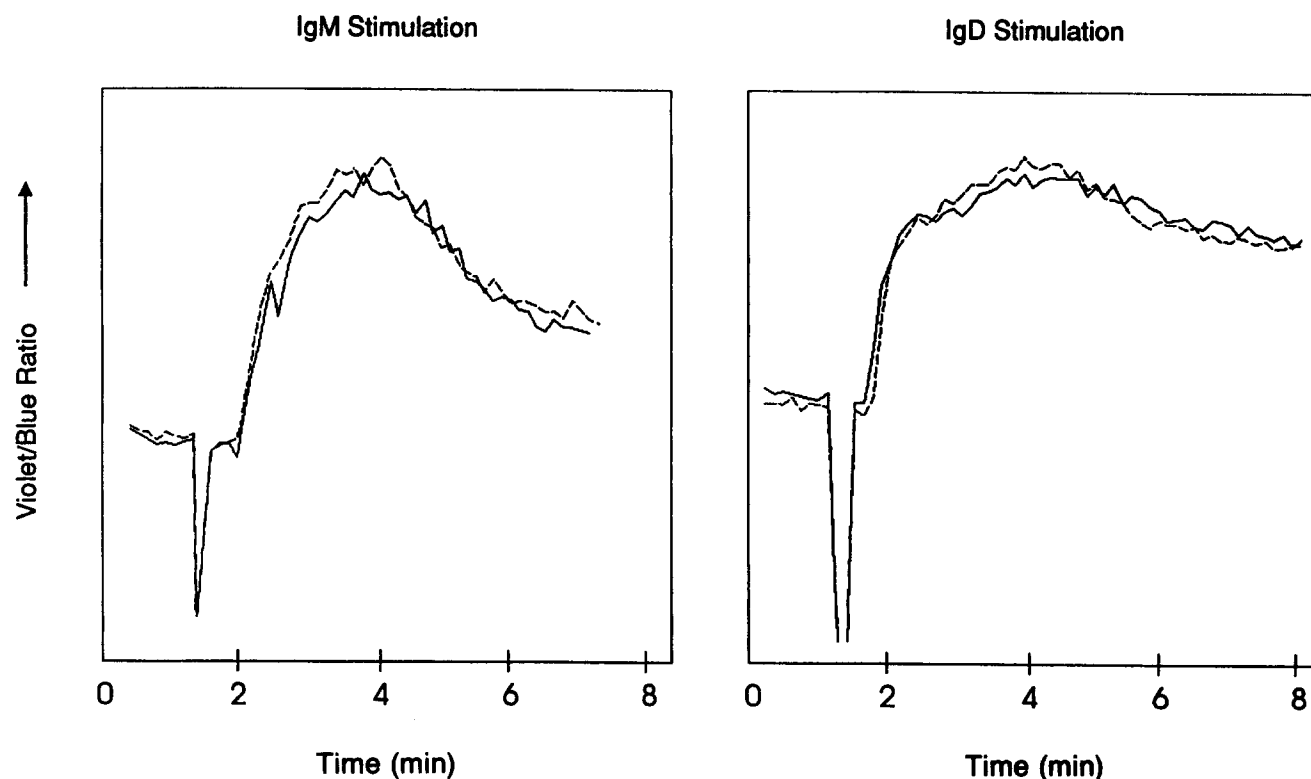
IgM Stimulation

IgD Stimulation

Figure 3. Ca^{2+} accumulation in *fyn*^Tbull splenocytes. Intracellular concentrations of Ca^{2+} were determined by flow cytometry of Indo1-labeled cells after stimulation with anti-IgM at 80 µg/ml or anti-IgD at 100 µg/ml. Calcium responses of B220 surface-stained populations from control (*solid line*) and *fyn*^Tbull animals (*dashed line*) are compared.
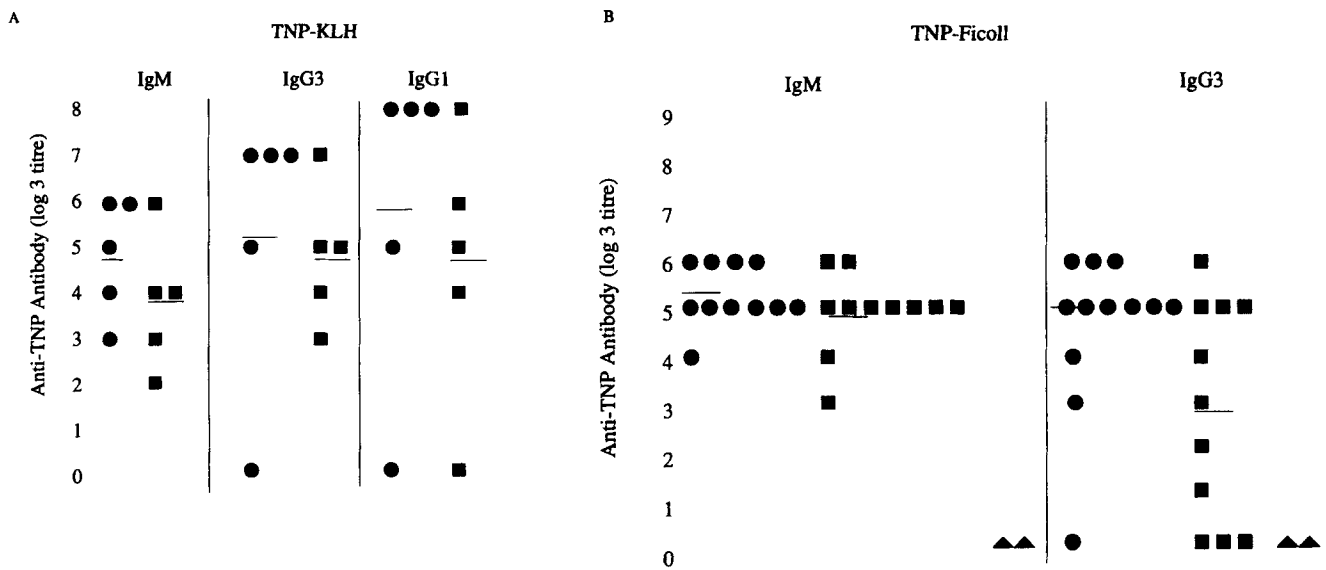
Figure 4. Antibody responses in *fyn*^Tnull mice. Control and *fyn*^Tnull mice were immunized with the thymic-dependent antigen TNP-KLH or the type 2 thymic-independent antigen TNP-Ficoll, and sera were collected after 7 d. The figure illustrates the serum titers of TNP-Ficoll–specific IgM and IgG3 and TNP-KLH–specific IgM, IgG1, and IgG3. Each symbol represents the response of an individual control (*circle*), *fyn*^Tnull (*square*), or CBA/N (*B, triangle*) animal. Horizontal bars represent mean responses. The mean IgG3 log₃ control response (4.58) differs significantly from that of *fyn*^Tnull mice (2.36; P <0.001 by Student's t test).

quences of deletion of the β_c chain in factor-dependent cell lines expressing mutant receptors (47, 48). A defect in IL-5 signaling in the *fyn*^Tnull background suggested that *fyn*^Tnull animals might exhibit analogous aberrations in GM-CSF signaling. To examine this hypothesis, the proliferative responses of bone marrow cells after stimulation with GM-CSF were assessed. Wild-type and *fyn*^Tnull bone marrow cells exhibited similar proliferative responses over a range of stimulatory doses of GM-CSF and IL-3 (data not shown). We conclude that the absence of p59^fyn in B cells leads to a selective and essentially absolute defect in IL-5 signaling.

IL-5–induced Activation of p77^btk. The observation that mutations in *fyn* and *btk* both compromise the response to IL-5, coupled with a previous report that these molecules associate in the yeast two-hybrid system (50) prompted us to seek biochemical evidence for an interaction between p59^fyn and p77^btk in B cells.

Since the in vitro kinase activity of p77^btk has been reported to increase after stimulation of B cell lines with IL-5 (51), we asked whether this activation requires p59^fyn expression. Splenic B cells were prepared by complement lysis of T cells and stimulated to blast by culture in LPS, a process that augments surface expression of the IL-5 receptor (52). These B cell blasts were stimulated with recombinant murine IL-5 and lysed as described. Immune complex kinase assays were performed, and autophosphorylation of p77^btk was quantitated using a phosphoimager. Fig. 6 *A* documents an IL-5–inducible increase in kinase activity in p77^btk immune complexes from both control and *fyn*^Tnull animals. Hence, the activation of p77^btk by IL-5 receptor ligation occurs independently of p59^fyn. Such an observation suggests that if p77^btk and p59^fyn contribute to a single pathway emanating from

the IL-5 receptor, then their relationship must be such that p77^btk is the IL-5 receptor–proximal element.

To document a physical association between p77^btk and p59^fyn, analogous to that reported in the yeast two-hybrid system (50), immune complexes of p59^fyn were prepared from lysates of splenic B cells and a kinase reaction was performed as described above. The components of the Fyn immune complex were subsequently solubilized in SDS, subjected to a second round of immunoprecipitation using p59^fyn- or p77^btk-specific antibodies and then resolved by PAGE. Using this protocol, we were unable to detect phosphorylated p77^btk among the components of p59^fyn immune complexes prepared from lysates of splenic B cells solubilized with either Brij96 (Fig. 6 *B*, lane 4), Triton X-100, or CHAPS (data not shown). In control experiments, p59^fyn was readily reprecipitable in the SDS-solubilized immunecomplex (Fig. 6 *B*, lane 1), suggesting that the failure to detect p77^btk was not a consequence of the solubilization regime.

Although we were unable to detect the presence of phosphorylated p77^btk in p59^fyn immune complexes from resting splenic B cells, it seemed possible that such an interaction might take place given the appropriate physiological stimulation. Hence, Triton X-100, CHAPS, or Brij 96 lysates were prepared from splenic B cells stimulated with IL-5 and subjected to immune complex analysis. Once again, however, we were unable to detect p77^btk in these Fyn immune complexes (Fig. 6 *B*, lanes 5 and 6).

Discussion

Preliminary biochemical evidence has implicated p59^fyn in signal transduction from both the B cell receptor and the
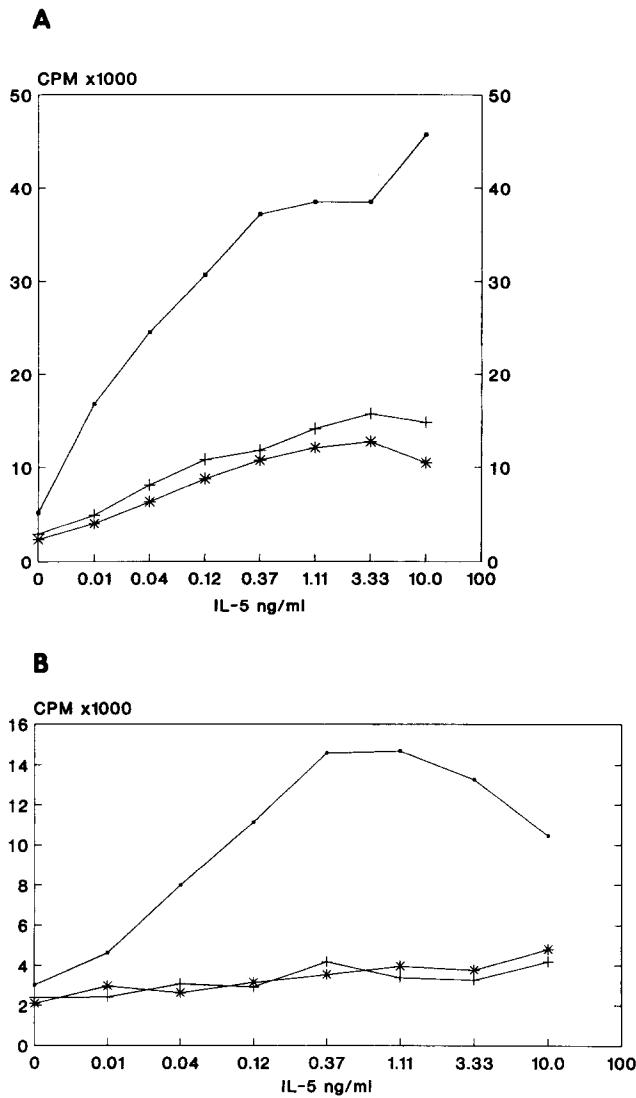
A

CPM x1000

B

CPM x1000

Figure 5. Impaired cytokine responses in *fyn*^Tnull mice. Splenic B cells were prepared from control or *fyn*^Tnull animals and stimulated with IL-5 and dextran sulfate (*A*) or CD40 ligand trimer (*B*). Proliferation was assessed after 48 h by the incorporation of ³[H]thymidine and is presented as counts per minute. Assays were performed in triplicate; the experiment shown is representative of two (*B*) or five (*A*) experiments. —⊞—, +/+; —+—, —/—; —*—, —/—.

T cell receptor: p59^fyn associates with both TCRs and BCRs, and p59^fyn activation is observed in both populations of cells after receptor engagement. However, the consequences of the targeted inactivation of p59^fynT differ radically in these two populations. CD4⁺8⁻ or CD4⁻8⁺ thymocytes that lack p59*fyn* are acutely refractile to TCR stimulation. In contrast, splenic B cells carrying an identical mutation mount a response to receptor stimulation that is difficult to distinguish from that of normal animals. Hence, p59^fyn is not an obligate participant in the BCR signal transduction pathway. Whether other kinases routinely subserve this function or simply act to replace p59^fyn in *fyn*^Tnull B cells cannot be ascertained unambiguously. Indeed, identification of the major kinases responsible for BCR signal transduction may require
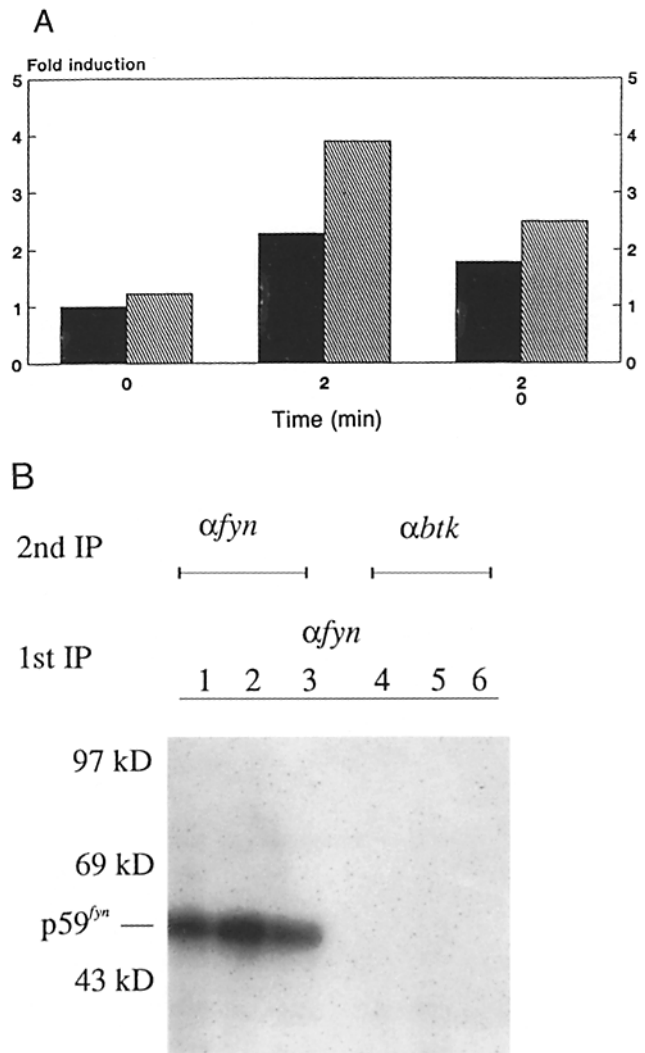
A

B

Figure 6. (*A*) Induction of p77^btk kinase activity by IL-5 B cell blasts cultured in IL-5 for 48 h were stimulated with rIL-5 at 100 ng/ml for the indicated periods of time, lysed, and p77^btk immunoprecipitated. The products of an in vitro kinase reaction were resolved by PAGE and the incorporation of ³²P(γ)ATP was evaluated by phosphoimager analysis. ■, +/+; ▩, —/—. (*B*) p77^btk is not detectable in p59^fyn immune complexes. B cell blasts from control animals were stimulated with rIL-5 at 140 ng/ml and lysed in 1% Brij 96. p59^fyn immune complexes were subjected to an in vitro kinase reaction and subsequently solubilized in SDS before reimmunoprecipitation with Fyn- or Btk-specific antiserum. Phosphorylated substrates were resolved by PAGE. Lanes *1* and *4*, unstimulated; lanes *2* and *5*, stimulation with IL-5 for 1 min; lanes *3* and *6*, stimulation with IL-5 for 5 min.

the targeted inactivation of the genes encoding all potential participants. Notwithstanding the seemingly normal responses of *fyn*^Tnull B cells to anti-IgM stimulation, these cells manifest a readily demonstrable defect in IL-5 responses in vitro, and in antigen responses in vivo.

The IL-5 receptor consists at minimum of α and β chains, the latter of which is shared with the receptors for GM-CSF and (in humans) IL-3 (46). Involvement of the Btk kinase in IL-5 signaling seems certain: Btk activity increases after IL-5 stimulation, and B cells from XID mice fail to respond

to IL-5 costimulation. Our studies demonstrate that p59^{fyn} also participates in this signal transduction process. The activation of Btk that occurs after IL-5 stimulation suggests that the failure to mount a mitogenic response to IL-5 is not a consequence of an intrinsic defect in the IL-5 receptor. Rather, the linkage of Btk activation to subsequent proliferative events appears to require p59^{fyn}.

Although bone marrow cell proliferative responses to GM-CSF (and IL-3) remain normal in *fyn*^{Tnull} mice, we cannot conclude that p59^{fyn} does not interact with the β_c chain. Levels of p59^{fyn} are exceedingly low in normal bone marrow cells (data not shown), and those cells that proliferate in response to GM-CSF may not actually express this protein. Hence, the normal response to GM-CSF and IL-3 does not imply that p59^{fyn} in B cells interacts with the unique α chain of the IL-5 receptor. Direct identification of a physical association of p59^{fyn} with the IL-5 receptor, if such exists, will require biochemical studies in cells engineered to express high levels of the receptor; IL-5 receptor levels are barely detectable on normal responding cells.

What, then, can be said of IL-5 receptor signaling in light of our results? First, two distinct PTKs, p77^{btk} and p59^{fyn}, are both required for satisfactory costimulation of anti-IgM–induced proliferation by IL-5 in normal B cells. Since a physical interaction between these two very different kinases has been detected using a yeast two-hybrid system (50), p59^{fyn} and p77^{btk} may regulate one another. However, the interaction between these two kinases, assessed either through coimmunoprecipitation or by an alteration in kinase activity, could not be demonstrated in normal B cells. Hence, we can only say that these two kinases act in concert. Obviously, if IL-5 receptor stimulation in fact initiates a true kinase cascade, p77^{btk} must regulate p59^{fyn} in the signaling pathway, and not vice-versa. Furthermore, since *fyn*^{Tnull} mice manifest only some of the immune aberrations seen in xid mice, Btk must act on targets other than p59^{fyn}. Together these observations suggest that Fyn and Btk both interact with the IL-5 receptor, but contribute to related parallel pathways.

Precedent exists for the simultaneous involvement of multiple nonreceptor PTKs in cytokine receptor signaling. In the IL-2 receptor, for example, interaction of the γ_c receptor subunit with the Jak3 kinase, and of the β receptor subunit with Jak1, Lck, and Syk (through different mechanisms in each case), provides for engagement of seemingly independent signaling pathways involving the transcription factors Myc and Fos/Jun (30, 53–59), and of the Bcl-2 survival factor (60). Thus, four different PTKs act together to induce changes in the proliferative and differentiative character of responder cells.

Our results and those of other groups suggest that the IL-5 receptor will couple in an analogous fashion to multiple parallel kinase pathways. The Jak2 kinase is activated as a consequence of IL-3, IL-5, and GM-CSF receptor engagement, and it subsequently becomes associated with the membrane proximal region of β_c, a region previously reported to be required for activation of c-*myc*, *pim*-1, and the execution of the mitogenic response (28, 61). A second domain within β_c is required for the activation of cytoplasmic tyrosine kinases and of the MAP kinase signaling cascade (47, 48, 62). In addition, a common motif has been defined in the unique α chains of the GM-CSF, IL-3, and IL-5 receptors, which is required for signal transduction in factor-dependent cell lines. Phosphorylation of Jak2 after GM-CSF stimulation also requires an activity associated with the unique α chain (62, 63). Hence, three distinct nonreceptor PTKs, Jak2, Btk, and Fyn, almost certainly act to convey the IL-5 signal. As a corollary, we suspect that the low basal proliferation characteristics of *fyn*^{Tnull} B cells (Fig. 2) results from an inability to respond to the cytokine mixture present in normal culture media. Thus, other as yet undefined cytokine receptor signaling pathways may act via p59^{fyn}.

Our studies demonstrate that p59^{fyn} expression is required for satisfactory IL-5–induced signaling in mouse B lymphocytes. A similar defect in humans could underlie rare cases of B cell immunodeficiency.

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