Involvement of p59fynT in Interleukin-5 Receptor Signaling

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Summary

Previous studies implicate the nonreceptor protein tyrosine kinase (PTK) p59fyn 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 p59fyn. Remarkably, antigen receptor signaling was only modestly defective in fyn-/- B cells. In contrast, signaling from the interleukin (IL)-5 receptor, which ordinarily provides a comitogenic stimulus with anti-immunoglobulin, was completely blocked. Our results document the importance of p59fyn 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 αβ 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-src is able to associate with the antigen receptor complex and becomes tyrosine phosphorylated after anti-Ig antibody stimulation of B cells (4, 10, 11). p70hck, 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 p70hck protein (14, 15).

Several members of the src family of protein tyrosine kinases, particularly p53/56hck, p59fyn, p56lck, and p56fyn 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 p59fyn 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 p59fyn via its SH2 domain, and results in an increase in kinase activity (21-23). p59fyn 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-
participate 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 p56lck and the β chain of the IL-2 receptor (30), and between p59fyn 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 (fynT), by targeted disruption (33). These fynTnull animals develop normally and are indistinguishable at a gross level from wild-type siblings. However they exhibit an essentially specific defect: mature thymocytes from fynTnull mice are acutely refractory 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 p59fyn. However, the absence of p59fyn in the B cell lineage is not without consequence: fynTnull mice show impaired B cell responses after immunization, and these fynTnull 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. fynTnull mice were generated as previously described and were maintained by brother × sister matings. Homozygous wild-type animals derived from the intercross of fynTnull 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). Percoll gradients were harvested by lysis 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-IgM (Southern Biotechnology Associates, Birmingham, AL), biotinylated anti-IgD (Becton Dickinson & Co., Mountain View, CA), and 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 fynTnull 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 ‧ 106 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 15 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 fynTnull 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 Laboratories) and stimulated with goat anti–mouse IgM (Fab′)2 (Organon Teknika) or sheep antimouse IgD (Binding Site Inc.) at the indicated doses. Data were analyzed using MTIME software as described previously (35).

Responses of fynTnull 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 p59fyn and p77bk. 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 LPS 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-cholamidopropyl]-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. p77bk was immunoprecipitated from one of these as previously described (14) and p59fyn immunoprecipitated from the second aliquot as a control. Phosphorylated substrates were resolved by PAGE.

Results

B Cell Development Proceeds Normally in the Absence of p59fyn. 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 p59fyn (31, 32) suggested that B cell development in fynTnull mice might be compromised. Using carefully prepared populations,
we found that p$^{59\text{fr}}$ is the Fyn isoform expressed in B cells and noted that $fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ animals. Spleens from $fyn^{\text{null}}$ 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 $p^{59\text{fr}}$.

Development of the B1 B cell lineage in the absence of $p^{59\text{fr}}$ was also examined. B1 B cells, which represent 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 Ig$^{\text{Mhigh}}$ 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 $fyn^{\text{null}}$ mutation (Fig. 1 C).

Signaling through the B Cell Antigen Receptor Proceeds Normally in $fyn^{\text{null}}$ Animals. In the absence of $p^{59\text{fr}}$, T lymphocyte development proceeds normally (33, 39). However, thymocytes from $fyn^{\text{null}}$ mice exhibit a substantially compromised response to stimulation through the TCR (33). We therefore evaluated B lymphocyte function in $fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ B cells. Fig. 2 illustrates the proliferative responses of splenic B cells from 12 $fyn^{\text{null}}$ animals stimulated with goat anti-mouse IgM at 50 $\mu$g/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, $fyn^{\text{null}}$ B cells were clearly capable of sustaining a robust response to stimulation through surface immunoglobulin. Titration of the stimulatory dose from 50–6.25 $\mu$g/ml did not perturb this response (Fig. 2 and data not shown). Thus, $p^{59\text{fr}}$ 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 $fyn^{\text{null}}$ 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. $fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ 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 $p^{59\text{fr}}$ (Fig. 3 and data not shown). In particular, the stimulation of splenocytes from wild-type and $fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ 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 $p^{59\text{fr}}$.

**Antibody Responses in $fyn^{\text{null}}$ Animals.** To evaluate the immunological competence of $fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ mice were comparable to those present in age-matched control animals (data not shown). $fyn^{\text{null}}$ 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, $fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ mice (33).

$fyn^{\text{null}}$ 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 $fyn^{\text{null}}$ 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 \textit{fyn}^\text{null} 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.
Mitogenic responses of fyn<sup>−/−</sup> 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 fyn<sup>−/−</sup> 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<sup>−/−</sup> Animals. The similarity of the B cell defect seen after immunization of fyn<sup>−/−</sup> 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<sup>−/−</sup> 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<sub>c</sub>) 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<sub>c</sub> chain, as shown by the detrimental conse-
Figure 4. Antibody responses in fynTnuH mice. Control and fynTnuL 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), fynTnuH (square), or CBA/N (triangle) animal. Horizontal bars represent mean responses. The mean IgG3 log3 control response (4.58) differs significantly from that of fynTnuH mice (2.36; P < 0.001 by Student’s t test).

Table 1: Antibody Responses to TNP-KLH and TNP-Ficoll

| Response | TNP-KLH | TNP-Ficoll |
|----------|---------|------------|
| IgM      | 8       | 6          |
| IgG3     | 7       | 5          |
| IgG1     | 4       | 3          |

Discussion

Preliminary biochemical evidence has implicated p59fyn in signal transduction from both the B cell receptor and the IL-5 receptor, then their relationship must be such that p77btk is the IL-5 receptor-proximal element.

To document a physical association between p77btk and p59fyn, analogous to that reported in the yeast two-hybrid system (50), immune complexes of p59fyn 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 p59fyn- or p77btk-specific antibodies and then resolved by PAGE. Using this protocol, we were unable to detect phosphorylated p77btk among the components of p59fyn immune complexes prepared from lysates of splenic B cells solubilized with either Brij 96 (Fig. 6 B, lane 4), Triton X-100, or CHAPS (data not shown). In control experiments, p59fyn was readily reprecipitable in the SDS-solubilized immune complex (Fig. 6 B, lane 1), suggesting that the failure to detect p77btk was not a consequence of the solubilization regime.

Although we were unable to detect the presence of phosphorylated p77btk in p59fyn 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 p77btk in these Fyn immune complexes (Fig. 6 B, lanes 5 and 6).

Discussion

Preliminary biochemical evidence has implicated p59fyn in signal transduction from both the B cell receptor and the
Figure 5. Impaired cytokine responses in fyn<sup>−/−</sup> mice. Splenic B cells were prepared from control or fyn<sup>−/−</sup> 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 [3H]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<sup>fr</sup> associates with both TCRs and BCRs, and p59<sup>fr</sup> activation is observed in both populations of cells after receptor engagement. However, the consequences of the targeted inactivation of p59<sup>fr</sup> differ radically in these two populations. CD4<sup>+</sup> or CD4<sup>+</sup> thymocytes that lack p59<sup>fyn</sup> 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<sup>fr</sup> is not an obligate participant in the BCR signal transduction pathway. Whether other kinases routinely subserve this function or simply act to replace p59<sup>fr</sup> in fyn<sup>−/−</sup> B cells cannot be ascertained unambiguously. Indeed, identification of the major kinases responsible for BCR signal transduction may require the targeted inactivation of the genes encoding all potential participants. Notwithstanding the seemingly normal responses of fyn<sup>−/−</sup> 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 of 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 p59fr 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 p59fr.

Although bone marrow cell proliferative responses to GM-CSF (and IL-3) remain normal in fyn−/−null mice, we cannot conclude that p59fr does not interact with the β chain. Levels of p59fr 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 p59fr in B cells interacts with the unique α chain of the IL-5 receptor. Direct identification of a physical association of p59fr 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, p77Btk and p59fr, 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), p59fr and p77Btk may regulate one another. However, the interaction between these two kinases, assessed either through coimmunoprecipitation or by an alteration in kinase activity, could contribute to related parallel pathways.

Hence, three distinct nonreceptor PTKs, Jak2, Btk, and Fyn, almost certainly act to convey the IL-5 signal. As a consequence of IL-5, Fyn and Btk both interact with the IL-5 receptor, for example, interaction of the γc 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 Fox/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 βc, 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 βc 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−/− null 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 p59fr.

Our studies demonstrate that p59fr 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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References

1. Gold, M.R., D.A. Law, and A.L. DeFranco. 1990. Stimulation of protein tyrosine phosphorylation by the B lymphocyte antigen receptor. Nature (Lond.). 345:810–813.

2. Campbell, M.A., and B.M. Sefton. 1990. Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. EMBO (Eur. Mol. Biol. Organ.) J. 9:2125–2131.

3. Brunswick, M., L.E. Samelson, and J.J. Mond. 1991. Surface
immunoglobulin crosslinking activates a tyrosine kinase pathway in B cells that is independent of protein kinase C. Proc. Natl. Acad. Sci. USA. 88:1311–1314.

4. Hutchcroft, J.E., M.L. Harrison, and R.L. Geahlen. 1991. B lymphocyte activation is accompanied by phosphorylation of a 72 kDa protein-tyrosine kinase. J. Biol. Chem. 266:14846–14849.

5. Justement, L.B., K.S. Campbell, N.C. Chien, and J.C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. Science (Wash. DC). 252:1839–1842.

6. Cambier, J.C., D.C. Morrison, M.M. Chien, and K.R. Lehnmann. 1991. Modeling of T cell contact-dependent B cell activation. IL-4 and antigen receptor ligation primes quiescent B cells to mobilize calcium in response to Igα cross-linking. J. Immunol. 146:2075–2082.

7. Lane, P.J.L., J.A. Ledbetter, F.M. McConnell, K. Draves, J. Deans, G. Schieven, and E.A. Clark. 1991. The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. J. Immunol. 146:715–722.

8. Mizuguchi, J., Y. Yamanashi, K. Ehara, T. Tamura, H. Narimatsu, Y. Goyokawa, H. Ikazawa, Y. Uehara, and T. Yamamoto. 1992. Tyrosine protein kinase is involved in anti-IgM-mediated signaling in BAL17 B lymphoma. J. Immunol. 148:689–694.

9. Cambier, J.C., C.M. Pleiman, and M.R. Clark. 1994. Signal transduction by the B cell antigen receptor and its coreceptors. Annu. Rev. Immunol. 12:457–486.

10. Taniguchi, T., T. Kobayashi, J. Kondo, K. Takahashi, H. Nakamura, J. Suzuki, R. Nagai, T. Yamada, S. Nakamura, and H. Yamamura. 1991. Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. J. Biol. Chem. 266:15790–15796.

11. Hutchcroft, J.E., M.L. Harrison, and R.L. Geahlen. 1992. Association of the 72-kDa protein-tyrosine kinase FTK72 with the B cell antigen receptor. J. Biol. Chem. 267:8613–8619.

12. Thomas, J.E., P. Sideras, C.I.E. Smith, I. Vorechovsky, V. Chapman, and W.E. Paul. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science (Wash. DC). 261:355–358.

13. Rawlings, D.J., D.C. Safran, S. Tsukada, D.A. Largaespada, J.C. Grimald, L. Cohen, R.N. Mohr, J.F. Bazan, M. Howard, N.G. Copeland et al. 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science (Wash. DC). 261:358–361.

14. Tsukada, S., D.C. Safran, D.J. Rawlings, O. Parolini, R.C. Allen, I. Kliukan, R.S. Sparkes, H. Kubagawa, T. Mohandas, S. Quan et al. 1993. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell. 72:279–290.

15. Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarström, C. Kinnon, R. Levin, M. Bobrow et al. 1993. The gene involved in X-linked agammaglobulinemia is a member of the src family of protein-tyrosine kinases. Nature (London). 361:226–233.

16. Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima. 1991. Association of B cell antigen receptor with protein tyrosine kinase lyn. Science (Wash. DC). 251:192–194.

17. Campbell, M., and B.M. Sefton. 1992. Association between B-lymphocyte membrane immunoglobulin and multiple members of the src family of protein tyrosine kinases. Mol. Cell. Biol. 12:2315–2321.

18. Burkhardt, A.L., M. Brunswick, J.B. Bolen, and J.J. Mond. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. Proc. Natl. Acad. Sci. USA. 88:7410–7414.

19. Saouaf, S.J., S. Mahajan, R.B. Rowley, S.A. Kut, J. Fargnoli, A.L. Burkhardt, T. Tsukada, O.N. Witte, and J.B. Bolen. 1994. Temporal differences in the activation of three classes of nontransmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. Proc. Natl. Acad. Sci. USA. 91:9524–9528.

20. Lin, J., and L.B. Justement. 1992. The MB-1/B29 heterodimer couples the B cell antigen receptor to multiple SRC family protein tyrosine kinases. J. Immunol. 149:1548–1555.

21. Clark, M.R., S.A. Johnson, and J.C. Cambier. 1994. Analysis of Igα-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-α stimulation of Fyn activity. EMBO (Eur. Mol. Biol. Organ.) J. 13:1911–1919.

22. Clark, M.R., K.S. Campbell, A. Kazlauskas, S.A. Johnson, M. Hertz, T.A. Potter, C. Pleiman, and J.C. Cambier. 1992. The B cell antigen receptor complex: association of Igα and Igβ with distinct cytoplasmic effectors. Science (Wash. DC). 258:123–126.

23. Pleiman, C.M., C. Abrams, L.T. Gauen, W. Bedzyk, J. Jongstra, A.S. Shaw, and J.C. Cambier. 1994. Distinct p53/p561y and p59fr domains associate with nonphosphorylated and phosphorylated Igα. Proc. Natl. Acad. Sci. USA. 91:4268–4272.

24. Pleiman, C.M., M.R. Clark, L.K.T. Gauen, S. Winitz, K.M. Coggeshall, G.L. Johnson, A.S. Shaw, and J.C. Cambier. 1993. Mapping of sites on the src family protein tyrosine kinases p551y, p59fr, and p561y which interact with the effector molecules phospholipase C-γ2, microtubute-associated protein kinase, GTpase-activating protein, and phosphatidylinositol 3-kinase. Mol. Cell. Biol. 13:5877–5887.

25. Peschon, J.J., P.J. Morrissey, K.I. Grinstein, F.J. Ramsdell, E. Maraskovsky, G. Linnik, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware, J.D. Meyer, and B.L. Davison. 1994. Early lymphocyte expansion is severely impaired in interleukin-7 receptor deficient mice. J. Exp. Med. 180:1955–1960.

26. Mahanty, S., and T.B. Nutman. 1993. The biology of interleukin-5 and its receptor. Cancer Invest. 11:624–634.

27. Mandler, R., C.C. Chu, W.E. Paul, E.E. Max, and C.M. Snapper. 1993. Interleukin 5 induces sa-sy1 DNA rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: a three-component model for Ig class switching. J. Exp. Med. 178:1577–1586.

28. Ihle, J.N., B.A. Wittuthun, F.W. Quelle, K. Yamamoto, W.E. Thierfelder, B. Kreider, and O. Silvennoien. 1994. Signaling by the cytokine receptor superfamilly: JAKs and STATs. Trends Biochem. 19:222–227.

29. Darnell, Jr., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science (Wash. DC). 264:1415–1421.

30. Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S.D. Levin, R.M. Perlmuter, and T. Tanaguchi. 1991. Interaction of the IL-2 receptor with the src family kinase p561k: identification of novel intermolecular association. Science (Wash. DC). 252:1523–1528.

31. Venkitaraman, A.R., and R.J. Cowling. 1992. Interleukin 7 receptor functions by recruiting the tyrosine kinase p59fr through a segment of its cytoplasmic tail. Proc. Natl. Acad. Sci. USA. 89:12083–12087.

32. Seckinger, P., and M. Fougereau. 1994. Activation of src family kinases in human pre-B cells by IL7. J. Immunol. 153:97–109.
33. Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59^Fi. Cell. 70:751–763.
34. Cooke, M.P., K.M. Abraham, K.A. Forbus, and R.M. Perlmutter. 1991. Regulation of T cell receptor signalling by a src family protein-tyrosine kinase (p59^Fi). Cell. 65:281–291.
35. Rabinovitch, P.S., C.H. June, A. Grossman, and J.A. Ledbetter. 1986. Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation: simultaneous use of indo-1 and immunofluorescence with flow cytometry. J. Immunol. 137:952–961.
36. Renshaw, B.R., W.C. Fanslow, R.J. Armitage, K.A. Campbell, D. Liggitt, B. Wright, B.L. Davison, and C.R. Maliszewski. 1994. Humoral immune responses in CD40 ligand-deficient mice. J. Exp. Med. 180:1899–1900.
37. Löffert, D., S. Schaal, A. Ehlich, R.R. Hardy, Y. Zou, W. Muller, and K. Rajewsky. 1994. Early B-cell development in mice lacking the thymic isoform of p59^Fi. J. Immunol. 153:501–538.
38. Kantor, A.B., and L.A. Herzenberg. 1993. Origin of murine B cell lineage. Annu. Rev. Immunol. 11:501–538.
39. Swan, K.A., J. Alberola-Ila, J.A. Gross, M.W. Appleby, K.A. Forbus, J.F. Thomas, and R.M. Perlmutter. 1995. Involvement of p21^w and p59^Fi in B cell activation. J. Exp. Med. 180:1183–1190.
40. Subbarao, B., and D.E. Mosier. 1983. Induction of B lymphocyte proliferation without antibody secretion by monoclonal anti-Lyb-2 antibody. J. Immunol. 130:2033–2037.
41. Scher, I. 1982. The CBA/N mouse strain: an experimental model illustrating the influence of the X-chromosome on immunity. Adv. Immunol. 33:2–64.
42. Perlmutter, R.M., M. Nahm, K.E. Stein, J. Slack, I. Zitrion, W.E. Paul, and J.M. Dave. 1979. Immunoglobulin subclass-specific immunofluorescence in mice with an X-linked B-lymphocyte defect. J. Exp. Med. 149:993–998.
43. Hitoshi, Y., E. Sonoda, Y. Kikuchi, S. Yonehara, H. Nakachi, and K. Takatsu. 1993. IL-5 receptor-positive B cells, but not eosinophils, are functionally and numerically increased in mice carrying the X-linked immune defect. Int. Immunol. 5:1183–1190.
44. Maliszewski, C.R., K. Grabstein, W.C. Fanslow, R. Armitage, M.K. Spriggs, and T.A. Sato. 1993. Recombinant CD40 ligand stimulation of murine B cell growth and differentiation: cooperative effects of cytokines. Eur. J. Immunol. 23:1044–1049.
45. Miyajima, A., A.L.F. Mui, T. Ogorochi, and K. Sakamaki. 1993. Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood. 82:1960–1974.
46. Sato, N., K. Sakamaki, N. Terada, K. Arai, and A. Miyajima. 1993. Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common beta subunit responsible for different signaling. EMBO (Eur. Mol. Biol. Organ.) J. 12:4181–4189.
47. Sato, N., K. Sakamaki, N. Terada, K. Arai, and A. Miyajima. 1993. Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common beta subunit responsible for different signaling. EMBO (Eur. Mol. Biol. Organ.) J. 12:4181–4189.
48. Sakamaki, K., I. Miyajima, T. Kitamura, and A. Miyajima. 1992. Critical cytoplasmic domains of the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. EMBO (Eur. Mol. Biol. Organ.) J. 11:3541–3549.
49. Jacobsen, S.E.W., F.W. Ruscetti, A.B. Roberts, and J.R. Keller. 1993. TGFB is a bidirectional modulator of cytokine receptor expression on murine bone marrow cells. J. Immunol. 151:4534–4544.
50. Cheng, G., Z.S. Ye, and D. Baltimore. 1994. Binding of Bruton’s tyrosine kinase to Fyn, Lyn or Hck through a src homology 3 domain-mediated interaction. Proc. Natl. Acad. Sci. USA. 91:8152–8155.
51. Sato, T., S. Katagiri, S. Takaki, Y. Kikuchi, Y. Hitoshi, S. Yonehara, S. Tsukada, D. Kitamura, T. Watanabe, W. Witte, and K. Takatsu. 1994. IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton’s tyrosine and Janus 2 kinases. J. Exp. Med. 180:2101–2111.
52. Mita, S., N. Harada, S. Naomi, Y. Hitoshi, K. Sakamoto, M. Akagi, A. Tominaga, and K. Takatsu. 1988. Receptors for T cell–replacing factor/interleukin 5. J. Exp. Med. 168:863–878.
53. Hatakeyama, M., H. Mori, T. Doi, and T. Taniguchi. 1989. A restricted cytoplasmic region of IL-2 receptor beta chain is essential for growth signal transduction but not for ligand binding and internalization. Cell. 59:837–845.
54. Shibuya, H., M. Yoneyama, J. Ninomiya-Tsujii, K. Matsumoto, and T. Taniguchi. 1992. IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. Cell. 70:57–67.
55. Minami, Y., T. Kono, K. Yamada, A. Kawahara, R.M. Perlmutter, and T. Taniguchi. 1993. Association of p56^k^ with IL-2 receptor beta chain is critical for the IL-2-induced activation of p56^k^, EMBO (Eur. Mol. Biol. Organ.) J. 12:759–768.
56. Johnston, J.A., M. Kawamura, R.A. Kirken, Y. Chen, T.B. Blake, K. Shibuya, J.R. Ortdal, D.W. McVicar, and J.J. O’Shea. 1994. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature (Lond.). 370:151–153.
57. Witthuhn, B.A., O. Silvennoinen, O. Miura, K.S. Lai, C. Cwik, E.T. Liu, and J.N. Ihle. 1994. Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. Nature (Lond.). 370:153–157.
58. Miyazaki, T., A. Kawahara, H. Fujii, Y. Nakagawa, Y. Minami, Z. Liu, I. Oishi, O. Silvennoinen, B.A. Witthuhn, J.N. Ihle, and T. Taniguchi. 1994. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. Science (Wash. DC). 266:1045–1047.
59. Russell, S.M., J.A. Johnston, M. Noguchi, M. Kawamura, C.M. Bacon, M. Friedmann, M. Berg, D.W. McVicar, B.A. Witthuhn, O. Silvennoinen et al. 1994. Interaction of IL-2Rbeta and gamma chains with Jak1 and Jak3: implications for XSCID and XCID. Science (Wash. DC). 266:1042–1045.
60. Miyazaki, T., Z. Liu, A. Kawahara, Y. Minami, K. Yamada, Y. Tsujimoto, E.L. Barsoumian, R.M. Perlmutter, and T. Taniguchi. 1995. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc and bck cooperate in hematopoietic cell proliferation. Cell. 8:223–231.
61. Quelle, F.W., N. Sato, B.A. Witthuhn, R.C. Inhorn, M. Eder, A. Miyajima, J.D. Griffin, and J.N. Ihle. 1994. JAK2 associates with the bc chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol. Cell. Biol. 14:4335–4341.
62. Weiss, M., C. Yokoyama, Y. Shikama, C. Naugle, B. Drucker, and C.A. Sieff. 1993. Human granulocyte-macrophage colony-stimulating factor receptor signal transduction requires the proximal cytoplasmic domains of the alpha and beta subunits. Blood. 82:3298–3306.
63. Takaki, S., H. Kanazawa, M. Shiiba, and K. Takatsu. 1994. A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor a chain and its function in IL-5-mediated growth signal transduction. Mol. Cell. Biol. 14:7404–7413.