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Immunology

Tyrosine phosphorylation of Blk and Fyn Src homology 2 domain-binding proteins occurs in response to antigen-receptor ligation in B cells and constitutively in pre-B cells

(pre-B receptor/intracellular signaling/tyrosine kinase)

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ABSTRACT **Proteins that bind to discrete domains of the Blk, Fyn, Lyn, and Btk protein tyrosine kinases were examined in pre-B cells that had not been subjected to any external stimulation, as well as in nonstimulated and antigen-receptor-ligated B cells. Proteins that bind to the Src homology 2 domains of Blk and Fyn were identified in B cells that had been activated with anti-IgM but were not identified in unstimulated B cells. A number of Blk and Fyn Src homology 2 domain-binding phosphoproteins were also observed in pre-B cells that had not been stimulated *in vitro*. The phosphoproteins seen in activated B cells potentially represent substrates that play a role in the pathway of antigen-receptor-mediated signaling. Distinct signaling pathways involving distinguishable kinase substrates may be relevant in pre-B-cell-receptor-mediated cell survival during ontogeny. These results indirectly support models that predict constitutive ligand-independent signaling by the pre-antigen receptor during lymphoid ontogeny.**

During B-lymphocyte ontogeny, pre-B cells that make in-frame variable region (V) to diversity–joining region (DJ) rearrangements at the μ heavy-chain locus receive a survival signal and are able to differentiate further; however cells that make nonproductive rearrangements at this locus fail to be selected and are lost. Membrane immunoglobulin heavy chains associate in pre-B cells with the ω and ι surrogate immunoglobulin light chains (1, 2), which are the protein products of the pre-B-cell-specific $\lambda 5$ and vPreB genes (3–7). The pre-B receptor is believed to provide a feedback signal to cells that have made successful rearrangements. Because in murine bone-marrow pre-B cells the membrane form of the immunoglobulin μ heavy chain (μ_m)–surrogate light-chain complex appears primarily intracellular (7), we suggested that a signal may well be generated constitutively from this receptor in the absence of a conventional ligand (2, 7, 8). Genetic experiments in which the membrane exons of the μ heavy chain (9) or the $\lambda 5$ gene (10) were deleted by homologous recombination led to similar arrests of ontogeny at the late pre-B-cell stage, confirming the hypothesis that signal transduction through this pre-antigen receptor may play a critical role in late pre-B-cell survival. A similar selection mechanism has now been identified in the T-cell lineage, wherein cells that make an in-frame rearrangement at the T-cell-receptor β-chain locus receive a signal that permits further differentiation (11, 12). In pre-T cells the β chain of the T-cell receptor covalently associates with a 33-kDa glycoprotein that is presumed to represent the T-cell-lineage equivalent of the ω chain (13).

In B cells, surface IgM functions as the antigen receptor. Associated with membrane immunoglobulin is a disulfide-linked heterodimer made up of two glycoproteins whose cytoplasmic tails contain motifs that play a role in linking receptors to cytoplasmic Src-family tyrosine kinases (14, 15). One of these proteins is the product of the *mb-1* gene and is often referred to as Igα; the other is the product of the B29 gene and is sometimes referred to as Igβ (14–17). The accessory α/β heterodimer associated with the B-cell receptor is also associated with the pre-B receptor (8, 14). A number of Src-family kinases have been reported to be associated with the B-cell receptor. These kinases include Lyn, Fyn, Blk, and Lck (18–20). A distinct tyrosine kinase called PTK72 or Syk, which possesses two Src homology 2 (SH2) domains and which lacks a Src homology 3 (SH3) domain, has also been found associated with the antigen receptor on B lymphocytes (21), although the structural basis for this association is very poorly understood. A tyrosine kinase that is mutated in patients with X-linked agammaglobulinemia and in the X-linked immune deficiency mouse has recently been cloned and is referred to as Bruton's tyrosine kinase or Btk (refs. 22–25; previously referred to as Atk and BPK). Although genetic evidence suggests that this kinase may be functionally linked to the pre-B and B-cell receptors, no biochemical link between these receptors and Btk has as yet been established.

A few interesting and conserved domain structures are found in a large number of proteins that play an important role in signal transduction. Two such structural motifs are the SH2 and SH3 domains (26, 27). In an attempt to examine the possibility that the pre-B receptor might signal constitutively, mimicking an antigen-activated B cell, we examined tyrosine-phosphorylated proteins that bind to the SH2 and SH3 domains of the Lyn, Fyn, Blk, and Btk tyrosine kinases in unstimulated and stimulated B cells and also in pre-B cells. We detected a number of proteins that bind to the SH2 domains of Blk and Fyn upon B-cell activation via the antigen receptor but that were not detected in nonactivated B cells. A subset of SH2 domain-binding proteins were identified as constitutively tyrosine-phosphorylated molecules in pre-B cells that had not been subjected to anti-IgM treatment.

MATERIALS AND METHODS

Glutathione *S*-Transferase (GST) Fusion Constructs. GST fusion constructs were made involving the SH2 and SH3 domains of Blk (28), Fyn (T) (29), Lyn (18), and Btk (23). Fusion constructs were also made representing the N-terminal domains of Btk and Blk. First-strand cDNA was synthesized by using random hexamers as primer and RNA from a pre-B-cell line, NFS 70.15, as template. Using the primers

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Abbreviations: GST, glutathione *S*-transferase; SH2 and SH3, Src-homology 2 and 3, respectively; μ_m, membrane form of the immunoglobulin μ heavy chain.

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listed below, the appropriate SH2 and SH3 domains were amplified by using the PCR, and amplified fragments were digested with *Bam*HI and *Eco*RI and subcloned into the PGEX2T vector (30). The restriction sites in the primers that follow are underlined: Blk SH2 domain (residues 118–214), 5′ primer 5′-GCCCGGATCCTGGTTCTTCAGGACCATCAGC-3′ and reverse primer 5′-CCGCGAATTCGGGCAGAGTCAACTTCTGACA-3′; Lyn SH2 domain (residues 129–226), 5′ primer 5′-GCCCGGATCCTGGTTCTTCAAGGACATAATA-3′ and reverse primer 5′-CCGCGAATTCGCATGCCTTCTCCAGTCTTCT-3′; Fyn SH2 domain (residues 149–241), 5′ primer 5′-GCCCGGATCCTGGTACTTTGGAAAACTTGGC-3′ and reverse primer 5′-CCGCGAATTCGTTAAAACACAAACCATCAGC-3′; Btk SH2 domain (residues 281–372), 5′ primer 5′-GCCCGGATCCTGGTATTCCAAGCACATGACT-3′ and reverse primer 5′-CCGCGAATTCCACAGGATATTTCAGCCTGGA-3′; Blk SH3 domain (residues 57–107), 5′ primer 5′-GCCCGGATCCGTGGTGGCTCTGTTTGACTAT-3′ and reverse primer 5′-CCGCGAATTCAAAGTTGCTGGGCACATAACC-3′; Lyn SH3 domain (residues 70–118), 5′ primer 5′-GCCCGGATCCGCCTTATACCCTTATGATGCC-3′ and reverse primer 5′-CCGCGAATTCCACGTAGTTGCTGGGGATGAA-3′; Fyn SH3 domain (residues 89–138), 5′ primer 5′-GCCCGGATCCGCGCTTTATGACTATGAAGCA-3′ and reverse primer 3′-CCGCGAATTCCAAGTAATTGCTGGGGATGAA-3′; Btk SH3 domain (residues 219–268), 5′ primer 5′-GCCCGGATCCGTCGTGGCCCTTTATGATTAC-3′ and reverse primer 5′-CCGCGAATTCATAGTTACTTGGGATGTAGCC-3′; Btk N terminus (residues 25–173), 5′ primer 5′-GCCCGGATCCTTCAAGAAGCGCCTGTTTCTC-3′ and reverse primer 5′-CCGCGAATTCTCCATTCCTGTTCTCCAAAAT-3′; Blk N terminus (residues 1–56), 5′ primer 5′-GCCCGGATCCATGGGGCTGCTGAGCAGCAAG-3′ and reverse primer 5′-CCGCGAATTCAAAACGCTCCTCTTCATCTGG-3′.

Cell Lines. NFS 70 (31) is a pro-B cell line that has not yet rearranged its μ locus (and which is transformed by a viral nuclear oncoprotein). This line was subcloned to yield pre-B subclones (expressing μ and surrogate light chains). One such subclone, NFS 70.15, was used in our studies. WEHI 231 is a surface IgM positive "immature" B-cell line (32) in which antigen-receptor ligation results in tyrosine phosphorylation followed by programmed cell death. Receptor ligation was done with 5×10^7 cells at 37°C in 200 μl of serum-free RPMI medium, using 10 μg of rabbit anti-mouse IgM (Zymed).

Affinity Isolation and *in Vitro* Kinase Assays. GST fusion proteins were expressed in *Escherichia coli* using 0.1 mM isopropyl thiogalactoside for induction, released by sonication, and purified on glutathione-Sepharose (33). Beads were washed four times in 0.5% Nonidet P-40/20 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA. For *in vitro* kinase assays, 5×10^6 lymphocytes, lysed in 1% Nonidet P-40 (Sigma), in 50 mM Tris, pH 8.0/120 mM NaCl/2 mM phenylmethylsulfonyl fluoride, were used for each assay. In general, 5 μg of GST fusion protein was used in each assay. Beads were incubated with the lysate for 2 hr at 4°C, washed four times with the above lysis buffer and once with the kinase buffer (10 mM Hepes, pH 7.0/10 mM $MgCl_2$/2 mM Na_3VO_4), and then resuspended in 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). The reaction mixture was incubated at 30°C for 15 min, the beads were washed four times with lysis buffer, and samples were analyzed on a 9% polyacrylamide/SDS gel.

Antiphosphotyrosine Immunoblot Assays. Binding of proteins in cell lysates to GST fusion proteins was done as described above. After separation on SDS/PAGE, samples were transferred to Immobilon-P (Millipore) membranes, and proteins containing phosphotyrosine were detected by using an antiphosphotyrosine monoclonal antibody (PY 20; Zymed) and the ECL detection system (Amersham).

RESULTS

One biochemical approach to dissecting the pathways involved in signal transduction through the pre-B-cell and B-cell receptors is to identify proteins that bind to association motifs in the tyrosine kinases associated with these receptors or which are presumed to play a role in signal transduction downstream of the receptor. For the antigen receptor it is obviously of interest to detect and identify proteins that are biochemically altered by receptor ligation. The identification of phosphoproteins (presumably phosphorylated by the kinase in question) that acquire the ability to bind to the SH2 or SH3 domains (or to the unique N-terminal "domains") of Blk, Lyn, Fyn, or Btk in activated B cells was one objective of this study. For pre-B cells, particularly because the μ_m–surrogate light-chain complex may signal in a ligand-independent fashion, we were interested in examining potential SH2- and SH3-binding proteins, even in the absence of any known external stimulus.

Two approaches were used for detecting domain-specific binding proteins. (*i*) Proteins that bind to GST fusion proteins containing these domains were isolated on a glutathione-Sepharose matrix, and after a washing step, [γ-^{32}P]ATP was incubated with the beads. If a protein kinase was bound directly to the beads via a domain-specific association or indirectly via an intermediary protein, phosphorylation of potential targets could conceivably occur during this incubation step. Proteins were separated with SDS/PAGE, and phosphorylated proteins were visualized by using autoradiography. With this exposure, proteins phosphorylated on serine, threonine, or tyrosine residues can be visualized; incubation in 1 M KOH at 60°C is used to preferentially cleave serine and threonine phosphates. This method depends upon the target protein either being a protein tyrosine kinase itself or being associated directly or indirectly with one. In general, the method detects proteins that can potentially be phosphorylated on tyrosine residues *in vitro* whether or not the event has actually occurred before lysis. An obvious limitation of such a method is that only a subset of domain-specific binding proteins is likely to be available for analysis.

(*ii*) The second method involved the binding of unlabeled cellular proteins to SH2, SH3, or N-terminal domains followed by the separation of these proteins on SDS/PAGE and an immunoblot analysis with an antiphosphotyrosine monoclonal antibody. This method focuses on proteins that were tyrosine-phosphorylated *in vivo* before lysis. Apart from the fact that detection is limited to tyrosine-phosphorylated proteins, another limitation of this approach is that antiphosphotyrosine monoclonal antibodies may fail to detect a subset of tyrosine-phosphorylated proteins upon immunoblot analysis.

***In Vitro* Kinase Assays Reveal Blk-SH2-Binding Proteins in Activated B Cells and Nonstimulated Pre-B Cells.** WEHI 231 is a B-cell line characteristic of the "immature" B-cell stage. Receptor ligation leads to the activation of signal transduction that depends on the activation of tyrosine phosphorylation (34). A role for the Blk kinase in membrane immunoglobulin-mediated signaling at this stage of differentiation has been suggested by antisense experiments (35). The choice of an appropriate pre-B-cell line for studying signal transduction has presented difficulties. Most available pre-B-cell lines are transformed by viral protein-tyrosine kinases, and examining tyrosine-phosphorylated proteins in these lines is difficult because of a relatively high background. Two commonly used non-Abelson-derived "pre-B" cell lines, NFS 5.3 and 70Z/3, appear to have been derived from more mature

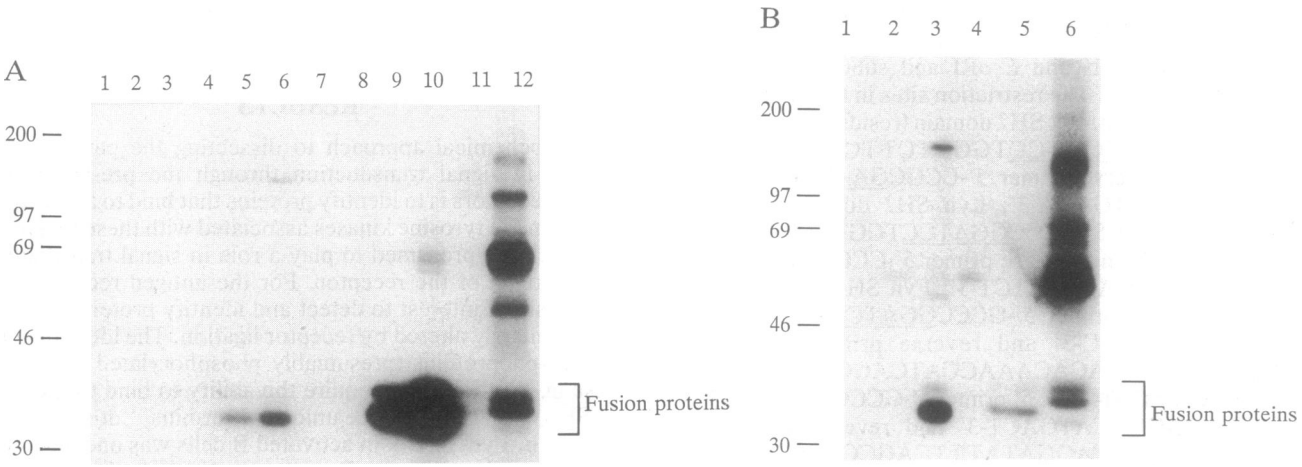

FIG. 1. (*A*) Binding of proteins from nonstimulated and anti-IgM-activated WEHI 231 B cells to discrete domains of Btk and Blk. Analysis by an *in vitro* kinase assay. Lysates from unstimulated (odd-numbered lanes) and anti-IgM-activated (even-numbered lanes) WEHI 231 cells were affinity-isolated by using the following GST fusion proteins and glutathione-Sepharose and analyzed after an *in vitro* kinase assay. Lanes: 1 and 2, GST; 3 and 4, GST–Btk-N terminus; 5 and 6, GST–Btk-SH3; 7 and 8, GST–Btk-SH2; 9 and 10, GST–Blk-N terminus; 11 and 12, GST–Blk-SH2. (*B*) Binding of proteins from nonstimulated NFS 70.15 pre-B cells to discrete domains of Btk and Blk. Analysis was as for *A*. Lanes: 1, GST; 2, GST–Btk-N terminus; 3, GST–Btk-SH3; 4, GST–Btk-SH2; 5, GST–Blk-N terminus; 6, GST–Blk-SH2.

B-stage tumors that have ceased to express their rearranged κ light chains. The NFS lines are derived by transformation with a nuclear oncoprotein. We subcloned NFS 70, a pro-B-cell line that has yet to rearrange its μ locus and derived pre-B-cell subclones (expressing largely intracellular μ-surrogate light-chain complexes). One such subclone, NFS 70.15, was used in these studies.

In initial experiments we examined, by an *in vitro* kinase assay, proteins that bound to the N-terminal, SH2, and SH3 domains of Btk and the N-terminal and SH2 domains of Blk, using lysates from unstimulated and anti-IgM-stimulated WEHI 231 cells. As can be seen in Fig. 1*A*, under the conditions of the assay used, no proteins were detected bound to the N terminus (lanes 3 and 4) or SH2 domains (lanes 7 and 8) of Btk. In unstimulated WEHI 231 cells a protein of ≈140 kDa was bound to the SH3 domain of Btk; a slight increase in binding was observed after receptor ligation (lanes 5 and 6). The most prominent proteins that were stimulated by receptor ligation appeared bound to the SH2 domain of Blk. Although an ≈130-kDa protein was the only protein seen bound to this domain in nonstimulated cells, anti-IgM stimulation led to a discernible increase in the binding of this protein and also the appearance in lysates of a prominent 72-kDa/77-kDa doublet and clearly detectable, though less prominent, 56-kDa and 180-kDa species (lanes 11 and 12). A somewhat diffuse 74- to 77-kDa species was seen bound to the N terminus of Blk in stimulated but not in nonstimulated cells. In experiments done by using the SH3 domains of Fyn, Lyn, and Blk, no discernible difference was seen in the patterns of binding proteins in unstimulated and stimulated cells (data not shown).

In "nonstimulated" NFS 70.15 pre-B cells, the most notable feature was that while no specific bands were seen bound to the N terminal or SH2 domains of Btk (Fig. 1*B*, lanes 2 and 4) or the N-terminal domain of Blk (lane 5), a number of prominent species were seen bound to the SH2 domain of Blk. The most prominent of these species was a 56-kDa protein, with additional prominent bands corresponding to proteins of 58 kDa, 65 kDa, 77 kDa, and 130 kDa. In this pre-B-cell line, no difference in protein binding was observed with anti-IgM stimulation (data not shown), which was perhaps not surprising, given that surface IgM is barely detectable in this cell line.

Blk-SH2- and Fyn-SH2-Binding Proteins Are Tyrosine-Phosphorylated in Receptor-Ligated B Cells and Constitutively Tyrosine-Phosphorylated in Pre-B Cells. To confirm that the SH2 domain of Blk associated with tyrosine-phosphorylated proteins derived from lysates of activated B cells and also from pre-B cells, we used an antiphosphotyrosine immunoblot assay for subsequent studies. As seen in Fig. 2, activation of B cells leads, as expected, to the phosphorylation on tyrosine residues of a number of proteins (lanes 2 and 3), the most prominent of which are also capable of binding to the SH2 domains of Blk (lanes 4 and 5) and Fyn (lanes 6 and 7). The most prominent species seen was a doublet in the 120- to 130-kDa size range and another doublet corresponding to 65-kDa and 72-kDa species. A fainter 77-kDa species was also visible.

A similar assay was performed on lysates from nonstimulated NFS 70.15 cells. In Fig. 3*A*, proteins immunoprecipitated from an antiphosphotyrosine Sepharose matrix (lane 2) were run alongside proteins eluted from a GST–Blk-SH2 affinity matrix and analyzed by a phosphotyrosine immunoblot approach. The majority of the phosphotyrosine-containing proteins immunoprecipitated also bound to the Blk-SH2 matrix. These proteins included major species of 44, 56, 62, 65, 72, 77, 120–130, and 180 kDa. In Fig. 3*B*, lysates from

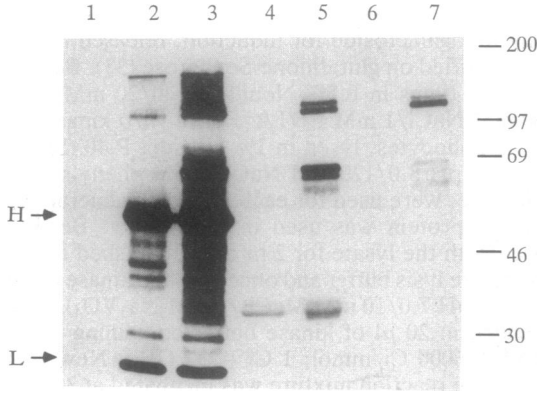

FIG. 2. Antiphosphotyrosine immunoblot analysis of proteins isolated from WEHI 231 lysates before and after anti-IgM activation. Lanes 1, 3, 5 and 7 contain proteins from anti-IgM-activated WEHI 231; lanes 2, 4, and 6 contain proteins from nonstimulated WEHI 231 cells. Lanes: 1, GST; 2 and 3, antiphosphotyrosine immunoprecipitation; 4 and 5, GST–Blk-SH2; 6 and 7, GST–Fyn-SH2. H, heavy chain; L, light chain.

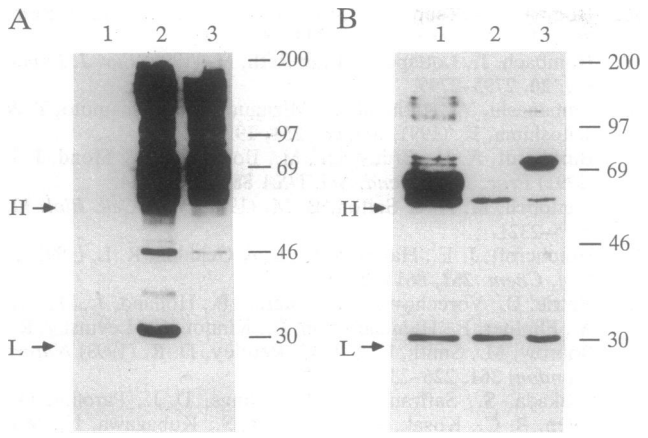

FIG. 3. (*A*) Antiphosphotyrosine immunoblot analysis of proteins isolated from NFS 70.15 pre-B cells. Lanes: 1, GST; 2, antiphosphotyrosine immunoprecipitation; 3, GST–Blk-SH2. H, heavy chain; L, light chain. (*B*) Comparison of constitutively phosphotyrosinylated proteins in NFS 70.15 with proteins phosphorylated on tyrosine residues after anti-IgM activation of WEHI 231. Proteins were analyzed by an antiphosphotyrosine immunoblot assay. Lanes: 1, NFS 70.15; 2, nonstimulated WEHI 231; 3, anti-IgM-activated WEHI 231. All samples were immunoprecipitated with covalently immobilized antiphosphotyrosine monoclonal antibodies before SDS/PAGE.

NFS 70.15, unstimulated WEHI 231 cells and anti-IgM-activated WEHI 231 cells were separately immunoprecipitated with an antiphosphotyrosine antibody and analyzed by an antiphosphotyrosine immunoblot assay. It is clear that the pattern of tyrosine phosphorylation in pre-B cells is distinct from that in receptor-ligated B cells, whereas in unstimulated B cells, when identical amounts of lysate are used, little tyrosine phosphorylation can be detected.

It has recently been demonstrated that there are differences in the patterns of phosphoproteins derived from a lysate of a mature B-cell line that bind to the SH2 domains of Blk, Fyn(T), and Lyn (36). We used the antiphosphotyrosine assay to examine the binding to a range of concentrations of the Blk-, Lyn-, Fyn-, and Btk-SH2 fusion proteins of phosphotyrosinylated proteins from unstimulated NFS 70.15 cells. The overall patterns of proteins that bound to the SH2 domains of Blk and Fyn were almost identical (Fig. 4). A

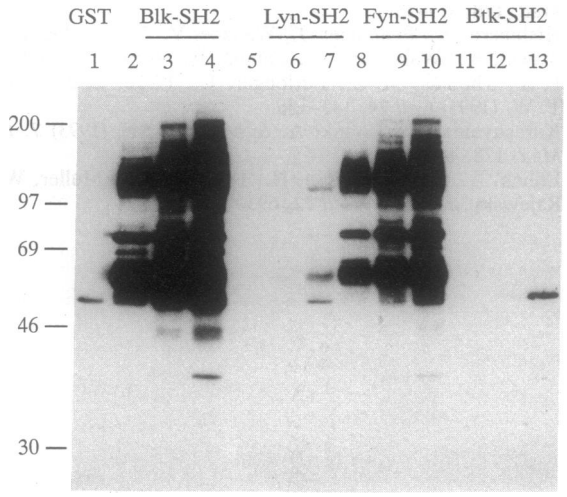

FIG. 4. Comparison and titration of SH2 domain-binding proteins from NFS 70.15 cells. Analysis by an antiphosphotyrosine immunoblot assay. Lanes 2, 5, 8, and 11 contain 1 μg of GST fusion protein; lanes 3, 6, 9 and 12 contain 5 μg of protein; lanes 1, 4, 7, 10, and 13 contain 20 μg of protein. Lanes: 1, GST; 2–4, GST–Blk-SH2; 5–7, GST–Lyn-SH2; 8–10, GST–Fyn-SH2; 11–13, GST–Btk-SH2.

qualitatively similar pattern at a substantially lower abundance was seen for the Lyn-SH2 domain, whereas no binding was observed to the SH2 domain of Btk.

DISCUSSION

A number of important distinctions appear between the signaling pathways used by membrane immunoglobulins in pre-B cells and in antigen-activated B cells. The pre-B receptor might signal from an intracellular location in a ligand-independent manner, and its requirement for surrogate light chains might be absolute (i.e., a conventional light chain may be unable to substitute for the surrogate chains). Signal transduction by the pre-B receptor may not have as stringent a requirement for the CD45 tyrosine phosphatase as has been noted for the B-cell antigen receptor. In addition, Btk may have distinct roles downstream of membrane immunoglobulin in the pre-B and B-cell pathways.

Pre-B cells have long been referred to as cells that contain intracellular or "cytoplasmic" μ heavy chains, and clearly surface μ_m is difficult to detect in these cells (7), although this is a matter of some debate (37). Indeed, while most signaling receptors monitor the external milieu of the cells in which they are expressed, the pre-B-cell and pre-T-cell receptors on individual lymphocytes are designed to lead to the selection of individual cells that have succeeded in making in-frame rearrangements. An intracellular-ligand model (where a putative ligand may be expressed in the endoplasmic reticulum itself) or a ligand-independent model (2, 7, 8) assumes that signal transduction would be constitutive soon after assembly. Although such a model does not demand a search for a ligand, several mechanistic questions remain to be answered. How does membrane immunoglobulin in a pre-B cell generate a signal? Is an intracellular crosslinking step required? A likely assumption is that membrane immunoglobulin in pre-B cells is crosslinked in the endoplasmic reticulum membrane itself in a surrogate light-chain-dependent manner. Premature expression of a human conventional λ light chain prevents pre-B-cell to B-cell maturation in a dose-dependent manner (38) at the late pre-B-cell stage, the stage at which both the μ_mT (homozygous membrane exon deleted) and λ5 −/− mice are also unable to differentiate any further. A potential interpretation of this finding is that a μ_m–conventional light-chain complex is not functionally equivalent to a μ_m–surrogate light-chain complex. It remains to be ascertained whether the pre-B receptor is physically in a crosslinked form and whether surrogate light chains play a direct role in this process.

Disruption of the sixth exon of CD45 by gene targeting led to compromised B-cell signaling in mature B cells but had little effect on the pre-B to B-cell transition itself (39). While this implies that the conventional B220 surface protein is required for signaling in later stages of B-cell differentiation, it might not be necessary for pre-B-cell signaling. It is, however, possible that although these mice lack cell-surface CD45 in the B-cell lineage, there might be an intracellular form of CD45 in these mice that is functional and necessary.

Mutations in the Btk-encoding gene that compromise kinase activity lead to X-linked agammaglobulinemia, a human X chromosome-linked recessive disease characterized by the failure of pre-B-cell to B-cell maturation. A point mutation in *btk*, which does not compromise kinase activity, permits maturation of pre-B cells to the B-cell stage but abrogates signal transduction in mature B cells in response to polyvalent stimuli, such as crosslinking of the antigen receptor. While Btk is presumed to be an important downstream effector of membrane immunoglobulin-mediated signaling in both the pre-B-cell and B-cell stages, it seems very likely that this kinase targets different substrates or is regulated in a different fashion at these stages of differentiation.

Although surrogate light chains may be expressed in noncovalent association with other proteins on the surface of Abelson-transformed pro-B-cell lines in which the μ locus is in germ-line configuration, it remains unclear whether these pro-B-cell complexes represent signaling receptors (40). Indeed, a major role for the $\omega/\lambda 5$ protein must certainly be at the late pre-B-cell stage because the developmental arrest in μ-membrane-exon-deleted homozygotes occurs at an apparently identical step as in the $\lambda 5-/-$ mice, with almost identical patterns of precursor-cell accumulation seen (41). The differences in the tyrosine-phosphorylated proteins that we have identified at the pre-B-cell and the B-cell stage support the notion of the presence of distinct signaling pathways through membrane immunoglobulin in pre-B cells and slightly more differentiated immature B cells. The presence of tyrosine-phosphorylated proteins that bind pre-B-receptor-associated Src-family kinase SH2 domains in an unstimulated pre-B-cell line provides supportive, although indirect, evidence for constitutive signal transduction through the pre-B-cell receptor. Even though the β chain–gp33 complex might be expressed at a detectable level on the surface of pre-T cells, it is extremely likely that a constitutive signal-transduction mechanism is also responsible for the pre-T-cell receptor-mediated selection of early T-lineage cells.

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