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Granulocyte colony-stimulating factor receptor signaling involves the formation of a three-component complex with Lyn and Syk protein-tyrosine kinases

(cytokines/hematopoietins/antigen receptor activation motif)

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ABSTRACT **Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein that critically regulates the viability, proliferation, and differentiation of granulocytic precursors and the function of neutrophils by signaling through its receptor. Cloning of the human G-CSF receptor (G-CSFR) cDNA has demonstrated sequence homology with other members of the hematopoietic/cytokine receptor superfamily. G-CSF stimulates the appearance of phosphotyrosine proteins in several types of human and murine myeloid cells. Since the receptor does not possess intrinsic tyrosine kinase activity, we hypothesized that G-CSFR interacts with and activates cytosolic protein-tyrosine kinases (PTKs). *In vitro* protein kinase assay of human G-CSFR immunoprecipitates demonstrated at least two tyrosine phosphoproteins, pp55 and pp70. We observed that G-CSF activated p53/p56*lyn*, a Src-related PTK, and p72*syk*, a non-Src-related PTK. Lyn and Syk were recovered in anti-G-CSFR immunoprecipitates; Lyn was detected in the absence of ligand. In addition, upon G-CSF stimulation, Lyn coimmunoprecipitated with Syk. Analysis of the G-CSFR amino acid sequence revealed a potential receptor activation motif for Syk. On the basis of immunoprecipitation and sequence analysis data, we propose that the human G-CSFR forms a three-component signaling complex with Lyn and Syk. Their sequential recruitment into the G-CSFR signaling complex demonstrates the coordinated involvement of two PTKs with a member of the hematopoietic/cytokine receptor superfamily.**

Granulocyte colony-stimulating factor (G-CSF) critically regulates the production and functional activity of neutrophilic granulocytes, the bulwark of host defense, and is emerging as the drug of choice for ameliorating chemotherapy-induced bone marrow suppression and congenital neutropenia disorders (1, 2). The receptor for G-CSF belongs to the newly described family of hematopoietin/cytokine receptors (3, 42). These receptors share the characteristics of no intrinsic enzymatic activity, a single transmembrane domain, and a cytokine receptor homology domain containing two sets of paired cysteines and a WSXWS motif. The hematopoietin receptors appear to transduce their signals via either a multichain complex—e.g., receptors for interleukin 2 (IL-2), granulocyte–macrophage (GM)-CSF, IL-3, IL-5, and IL-6—or a homodimeric complex—e.g., receptors for G-CSF and growth hormone (4, 5). A subset of receptor molecules for hematopoietins (G-CSF, IL-6, and leukemia inhibitory factor/oncostatin M) demonstrates homology in their extracellular region (4). Little is known about the mechanisms of signal transduction for the G-CSF receptor (G-CSFR) and this subset of receptors. While G-CSF has been demonstrated to stimulate the Na^+/H^+ exchanger in human endothelial cells (6), G-CSF priming of neutrophils does not result in changes in resting transmembrane electrical potential, levels of intracellular free calcium, cytosolic pH, or protein kinase C translocation (7). In the murine leukemia cell line NFS-60, G-CSF leads to increased binding of radiolabeled guanosine triphosphate to isolated cell membranes (8), implicating a role for guanine nucleotide-binding proteins in G-CSF signaling. More recently, G-CSF stimulation has been demonstrated to result in early phosphorylation on tyrosine of proteins of 55–60 kDa in neutrophils and the murine factor-dependent myeloid cell lines 32Dc13 and NFS-60 (ref. 9; D.J.T., unpublished data); 73–75 kDa in neutrophils, HL-60 cells, and 32Dc13 cells (ref. 10; D.J.T., unpublished data); 80–90 kDa in neutrophils and 32Dc13 cells (ref. 11; D.J.T., unpublished data); 97 kDa in the factor-dependent human leukemic cell line AML-193 (12); and 115 kDa in neutrophils (13). These results implicate a role for nonreceptor protein-tyrosine kinases (PTKs) in G-CSF signaling. The identity of the PTK(s) associated with and activated by the G-CSFR is unknown.

MATERIALS AND METHODS

Cells. Neutrophils were obtained from healthy adult volunteers as described. Neutrophils were studied within 6 hr of venipuncture. The murine cell line 32Dc13 was grown in Iscove's modified Dulbecco's medium (IMDM; GIBCO/BRL) containing 10% fetal calf serum and 10% WEHI-conditioned medium at 37°C with 5% CO_2, as described (14). Cells were washed twice with sterile PBS and resuspended in serum and factor-free IMDM supplemented with insulin and transferrin for 4–10 hr at 37°C with 5% CO_2.

Reagents. Anti-Syk antiserum was raised in rabbits, using either the C-terminal 28 amino acids of the porcine sequence or a fusion protein containing the N-terminal unique sequence and the first SH2 domain of porcine Syk (15). The former antiserum was superior for immune complex assays (S.J.C., unpublished data) but did not immunoblot (R.L.G., unpublished data); the latter antiserum did immunoblot Syk

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage CSF; G-CSFR, G-CSF receptor; PTK, protein-tyrosine kinase; RT, room temperature; PVDF, poly(vinylidene fluoride).

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(J.B.B., unpublished data). Anti-Lyn antiserum was raised in rabbits using the fusion protein containing the complete human sequence (16). Antisera against the other Src-related tyrosine kinases were raised against fusion proteins containing the unique N-terminal region and have been described elsewhere (17). Fes antiserum was provided by James R. Downing (St. Judes Research Hospital). Btk antiserum was provided by Owen Witte (University of California, Los Angeles, Medical School) (18). Immunoprecipitating antisera against the human G-CSFR were prepared by immunizing rabbits with a maltose binding protein/G-CSFR fusion protein containing the external domain of the human G-CSFR from amino acids 48 to 316. The cDNA encoding this portion of the human G-CSFR was derived from a nearly full-length cDNA isolated from a HL-60 cDNA library (19). The original cDNA was sequenced and confirmed to be derived from class I G-CSFR mRNA (D.J.T., unpublished data). The antiserum specifically stained BAF/BO3 cells transfected with the human G-CSFR (20) and human neutrophils; it also augmented G-CSF-induced proliferation of G-CSFR-transfected BAF/BO3 cells and identified the G-CSFR in immunoblots of membrane preparations of these cells (D.J.T., unpublished data). Recombinant human G-CSF was produced by Amgen Biologicals (Neupogen).

***In Vitro* Protein Kinase Studies.** Neutrophils and 32Dc13 cells were stimulated with G-CSF (100 ng/ml) for defined time periods. The cells were pulse centrifuged and resuspended in lysis buffer [10 mM Tris·HCl, pH 7.4/137 mM NaCl/1 mM $CaCl_2$/1% Nonidet P-40/10% (vol/vol) glycerol/2 mM $NaVO_4$/1 mM phenylmethylsulfonyl fluoride/10 mg of aprotinin per ml/10 mg of leupeptin per ml] and rocked for 30 min at 4°C. Samples were centrifuged at 14,000 × *g* for 15 min at 4°C. Rabbit polyclonal antisera directed against Src, Fyn, Yes, Lyn, Blk, Hck, Fgr, Lck, Fes, and Btk were used at a 1:100 dilution; anti-G-CSFR antiserum was used at a 1:50 dilution. Antibody/lysate mixtures were incubated overnight at 4°C. A 50% slurry of protein A-Sepharose (Sigma) was added for 45 min at 4°C. Successive washes were performed with PBS with 1% Nonidet P-40 followed by 100 mM Tris·HCl, pH 7.4/500 mM LiCl and 10 mM Tris·HCl, pH 7.4/1 mM EDTA/100 mM NaCl (TEN) for the Src-related kinases, Fes, and Btk immunoprecipitates. Syk immunoprecipitates were washed in PBS with 1% Nonidet P-40 followed by TEN. G-CSFR immunoprecipitates were washed in TEN buffer. Reactions were carried out for 20 min at room temperature (RT) in buffer consisting of 40 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μM ATP, and 10–20 μCi of [γ-^{32}P]ATP per sample (1 Ci = 37 GBq) and stopped with the addition of 2× Laemmli buffer. Samples were boiled and separated by SDS/PAGE on a 10% gel (for Src-related, Btk, and Syk kinase assays) or a 7.5% gel (for G-CSFR-associated kinases), and phosphoproteins were identified by autoradiography.

Phosphoamino Acid Analysis. Fifty million neutrophils were stimulated by G-CSF for 1 min at 37°C. Cells were lysed, and G-CSFR immunoprecipitates were prepared. The immune complex kinase assay was performed, and its products were resolved by SDS/PAGE. Proteins were transferred onto poly(vinylidene difluoride) (PVDF) paper. Phosphoproteins were identified by autoradiography. Bands corresponding to 55, 70, and 80 kDa were cut out of the PVDF membrane and were digested in the presence of 6M HCl for 60 min at 100°C as described (21). The supernatant was collected, lyophilized, and resuspended in 10 μl of buffer (pH 1.9), which contained phosphotyrosine, phosphoserine, and phosphothreonine. Each was spotted onto cellulose TLC plates and electrophoresed for 20 min at 1.5 kV in the first direction. After drying, buffer (pH 5.5) was substituted, and the plate was rotated 90° counterclockwise and electrophoresed for 16 min at 1.3 kV. After drying, phosphoamino acid standards were identified by 0.25% ninhydrin spray and baking at 65°C. Radiolabeled phosphoamino acids were revealed by autoradiography with Kodak X-Omat AR film (3-day exposure at −70°C).

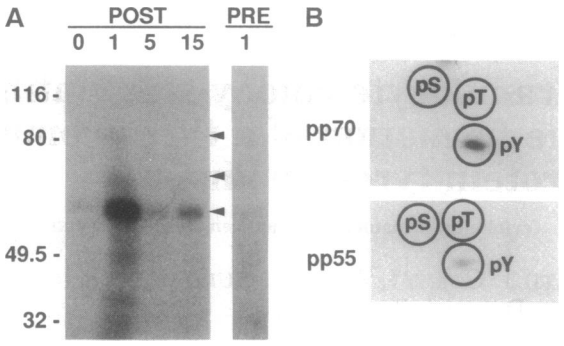

FIG. 1. Coimmunoprecipitation of PTK activity with G-CSFR. (*A*) Aliquots of neutrophils (10^6) were treated for the indicated times (minutes) at 37°C with human G-CSF (100 ng/ml) and lysed with 1% Nonidet P-40. *In vitro* kinase assays were performed on immunoprecipitates made with pre- and postimmune anti-G-CSFR antisera at the times indicated. Arrowheads indicate phosphoproteins of 55, 70, and 80 kDa that appeared within 1 min of G-CSF stimulation. (*B*) pp55 and pp70 were cut out from the PVDF membrane and digested in 6 M HCl and electrophoresed on a TLC plate in the presence of unlabeled phosphotyrosine (pY), phosphoserine (pS), and phosphothreonine (pT). Circles indicate the region of migration of the control unlabeled phosphoamino acids superimposed over the autoradiograph of the plate.

Immunoblotting. Neutrophil lysates were prepared as described above. After addition of 2× Laemmli buffer, samples were boiled for 5 min and separated by SDS/PAGE using a 10% gel. Proteins were transferred onto PVDF membranes. Membranes were blocked with PBS containing 5% nonfat dry milk for 2 hr at RT. Primary antibody incubations were performed at RT for 2 hr. The titers of antibodies used were 1:100 for anti-Lyn antiserum and 1:50 for anti-Syk antiserum. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-murine or goat anti-rabbit immunoglobulin (Bio-Rad). Each was used at a 1:3000 dilution for 45 min at RT. The immunoblot was developed by the chemiluminescence method following the manufacturer's guidelines (Amersham).

RESULTS AND DISCUSSION

To determine whether there is recruitment of PTK into a signaling complex with the G-CSFR, we performed *in vitro* protein kinase assays on neutrophil lysates that had been immunoprecipitated with anti-G-CSFR antiserum. Three

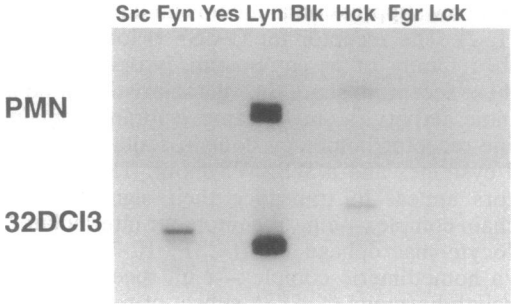

FIG. 2. Identification of SRC-related PTK in neutrophils (PMN) and 32Dc13. Antisera were used to immunoprecipitate specific Src-related PTK. Immunoprecipitates were incubated with ATP reaction buffer including [γ-^{32}P]ATP. Reaction products were separated by SDS/PAGE using a 7.5% gel that was dried for autoradiography.

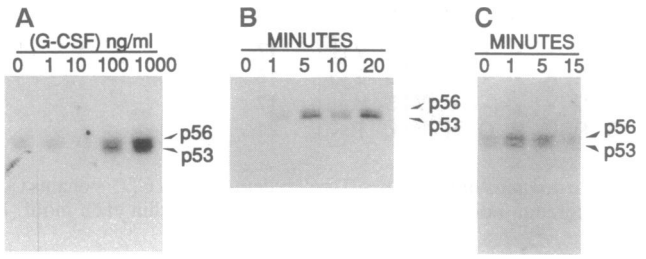

FIG. 3. Immune complex protein kinase assays of Lyn immunoprecipitates from neutrophils and 32Dc13 cells. Equivalent numbers (10^6) of neutrophils (*A* and *B*) or 32Dc13 cells (*C*) were stimulated with G-CSF for 10 min at 37°C at the indicated concentrations (*A*) or with G-CSF (100 ng/ml) for the indicated times (*B* and *C*). Cells were lysed and anti-Lyn immunoprecipitates were prepared using anti-Lyn antisera from rabbits immunized with the fusion protein containing the complete sequence. PTK assays were performed and their products were resolved by SDS/PAGE. Gels were dried and autoradiographed.

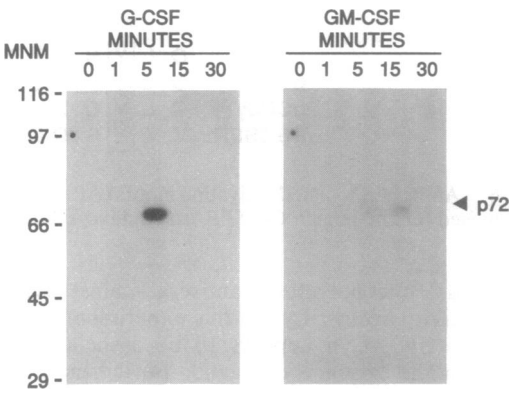

FIG. 4. Immune complex protein kinase assays of Syk immunoprecipitates of neutrophils (MNM) stimulated with G-CSF or GM-CSF. Equivalent numbers of neutrophils (10^6) were stimulated with G-CSF (*Left*) (100 ng/ml) or GM-CSF (*Right*) (50 ng/ml) for the times indicated. Cells were lysed and anti-Syk immunoprecipitates were prepared using anti-Syk antiserum raised in rabbits immunized with the C-terminal 28 amino acids of the porcine Syk sequence (15). PTK assays were performed and their products were resolved by gel electrophoresis. Gels were dried and autoradiographed. Numbers on left are kDa.

phosphorylated bands corresponding to 55, 70, and 80 kDa appeared reproducibly, with maximal phosphorylation occurring at 1–5 min (Fig. 1*A*). Phosphoamino acid analysis of bands corresponding to these three phosphoproteins demonstrated phosphorylation on only tyrosine residues (Fig. 1*B*; S.J.C., unpublished data for pp80). The molecular masses of these phosphotyrosine proteins suggested the possibility that the G-CSFR associates with and activates autophosphorylation of nonreceptor PTK.

Nonreceptor PTKs belong to one of eight general families (22). Of these, the Src-related PTKs most often have been found to associate with hematopoietic cell surface receptors (23). We prepared lysates from human neutrophils and 32Dc13 cells and tested them for baseline expression of Src-related PTKs. Basal *in vitro* PTK activity was found for only Lyn and Hck in neutrophils and for Lyn, Fyn, and Hck in 32Dc13 cells (Fig. 2). The Lyn doublet represents alternatively spliced products (24), the significance of which has not yet been demonstrated. We also tested for Fes and Btk activities. Neither activity was detected in neutrophils (S.J.C., unpublished data).

Lysates from G-CSF-stimulated neutrophils and 32Dc13 cells were immunoprecipitated with antiserum directed against the three candidate Src-like kinases (Lyn, Fyn, and Hck), and immune complex kinase assays were performed. Of the three, only Lyn demonstrated a dose- and time-dependent increase in phosphorylation in neutrophils and 32Dc13 cells (Fig. 3; S.J.C., unpublished data for Hck and Fyn). In addition to Lyn, the non-Src-related PTK, Syk, has recently been implicated in B-cell and mast-cell activation (25–27). Because Syk has been found in cells of myeloid lineage and is activated in HL-60 cells during Fc-γ signaling (28, 29), we investigated whether G-CSF also activated Syk. Furthermore, since GM-CSF also activated Lyn in neutrophils (30), we compared Syk activity in response to G-CSF versus GM-CSF (Fig. 4). Interestingly, G-CSF reproducibly activated Syk rapidly and transiently at 1–5 min, whereas GM-CSF's stimulation of Syk was weaker and delayed (5–15 min).

We hypothesized on the basis of their molecular mass and phosphorylation on tyrosine that G-CSFR-associated pp55 might be Lyn and pp70 might be Syk. We performed a series of coimmunoprecipitation studies to determine whether a physical association existed between these three signaling molecules. Since antisera against G-CSFR immunoprecipitates but does not blot the G-CSFR from human neutrophils (D.J.T., unpublished data), we examined G-CSFR immunoprecipitates for the presence of Lyn or Syk. Both Lyn and Syk coimmunoprecipitated with the G-CSFR (Fig. 5 *A* and *B*). Since the SH2 domain of Lyn and the first SH2 domain of Syk share substantial homology (47% within the first 30

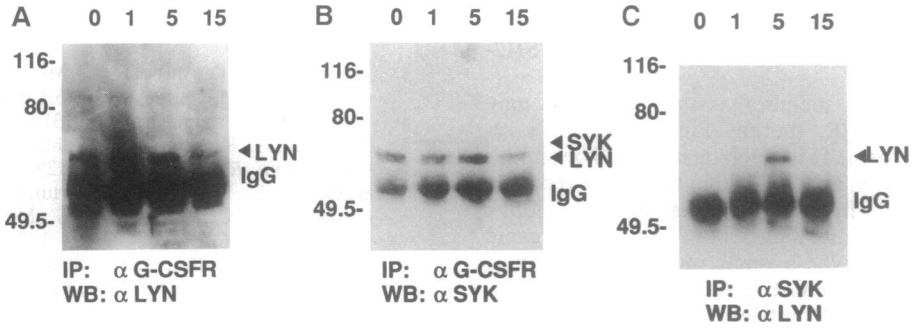

FIG. 5. Lyn and Syk associate and coimmunoprecipitate with the human G-CSFR. Lysates from neutrophils stimulated with G-CSF (100 ng/ml) for the times indicated were immunoprecipitated with anti-G-CSFR antisera, separated by SDS/PAGE, blotted with anti-Lyn antiserum (*A*) (1:100) or anti-Syk antiserum (*B*) (1:75). The anti-Syk antiserum used for immunoblotting was generated against the N-terminal unique portion of the porcine Syk sequence (15). Secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio-Rad) at 1:3000 dilution for 45 min at RT. The immunoblot was developed by the chemiluminescence method following the manufacturer's guidelines (Amersham). (*C*) Lysates of neutrophils stimulated with G-CSF (100 ng/ml) for the times indicated were immunoprecipitated with anti-Syk antisera. Immunoprecipitates were separated by SDS/PAGE, transferred to PVDF membranes, and developed with anti-Lyn antisera as described above. IP, immunoprecipitate; WB, Western blot. Numbers on left are kDa.

FIG. 6. Antigen-associated activation motifs (ARAMs) within the cytoplasmic domains of the human T-cell receptor ζ and CD3 γ chains (33) and proposed for the human G-CSFR from amino acids 727 to 747. Far-right column indicates number of amino acids within each motif. h, Human.

amino acids), the polyclonal antisera against Syk showed cross-reactivity against Lyn in this experiment (Fig. 5*B*). In contrast to Syk, Lyn appears to be associated with the G-CSFR in the absence of ligand. Furthermore, Lyn appeared in Syk immunoprecipitates in a time-dependent course similar to Syk phosphorylation (Fig. 5*C*). These data indicate that pp55 is Lyn and pp70 is Syk.

In a recent report demonstrating the *in vitro* association of Jak2 with fusion proteins containing the cytoplasmic domain of the erythropoietin receptor (31), the authors mentioned that G-CSF activated Jak2. In our G-CSFR immunoprecipitation studies, we did not observe a phosphorylated protein corresponding to the molecular mass of Jak2 (130 kDa) associated with the native G-GSFR in normal human neutrophils.

On the basis of our findings, we propose that the minimal signaling complex for G-CSF consists of three components. Two of the three components, G-CSFR and Lyn, appear to interact without ligand. Upon ligand binding, G-CSFR forms a dimer (4). Dimerization activates Lyn, possibly in a manner analogous to activation of the kinase portion of PTK-containing receptors (32). Syk associates with the complex resulting in its rapid and transient activation.

Recruitment of Syk may occur through Lyn or G-CSFR. The N-terminal half of Syk contains two SH2 domains while Lyn contains one SH2 domain. Lyn and Syk may associate via a phosphotyrosine–SH2 interaction (33). Analysis of the amino acid sequence of the cytosolic domain of the human G-CSFR, however, suggests a potential phosphotyrosine site for SH2 recognition by Syk. Syk and its related PTK, ZAP-70, have been found to associate with signaling complexes found in B and T cells, mast cells, and natural killer cells (34). Mutational analysis strongly suggests that an antigen receptor activation or homology motif (ARAM or ARH motif) exists that is recognized by ZAP-70 and possibly by Syk. This motif is based on two repeated SH2 binding sequences, YXXL/I, separated by 7 or 8 amino acids (34, 35). Such a motif may be found between amino acid residues 727 and 747 of the human G-CSFR (Fig. 6). In this case, cysteine (nonpolar) is substituted for leucine or isoleucine (each nonpolar) in the fourth position of the second SH2-binding site; furthermore, 11 amino acids separate the two SH2-binding domains.

It is also possible that membrane-associated Lyn may phosphorylate the G-CSFR, thereby facilitating Syk's recruitment from the cytosol. This mechanism would be similar to that observed with Fyn and ZAP-70 in the T-cell receptor signaling complex (33). In preliminary studies of G-CSF-stimulated neutrophils radiolabeled *in vivo* with inorganic phosphate, we have observed a single radiolabeled band of the size corresponding to the G-CSFR specifically immunoprecipitated by anti-G-CSFR antisera (S.J.C., unpublished data). In addition, another group has commented recently that the murine G-CSFR is tyrosine phosphorylated (36).

The potential G-CSFR ARAM is located in a region of the receptor near the C terminus downstream from the region essential for proliferation (18). However, this region has recently been demonstrated to be critical for G-CSF-induced myeloid differentiation (36, 37). Alternatively, a similarly located phosphotyrosine site is found in the erythropoietin receptor in an area associated with down-regulation of the proliferative signal (38). The transient occupancy of this potential phosphotyrosine residue by Syk might result in deregulation of a negative growth signal.

The basis for the constitutive association of Lyn with the G-CSFR is unknown. Lyn contains an SH3 domain while G-CSFR contains a proline-rich region (box 1) within its intracellular domain that is shared by several members of the hematopoietin receptor family (39, 40). It is possible that an SH3–box 1 interaction contributes to the Lyn/G-CSFR association. Mutational analysis to map domains of the human G-CSFR that interact with Lyn and Syk and to determine their role in proliferation and differentiation remains to be done.

Of note, Law *et al.* (41) have recently proposed a model of B-cell antigen receptor signaling based on transfection studies using chimeric constructs composed of three components: the μ heavy chain (extracellular), the CD8 antigen (transmembrane and proximal cytoplasmic), and a portion of the immunoglobulin α or β chains containing the antigen receptor homology motif (cytoplasmic tail). Their results suggest that B-cell antigen binding and receptor cross-linking results in sequential recruitment and activation of either Fyn or Lyn followed by Syk.

The identity of the G-CSFR-associated tyrosine phosphoprotein, pp80, is uncertain. It may represent a third PTK. Of note, a tyrosine phosphorylated protein of 80 kDa has been reported by others in neutrophils after G-CSF stimulation (11).

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