Binding of src-like Kinases to the β-Subunit of the Interleukin-3 Receptor

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We have previously shown that stimulation of 32D cl3 cells with interleukin (IL-3) results in the activation of three src-like tyrosine kinases, fyn, hck, and lyn. The β subunit of the IL-3 receptor co-immunoprecipitated with hck in lysates of both unstimulated and IL-3-stimulated cells; however, the β subunit did not precipitate with either fyn or lyn. The association of these three kinases with the β subunit of the IL-3 receptor was further investigated using bacterial fusion proteins encoding the unique, SH3, and SH2 domains of these three kinases. Fusion proteins of both hck and fyn bound to a 150-kDa tyrosine-phosphorylated protein present in lysates of IL-3-stimulated cells. This protein was identified as the β subunit of the IL-3 receptor by immunoblotting with an anti-β antibody. Glutathione S-transferase (GST) fusion proteins containing the SH2 domain of hck bound to the β subunit although the amount of β subunit that bound to the SH2 domain alone was only 30% of that which bound to the fusion protein containing the unique, SH3, and SH2 domains. This indicates that the SH2 domain is one of the motifs involved in binding hck to the β subunit. A GST fusion protein encoding a 236-amino acid region of the cytoplasmic tail of the β subunit, which contained four tyrosine residues, bound to hck and fyn. Binding to both proteins was dramatically increased when the GST-β fusion protein was tyrosine-phosphorylated. Far Western blot analysis was used to demonstrate the binding of the unique, SH3, and SH2 domains of hck to this 236-amino acid region of the β subunit; tyrosine phosphorylation of this protein increased the binding of both the unique region and the SH2 domain probes. These data indicate that binding of hck to the β subunit is mediated by both phosphotyrosine-dependent and -independent mechanisms.

Stimulation of cytokine family receptors with their ligands results in the rapid tyrosine phosphorylation of cellular proteins despite the fact that these receptors do not contain intrinsic protein tyrosine kinase activity (1–8). This contradiction has been explained by the activation of nonreceptor tyrosine kinases following ligand binding. Activation of both the Janus and src families of tyrosine kinases have been observed with a variety of different cytokines (9–21). Investigations with cell lines lacking specific Janus family members have indicated that they are critical in stimulating proliferation in response to some cytokines (22, 23). Similar studies have not been conducted to date with src-like kinases. Therefore, the precise role of src-like kinases in cytokine-mediated signaling events and cytokine-mediated proliferation has not yet been determined.

We have been investigating the activation of src-like kinases in signal transduction by the IL-3 receptor. Following IL-3 stimulation of the murine myeloid cell line 32D cl3, we have observed the activation of three src-like kinases: fyn, hck, and lyn (11). The catalytic activation of each kinase was rapid and transient. Over-expression of these three kinases in 32D cl3 cells resulted in an elevated level of tyrosine phosphorylation and kinase activity. Over-expression of c-src in the same cells, however, did not result in the activation of this kinase, suggesting specificity in which src-like kinases are activated by the IL-3 receptor (11).

In our previous studies, we observed the apparent association of hck with a 150-kDa protein that became tyrosine-phosphorylated following stimulation of cells with IL-3 (11). Although we suggested that this protein might represent the β subunit of the IL-3 receptor, this was not proven by immunoblotting. There are at least five proteins with molecular masses between 120,000 and 150,000 Da that become phosphorylated on tyrosine residues following IL-3 stimulation. These proteins include cbl (24), JAK2 (12), the β subunit (25, 26), SHIP (27, 28), and CAS. The latter two proteins migrate near the upper region of the 120–150,000 Da molecular mass range with a migration pattern similar to that observed in our previous study (11). In this report, we demonstrate that the β subunit co-immunoprecipitates with hck but not with fyn or lyn, that fusion proteins encoding the unique, SH3, and SH2 domains of either hck or fyn bound to the β subunit, and that hck and fyn bind to a specific region of the receptor in both phosphotyrosine-dependent and -independent manners. The potential role for this binding in regulation of kinase activation and signaling mechanisms is discussed.

MATERIALS AND METHODS

Cells and Cell Culture—The 32D cl3 cell line was obtained from Dr. Joel Greenberger (University of Pittsburgh, Pittsburgh, PA) and was maintained as described (29). All media components were obtained from Life Technologies, Inc. (Gaithersburg, MD). Recombinant murine IL-3 was obtained from Collaborative Biotechnology (Bedford, MA). Generation and characterization of the 32D/fyn and 32D/hck cell lines is described elsewhere (11).

Construction of Bacterial Expression Vectors—Bacterial expression vectors were constructed by PCR using synthetic oligonucleotides. 

1 The abbreviations used are: IL-3, interleukin-3; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine-based activation motifs; PCR, polymerase chain reaction; GST-pβ, the tyrosine-phosphorylated form of GST-β; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence lighting.

2 S. M. Anderson, S. C. Wu, and B. L. Koch, manuscript in preparation.
vectors encoding different regions of fyn, hck, or lyn coupled to glutathione S-transferase (GST) were either constructed for this study or were provided by other investigators. The following expression vectors were obtained from Dr. John Cambier (National Jewish Center for Immunology and Respiratory Diseases Denver, CO): FYN-1-255, FYN-SH2, FYN-SH3, and FYN-1-27 (30). GST-HCK-(1-61) was obtained from Dr. Clifford Lowell (University of California, San Francisco, CA) (31). The unique, SH3, and SH2 domains of fyn, hck, and lyn were amplified by PCR and cloned into GEX-2T. The unique, SH3, and SH2 domains of each construct are as follows. FYN was inserted between the BamHI and EcoRI sites (sense primer 5'-AGAGTACGGCCTGTCAGTACGTT-3', antisense primer 5'-GAAGATCTGGCCTGTGTGCAATG-3'); HCK was inserted between the BamHI and EcoRI sites (sense primer 5'-GAAGATCTAATGACAGTGTTCTTCCAGTGC-3', antisense primer 5'-GAGAATACGGCCTGTCAGTACGTT-3'); and Lyn was inserted between the BamHI and EcoRI sites (sense primer 5'-GAGATCTCGTGAATTGATATCATACAAA-3', antisense primer 5'-GAGAATTCGGCCACACTAATGCAC-3'). The SH2 domain of hck was amplified by PCR (sense primer 5'-AGAGTATTTAGCGGACACCTCAGGACAG-3', antisense primer 5'-GAATCCGAAGAAGCTGTTCTTCCGCCC-3'). The SH3 domain of hck was inserted between the BgII and EcoRI sites (sense primer 5'-GTGATGATCTATGGAGACAGAGGAGTGGTT-3', antisense primer 5'-GAAGATCTGGCCTGTGTGCAATG-3'). The GEX expression vectors were either introduced into competent GEX-3X and is referred to as (Schleicher and Schuell). This step also served to remove the free glutathione-

binding with into pCR2, and plasmid DNA purified. The SH2 domain was released by sense primer 5'-GATCTGGATGTATTAAATCAAAA-3'. The SH3 domain was amplified by PCR (sense primer 5'-CTCAGGACACCTCAGGACAG-3', antisense primer 5'-GAATCCGAAGAAGCTGTTCTTCCGCCC-3'). The SH2 domain of hck was inserted between the BgII and EcoRI sites (sense primer 5'-GTGATGATCTATGGAGACAGAGGAGTGGTT-3', antisense primer 5'-GAAGATCTGGCCTGTGTGCAATG-3'). The unique, SH3, and SH2 domains of fyn, hck, and lyn were amplified by PCR and cloned into GEX-2T. The unique, SH3, and SH2 domains of each construct are as follows. FYN was inserted between the BamHI and EcoRI sites (sense primer 5'-AGAGTACGGCCTGTCAGTACGTT-3', antisense primer 5'-GAAGATCTGGCCTGTGTGCAATG-3'); HCK was inserted between the BamHI and EcoRI sites (sense primer 5'-GAAGATCTAATGACAGTGTTCTTCCAGTGC-3', antisense primer 5'-GAGAATACGGCCTGTCAGTACGTT-3'); and Lyn was inserted between the BamHI and EcoRI sites (sense primer 5'-GAGATCTCGTGAATTGATATCATACAAA-3', antisense primer 5'-GAGAATTCGGCCACACTAATGCAC-3'). The SH2 domain of hck was amplified by PCR (sense primer 5'-AGAGTATTTAGCGGACACCTCAGGACAG-3', antisense primer 5'-GAATCCGAAGAAGCTGTTCTTCCGCCC-3'). The SH3 domain of hck was inserted between the BgII and EcoRI sites (sense primer 5'-GTGATGATCTATGGAGACAGAGGAGTGGTT-3', antisense primer 5'-GAAGATCTGGCCTGTGTGCAATG-3'). The GEX expression vectors were either introduced into competent DH5α bacteria or into TBK1 or TTKX1 bacteria (Stratagene, La Jolla, CA). The latter two strains of bacteria contain an inducible elk1 tyrosine kinase and allow for the production of tyrosine-phosphorylated bacterial fusion proteins. Expression of GST-fusion proteins in DH5α was induced as follows. Individual colonies picked from plates were grown in 50 ml of Luria Broth overnight. The cultures were diluted 1:20 and grown for 2 h, after which isopropyl-1-thio-D-galactopyranoside was added to a final concentration of 0.1 mM. The cultures were then grown for an additional 4 h before protein purification. Expression of the fusion proteins in TBK1 or TTKX1 cells was accomplished as follows. Colonies picked from plates were grown in 100 ml of Luria Broth overnight. Cultures were diluted 1:10 and allowed to grow for 2 h, and isopropyl-1-thio-D-galactopyranoside was added to a final concentration of 0.1 mM. After an additional 2 h, indoleacrylic acid was added at a final concentration of 50 μM, and the cultures allowed to grow for 2 h. Both phosphorylated and nonphosphorylated proteins were purified by the same means. Briefly, bacteria were pelleted at 6,000 rpm for 10 min in a Beckman JA10 rotor. The pelleted cells were resuspended in 20 ml of lysis buffer (50 mM Tris, pH 8.0, 0.2 mM EDTA) containing 100 units/ml aprotinin and lysed by sonication. To aid in protein solubilization, Triton X-100 was added to a final concentration of 1% and incubated for 30 min at 4 °C. Cellular debris was removed by centrifugation at 9,500 rpm for 10 min. Supernatants were incubated with 2 ml of a 50% slurry of glutathione-agarose beads (Sigma) overnight at 4 °C. The beads were isolated by centrifugation, and washed with lysis buffer three times. The fusion protein was removed from the beads by the addition of free reduced glutathione. Excess glutathione was removed from the isolated fusion protein by centrifugation using Centrex UF-2 10 K Spin Columns (Schleicher & Schuell). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

Immunopurification of Tyrosine-phosphorylated GST Fusion Proteins—Agarose-conjugated 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) was loaded onto Poly-Prep columns (Bio-Rad) and washed with phosphate-buffered saline. Concentrated fusion protein was added to the column and eluted with 100 mM phenyl phosphate in phosphate-buffered saline. Column fractions were analyzed by SDS-PAGE, and the peak fractions were concentrated using Centrex UF2 columns (Schleicher & Schuell). This step also served to remove the free phenyl phosphate.

Immunoprecipitation and Immuno blotting—Immunoprecipitation was performed as described previously (11). Rabbit polyclonal antibodies to fyn and anti-phosphotyrosine antibody 4G10 coupled to agarose beads were obtained from Upstate Biotechnology, Inc. Antibodies to hck and GST were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Additional antibodies to fyn and lyn were obtained from Transduction Laboratory (Lexington, KY). A polyclonal antibody to GST-β was raised in rabbits and affinity purified. A monoclonal antibody to GST-β was also prepared by conventional hybridoma technology. Monoclonal antibody 377C2 was used in these studies. Immunoprecipitated proteins were resolved on SDS-PAGE gels and electro-transferred to Immobilon membrane (Millipore, Bedford, MA). Immoblotting was conducted as described using the enhanced chemiluminescence lighting (ECL) system according to the manufacturer’s recommendations (Amersham Life Sciences Inc.).

GST-binding Assays and Far Western Blotting—Cell extracts were prepared from either unstimulated or IL-3-stimulated 32D cl3 cells by lysing in RIPA (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 0.5% Triton X-100, 0.25% sodium deoxylcholate) supplemented with 100 units/ml aprotinin and 1 mM sodium orthovanadate (Calbiochem, La Jolla, CA) and by removing particulate matter by centrifugation for 30 min at 13,000 rpm in a refrigerated Savant microfuge. Each binding assay consisted of the lysis from 2 x 10^6 cells. Protein concentrations were determined by the Bio-Rad 535 nm assay (Bio-Rad) using albumin as a standard. The GST proteins were then added directly to the clarified lysates and allowed to incubate for 1 h at 4 °C. The concentration of GST fusion protein added varied in each study, and the amount used in each experiment is indicated in each figure legend. The complexed proteins were isolated by the addition of 50 μl of glutathione-agarose beads for 30 min, and the protein complexes were washed three times with RIPA buffer with aprotinin. Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted as described.

Far Western blot analysis was conducted by running proteins of interest on an SDS gel, transferring the proteins to Immobilon, and probing with a filter with a biotinylated bacterial fusion protein. Complexes were visualized by probing with streptavidin-horseradish peroxidase (Amersham) and performing the ECL protocol. All procedures were as described for immunoblotting, and biotinylation was accomplished with a kit from Amersham used according to the manufacturer’s recommendations.

RESULTS

Binding of hck and fyn to the β Subunit of the IL-3 Receptor—In our previous studies, we examined the activation of src-like kinases following IL-3 stimulation of 32D cl3 cells (11). In those studies, we observed that a 150-kDa tyrosine-phosphorylated protein co-immunoprecipitated with hck in anti-hck immunoprecipitates. The size of this protein suggested to us that it might represent the β subunit of the IL-3 receptor; however, this was not demonstrated by immunoblotting due to the poor quality of the anti-β antibody preparations available at that time. Since that time, we have prepared an anti-β subunit monoclonal antibody for use in immunoblotting studies. Furthermore, it has recently become clear that there are at least two other proteins with molecular masses of 140–150,000 Da that become tyrosine-phosphorylated following IL-3 stimulation. These proteins include SHIP (27, 32) and CAS.2 To confirm that the 150-kDa tyrosine-phosphorylated protein that associates with hck was the β subunit, we again performed immunoprecipitation studies. Nonimmune serum and antibodies directed against hck, fyn, and lyn were added to lysates from unstimulated and IL-3-stimulated 32D cl3 cells. These immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting using the anti-β subunit antibody 377C2. As shown in Fig. 1, the anti-hck antisera immunoprecipitates a protein of 150 kDa from lysates of unstimulated and stimulated 32D cl3 cells (Fig. 1, compare lanes 3–4 with 9–10). Based upon the reactivity of this protein with the anti-β monoclonal antibody, this protein represents the β subunit of the IL-3 receptor. The anti-hck antisera immunoprecipitated approximately 2- or 3-fold more β subunit from the lysates of IL-3-stimulated cells.
fig. 1. Co-immunoprecipitation of the β subunit with hck. 32D cl3 cells were cultured in the absence of IL-3 for 16 h (lanes marked −) and then stimulated with IL-3 for 10 min (lanes marked +). The cells were lysed and incubated with either non-immune (NIS), anti-hck, anti-fyn, or anti-lyn antisera. The antibodies used are indicated at the top of each pair of lanes. Immune complexes were isolated and separated by SDS-PAGE. Whole cell lysates (lanes marked WCL) were run alongside the immunoprecipitation reactions as a positive control for the immunoblotting reaction. The immunoblot was probed with the anti-β subunit monoclonal antibody 377C2. The position of the β subunit is indicated by an arrowhead (>) on the right side of the figure.

compared with the amount of the protein that co-precipitated with hck from lysates of unstimulated cells, as determined by densitometry. Conversely, this protein did not appear in immunoprecipitates using nonimmune, anti-fyn, or anti-lyn sera. These data confirm our previous hypothesis that the β subunit of the IL-3 receptor co-immunoprecipitates with hck but not with fyn or lyn. These new results also indicate that hck may be associated with the β subunit in the absence of IL-3 stimulation (Fig. 1, lane 3). This possibility was not explored in our previous work and suggests that hck may be constitutively associated with the IL-3 receptor.

To further explore this association between src-like kinases and the β subunit, we utilized GST-fusion proteins encoding the unique, SH3, and SH2 domains of hck, fyn, and lyn to determine whether they bound to any tyrosine-phosphorylated proteins in IL-3-stimulated 32D cl3 cells. GST, GST-HCK, GST-FYN, or GST-LYN were added to lysates of IL-3-stimulated 32D cl3 cells at a final concentration of 2 μM. A specific set of tyrosine-phosphorylated proteins were observed to bind to GST-HCK, GST-FYN, and GST-LYN; however, none of these proteins bound to GST alone (Fig. 2A, compare lane 1 with 2–4). Of greatest interest was the 150-kDa band present in lanes 2 and 3 of Fig. 2A. While this band most likely comprises several proteins, its size suggests that it may contain the β subunit of the IL-3 receptor.

Several other tyrosine-phosphorylated proteins were also observed to bind, including a protein of 120 kDa that bound to both GST-HCK and GST-LYN but to a lesser extent to GST-FYN in this study (Fig. 2B). This protein is the bcl protooncogene, based upon its size and immunoblotting studies (24). In addition, a protein of approximately 56 kDa was noted to bind to both GST-HCK and GST-FYN but not to GST-LYN (data not shown). Based upon its apparent molecular mass and immunoblotting studies, this protein appears to be the SH2-containing adapter protein Shc (data not shown).

To investigate whether the 150-kDa band contains the β subunit, immunoblotting was performed using a monoclonal antibody directed against the β subunit of the IL-3 receptor. Lysates of unstimulated and IL-3-stimulated cells were used in a binding assay with the GST, GST-HCK, GST-FYN, or GST-LYN fusion proteins, and the bound proteins were subjected to immunoblot analysis with the anti-β antibody. A major band of 150 kDa was observed to bind to GST-HCK and GST-FYN in lysates of IL-3-stimulated cells but not in lysates of unstimulated cells (Fig. 2B, lanes 3–6). This band co-migrated with the 150-kDa band present in whole cell lysates of both unstimulated and stimulated cells (Fig. 2B, lanes 9 and 10). This protein was not observed to bind to either GST or GST-LYN fusion proteins regardless of whether lysates of unstimulated or stimulated cells were used in the binding reactions. The amount of β subunit that bound to GST-FYN was only 50% of that which bound to GST-HCK. This clearly indicates that the β subunit of the IL-3 receptor is among the tyrosine-phosphorylated proteins present in lysates of IL-3-stimulated cells that bind to GST-HCK and GST-FYN.

The Interaction between the β Subunit and hck Is Mediated by the SH2 Domain.—A major mechanism for interaction with tyrosine-phosphorylated proteins is by means of SH2 domains, which are known to bind to phosphotyrosine residues in a sequence-specific context. To investigate whether the SH2 domains of hck and fyn are involved in the binding of these proteins with the β subunit, binding studies were performed with a series of bacterial fusion proteins containing individual domains of either hck or fyn. Proteins included in this study were GST-HCK (which spans amino acids 1–255), GST-HCK-(1–61) (which spanned amino acids 1–61, including most of the unique region), GST-HCK-SH3, GST-HCK-SH2, GST-FYN, GST-FYN-(1–27), GST-FYN-SH3, and GST-FYN-SH2. The 150-kDa band corresponding to the β subunit bound only to the GST-HCK and GST-HCK-SH2 fusion proteins. The amount of the β sub-
unit that bound to GST-HCK-SH2 was only 30% of that which bound to GST-HCK (Fig. 3, top panel; fourth and tenth lanes). This indicates that the SH2 domain of hck can bind to the tyrosine-phosphorylated β subunit on its own; however, high affinity binding of GST-HCK to the β subunit may require the interaction of multiple domains of HCK with the β subunit.

In contrast to the results obtained with hck, it appears that the binding of fyn to the β subunit is more complex and requires multiple regions of fyn. Although GST-FYN, which includes the unique, SH3, and SH2 domains, bound the β subunit, none of the individual domains bound to the β subunit (Fig. 3, bottom panel). Therefore, unlike hck, no single domain can be identified as mediating binding to the β subunit.

Tyrosine-phosphorylated β Subunit Fusion Proteins Bind to hck and fyn—The above studies clearly suggest that the binding of hck to the β subunit involves recognition of phosphorylated tyrosine residues by the SH2 domain of this kinase. To provide further support that the β subunit was capable of binding to hck and fyn, we prepared a GST fusion protein encoding a region of the β subunit that contains four tyrosine residues. This region is immediately C-terminal to the box 1 sequence and spans amino acids 481 to 716 of the mouse β subunit. This fusion protein was prepared in both nonphosphorylated and tyrosine-phosphorylated versions by growing the cell lysates from 32D cl3 cells (data not shown). Binding of fyn to GST-pβ was tyrosine-phosphorylated (data not shown); therefore, we do not believe that the binding of hck to GST-β subunit reflects the fact that the GST-β fusion protein may have been phosphorylated by hck or other src-like kinases.

The conditions used above to demonstrate the binding of GST-β to hck did not reveal binding of GST-β or GST-pβ to fyn (data not shown). Binding of fyn to GST-pβ could be detected when lysates of 32D/fyn cells were used instead of 32D cl3 cells (Fig. 4B). These cells have been described previously as having elevated levels of fyn due to expression of a fyn-encoding retrovirus (11). No binding of fyn to GST-β was detected although fyn did bind to GST-pβ (Fig. 4B). By densitometry, we estimate there to be a 10-fold increase in the amount of fyn bound to GST-pβ, compared with that bound to GST-β (Fig. 4B). These results suggest that both hck and fyn can bind in solution to a 236-amino acid region of the β subunit that contains these four tyrosine residues although there may be some differences in the binding of these two proteins since we could only detect binding of fyn with lysates of cells that overexpress this protein.

As a second approach to demonstrate the binding of hck to both the tyrosine-phosphorylated and nonphosphorylated β subunit, we utilized Far Western blot analysis. Varying amounts (from 0.1 to 10 μg) of GST-β and GST-pβ were run on an SDS gel, transferred to Immobilon membrane, and the filter...
was probed with biotinylated GST-HCK. Based upon the amount of signal obtained with the ECL reaction, it appears that there is at least 5- to 10-fold more GST-HCK bound to GST-pβ than to GST-β (Fig. 5). The extent of tyrosine phosphorylation of the GST-β protein is indicated by the anti-phosphotyrosine blot shown in the bottom panel of Fig. 5. The faint band observed in the anti-phosphotyrosine blot in the lanes containing the nonphosphorylated protein did not change in intensity as the protein concentration changed, and it extended beyond the lanes that contained protein, indicating that it is most likely due to a nonspecific reaction. Binding of the hck probe to the GST-β protein that did not contain phosphorylated tyrosine residues is consistent with the observation described above in Fig. 4. These results suggest that there are both phosphotyrosine-dependent and -independent interactions between hck and the β subunit.

A Far Western blot approach was also used to examine the ability of the different domains of hck to bind to GST-β and GST-pβ. In this study, GST, GST-β, and GST-pβ were resolved on a gel and immunoblotted, and the immunoblots were probed with biotinylated fusion proteins comprising the individual domains of hck (Fig. 6). None of the hck probes bound to a significant degree to GST alone (Fig. 6, and data not shown). Consistent with the results described in Figs. 4 and 5 above, GST-HCK bound to both GST-β and GST-pβ, and there was a 4-fold increase in the binding of GST-HCK to GST-pβ compared with the amount of binding to GST-β (Fig. 6, first panel). The GST-HCK-SH3 probe bound almost equally to GST-β and GST-pβ; densitometric scanning indicates a 1.5-fold increase in the binding of GST-HCK-SH3 to GST-pβ (Fig. 6, third panel). This is consistent with the SH3 domain of hck binding primarily to proline-rich sequences present in this region of the β subunit. The GST-HCK-SH2 probe bound to both GST-β and GST-pβ; however, there was an apparent 4.5-fold increase in the amount of probe binding to the GST-pβ consistent with interaction of the SH2 domain with phosphorylated tyrosine residues (Fig. 6, fourth panel). We were surprised to observe that the unique region probe displayed phosphorylation-dependent binding; that is, the probe bound to both GST-β and GST-pβ.

However, there was a 2.6-fold increase in the amount of binding to GST-pβ compared with the amount of GST-HCK-1–61 that bound to GST-β (Fig. 6, second panel). It is not clear whether this represents actual binding of the unique region probe to phosphotyrosine, whether this reflects its binding to regions of charged amino acids as thought to contribute to the association of the unique region of src-like kinases to the plasma membrane (33, 34), or whether there is another determinant recognized by the unique region.

DISCUSSION

In this manuscript, we have described the association of two different src-like kinases, hck and fyn, with the β subunit of the IL-3 receptor. Evidence supporting this association includes: 1) the co-immunoprecipitation of the β subunit of the IL-3 receptor with anti-hck antisera, as determined by immunoblotting using an anti-β subunit monoclonal antibody; 2) the binding of GST fusion proteins encoding the unique, SH3, and SH2 domains of hck and fyn appears to be specific since a similar GST fusion protein encoding a 236-amino acid region of the β subunit binds to both hck and fyn, and phosphorylation of this fusion protein increases binding to these kinases; and 5) specific domains of hck bind to this same 236-amino acid region of the β subunit in both phosphotyrosine-dependent and -independent manners. The interaction of hck and fyn appears to be specific since a similar GST fusion protein encoding the unique, SH3, and SH2 domains of the lyn tyrosine kinase does not bind to the β subunit.

We were led to initiate these studies because we had previously observed the co-immunoprecipitation of a 150-kDa tyrosine-phosphorylated protein with hck in anti-hck immunoprecipitates (11). As noted above, we did not previously identify the 150-kDa protein as the β subunit and there are several other tyrosine-phosphorylated proteins that migrate at this molecular mass, including CAS (35) and SHIP (27, 28). In this manuscript, we demonstrate that hck immunoprecipitates with the β subunit through the use of a newly prepared anti-β subunit monoclonal antibody. In addition, we discovered that the β subunit co-immunoprecipitates with hck in lysates from unstimulated 32D cI3 cells. This observation was unexpected, particularly since the GST-HCK fusion protein (comprising the unique, SH3, and SH2 domains of hck) does not bind to the β subunit in lysates prepared from unstimulated cells (Fig. 2B, lane 3). It is unclear why these experiments give paradoxical results; however, it is possible that the kinase domain of hck contributes to the formation of the stable complex with the β subunit and that this is particularly important in unstimulated cells.

In contrast to studies using anti-hck antiserum, the β sub-
unit did co-immunoprecipitate with anti-fyn or anti-lyn under the same conditions that it was observed to associate with hck (Fig. 1 and Ref. 11). In light of the lack of co-immunoprecipitation of the β subunit with fyn, it was surprising to observe that the GST-FYN fusion protein bound to the β subunit. The lack of co-precipitation of fyn and the β subunit may reflect the fact that the epitope recognized by the anti-fyn antibody could be obscured by its interaction with the β subunit, or alternatively, the addition of a relatively high concentration of the GST-FYN fusion protein resulted in the detection of a relatively weak binding between the two proteins. We favor the latter possibility because other experiments presented in this paper, particularly Figs. 3–5, indicate that there are substantial differences in the interaction between the β subunit and either hck or fyn.

The phosphorylation of tyrosine residues is critical in the activation of signal transduction pathways. Phosphorylated tyrosine residues serve as binding sites for SH2 domains present in signaling molecules. The binding of these signaling molecules to phosphotyrosine residues on growth factor receptors is thought to be critical in the activation of these signaling molecules through their subsequent tyrosine phosphorylation by the kinases associated with that receptor. SH2 domains are critical in this process, as they confer the ability to bind to a specific phosphorylated tyrosine residue on an activated receptor molecule. It has become clear that the amino acids flanking a phosphorylated tyrosine residue are critical in determining which SH2 domains will bind to that site. The 236-amino acid region to which hck and fyn bind contains four tyrosine residues, three of which are also present in the human β, subunit (1–3). The first tyrosine residue (Tyr529) is not present in the human β subunit, suggesting that either it is not critical in signaling events or that its function in the human β subunit is replaced by another tyrosine residue. Table I lists the four tyrosine residues found in this region of the mouse β, and β, receptors (4). Of the four sites listed, none fit consensus sequences established for known SH2 domains (36–38). One site, NGPY595, appears to match the consensus binding site for the SH3 domains of src-like kinases. Data that might serve as binding sites for the SH3 domains of either hck or fyn, however, none of these sequences match the consensus sequence for SH3 domains of src-like kinases. Data presented above suggest that hck can bind to this region of the β subunit in a phosphotyrosine-independent manner that would be consistent with the binding of the SH3 domain to proline-rich sequences. Future studies will be directed at identifying the putative binding site for the hck SH3 domain in this region of the β subunit.

The role of the unique domain of src-like kinases is poorly defined to date, and it is unclear what motifs the unique domain might interact with or bind. The unique domain of hck has been shown to bind to cysteine residues in the cytoplasmic tails of CD4 and CD8 (42). Cambier and co-workers (43) have demonstrated that the first ten amino acids of fyn and lyn bind to the amino acids DSCM present in the ITAM motif of Ig-α. Binding to the DSCM motif is of low affinity and phosphotyrosine-independent; however, it appears to be important in activation of specific src-like kinases by Ig-α. While there are no DSCM motifs in the β, or β, subunits, this does not rule out that a similar interaction occurs between hck or fyn and the β subunit.

We suggest that hck is constitutively associated with the β subunit of the IL-3 receptor through the binding of its SH3 domain, and perhaps also the unique domain, to specific recognition sequences that do not contain phosphorylated tyrosine residues. We believe that hck associated with the β subunit in this context is inactive either due to phosphorylation of the C-terminal tyrosine residue that regulates the catalytic activation of src-like kinases or because it is held by the β subunit in an inactive conformation. Following ligand binding to the receptor, the JAK2 tyrosine kinase is activated and is thought to phosphorylate the cytoplasmic tail of the β subunit. Following phosphorylation of the β subunit, we hypothesize that the SH2 domains of hck and fyn now bind to a specific phosphotyrosine residue resulting in their activation, presumably due to an alteration in the conformation of the kinase. The binding of the SH2 domain of hck to the phosphorylated β subunit may be the critical event in its activation, and activation of hck may not involve the dephosphorylation of the regulatory C-terminal tyrosine. This model is analogous to the activation of src-like kinases following their binding to phosphorylated ITAM motifs in either the T-cell or B-cell receptor (44, 45). The activation of hck and fyn is thus dependent upon JAK2 activation since we believe that phosphorylation of the receptor is accomplished by JAK2. Future studies will address the ability of phosphorylated peptides to activate hck and fyn, the role of phosphatases in the activation of src-like kinases, and the putative sites of interaction between the β subunit and the unique and SH3 domains of hck and fyn.

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