BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 117–123 (1998)
ARTICLE NO. RC989441

The Src-like Tyrosine Kinase Hck Is Activated by Granulocyte Colony-Stimulating Factor (G-CSF) and Docks to the Activated G-CSF Receptor

Alister C. Ward,*[,1] Jennifer L. Monkhouse,† Xavier F. Csar,†
Ivo P. Touw,* and Paul A. Bello‡
**Institute of Hematology, Erasmus University Rotterdam, The Netherlands; †Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Parkville, Australia; and ‡Faculte de Pharmacie, Universite de Montpellier I, Montpellier, France*

Received August 24, 1998

Activation of the granulocyte colony-stimulating factor receptor (G-CSF-R) leads to tyrosine-phosphorylation of multiple cytoplasmic components. To date, the kinases Jak1, Jak2, Tyk2, Lyn, and Syk have been implicated in this process. However, it is unknown if other kinases might be involved in the diverse responses from the G-CSF-R, which include mitogenesis, survival, differentiation, and functional activation of responsive cells. The hematopoietic cell kinase (Hck) is a member of the Src-family of kinases known to be expressed in cells of the granulocytic lineage. It also interacts with the gp130 subunit of the LIF/IL-6 receptors, which is closely related to the G-CSF-R, and so represents a good candidate for mediating at least some of the downstream signaling from the G-CSF-R. Therefore, we investigated the activation of Hck by the G-CSF-R in intact cells as well as *in vitro.* These studies revealed recruitment of Hck to activated G-CSF-R, mediated by direct binding via its SH2 domain to multiple phosphotyrosines of the receptor. In addition, we show that Hck becomes activated upon G-CSF treatment and is, in turn, able to phosphorylate the G-CSF-R, indicating a clear functional and physical involvement in G-CSF signaling.

Key Words: **Src kinase; cytokine receptor; Hck, G-CSF-R.**

The granulocyte colony-stimulating factor receptor (G-CSF-R) is a member of the hematopoietin receptor superfamily which forms homodimeric complexes upon ligand binding (1, 2). The G-CSF-R stimulates the proliferation, survival, maturation and functional activation of cells of the granulocytic lineage and is therefore pivotal in the regulation of granulopoiesis (3–5). Like other hematopoietin receptors, the G-CSF-R lacks intrinsic tyrosine kinase activity, but has been shown to activate multiple cytoplasmic tyrosine kinases, including the Janus tyrosine kinases Jak1, Jak2, and Tyk2 (6–10), the Src-family kinase Lyn, and Syk (11, 12). Of these, Lyn and Jak1 seem to be constitutively-associated, while Syk and Jak2 are recruited to the activated receptor complex (6, 11). However, the full repertoire of kinases involved in the diverse signaling of the G-CSF-R at different stages of granulocytic development remains unknown.

The hematopoietic cell kinase (Hck), a member of the Src family of membrane-associated, intracellular protein tyrosine kinases, is expressed primarily in hematopoietic cells of the myeloid and B-cell lineage, as well as in undifferentiated ES cells (13–16). In both the granulocytic and monocytic lineages, Hck expression is highest in more differentiated cells, suggesting that the protein might function in myeloid differentiation or activation (17, 18). In support of this idea, it has been shown that Hck is vital in the response of macrophages to LPS and interferon-γ (19), while it is also a component of the FcγRI receptor mediated signaling in human granulocytes (20). In contrast, expression of activated or wild-type Hck in 32Dcl3 myeloid cells blocked granulocytic differentiation in response to G-CSF, while prolonging survival (21). Hence, like the

[1] To whom correspondence should be addressed at Institute of Hematology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Fax: 31-10-4362315. E-mail: ward@hema.fgg.eur.nl.

0006-291X/98 $25.00

G-CSF-R itself, Hck may have distinct roles at various stages of granulocytic development.

Hck has been shown to be physically and functionally associated with the gp130 subunit of the LIF/IL-6 receptor (16), which is closely related structurally to the G-CSF-R. Therefore, we sought to investigate whether Hck might interact with the G-CSF-R complex. In this report we show that Hck is activated by stimulation with G-CSF in intact cells, and is able to directly phosphorylate a G-CSF-R fusion protein *in vitro*. In addition, Hck shows inducible recruitment to the G-CSF-R complex in intact cells. Finally, using a tyrosine-phosphorylated G-CSF-R fusion protein, we show that Hck is able to bind directly to the G-CSF-R in a tyrosine-dependent manner, via its Src-homology 2 (SH2) domain. Together these data demonstrate a clear physical and functional interaction of Hck with the G-CSF-R.

MATERIALS AND METHODS

Cells and culture conditions. The IL-3-dependent murine myeloid cell line NFS-60 (22) was maintained in DMEM medium supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng of human G-CSF per ml at 37°C and 5% (v/v) CO_2.

Plasmid construction. A DNA fragment corresponding to the region coding for the cytoplasmic domain of the murine G-CSF receptor (aa 632-812) was amplified from plasmid pJ17 (1) by PCR with the oligonucleotide primers 5′-CTGGATCCTTCTGGTCAGAT and 5′-CTGAATTCTAGAAACCCCCT. This fragment was then cleaved with BamHI and EcoRI and subcloned into pGEX2TK (Pharmacia, Uppsala, Sweden), to generate a plasmid designated p2TK-GR. In addition, the following GST-fusion constructs were made utilizing standard PCR protocols to amplify the appropriate coding regions, which were cloned into pGEX-2T or pGEX-2TH: Lck-SH2 (aa 126–223 of murine Lck), Lyn-SH2 (aa 128–225 of murine Lyn), Fyn-SH2 (aa 148–245 of human Fyn), Yes-SH2 (aa 156–253 of chicken Yes), Src-SH2 (aa 145–248 of viral Src), Hck-SH2 (aa 142–250 of murine Hck), Hck-SH3 (aa 83–132), Hck-USH3 (aa 1–132), Hck-SH3SH2 (aa 83–250), and Hck-USH3SH2 (aa 1–250). The authenticity of all constructs was verified by DNA sequencing.

Expression of GST-fusion proteins. For routine expression, plasmids were transformed into the *E. coli* strain XL-1 Blue (Stratagene, La Jolla, CA). Exponentially growing cultures were induced with 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 4 h at 37°C. The cells were then washed in TBS and lysed by sonication in TEND (TBS, 1 mM EDTA, 0.5% NP-40, 1 mM DTT). The lysate was centrifuged at 12,000 rpm for 20 min and incubated with glutathione Sepharose 4B beads (Pharmacia; AMRAD, Melbourne, Australia) for 30 min. The beads were washed extensively with TEND, before elution of the purified fusion proteins with 25 mM Glutathione in TE. For the production of tyrosine-phosphorylated G-CSF-R cytoplasmic domain, plasmid p2TK-GR was introduced into the strain TKX1 (Stratagene), which contains an inducible tyrosine kinase. Fusion protein was produced as described above, except a second induction was carried out in TK induction media for 2 h at 30°C, according to the manufacturer's instructions. The protein was then ^{32}P-labeled with heart muscle kinase and cleaved with thrombin to remove the GST tag, as described previously (23, 24).

Far Western blot analysis. GST fusions were purified as described above, then electrophoresed on 10% SDS–PAGE gels (25), and electrophoretically transferred to Hybond-C membranes (Amersham Nederland BV, Den Bosch, The Netherlands; Amersham, Sydney, Australia). These were processed through a denaturation–renaturation cycle (26) and probed with the ^{32}P-labeled G-CSF-R, as described (24).

Preparation of cell lysates, immunoprecipitation and Western blotting. Before stimulation, cells were deprived of G-CSF for 14 h at 37°C, then stimulated with 100 ng/ml G-CSF. Subsequently, cells were pelleted and lysed by incubation for 30 min at 4°C in lysis buffer (20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% Nonidet P-40, 10% glycerol, 2 mM Na_3VO_4, 1 mM Pefabloc SC, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml bacitracin and 50 μg/ml iodoacetamide). Insoluble materials were removed by centrifugation at 4°C for 15 min at 15,000*g*. Immunoprecipitations were performed on the clarified cell lysates by incubation overnight at 4°C with anti-Hck antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) were then added for 1 h at 4°C. After washing the beads with ice-cold lysis buffer 4 times, and once with PBS, bound proteins were eluted by boiling for 5 min in sodium dodecyl sulphate (SDS) sample buffer. Following SDS–polyacrylamide gel electrophoresis (SDS–PAGE), proteins were transferred onto nitrocellulose (0.2 μm; Schleicher & Schuell, Dassel, Germany). Filters were blocked by incubation in TBST (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-20) containing 0.6% (w/v) BSA. Antibodies used for Western blotting were anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) and anti-G-CSF-R (Santa Cruz) and were diluted in TBST containing 0.6% (w/v) BSA. After washing with TBST, immune complexes were detected with horseradish peroxidase-conjugated species-specific antiserum (DAKO, Glostrup, Denmark), followed by enhanced chemiluminescence reaction (DuPont, Boston, MA). In some instances, membranes were stripped in 62.5 mM Tris–HCl pH 6.7, 2% SDS and 100 mM β-mercaptoethanol at 50°C for 30 min, reblocked, washed, and reprobed.

Kinase assays. Immunoprecipitation-kinase assays were performed by incubating immunoprecipitate pellets for 20 min at 25°C in 50 μl kinase assay buffer containing 20 mM HEPES pH 7.4, 10 mM $MnCl_2$, 0.2 mM ATP, 0.2 mM NaF, 0.1 mM sodium orthovanadate and 10 μCi (γ-^{32}P)-ATP. In some experiments, 1 μg enolase (Sigma) or GST-G-CSF-R were added as exogenous substrates. Reactions were stopped by the addition of SDS–PAGE sample buffer. Samples were boiled for 5 min and subjected to SDS–PAGE, stained with Coomassie Brilliant Blue R-250 (Bio-RAD Laboratories, Richmond, CA), dried and exposed to X-ray film.

RESULTS

Activation of Hck following G-CSF treatment. Previous studies have shown G-CSF-mediated activation of the Src kinase, Lyn, as well as other non-receptor tyrosine kinases, Jak1, Jak2, Tyk2 and Syk (6–12). However, it was unknown if other kinases might also be involved. As a first step to investigating a potential role for alternate kinases in G-CSF-R signaling, we determined whether other Src-family kinases were activated following G-CSF treatment of murine NFS-60 cells, which have provided a convenient model for the study of signaling pathways from the G-CSF-R (27, 28). Stimulation of NFS-60 cells with G-CSF resulted in a rapid and transient activation of Hck, as shown in an immunoprecipitation-autokinase assay, with a peak of

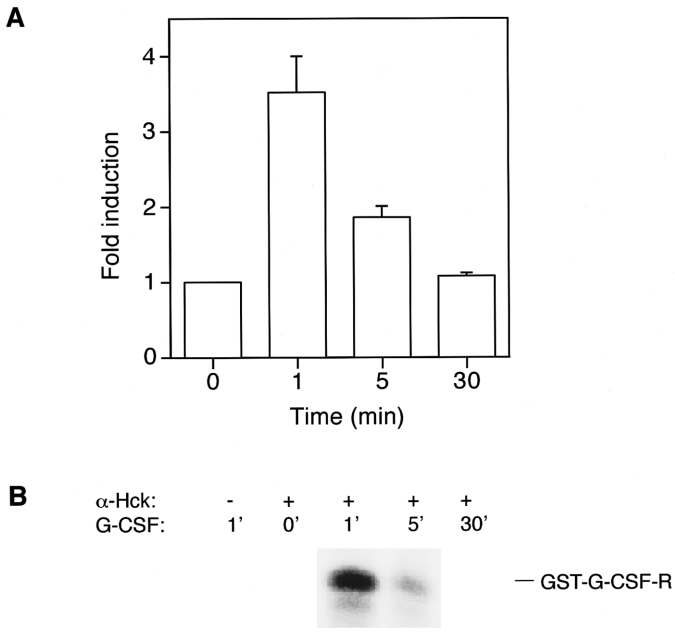

FIG. 1. Time course of G-CSF-dependent activation of Hck and phosphorylation of recombinant G-CSF-R *in vitro*. (A) Quiescent NFS-60 cells were stimulated with 100 ng/ml G-CSF for the indicated times, and cell lysates analyzed for autophosphorylation activity following immunoprecipitation with anti-Hck. Reaction products were separated by SDS–PAGE, with gels stained with Coomassie before drying and autoradiography, with the intensity of radiolabeled p56/p59Hck quantified with a phosphoimager. Values shown are the mean and standard error of three independent experiments. (B) Anti-Hck immunoprecipitates of NFS-60 cell lysates produced as described in Fig. 1A were subjected to an exogenous kinase assay utilizing GST-G-CSF-R as a substrate. These were analyzed by SDS–PAGE, with gels stained with Coomassie before drying and autoradiography. The position of the GST-G-CSF-R is indicated. The gel shown is a representative of three independent experiments.

3.7-fold induction at 1 min (Fig. 1A). While this represents the values for total Hck, the fold-stimulation was similar for each of the isoforms (data not shown). Western blot analysis was used to standardize for equal loading of Hck. In agreement with other studies (11, 12), activation of Lyn was also observed, and with similar kinetics. In contrast, no activation of Src, Fyn or Yes was seen (data not shown).

Phosphorylation of GST-G-CSF-R by Hck in vitro. In preliminary studies aimed at detecting kinases able to phosphorylate the G-CSF-R, we incubated a recombinant GST-G-CSF-R fusion protein with lysates from NFS-60 cells stimulated with G-CSF for different times. Strong, inducible phosphorylation of the fusion protein was observed, following similar kinetics to Hck activation (data not shown), which suggested that at least one of the kinases responsible for G-CSF-R phosphorylation might be Hck. Therefore, we added the GST-G-CSF-R as an exogenous substrate in an anti-Hck immunoprecipitation kinase assay. Under these conditions, the fusion protein was strongly phosphorylated with the same kinetics as Hck autokinase activity (Fig. 1B). This suggests that the G-CSF-R may represent an *in vivo* target of Hck.

Hck and G-CSF-R co-immunoprecipitate following G-CSF stimulation. Since Hck was activated so rapidly by G-CSF treatment, we wondered if it was physically associated with the G-CSF-R complex, as is the case with the gp130 component of the LIF-R complex (16), and the β-subunit of the IL-3 receptor (29). Immunoprecipitation experiments with anti-Hck followed by probing with antiphosphotyrosine antibody revealed major bands at 56/59 kDa, representing Hck. On a longer exposure, the only reproducible banding observed was of a size consistent with the G-CSF-R. Re-probing with anti-G-CSF-R antisera confirmed its identity (Fig. 2). This inducible interaction is in contrast to the constitutive association of another family member, Lyn, to the G-CSF-R (11).

Hck binds directly to the G-CSF-R via its SH2 domain. Given the strong, inducible association of Hck with the G-CSF-R, we sought to determine if this involved a direct interaction, or occurred through an intermediate. To investigate this further, we produced a ^{32}P-labeled, tyrosine-phosphorylated GST-G-CSF-R cytoplasmic fusion protein and, after cleavage to remove the GST-tag, used this to probe isolated domains derived from Hck (Fig. 3A) which were produced in bacteria and immobilised on nitrocellulose. Only constructs containing the Src-homology 2 (SH2) domain of Hck bound to the receptor, with the SH3 and unique (U) domains not required for binding, although the SH3-SH2 construct bound slightly less than either the SH2 or U-SH3-SH2 constructs (Fig. 3B). As controls, neither GST or non-tyrosine-phosphorylated GST-G-CSF-R showed any binding (data not shown). These data demonstrate direct binding of the SH2 domain of Hck with specific phosphotyrosines of the G-CSF-R.

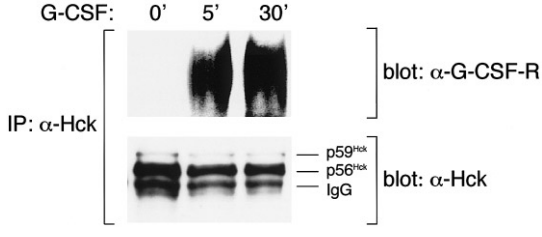

FIG. 2. Co-immunoprecipitation of Hck with the G-CSF-R. Anti-Hck immunoprecipitates of NFS-60 cell lysates produced as described in the legend to Fig. 1A were separated by SDS–PAGE, before transferring to nitrocellulose. The Western blot was hybridized with anti-G-CSF-R, before stripping and reprobing with anti-Hck antibodies to confirm equal loading of Hck. Similar results were obtained in two other experiments.

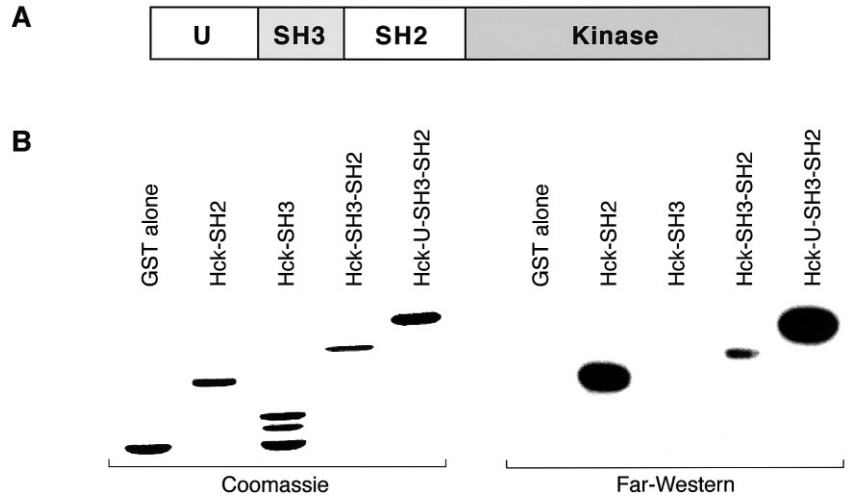

FIG. 3. Analysis of interactions between tyrosine-phosphorylated GST-G-CSF-R and isolated Hck domains. (A) Schematic representation of Hck with domain structure indicated; U: unique region; SH3: Src homology 3 region; SH2: Src homology 2 region; kinase: tyrosine kinase catalytic domain. (B) Purified GST-fusions with the indicated Hck domains were separated by SDS–PAGE and transferred to nitrocellulose before probing with ^{32}P-labeled, tyrosine-phosphorylated GST-G-CSF-R, and subsequent autoradiography. This result was repeated.

This is different to the interaction of Hck with, for instance, the βc subunit of IL-3 receptor, which involves both tyrosine-dependent and independent mechanisms involving the unique, SH3 and SH2 domains (29). Finally, to test the specificity of binding of the Hck SH2 domain, we also examined the SH2 domains from a number of Src family members (Fig. 3B, Fig. 4). In addition to Hck, only Lck showed binding to the phosphorylated G-CSF-R cytoplasmic domain protein.

DISCUSSION

In this report we show recruitment of Hck to activated G-CSF-R in NFS-60 cells, mediated by direct binding via its SH2 domain to multiple phosphotyrosines of the receptor. In addition, we show that Hck becomes activated upon G-CSF treatment and is, in turn, able to phosphorylate the G-CSF-R. This has a number of implications for our understanding of G-CSF signaling.

First, given the array of kinases activated by the G-CSF-R, an important question is which of these are required for G-CSF signaling. In this regard, overexpression and knockout studies have provided important answers. English has shown that enforced expression of constitutively-active Hck completely blocks G-CSF-mediated granulocytic differentiation of 32Dcl3 (21), while a recent paper has shown that Lyn was absolutely required for mitogenic signaling from the G-CSF receptor (12). In the latter report, Lyn-deficient, but not Syk-deficient, cell-lines failed to incorporate thymidine in response to G-CSF, although Jak1 and Jak2 were still activated. In addition, expression of kinase-inactive Lyn, but not kinase-inactive Jak2, also blocked G-CSF-induced thymidine incorporation. This is in agreement with studies on Jak1 and Jak2 knockouts which showed a normal G-CSF response (30, 31), and other reports which failed to find Jak activation in response to G-CSF in human neutrophils (32, 33). However, other studies in Jak-deficient cell lines have indicated that Jak1 is critical with Jak2 and Tyk2

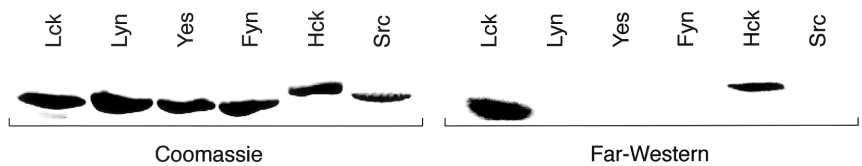

FIG. 4. Far Western blot analysis of tyrosine-phosphorylated GST-G-CSF-R binding to the SH2 domains from different Src family members. Purified GST-SH2 domain fusions were subjected to Far-Western analysis, as described in the legend to Fig. 3B. This result was confirmed in an independent experiment.

fulfilling more minor roles (10). Taken together, these studies suggest that the relative importance of the various kinases which associate with the G-CSF-R may change depending on the cell type, and the cellular response, such that at times a particular kinase is critical, while at other times it is redundant.

Second, Hck clearly fulfills several important roles in the proper functioning of mature cells, especially neutrophils and macrophages. Indeed, its expression increases during granulocytic differentiation of NB4 cells (34) and monocytic differentiation of cell lines (35). In neutrophils it is activated by a range of stimuli, including GM-CSF, β_2 and β_3 integrins, CD63, CD50 (ICAM-3), hypotonic challenge, opsonized zymosan and calcium ionophore (34, 36-40), and is associated with the FcγRI receptor upon receptor engagement (41). Hck also becomes activated by CD45 in macrophages (42). Furthermore, Hck/Fgr deficient mice show defective neutrophil adhesion functions (37), while there is evidence for a direct role for Hck in degranulation (34). These data suggest that Hck is a central player in the activation of these cells. However, other data suggests that Hck fulfills quite a different role in less mature cells, where it is involved in mitogenic signaling. Thus, increased Hck expression increases GM-CSF-mediated proliferative responses (43), while expression in ES cells reduces the concentration of LIF require to maintain them in a proliferative, rather than differentiating state (16). In addition, we have seen expression of Hck in cell-lines which proliferate in response to G-CSF (for example, NFS-60 and Ba/F3 cells), but not in 32D cells, which differentiate in response to G-CSF (data not shown), and, as described earlier, enforced Hck expression blocked granulocytic differentiation in response to G-CSF in these cells (21). This suggests that Hck provides different functions in immature and mature cells, and so could conceivably contribute to both mitogenic and activation signaling from the G-CSF-R depending on cell type.

We have conclusively shown direct binding of Hck via its SH2 domain to tyrosine-phosphorylated G-CSF-R, and phosphorylation of the receptor by Hck *in vitro*. Analysis of the sequences surrounding the four cytoplasmic tyrosine residues of the G-CSF-R can offer some explanation for these observations (Table 1). First, Y763 closely matches the YEEI consensus binding site for Src family members (44), and so is a good candidate for Hck binding. However, when we probed Hck-SH2 blots with a GST-G-CSF-R fusion in which this tyrosine was mutated to phenylalanine, some binding was still seen (data not shown). This suggests that Hck interacts with at least one other phosphotyrosine of the G-CSF-R. It is not obvious from the sequence which is the most likely, since Hck is apparently able to dock to phosphotyrosines in a diverse array of sequence contexts if we consider, for example, its binding to gp130 (16), and the β-subunit of the IL-3 receptor (29). However, Y728 matches the optimal Lck phosphorylation site (I/V/L-Y-G-X-L/V/F/I), and since it is known that Src kinases preferentially phosphorylate peptides recognized by their SH2 domains (45), Y728 represents a likely second site. With regard to the ability of Hck to phosphorylate the G-CSF-R *in vitro*, both Y728 and Y763 fit the Src consensus phosphorylation site (Y-hydrophilic-hydrophilic-hydrophobic), while the sequence adjacent to Y728 is also close to the YGVV of cdc-20, a known Hck substrate (46), and as mentioned above also matches the Lck optimal site.

TABLE 1

Sequence Context of the Four Cytoplasmic Tyrosines of the Murine G-CSF-R

Position	Sequence
703	LVQA**Y**VLQG
728	DQVL**Y**GQVL
743	GVMQ**Y**IRSD
763	SPKS**Y**ENIW

Finally, it was of interest that the Lyn SH2 domain did not bind to tyrosine-phosphorylated G-CSF-R. This is consistent with other data that Src SH2 domains show overlapping, but not identical binding (45, 46). In addition, as discussed earlier, Lyn kinase activity is stimulated by G-CSF in human neutrophils, but Lyn appears to be constitutively-associated with the receptor (11). Thus, it is perhaps not surprising that it does not bind via its SH2 domain, but rather uses other domains to facilitate non-tyrosine-dependent binding. Therefore, it appears that the scenario of Src-kinase activation by the G-CSF-R is similar to that observed with the FcγRIIa receptor, in which Lyn is constitutively-associated with the receptor, while Hck is recruited only after receptor activation (47). This suggests that Lyn might be important for the initial receptor phosphorylation which creates a docking site(s) for Hck. This would not only facilitate the association of Hck with the G-CSF-R complex, but also remove the inhibition of Hck kinase activity as a consequence of intramolecular interactions between its SH2 linker and SH3 domains (48). However, since we found that association of Hck with the G-CSF-R continues well after the peak of Hck kinase activity, additional mechanisms to control Hck activation, rather than simply receptor docking are likely to exist. In addition, we consistently observed that the SH3-SH2 construct bound less efficiently than the SH2 or U-SH3-SH2 constructs. From the crystal structure of Hck, it is known that the SH3 domain interacts with the SH2-linker peptide in a manner to block accessi-

bility to the SH2 domain (48). Since the constructs used contain part of this linker peptide, it is feasible that some residual SH3-linker interaction inhibits the SH2/phosphotyrosine interactions. The addition of the U region must then relieve this inhibition. Therefore, other structural constraints are likely to be important in determining the accessibility of SH2 domains to particular docking sites, as also suggested by others (46, 48).

ACKNOWLEDGMENTS

This work was supported by an EMBO Long-Term Fellowship (A.C.W.) and grants from the National Health and Medical Research Council of Australia, The Nederlandse Organisatie voor Wetenschappelijke Onderzoek (N.W.O.), and the Dutch Cancer Society. The authors gratefully thank Marieke von Lindern for critical reading of the manuscript and Karola van Rooyen for her skillful production of figures.

REFERENCES

1. Fukunaga, R., Ishizaka Ikeda, E., Seto, Y., and Nagata, S. (1990) *Cell* **61,** 341–350.
2. De Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255,** 306–312.
3. Nicola, N. A. (1989) *Annu. Rev. Biochem.* **58,** 45–77.
4. Demetri, G. D., and Griffin, J. D. (1991) *Blood* **78,** 2791–2808.
5. Avalos, B. R. (1996) *Blood* **88,** 761–777.
6. Nicholson, S. E., Oates, A. C., Harpur, A. G., Ziemiecki, A., Wilks, A. F., and Layton, J. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2985–2988.
7. Nicholson, S. E., Starr, R., Novak, U., Hilton, D. J., and Layton, J. E. (1996) *J. Biol. Chem.* **271,** 26947–26953.
8. Barge, R. M., de Koning, J. P., Pouwels, K., Dong, F., Lowenberg, B., and Touw, I. P. (1996) *Blood* **87,** 2148–2153.
9. Novak, U., Ward, A. C., Hertzog, P. J., Hamilton, J. A., and Paradiso, L. (1996) *Growth Factors* **13,** 251–260.
10. Shimoda, K., Feng, J., Murakami, H., Nagata, S., Watling, D., Rogers, N. C., Stark, G. R., Kerr, I. M., and Ihle, J. N. (1997) *Blood* **90,** 597–604.
11. Corey, S. J., Burkhardt, A. L., Bolen, J. B., Geahlen, R. L., Tkatch, L. S., and Tweardy, D. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4683–4687.
12. Corey, S. J., Dombrosky-Ferlan, P. M., Zuo, S., Krohn, E., Donnenberg, A. D., Zorich, P., Romero, G., Takata, M., and Kurosaki, T. (1998) *J. Biol. Chem.* **273,** 3230–3235.
13. Holtzman, D. A., Cook, W., and Dunn, A. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8325–8329.
14. Ziegler, S. F., Marth, J. D., Lewis, D. B., and Perlmutter, R. M. (1987) *Mol. Cell. Biol.* **7,** 2276–2285.
15. Quintrell, N., Lebo, R., Varmus, H., Bishop, J. M., Pettenati, M. J., Le Beau, M. M., Diaz, M. O., and Rowley, J. D. (1987) *Mol. Cell. Biol.* **7,** 2267–2275.
16. Ernst, M., Gearing, D. P., and Dunn, A. R. (1994) *EMBO J.* **13,** 1574–1584.
17. Willman, C. L., Stewart, C. C., Longacre, T. L., Head, D. R., Habbersett, R., Ziegler, S. F., and Perlmutter, R. M. (1991) *Blood* **77,** 726–734.
18. Lichtenberg, U., Quintrell, N., and Bishop, J. M. (1992) *Oncogene* **7,** 849–858.
19. English, B. K., Ihle, J. N., Myracle, A., and Yi, T. (1993) *J. Exp. Med.* **178,** 1017–1022.
20. Durden, D. L., Kim, H. M., Calore, B., and Liu, Y. (1995) *J. Immunol.* **154,** 4039–4047.
21. English, B. K. (1996) *J. Leuk. Biol.* **60,** 667–673.
22. Palacios, R., and Steinmetz, M. (1985) *Cell* **41,** 727–734.
23. Smith, D. B., and Johnson, K. S. (1988) *Gene* **67,** 31–40.
24. Kaelin, W. G., Jr., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., *et al.* (1992) *Cell* **70,** 351–364.
25. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
26. Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschulz, W. H., and McKnight, S. L. (1988) *Genes Dev.* **2,** 801–806.
27. Ward, A. C., Csar, X. F., Hoffmann, B. W., and Hamilton, J. A. (1996) *Biochem. Biophys. Res. Commun.* **224,** 10–16.
28. Csar, X. F., Ward, A. C., Hoffmann, B. W., Guy, G. G., and Hamilton, J. A. (1997) *Biochem. J.* **322,** 79–87.
29. Burton, E. A., Hunter, S., Wu, S. C., and Anderson, S. M. (1997) *J. Biol. Chem.* **272,** 16189–16195.
30. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J.-C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deurson, J. M., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93,** 385–395.
31. Rodig, S. J., Meraz, M. A., White, J. M., Lampe, P. A., Riley, J. K., Arthur, C. D., King, K. L., Sheehan, K. C. F., Yin, L., Pennica, D., Johnson, E. M., and Schreiber, R. D. (1998) *Cell* **93,** 373–383.
32. Shimoda, K., Iwasaki, H., Okamura, S., Ohno, Y., Kubota, A., Arimura, F., Otsuka, T., and Niho, Y. (1994) *Biochem Biophys. Res. Commun.* **203,** 922–928.
33. Avalos, B. R., Parker, J. M., Ware, D. A., Hunter, M. G., Sibert, K. A., and Druker, B. J. (1997) *Exp. Hematol.* **25,** 160–168.
34. Welch, H., and Maridonneau-Parini, I. (1997) *J. Biol. Chem.* **272,** 102–109.
35. Matikainen, S., and Hurme, M. (1994) *Int. J. Cancer* **57,** 98–103.
36. Wei, S., Liu, J. H., Epling-Burnette, P. K., Gamero, A. M., Ussery, D., Pearson, E. W., Elkabani, M. E., Diaz, J. I., and Djeu, J. Y. (1996) *J. Immunol.* **157,** 5155–5162.
37. Lowell, C. A., Fumagalli, L., and Berton, G. (1996) *J. Cell. Biol.* **133,** 895–910.
38. Skubitz, K. M., Campbell, K. D., Iida, J., and Skubitz, A. P. (1996) *J. Immunol.* **157,** 3617–3626.
39. Skubitz, K. M., Campbell, K. D., and Skubitz, A. P. (1997) *J. Immunol.* **159,** 820–828.
40. Krump, E., Nikitas, K., and Grinstein, S. (1997) *J. Biol. Chem.* **272,** 17303–17311.
41. Wang, A. V., Scholl, P. R., and Geha, R. S. (1994) *J. Exp. Med.* **180,** 1165–1170.
42. Roach, T., Slater, S., Koval, M., White, L., McFarland, E. C., Okumura, M., Thomas, M., and Brown, E. (1997) *Curr. Biol.* **7,** 408–417.
43. Linnekin, D., Howard, O. M., Park, L., Farrar, W., Ferris, D., and Longo, D. L. (1994) *Blood* **84,** 94–103.
44. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72,** 767–778.
45. Songyang, Z., Carraway, K. L., III, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. L., Ponder, B. A. J.,

Mayer, B. J., and Cantley, L. C. (1995) *Nature* **373,** 536–539.

46. Sicilia, R. J., Hibbs, M. L., Bello, P. A., Bjorge, J. D., Fujita, D. J., Stanley, I. J., Dunn, A. R., and Cheng, H.-C. (1998) *J. Biol. Chem.*, in press.
47. Ibarrola, I., Vossebeld, P. J., Homburg, C. H., Thelen, M., Roos, D., and Verhoeven, A. J. (1997) *Biochim. Biophys. Acta* **1357,** 348–358.
48. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) *Nature* **385,** 602–609.