Direct Association of Csk Homologous Kinase (CHK) with the Diphosphorylated Site Tyr<sup>568/570</sup> of the Activated c-KIT in Megakaryocytes*

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The Csk homologous kinase (CHK), formerly MATK, has previously been shown to bind to activated c-KIT. In this report, we characterize the binding of SH2<sup>CHK</sup> to specific phosphotyrosine sites on the c-KIT protein sequence. Phosphopeptide inhibition of the in vitro interaction of SH2<sup>CHK</sup>-glutathione S-transferase fusion protein/c-KIT from SCF/KL-treated Mo7e megakaryocytic cells indicated that two sites on c-KIT were able to bind SH2<sup>CHK</sup>. These sites were the Tyr<sup>568/570</sup> diphosphorylated sequence and the monophosphorylated Tyr<sup>721</sup> sequence.

To confirm this, we precipitated native CHK from cellular extracts using phosphorylated peptides linked to Affi-Gel 15. In addition, purified SH2<sup>CHK</sup>-glutathione S-transferase fusion protein was precipitated with the same peptide beads. All of the peptide bead-binding studies were consistent with the direct binding of SH2<sup>CHK</sup> to phosphorylated Tyr<sup>568/570</sup> and Tyr<sup>721</sup> sites. Binding of FYN and SHC to the diphosphorylated Tyr<sup>568/570</sup> site was observed, while binding of Csk to this site was not observed. The SH2<sup>CHK</sup> binding to the two sites is direct and not through phosphorylated intermediates such as FYN or SHC. Site-directed mutagenesis of the full-length c-KIT cDNA followed by transient transfection indicated that only the Tyr<sup>568/570</sup>, and not the Tyr<sup>721</sup>, is able to bind SH2<sup>CHK</sup>. This indicates that CHK binds to the same site on c-KIT to which FYN binds, possibly bringing the two into proximity on associated c-KIT subunits and leading to the down-regulation of FYN by CHK.

The Csk homologous kinase (CHK),<sup>1</sup> previously referred to as megakaryocyte-associated tyrosine kinase (MATK), is a recently identified protein tyrosine kinase found predominantly in human brain and hematopoietic cells (1, 2). The kinase is composed of 527 amino acids and has highest homology (~50% identity overall) with the human Csk, a kinase which is known to phosphorylate the C-terminal tyrosine of SRC family kinases. Such phosphorylation results in the inactivation of these SRC kinases (3–6). It has been shown that CHK is also able to phosphorylate the C-terminal tyrosine of pp60<sup>SRC</sup> (2, 7). Thus, in certain situations, CHK might be a physiological regulator of SRC kinase activity. Like Csk, CHK contains SH3, SH2, and tyrosine kinase domains positioned from the N terminus to the C terminus. Both kinases lack N-terminal myristoylation sites and autophosphorylation sites.

Murine counterparts to CHK have been found by a number of investigators. These have been designated as Ntk, which is cloned from mouse fetal thymus (8), and Ctk, which is cloned from mouse brain (9). The Ntk protein is reported to be of two molecular weights, 52 and 56 kilodaltons, and results from alternate transcriptional splicing, while the Ctk was reported to be of only 52 kilodaltons. It is the 56-kilodalton murine form which most closely resembles human CHK. Human CHK has also been cloned by Sakano et al. (10) from megakaryoblastic leukemia cells and designated HYL. Another human CHK-like form possessing a molecular weight of 57 kilodaltons (designated LSK), which is nearly identical to CHK, was identified in normal killer cells and in activated T cells (11).

We recently found that in CMK cells stimulated by stem cell factor/kit ligand (SCF/KL), CHK associates with tyrosine phosphorylated c-KIT through its SH2 domain (12). c-KIT functions as a growth factor receptor for myeloid and erythroid lineages and promotes the survival of primitive stem cells (13). Mice with defective c-KIT (W) are anemic and deficient in hematopoietic progenitor cells (14).

Since the SRC family kinase FYN is also known to bind to the related PDGF receptor (15), we hypothesized that the binding of CHK by c-KIT would bring CHK into proximity to the bound SRC kinases. In this report, we have characterized the binding of the SH2<sup>CHK</sup> domain to specific tyrosine phosphorylated sites on c-KIT by phosphopeptide inhibition of c-KIT/CHK<sup>SH2</sup>, by binding of SH2<sup>CHK</sup> or native CHK to phosphopeptide beads, and by site-specific mutagenesis of c-KIT cDNA. Interestingly, we also found that the SRC family kinase FYN binds to one of these sites on c-KIT through its SH2 domain. The adapter protein SHC was similarly shown to bind to this site. These findings are in agreement with previous studies on the PDGF receptor (15–17). Our results indicate that SH2<sup>CHK</sup> binds directly to c-KIT at the Tyr<sup>568/570</sup> site and does not bind through intermediates such as FYN or SHC. Thus, it is likely that the biological effect of CHK in hematopoietic cells is through direct, site-specific binding to c-KIT.
Materials—Tyrosine-phosphorylated and nonphosphorylated synthetic peptides were obtained from the Dana Farber Cancer Institute Molecular Biology Core Facility (Boston, MA). Peptides were analyzed for purity by high pressure liquid chromatography, mass spectrometry, and amino acid analysis. SH2^{GSH}-GST fusion protein was prepared as described previously (12). PKA kinase pS8A SH2-GST fusion protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- c-KIT polyclonal antibody was generously provided by Dr. B. Bennett, Amgen Inc. ( Thousand Oaks, CA). Interleukin-3 and GM-CSF were purchased from R & D Systems ( Minneapolis, MN). All other reagents were from Sigma unless otherwise specified. Factor-dependent megakaryocytic Mo7e cells were grown in RPMI 1640 medium ( Mediatech, Washington, D. C.) including 20% fetal bovine serum, 10 ng/ml interleukin-3, 10 ng/ml GM-CSF, 0.1% fetal bovine serum, and 10 μg/ml streptomycin. Prior to stimulation with SCF/KL, cells were starved for 15 h in RPMI medium with 1% fetal bovine serum, 100 IU/ml penicillin, and 10 μg/ml streptomycin.

**EXPERIMENTAL PROCEDURES**

**Construction of Wild Type and Mutant c-KIT Mammalian Expression Vectors—**Site-directed mutagenesis of full-length c-KIT cDNA in the pcDNV vector (19) (a gift of Dr. Yosef Yarden, Weizmann Institute, Rehovot, Israel) was performed using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). In order to change selected tyrosines to phenylalanines, we used the following synthetic oligonucleotides: GAG GAG ATA AAT GGA AAC ATC TTG TTC ATG CAC CCA ACA C (Tyrm → Phe) and GC AGC GAT AGT ACT AAT GAG TTC ATG AGC ATG AAA CTC GGA G (Tytr → Phe). The wild-type and mutant c-KIT inserts were excised from the pcDNV vector with BamHI/XhoI excision enzymes and inserted into pcDNA3 vectors for use in the transient transfection experiments.

**Transient Transfection of Wild Type and Mutant c-KIT Expression Vectors—**Wild type and mutant c-KIT pcDNA3 vectors were co-transfected into COS 7 cells along with a β-galactosidase pSV reporter plasmid (Promega, Madison, WI). For each 10-cm plate of COS 7 cells, 10 μg of c-KIT pcDNA3 plus 5 μg of β-galactosidase pSV DNA were co-transfected using LipofectAMINE reagent (Life Technologies, Inc.). After 5 h of transfection, full medium was added, and cells were continued in culture for another 48–72 h. At this time, cells were serum-starved, stimulated with SCF/KL (10 min, 350 ng/ml), and harvested into lysis buffer (as described in the previous section). Supernatants were then subjected to precipitation by SH2^{GSH}-GST fusion protein as described in the previous section. Glutathione-Sepharose precipitates were centrifuged and transferred to nitrocellulose filters by Western blotting with c-KIT polyclonal antibody. In each case, amounts of extract used in precipitation were normalized for the expression of β-galactosidase as measured by an O-nitrophenyl β-D-galactopyranoside reporter assay (Promega).

**RESULTS**

**Inhibition of SH2^{GSH}/c-KIT Interaction by Tyrosine-phosphorylated Peptides—**In order to identify the tyrosine phosphorylation on c-KIT which is critical for SH2^{GSH} association, we synthesized a series of tyrosine-phosphorylated heptapeptides derived from the cytoplasmic portion of human c-KIT. Since SH2 domains usually associate with residues on the C-terminal side of phosphotyrosine, we chose peptide sequences with tyrosine as the N-terminal residue. We did not include phosphotyrosine sites within the two catalytic domains of c-KIT. These peptides were used to inhibit the interaction of c-KIT from SCF/KL-activated Mo7e cells and the SH2^{GSH} GST fusion protein as described under Experimental Procedures. Complexes of c-KIT and SH2^{GSH} GST were shown by the presence of c-KIT in washed SH2^{GSH} GST precipitates (Fig. 1, A and B). Of the peptides that inhibited complex formation, the most significant was the YMDMKPG peptide (Fig. 1A, lane 10) with approximately 80% inhibition of association at 100 μM peptide. The nonphosphorylated YMDMKPG peptide failed to inhibit complex formation in this assay (Fig. 2, lane 5). We also found a slight inhibition by the peptide YVYIDPT (Fig. 1, lane 9). Recently, it has been reported that analogous peptide sequence in the PDGF receptor is a site for binding of FYN (15) and of SHC (16, 17). In the case of FYN, binding was significantly greater when both tyrosines of the sequence were phosphorylated. We tested whether this would be the case with SH2^{GSH} binding to c-KIT. As shown in Fig. 2, the diphosphorylated peptide YYVYIDPT (lane 1) gave significantly greater inhibition than did the monophosphorylated YVYVYIDPT (lane 2). Furthermore, the monophosphorylated Y{sup IDPT} failed to

three times). SDS sample buffer was added to the washed precipitates, and the samples were subjected to SDS-PAGE followed by transfer to nitrocellulose and immunoblotting as described above with anti-GST as the primary antibody. In order to test binding of native CHK to the peptide beads, 15 μl of beads were incubated with 1 ml of Mo7e extract (20 × 10⁶ cells equivalent) as described in the previous section. Extracts were incubated for 1.5 h at 5°C, with gentle mixing. Precipitates were treated with SDS sample buffer and subjected to SDS-PAGE/Western blotting with anti-CHK or other antibodies, followed by the appropriate secondary antibody and ECL development. Nitrocellulose filters were stripped for reblotting by incubating in 0.2 M glycine, 0.5 M NaCl, pH 2.8. After neutralization with Tris base, the blots were extensively washed with deionized water.
Fig. 1. Inhibition of SH2<sup>CHK</sup>/c-KIT by c-KIT phosphopeptides. Extracts from SCF/KL-stimulated Mo7e cells were added to SH2<sup>CHK</sup>, which was preincubated individually with each of nine different c-KIT phosphopeptides as indicated. The final concentration of peptide was 100 μM for each incubation. Panel A shows Western immunoblotting with PY20 anti-phosphotyrosine antibody. Panel B shows a similar transfer blotted with anti-c-KIT antibody.

Fig. 2. Phosphorylation-dependent inhibition of SH2<sup>CHK</sup>/c-KIT by monophosphorylated Tyr<sup>721</sup> and diphosphorylated Tyr<sup>568/570</sup> peptides. Extracts from SCF/KL stimulated Mo7e cells were incubated with SH2<sup>CHK</sup> along with phosphorylated or nonphosphorylated peptides as indicated above each lane. Western immunoblotting was performed with PY20 anti-phosphotyrosine antibody.

Fig. 3. Concentration dependence of inhibition of SH2<sup>CHK</sup>/c-KIT and SH3-SH2<sup>CHK</sup>/c-KIT by phosphopeptides. Extracts from SCF/KL-stimulated Mo7e cells were incubated with either SH2<sup>CHK</sup>-GST (panels A and C) or with SH3-SH2<sup>CHK</sup>-GST (panels B and D) in the presence of increasing concentrations of Y*VY*IDPT (panels A and B) or Y*MDMKPG (panels C and D). Western immunoblotting was with anti-c-KIT antibody.

Fig. 4. Association of SH2<sup>CHK</sup> to phosphopeptides linked to beads. Panel A, SH2<sup>CHK</sup>-GST fusion protein was incubated with phosphorylated and nonphosphorylated peptides linked to Affi-Gel 15 beads. Washed precipitates were run on SDS-PAGE, and transfers were blotted with anti-GST antibody. Panel B, GST protein alone was incubated with the same phosphorylated and nonphosphorylated peptides linked to beads. As in panel A, washed precipitates were run on SDS-PAGE, and transfers were blotted with anti-GST antibody.

Binding of SH2<sup>CHK</sup> and Native CHK to Immobilized Peptides—In order to further test the binding of CHK to the phosphorylated Tyr<sup>721</sup> and Tyr<sup>568/570</sup> sites, we linked these peptides to Affi-Gel 15 beads, and the association of either SH2<sup>CHK</sup>-GST fusion protein or native CHK from crude extracts of Mo7e cells was analyzed. As is shown in Fig. 4A, SH2<sup>CHK</sup>-GST-GST associated in a phosphotyrosine-dependent manner to Y*MDMKPG and Y*VY*IDPT linked to beads as shown by an anti-GST immunoblot of the precipitates. In a control precipitation, GST alone did not associate with the beads (Fig. 4B). A non-receptor peptide derived from the Tyr<sup>416</sup> phosphorylation site of FYN (IEDNEY*TARQGA) showed very little phosphate-dependent association to SH2<sup>CHK</sup>. We also observed association of SH2<sup>CHK</sup> to the phosphorylated C-terminal tyrosine peptide of SRC kinases (EPQY*QPGENL) (Fig. 4A, lane 8). When we looked at the association of native CHK to peptide beads as shown by anti-CHK immunoblotting, we observed a similar specificity (Fig. 5A). This specificity was in agreement with the peptide inhibition experiments and also indicated that the association was by direct binding of the SH2<sup>CHK</sup> domain to the tyrosine-phosphorylated site(s) on c-KIT.

Possible Association of SH2<sup>CHK</sup> to c-KIT through Other Components—We have shown by peptide inhibition that the PI3-kinase SH2<sup>CHK</sup> binds specifically to the Y*MDMKPG peptide as does SH2<sup>CHK</sup> (data not shown). To confirm that CHK binding to c-KIT is not indirectly via PI3-kinase p85, we stripped and rebotted the transfers from Fig. 1 with anti-p85 subunit antibody (Fig. 6). The distribution of p85 in this blot was similar to the distribution of c-KIT seen in Fig. 1. Thus the lack of p85 in the Y*MDMKPG-inhibited precipitate indicates that both p85 and CHK are competing for the same site. If CHK was binding through p85, inhibition with Y*MDMKPG would disorderly associate only c-KIT from the complex and would retain p85 in the SH3-SH2<sup>CHK</sup>-GST precipitate. In the case of the phosphorylated Tyr<sup>568/570</sup> site, we have demonstrated by immunoblotting that association of crude extracts to peptide beads resulted in not only CHK, but also FYN and SHC binding at this di-phosphorylated site (Fig. 5, B and C). Csk did not associate with the di-phosphorylated site and only slightly associated with the monophosphorylated site (Fig. 5D, left panel). This result was observed in spite of having a large amount of Csk in the crude extract (Fig. 5D, right panel). In order to confirm that the CHK
association at this site was direct, and not through either FYN or SHC, we studied the association of Mo7e extracts to peptide beads with SCF/KL-activated and nonactivated extracts. As can be seen in Fig. 7, there was no observable difference in the association of CHK to the beads when comparing SCF/KL-activated and nonactivated extracts. Thus, it is unlikely that CHK associated with c-KIT through another component which itself was tyrosine-phosphorylated in the course of activation.

Experiments attempting to co-precipitate SHC and CHK were negative (data not shown). Experiments attempting to inhibit the SH2CHK/c-KIT receptor complex with phosphopeptides present in SRC kinases were also negative (data not shown), again emphasizing that FYN and SHC were not intermediaries in CHK/c-KIT binding.

**Effect of Site-directed Mutagenesis of c-KIT on Association with SH2**

In order to confirm the significance of the binding of SH2^{CHK} to the activated c-KIT at the Tyr721 and Tyr^{568/570} sites, we constructed mutant c-KIT cDNAs for either Tyr^{721} → Phe (designated 2162 c-KIT) or Tyr^{568} → Phe (designated 1702 c-KIT) and attempted to demonstrate whether these mutant proteins were still able to associate with the expressed protein in CHK/c-KIT binding.

*FIG. 5. Association of native CHK and other extract components to phosphopeptide beads.* Extracts from SCF/KL-stimulated Mo7e cells were incubated with phosphorylated and nonphosphorylated peptides linked to Affi-Gel 15 beads. Washed precipitates were run on SDS-PAGE. Transfers were immunoblotted with anti-CHK (panel A), anti-FYN (panel B), anti-SHC (panel C), and anti-Csk (panel D). Arrows indicate the presence of the associated proteins.

*FIG. 6. Detection of the PI3-kinase p85 subunit in c-KIT/SH2^{CHK} precipitates inhibited by c-KIT phosphopeptides.* An immunoblot from Fig. 1 containing c-KIT/SH2^{CHK} precipitates inhibited by phosphopeptides was stripped and reblotted with anti-PI3-kinase p85 antibody.

*FIG. 7. Lack of dependence of CHK/c-KIT phosphopeptide association on activation of cells by SCF/KL.* Extracts from Mo7e cells activated by SCF/KL (left 3 lanes of panels A and B) or not activated by SCF/KL (right 3 lanes of panels A and B) were associated with the phosphorylated or nonphosphorylated peptides linked to beads as indicated above each lane. Phosphorylated peptide associations were conducted in duplicate, while nonphosphorylated peptide associations were done singly. Western immunoblotting was performed with anti-CHK antibody; the arrow indicates the position of the CHK protein.

In this report, we have identified the sites of binding of the SH2^{CHK} domain in the activated human c-KIT. Initial studies involving phosphopeptide inhibition indicated that it was phototyrosine antibody, comparable SCF/KL-dependent phosphorylation was observed for wild type and both mutant c-KIT proteins (data not shown). This indicated that the expressed protein is capable of ligand stimulated tyrosine autophosphorylation even with the replacement of the specific tyrosines by phenylalanines. When wild type c-KIT-expressing cell extracts were incubated with SH2^{CHK}-GST, c-KIT associated in an SCF/KL-dependent manner as was observed with endogenous c-KIT (Fig. 8A, lanes 1 an 2). As expected, no c-KIT association was observed in the GST control precipitation (Fig. 8A, lane 8). In these experiments, when SH2^{CHK} was incubated with either of the two mutant c-KIT-expressing extracts, the SCF/KL-activated Tyr^{568} → Phe c-KIT was completely unable to associate (lanes 3 and 4), while the Tyr^{721} → Phe c-KIT still retained the ability to associate with SH2^{CHK}-GST in an SCF/KL-dependent manner (lanes 5 and 6).

**DISCUSSION**

In this report, we have identified the sites of binding of the SH2^{CHK} domain in the activated human c-KIT. Initial studies involving phosphopeptide inhibition indicated that it was
from cell extracts as well as a bacterial SH2<supCHK</sup>-GST fusion protein can bind to phosphorylated peptides linked to beads. This was an unequivocal indication that the CHK/c-KIT interaction was direct and not a result of binding via intermediates in the crude extracts. The possibility of intermediates in the binding was a concern because the sites identified are known to bind other SH2-containing proteins. The Tyr<sup>721</sup> site is known to bind the p85 regulatory subunit of the PI3-kinase (20). In the PDGF receptor, the analogous Tyr<sup>570</sup> juxtamembrane site is known to bind SH2 domains of SHC and SRC family kinases (15–17). The demonstration of direct binding could not completely rule out an additional association via FYN or SHC. Our results of blotting the peptide bead/lysate precipitates with anti-SHC and anti-FYN confirmed that indeed, SHC and FYN were binding to the juxtamembrane phosphorylated Tyr<sup>568/570</sup> sequence of c-KIT. If these or other components were to serve as intermediates or adapters in the binding of SH2<supCHK</sup> to c-KIT, they themselves would have to be phosphorylated. However, nearly all tyrosine phosphorylation occurs after growth factor stimulation. Thus, the fact that there was no difference in association of peptide beads to CHK from SCF/KL-activated or nonactivated cells indicated that, again, an intermediate was unlikely. Therefore, it was reasonable to expect that we would not be able to co-precipitate SHC and SH2<supCHK</sup>, or that SRC phosphorylation site peptides would not interfere with SH2<supCHK</sup>/c-KIT interaction.

It is also notable that blotting the precipitates of extracts and peptide beads with anti-Csk did not show significant binding to the Tyr<sup>568/570</sup> site, but did show some weak binding to the Tyr<sup>721</sup> site. This observation is in spite of the fact that a significant amount of Csk was present in the crude lysates of these cells. Specificity of SH2<sup>Csk</sup> as determined by peptide libraries (19) shows a preference for Y*TKM. The similarity to the site Y*MDMKPG particularly at the +1 and +3 positions may explain the slight binding to this peptide. Csk has been shown to bind to tyrosine-phosphorylated paclitaxin, tensin, and focal adhesion kinase (FAK) (21, 22). This emphasizes the role of Csk in the regulation of SRC kinases located in focal adhesions. There have been no reports in the literature of Csk having the ability to bind to growth factor activated receptor tyrosine kinases. Thus, it is more likely that SRC family kinases bound to growth factor receptors such as c-KIT are phosphorylated and down-regulated by CHK and not Csk.

The implication of the c-KIT site directed mutagenesis is that in the association of SH2<supCHK</sup> to intact c-KIT, only the Tyr<sup>568/570</sup> site is effectively able to bind while the Tyr<sup>721</sup> site is not able to bind this SH2. This is in apparent contradiction to the data involving phosphopeptide inhibition and direct binding of CHK to phosphopeptide beads. A possible explanation for this is that the heptapeptides used in the experiments do not accurately reflect the 3-dimensional structure of the native c-KIT. Thus, the Y*MDMKPG peptide may resemble the Y*VY*IDPT peptide sufficiently such that it could compete for the Tyr<sup>568/570</sup> site. It may also be that this Y*MDMKPG peptide linked to beads, is able to bind to the SH2<supCHK</sup> even though the native c-KIT is unable to bind to SH2<supCHK</sup> at the Tyr<sup>721</sup> site. The mutagenesis experiment on the other hand does not rely on peptides to distinguish the binding and more accurately reflects the specificities of the protein-protein interactions. Furthermore, the mutation experiment indicates that there are no other sites on c-KIT that are able to bind the SH2<supCHK</sup> since binding was completely abolished by the mutation at the Tyr<sup>568</sup> site. Thus, the data taken together indicate that it is only the Tyr<sup>568/570</sup> site that is able to bind CHK through its SH2 domain.

A potential model for the regulation of SRC kinase by CHK
involves the SCF/KL stimulated dimerization of c-KIT, one bearing an activated SRC kinase and the other bearing the CHK kinase. This dimerization would bring activated SRC kinase into proximity to CHK, leading to the down-regulation of SRC kinase activity. Further studies will be needed to establish whether SRC kinase bound to c-KIT is the primary target for CHK, and to elucidate the downstream effects resulting from these interactions.

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