Crkl Is Constitutively Tyrosine Phosphorylated in Platelets From Chronic Myelogenous Leukemia Patients and Inducibly Phosphorylated in Normal Platelets Stimulated by Thrombopoietin

By Atsushi Oda, Yoshitaka Miyakawa, Brian J. Druker, Akaru Ishida, Katsutoshi Ozaki, Hideya Ohashi, Masatoshi Wakui, Makoto Handa, Kiyoko Watanabe, Shinichiro Okamoto, and Yasuo Ikeda

Platelet functions such as aggregation and clot retraction are often abnormal in chronic myelogenous leukemia (CML) patients. However, the molecular mechanisms of these altered functions are unknown. As expression of the p210(bcr-abl) oncoprotein, a constitutively active tyrosine kinase, is known to have an essential role in the pathogenesis of CML and tyrosine phosphorylation is intimately involved in various aspects of platelet activation, we examined the pattern of protein tyrosine phosphorylation in platelets from 15 CML patients by immunoblotting with a monoclonal antiphosphotyrosine antibody (4G10). Before and after stimulation with thrombin, the only consistent difference between normal and CML platelets was the presence of a tyrosine phosphorylated protein with a relative molecular weight of 39 kD. This tyrosine phosphorylated protein was identified as crkl, an SH2, SH3 containing adapter protein. Thus, as previously demonstrated for neutrophils from CML patients, tyrosine phosphorylation of p39crkl persists in mature platelets. No tyrosine phosphorylation of crkl was detected following stimulation with thrombin in normal platelets. However, crkl became incorporated into the Triton X-100 insoluble residue following thrombin stimulation in a manner dependent on platelet aggregation. Further, we found that crkl is an endogenous substrate for calpain, a protease that may be involved in postaggregation signaling processes. This suggests that crkl may be involved in the reorganization of the cytoskeleton during normal platelet aggregation and its tyrosine phosphorylation in CML platelets may contribute to the abnormal platelet function in CML patients. Finally, we found that thrombopoietin induces tyrosine phosphorylation of crkl in normal platelets and FDCP cells genetically engineered to express human c-Mpl. This suggests that crkl can be phosphorylated by a kinase other than p210(bcr-abl) and that crkl may have a role in signaling by thrombopoietin.

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HRONIC MYELOGENOUS leukemia (CML) is characterized by the presence of the Philadelphia (Ph') chromosome, which arises as a result of a specific translocation, t(9;22).12 This translocation leads to the juxtaposition of the abl proto-oncogene and the bcr gene, which generates a chimeric bcr-abl mRNA, and ultimately a 210-kD protein (p210bcr-abl).13 p210bcr-abl has elevated tyrosine kinase activity, which may be essential for the pathogenesis of CML, partially through the dysregulation of protein tyrosine phosphorylation in a pluripotent stem cell.14

Clinically, CML is characterized by a stable phase in which there is a massive expansion of myeloid cells. At least half of the patients will also have an elevated platelet count. This underlies the defect in CML being in a pluripotent stem cell and the finding of the Ph' chromosome in cells of the myeloid, erythroid, and megakaryocytic lineage.

Platelet functions such as aggregation, expression of procoagulant surfaces, and clot retraction are often abnormal in CML patients.3-5 However, the molecular mechanism of the platelet dysfunction is unknown. Because protein tyrosine phosphorylation is intimately involved in various aspects of platelet activation,6-12 we examined protein tyrosine phosphorylation in platelets from CML patients. Here, we report that crkl, a recently cloned SH2/SH3 adapter protein,15-17 is present in human platelets and is constitutively tyrosine phosphorylated in platelets from CML patients. Further, we found that crkl became incorporated into the Triton X-100 insoluble residue in a manner dependent on platelet aggregation, suggesting that crkl may participate in the reorganization of the cytoskeleton during and after aggregation. Further, like many proteins incorporated into the Triton X-100 insoluble residue, including glycoprotein IIIa, p60c-Kit, tafin, and actin binding protein, we found that crkl is an endogenous substrate for calpain.18-24

Further, we show that thrombopoietin induces tyrosine phosphorylation of crkl in normal platelets and FDCP-hMPL5 cells,11 which were genetically engineered to express human c-Mpl. This suggests that crkl can be phosphorylated in the absence of p210bcr-abl and that crkl may have a role in signaling by thrombopoietin.

MATERIALS AND METHODS

Materials. Prostaglandin E1 (PGE1), A23187, dibucaine, diethyl sulfoxide (DMSO), epinephrine, aspirin, apyrase (type VIII), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), sodium dodecyl sulfate (SDS), 2-mercaptoethanol, sodium orthovanadate, chicken egg albumin, protein A-Sepharose, Triton X-100, and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St Louis, MO). Polyvinylidene difluoride (PVDF) membranes (pore size, 0.45 mm) were from Millipore Corp (Bedford, MA). SDS-PAGE molecular standards were from Amersham (Ar-

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Crkl AND PLATELETS

Table 1

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Fig 1. A novel tyrosine phosphorylated protein in platelets from CML patients. Normal platelets and those from CML patients were lysed by the addition of an equal amount of 2× SDS-sample buffer before and after exposure to thrombin (1 U/mL) for the indicated time. After boiling for 5 minutes, proteins were separated by 10% or 12% SDS-PAGE and transferred onto nitrocellulose membranes. Tyrosine phosphorylated proteins were detected with 4G10 (a monoclonal anti-phosphotyrosine antibody). Bands were visualized using alkaline phosphatase-conjugated second antibody and NBT/BCIP. The arrows indicate the relative position of the novel band found in CML patients. These three samples are representative of the results from 15 CML and 10 normal controls. (A) Lane 1, resting normal human platelets. Lanes 2 and 3 normal human platelets 15 seconds, 1 minute after stimulation with thrombin (1 U/mL). Lane 4, resting CML platelets. Lanes 5 and 6, CML platelets 15 seconds, 1 minute after stimulation with thrombin (1 U/mL). (B) Lanes are the same as in (A). (C) Lane 1, resting CML platelets. Lanes 2 to 4, CML platelets 15 seconds, 1 minute, 5 minutes after stimulation with thrombin (1 U/mL). Lane 5, resting normal human platelets. Lanes 6 to 8, normal human platelets 15 seconds, 1 minute, 5 minutes after stimulation with thrombin (1 U/mL).

Platelet preparation. Blood from 15 CML patients and 10 healthy volunteers was drawn by venipuncture into 1/10 volume of 3.8% (wt/vol) trisodium citrate and gently mixed. Before drawing blood, informed consent was obtained. All 15 patients were BCR-ABL positive as demonstrated by reverse transcriptional polymerase chain reaction (RT-PCR) on peripheral blood samples. Two patients were negative for Ph' by cytogenetic study. Platelet counts were from 2.6 × 10^9 cells/mL to 6.7 × 10^9 cells/mL (mean, 4.6 × 10^9 cells/mL). Platelet-rich plasma (PRP) was prepared by centrifuging the whole blood at 200g for 20 minutes and aspirating PRP. PRP was incubated with aspirin (2 mmol/L) for 30 minutes at room temperature. PGE_2 (1 μmol/L) was added from a stock solution in absolute ethanol (1 mmol/L). The PRP was spun at 800g to form a soft platelet pellet. The pellet was resuspended in 1 mL of a modified HEPES-Tyrode buffer (129 mmol/L NaCl, 8.9 mmol/L NaHCO_3, 0.8 mmol/L KH_2PO_4, 0.8 mmol/L MgCl_2, 5.6 mmol/L dextrose, and 10 mmol/L HEPES, pH 7.4) also containing apyrase (2 U/mL) and was washed twice with this buffer. Platelets were resuspended in the same buffer at a concentration of 3 × 10^9 cells/mL with apyrase (2 U/mL) containing 1 mmol/L CaCl_2 at 37°C. The contaminating white blood cells were counted and were regularly less than 0.01% of platelets. If the contaminating white cell count exceeded this value, the cell suspension was centrifuged at 200g for 5 minutes until this value of the supernatant was less than 0.01%.

Gel electrophoresis and Western blotting. Platelet stimulation was terminated by the addition of an equal volume of 2 times concentrated Laemmli's sample buffer (10% glycerol, 1% SDS, 5% 2-
TBST. Antibody reactions were detected with NBT/BCIP or chemiluminescence according to the manufacturer’s instructions.

**Immunoprecipitation.** Cells were lysed by the addition of an equal amount of 2× SDS-sample buffer. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After the transfer, the PVDF membrane was cut in the middle of lane 3. The left half was probed with 4G10 and the right half with antiannexin II. Bands were visualized using alkaline phosphatase-conjugated second antibody and NBT/BCIP. Lanes 1 and 5, platelets from a CML patient (1.5 × 10⁶ cells/lane). Lanes 2 to 4, K562 cells (3 × 10⁶ cells/lane). The (*) indicates the relative migration of the novel 39-kD tyrosine phosphorylated protein found in CML platelets.

**Probe: Anti-PY Anti-Annexin II**

Fig 2. The novel tyrosine phosphorylated protein in platelets from CML patients comigrates with the most salient tyrosine phosphorylated protein in K562 cells, but not with annexin II. Platelets were lysed by the addition of an equal amount of 2× SDS-sample buffer. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After the transfer, the PVDF membrane was cut in the middle of lane 3. The left half was probed with 4G10 and the right half with antiannexin II. Bands were visualized using alkaline phosphatase-conjugated second antibody and NBT/BCIP. Lanes 1 and 5, platelets from a CML patient (1.5 × 10⁶ cells/lane). Lanes 2 to 4, K562 cells (3 × 10⁶ cells/lane). The (*) indicates the relative migration of the novel 39-kD tyrosine phosphorylated protein found in CML platelets.

**Fig 3.** Anti-crkl antisera recognize the same protein in lysates from normal human platelets and K562 cells. Platelets were lysed by the addition of an equal amount of 2× SDS-sample buffer. After boiling for 5 minutes, proteins were separated by SDS-PAGE and transferred onto PVDF membranes. crkl was detected using a specific antibody. Right lane, K562 cells (10⁶ cells/lane); left lane, resting normal human platelets (1.5 × 10⁶ cells/lane).
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RESULTS

Using anti-phosphotyrosine immunoblotting, we compared the pattern of phosphotyrosine-containing proteins in platelets from 15 CML patients with normal controls. As shown in Fig 1, the only consistent difference was the presence of a 39-kD tyrosine phosphorylated protein in platelets from the CML patients. The degree of tyrosine phosphorylation of the 39-kD protein did not seem to be affected by thrombin stimulation. In platelets from normal controls, there was no comigrating tyrosine phosphorylated protein even after stimulation with thrombin. The difference observed was not due to contaminating neutrophils, as these represented less than 0.01% of the platelets used and a corresponding number of neutrophils did not yield a detectable signal (data not shown).

A previous report suggested that platelets from CML patients express annexin II, unlike those from normal controls, and annexin II was strongly phosphorylated in platelets from CML patients and in K562 cells, a Phi positive cell line. Comparing the pattern of tyrosine phosphorylated proteins of K562 cells and CML platelets, we found that the most prominently tyrosine phosphorylated 39-kD protein in K562 cells comigrated with the 39-kD tyrosine phosphorylated protein of CML platelets (Fig 2). Although the annexin II antisera detected a protein in lysates of K562 cells, we were unable to detect annexin II in CML platelet samples. In addition, the 39-kD tyrosine phosphorylated protein in CML platelets and the comigrating prominently tyrosine phosphorylated protein in lysates of K562 cells did not comigrate with the annexin II band detected in lysates of K562 cells (Fig 2).

Recent studies have demonstrated that the prominently tyrosine phosphorylated 39-kD protein in K562 cells is crkl, an SH2/SH3 adaptor protein.
A

1 2 3 4 5 6 7

M. W. (kDa)

- 46

- crkl

- 30

B

1 2 3 4 5 6 7

M. W. (kDa)

- 200

- GP IIbα

- 97.4

Fig 5. Association of crkl (A) and GPllb (B) with the Triton X-100 insoluble residue. Normal human platelets were lysed with Triton X-100-EGTA buffer before or after stimulation with thrombin (1 U/mL) with or without stirring. Lysates were separated into soluble and insoluble fractions. Proteins from each fraction were separated by 10% SDS-PAGE and then exposed to (A) crkl antisera or (B) anti-GPllb monoclonal antibody. Lane 1, Triton X-100 soluble residue of resting cells (0.75 x 10⁶ cells). Lane 2, Triton X-100 insoluble residue of resting cells (13.0 x 10⁶ cells). Lanes 3 to 6, Triton X-100 insoluble residue from 3.0 x 10⁶ cells, 15 seconds, 1 minute, 5 minutes and 10 minutes after exposure to thrombin (1 U/mL) with stirring. Lane 7, Triton X-100 insoluble residue of cells (3.0 x 10⁶ cells), stimulated for 10 minutes with thrombin without stirring. It should be noted, we added EGTA and EDTA before lysis of platelets to prevent artificial cleavage.²³

To determine whether crkl is tyrosine phosphorylated in CML platelets, immunoprecipitation of crkl followed by antiphosphotyrosine immunoblotting was performed. As demonstrated in Fig 4, crkl is tyrosine phosphorylated in CML platelets, but not platelets from normal controls. As previously reported,¹³-¹⁵ the phosphorylated crkl migrates more slowly than the unphosphorylated form (Fig 4A). As has been described for neutrophils, we could not detect expression of p210⁷ BCR-ABL (Fig 4B).

Although the function of crkl in normal hematopoietic cells is unknown, it has been shown that many signaling molecules including p60⁷ src become incorporated into the integrin rich cytoskeleton in a manner dependent on platelet aggregation.⁷,¹² Therefore, we tested the possibility that crkl may also be incorporated into the cytoskeleton during platelet aggregation. In resting platelets, most of the crkl was present in the supernatant fraction and crkl became associated with the Triton X-100 insoluble residue after stimulation of platelets with thrombin with stirring to induce platelet aggregation (Fig 5A). The association was inhibited by omitting stirring to minimize platelet aggregation (Fig 5A, lane 7), as reported for p60⁷ src and αIIbβ3 (Fig 5B).⁷,¹²

Activation of the proteolytic enzyme, µ-calpain, is known to occur during platelet aggregation.¹⁸-²⁴ The activation of µ-calpain is accompanied by cleavage of the enzyme, although it is unclear whether cleavage is necessary for activation.¹⁸,¹⁹ Thus, calpain is activated and cleaved in platelets stimulated by thrombin with stirring to induce platelet aggregation. When stirring is omitted to minimize aggregation, calpain is not activated. Calpain can also be activated by treatment of platelets with A23187 and dibucaine. EGTA inhibits the activation induced by A23187, but not by dibucaine, consistent with their requirement or lack of requirement for extracellular calcium,¹⁸-²⁵ whereas calpeptin, a soluble peptide inhibitor of calpain, inhibits activation by various stimuli.²⁴ Under all of the above conditions where calpain was activated, there was a significant loss of crkl immunoreactivity, suggesting that crkl is a substrate for calpain. The amount of GPIIbα was unaffected, suggesting that the loss of crkl was specific (Fig 6C).

We and others reported that treatment of human platelets or other cells expressing c-Mpl, a thrombopoietin receptor, with thrombopoietin led to tyrosine phosphorylation of several intracellular proteins with relative molecular weights of 130 to 120, 95, 85, and 52 kD.¹⁰,²⁸-³⁵ Using specific antisera, we have identified Jak2, Tyk2, Shc, Stat3, and Stat5 as five of the proteins that were inducibly tyrosine phosphorylated in platelets in re-
Fig 6. Cleavage of calpain and crkl in human platelets. Cleavage of the 80-kD subunit of \( \mu \)-calpain in human platelets. Platelets were incubated with either calpeptin or DMSO (vehicle of calpeptin, final 0.1%) for 5 minutes. Platelets were then treated with A23187 (1 \( \mu \)mol/L, for 5 minutes), or dibucaine (1 mmol/L, for 15 minutes) in the presence 1 mmol/L CaCl\(_2\) or 5 mmol/L EGTA. Whole platelet lysates (1.5 \( \times \) 10\(^7\) cells/lane) were analyzed by 10% SDS-PAGE. Separated proteins were electrophoretically transferred from the gel onto nitrocellulose membranes. Calpain (A) or crkl (B) was detected by Western blotting with specific antibodies. The arrow indicates the relative position of the intact 80 kD subunit of \( \mu \)-calpain (A) or 39 kD crkl (B).

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Lane 1, control, DMSO (final 0.2%); lane 2, A23187 (1 \( \mu \)mol/L, for 5 minutes) + 1 mmol/L CaCl\(_2\); lane 3, A23187 (1 \( \mu \)mol/L, for 5 minutes) + 5 mmol/L EGTA; lane 4, A23187 (1 \( \mu \)mol/L, for 5 minutes) + 1 mmol/L CaCl\(_2\) + calpeptin (20 \( \mu \)mol/L); lane 5, dibucaine (1 mmol/L, for 15 minutes) + 1 mmol/L CaCl\(_2\); lane 6, dibucaine (1 mmol/L, for 15 minutes) + 5 mmol/L EGTA; lane 7, dibucaine (1 mmol/L, for 15 minutes) + 1 mmol/L CaCl\(_2\) + calpeptin (20 \( \mu \)mol/L).

Response to thrombopoietin (concentration, 1 to 100 ng/mL). Thus, platelets express a functional thrombopoietin receptor capable of generating a biochemical response and transmitting a signal. Accordingly, we examined whether crkl became tyrosine phosphorylated following treatment of platelets with thrombopoietin. Platelets, treated with thrombopoietin (100 ng/mL) for various times, were lysed in a buffer containing 1% Triton X-100 and immunoprecipitated with specific crkl antisera. The same amount of a 39-kD protein recognized by crkl antisera was immunoprecipitated from all cellular lysates (Fig 7A). Following treatment with thrombopoietin, this same protein was tyrosine phosphorylated (Fig 7A). Thus, crkl becomes tyrosine phosphorylated following thrombopoietin treatment of human platelets. The crkl immunoprecipitates also contained a protein with a molecular weight of approximately 100 kD, which was inducibly tyrosine phosphorylated in platelets, suggesting that this protein is associated with crkl (Fig 7A). We have been unsuccessful in our attempts to identify the protein.

To further confirm the link between thrombopoietin stimulation and crkl tyrosine phosphorylation, we examined whether crkl was tyrosine phosphorylated in FDCP-2 cells expressing human c-Mpl (FDCP-hMPL5). FDCP-hMPL5 cells were treated with thrombopoietin (100 ng/mL) for various times and were lysed in a buffer containing 1% Triton X-100. The same amount of a 39-kD protein recognized by crkl antisera was immunoprecipitated from all cellular lysates (Fig 7B, lower panel). This same protein became tyrosine phosphorylated after treatment with thrombopoietin (Fig 7B, upper panel).

DISCUSSION

In this study, we immunologically identified crkl in human platelets and demonstrated that this protein is constitutively tyrosine phosphorylated in platelets from CML patients, but not normal controls. There are no other obvious differences in the pattern of phosphotyrosine-containing proteins between CML and normal platelets. Although Shibata et al.

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GP IIba (upper panel) or crkl (lower panel) were detected by Western blotting as described in Figs 5 and 6B.

CML platelets and K562 cells. Indeed, this prominently tyrosine phosphorylated protein was recently identified as crkl by three independent groups. Although we cannot rule out the possibility that another kinase is activated by bcr-abl expression and its activation persists. Perhaps this could be the same kinase responsible for the tyrosine phosphorylation of crkl induced by thrombopoietin stimulation.

The function of crkl is unknown. Our data suggest that crkl may have a role in the reorganization of the cytoskeleton during platelet aggregation. Further, we found that crkl is an endogenous substrate for calpain and is cleaved during aggregation. In view of the recent report that integrin β3 subunit is also cleaved during platelet aggregation, it is possible that crkl, β3, and calpain may colocalize during platelet aggregation and that crkl and β3 are cleaved by the same protease. Although the degree of cleavage of crkl in aggregated platelets seems to be small, this is generally true for most of the substrates for calpain in platelets. For example, most of the talin or actin binding protein are cleaved in platelets treated with calcium ionophores or dibucaine in the presence of extracellular calcium ion. On the other hand, during platelet aggregation, the maximum degree of cleavage of them is less than 10%. However, it has been suggested that this small degree of cleavage may have a role in the shedding of procoagulant microparticles from platelets.

Interestingly, similar to CML platelets, granulocyte from CML patients have defective chemotaxis and adherence. The molecular basis for the defect is poorly understood. However, it has been reported that crkl may bind to a focal adhesion protein, paxillin. Because persistent tyrosine phosphorylation of crkl is a common feature for both platelets and granulocyte, it is attractive to speculate that constitutively tyrosine-phosphorylated crkl may contribute to the impaired adhesive capacity of these cells. Recently, crkl was shown to be associated with C3G, a guanine nucleotide exchange factor for rap proteins in T cells. Whether platelets express C3G or not is unknown. However, rap1B is abundantly expressed in platelets and redistributes to the cytoskeleton during platelet aggregation. Thus, it is possible that crkl may have a role in the regulation of the functions of small molecular GTPase proteins, like rap1B, in platelets. crkl may also have a role in various signal transduction systems through its inducible association with p120Cbl. Although platelets express and become tyrosine phosphorylated following treatment with thrombopoietin, we have been unable to detect an association between p120Cbl and crkl. Rather, crkl appears to associate with an unidentified 100-kD tyrosine phosphorylated protein.

Another more intriguing function of crkl has been suggested by the observation of thrombopoietin-stimulated...
Tyrosine phosphorylation of crkl in normal platelets as well as in FDCP-hMPL5 cells (Fig 7). This observation indicates that crkl can be tyrosine phosphorylated in the absence of p210BCR-ABL and this also suggests a possible role for crkl in normal megakaryopoiesis/thrombopoiesis, as thrombopoietin is essential for these processes. Thus, tyrosine phosphorylation of crkl is not limited to the pathological conditions like CML. We and others have also shown that similar to tyrosine phosphorylation induced by thrombopoietin in platelets, the treatment of platelets with thrombopoietin primes platelets for aggregation. Thus, it is also possible that crkl, which we
have shown here to be inducibly tyrosine phosphorylated following thrombopoietin stimulation, may have a role in priming platelets for aggregation.

ACKNOWLEDGMENT

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