Human leukemic cell line K562 is induced to differentiate into the megakaryocytic lineage by stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA). We demonstrate here that TPA stimulation increases tyrosine phosphorylation of an 80-kDa protein at an early stage of megakaryocytic differentiation and that this 80-kDa protein is identical with cortactin. Since tyrosine kinase Syk was activated by TPA stimulation, we examined the possibility that cortactin is a potential substrate of Syk in K562 cells. TPA-induced tyrosine phosphorylation of cortactin was decreased profoundly by overexpression of dominant-negative Syk. Furthermore, cortactin was associated with Syk even before TPA stimulation. Since cortactin was previously referred as an 80-85-kilodalton pp60CSK substrate, we examined the association between Src and cortactin, whereas its association could not be detected. These data suggest that Syk phosphorylates cortactin in K562 cells upon TPA treatment.

The generation of functional cells of the hematopoietic system is a complex process requiring both the constant production of large numbers of differentiated cells and the maintenance of primitive precursor cells. As model systems to investigate the mechanisms of hematopoietic differentiation, several hematopoietic cell lines that can be induced to various cell lineages have been used. In these cell lines, K562 cells, established from a patient with chronic myeloid leukemia in blast crisis (1), have a potential to differentiate into a variety of hematopoietic cell lineages (2–9). Several lines of evidence indicate that this cell line can be differentiated into megakaryocytic lineage by stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) (6, 10). The expression of platelet glycoprotein IIIa (GP IIIa) and thromboxane A2 receptor, which have been used as a marker of megakaryocytic differentiation, is strongly enhanced on the surface of K562 cells upon treatment with TPA (11, 12). Furthermore, during TPA induction, nuclear DNA ploidy of these cells is increased to 4–16n simultaneously with an increase in cell volume (13, 14). K562 cells can also be induced to undergo erythroid differentiation by various compounds, including hemin (2). When K562 cells are treated by hemin, the transcription of c-ř, e-, γ-, and α-globin mRNA is increased (15, 16) and hemoglobin is accumulated (2, 17).

Evidence has been accumulating that protein tyrosine phosphorylation and dephosphorylation play important roles in a variety of processes, leading to cell growth and differentiation in hematopoietic cells. Indeed, K562 cells transfected with c-řes, one of the non-receptor-type protein-tyrosine kinases, undergo myeloid differentiation (18). In contrast, erythroid differentiation of these cells can be induced by herbimycin A, an inhibitor of tyrosine kinase (19). A non-receptor-type protein-tyrosine kinase Syk is expressed in almost all the hematopoietic cells. Although the functions of Syk in mast cell, B cell, and platelet activation have been elucidated extensively (20), its roles in hematopoietic cell differentiation remain to be addressed. We found here that an 80-kilodalton (kDa) protein, cortactin, is tyrosine-phosphorylated in K562 cells upon TPA treatment, and cortactin associates physically and functionally with Syk. These findings suggest that Syk mediates tyrosine phosphorylation of cortactin at an early stage of megakaryocytic differentiation in K562 cells.

**EXPERIMENTAL PROCEDURES**

Cell Culture—K562 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Passages were performed every 3 days when the cells were in exponential phase of growth. The cells were induced to differentiation by adding 10 nM TPA and harvested at the indicated times. The differentiation of stimulated cells was confirmed by Giemsa staining.

Antibodies—Antibody (Ab) against porcine Syk was generated by immunizing rabbit with synthesized peptides as described (21). The sequence of synthesized peptides to generate anti-porcine Syk Ab is not conserved in human Syk; so this Ab reacts specifically with porcine Syk. Monodonal Ab (mAb) against human Syk (101) was obtained from Wako Chemicals, Tokyo, Japan. Anti-human retinoblastoma protein (Rb) mAb (G99–2005) was purchased from Pharmingen. Anti-phosphotyrosine mAb (4G10), anti-cortactin mAb (anti-p80/85 pp60CSK substrate, 4F11), and anti-Src mAb (GD11) were purchased from Upstate Biotechnology, Inc.

Generation of Dominant-negative Mutant—A point mutation (Lys395→Arg) in the ATP binding site of porcine syk cDNA was created by polymerase chain reaction as described (22), and this mutated cDNA was inserted into the EcoRI site of pApuro vector, harboring the chicken actin promoter and puromycin-resistant gene. This plasmid was linearized and transfected into human hematopoietic cell line K562 cells by electroporation using Electroporator II (Milnuscience) at 300 V, 1000 microfarads, and selected in the presence of 1.0 µg/ml puromycin. Cells
A report that cortactin is tyrosine-phosphorylated after stimulation of platelets with thrombin (23), together with the observation that this stimulant activates Syk (21), prompted us to examine the involvement of Syk in TPA-induced tyrosine phosphorylation in K562 cells. We first examined whether TPA activates Syk in this cell line or not. After TPA stimulation, cells were solubilized with Nonidet P-40 lysis buffer at the indicated times, and Syk was immunoprecipitated by anti-human Syk mAb. Then, in an in vitro kinase assay was performed with an exogenous substrate H2B histone. Syk kinase activity was increased within 1 min and reached to a maximum at 5 min after TPA stimulation (Fig. 3A). The amount of precipitated Syk did not change throughout the time course judging from the immunoblot analysis with anti-human Syk mAb (Fig. 3B).

To address the relation between Syk activation and phosphorylation of cortactin in TPA-treated K562 cells, cell lines expressing a dominant-negative mutant form of Syk were established. A point mutation was created in the ATP binding site of porcine Syk cDNA, leading to loss of its kinase activity, Syk(K225A) (22). This mutated cDNA was transfected into K562 cells, and stable transformants were isolated in the presence of puromycin. Expression of mutated porcine Syk was examined by immunoblot analysis with anti-Syk Ab which recognizes only porcine species, and 4 stable transformants were cloned. Fig. 4A shows the expression of mutated porcine Syk in 2 clones of Syk(K225A) expressing cells. To examine whether overexpression of Syk(K225A) affects tyrosine phosphorylation in K562 cells, whole cell lysates of stimulated and unstimulated cells were subjected to immunoblot analysis with anti-phosphotyrosine mAb. In parental K562 cells, an 80-kDa protein was tyrosine-phosphorylated by TPA stimulation, whereas in 4 transformants expressing Syk(K225A), this induction was abolished. Fig. 4B shows tyrosine phosphorylation of the 80-kDa protein in parental K562 cells and 2 clones of Syk(K225A) expressing cells. To confirm that this 80-kDa protein is cortactin, cell lysates from parental K562 cells and transformant expressing Syk(K225A) were immunoprecipitated by anti-cortactin Ab and probed with anti-phosphotyrosine mAb (Fig. 4C). Consistent with a whole cell lysate data, TPA-induced tyrosine phosphorylation of cortactin was not observed in transformant expressing Syk(K225A). These data demonstrate a strong correlation between Syk activity and TPA-induced tyrosine phosphorylation of cortactin.

To get insights of whether Syk directly or indirectly phosphorylates cortactin upon TPA treatment, co-immunoprecipitation experiments were performed. K562 cells were stimulated and Syk was immunoprecipitated as described in Fig. 3 with a modification that digitonin lysis buffer was used to solubilize cells instead of Nonidet P-40 lysis buffer. Immunoprecipitates were subjected to immunoblot analysis with anti-cortactin mAb. As shown in Fig. 5, cortactin was co-immunoprecipitated with Syk both before and after stimulation. The amount of cortactin was not different throughout the time course. Although the isotype of anti-human Syk mAb has not been determined, we conveniently used anti-human Rb mAb for negative control immunoprecipitation to exclude the possibility that cortactin binds nonspecifically to protein A. Co-
precipitation of cortactin with Syk was also found when Non-
idet P-40 lysis buffer was used to solubilize cell lysates (data
not shown), demonstrating that this association is stable. The
amount of precipitated Syk did not differ throughout the time
course (data not shown). Syk could not be detected in the
precipitates with anti-cortactin mAb. One possibility is that an
anti-human Syk mAb is not so suitable for immunoblot analy-
sis, we could not detect a small amount of co-precipitated Syk.
Another possibility is that the amount of cortactin might be
greater than that of Syk in this cell, and cortactin that does not
associate with Syk might be present. So, a detectable amount
of Syk could not be precipitated with cortactin.

Since cortactin has been reported to be a potential substrate
of Src, we assessed the kinase activity of Src following TPA
stimulation in wild type cells and Syk(K−/−) expressing cells. As
shown in Fig. 6A, both in K562 cells and transformant expressing
Syk(K−/−), autophosphorylation of Src and phosphorylation
of enolase were increased after addition of TPA, suggesting
that Syk activity does not affect TPA-induced activation of Src.
The amount of precipitated Src did not differ in each sample
(Fig. 6B). In the same condition for detecting the association of
cortactin with Syk, its association with Src could not be de-
tected (data not shown).

DISCUSSION

In this study, we demonstrate that cortactin is tyrosine-
phosphorylated following TPA stimulation in K562 cells by
using immunoprecipitation experiments. TPA is well known to
be an activator of protein kinase C, suggesting that the protein
kinase Cactivates tyrosine kinase(s), leading to the induction
of tyrosine phosphorylation of cortactin. TPA-induced tyrosine
phosphorylation was reported (24–32), whereas it was not nec-
essarily elucidated whether this process is mediated by protein
kinase C. It was reported that the biological effects by TPA
cannot be explained completely by protein kinase C activation
in K562 cells (33–36). It is also possible that TPA-induced
tyrosine phosphorylation of cortactin is independent of protein
kinase C activation.

Cortactin was initially described as an 80/85-kilodalton
pp60srcsubstrate. It became phosphorylated on tyrosine resi-
dues in v-Src-transformed chicken embryo (CE) cells (37). In
normal cells, cortactin is known to be tyrosine-phosphorylated
by various stimulants including growth factors and thrombin
(23, 38–40). Since Src is recruited to growth factor receptors
possessing tyrosine kinase in those cytoplasmic domains by
SH2 domain/phosphotyrosine interactions and consequently
activated, it was proposed that cortactin is phosphorylated by
this activated Src (40). However, it has been noted previously
Association of Cortactin with Syk

Fig. 4. Expression of kinase-negative Syk in K562 cells. K562 cells were transfected with kinase-negative porcine syk cDNA in which a point mutation was created in the ATP binding site. Transfected cells were selected in the presence of puromycin and cloned by limiting dilution. A, expression of transfected cDNA. Whole cell lysates of wild type K562 (Wt) and 2 clones of Syk(K-) transfected cells were analyzed by immunoblot analysis with anti-porcine Syk Ab. B, tyrosine phosphorylation of wild type K562 (Wt) and 2 clones of Syk(K-) transfected cells following TPA stimulation. Whole cell lysates of nonstimulated cells and TPA-stimulated cells were subjected to immunoblot analysis with anti-phosphotyrosine mAb. Stimulation was performed by adding 10 nM TPA for 20 min. The positions of the molecular markers are shown to the left in kDa. The arrowhead indicates the position of 80 kDa C, tyrosine phosphorylation of cortactin in wild type and Syk(K-) expressing cells. a, cortactin before and after stimulation in these cells was immunoprecipitated, followed by immunoblot analysis with anti-phosphotyrosine mAb. b, each immunoprecipitated sample was subjected to immunoblot analysis with anti-cortactin mAb. The positions of cortactin and immunoglobulin heavy chain are indicated.

Fig. 5. Association of cortactin with Syk. K562 cells were stim-ulated and Syk was immunoprecipitated as described in Fig. 3 with a modification that digitonin lysis buffer was used instead of Nonidet P-40 lysis buffer. Immunoprecipitates were subjected to immunoblot analysis with anti-cortactin mAb. For irrelevant Ab negative control, anti-Rb mAb was used. The positions of cortactin and immunoglobulin heavy chain are indicated.

that cortactin could not be detected in Src immunoprecipitates, and involvement of other tyrosine kinase(s) in tyrosine phosphorylation of cortactin has been suggested (37, 41–43). In platelets, tyrosine phosphorylation of cortactin and activation of Syk were induced by thrombin treatment, although a direct correlation between these events was not clarified. We show here a possibility that cortactin is a substrate of Syk upon TPA stimulation by the following evidence. 1) Syk is activated by TPA stimulation, and, following the activation of Syk, cortactin is tyrosine-phosphorylated. 2) Expression of dominant-negative Syk abolishes the TPA-induced tyrosine phosphorylation of cortactin. 3) Cortactin is associated with Syk even before TPA stimulation. Since Src is also activated by TPA stimulation, it is possible that cortactin is a direct substrate of Src in TPA-treated K562 cells. However, in contrast to the association of cortactin with Syk, we could not detect the association of cortactin with Src, suggesting that it is unlikely that Src phosphorylates cortactin directly.

An interaction between Syk and Src family protein-tyrosine kinases has been proposed (44). Coexpression of Src family protein-tyrosine kinases and Syk leads to a remarkable increase in net tyrosine phosphorylation, whereas Src family protein-tyrosine kinases or Syk alone induce only marginal phosphorylation in COS cells. Our finding that TPA-induced Src activation is not affected by overexpression of dominant-negative Syk may exclude the possibility that Syk activates Src kinase activity, resulting in tyrosine phosphorylation of cortactin. It would be less possible that Syk and Src are activated independently, considering the close relationship between Syk and Src or Src and cortactin. Src might be upstream to Syk.

As mentioned, TPA treatment of K562 cells induces the increase of nuclear DNA ploidy and cell volume. Cortactin is concentrated in a cytoskeleton-associated structure that is rich in focal adhesion proteins (37). Since cortactin is able to associate with F-actin through its tandem helix-turn-helix domain (41), it may be conceivable that cortactin is involved in this TPA-mediated morphological change.

Cortactin is related to a putative transcriptional factor HS1 (45). This protein is specifically expressed in hematopoietic cells and functions as a major substrate of protein-tyrosine kinases(s) involved in B-cell antigen receptor-mediated signaling. Tyrosine-phosphorylated HS1 is demonstrated to be located mainly in nucleus, proposing the idea that HS1 is translocated from the cytoskeleton to the nucleus through its phosphorylation (46, 47). Similarly, cortactin may be transported to the nucleus through its tyrosine phosphorylation where it may be involved in nuclear events such as transcriptional regulation, leading to differentiation into megakaryocytic lineage. Although these notions await further investiga-
tion, the present study demonstrates that cortactin may be the substrate of Syk rather than Src.

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