Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase

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In this study, we examined the molecular mechanism of erythropoietin-initiated signal transduction of erythroid differentiation through Src and phosphatidylinositol 3-kinase (PI3-kinase). Antisense oligonucleotides against *src* but not *lyn* inhibited the formation of erythropoietin-dependent colonies derived from human bone marrow cells and erythropoietin-induced differentiation of K562 human erythroleukaemia cells. Antisense p85 α oligonucleotide or LY294002, a selective inhibitor of PI3-kinase, independently inhibited the formation of erythropoietin-dependent colonies. In K562 cells, Src associated with PI3-kinase in response to erythropoietin. Antisense *src* RNA expression in K562 cells inhibited the erythropoietin-induced activation of PI3-kinase and its association with erythropoietin receptor. PP1, a selective inhibitor of the Src family, reduced erythropoietin-induced tyrosine phosphorylation of erythropoietin receptor and its association with PI3-kinase in F-36P human erythroleukaemia cells. The coexpression experiments and *in vitro* kinase assay further demonstrated that Src directly tyrosine-phosphorylated erythropoietin receptor, and associated with PI3-kinase. *In vitro* binding experiments proved that glutathione S-transferase–p85 α N- or C-terminal SH2 domains independently bound to erythropoietin receptor, which was tyrosine-phosphorylated by Src. Taken together, Src transduces the erythropoietin-induced erythroid differentiation signals by regulating PI3-kinase activity.

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Introduction

Erythropoietin (EPO) is a cytokine that regulates the proliferation and differentiation of erythroid progenitor cells. The binding of EPO to EPO receptor (EPOR) rapidly leads to tyrosine phosphorylation of EPOR and Janus kinase 2 (Jak2) (Witthuhn *et al.*, 1993; Miura *et al.*, 1994b). Subsequently, a set of signalling molecules,

including the signal transducers and activators of transcription 5 (STAT5) (Damen *et al.*, 1995b) and protein tyrosine phosphatases (Klingmüller *et al.*, 1995; Yi *et al.*, 1995) are tyrosine-phosphorylated and activated. Protein tyrosine kinases (PTKs), except for the Jak family, including c-Fps/Fes (Hanazono *et al.*, 1993), Tec (Machide *et al.*, 1995), Syk (Duprez *et al.*, 1998), and Lyn (Tilbrook *et al.*, 1997; Chin *et al.*, 1998), have been found to be tyrosine-phosphorylated and activated by EPOR stimulation. However, the function of these PTKs in EPOR-mediated signalling remains unclear.

The proto-oncogene, *c-src*, is a normal cellular counterpart of *v-src* that is an oncogene of the Rous sarcoma virus. The product of the *c-src* gene is pp60^{*c-src*} (Src), which expresses PTK activity and contains a Src homology (SH) 2 domain and an SH3 domain. Src is widely expressed in various tissues. It has been implicated in playing important roles in various cell functions including cell proliferation, haematopoietic and neural cell differentiation, and cell–cell contact (Cooper *et al.*, 1993). The activated Src phosphorylates on the tyrosine residues of a number of its substrates, such as RasGAP, pp125^{FAK} and cytoskeletal proteins (Wu *et al.*, 1991; Schaller *et al.*, 1994). In addition, the association of the SH3 domain of Src with the proline-rich regions of downstream regulatory molecules such as phosphatidylinositol 3-kinase (PI3-kinase) is involved in Src-mediated signal transduction (Liu *et al.*, 1993). However, the role of Src in EPOR-mediated signalling remains to be elucidated.

PI3-kinase is a heterodimeric enzyme that converts the three phosphoinositides of the canonical PI pathway [PI, PI(4)P and PI(4,5)P₂] to produce PI(3)P, PI(3,4)P₂ and PI(3,4,5)P₃, respectively (Fruman *et al.*, 1998). It consists of a regulatory subunit, p85, which has two SH2 domains and an SH3 domain, and a catalytic subunit, p110. Recently, PI3-kinase was found to be associated with EPOR and activated in EPO-responsive cells (Damen *et al.*, 1993, 1995a; He *et al.*, 1993; Miura *et al.*, 1994a). The role of PI3-kinase in EPOR-mediated signalling is controversial. Damen *et al.* (1995a) have shown that PI3-kinase may function in growth signal transduction from EPOR because a selective PI3-kinase inhibitor, wortmannin, inhibits the EPO-induced proliferation of DA-3 cells expressing high levels of EPOR. In contrast, Miura *et al.* (1994a) have shown that the C-terminus-deleted EPOR, which lacks the ability to associate with, and activate, PI3-kinase, is still mitogenically active. Recently, it has been reported that PI3-kinase-mediated signalling plays an important role in the proliferation and differentiation of mouse fetal liver progenitors of erythroid colony-forming units (CFU-E) (Klingmüller *et al.*, 1997).

The K562 human erythroleukaemia cell line is a useful tool for the analysis of EPOR-mediated differentiation signalling because EPO scarcely stimulates K562 cell

proliferation, but induces erythroid differentiation in the cells as shown previously (Kitanaka *et al.*, 1994). The decrease in the amount of Src, by inducing antisense *src* RNA expression in K562 cells, interferes with both haemoglobin synthesis and glycophorin A expression promoted by EPO (Kitanaka *et al.*, 1994). In addition, suppression of PI3-kinase activity by wortmannin reduces the expression of erythroid differentiation phenotypes in the cells (Kubota *et al.*, 1996). Thus, these results indicate that both Src and PI3-kinase are involved in erythroid differentiation signal transduction mediated by EPO-activated EPOR in K562 cells.

To elucidate more fully the mechanism of the signal transduction involved in EPO-induced erythroid differentiation, we have focused on the roles of Src and PI3-kinase. In the present study, we demonstrated that Src lies immediately upstream of PI3-kinase in the signal transduction of erythroid differentiation induced by EPO, and regulates EPO-induced activation of PI3-kinase through the tyrosine phosphorylation of EPOR, leading to the association of PI3-kinase and activated EPOR. In addition, the association of Src with PI3-kinase may be involved in the EPO-induced activation of PI3-kinase. Src and PI3-kinase also play crucial roles in the EPO-dependent erythroid differentiation of human haematopoietic progenitor cells. Therefore, differentiation signals from EPOR are transmitted to PI3-kinase through Src, with Src appearing to be one of the key PTKs that regulate the signal transduction of EPO-induced erythroid differentiation.

Results

EPO-induced erythroid differentiation of K562 cells inhibited by reduction of *src* expression but not *lyn*

We have previously reported that the amount of Src was reduced in K562-ASRC cells treated with dexamethasone in order to induce antisense *src* RNA expression, and that reduction of Src inhibited EPO-induced haemoglobin synthesis in these cells (Kitanaka *et al.*, 1994). However, the EPO-induced haemoglobin synthesis in K562-pMSG cells used as a control is not inhibited by dexamethasone treatment. In order to confirm that Src is involved in EPO-induced haemoglobin synthesis in K562 cells, we examined the effect of antisense *src* oligonucleotide on the induction of benzidine-positive cells by EPO. An increase in benzidine-positive cells induced by EPO was suppressed by treatment with antisense *src* but not with control oligonucleotides (Figure 1A). Antisense *src* oligonucleotide specifically suppressed the expression of *src* but not *lyn* in the cells (Figure 1B). In contrast, treatment with antisense *lyn* oligonucleotide was unable to inhibit haemoglobin synthesis in K562 cells induced by EPO, although the expression of *lyn* was suppressed by the treatment (Figure 1C and D).

The proliferation of K562 cells was slightly inhibited by the treatment with antisense *src* or *lyn* oligonucleotides. EPO did not restore the inhibitory effects of these antisense oligonucleotides on cell proliferation (data not shown).

Erythroid differentiation of K562 cells inhibited by antisense p85 α oligonucleotide

As shown in our previous report (Kubota *et al.*, 1996), PI3-kinase is likely to be involved in the signal transduction of EPO-induced erythroid differentiation, since wortmannin inhibits the EPO-induced differentiation of K562 cells. To confirm this, we examined the effect of antisense p85 α oligonucleotide on EPO-induced haemoglobin synthesis in K562 cells (Figure 2A). Antisense p85 α oligonucleotide inhibited EPO-induced haemoglobin synthesis in the cells. Sense p85 α or scramble oligonucleotide as a control did not suppress the increase in benzidine-positive cells in response to EPO. As shown in Figure 2B, p85 α expression in K562 cells was suppressed by treatment with antisense p85 α but not with control oligonucleotides. Antisense p85 α oligonucleotide at the concentration used in this study was unable to inhibit the proliferation of K562 cells in the presence or absence of EPO (data not shown).

In addition, LY294002, a selective inhibitor of PI3-kinase, inhibited EPO-induced haemoglobin synthesis in K562 cells in a dose-dependent manner (Figure 2C). A concentration of 1 μ M LY294002 inhibited haemoglobin synthesis in K562 cells, but not cell proliferation. LY294002 at higher concentrations suppressed the proliferation and haemoglobin synthesis of K562 cells.

Effects of antisense oligonucleotides on the formation of CFU-E

The findings described above indicate that the EPOR-mediated erythroid differentiation signal is transduced by Src and PI3-kinase in K562 cells. Therefore, to determine whether Src and PI3-kinase are involved in the erythroid differentiation of normal human haematopoietic progenitor cells, we examined the effect of antisense oligonucleotide against *src*, *lyn* or p85 α on the formation of CFU-E colonies (Figure 3). The number of CFU-E colonies derived from bone marrow cells treated with antisense *src* oligonucleotide was decreased to 37.2% of that of CFU-E colonies treated with EPO alone. Control oligonucleotide of *src* did not suppress the formation of CFU-E colonies (Figure 3A). Since the human bone marrow mononuclear cells were subjected to the experiments concerning erythroid colony formation assay two days after oligonucleotide treatment, we examined the expression of *src* in the cells. As shown in Figure 3B, the expression of *src* in the bone marrow mononuclear cells was inhibited by 2 days treatment with antisense *src* but was not inhibited by the control oligonucleotides. These oligonucleotides did not affect the levels of *lyn* expression in the cells. The formation of CFU-E colonies was not reduced by treatment with antisense *lyn* oligonucleotide (Figure 3A), although *lyn* expression in the bone marrow mononuclear cells was suppressed by treatment with them (Figure 3B).

The number of CFU-E colonies was decreased to 64.6% of that of CFU-E colonies treated with EPO alone after treatment with antisense p85 α oligonucleotide (Figure 3C). In contrast, control oligonucleotides did not affect the EPO-induced formation of CFU-E colonies. The expression of p85 α in the bone marrow mononuclear cells was inhibited by treatment with antisense p85 α oligonucleotide but was not affected by the control oligonucleotides (Figure 3D).

Fig. 1. Antisense *src* but not *lyn* oligonucleotides inhibit EPO-induced haemoglobin synthesis in K562 cells. K562 cells were treated with 4 μ M antisense (AS) *src* (A), *lyn* (C) or control (C) oligonucleotide for 48 h before EPO stimulation. During the stimulation with EPO for 72 h, the medium was replenished daily with each oligonucleotide. The cells were then subjected to benzidine staining 72 h after EPO stimulation in order to detect the cells producing haemoglobin. The value represents the mean $\pm$ SD ($n = 3$). Total RNA was isolated from K562 cells treated as described above. The expression of *src* (B) and *lyn* (D) in the cells was analysed by means of RT-PCR using specific primers. The expression of β -actin was used as a control. The arrows indicate the positions of the *src*, *lyn* and β -actin cDNA products. Molecular weight markers are shown in the left lane.

In addition, LY294002 inhibited the formation of CFU-E in a dose-dependent manner (CFU-E colonies; 450 ± 29 without LY294002, 331 ± 10 and 166 ± 13 with LY294002 at 5 and 50 μ M, respectively).

PI3-kinase activity in α PY immunoprecipitates

The findings described above indicate that Src and PI3-kinase are involved in the signal transduction of EPO-induced erythroid differentiation. The Src family of non-receptor tyrosine kinases, Lck and Fyn, have been proposed as potential mediators of the IL-2-dependent activation of PI3-kinase (Taichman *et al.*, 1993; Karnitz *et al.*, 1994). Therefore, to elucidate the mechanism of EPOR-mediated signal transduction involving Src and PI3-kinase, we examined the role of Src in the EPO-induced activation of PI3-kinase in K562 cells.

Treatment with EPO augmented PI3-kinase activity in the anti-phosphotyrosine (α PY) immunoprecipitates from

the lysates of K562-ASRC and K562-pMSG cells (Figure 4), while wortmannin interfered with EPO-enhanced PI3-kinase activity in both cells (Figure 4). Induction of antisense *src* RNA expression in K562-ASRC cells by treatment with dexamethasone completely inhibited EPO-induced activation of PI3-kinase (Figure 4A). In contrast, treatment with dexamethasone was unable to inhibit the increase in PI3-kinase activity in EPO-treated K562-pMSG cells (Figure 4B).

Effect of wortmannin on Src PTK activity

To determine whether wortmannin affects the EPO-induced activation of Src PTK, an *in vitro* kinase assay of α Src immunoprecipitates was carried out. It was seen that wortmannin did not inhibit EPO-enhanced Src PTK activity (Figure 5A and C, lanes 2 and 6) nor did it affect the levels of Src expression in K562-ASRC or K562-pMSG cells (Figure 5B and D, lanes 2 and 6).

Fig. 2. Antisense p85 α oligonucleotide and LY294002 inhibit EPO-induced haemoglobin synthesis in K562 cells. (A) K562 cells were treated with 4 μ M antisense (AS) p85 α or control (C) oligonucleotides as described in Figure 1. Each value represents the mean $\pm$ SD ($n = 3$). (B) RT-PCR analysis of p85 α expression in K562 cells treated with antisense p85 α or control oligonucleotides. The arrows indicate the positions of p85 α and β -actin cDNA products. (C) K562 cells were treated with various concentrations of LY294002. The cells were stimulated with 4 U/ml EPO 20 min after adding LY294002. The cells were then cultured for three days. Haemoglobin synthesis in the cells was analysed by means of benzidine staining.

EPO-induced activation of Src was not detected in K562-ASRC cells treated with dexamethasone in order to induce antisense *src* RNA expression (Figure 5A, lanes 2 and 4), mainly because of the decrease in the amount of Src in the cells (Figure 5B, lanes 2 and 4). Treatment with dexamethasone did not inhibit EPO-enhanced Src PTK activity in K562-pMSG cells (Figure 5C, lanes 2 and 4). As described above, suppression of Src expression abrogates EPO-induced activation of PI3-kinase in K562-ASRC cells. On the other hand, wortmannin does not inhibit EPO-induced activation of Src in either K562-ASRC or K562-pMSG cells. These findings indicate that PI3-kinase lies downstream of Src in the signal transduction pathways involved in EPO-induced erythroid differentiation.

Association between Src and PI3-kinase in vivo

To determine how Src regulates PI3-kinase activity in signalling mediated by EPOR, we examined whether EPO induces the association between Src and PI3-kinase in K562 cells. Src was seen to associate with p85 α in EPO-treated K562-ASRC and K562-pMSG cells but not in the cells without EPO treatment (Figure 6A–D, lanes 1 and 2). Wortmannin did not inhibit the co-immunoprecipitation of Src with p85 α in either EPO-treated K562-ASRC or K562-pMSG cells (Figure 6A and B, lanes 2 and 3; C and D, lanes 2 and 4). After treatment with dexamethasone, however, Src was not detected in α p85 immunoprecipitates from K562-ASRC cells (Figure 6A, lanes 5 and 6). p85 α was also undetected in α Src immunoprecipitates from the cells treated with dexamethasone (Figure 6C, lanes 5 and 6). These are mainly due to suppression of Src expression in the cells. Src was co-immunoprecipitated with p85 α from the lysates of EPO-treated K562-pMSG cells even after dexamethasone treatment (Figure 6B, lanes 2 and 5; D, lanes 2 and 6). The same amount of p85 α or Src was immunoprecipitated from each sample as shown in Figure 6A and B, or C and D, respectively. These findings indicate that EPO induces the association between Src and p85 α in K562 cells, and that PI3-kinase activity is not required for this association.

Moreover, PI3-kinase activity was enhanced in α Src immunoprecipitate from the lysate of K562-ASRC cells treated with EPO (Figure 7A, lanes 1 and 2). This enhancement of PI3-kinase activity was inhibited by wortmannin treatment (Figure 7A, lanes 2 and 4). PI3-kinase activity was also enhanced in α Src immunoprecipitate from the lysate of EPO-treated K562-pMSG cells even after dexamethasone treatment (data not shown).

Physical association between PI3-kinase and EPOR

It has been previously demonstrated both *in vivo* and *in vitro* that EPO induces the association between PI3-kinase and EPOR (Damen *et al.*, 1993, 1995a; He *et al.*, 1993; Miura *et al.*, 1994a). Therefore, we examined whether p85 α associates with EPOR in K562 cells treated with EPO and whether antisense *src* RNA expression and wortmannin affect the EPO-induced association between p85 α and EPOR. EPOR was detected in α p85 immunoprecipitates from the lysates of EPO-treated K562-ASRC cells but not from those of the cells without EPO treatment (Figure 8A, lanes 1 and 2). The EPO-induced association between EPOR and p85 α in K562-ASRC cells was

Fig. 3. Effect of antisense *src*, *lyn*, or p85 α oligonucleotide on erythroid colony growth. After bone marrow mononuclear cells (5×10^4 cells) were treated with antisense (AS) *src*, *lyn*, p85 α or control (C) oligonucleotides for 48 h, they were plated into 1 ml of 0.9% methylcellulose with 4 U/ml EPO in the presence of each oligonucleotide. After 7 days incubation, the number of erythroid colonies was counted under a microscope (A and C). The value represents the mean $\pm$ SD ($n = 3$). After bone marrow mononuclear cells (5×10^4 cells) were treated with antisense *src*, *lyn*, p85 α or control oligonucleotide for 48 h, total RNA was isolated from the cells. Total RNA was then subjected to RT-PCR analysis using each specific primer set to detect the expression of *src*, *lyn* or p85 α in the cells (B and D).

inhibited by treatment with dexamethasone but not with wortmannin (Figure 8A, lanes 3 and 5). EPOR was immunoprecipitated with p85 α from the lysates of EPO-treated K562-pMSG cells even after treatment with dexamethasone (Figure 8C, lanes 2 and 5). The same amount of p85 α was immunoprecipitated from each lysate of K562-ASRC or K562-pMSG cells (Figure 8B and D).

EPOR tyrosine phosphorylation induced by EPO and its association with p85 α are suppressed by inhibition of Src PTK activity

Suppression of Src expression in K562 cells resulted in the inhibition of the EPO-induced association between p85 α and EPOR (Figure 8), suggesting that Src regulates the binding of p85 α to tyrosine-phosphorylated EPOR. To address the mechanism by which Src regulates the EPO-induced association between p85 α and EPOR, we examined the effect of PP1, a selective inhibitor of the Src family PTKs, on the EPO-induced tyrosine phosphorylation of EPOR and its association with p85 α in a human erythroleukaemia cell line, F-36P, which was

differentiated into the erythroid lineage in response to EPO (Chiba *et al.*, 1991). As shown in Figure 9A, PP1 reduced the EPO-induced tyrosine phosphorylation of EPOR in a dose-dependent manner. Consistently, the amount of p85 α co-immunoprecipitated with EPOR was reduced by increasing concentrations of PP1 (Figure 9B). In contrast, PP3, an inactive analogue of PP1, had no effects on the EPO-induced tyrosine phosphorylation of EPOR nor on its association with p85 α (Figure 9A and B). PP1 had little effect on the *in vivo* tyrosine phosphorylation of Jak2 even at a high concentration of 100 μ M, at which its inhibitory effect on Jak2 kinase activity *in vitro* has been reported (Hanke *et al.*, 1996; Okutani *et al.*, 2001; data not shown). These findings indicate that Src regulates the binding of p85 α to tyrosine-phosphorylated and activated EPOR, independently of Jak2.

Src tyrosine-phosphorylates EPOR

To further explore the possibility that Src tyrosine-phosphorylates EPOR, EPOR was coexpressed with the wild-type or kinase-inactive Src in COS7 cells. The

Fig. 4. Effects of antisense *src* RNA expression and wortmannin on EPO-induced activation of PI3-kinase. After K562-ASRC (A) and K562-pMSG (B) cells were incubated with 2 μ M dexamethasone for 2 days or 100 nM wortmannin at 37°C for 30 min, they were stimulated with 30 U/ml EPO at 37°C for 5 min. The cell lysates (1×10^7 cells) were then immunoprecipitated with α PY antibody and *in vitro* PI3-kinase assay on the immune complex was carried out as described in Materials and methods. The radiolabelled lipids were detected and quantified using a Bioimaging Analyzer BAS2000. The numbers shown represent the percentage of PI3-kinase activity in each α PY immunoprecipitate compared with that from K562-ASRC or K562-pMSG cells treated with EPO alone. PIP, phosphatidylinositol-3-phosphate; Ori., origin.

wild-type Src, but not the kinase-inactive one, tyrosine-phosphorylated EPOR (Figure 10A). Levels of tyrosine phosphorylation of EPOR expressed in COS7 cells were only marginally enhanced by EPO stimulation (data not shown). As shown in Figure 10A, both the wild-type and kinase-inactive Src physically associated with EPOR. However, the amount of wild-type Src associated with EPOR was significantly augmented compared with that of the kinase-inactive Src associated with EPOR. This may be due to the tyrosine phosphorylation of EPOR induced by Src.

In addition, *in vitro* kinase assay showed that recombinant Src (Upstate Biotechnology, Lake Placid, NY) directly tyrosine-phosphorylated EPOR immunoprecipitated from

Fig. 5. Effects of antisense *src* RNA expression and wortmannin on EPO-induced activation of Src. K562-ASRC (A and B) and K562-pMSG (C and D) cells were treated with dexamethasone, wortmannin or EPO as described in Figure 4. The cell lysates (2×10^7 cells) were immunoprecipitated with α Src antibody and the samples were divided into two. *In vitro* kinase assay of the immune complex was then performed using one half of them as described in Materials and methods (A and C). The other half was subjected to western blotting analysis using α Src antibody (B and D).

the lysates of COS7 cells that had been transfected with EPOR (Figure 10B).

GST-p85 α fusion proteins bind to EPOR *in vitro*

To explore the mechanism of p85 α binding to EPOR, the *in vitro* binding of glutathione S-transferase (GST)-p85 α fusion proteins to EPOR was examined. As shown in Figure 10C, GST-p85 α N- and C-terminal SH2 domains independently bound to EPOR tyrosine-phosphorylated by wild-type Src coexpressed with EPOR in COS7 cells. The GST-p85 α N-terminal SH2 domain did not associate with unphosphorylated EPOR. To some extent, the unphosphorylated EPOR was precipitated with the GST-p85 α C-terminal SH2 domain from the lysate of COS7 cells cotransfected with EPOR and kinase-inactive Src as well as from that of the cells transfected with EPOR alone. However, the amount of EPOR precipitated with the GST-p85 α C-terminal SH2 domain was significantly augmented after the tyrosine phosphorylation of EPOR induced by wild-type Src. In addition, the GST-p85 α SH2 domains did not precipitate Src from the lysates of COS7 cells transfected with wild-type Src alone (data not

Fig. 6. Association between Src and PI3-kinase *in vivo*. The lysates of K562-ASRC (**A** and **C**) and K562-pMSG (**B** and **D**) cells were prepared as described in Figure 4. The cell lysates were immunoprecipitated with α p85 or α Src antibody followed by western blotting analyses using α Src and α p85 antibodies.

shown). These results indicate that the GST-p85 α SH2 domains bind directly to EPOR tyrosine-phosphorylated by Src.

Discussion

In the present study, we demonstrated that Src directly tyrosine-phosphorylates EPOR, and that PI3-kinase is activated by binding of p85 α SH2 domains to tyrosine residue(s) in EPOR phosphorylated by Src. In addition, the association of PI3-kinase with Src induced by EPO treatment may activate PI3-kinase. Src and PI3-kinase also function in the EPO-dependent erythroid development of human haematopoietic progenitor cells.

As shown in the present study, antisense p85 α oligonucleotide or LY294002, a more selective inhibitor of PI3-kinase than wortmannin, inhibited the EPO-induced erythroid differentiation of K562 cells. Moreover, the inhibition of EPO-enhanced PI3-kinase activity by the suppression of Src expression is consistent with the suppression of the erythroid differentiation of K562 cells. These findings strongly support the hypothesis that PI3-kinase is involved in the signalling pathway of

erythroid differentiation mediated by activated EPOR in K562 cells.

p85 α -deficient mice show defects in B-cell development but not in erythropoiesis (Fruman *et al.*, 1999; Suzuki *et al.*, 1999). However, as shown in the present study, p85 α is involved in the EPO-dependent erythroid differentiation of haematopoietic progenitor cells derived from adult human bone marrow cells. Although the precise reason for this discrepancy is unknown, other molecules of the PI3-kinase family or other signalling molecules may compensate for the defect of p85 α function in erythropoiesis in the early development of p85 α -deficient mice, but may not completely compensate for the defect in EPO-dependent erythropoiesis in adults. Recently, Klingmüller *et al.* (1997) have shown that tyrosine 479 of mouse EPOR corresponding to tyrosine 504 of human EPOR may be involved in transducing the signals for the proliferation and differentiation of erythroid progenitor cells. The expression of EPOR containing only one cytoplasmic tyrosine residue, tyrosine 479, in fetal liver progenitor cells of EPOR-deficient mice restores ~90% of CFU-E formation compared with that in haematopoietic cells from wild-type EPOR-transfected EPOR-deficient mice.

Fig. 7. PI3-kinase activity in αSrc immunoprecipitates. The lysates of K562-ASRC cells were prepared as described in Figure 4. After the cell lysates (2×10^7 cells) were immunoprecipitated with αSrc antibody, the immunoprecipitates were divided in two. One half was subjected to *in vitro* PI3-kinase assay (A), while the other half was subjected to western blotting analysis using αSrc antibody (B). The numbers shown represent the percentage of PI3-kinase activity in each αPY immunoprecipitate compared with that from K562-ASRC cells treated with EPO alone. PIP, phosphatidylinositol-3-phosphate; Ori., origin.

This finding indicates that PI3-kinase is involved in the erythroid differentiation of haematopoietic cells, since tyrosine 479 is the binding site of p85α to EPOR and its binding activates PI3-kinase. In addition, their findings indicate that the PI3-kinase-independent pathway also plays a role in transducing the signals implicated in the erythroid differentiation of haematopoietic progenitor cells, because mutant EPOR lacking tyrosine 479 supports the formation of ~50% of the number of CFU-E colonies achieved by wild-type EPOR.

The mechanism of PI3-kinase activation may be based on the interaction between PI3-kinase and EPOR because EPOR was co-immunoprecipitated with PI3-kinase from the lysates of K562 and F-36P cells treated with EPO, as described previously (Damen *et al.*, 1993; He *et al.*, 1993; Miura *et al.*, 1994a). The coexpression experiments in COS7 cells and *in vitro* kinase assay demonstrated that Src directly tyrosine-phosphorylated EPOR. In addition, PP1, a selective inhibitor of Src PTK, suppressed the EPO-induced tyrosine phosphorylation of EPOR in F-36P cells

Fig. 8. EPO-induced association between p85α and EPOR. The lysates (4×10^7 cells) of K562-ASRC (A and B) and K562-pMSG (C and D) cells were prepared as described in Figure 4. The αp85 immunoprecipitates from these cell lysates were analysed by western blotting using αEPOR antibody (A and C). The same blots were reprobed with αp85 antibody (B and D).

Fig. 9. PP1 dose-dependently inhibits EPO-induced tyrosine phosphorylation of EPOR and association of p85α with EPOR in F-36P cells. Serum- and cytokine-starved F-36P cells were treated with increasing concentrations of PP1 or PP3 for 1 h followed by stimulation with 100 U/ml EPO at 37°C for 5 min. The cell lysates were then subjected to immunoprecipitation with αEPOR antibody followed by western blotting analysis using the indicated antibodies. The positions of EPOR and p85α are indicated.

Fig. 10. Src tyrosine-phosphorylates EPOR and induces its association with GST-p85 α SH2 domains *in vitro*. (A) EPOR and wild-type or kinase-inactive Src were transiently coexpressed in COS7 cells. Two days after transfection, cell lysates were subjected to immunoprecipitation with α EPOR antibody followed by western blotting analysis using the indicated antibodies. (B) *In vitro* PTK assay was performed by using recombinant Src and α EPOR immunoprecipitates from the lysates of COS7 cells transfected with EPOR alone. The samples were then subjected to western blotting analysis using the indicated antibodies. (C) Cell lysates prepared as described above were incubated with agarose-conjugated GST-p85 α N- or C-terminal SH2 domains (N or C, respectively) at 4°C for 4 h. GST-agarose (G) was used as a control. The proteins precipitated with the agarose beads were analysed by western blotting using the indicated antibodies.

in a dose-dependent manner. This inhibitory effect of PP1 was consistent with the reduction of the association between p85 α and EPOR. Therefore, Src most likely provides the binding site(s) for p85 α in EPOR by means of the tyrosine phosphorylation of EPOR. *In vitro* binding studies using GST-p85 α fusion proteins further showed that both N- and C-terminal SH2 domains of p85 α bound to EPOR that had been tyrosine-phosphorylated by the wild-type Src. The GST-p85 α C-terminal SH2 domain bound to unphosphorylated EPOR to some extent. The precise reason for this interaction is unknown.

Damen *et al.* (1995a) have shown that after EPO treatment, PI3-kinase activity in α PY but not in α EPOR immunoprecipitates was increased in DA-3 cells expressing mutant EPOR, bearing phenylalanine 503 substituted for tyrosine, as well as in cells expressing wild-type EPOR. Therefore, although the binding of p85 α to tyrosine 503 in EPOR activated by EPO may enhance PI3-kinase activity, this interaction is unlikely to be essential for the EPO-induced activation of PI3-kinase. Another mechanism of PI3-kinase activation induced by EPO treatment should function in the cells. As shown in the present study, the EPO-induced association between Src and p85 α recruits PI3-kinase to the plasma membrane in order to activate it, because Src is anchored to the inner surface of the plasma membrane, in which substrates for PI3-kinase are abundantly distributed. The Src and p85 α coexpression experiment in COS7 cells further demonstrated that Src associates with p85 α (data not shown). In addition, an *in vitro* binding experiment showed that Src did not bind to GST-p85 α SH2 domains. The SH3 domains of members of the Src family bind directly to two proline-rich sequences in p85 α (Liu *et al.*, 1993; Taichman *et al.*, 1993; Karnitz *et al.*, 1994). Taken together, the direct interaction between the Src SH3 domain and proline-rich sequences in p85 α may be involved in the activation of PI3-kinase. Alternatively, since several tyrosine-phosphorylated proteins were coprecipitated in α p85 immunoprecipitates from the lysates of K562-ASRC cells treated with EPO (data not shown), these proteins may intervene in the association between p85 α and Src.

Since EPOR was coprecipitated in α Src immunoprecipitates from the lysates of K562-ASRC and K562-pMSG cells after EPO treatment (data not shown), EPO treatment appears to induce the association of Src with EPOR. The coexpression experiments in COS7 cells further showed that Src binds to unphosphorylated EPOR, and that the association of Src with EPOR is augmented after EPOR is tyrosine-phosphorylated in COS7 cells cotransfected with the wild-type Src and EPOR. Recently, Chin *et al.* (1998) reported that the tyrosine kinase domain of Lyn binds to the unphosphorylated mouse EPOR, and that its SH2 domain binds directly to mouse EPOR through its interaction with phosphorylated tyrosine 464 or 479 after EPO stimulation. Since the amino acid sequences following tyrosine 464 in mouse EPOR and tyrosine 488 in human EPOR are identical to the consensus sequence for the binding of the Src SH2 domain (phosphotyrosine-acidic-acidic-hydrophobic) (Songyang *et al.*, 1993), Src may bind directly to these phosphorylated tyrosine residues in EPOR through its SH2 domain, after EPO stimulation.

Although *src*-deficient mice have normal levels of erythrocytes (Soriano *et al.*, 1991), mice deficient in both *hck* and *src* have haematopoietic deficiencies characterized by anaemia and leukocytopenia (Varmus *et al.*, 1994). Therefore, Src should cooperate with other members of the Src family PTKs to provide the signals for normal haematopoietic development including erythropoiesis. In the present study using bone marrow cells from normal human adults, Src but not Lyn was seen to play an essential role in the EPO-dependent erythroid differentiation of these cells. In contrast, Tilbrook *et al.* (1997) have demonstrated that Lyn plays an essential role in the EPO-initiated differentiation of J2E immature mouse erythroid cells, although *lyn*-deficient mice express normal erythropoiesis (Hibbs *et al.*, 1995). Recently, Tilbrook *et al.* (2001) have demonstrated that ectopic expression of Lyn in mouse fetal liver cells promotes the formation of CFU-E colonies. Although the precise reason for this discrepancy is unknown, Src in human EPOR-mediated signalling may substitute for the function of Lyn in mouse erythroid progenitor cells. Therefore, haematopoietic progenitor cells can overcome functional defect(s) of the Src family PTK(s) early in their development through compensation with other PTKs, but are unable to compensate for the fault(s) as they become more committed to and enter the final stages of differentiation.

In conclusion, EPOR-mediated signals involved in the erythroid differentiation of K562 and normal human erythroid progenitor cells are likely to be predominantly transduced through Src and PI3-kinase. A model of the Src- and PI3-kinase-mediated signalling mechanism from EPOR is as follows: after EPO binds to its receptors, EPOR is tyrosine-phosphorylated and Src is activated. Then Src also phosphorylates EPOR on tyrosine residue(s) to provide the binding site(s) for PI3-kinase. PI3-kinase binds to phosphorylated tyrosine residue(s) in EPOR through the p85 α SH2 domains, and to the Src SH3 domain probably through the proline-rich regions in p85 α . The recruitment of PI3-kinase to the plasma membrane allows it to be activated, and the differentiation signals are then transmitted downstream from PI3-kinase. Further experiments are required, however, to determine the signalling molecules functioning downstream of PI3-kinase in the signal transduction of EPO-induced erythroid differentiation.

Materials and methods

Reagents

Recombinant human EPO was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Wortmannin and reagents for PI3-kinase assay were purchased from Sigma (St Louis, MO). LY294002 and PP1 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). PP3 was from Calbiochem (San Diego, CA). α PY monoclonal antibody, α p85 and α EPOR polyclonal antibodies were from Upstate Biotechnology. α Src monoclonal antibody was from Oncogene Science (Cambridge, MA). α EPOR and α Src polyclonal antibodies were from Santa Cruz (Santa Cruz, CA). All other agents were purchased from commercial sources.

Expression vectors for the murine wild-type (pUSE *src*-wt) and kinase-inactive Src with a mutation of lysine 297 to arginine (pUSE *src*K297R) were purchased from Upstate Biotechnology (Barone *et al.*, 1995). Expression vectors for the mouse EPOR (pXM-EPOR) and human p85 α (pRK-p85 α) were generous gifts from Drs O.Miura and A.Ullrich, respectively. Agarose-conjugated GST fusion protein containing human p85 α N- or C-terminal SH2 domain was from Upstate Biotechnology.

Establishment of stable transformant cells

Stable transformants of K562 cells transfected with non-recombinant pMSG plasmid (Amersham Pharmacia, UK) or pMSG recombined with *v-src* fragment (800 bp) (a gift of the Japanese Cancer Resources Bank, Tokyo, Japan) in antisense direction (K562-pMSG and K562-ASRC cells, respectively) were established as described previously (Kitanaka *et al.*, 1994). We have already confirmed that antisense *src* RNA expression does not alter the levels of Lyn, Fyn and Yes, members of the Src family PTKs in K562-ASRC cells (data not shown).

Treatment of F-36P and the transformants of K562 cells with chemicals and EPO

The transformants were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (the growth medium). Since our previous report (Kitanaka *et al.*, 1994) demonstrated that endogenous Src expression in K562-ASRC cells is significantly diminished after treatment with dexamethasone, cells treated with 2 μ M dexamethasone for 2 days were subjected to the following experiments. The cells were treated with 100 nM wortmannin at 37°C for 30 min. The cells as control were treated with the same volume of ethanol (0.1% v/v) for 2 days and dimethyl sulfoxide (0.1% v/v) at 37°C for 30 min. After treatment with dexamethasone and wortmannin, the cells were incubated with 30 U/ml EPO at 37°C for 5 min.

F-36P human erythroleukaemia cells, which are IL-3- or GM-CSF-dependent, were provided by RIKEN Cell Bank (Tsukuba, Japan). In the experiments using F-36P cells, the cells were serum and cytokine starved for 16 h, and then treated with the indicated concentrations of PP1 or PP3 for 1 h, followed by stimulation with 100 U/ml EPO at 37°C for 5 min.

Immunoprecipitation and western blotting analysis

The cells prepared as described above were harvested and lysed in lysis buffer (Kubota *et al.*, 1998). The cell lysates were subjected to immunoprecipitation as described previously (Kubota *et al.*, 1998). Samples were subjected to SDS-PAGE, and separated proteins were electrophoretically transferred to PVDF membrane.

For western blotting analysis, PVDF membranes pre-incubated with 5% non-fat milk powder solution were probed with specific antibodies followed by detection using an enhanced chemiluminescence system (Amersham Pharmacia).

PI3-kinase assay

PI3-kinase assay was performed as described previously (Kubota *et al.*, 1998). The radiolabelled lipids were detected and quantified using a Bioimaging Analyzer BAS2000 (Fuji Film, Tokyo, Japan).

In vitro Src PTK assay

The cell lysates prepared as described above were immunoprecipitated with α Src monoclonal antibody. *In vitro* PTK assay of the immune complexes was performed as described previously (Kitanaka *et al.*, 1998). The gels were treated with 1 M KOH to remove serine/threonine-bound phosphates as described previously (Eriksson *et al.*, 1992). The enolase band phosphorylated on tyrosine residue(s) was detected and quantified by means of the Bioimaging Analyzer BAS2000. In some experiments, α PY immunoblotting was performed to detect the Src-promoted tyrosine phosphorylation of EPOR (Okutani *et al.*, 2001).

Transient expression in COS7 cells

Transfection of expression vectors into COS7 cells was performed using the SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two days after transfection, cells were left unstimulated or stimulated with EPO, solubilized and subjected to immunoprecipitation followed by western blotting analysis.

In vitro binding studies using GST-p85 α fusion proteins

Expression vectors for EPOR and wild-type or kinase-inactive Src were cotransfected in COS7 cells. Cell lysates were incubated with agarose-conjugated GST-p85 α N- or C-terminal SH2 domain at 4°C for 4 h. Proteins bound to GST-p85 α SH2 domains were eluted by boiling and subjected to western blotting analysis.

Oligonucleotides

Oligonucleotides against human p85 α were synthesized by Bex Co. (Tokyo, Japan), as described previously (Skorski *et al.*, 1995). The sequence of p85 α antisense, scramble and sense phosphorothioate oligonucleotides were 5'-GTACTGGTACCCTCAGCACTCAT-3', 5'-GTACTGCGACTCCCATGACTCAT-3' and 5'-ATGAGTGCTG-AGGGGTACCAGTAC-3', respectively. They correspond to codons

1–8 of the published human cDNA sequence. Computer search (FASTA) indicated that the p85 α antisense oligonucleotide used in these studies is not complementary to any other known gene sequence.

Antisense oligonucleotides and controls directed to human *src* and *lyn* have been designed and manufactured by BIOGNOSTIK (Göttingen, Germany). The sequences of these phosphorothioate oligonucleotides were as follows: *src* oligomers, 5'-ATAGAGGGCCACAAAGGT-3' (antisense) and 5'-ACTACGACCTACGTGAC-3' (random control); *lyn* oligomers, 5'-CTTTGGAAGCAGCACTTT-3' (antisense) and 5'-GGATTACCTATTGCTGG-3' (random control).

RT-PCR analysis

After total RNA was isolated from K562 cells, bone marrow mononuclear cells and erythroid colonies, cDNA was synthesized from 1 μ g of total RNA with 5 U of avian myeloblastosis virus reverse transcriptase XL (Life Science, Rockville, MD). For amplification of the cDNA products, the oligodeoxynucleotide primers used were as follows: p85 α primers, 5'-ATTGACTTGCACCTGGGTGAC-3' (sense) and 5'-GGAGCTCTGTGTTCTGTATAG-3' (antisense); *src* primers, 5'-GACCTGTCCTTCAAGAAAG-3' (sense) and 5'-GTTGCTGGGGATGTAGCCT-3' (antisense); *lyn* primers, 5'-GACAGCTTGAGTGACGATG-3' (sense) and 5'-AGGGACTTGTGCTTTCCACC-3' (antisense); β -actin primers, 5'-CGGAACCGCTCATTGCC-3' (sense) and 5'-ACCCACACTGTGCCATCTA-3' (antisense). Each primer was added at a final concentration of 0.2 μ M in a total volume of 50 μ l. After *Taq* polymerase (Takara, Kyoto, Japan) was added to the samples, 25 or 30 cycles of amplification were performed in a DNA thermal cycler using the following step programs: 1 min at 94°C for denaturing, 1 min at 55°C (*src*, β -actin), 58°C (*lyn*) or 62°C (p85 α) for annealing, and 2 min at 72°C for extending. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide. Two forms of *lyn* cDNA were amplified by RT-PCR using primers since their positions include the region deleted in the alternative form of *lyn*. A single form of cDNA was amplified by other primer sets (p85 α , *src* and β -actin).

Treatment of K562 cells with oligonucleotides

K562 cells (2×10^4) were seeded into each well of a 24-well dish containing 500 μ l of the growth medium. Oligonucleotides were then added to the cell suspension to 4 μ M final concentration. The medium was replenished daily with each oligonucleotide. The number of cells was counted on days three and five.

For erythroid differentiation studies, K562 cells were treated with 4 μ M oligonucleotides for 48 h as described above. The cells were then treated with 4 U/ml EPO in the presence of oligonucleotides for 3 days and subjected to benzidine staining. The same doses of oligonucleotides were added daily to the medium.

Colony assay

Bone marrow cells were obtained from healthy volunteers after obtaining their informed consent. Mononuclear cells were separated on a Ficoll-PaqueTM (Amersham Pharmacia) density gradient. After removing the adherent cells, the cells were subjected to the following experiments. 5×10^4 cells in 200 μ l of the growth medium were seeded into each well of a 96-well dish. Control or antisense oligonucleotides were added to them at a concentration of 4 μ M at the beginning of culture and 24 and 48 h later. The cells were incubated for 24 h after adding the last dose of oligonucleotides and then plated in the semisolid methylcellulose medium (MethoCult H4230, Stem Cell Technologies, Vancouver) containing 4 U/ml EPO and 4 μ M oligonucleotides in triplicate 35 mm petri dishes, without washing. The number of CFU-E colonies was counted under contrast microscopy 7 days after incubation.

In the experiments using LY294002, the bone marrow mononuclear cells were plated in a semisolid methylcellulose medium containing 4 U/ml EPO and increasing concentrations of LY294002. The same volume of dimethyl sulfoxide was used as a control.

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