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The Proprotein Convertase PACE4 Is Upregulated by PDGF-BB in Megakaryocytes: Gene Expression of PACE4 and Furin Is Regulated Differently in Dami Cells[1]

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The differentiation of megakaryocytes into platelets is highly regulated by many cytokines and growth factors. PACE4 and furin are Ca^{2+}-dependent serine endoproteases belonging to the subtilisin-like proprotein convertase (SPC) family. These enzymes are involved in the proteolytic activation of proteins that play essential roles in cell growth and differentiation. In this study, we examined the expression of PACE4 and furin during the differentiation of megakaryoblastic cell lines, Dami and HEL cells, induced by phorbol 12-myristate 13-acetate (PMA). PMA stimulates not only the expression of platelet-derived growth factor-B (PDGF-B) mRNA, but also PACE4 mRNA in these cell lines. The expression of PACE4 transcripts (both the PACE4A and PACE4C/CS isoforms) was upregulated more than 4-fold by PMA. Moreover, direct treatment with PDGF-BB also resulted in an increase in the level of PACE4 mRNA. Further, the effect of PDGF-BB on PACE4 expression was confirmed by promoter assay of the PACE4 gene. Although the furin mRNA level was increased by TGF-β1 in Dami cells, it was not affected by PDGF-BB. These results indicate for the first time that PACE4 expression is specifically upregulated by PDGF-BB in differentiated megakaryoblasts, suggesting a unique role for PACE4 in platelet production.

Key words: megakaryocytes, PACE4, PDGF-BB, proprotein convertase, transcriptional regulation.

Megakaryocytes originate in the bone marrow from pluripotent hematopoietic stem cells through a differentiation process that involves stem cell commitment, nuclear polyploidization and cytoplasmic maturation leading to the production of platelets (*1–3*). Several cytokines and growth factors synergistically promote proliferation and differentiation in the bone marrow. To date, a number of megakaryoblastic cell lines, such as Dami cells, have been characterized (*1, 4*). They can be induced to undergo further differentiation to varying degrees in the presence of cytokines and growth factors, or by a phorbol esters such as phorbol 12-myristate 13-acetate[3] (PMA) (*1, 2*). It was previously shown that the amounts of megakaryocytic markers, especially platelet α-granule proteins and platelet membrane glycoproteins, increased markedly in PMA-treated megakaryoblasts following the accumulation of mRNA. PDGF and TGF-β1 are known to be stored in α-granules and secreted under physiological conditions (*5, 6*). Mice lacking PDGF or its receptor exhibit multiple hematologic disorders including thrombocytopenia (*7, 8*), and TGF-β1 is suggested to modulate thrombopoietin-mediated effects on megakaryocytic proliferation (*9*). Thus these growth factors are crucial for megakaryocytopoiesis. A common feature of PDGF and TGF-β–related growth factors is that their active forms are dimers of a carboxy-terminal fragment cleaved from a larger precursor (*10–14*). Therefore their biological activities are determined by proteolytic maturation. Generally the sequence of the processing site is Arg-Xaa-Arg/Lys-Arg, which is a potential cleavage motif for subtilisin-like proprotein convertase (SPC) family proteases. To date, seven members of this family have been identified in mammals: furin, PC1/3, PC2, PACE4, PC4, PC5/6, and PC7/8/LPC (*15–17*). Recent genetic studies have indicated the involvement of PACE4 and furin in the regulation of the biological activities of TGF-β–related factors such as bone morphogenetic proteins (BMPs) during embryogenic development (*18–23*). Previously, Blanchette *et al.* demonstrated that TGF-β1 upregulates its own processing enzyme, furin, in various cell lines (*24, 25*), and Dubois *et al.* reported that both furin and TGF-β1 are expressed in megakaryocytes (*26*). However, little information has been published about the expression of PACE4 in differentiating megakaryoblasts. It was reported that both PACE4 and furin have similar substrate specificities and overlapping expressions, as shown in proalbumin processing in HepG2 cells (*27*). Although PACE4 was long considered to substi-

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Abbreviations: SPC, subtilisin-like proprotein convertase; PMA, phorbol 12-myristate 13-acetate; PDGF-BB, platelet-derived growth factor–BB homodimer; TGF-β1, transforming growth factor–β1; GPIbα, glycoprotein Ib α chain.

tute for or supplement the activity of furin, these enzymes actually have different properties as follows. Furin is found in the trans-Golgi network as a membrane-bound enzyme and exhibits a ubiquitous distribution, whereas PACE4 is a secretory enzyme that shows cell-specific expression (*15–17, 28–31*). In addition, we recently succeeded in bio-engineering specific protein-based inhibitors to discriminate between PACE4 and furin (*32*), and several reports have shown that in substrate processing, the activity of PACE4 is much more limited than that of furin (*33–35*). These findings clearly show a difference in cleavage specificity. On the other hand, the genes for these enzymes are located close together on chromosome 15 in humans and chromosome 7 in mice, suggesting a local duplication during evolution of the SPC family (*36, 37*). However, the overall gene structure is quite different (*38–41*). Recently we found that PACE4 is expressed in human megakaryoblastic cell lines, Dami and HEL cells. In this study, in an attempt to clarify whether PACE4 is needed to achieve platelet production, we analyzed the expression of PACE4 in differentiating megakaryoblasts. The results demonstrate for the first time that PDGF-BB is a major positive regulator of PACE4 expression during megakaryocytopoiesis.

MATERIALS AND METHODS

Growth Factors and Chemical Reagents—Phorbol 12-myristate 13-acetate (PMA) and human transforming growth factor–β1 (TGF-β1) were purchased from Sigma (St. Louis, MO). Human platelet-derived growth factor–BB (PDGF-BB) was from Genzyme/Techne (Minneapolis, MN). Wortmannin was from Calbiochem (La Jolla, CA). LY-294002 was from Upstate Biotechnology (Lake Placid, NY). PD98059 was from Alexis Biochemicals (San Diego, CA). PMA was diluted to 100 μM with dimethyl sulfoxide (DMSO), growth factors were dissolved in 4 mM HCl containing 0.1% BSA, protein kinase inhibitors were dissolved in DMSO, and stored at –80°C until use. The other reagents were of the highest grade available.

Cell Culture and Stimulation—Dami cells, kindly provided by Prof. S. Yamamoto (The University of Tokushima, School of Medicine, Tokushima), were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% horse serum. HEL cells, kindly provided by Prof. N. Ueda (Kagawa Medical University, Dept. Biochem., Kagawa), were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added to all media. The cells were cultured in a humidified 5% CO_2 atmosphere. For treatment with various reagents, Dami and HEL cells were resuspended in fresh culture medium supplemented with 5% serum containing PMA (100 nM), TGF-β1 (1 or 10 ng/ml), or PDGF-BB (1 or 10 ng/ml) at a density of 2×10^5 cells/ml. HEK 293 cells were cultured until 80% confluent following stimulation with PDGF-BB (50 ng/ml) in fresh culture medium containing 5% serum for 6 h. For the inhibition experiments, the cells were pretreated in the absence or presence of wortmannin (0.1 μM), LY294002 (10 μM), PD98059 (10 μM), or DMSO for 10 min before stimulation with PDGF-BB (1 ng/ml) for 6 h.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from the cells using ISOGEN (Nippon Gene, Toyama) according to the manufacturer's instructions. For Northern blotting, total RNA (10 μg) was resolved by electrophoresis in a 1% agarose gel containing 6.7% formaldehyde and then transferred to a Hybond-N+ nylon membrane (Amersham BioSciences, Buckinghamshire, UK). The membranes were prehybridized at 65°C for 3 h in Rapid-hyb buffer (Amersham BioSciences), and then hybridized with radiolabeled cDNA probe at 65°C. The cDNA probes were labeled with [α-^{32}P]dCTP (Amersham BioSciences) using a BcaBEST™ labeling kit (Takara, Kyoto). The sites of the cDNA probes were as follows: PACE4 (corresponding to nucleotides 1372 to 1764 in the human PACE4A-I cDNA), PACE4C/CS (nucleotides 1974 to 2283), PACE4D (–546 to –126), furin (1561 to 2050), PC5/6 (1801 to 2385), PC7/8 (1315 to 1992), GPIbα (–2 to 313) PDGF-B (–9 to 731), and TGF-β1 (837 to 1176). The minus (–) sign is relative to the initiator ATG. After hybridization, the membranes were washed with 2× SSPE (1× SSPE: 10 mM sodium phosphate containing 0.15 M NaCl and 1 mM EDTA, pH 7.4) containing 0.1% SDS at room temperature, and with 1× SSPE containing 0.1% SDS at 65°C. The blots were exposed to X-ray film (Konica, Tokyo) with an intensifying screen at –80°C. The relative intensities of the bands were determined in comparison with the invariant GAPDH signal on the same membrane.

Transfection and Gene Reporter Assays—The reporter plasmid containing 2.0 kb of the 5′-flanking region of the human PACE4 gene was used as described previously (*42*). Dami cells were cultured in the presence of 100 nM PMA for 4 h and transiently transfected with reporter plasmid using FuGENE™6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. FuGENE6 reagent (3 μl) was incubated in 97 μl of IMDM at room temperature. After 5 min, reporter plasmid (0.65 μg) and pGL3-β-galactosidase (0.35 μg) were added and incubated for 30 min at room temperature. Then the FuGENE6-plasmid mixture (100 μl) was added to the cells and incubated at 37°C. After 42 h, the medium was replaced with fresh IMDM containing 5% horse serum and the cells were treated with PDGF-BB (10 ng/ml) for 6 h. The luciferase activity in cell extracts was assayed as described previously (*42*). The luciferase activity was normalized for transfection efficiency by the measurement of β-galactosidase activity. The results are presented as the mean of the luciferase activity ± SD from a single experiment performed in triplicate.

RESULTS

Expression of mRNAs for SPC Family Members in Dami Cells—We first examined the expression profile of SPC family members in Dami cells by Northern blotting. As shown in Fig. 1A, furin (4.4 kb; panel 2) and PC7/8 (4.4 kb; panel 4) mRNAs were expressed in Dami cells, whereas PC5/6 mRNA was undetectable (panel 3). PACE4 transcripts were detected as 4.4 kb and 3.0 kb bands using a common PACE4 cDNA probe (panel 1). The 4.4 kb band was also detected in human hepatoma HepG2 cells, which express PACE4A-II as the major isoform (*27*). The 3.0 kb transcript was not detected in HepG2 cells. Previously, we showed that multiple PACE4 transcripts (A-I, A-II, B, C, CS, D, E-I, and E-II) are produced by the alternative splic-

ing of a single gene (*41*). The carboxy terminal region varies in length and sequence among these isoforms. To determine the origin of the 3.0 kb band, Northern blot analysis was further performed using specific probes for PACE4 isoforms (Fig. 1B). This smaller transcript hybridized with a PACE4C/CS-specific probe (panel 2), but not with a PACE4D probe (panel 3). Consistent with the results of Northern blot analysis, PACE4A-II and PACE4C/CS were identified by a ribonuclease protection assay (data not shown). These results indicate that PACE4A-II, PACE4C (or PACE4CS), furin, and PC7/8 mRNAs are expressed in Dami cells.

Effect of PMA on PACE4, Furin, and PC7/8 mRNA Expression in Dami Cells—Dami cells were cultured in the presence of 100 nM PMA and the time course of the expression of SPCs, PDGF-B, and TGF-β1 mRNA was analyzed by Northern blotting. The morphology of Dami cells changed markedly after exposure to PMA, with increased adherence to the tissue culture dishes and cell spreading. The cell nuclei became larger and lobulated (Fig. 2A). In addition, the differentiation of Dami cells induced by PMA was confirmed by an increase in the megakaryocyte phenotypic marker, platelet glycoprotein GPIbα mRNA (Fig. 2A). As shown in Fig. 2B, the level of GAPDH was unchanged, indicating that PMA treatment does not cause a general increase in cellular gene expression. The basal level of PDGF-B mRNA in untreated Dami cells is very low, and the PMA treatment resulted in a great increase from day 1 with a maximal effect after 2 days (Fig. 2B). Similarly, the expression of TGF-β1 mRNA increased in response to PMA (Fig. 2B). In contrast, PDGF-A mRNA barely detectable in the untreated and PMA-treated Dami cells (data not shown). Subsequently, PMA slowly induced the accumulation of two PACE4 transcripts (PACE4A-II and PACE4C/CS isoforms), and its expression increased markedly from day 2 (Fig. 2, B and C). The accumulation of both transcripts was augmented 4.3-fold at 3 days in the presence of PMA, as compared to the level at zero time. PMA did not affect the ratio of PACE4A-II to PACE4C/CS. Furin mRNA expression also increased gradually over a period of 3 days (Fig. 2, B and C). The expression of PC7/8 mRNA was unchanged (Fig. 2, B and C). Thus PACE4 mRNAs are the most increased by PMA among the SPCs examined. Moreover, the sequential accumulation of PDGF-B and PACE4 mRNA, suggests a positive effect of PDGF-BB on the transcription of PACE4.

Effect of PDGF-BB on PACE4 and Furin mRNA Expression in Dami and HEL Cells—We further analyzed the direct effect of PDGF-BB on PACE4 mRNA expression. When Dami cells were cultured with various concentrations of PDGF-BB, the maximal effect was detectable at 1–10 ng/ml (data not shown), and no morphological changes were observed after treatment of PDGF-BB (Fig. 3A). Both transcripts (PACE4A-II and C/CS) were augmented simi-

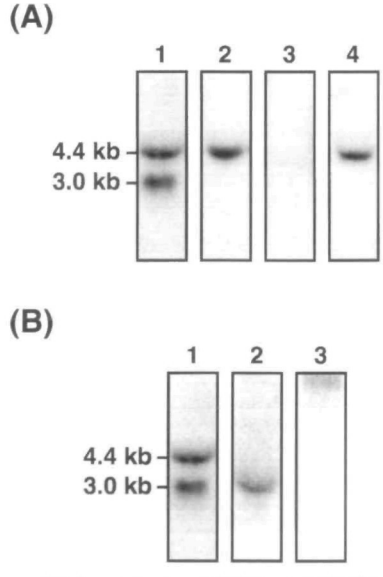

Fig. 1. **Northern blot analysis of SPC and PACE4 isoform expression in Dami cells.** (A) Ten micrograms of total RNA from Dami cells was analyzed by Northern blotting as described under "MATERIALS AND METHODS." The blots were hybridized with cDNA probes, as follows: PACE4 (panel 1), furin (panel 2), PC5/6 (panel 3), and PC7/8 (panel 4). (B) The blots were hybridized with cDNA probes: PACE4 (lane 1), PACE4C/CS (lane 2), and PACE4D (lane 3).

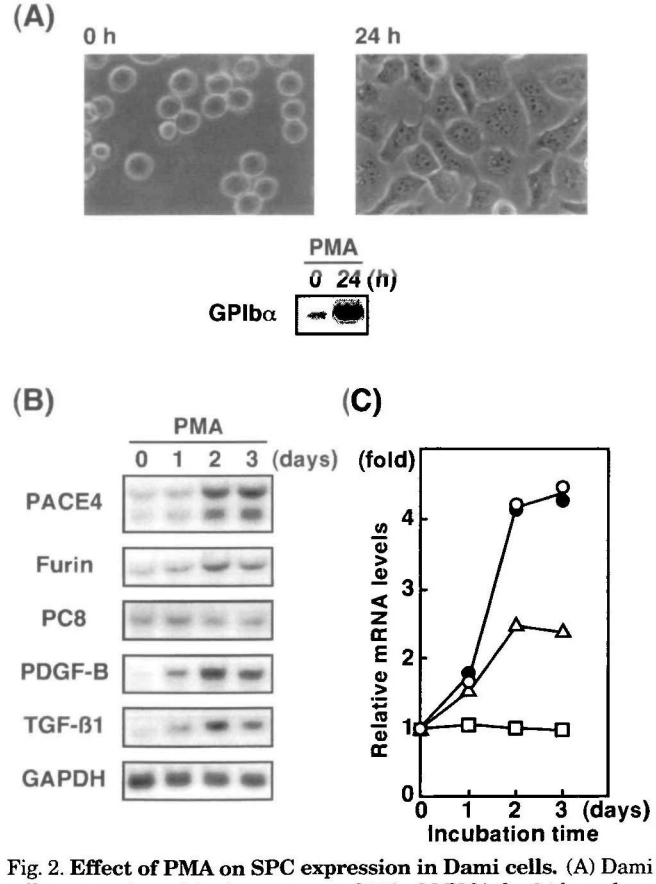

Fig. 2. **Effect of PMA on SPC expression in Dami cells.** (A) Dami cells were cultured in the presence of 100 nM PMA for 24 h, and examined under phase-contrast optics at a magnification of ×200 (upper). Then total RNA was isolated and analyzed for GPIbα mRNA by Northern blotting (lower). (B) The cells were cultured with PMA for the periods indicated. Total RNA was isolated and analyzed by Northern blotting. The blots were hybridized with PACE4, furin, PC7/8, PDGF-B, TGF-β1, and GAPDH cDNA probes. (B) The densities of the PACE4A-II (open circles), PACE4C/CS (closed circles), furin (open triangles), and PC7/8 (open squares) mRNA bands were quantified using an imaging analyzer BAS-1500. The data are expressed relative to the level at zero time after being normalized to the amount of GAPDH mRNA.

larly by treatment with PDGF-BB. In the time-course analysis, the level of PACE4 transcripts was increased slightly at 2 h, and reached a peak at 4–8 h (Fig. 3A). The accumulation of PACE4 mRNA was increased approximately 4-fold at 8 h after treatment with PDGF-BB as compared with the control. However, the furin mRNA level was not influenced at all by PDGF-BB treatment (Fig. 3A). Similar results were obtained when Dami cells were treated with PDGF-BB in serum-free medium (data not shown). To examine the cell specificity of the PDGF-BB effect on PACE4 expression, another megakaryoblastic cell line, HEL cells, was analyzed, because these cells respond to phorbol ester by undergoing terminal differentiation as in the case of Dami cells (*43, 44*). PACE4 transcripts were increased 4.4-fold by PDGF-BB stimulation, while the level of furin mRNA was slightly decreased in HEL cells (Fig. 3B). In contrast, PDGF-BB had no effect on the expression of PACE4 transcripts in HEK 293 cells (Fig. 3B) or Swiss 3T3 fibroblasts (data not shown). Thus these results indicate that only the mRNAs of PACE4 (PACE4A-II and C/CS) are modulated by PDGF-BB among the SPCs expressed in PMA-differentiated megakaryoblasts.

Effect of TGF-β1 on PACE4 and Furin mRNA Expression in Dami Cells—Subsequently, we examined the direct effect of TGF-β1 on PACE4 mRNA expression in Dami cells. TGF-β1 is known to upregulate the expression of furin, its own processing enzyme (*24, 25*). Although furin mRNA expression was increased by TGF-β1 treatment, the PACE4 mRNA level was unchanged (Fig. 4).

PDGF-BB Induces Promoter Activity of the PACE4 Gene

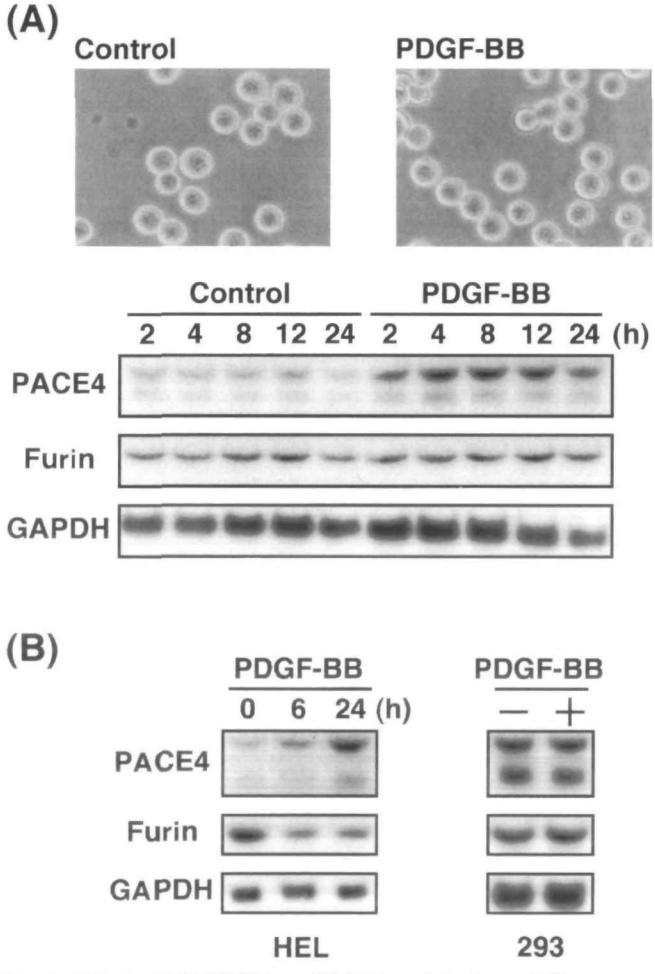

Fig. 3. **Effect of PDGF-BB on PACE4 and furin mRNA expression.** (A) Dami cells were cultured in the presence of 10 ng/ml of PDGF-BB, and examined under phase-contrast optics at ×200 magnification (upper). The cells were lysed at the times indicated, and total RNA was isolated and analyzed by Northern blotting (lower). (B) HEL cells were cultured in the presence of 1 ng/ml of PDGF-BB for 6 and 24 h. HEK 293 cells were cultured in the presence of 50 ng/ml of PDGF-BB for 6 h. Total RNA was isolated and analyzed for PACE4 and furin mRNAs by Northern blotting.

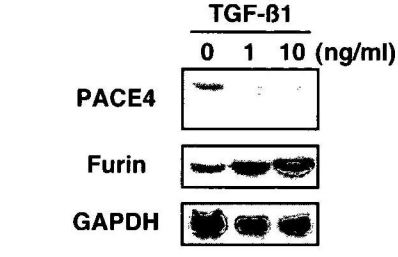

Fig. 4. **Effect of TGF-β1 on PACE4 and furin mRNA expression in Dami cells.** Dami cells were incubated in the presence (1 or 10 ng/ml) of TGF-β1 for 24 h. Total RNA was isolated and analyzed for PACE4 and furin mRNAs by Northern blotting.

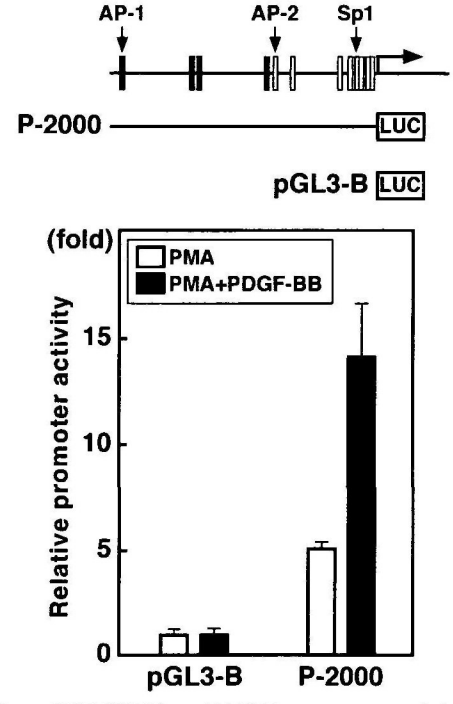

Fig. 5. **Effect of PDGF-BB on PACE4 promoter activity in Dami cells.** A construct P-2000 containing 2.0 kb of the 5′-flanking region was ligated into a luciferase plasmid (pGL3-Basic). Putative regulatory elements are shown at the top of the figure. The transcription start site, –314 in HepG2 cells, is indicated by an arrow. PMA-treated Dami cells were transfected with the reporter gene (P-2000) using FuGENE6 reagent as described under "MATERIALS AND METHODS," and cultured in the absence or presence of PDGF-BB (10 ng/ml) for 6 h. The luciferase activity was expressed relative to the luciferase activity of the promoterless construct (pGL3-B), and normalized to the β-galactosidase activity. The experiment was repeated twice with similar results.

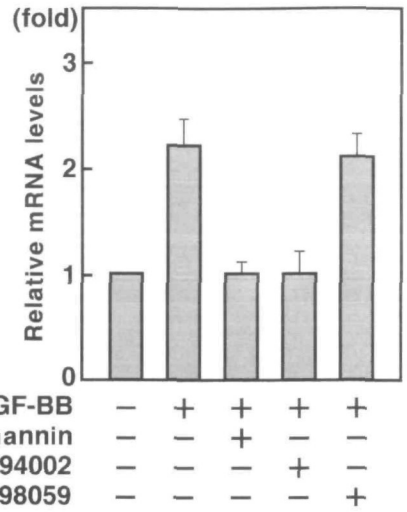

Fig. 6. **Effects of protein kinase inhibitors on PDGF-BB–mediated PACE4 expression.** Dami cells were pretreated in the absence or presence of wortmannin (0.1 μM), LY294002 (10 μM), PD98059 (10 μM), or DMSO for 10 min before stimulation with PDGF-BB (1 ng/ml) for 6 h. Total RNA was isolated and analyzed for PACE4 mRNA by Northern blotting. The results of quantitative analysis of blotted bands obtained in three independent experiments are shown. Each value is expressed as the mean ± SD.

in Dami Cells—To confirm the effect of PDGF-BB on the transcription of PACE4 mRNA, we examined whether PDGF-BB can stimulate the promoter activity of the PACE4 gene through transient transfection with a PACE4 promoter/luciferase reporter gene construct. In this experiment, adherent PMA-treated Dami cells were used to increase the efficiency of transfection. As shown in Fig. 5, the P-2000 construct with 2.0 kb of the 5′-flanking region of the human PACE4 gene fused to the promoterless luciferase reporter gene was used to transfect Dami cells. The cells were cultured in the absence and presence of PDGF-BB after transfection and the luciferase activity in the cells was assayed. The treatment with PDGF-BB produced a marked increase (13.6-fold) in luciferase activity as compared with the control cells (4.9-fold). The luciferase activity of the control cells was also increased. Endogenous PDGF-BB synthesized in the cells might activate the promoter because PMA stimulated the synthesis of PDGF-BB as mentioned above.

Effect of Protein Kinase Inhibitors on the PDGF-BB-mediated Inducion of PACE4—To study the signal transduction pathways underlying the upregulating effect of PDGF-BB on PACE4 expression, we examined the effect of protein kinase inhibitors such as wortmannin and LY-294002, two unrelated inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase) and PD98059, a mitogen-activated protein kinase (MAP-kinase) inhibitor (*45–48*). As shown in Fig. 6, incubation of Dami cells with 0.1 μM wortmannin and 10 μM LY294002 blocked the effect of PDGF-BB on PACE4 mRNA levels, although incubation with 10 μM PD98059 had no such effect. These results indicate that the effect of PDGF-BB on PACE4 expression in Dami cells is exerted through a signal transduction pathway involving PI 3-kinase activity.

DISCUSSION

Recent studies showed the involvement of both PACE4 and furin in the endogenous processing of TGF-β–related proteins required for cell differentiation (*18–23*). The significance of these enzymes during embryogenic development was clearly demonstrated by knockout experiments in mice. Mouse embryos lacking PACE4 develop an ambiguous situs combined with left pulmonary isomerism and/or display varying degrees of holoprosencephaly, whereas furin-deficient embryos fail to undergo axial rotation and develop severe ventral closure and heart morphogenesis defects (*49–51*). Thus neither PACE4 nor furin can compensate for the loss of the other enzyme, indicating distinct roles for these proteins. Understanding the difference in the transcriptional regulatory mechanisms of PACE4 and furin is another approach to clarifying their roles. Recently, we reported that PACE4 gene expression is suppressed by the mammalian achaete-scute homolog-1 (MASH-1) transcription factor in neuroblastoma cell lines (*52*). However the upregulating factors have not yet been identified. In the present study, we compared the regulatory mechanisms of PACE4 and furin gene expression during the differentiation of the megakaryoblastic cell lines, Dami and HEL cells. These cells express three kinds of SPC family proteases, PACE4, furin, and PC7/8. In addition, two kinds of PACE4 transcripts were detected. The 4.4 and 3.0 kb mRNAs were identified as PACE4A-II and PACE4C/CS transcripts by Northern blotting and ribonuclease protection assay. PACE4A contains a signal peptide, a propeptide, a subtilisin-like catalytic domain (SCD), a homoB domain and a cysteine-rich region (CRR). PACE4C lacks a carboxy-terminal region in the homoB domain and the entire CRR (*53*). PACE4CS is a C-terminally truncated form of PACE4C (*54*). The sizes of the mRNAs coding PACE4C and CS are almost the same. Although PACE4C and PACE4CS were shown to be inactive enzymes by coexpression experiments, their roles are still unknown (*54, 55*). The levels of both PACE4 transcripts (PACE4A-II and C/CS) were increased more than 4-fold after treatment with PMA for 2 days, whereas the furin mRNA level showed only a slight increase, and the level of PC7/8 mRNA was unchanged. More importantly, we showed that PDGF-BB increased PACE4 expression in a time-dependent manner, whereas it had no effect on furin expression. The upregulation of the expression of PACE4 transcripts by treatment with PDGF-BB was confirmed in another megakaryoblastic cell line, HEL cells. By contrast, direct treatment with TGF-β1 enhanced furin mRNA expression, however, PACE4 expression was almost unchanged.

PDGF is a potent stimulant causing mesenchymal cell proliferation, migration, and altered cell metabolism *in vitro* and *in vivo* (*56–58*). Moreover, PDGF also stimulates hematopoiesis by promoting granulopoiesis (*59*), erythropoiesis (*60, 61*) and megakaryocytopoiesis (*62*). A crucial role for PDGF in megakaryocytopoiesis and platelet production was suggested by the generation of mice deficient in PDGF-B and its receptor, PDGF receptor–β (PDGFR-β). These mice exhibit multiple hematologic disorders including thrombocytopenia (*7, 8*). In addition, megakaryocytes have been found to express PDGF and the PDGF receptor (*63*). Therefore, these results suggest that PACE4 gene ex-

pression is upregulated by PDGF-BB in an autocrine manner or synergistically with other factors. The binding of PDGF to its receptor results in the activation of several intracellular signaling pathways, including the Ras/MAP-kinase pathway and PI 3-kinase pathway. Exposure of megakaryocytes to PDGF-BB markedly induces the expression of the immediate-early gene, and significantly stimulates cell proliferation and colony formation (*63*). PDGF-BB is known to activate nuclear transcription factors such as Fos, Jun, Stat and Ets in other cellular systems (*64, 65*). The promoter region of the human PACE4 gene contains several potential binding sites for the transcription factors AP-1, AP-2, and Sp1, which are candidate mediators of PDGF-BB signaling. Further analysis is necessary to elucidate the sequence in the 5′-flanking region of the PACE4 gene important for the induction by PDGF-BB.

Although TGF-β1 regulates the expression of its own converting enzyme, furin (*24, 25*), it is unlikely that pro-PDGF-BB is activated by PACE4. Coexpression experiments indicated that pro-PDGF-BB is a poor substrate for PACE4 (data not shown). On the other hand, the levels of von Willebrand factor (vWF) mRNA and mature protein were increased in Dami cells as were PDGF-BB and PACE4 (*4, 66*). vWF is initially synthesized as pro-vWF, a larger precursor form, and then converted to mature vWF through cleavage of the propeptide at a dibasic site. It was shown that PACE4 is able to process the precursor form of vWF to the mature form in coexpression experiments (*35, 55*). It is suggested that pro-vWF is a candidate for the endogenous substrate of PACE4. To date, however, the physiological substrate of PACE4 has yet to be identified.

In conclusion, we revealed for the first time that PACE4 mRNA expression is selectively upregulated by PDGF-BB in megakaryoblasts, unlike the expression of furin mRNA. Our results indicate that PACE4 and furin play distinct roles in megakaryocyte differentiation. Further study of the induction of PACE4 mRNA will afford new insight into the mechanism of the differentiation of megakaryocytes into platelets.

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