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Expression and Function of the Collagen Receptor GPVI during Megakaryocyte Maturation*

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In this report, the expression and function of the platelet collagen receptor glycoprotein VI (GPVI) were studied in human megakaryocytes during differentiation and maturation of mobilized blood and cord blood derived $CD34^+$ cells. By flow cytometry, using an anti-GPVI monoclonal antibody or convulxin, a GPVI-specific ligand, GPVI was detected only on $CD41^+$ cells including some $CD41^+/CD34^+$ cells, suggesting expression at a stage of differentiation similar to CD41. These results were confirmed at the mRNA level using reverse transcription-polymerase chain reaction. GPVI expression was low during megakaryocytic differentiation but increased in the more mature megakaryocytes ($CD41^{high}$). As in platelets, megakaryocyte GPVI associates with the Fc receptor γ chain (FcRγ). The FcR γ chain was detected at the RNA and protein level at all stages of megakaryocyte maturation preceding the expression of GPVI. The other collagen receptor, $\alpha_2\beta_1$ integrin (CD49b/CD29), had a pattern of expression similar to GPVI. Megakaryocytic GPVI was recognized as a 55-kDa protein by immunoblotting and ligand blotting, and thus it presented a slightly lower apparent molecular mass than platelet GPVI (58 kDa). Megakaryocytes began to adhere to immobilized convulxin via GPVI after only 8–10 days of culture, at a time when megakaryocytes were maturing. At this stage of maturation, they also adhered to immobilized collagen by $\alpha_2\beta_1$ integrin-dependent and -independent mechanisms. Convulxin induced a very similar pattern of protein tyrosine phosphorylation in megakaryocytes and platelets including Syk, FcRγ, and $PLC_{\gamma 2}$. Our results showed that GPVI is expressed early during megakaryocytic differentiation but functionally allows megakaryocyte adherence to collagen only at late stages of differentiation when its expression increases.

At sites of vascular injury, platelets adhere and are activated by contact with collagen fibers exposed with the subendothelium matrix leading to thrombus formation. Several collagen receptors are present on platelets: integrin $\alpha_2\beta_1$ (CD49b/CD29), GPIV[1] (CD36), GPVI, p65, and a recently cloned type III collagen receptor (1, 2). The direct interaction between collagen and integrin $\alpha_2\beta_1$ adheres platelets at the collagen surface, but platelet activation and thrombus formation and attachment are subsequently mainly supported by GPVI. In addition to collagen, two GPVI-specific ligands have been described: collagen-related peptides (3) and convulxin, a snake venom protein (4). GPVI has recently been characterized as a new member of the immunoglobulin superfamily of cell-associated receptors, in which expression is restricted to the hematopoietic lineage (5–7). It is coexpressed as a noncovalent complex with the common immunoglobulin receptor γ (FcRγ) chain, which acts as the signaling subunit of the complex (8, 9) As a consequence, signaling pathways coupled to GPVI dimerization are identical to those coupled to other immune receptors; tyrosine phosphorylation of the FcR γ chain on its immunoreceptor tyrosine-based motif (ITAM) by a Src familly kinase (8, 10, 11) allows recruitment by the SH2 (Src homology 2) domain of $P72^{Syk}$ that is in turn phosphorylated and activated. Downstream of Syk, $PLC_{\gamma 2}$ is activated, leading to platelet aggregation and dense granule secretion (12).

Expression of platelet receptors during megakaryocyte differentiation discriminates different stages of megakaryocyte maturation (13, 14). GPIIb/GPIIIa (CD41/CD61) are platelet proteins that are the first expressed during megakaryocyte differentiation, although their expression is not entirely specific of this cell lineage (15, 16). A fraction of megakaryocyte progenitors, colony-forming unit megauaryocytes, express GPIIb and GPIIIa (17). Later in differentiation, promegakaryoblasts and megakaryoblasts also express the GPIb·GPIX complex (CD42) and CD36. More recently, it has been shown that GPV, in which the promoter has been cloned, is expressed during megakaryocyte differentiation even later than the GPIb·GPIX complex (18).

Our study was designed to characterize GPVI expression during megakaryocytopoiesis and to determine more precisely the kinetics of GPVI expression and its functionality in megakaryocytes. Cell surface expression of GPVI was studied by flow cytometry in comparison with cell surface expression of CD49b and by blotting experiments using specific antibodies or one specific ligand, convulxin. Expression of GPVI and FcR γ chain mRNA was studied by RT-PCR. Functional characterization was performed by measuring adhesion to immobilized collagen or convulxin and by studying the signals coupled to GPVI. Our results indicate that GPVI is expressed at an early stage of megakaryocyte differentiation but at low levels and that it is functional in mature megakaryocytes, suggesting that

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[1] The abbreviations used are: GP, glycoprotein; FcRγ Fc receptor γ chain; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; BSA, bovine serum albumin; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; TPO, thrombopoietin.

GPVI may regulate thrombopoiesis and platelet release *in vivo* through its interactions with collagen.

EXPERIMENTAL PROCEDURES

Materials—Convulxin was purified from the venom of *Crotalus durissus terrificus* (from Fundação Ezequiel Dias (FUNED), Minas Gerais, Brazil or Latoxan, Valence, France) as previously described (19). Convulxin was labeled with ^{125}I using the Iodogen procedure (Pierce) and Na^{125}I (Amersham Pharmacia Biotech) or was coupled to fluorescein (FITC) as previously described (7).

The anti-GPVI monoclonal antibody (mAb), 3J24·2, was produced by immunizing Balb/c mice with the DNA encoding a fusion protein corresponding to the extracellular domain of GPVI (residues 1–269) fused at its C terminus via a 3 Ala linker to the human IgG_1 Fc sequence (shGPVI-Fc)[7] using the Rapid Immunization Gene Gun delivery as described (20). Mice were boosted with 100 μg of intravenous shGPVI-Fc 4 days prior to fusion. Spleen cells of one mouse were fused with SP2/0 myeloma cells using PEG 1500, and hybridoma lines were screened for secretion of GPVI-specific antibodies by enzyme-linked immunoassay on plate-bound shGPVI-Fc. Antibodies were isotyped, and 3J24·2 was shown to be IgG_1. Selected cell lines were cloned using ClonalCell™-HY medium D (Stem Cell Technology, Vancouver, British Columbia, Canada). Ascitic fluids were produced, and antibodies were purified by chromatography on protein A-Sepharose (Amersham Pharmacia Biotech). Purified 3J24·2 does not inhibit collagen- or convulxin-induced platelet activation but behaves as a mild platelet agonist inducing a low level of aggregation (~10%) after prolonged incubation (15 min) at 37 °C with stirring. 3J24·2 recognizes nonreduced GPVI in immunoblotting experiments. 3J24·2 was conjugated to FITC by the same method as used for convulxin (7). Polyclonal anti-GPVI IgGs were purified from a patient plasma (21), kindly provided by Dr. M. Okuma, and Fab fragments were from the same batch as in previous studies (4). The anti-integrin $\alpha_2\beta_1$ monoclonal antibody was kindly provided by Dr. B Coller (Mount Sinai Medical Center, New York, NY) (22).

Collagen type I (from equine tendon) or collagen from fetal calf skin was purchased from Chrono-Log Corp. (Haverton, PA) or Horm (Hormon-Chemie, Munich, Germany) and Bio/Data Corporation (Horsham, PA), respectively. The following mAbs were purchased from Beckman-Coulter (Marseille, France): FITC anti-CD49b mAb (anti-integrin α_2; clone Gi9), R-PE anti-CD41 mAb, R-PE-Cy5 anti-CD34 mAb and FITC anti-CD42b mAbs. The anti-PY 4G10 was obtained from Upstate Biotechnology (Euromedex, Souffelweyersheim, France) as the mAb directed against the FcR γ chain that was conjugated to FITC. Anti-phosphotyrosine (PY20), anti-Syk (LR Syk), and anti-PLC$_{\gamma 2}$ (Q20) mAbs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The peroxidase-coupled sheep anti-mouse IgG and the chemiluminescent reagent ECL were from Amersham Pharmacia Biotech. Phenylarsine oxide, vanadate, leupeptin, PMSF, aprotinin, and Nonidet P-40 were obtained from Sigma, and polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, MA).

Purification of CD34$^+$ Cells—After obtaining informed consent, an aliquot of leukapheresis from adult patients, after mobilization by chemotherapy and granulocyte/colony-stimulating factor, was obtained for research purposes. Human umbilical blood cells were obtained from full term deliveries under guidelines established by the Ethical Committee. Cells were separated over a Ficoll-metrizoate gradient (Lymphoprep, Nycomed Pharma, Oslo, Norway). CD34$^+$ cells were then isolated by a positive selection using the Miltenyi immunomagnetic bead technique according to the manufacturer's protocol. The purity was about 90% after two passages through the column as estimated by flow cytometry.

In Vitro Liquid Cultures of Megakaryocytes from CD34$^+$ Cells—CD34$^+$ cells were grown for 5–14 days in "serum-free" Iscove's modified Dulbecco's medium (Life Technologies, Inc.) prepared as previously reported (23). The medium was supplemented with a combination of pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF 10 ng/ml, a generous gift from Kirin, Tokyo, Japan), a truncated form of thrombopoietin (TPO), and 50 ng/ml recombinant human stem cell factor (a generous gift from Amgen, Thousand Oaks, CA) for flow cytometry or with PEG-rHuMGDF alone for biochemical and adhesion experiments.

Cell Sorting of Different CD41 Subsets—Megakaryocytes at different stages of differentiation were obtained after 6 days of culture. Cells were incubated with a mixture of a FITC anti-CD42a, R-PE anti-CD41a, and R-PE-Cy5 anti-CD34 mAbs for 30 min at 4 °C in their culture medium. Cells were washed in culture medium and sorted into the following five populations using a FACS Vantage flow cytometer (Becton Dickinson) equipped with an argon laser (Coherent Radiation, Palo Alto, CA) and a 100-μm nozzle: CD34$^+$CD41a$^-$, CD34$^+$CD41a$^+$CD42a$^-$, CD34$^-$CD41a$^+$CD42a$^-$, CD34$^-$CD41a$^+$CD42a$^+$, and the entire CD41^{high} cells corresponding to mature megakaryocytes. Each cell fraction except the CD41^{high} one was re-sorted to ensure a purity level greater than 97%.

RT-PCR—RNA was extracted according to the technique of Chomczynsky and Sacchi (24). Total RNA (500 ng-1 μg) was denatured at 70 °C for 10 min and then reverse-transcribed with avian myeloblastosis leukemia virus reverse transcriptase (20 units, Promega, Madison, WI) at 42 °C for 1 h with random hexanucleotides (200 ng, Amersham Pharmacia Biotech) as primers in a final volume of 50 μl.

After reverse transcription, each sample was subjected to amplification of GPVI, FcR γ chain, or β_2 microglobulin cDNA. The sequences of the specific primers for GPVI, FcR γ chain, and β_2 microglobulin were: 5′-TTCTGTCTTGGGCTGTGTCTG-3′, 5′-AGATGATTCCAGCAGTGGTCTT-3′, and 5′-CCTGAAGCTGACAGCATTCGG-3′ for sense primers, respectively, and 5′-CCCGCCAGGATTATTAGGATC-3′, 5′-TCTTTAACAGGGAGGAGGAACC-3′, and 5′-CTCCTAGAGCTACCTGTGGAG-3′ for antisense primers, respectively.

PCR was performed as previously reported in a 25-μl reaction mixture (containing 30 pmol of primers for GPVI and FcR γ chain amplifications and 10 pmol of primers for β_2 microglobulin amplification) in ATGC buffer. The reaction mixture was subjected to denaturation for 5 min at 95 °C and then amplified by 35 cycles as previouly reported (7). PCR products (9 μl) were electrophoresed on a 2% agarose gel. Fragments were visualized by illumination after ethidium bromide staining. MassRuler DNA Ladder, low range (MBI, Fermentas Vilnius, Lithuania) was used as a size marker.

Flow Cytometry—Cells were studied from day 5 to day 14 of culture. To study GPVI during megakaryocyte maturation, cells were simultaneously labeled for 30 min with R-PE-Cy5 anti-CD34 mAb, R-PE anti-CD41 mAb, and a FITC anti-CD42 or FITC anti-GPVI mAb (3J24-2) or FITC anti-CD49b mAb and subsequently washed. Isotype IgGs were used as controls. In some experiments, cells were labeled by the R-PE anti-CD41 mAb or R-PE anti-CD34 mAb and FITC-convulxin (20 nM). Nonspecific labeling was measured using FITC-coupled bothrojaracin (20 nM), another protein from the same C-type lectin snake venom protein family as convulxin but which does not bind to platelets (7). For studying the expression of the FcR γ chain, cells were incubated with R-PE CD41, washed, fixed by 0.5% paraformaldehyde for 15 min, and then permeabilized by 0.1% Triton X-100, washed, and incubated with the FITC anti-FcR γ chain antibody. Cells were then analyzed by flow cytometry using a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Adhesion to Immobilized Collagen or Convulxin—Adhesion experiments were performed using cells cultured in the presence of TPO alone, a culture condition that allows the growth of megakaryocytes with a high level of purity (about 90%). Adhesion was measured in static conditions on microtitration plates as previously described (4, 7). Briefly, collagen (2 μg) and convulxin (1.5 μg) were immobilized on Immulon II plates. After saturation with BSA, megakaryocytes were added for 60 min at 37 °C in the presence or the absence of 10 mM EDTA. Megakaryocyte adherence was quantified by measuring alkaline phosphatase activity as follows: 100 μl of 0.1 M sodium citrate, pH 5.4, containing 0.3% v/v Triton and 7 mM of paranitrophenylphosphate were added to emptied wells. Reaction was stopped with 1 M NaOH, and optical density was measured at 405 nm. Nonspecific adhesion was measured using wells coated with BSA. The percentage of adherent cells was calculated from a calibration curve performed using cells in suspension.

Immunoblotting and Ligand Blotting—Cells (2.10^6/ml) were lysed in a radioimmune precipitation buffer composed of 1% v/v Nonidet P-40 in 12 mM Tris, 300 mM NaCl, 12 mM EDTA containing 0.2 mM PMSF, 2 μM leupeptin, and 5 Kallikrein inhibitory units (KIU) of aprotinin. Proteins (40 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (6, 19). Membranes were incubated with polyclonal anti-GPVI IgG or with 3J24-2 or the anti-FcR γ chain mAbs. IgGs were detected with peroxidase-coupled protein A for the polyclonal antibody or peroxidase-coupled goat anti-mouse IgG for the mAbs and revealed by chemiluminescence. Ligand blotting was performed as previously reported (4, 7) using ^{125}I-labeled convulxin and autoradiography.

Tyrosyl phosphorylations were studied on cells cultured in the presence of TPO alone. Megakaryocytes (1.10^6 cells/ml) in Hanks buffer containing 2 mg/ml BSA were incubated in an aggregometer at 37 °C under stirring conditions (1100 rpm). Convulxin (10 nM) or collagen (100 μg/ml) was added to the suspension for 6 min. Incubation was

stopped by the addition of 100 μl from a mixture containing 65 mM EDTA, 33 mM vanadate, and 2 μM phenylarsin oxide (PAO) (25). Samples were centrifuged for 1 min at 13,000 × *g*. Pellets were then lysed in a buffer containing 1% v/v Triton, 0.1% w/v SDS, 5% β-mercaptoethanol, 4.2 mM EDTA, 24 mM vanadate, 4 μM PAO, 0.25 mg/ml pefabloc, 1.5 mM PMSF, 500 KIU/ml aprotinin, and 5 μg/ml leupeptin (Sigma). Proteins were separated by SDS- polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were incubated with the anti-phosphotyrosine mAb PY20 followed by peroxidase-coupled anti-mouse IgGs and chemiluminescence.

Immunoprecipitation—Megakaryocytes (0.5×10^6) or platelets (2.5×10^8) were lysed by the addition of a radioimmune precipitation buffer containing 1% (v/v) Nonidet P-40, 5 mM vanadate, 2 mM PMSF, 0.25 mg/ml pefabloc, 500 KIU/ml aprotinin, and 5 μg/ml leupeptin. Samples were precleared by incubation with protein A-Sepharose for 15 min at 4 °C and centrifuged to reduce nonspecific signal (25). Lysates were incubated with anti-$PLC_{\gamma 2}$ mAb, anti-Syk mAb, or 3J24-2 mAb overnight at 4 °C and then incubated with the protein A/G-Sepharose for 2 h at 4 °C. Immunoprecipitated proteins were solubilized by 2% (w/v) SDS, reduced with 5% (v/v) 2-mercaptoethanol, except for GPVI immunoprecipitation, and finally transferred to PVDF.

RESULTS

Study of GPVI Expression during Megakaryocyte Differentiation—To determine expression of GPVI during megakaryocyte differentiation, we used a new mAb called 3J24·2 directed against human GPVI and the binding of a specific ligand, convulxin. Megakaryocyte cultures were performed from $CD34^+$ cells isolated from cord blood or leukapheresis and were

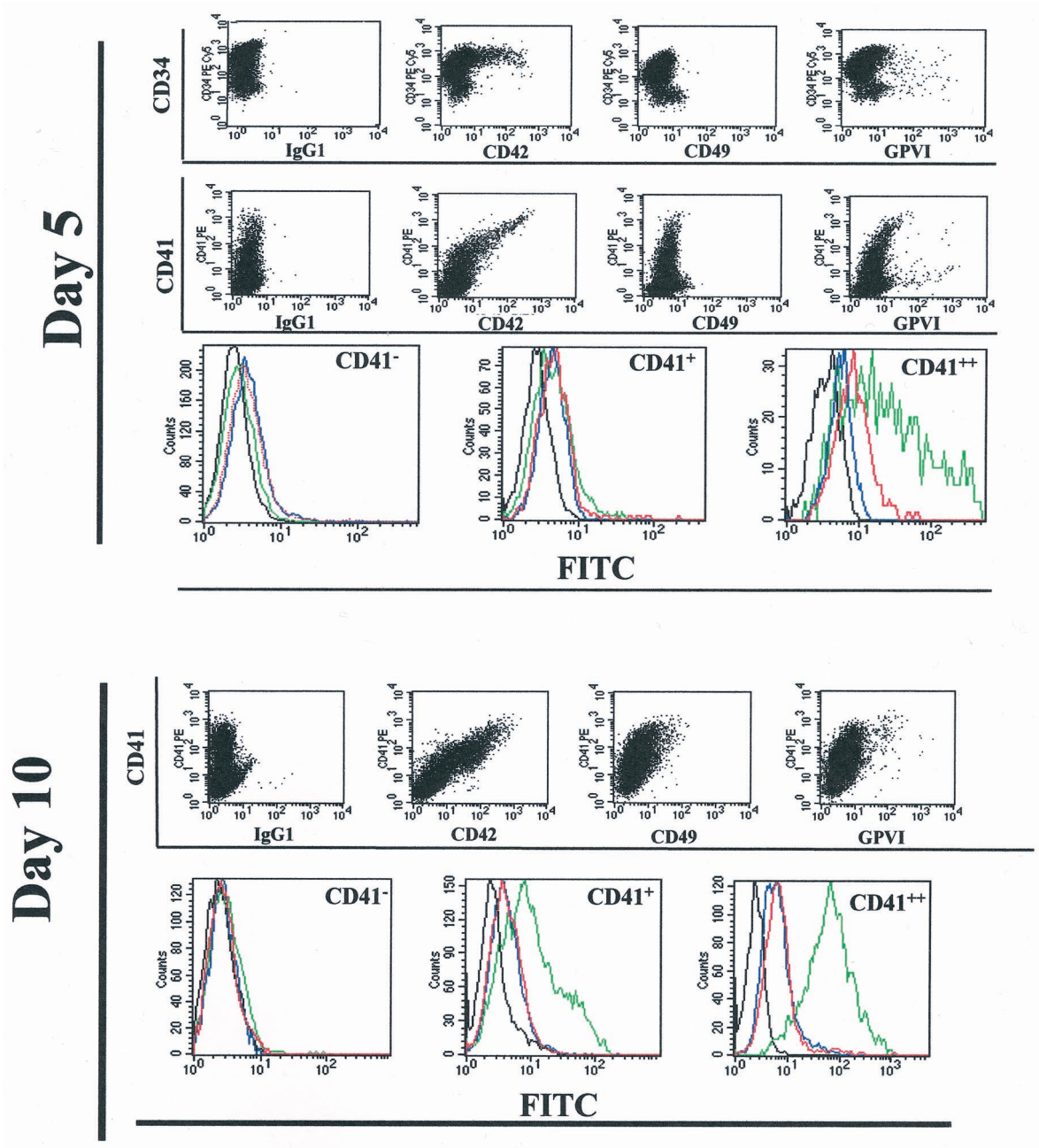

FIG. 1. **Expression of GPVI and $\alpha_2\beta_1$ integrin using flow cytometry on mobilized peripheral blood $CD34^+$.** Cells were analyzed on a FACSort flow cytometer at day 5 or 10 in *in vitro* culture. Cells (100,000 cells/50 μl) were incubated for 30 min at 4 °C with R-PE-Cy5 anti-CD34 mAb, R-PE anti-CD41 mAb, FITC anti-CD42 mAb, FITC anti-CD49b mAb, or FITC anti-GPVI mAb as described under "Experimental Procedures," and controls were performed with R-PE-Cy5 IgG_1 mouse antibody, R-PE IgG_1 mouse antibody, or FITC IgG_1 mouse antibody. Histograms of fluorescence were determined in CD41 negative cells (*$CD41^-$*), immature megakaryocytic cells (*$CD41^+$*), and more mature megakaryocytic cells (*$CD41^{2+}$*). FITC anti-CD42 labeling (*green line*), FITC anti-CD49b labeling (*blue line*), and FITC anti-GPVI labeling (*red line*) were compared with control FITC IgG_1 mouse antibody (*black line*). The results are representative of five independent experiments.

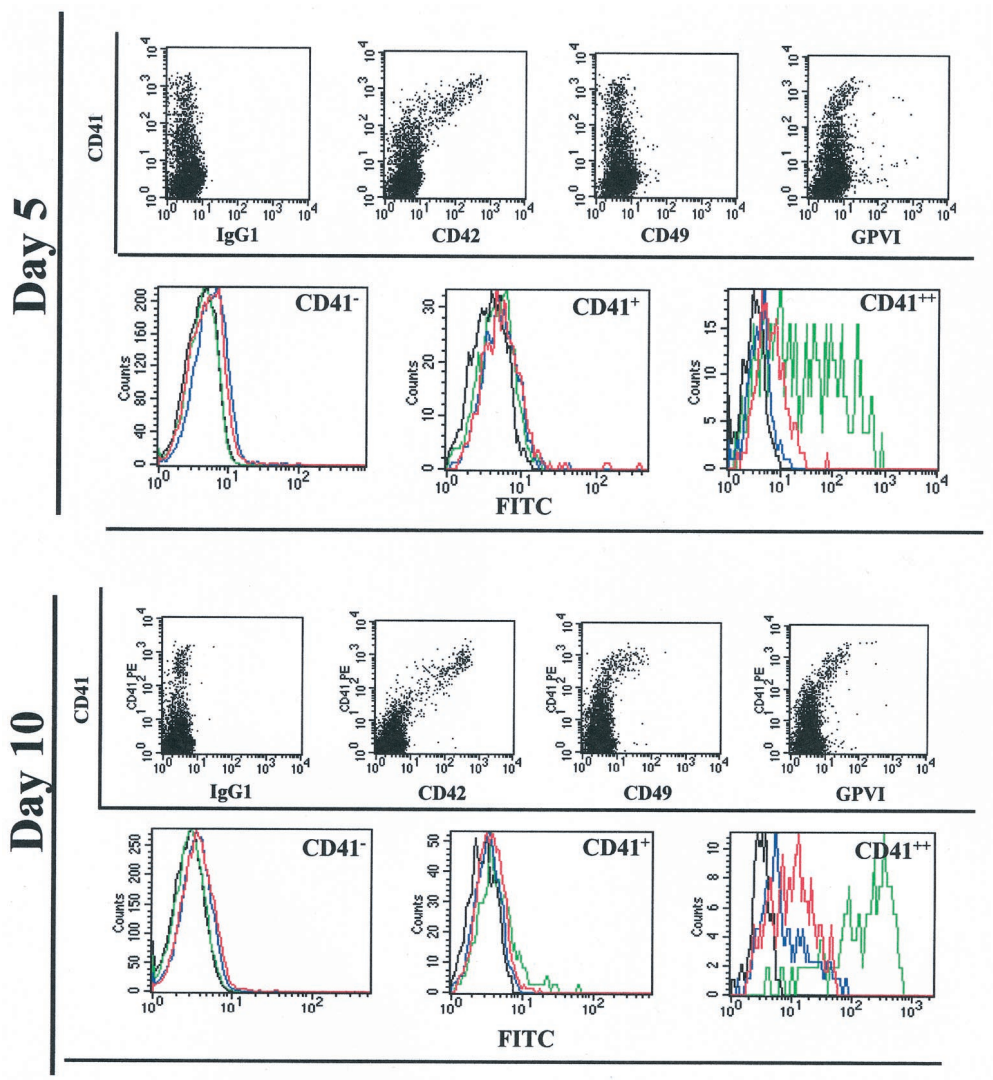

FIG. 2. **Expression of GPVI and $\alpha_2\beta_1$ integrin using flow cytometry on cord blood CD34$^+$.** Experiments were performed as described in Fig. 1. Cultures were performed from cord blood CD34$^+$ cells and analyzed at days 5 and 10. Histograms of fluorescence were determined in CD41$^-$, CD41$^+$, and CD41^{2+} cells. FITC anti-CD42 labeling (*green line*), FITC anti-CD49b labeling (*blue line*), and FITC anti-GPVI labeling (*red line*) were compared with control FITC IgG_1 mouse antibody (*black line*). The results are representative of three independent experiments.

studied at different days of culture from days 5 to 14. To analyze the precise stage at which GPVI is expressed, a triple staining technique was performed using the CD34 and CD41 antigens as markers of differentiation; CD41$^+$ cells corresponded to immature megakaryocytic cells, whereas CD41^{2+} cells to mature megakaryocytes. In addition, expression of GPVI was compared with that of another collagen receptor, CD49b ($\alpha_2\beta_1$integrin), and with GPIbα.

Using megakaryocytes derived *in vitro* by culture in TPO, GPVI was present only on the CD41$^+$ cell subset. GPVI was expressed on CD41+ cells regardless of time in culture (5, 10 (Figs. 1 and 2), or 14 days (data not shown)). Expression was also not dependent on the source of CD34$^+$ cells, blood-mobilized cells (Fig. 1), or cord blood (Fig. 2). GPVI was also present on both CD34$^+$ cells (Figs. 1 and 3) but only in the CD34$^+$CD41$^+$ cell subset. However, expression of GPVI was low during megakaryocytic differentiation and was just over the background in the CD41$^+$ cells (see histograms in Figs. 1–3). Expression of GPVI increased with maturation in parallel with the augmentation of CD41 antigen on the cell surface (see dot plots in Figs. 1–3). CD49b had a similar pattern of expression to GPVI in these cultures with a weak expression in the CD41$^+$ cells and increased expression in the CD41^{2+} cells (Figs. 1–3). However, there was a great variation in the expression of CD49b. In some experiments, the level of CD49b was at the threshold of detection (Figs. 1 and 2), whereas in other experiments it was at an intermediate point between the level of CD42 and GPVI (Fig. 3). This heterogeneity in the expression of CD49b might be the consequence of CD49b polymorphisms(26). Expression of CD42 was slightly different from that of GPVI. Indeed, its level was much higher and increased linearly with the CD41 antigen (Figs. 1–3). However, it appears, especially in experiments with cord blood cells, that a fraction of CD41$^+$ cells did not express CD42 (see Fig. 2, *Day 5*).

Together, these results indicate a very weak expression of GPVI and CD49b on immature megakaryocytes. The two molecules increase at the end of the maturation concurrent with CD41 expression, but their expression remains much weaker than that of CD42. To confirm that GPVI was synthesized early in differentiation, RT-PCR analysis was performed on different cell fractions sorted on the expression of CD34, CD41, and CD42. GPVI mRNA was detected in all CD41$^+$ cell fractions including those that also express CD34, whereas it was not detected in the CD41$^-$ cell fraction (Fig. 4*A*).

To determine whether functional binding of megakaryocyte GPVI to a specific ligand takes place, flow cytometry was per-

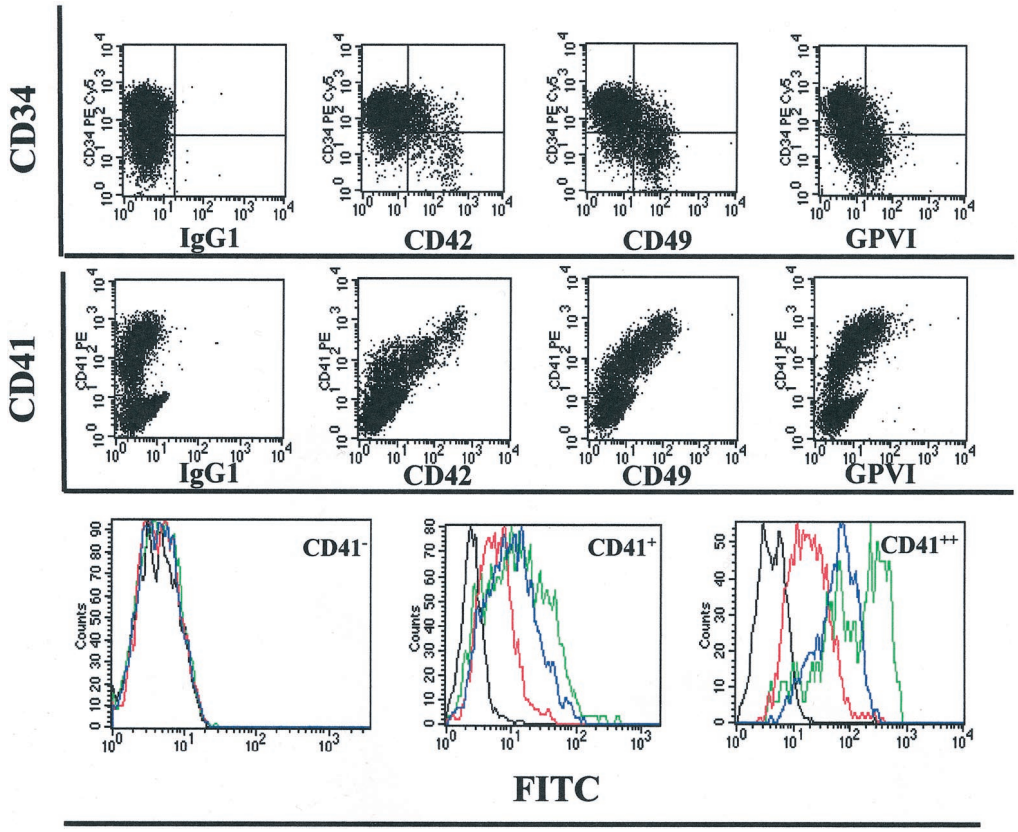

FIG. 3. **Differences in CD49b expression among patients.** Similar experiments as in Fig. 1 were performed on the cells from another donor. A higher level of CD49b expression is observed, probably reflecting integrin $\alpha_2\beta_1$ polymorphisms (29). Of seven individuals, CD49b expression was only found highly expressed in two.

formed using FITC-convulxin and R-PE anti-CD41 mAb or R-PE anti-CD34 mAb. The results confirmed those obtained with the anti-GPVI mAb; FITC-convulxin labeled $CD41^+$ cells just over the threshold when compared with the control snake venom protein, FITC-coupled bothrojaracin (Fig. 5, *Day 8*). This level of binding was low but much more pronounced in mature megakaryocytes ($CD41^{2+}$) (Fig. 5). Altogether, these results indicate that GPVI is expressed at low levels during megakaryocyte differentiation and might be functional.

Megakaryocyte Express GPVI as a 55-kDa Protein Associated with the FcR γ Chain—Expression of GPVI by megakaryocytes was further characterized by immunoblotting and ligand blotting and by immunoprecipitation. As previously reported, platelet GPVI was detected as a 58-kDa band protein with both the anti-GPVI mAb (Fig. 6, *lane a*) and ^{125}I-convulxin (Fig. 6, *lane d*). The anti-GPVI mAb (Fig. 6, *lane b*) as well as ^{125}I-convulxin (Fig. 6, *lane e*) both labeled a 55-kDa band in megakaryocyte lysates, suggesting that GPVI expressed by megakaryocytes has a slightly lower molecular mass than platelet GPVI. One characteristic behavior of platelet GPVI as of recombinant GPVI in immunoblotting experiments is that convulxin blocks the binding of polyclonal anti-GPVI antibodies. (4, 7) The same observation is made for megakaryocyte GPVI (Fig. 6, *lanes g–h*). At least, 3J24.2 precipitated a 56-kDa protein from megakaryocyte lysates, which was recognized by ^{125}I-convulxin (Fig. 6, *lanes c* and *d*). Together, these results indicate that megakaryocytes produce GPVI.

In platelets, FcR γ chain is associated as a noncovalent complex with GPVI and acts as the signaling chain (8, 11). We checked whether the FcR γ chain is also expressed by adult or neonate $CD34^+$-derived megakaryocytes. Using FITC-coupled antibody, which binds to the intracellular domain of the γ chain and permeabilized cells in flow cytometry studies, FcR γ chain

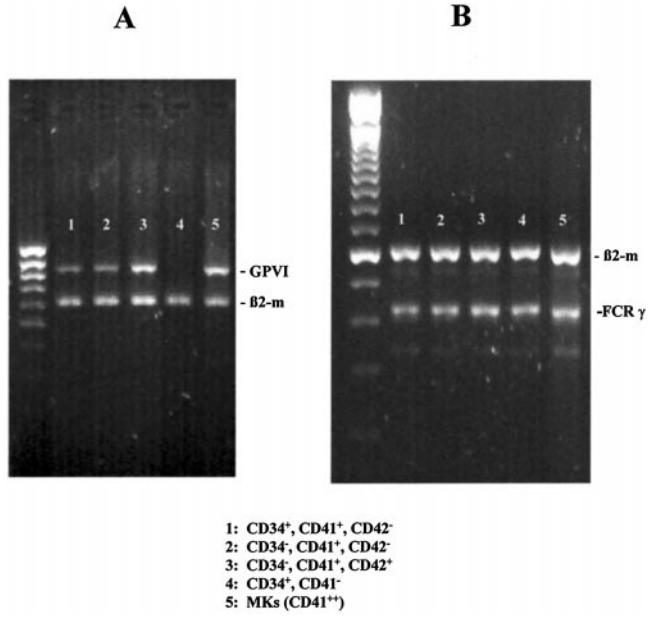

FIG. 4. **Expression of GPVI using RT-PCR.** Megakaryocytes at different stage of differentiation were obtained after 6 days of culture and were sorted according to their immunophenotype into five different populations: 1) $CD34^+CD41a^+CD42^-$, 2) $CD34^-CD41a^+CD42a^-$, 3) $CD34^-CD41a^{2+}CD42a^{2+}$, 4) $CD34^+CD41a^-$, and 5) the entire $CD41^{2+}$ cell population, corresponding to mature megakaryocytes. GPVI and β_2 microglobulin ($\beta 2$) transcripts were amplified in *panel A*. The high molecular weight fragment (830 bp) is generated from GPVI primers, and the low molecular weight fragment (603 bp) is generated from the β_2 microglobulin primers. FcR γ chain and β_2 microglobulin transcripts were amplified in *panel B*. The high molecular weight fragment (603 bp) is generated from the β_2 microglobulin primers, and the low molecular weight fragment (429 bp) is generated from the FcR γ chain primers.

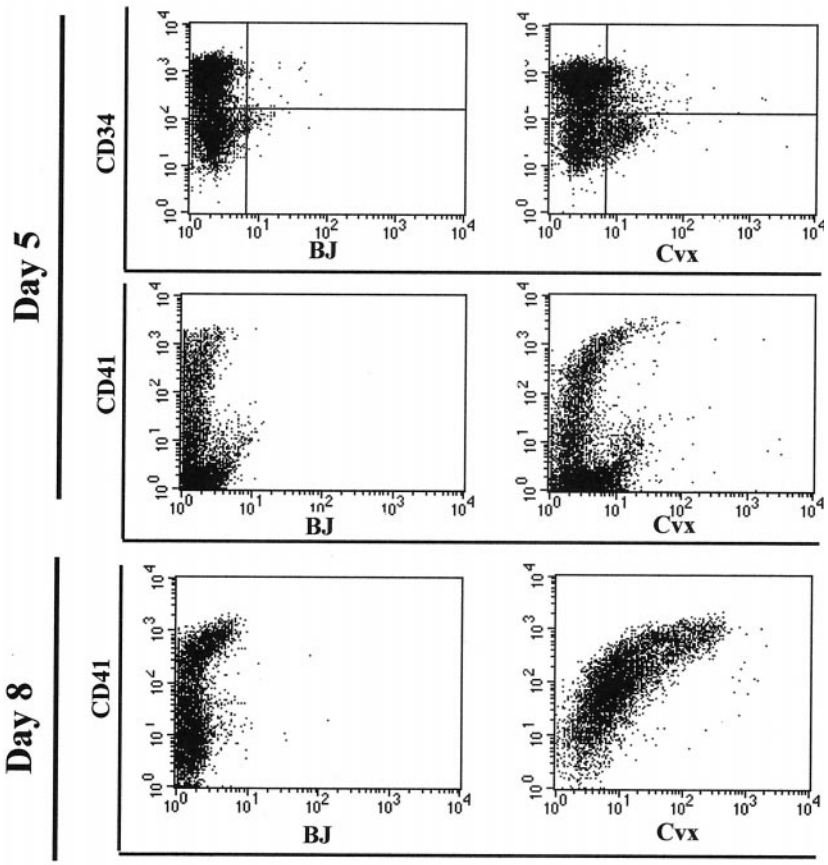

FIG. 5. **Expression of GPVI by mobilized CD34$^+$ using FITC-convulxin.** Cells (100,000 cells/50 μl) were incubated for 30 min at 4 °C with either R-PE anti-CD34 mAb or R-PE anti-CD41 mAb and FITC-convulxin. Controls were performed with R-PE-IgG_1 mouse antibody or with FITC-bothrojaracin. Cells were analyzed at day 5 or day 8 of mobilized blood CD34$^+$ cell cultures.

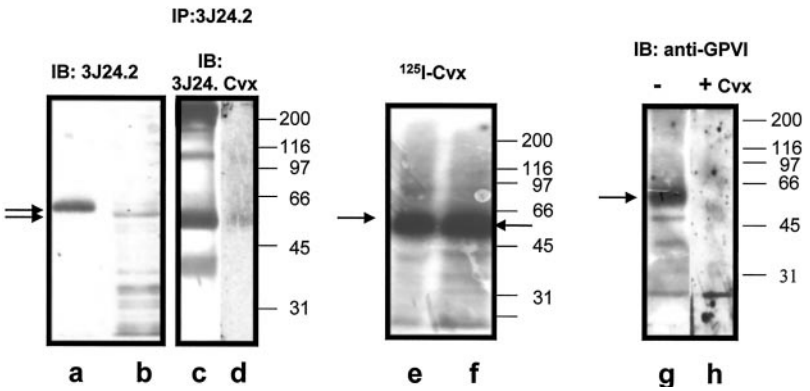

FIG. 6. **Expression of GPVI using immunoblotting, immunoprecipitation, and ligand blotting.** Megakaryocyte (*lanes b* and *f–h*) and platelet (*lanes a* and *e*) proteins were solubilized in SDS, separated on 10% acrylamide slab gels, and transferred to PVDF membranes. Alternatively, megakaryocyte proteins were immunoprecipitated by the anti-GPVI mAb 3J24.2 before electrophoresis (*lanes c* and *d*). Proteins were detected by immunoblotting with 3J24.2 (*lanes a–c*), ligand blotting with ^{125}I-convulxin (*lanes d–f*), or immunoblotting with the polyclonal anti-GPVI antibody in the absence (*lane g*) or presence of cold convulxin (*lane h*). Detection was performed using peroxidase-coupled anti-mouse IgGs for 3J24.2, peroxidase-coupled protein A for the polyclonal antibody, and chemiluminescence or direct autoradiography for ^{125}I-convulxin.

was detected in all cells, *i.e.* in CD41$^-$ cells as well as in CD41$^+$ cells (Fig. 7). Accordingly, FcR γ chain mRNA was detected by RT-PCR in megakaryocytes, whatever their stage of maturation, as well as in CD34$^+$CD41$^-$ cells. By immunoblotting with the anti-FcR γ chain mAb, an intense doublet corresponding to FcRγ was detected in megakaryocyte lysates (Fig. 7). Furthermore, as already observed with platelet lysates (9) or for recombinant GPVI (7), FcRγ was coprecipitated with GPVI, indicating that both proteins are noncovalently associated in megakaryocytes (Fig. 7)

These results indicate that megakaryocytes express GPVI as a complex with its signaling subunit FcR γ chain. Thus, in subsequent experiments we investigated whether the signaling pathway coupled to GPVI was functional in megakaryocytes.

Megakaryocyte Adhesion to Immobilized Collagen or Convulxin Involves GPVI and Integrin $\alpha_2\beta_1$—GPVI is responsible for platelet adhesion to immobilized convulxin and contributes to the Mg^{2+}-independent platelet adhesion to collagen in a static model of adherence (27, 28), whereas $\alpha_2\beta_1$ integrin serves as a Mg^{2+}-dependent platelet receptor for type I collagen both in static conditions and in flow (22, 29).

At day 6 of culture, the number of megakaryocytes that adhered to immobilized collagen or convulxin was very low, only slightly above the level of nonspecific binding to immobilized BSA (Fig. 8*A*). Later in the maturation, adhesion of megakaryocytes to immobilized collagen or convulxin increased (12- and 22-fold, respectively, at day 9; 18- and 30-fold at day 14; Fig. 8, *B* and *C*). Adhesion to immobilized convulxin was inhibited in part by the polyclonal anti-GPVI Fab fragments (data not shown) as already observed for platelet adhesion to immobilized convulxin (4). Megakaryocyte adhesion to immobilized collagen was reduced in the presence of the anti-inte-

grin $\alpha_2\beta_1$ mAb 6F1 (data not shown), suggesting that the integrin is involved in adhesion (22). In fact, EDTA also reduced megakaryocyte adhesion to collagen, and its inhibitory effect increased with maturation increasing from 60% at day 9 to 80% at day 14 of culture. Together, these results suggest that the combined expression of GPVI and $\alpha_2\beta_1$ integrin is too low at the earlier stage of maturation to allow the multiple interactions required for adhesion to the immobilized collagen. Later in the maturation, adhesion of megakaryocytes to convulxin indicates that GPVI is functional and adhesion to collagen may be via either GPVI or $\alpha_2\beta_1$. However, as shown by the greater inhibitory effect of EDTA at day 14 compared with day 9, $\alpha_2\beta_1$ integrin is the major contributor to megakaryocyte adhesion to collagen later in differentiation.

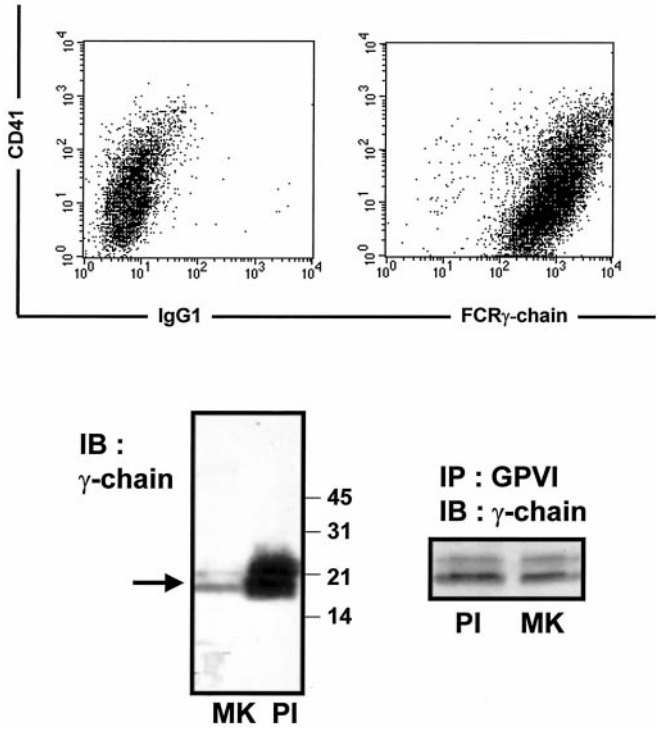

FIG. 7. **Expression of the FcR γ chain using flow cytometry on mobilized CD34$^+$ and immunoblotting.** Cells (100,000 cells/50 μl) were incubated for 30 min at 4 °C with FITC anti-FcRγ polyclonal antibody after permeabilization with 0.1% Triton X-100. Controls were performed with FITC IgG_1 mouse antibody. Mobilized blood CD34$^+$ cells were analyzed at day 10 of culture. Three experiments were performed, which gave identical results. *Lower left*, megakaryocyte (*MK*) and platelet (*Pl*) proteins were separated on 12% acrylamide gels, transferred onto PVDF membranes, and probed with the polyclonal anti-FcR γ chain antibody. Proteins were revealed using a peroxidase-coupled secondary antibody and chemiluminescence. FcR γ chain migrates as a doublet. *Lower right*, GPVI was immunoprecipitated (*IP*) from platelet or megakaryocyte lysates using 3J24.2, and precipitated proteins were immunoblotted (*IB*) using the anti-FcR γ chain antibody.

Collagen and Convulxin Trigger Protein Tyrosine Phosphorylations in Megakaryocytes—GPVI dimerization by its ligands is coupled to a very potent signaling pathway, which involves activation of a cascade of tyrosine kinases and which culminates in the phosphorylation and activation of $PLC_{\gamma 2}$ leading to platelet activation (8, 30, 31). To further characterize the functional state of GPVI in megakaryocytes, we analyzed protein tyrosine phosphorylation in response to convulxin or collagen. Adult CD34$^+$-derived megakaryocytes of the same culture were studied sequentially. This approach allowed us to study the pattern of tyrosine phosphorylation according to maturation because cultures are partially synchronized (see Figs. 1 and 2). The addition of convulxin or collagen to the cell suspension at 37 °C with stirring resulted in a slight transient increase in light transmission comparable with the light transmission change observed during platelet shape change (not shown). The pattern of tyrosyl-phosphorylated proteins from day 9 cultured megakaryocytes and from platelets is shown in Fig. 9*A*. Before activation, some proteins were phosphorylated in both platelets (*lane 4*) and megakaryocytes (*lane 1*), most notably a doublet at 55 kDa, and an additional low molecular mass protein (~30 kDa) was detected in megakaryocytes. In both cell types, additional proteins were phosphorylated after incubation with convulxin to give bands at 145, 120, 105, and 72 kDa. However, a phosphorylated 38-kDa protein was detected in platelets as in previous studies (25, 30) but not in megakaryocytes. Megakaryocyte incubation with collagen also resulted in the tyrosine phosphorylation of these proteins but to a much lower extent than convulxin, as already reported for platelets (25).

The pattern of phosphorylation obtained with megakaryocytes from the same culture at different stages of maturation is shown in Fig. 9*B*. With equal sample loading (5×10^5 cells in each sample) and exposure, differences in the intensity of phosphorylated bands were observed according to maturation. Convulxin- or collagen-induced protein tyrosine phosphorylations were very faint at day 6. The intensity of the signal greatly increased at day 9 parallel to GPVI expression but decreased at day 14 (Fig. 9*C*), suggesting that the efficiency of GPVI-coupled signals decreases at this late stage of maturation. In platelets, $p72^{Syk}$ and $PLC_{\gamma 2}$ are tyrosine-phosphorylated upon GPVI activation by convulxin (25, 30, 32). Among the different proteins phosphorylated upon incubation of megakaryocytes with collagen or convulxin, bands with the same migration as $p72^{Syk}$ and $PLC_{\gamma 2}$ were also observed at 72 and 145 kDa, respectively. $p72^{Syk}$ and $PLC_{\gamma 2}$ were immunoprecipitated from megakaryocytes (day 10 culture) or from platelets, before or after stimulation by convulxin or collagen, and analyzed for their content in phosphotyrosine (Fig. 10). $PLC_{\gamma 2}$ was tyrosine-phosphorylated only upon megakaryocyte and platelet activation by collagen or convulxin. $p72^{Syk}$ was very slightly phosphorylated in nonactivated megakaryocytes and platelets, but the intensity of the band in-

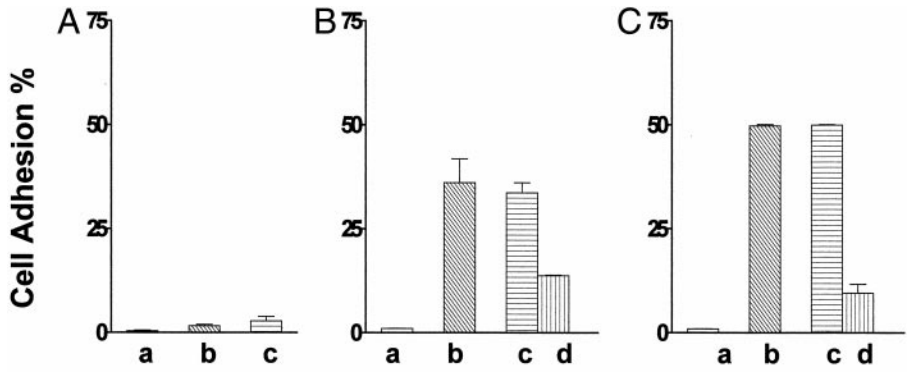

FIG. 8. **Adhesion of cultured megakaryocytes to immobilized convulxin and immobilized collagen.** Megakaryocytes (200,000 cells/ml) obtained from blood-mobilized CD34$^+$ cells cultured in the presence of TPO at day 6 (*A*), day 9 (*B*), or day 14 (*C*) were added to microtitration plates coated with convulxin or collagen type I. After washing, detection of adherent megakaryocytes was performed by measuring alkaline phosphatase activity. Nonspecific adhesion was detected on BSA-coated wells (*lane a*). Megakaryocytes were incubated with immobilized convulxin (*lane b*) or with immobilized-collagen in the absence (*lane c*) or presence of EDTA (*lane d*) for 1 h at 37 °C. The results are the mean ± S.E. of triplicate experiments.

creased upon incubation with collagen or convulxin. In addition, a phosphorylated low molecular weight protein corresponding to FcRγ was coprecipitated with Syk upon activation of megakaryocytes and platelets by collagen or by convulxin, indicating that Syk is recruited by phosphorylated FcR γ chain upon GPVI activation. Together, these results indicate that the signaling pathway coupled to GPVI is functional in megakaryocytes.

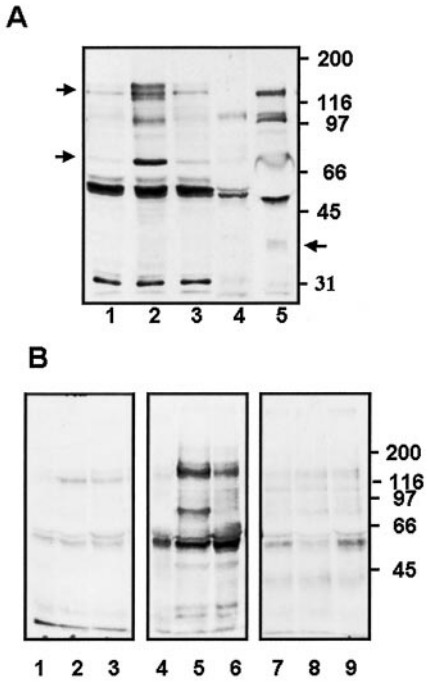

FIG. 9. **Convulxin- and collagen-triggered protein tyrosine phosphorylation in human megakaryocytes.** In *A*, megakaryocytes obtained from $CD34^+$ cells cultured in the presence of TPO for 9 days were stimulated with 10 nM convulxin or 100 μg/ml collagen for 6 min at 37° with agitation. Proteins from nonstimulated (*lane 1*) or convulxin (*lane 2*)- or collagen-stimulated (*lane 3*) megakaryocytes were separated using 8.5% acrylamide gels, transferred on PVDF membranes, and probed by immunoblotting with the anti-phosphotyrosine antibody PY20. Detection was performed using a peroxidase-coupled anti-mouse IgG antibody and chemiluminescence. In *lanes 4* and *5*, proteins from nonstimulated or convulxin-stimulated platelets were analyzed using the same method. In *B*, changes in convulxin- or collagen-induced phosphorylation were studied according to megakaryocyte maturation. $CD34^+$-derived cells were cultured in the presence of TPO (5×10^5 cells/sample) for 6 (*lanes 1, 2*, and *3*), 9 (*lanes 4, 5*, and *6*), or 14 days (*lanes 7, 8*, and *9*) and were not stimulated (*lanes 1, 4*, and *7*) or stimulated with 10 nM convulxin (*lanes 2, 5*, and *8*) or 100 μg/ml collagen (*lanes 3, 6*, and *9*). Samples were prepared and analyzed as described in *A*.

DISCUSSION

Megakaryocyte maturation occurs in the marrow, whereas proplatelet formation and platelet release takes place in the blood stream either in marrow sinusoids or in the general circulation (33). It is thus likely that specific mechanisms exist for the adhesion of progenitors to bone marrow stroma cells or to extracellular matrix proteins during maturation. On the other hand, to release platelets, megakaryocytes have to migrate through the endothelial barrier and cross the extracellular matrix, where they may interact with adhesive proteins such as fibronectin and collagen. In platelets, the synergy of the two collagen receptors $\alpha_2\beta_1$ and GPVI is important in mediating adhesion and in subsequent platelet secretion and aggregation (31). The precise expression of these two proteins and their function during megakaryocyte differentiation are poorly understood. Recently, GPVI has been cloned, and this collagen receptor seems to be specific to the megakaryocyte/platelet lineage (5, 7). Thus, it is now possible to study the expression and function of GPVI during megakaryocyte maturation.

Based on the response to collagen-related peptides, GPVI signaling has been proposed to occur only in mature megakaryocytes (34–36). Because FcR γ chain is expressed in several megakaryocytic cell lines in which GPVI has not been detected, it was suggested that GPVI could be a late marker of megakaryocyte differentiation in contrast to FcR γ chain (37). Here we have directly studied the expression of GPVI during megakaryocytic differentiation using a culture system from $CD34^+$ cell. By flow cytometry, the pattern of GPVI expression partially mimicked that of CD41. Indeed, GPVI expression was found only in cells expressing CD41 including $CD41^{low}$ cells. However, expression was weak, just at the threshold of detection, in these immature cells, and expression of GPVI is seen clearly only in the brightest $CD41^+$ cells corresponding to mature megakaryocytes. The fact that GPVI was expressed at a low level in immature megakaryocytic cells, including those expressing CD34, was confirmed by RT-PCR after cell sorting. Interestingly, expression of GPVI was quite similar to that of CD49b, which was also considered as a late marker of megakaryocyte differentiation. There is increasing evidence that the $CD41^{low}$ cell population is not committed to the megakaryocytic lineage. Thus, it is possible that the GPVI expression is not entirely specific to the megakaryocytic lineage and might be expressed at low level on nonmegakaryocytic progenitor cells. However, preliminary results suggest that the majority of the $CD34^+GPVI^+$ cells are late megakaryocyte progenitors.[2]

Studies of the promoter regions and of transgenic mice have shown that platelet-restricted genes are controlled by two types

[2] A.-H. Lagrue-Lak-Hal, N. Debili, G. Kingbury,, C. Lecut, J.-P. Le Couedic, J.-L. Villeval, M. Jandrot-Perrus, and W. Vainchenker, unpublished results.

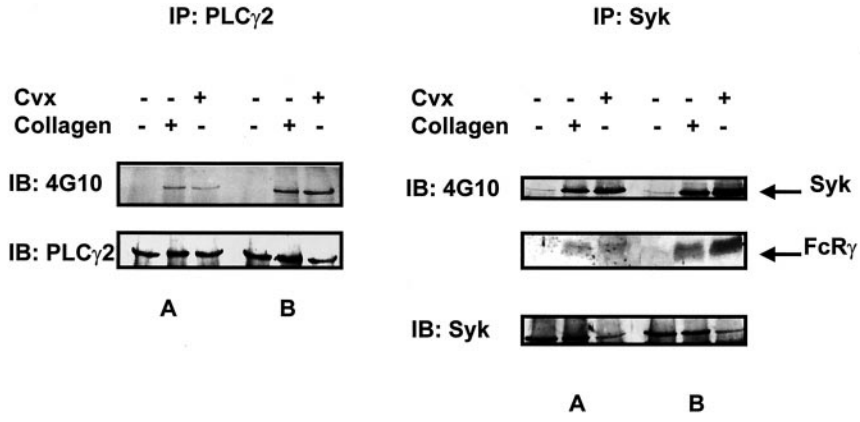

FIG. 10. **Convulxin-induced $PLC_{\gamma 2}$, Syk, and FcR γ chain phosphorylation in human megakaryocytes.** Proteins from megakaryocytes (*lanes A*) or platelets (*lanes B*), nonstimulated or stimulated by collagen or convulxin, were immunoprecipitated using anti-$PLC_{\gamma 2}$ or anti-Syk antibodies. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to PVDF membranes. Membranes were probed with the anti-phosphotyrosine 4G10 antibody, stripped, and reprobed with the anti-$PLC_{\gamma 2}$ antibody or the anti-Syk antibody, respectively. Detection was performed using secondary peroxidase-coupled antibodies and chemiluminescence.

of molecular regulation (37–39). The main platelet-restricted genes such those of the GPIb complex, GPIIb and PF4, contain binding sites in their promoters for GATA and Ets transcription factors. Expression of megakaryocyte-specific genes is down-regulated in GATA-$1^{-/-}$ mice (40). A second set of genes, which includes thromboxane synthase and β1 tubulin, is regulated by $p45^{NF\text{-}E2}$ (41–43). Expression of these genes is totally inhibited in the $p45^{NF\text{-}E2-/-}$ megakaryocytes leading to the absence of proplatelet formation (38, 42, 43). It has been suggested that this set of genes is involved in platelet functions and is expressed at late stages of differentiation. Recently, we investigated the expression of thomboxane synthase during human megakaryocyte differentiation and also found that its expression started early during differentiation in contrast to β1 tubulin (44). The pattern of thromboxane synthase, GPVI, and CD49b expression in megakaryocyte differentiation has many similarities because the level of these proteins markedly increased in the more mature megakaryocytes. It has been shown that megakaryocyte-specific enhancer of the α_2 integrin gene contains two tandem AP1 binding sites that are consensus sites for $p45^{NF\text{-}E2}$ (45, 46). However, presently there is no evidence that $p45^{NF\text{-}E2}$ regulates transcription of the α_2 integrin gene. The promoter of the human GPVI gene is still unknown, and the identity of the regulatory factors remains to be determined.

Polymorphisms in the α_2 gene have been identified and shown to result in a variability of $\alpha_2\beta_1$ integrin density at the platelet surface (26, 47). Polymorphisms are obvious even in megakaryocytes, as indicated by the heterogeneous expression of CD49b by megakaryocytes from the different donors that we have analyzed.

Platelet membrane GPVI migrates with an apparent molecular mass of 58 kDa under nonreducing conditions. The human protein contains one *N*-glycosylation site and several presumptive sites for *O*-glycosylation on its extracellular domain (5, 7), and the predicted molecular mass of the core protein is ~40 kDa. GPVI detected in megakaryocyte lysates by immunoblotting or ligand blotting migrated with an apparent molecular mass of 55–56 kDa, which is slightly lower than the mass of platelet GPVI but slightly higher than the mass of *N*-deglycosylated GPVI (54 kDa) when runned in parallel (data not shown); this suggests that GPVI undergoes a final processing step at the end of megakaryocyte maturation.

Despite the fact that the GPVI molecular mass is slightly different in megakaryocytes than in platelets, it associates with the FcR γ chain and is functional in megakaryocytes. Expression of GPVI allows megakaryocytes to bind to convulxin in solution and to adhere to immobilized convulxin and immobilized collagen in a Mg^{2+}-independent manner. Furthermore, collagen and convulxin trigger GPVI-coupled signals in megakaryocytes. This finding is in agreement with the results of Melford *et al.* (34) and Mountford *et al.* (36) showing that collagen-related peptides induces a rise in intracellular $[Ca^{2+}]$ in mature murine megakaryocytes and protein tyrosine phosphorylation in human megakaryocytes. At the earlier stages of maturation (day 5–6), the amount of GPVI expressed on megakaryocyte surface is probably too low for stable adhesion to immobilized convulxin or collagen. However, it signifies that several proteins are tyrosine-phosphorylated upon incubation with convulxin in solution, indicating that GPVI dimerization occurs in these conditions. Thus, our results indicate that, as soon as it is expressed, GPVI has the potential to bind to the ubiquitously expressed FcR γ chain and to trigger signaling. Later in differentiation (day 9), an increase in GPVI expression allows megakaryocyte adhesion to immobilized convulxin. At the end of maturation, the decreased level of GPVI-coupled protein tyrosine phosphorylation suggests that GPVI might be uncoupled from its signaling pathway by a mechanism that remains to be determined. At the same moment, the role of $\alpha_2\beta_1$ integrin appears to become predominant in cellular interaction with collagen. These results are in agreement with the respective role of $\alpha_2\beta_1$ integrin and GPVI in platelets. It has been already shown that $\alpha_2\beta_1$ integrin plays a major role in mature megakaryocyte (48) and megakaryocytic cell line adhesion (46). In mature mouse megakaryocytes, the cross-linking of $\alpha_2\beta_1$ integrin has been reported to increase intracellular $[Ca^{2+}]$ via calcium influx and independently of Src and Syk activation (35). From these results, it was suggested that collagen triggers $[Ca^{2+}]_i$ increase in mature megakaryocytes via multiple receptors including GPVI, causing calcium mobilization and $\alpha_2\beta_1$ integrin, which stimulates an influx of extracellular calcium (35). Our observations that $\alpha_2\beta_1$ integrin involvement in collagen-mediated megakaryocytes adhesion increases with maturation and that simultaneously GPVI-triggered signals decrease at the latest stages of maturation provide further evidence that $\alpha_2\beta_1$ integrin may be more important than GPVI for mature megakaryocyte adhesion. Collagen interaction with megakaryocytes and platelets may have different physiological consequences. Although collagen interaction with platelets is associated with activation, leading them to an irreversible process, collagen interactions with megakaryocytes may have a different role depending on the stage of maturation. One can hypothesize that collagen contributes to localizing the immature progenitors in bone marrow at sites where conditions for proliferation and maturation are optimal. Alternatively, at the end of maturation, collagen could contribute to cell migration of mature megakaryocytes and to their subsequent exit into circulation. Whatever its role, when platelets are released they must be fully functional, which means that no activation processes between GPVI and collagen should happen during their formation. How this occurs remains to be understood. Some types of collagen do not activate platelets, and they may trigger adhesion without activation in the sites of platelet release. Alternatively, the signaling pathway of the GPVI·FcRγ complex may be inefficient or inhibited in megakaryocytes and young platelets.

In conclusion, our results indicate that collagen receptors like GPVI and CD49b are expressed by megakaryocytes at an early stage of maturation and that they are fully functional later in differentiation. The characterization of their role in megakaryocyte interactions with collagen should allow a better comprehension of diseases such as platelet production defects.

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