Developmentally regulated expression of a 78 kDa erythroblast membrane glycoprotein immunologically related to the platelet thrombospondin receptor

Nelly KIEFFER,*|| Ali BETTAIEB,* Chantal LEGRAND,† Laure COULOMBEL,‡ William VAINCHENKER,* Lena EDELMAN§ and Janine BRETON-GORIUS*

*INSERM U91/CNRS UA 607, Hôpital Henri Mondor, Créteil, †INSERM U150/CNRS UA 334 Hôpital Lariboisière, Paris, ‡Laboratoire d'Hématologie et d'Histoembryologie-cytogénétique, Kremlin-Bicêtre, and §Institut Pasteur, Paris, France

We have previously described a monoclonal antibody (FA6-152), obtained by immunizing mice with fetal human erythrocytes [Edelman, Vinci, Villeval, Vainchenker, Henri, Miglierina, Rouger, Reviron, Breton-Gorius, Sureau & Edelman (1986) *Blood* 67, 56–63]. The antibody labelled fetal, but not adult, erythrocytes and bound to both fetal and adult platelets and monocytes. In the present study we have characterized the antigen recognized by FA6-152 on human platelets and on cells of the erythroid lineage at different stages of maturation. FA6-152 precipitated a chymotrypsin-resistant 88 kDa sialoglycoprotein from both iodinated and periodate/ NaB^3H_4 -surface-labelled platelets which corresponds to glycoprotein IV, the platelet thrombospondin (TSP) receptor. After neuraminidase treatment, a shift of the apparent molecular mass from 88 kDa to 85 kDa was observed. Scatchard analysis revealed that ^{125}I -FA6-152 bound saturably with high affinity to a single class of platelet binding sites (K_d 6.4 ± 0.6 nM). The number of FA6-152 IgG molecules bound per platelet was 25400 ± 8800 ($n = 4$) and did not change upon thrombin activation of platelets. At low doses of α -thrombin (0.025 unit), FA6-152 inhibited platelet aggregation as well as endogenous TSP binding to the platelet surface. Immunofluorescence labelling of bone-marrow cells and of cultures *in vitro* of burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E) revealed that the FA6-152 antigen is a very early marker of erythroid differentiation and that its expression declines during maturation. Immunochemical identification of the FA6-152 antigen on fetal erythroblasts and fetal mature erythrocytes revealed a 78 kDa glycoprotein migrating just in front of the glycophorin A dimer. The antigen, which was absent from adult mature erythrocytes, was also detected in human erythroleukaemic (HEL) cells where FA6-152 precipitated two bands of molecular mass 85 and 88 kDa. Our data establish the existence of a previously unidentified 78 kDa erythroblast cell-surface glycoprotein whose expression is developmentally regulated during erythroid differentiation and which is immunologically related to the 88 kDa platelet TSP receptor.

INTRODUCTION

In many developmental systems, membrane glycoproteins have been identified which appear at the cell surface in concert with an initial requirement for cell–cell adhesion. In the bone marrow, normal haematopoietic cell regulation requires the interaction of haematopoietic progenitor cells with marrow stromal cells, which are essential for the lodging, proliferation and eventual differentiation of these progenitors into mature blood cells (Dexter, 1979). Erythroid cells exhibit selective binding to fibronectin through a 140 kDa fibronectin receptor. During differentiation of erythroblasts to reticulocytes *in vivo* (Patel & Lodish, 1986) or of murine erythroleukaemic (MEL) cells *in vitro* (Giancotti *et al.*, 1986), the fibronectin receptor is lost from the surface of the cells, leading to a loss of adhesiveness to fibronectin. Coulombel *et al.* (1988) recently reported incomplete inhibition, by anti-fibronectin antibodies, of human-

erythroid-progenitor-cell adhesion to extracellular matrixes secreted by human marrow fibroblasts, suggesting that other matrix molecules might be involved in erythroid-progenitor attachment.

Like fibronectin, TSP is a multifunctional glycoprotein which binds to a large number of macromolecules and mediates cell adhesion *in vitro* [for a review, see Lawler (1986)]. Current data have now provided evidence that TSP is synthesized and secreted by a variety of cells, including endothelial cells (Mosher *et al.*, 1982), fibroblasts (Jaffe *et al.*, 1983), smooth-muscle cells (Raugi *et al.*, 1982), glial cells (Asch *et al.*, 1986), monocytes and macrophages (Jaffe *et al.*, 1985), and is incorporated into the extracellular matrix of growing cells (Mumby *et al.*, 1984). In addition, specific binding of ^{125}I -TSP to platelets (Wolff *et al.*, 1986), endothelial cells (Murphy-Ullrich & Mosher, 1987), fibroblasts (McKeown-Longo *et al.*, 1984) and human tumour-cell lines (Asch *et al.*, 1987) has been reported. TSP is therefore thought to be implicated

Abbreviations used: TSP, thrombospondin; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; MEL, murine erythroleukaemic (cell line); HEL, human erythroleukaemic cell line; GP, glycoprotein; BSA, bovine serum albumin; PBS, phosphate-buffered saline (composition and pH given in the text); PHA-LCM, phytohaemagglutinin/leucocyte-conditioned medium; Epo, erythropoietin; Tos-Lys- CH_2Cl , tosyl-lysylchloromethane ('TLCK'); PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; GPA, glycophorin A.

|| Present address and address for correspondence and reprint requests: COR Therapeutics Inc., 256 East Grand Avenue, Suite 80, South San Francisco, CA 94080, U.S.A.

in numerous cell-cell or cell-matrix interactions, promoting platelet aggregation (Leung, 1984), platelet-monocyte/macrophage interaction (Silverstein & Nachman, 1987), cell-substratum attachment and spreading (Tuszynski *et al.*, 1987), as well as adherence of *Plasmodium falciparum* (a malarial parasite)-infected erythrocytes to endothelial cells (Roberts *et al.*, 1985a,b). A role for TSP in cytoadhesion of human haematopoietic progenitor cells to the medullar microenvironment has recently been suggested by Long *et al.* (1987), who demonstrated specific attachment of haematopoietic progenitor cells to immobilized purified TSP. So far, no cell-lineage-specific TSP receptor has been identified. In contrast, cell-surface sulphated glycolipids (Roberts *et al.*, 1985a,b) and heparan sulphate proteoglycans (McKeown-Longo *et al.*, 1984; Murphy-Ullrich & Mosher, 1987) have been reported to function as TSP-binding sites.

Recently, a monoclonal antibody (OKM5), reactive with platelets, endothelial cells and monocytes and identifying an 88 kDa cell-surface glycoprotein on C32 melanoma cells (Barnwell *et al.*, 1985) has been shown to immunoprecipitate the 88 kDa glycoprotein (GP) IV from human platelets (Asch *et al.*, 1987). The antibody has further been used by Asch *et al.* (1987) to demonstrate that platelet GP IV functions as a cell-surface TSP receptor.

In the present study we used a monoclonal antibody (FA6-152) reacting with cells of the erythroid and megakaryocytic lineage to demonstrate the existence of a previously unidentified 78 kDa erythroid cell-surface glycoprotein whose expression is developmentally regulated during erythroid differentiation and which is immunologically related to the 88 kDa platelet TSP receptor.

MATERIALS AND METHODS

Cell isolation and cell culture

Platelets. Blood (6 vol.) from healthy adult volunteers was taken into acid/citrate/dextrose (130 mM-citric acid/126 mM-trisodium citrate/110 mM dextrose) (1 vol.) and centrifuged at 120 *g* for 15 min at 20 °C. Platelet-rich-plasma was recovered and platelets isolated by repeated centrifugations in a modified Tyrode's buffer pH 6.5 [103 mM-NaCl/36 mM-citric acid/5 mM-KCl/2 mM-CaCl₂/1 mM-MgCl₂/5 mM-glucose/bovine serum albumin (BSA) (3.5 mg/ml; fraction V, Sigma, St. Louis, MO, U.S.A.)], containing 25 µg of apyrase (Sigma, grade I)/ml and 100 nM-prostaglandin E1 (Sigma) as previously described (Legrand *et al.*, 1985). The pellet was resuspended to a final platelet count of 2.5×10^8 platelets/ml in Tyrode's buffer, pH 7.4, containing 5 mM-Hepes and BSA (3.5 mg/ml).

Human fetal erythrocytes. Fetal blood samples were obtained by fetoscopy performed for antenatal diagnosis. Blood was collected into heparin and centrifuged for 10 min at 1800 *g*. The erythrocyte pellet was recovered and washed three times in phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/4.3 mM-Na₂HPO₄·7H₂O/1.4 mM-KH₂PO₄, pH 7.3). The pellet was finally resuspended at 10^9 cells per ml in PBS.

Human fetal erythroid precursor cells. Fetal erythroid precursor cells were isolated from human fetal liver recovered 1 h after delivery from therapeutic abortion. The liver was placed into a Petri dish containing 5 ml of PBS and the liver cells isolated by gentle teasing of the tissue. The cell suspension was then transferred to a conical plastic tube and residual tissue aggregates eliminated after centrifugation of the cell suspension for 1 min at 50 *g*.

HEL cells. HEL cells (Martin & Papayannopoulou, 1982) were cultured in RPMI medium (Boehringer, Mannheim, Germany) with 10% (v/v) heat-inactivated fetal-calf serum (Boehringer) in a fully humidified atmosphere with 5% CO₂. Passages were performed every 3 days, and the cells were studied in the exponential phase of growth.

Clonogenic assays and indirect immunofluorescence

Clonogenic assays for blood BFU-E were performed by the plasma clot technique as described by McLeod *et al.* (1974). The stimulating factors were phytohaemagglutinin/leucocyte-conditioned medium (PHA-LCM) plus 1 unit of porcine erythropoietin (Epo; CNTS, Paris, France)/ml. Fluorescence labelling with FA6-152 was performed directly in the culture dishes at day 6 or 7 of culture for immature erythroblasts and at day 12 of culture for mature cells as previously described (Vinci *et al.*, 1984).

Cell labelling

Surface labelling. Galactose and *N*-acetylgalactosamine residues on the cell surface were labelled by the neuraminidase/galactose oxidase/NaB³H₄ method (Gahmberg & Hakomori, 1973) and sialic acid residues by the periodate/NaB³H₄ method (Gahmberg & Andersson, 1977); cell-surface-protein iodination was performed using the lactoperoxidase-catalysed ¹²⁵I-iodination procedure (Phillips & Agin, 1977). For each labelling, 10⁹ washed platelets or erythrocytes and 5×10^7 washed erythroblasts were used.

Metabolic radiolabelling of HEL cells. Steady-state labelling of HEL cells was performed by using [³⁵S]-methionine (Amersham International, Amersham, Bucks., U.K.; sp. radioactivity > 800 Ci/mmol) as previously described by us (Kieffer *et al.*, 1986). Briefly, cells in the exponential phase of growth were washed once in RPMI 1640 medium and resuspended at 2.5×10^6 cells/ml in methionine-free medium supplemented with 10% (v/v) heat-inactivated dialysed fetal-calf serum. After 10 min at 37 °C, [³⁵S]-methionine (50 µCi/ml) was added and the cells cultured for 3 h at 37 °C.

Chymotrypsin digestion of surface-labelled platelets

Washed platelets were resuspended at 10^9 platelets/ml in digestion buffer, pH 7.4 (10 mM-Tris/HCl/150 mM-NaCl/1 mM-EDTA) and incubated for 2 min at 37 °C. Tosyl-lslylchloromethane (Tos-Lys-CH₂Cl)-treated chymotrypsin from bovine pancreas (Sigma) was dissolved at 200 µM in the same buffer and added to 1 ml aliquots of the platelet suspension to give a final concentration of 10 µM. After 10 min at 37 °C, phenylmethanesulphonyl fluoride (PMSF; Sigma; 100 mM; freshly prepared in ethanol) was added to a final concentration of 2 mM. Platelets were then pelleted by

centrifugation for 15 min at 1200 *g*. The supernatant and platelet pellet were separately recovered.

Monoclonal antibodies and protein iodination

The monoclonal mouse antibody FA6-152 (IgG1) was purified from mouse ascites by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The monoclonal rat IgG2a antibody 5G11 (Kieffer *et al.*, 1988), which does not bind to protein A, was purified from rat ascites by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-Trisacryl chromatography as described by Bazin *et al.* (1974). Purified IgGs were radiolabelled with Na^{251}I by the chloramine-T method to a specific radioactivity of ≈ 500 c.p.m./ng as previously described by Hunter & Greenwood (1962). Radiolabelled IgGs were separated from residual free iodide by passage through a Sephadex G-25 column, saturated with BSA and equilibrated with PBS. More than 98% of the radioactivity was precipitated by 20% (w/v) trichloroacetic acid. Protein concentration was determined spectrophotometrically by using an absorption coefficient ($A_{280}^{1\%}$) of 1.48. BSA was added to a final concentration of 2 mg/ml, and the samples were stored at -80°C before use.

The monoclonal anti-(glycophorin A) antibody (LICR-LON-R10) was kindly provided by Dr. P. A. W. Edwards (Ludwig Institute for Cancer Research, The Haddow Laboratories, Sutton, Surrey, U.K.). The monoclonal antibody OKM1, directed against the C3bi receptor on monocytes, was obtained from the American Type Culture Collection, Rockville, MD, U.S.A., and served as a negative control.

Platelet binding assays

FA6-152 binding to washed human platelets. Washed non-activated platelet samples ($1.25 \times 10^8/\text{ml}$) were incubated for 30 min at room temperature with increasing concentrations of ^{125}I -FA6-152 as detailed in the legend to Fig. 3 (below). At the end of the incubation, duplicate 0.1 ml aliquots of each sample were layered on 0.5 ml of 20% (w/w) sucrose and centrifuged for 5 min at 12000 *g* in an Eppendorf centrifuge. The supernatants were aspirated, and the radioactivity associated with the pellets was measured in a γ -radiation counter (Beckman Instruments, Fullerton, CA, U.S.A.). Non-specific binding was determined by measuring residual radioactivity in the presence of a 20-fold excess of unlabelled antibody.

Effect of FA6-152 on TSP binding to the surface of thrombin-activated platelets. TSP expression on the surface of thrombin-stimulated platelets was measured by using ^{125}I -5G11 as previously described (Legrand *et al.*, 1988). Unstirred washed platelet samples ($2.5 \times 10^8/\text{ml}$) were incubated with 1 μg of FA6-IgG₁ or an irrelevant mouse IgG₁ for 5 min at room temperature before platelet activation. Platelets were then activated for 3 min at 37°C with various amounts of α -thrombin. At the end of the incubation time, hirudin (Sigma) was added in a 10-fold excess (unit/unit). Platelets were diluted to $1.25 \times 10^8/\text{ml}$ and further incubated for 30 min at room temperature with ^{125}I -5G11 (20 $\mu\text{g}/\text{ml}$). Bound radioactivity was measured as described for ^{125}I -FA6-152 binding.

Immunoprecipitation experiments and SDS/polyacrylamide-gel electrophoresis (PAGE)

Immunoprecipitation experiments were performed as previously described by us (Kieffer *et al.*, 1986). Briefly, radiolabelled cells were lysed by resuspending the cell pellet in lysis buffer (10 mM-Tris/150 mM-NaCl/1% Triton X-100/2 mM-PMSF, pH 7.4). Triton X-100-insoluble material was removed by centrifuging the samples at 100000 *g* for 30 min at 4°C . Samples (200 μg of protein in 200 μl) were incubated at 4°C with 10 μg of FA6-152 IgG. After an incubation of 3 h, 25 μl of protein A-Sepharose CL-4B was added and the incubation continued for 1 h at 4°C . Protein A-Sepharose beads were washed six times with lysis buffer, and the final pellet was resuspended in 50 μl of 10 mM-Tris/HCl, pH 7.4, containing 2% (w/v) SDS and, when required, 5% 2-mercaptoethanol. Bound proteins were eluted by heating the suspension at 100°C for 5 min. The immune complexes were analysed by SDS/PAGE on 7–12%-(w/v)-polyacrylamide gradient slab gels as described by Laemmli (1970). After electrophoresis, the gels were fixed, processed for fluorography with Amplify (Amersham International), dried and exposed for autoradiography at -80°C to Kodak XAR-5 films (Kodak-Pathé, Paris, France).

RESULTS

Immunochemical identification of the FA6-152 antigen on human platelets

The monoclonal antibody FA6-152, which was generated by immunizing mice with fetal erythrocytes, has previously been shown to react with both human erythroid cells and platelets (Edelman *et al.*, 1986). In the present study we have characterized the antigen identified by FA6-152. Immunoprecipitation experiments were performed using extracts of ^3H - or ^{125}I -surface-labelled platelets. The precipitates were analysed by SDS/PAGE, followed by fluorography. As Fig. 1 shows, FA6-152 precipitated a radiolabelled polypeptide from periodate/ NaB^3H_4 -labelled platelets migrating with an apparent M_r of 88 kDa. An 88 kDa polypeptide was also precipitated when ^{125}I -labelled platelets were tested (result not shown). When the neuraminidase/galactose oxidase/ NaB^3H_4 labelling procedure was used, which incorporates ^3H into penultimate galactose residues, an 85 kDa polypeptide was observed. It is noteworthy that, in contrast with GP Ib, the major sialoglycoprotein of the platelet membrane, whose electrophoretic mobility decreases after neuraminidase treatment, the electrophoretic mobility of the FA6-152 antigen increased after cleavage of sialic acid. Reducing or non-reducing conditions did not affect the apparent molecular mass of the precipitated band. Since the erythroid-cell FA6-152 antigen has previously been shown to be resistant to proteolytic cleavage (Edelman *et al.*, 1986), we used this property to characterize the platelet antigen further. Immunoprecipitation experiments were performed on the pellet and supernatant of untreated and chymotrypsin-treated ^3H -labelled platelets (Fig. 2). Chymotrypsin treatment of whole ^3H -surface-labelled platelets releases two major labelled glycopolypeptides, namely glyocalicin, the hydrophilic cleavage product of platelet GP Ib, and a large proteolytic fragment released from GP V (Berndt & Phillips, 1981). When samples of both

Fig. 1. Fluorography pattern of a polyacrylamide slab gel of immunoprecipitates obtained with the monoclonal antibody FA6-152 from ^3H -surface-labelled platelets

Washed platelets were surface-labelled by the periodate/ NaB^3H_4 method or the neuraminidase/galactose oxidase/ NaB^3H_4 method. Immunoprecipitations were performed by using $10\text{ }\mu\text{g}$ of FA6-152 IgG as described in the Materials and methods section and the precipitates resolved on 7–12% gradient acrylamide slab gels in the presence of 5% 2-mercaptoethanol. Lane A, a sample ($100\text{ }\mu\text{g}$ of protein) of periodate/ NaB^3H_4 -labelled platelets; lane B, immunoprecipitate from periodate/ NaB^3H_4 -labelled platelets; lane C, a sample ($100\text{ }\mu\text{g}$ of protein) of neuraminidase/galactose oxidase/ NaB^3H_4 -labelled platelets; lane D, immunoprecipitate from neuraminidase/galactose oxidase/ NaB^3H_4 -labelled platelets. The migration of [^{14}C]methylated standard molecular-mass markers is indicated. From top to bottom: myosin, 200 kDa; phosphorylase *b*, 92 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa). '88k' and '85k' are the 88 and 85 kDa glycoproteins referred to in the text.

the chymotrypsin-treated platelet pellet and the supernatant were tested, FA6-152 precipitated an 88 kDa polypeptide from the platelet pellet (lane F), whereas no band was precipitated from the supernatant (lane H). Taken together, these results provide evidence that the platelet FA6-152 antigen is an 88 kDa single-chain cell-surface glycoprotein that is resistant to chymotrypsin digestion and thus has biochemical characteristics identical with those of platelet GP IV.

FA6-152 binding to human platelets

To characterize the FA6-152 antigen further, we studied the direct binding of ^{125}I -FA6-152 to human platelets. Fig. 3 illustrates a representative binding experiment. Half-maximum binding occurred within 2 min, and binding was complete after 10 min. Saturation was obtained with $1.5\text{ }\mu\text{g}$ of IgG per 10^8 platelets. Scatchard analysis revealed that ^{125}I -FA6-152 bound with high affinity to a single class of binding sites ($23\,730$ molecules/platelet; $K_d\ 6.4 \pm 0.6\text{ nM}$). On the basis of data

Fig. 2. Fluorography pattern of a polyacrylamide slab gel of immunoprecipitates obtained with the monoclonal antibody FA6-152 from ^3H -surface-labelled platelets before and after chymotrypsin treatment

Washed ^3H -surface-labelled platelets were treated with $10\text{ }\mu\text{M}$ -chymotrypsin for 10 min at $37\text{ }^\circ\text{C}$ as described in the Materials and methods section. Platelet pellet and supernatant were separately recovered and processed for immunoprecipitation experiments with FA6-152 antibody. The samples were subjected to 7–12% gradient acrylamide slab gels in the presence of 5% 2-mercaptoethanol. Lanes A and B, total profile of ^3H -surface labelled platelets (' ^3H ') before (A) and after (B) chymotrypsin treatment; lanes C and D, supernatant ('S') of ^3H -surface-labelled platelets before (C) and after (D) chymotrypsin treatment; lanes E and F, immunoprecipitate ('P') obtained with FA6-152 from Triton X-100-solubilized labelled platelets before (E) and after (F) chymotrypsin treatment; lanes G and H, immunoprecipitation ('IP') performed with FA6-152 on supernatants ('S') of platelets before (G) and after (H) chymotrypsin treatment. Standard molecular-mass markers are those indicated in the legend to Fig. 1 (but also '14', which is lysozyme, 14.3 kDa). The arrows on the left indicate the two major glycopolypeptides released by chymotrypsin.

derived from four different experiments, the number of FA6-152 molecules bound per platelet was $25\,400 \pm 8\,800$ ($n = 4$). This number was not modified on thrombin stimulation (Table 1).

Effect of FA6-152 on platelet aggregation and on endogenous TSP binding to thrombin-activated platelets

Since platelet GP IV has been reported to be the putative TSP receptor, we wondered whether FA6-152 had an effect on platelet aggregation and endogenous TSP binding to thrombin-activated platelets. Initial studies revealed that the antibody by itself was able to induce platelet aggregation and secretion when used at a concentration of more than $1\text{ }\mu\text{g}/\text{ml}$. All subsequent studies were therefore performed at an antibody concentration not exceeding $1\text{ }\mu\text{g}/\text{ml}$. Under these experimental conditions, FA6-152 inhibited platelet aggregation induced by low doses of α -thrombin

Fig. 3. ¹²⁵I-FA6-152 binding to human platelets

(a) Washed platelets were resuspended at 1.25×10^8 platelets/ml in Tyrode's buffer containing 3.5 mg of BSA/ml and incubated with increasing amounts of ¹²⁵I-FA6-152. After a 30 min incubation at room temperature, bound ¹²⁵I-FA6-152 was determined as described in the Materials and methods section. (b) Scatchard analysis of the binding data.

Table 1. Effect of FA6-152 on the surface expression of platelet thrombospondin

Platelets (2.5×10^8 /ml) were incubated for 3 min at 37 °C with various concentrations of α -thrombin in the presence of FA6-152 IgG₁ (1 μg/ml) or irrelevant mouse IgG₁. Hirudin was added in a 10-fold excess (unit/unit). Platelets were diluted to 1.25×10^8 /ml and incubated for 30 min at room temperature with ¹²⁵I-FA6-152 (2.5 μg/ml) or ¹²⁵I-5G11 (20 μg/ml). Results are means $\pm$ S.D. for four experiments.

[α -Thrombin] (unit/ml)	¹²⁵ I-FA6 binding molecules/ platelet	¹²⁵ I-5G11 binding (molecules/platelet) in the presence of:	
		Control IgG ₁ (1 μg/ml)	FA6-IgG ₁ (1 μg/ml)
0	17100 $\pm$ 1100	1600 $\pm$ 1400	2000 $\pm$ 1500
0.025	—	19100 $\pm$ 4100	10850 $\pm$ 2500
0.050	—	23700 $\pm$ 3500	11300 $\pm$ 2700
0.100	17300 $\pm$ 3800	31100 $\pm$ 5600	29400 $\pm$ 7800

(< 0.05 unit/ml). When endogenous TSP binding to the platelet surface was measured in the presence of FA6-152, 50% inhibition was observed (Table 1). However, this inhibitory effect was overcome when higher concentrations of α -thrombin (0.1 unit) were used for platelet activation.

Expression of the FA6-152 antigen on human erythroid precursor cells

As previously reported by Edelman *et al.* (1986), erythroblasts at all stages of differentiation discernible in the marrow were strongly labelled by FA6-152, whereas only a faint fluorescence was observed on early circulating reticulocytes and none could be detected on mature erythrocytes. In contrast, peripheral-blood monocytes and platelets were heavily labelled (results not shown). When the reactivity of FA6-152 was studied on cultures *in vitro* of blood progenitor-derived colonies, FA6-152

Fig. 4. Immunofluorescence staining of cultures *in vitro* of blood BFU-E colonies

BFU-E-derived colonies were grown in plasma clot and stained at day 6 of culture with FA6-152, followed by fluorescein isothiocyanate-goat anti-mouse F(ab')₂.

reacted with erythroid colonies as early as day 6 of culture. Fig. 4 displays a BFU-E colony stained with the FA6-152 antibody at day 6 of culture. The reactivity of FA6-152 was not exclusively restricted to the erythroid lineage, since megakaryocytic colonies as well as monocyte/macrophage colonies were also stained (results not shown).

Immunochemical identification of the FA6-152 antigen on cells of the erythroid lineage at different stages of maturation

To identify the FA6-152 antigen on the surface of cells of the erythroid lineage, immunoprecipitation experiments were performed on human erythroid cells at different stages of maturation: mature adult erythrocytes, mature fetal erythrocytes and fetal erythroblasts, as well as cells of the human erythroleukaemic cell line HEL. Periodate/NaB³H₄ labelling of mature adult erythrocytes identifies two major bands corresponding to glycophorin A (GPA) monomer (PAS 2) and GPA dimer (PAS 1). Monomeric GPA has an apparent M_r of 40000 under standard SDS/PAGE conditions; however, with high

Fig. 5. Fluorography pattern of a polyacrylamide gel of immunoprecipitates obtained with the monoclonal antibodies FA6-152 and LIRC-LON-R10 from ^3H -surface-labelled cells of the erythroid lineage

Human adult erythrocytes, fetal erythrocytes, fetal erythroblasts and HEL cells were isolated and radiolabelled as described in the Materials and methods section. Immunoprecipitations were performed with the monoclonal antibodies FA6-152 and LIRC-LON-R10 and the precipitates resolved on 7–12 % gradient acrylamide slab gels in the presence of 5 % 2-mercaptoethanol. Lane A, total profile of periodate/ NaB^3H_4 -labelled mature adult erythrocytes; lane B, immunoprecipitate obtained with FA6-152; lane C, immunoprecipitate obtained with LIRC-LON-R10; lane D, total profile of periodate/ NaB^3H_4 -labelled mature fetal erythrocytes; lane E, immunoprecipitate obtained with OKM1 serving as a negative control; lane F, immunoprecipitate obtained with FA6-152; lane G, total profile of periodate/ NaB^3H_4 -surface-labelled fetal erythroblasts; lane H, immunoprecipitate obtained with FA6-152; lane I, immunoprecipitate obtained with FA6-152 from [^{35}S]methionine-labelled HEL cells; lane J, immunoprecipitate obtained with OKM1. Molecular-mass markers are those mentioned in the legends to Figs. 1 and 2.

concentrations of GPA, such as occur in the erythrocyte membrane, a substantial amount of GPA dimerization occurs during cell solubilization and sample processing, with GPA dimer migrating at an apparent M_r of 80000. Fig. 5, lane A, shows the surface profile of ^3H -labelled adult peripheral-blood erythrocytes. GPA monomers and dimers were immunoprecipitated with the monoclonal antibody LIRC-LON-R10 and had an electrophoretic mobility corresponding to 40 and 80 kDa respectively (lane C). When the FA6-152 antibody was tested, no band was precipitated, confirming the absence of the FA6-152 antigen on mature adult erythrocytes (lane B). Lane F shows the reactivity of FA6-152 with mature fetal erythrocytes. Owing to the heavy labelling of GPA in this sample, as shown in lane D, non-specific entrapment of GPA within the FA6-152 immunoprecipitate could not be avoided. Still, the result in lane F clearly demonstrates the presence of a specific band of M_r 78000 migrating just in front of the GPA dimer. This band was not detected when the experiment was performed with OKM1 serving as a negative control (lane E). Lane G displays the radiolabelled fetal erythroblast surface profile and lane H the immunoprecipitate obtained with FA6-152. This time a unique 78 kDa band was clearly visible. When FA6-152 was tested on metabolically labelled HEL cells (lane I), two bands were precipitated with apparent molecular-mass values of 88 and 85 kDa respectively.

DISCUSSION

In the present paper we have provided evidence that the FA6-152 antigen, which is expressed in erythroid

precursor cells and human platelets, corresponds to GP IV, the platelet TSP receptor. Thus FA6-152 precipitates an 88 kDa single-chain glycoprotein from both ^3H - and ^{125}I -surface-labelled platelets. Two platelet glycoproteins, GP IV (also referred to as 'GP IIIb' by some authors) and GP V, could correspond to the FA6-152 antigen, since they have similar biochemical characteristics: both glycoproteins are single-chain polypeptides with very close apparent molecular masses (88 as against 82 kDa) (Berndt & Phillips, 1981; Tsuji & Osawa, 1986). However, in contrast with GP IV, whose major characteristic is to be highly resistant to proteinase cleavage, GP V is a substrate for thrombin and is easily released from the cell surface by chymotrypsin treatment (Wicki & Clemetson, 1985). The fact that the FA6-152 antigen was resistant to chymotrypsin treatment provides evidence that it does correspond to platelet GP IV, and excludes GP V as the possible antigen.

There is now increasing evidence that platelet GP IV functions as a cell-surface TSP receptor. Asch *et al.* (1987) have demonstrated a direct interaction of TSP with GP IV and shown that a monoclonal antibody reacting with GP IV (OKM5) inhibits endogenous TSP binding to thrombin- or ionophore-stimulated human platelets. Using an anti-TSP monoclonal antibody (5G11), we have recently been able to precipitate platelet GP IV from Triton X-100-solubilized thrombin-stimulated platelets, demonstrating the presence of TSP-GPIV ligand-receptor complexes (Kieffer *et al.*, 1988). Here, we used the FA6-152 antibody to characterize further the functional role of platelet GP IV. FA6-152 binding studies revealed an average of 25400 ± 8800 binding sites on both non-activated and thrombin-activated platelets.

The cell-surface density of GP IV is thus similar to that measured for another platelet membrane sialoglycoprotein, namely platelet GP Ib (Ruan *et al.*, 1981; Collier *et al.*, 1983). It is noteworthy that the number of FA6-152 binding sites is also close to values reported for ^{125}I -TSP binding to platelets (Wolff *et al.*, 1986), or the surface expression of endogenous TSP upon thrombin stimulation (Aiken *et al.*, 1986; Legrand *et al.*, 1988; Boukerche & McGregor, 1988). To investigate further the role of GP IV in platelet surface TSP binding, we studied the effect of FA6-152 on endogenous TSP binding to human platelets. Preincubation of platelets with FA6-152 IgG (1 $\mu\text{g}/\text{ml}$) (i.e. its K_d value) resulted in a 50% inhibition of both the surface expression of TSP and platelet aggregation induced by low doses of α -thrombin. Since with higher doses of thrombin (0.1 unit), the FA6-152 induced inhibitory effect was overcome, our results suggest that GP IV is not the unique platelet TSP receptor. Indeed, Aiken *et al.* (1987a) have shown that more than one class of TSP binding sites exist at the platelet surface. Furthermore, a recent study by Tuszynski *et al.* (1988) has emphasized that GP IV is a receptor for TSP on unstimulated platelets and that other receptors are made available by thrombin stimulation. Since fibrinogen has been shown to bind TSP (Leung & Nachman, 1982), thrombin-induced surface-bound fibrinogen molecules have to be considered as possible TSP-binding sites.

Immunofluorescence labelling studies performed with FA6-152 on bone-marrow cells and on cultures *in vitro* of BFU-E and CFU-E revealed that the FA6-152 antigen is a very early marker of erythroid differentiation and that its expression declines during maturation (Edelman *et al.*, 1986). Using immunochemical techniques we have now been able to characterize the FA6-152 antigen on human fetal erythroblasts as well as fetal reticulocytes. Our results demonstrate the existence of a previously unidentified 78 kDa surface glycoprotein in the erythroblast-cell membrane and its absence from mature adult erythrocytes. Under identical SDS/PAGE conditions, we observed a slight difference between the electrophoretic mobility (as expressed by M_r) of erythroid GP IV (78 kDa) and that of platelet GP IV (88 kDa). This result suggests that cell-lineage-specific differences in the carbohydrate moiety of the oligosaccharide chains might exist. In support of this hypothesis is the decrease in the apparent molecular mass of platelet GP IV from 88 to 85 kDa after neuraminidase treatment. Interestingly, when immunoprecipitations were performed on [^{35}S]methionine-labelled HEL cells, which express both erythroid and megakaryocytic cell-surface markers, FA6-152 precipitated radiolabelled polypeptides of M_r 85000 and 88000, two molecular masses observed for the platelet phenotype of GP IV.

The functional role of GP IV on erythroid cells is so far unknown. However, the developmentally regulated expression of GP IV, which parallels that observed for the fibronectin receptor (Patel & Lodish, 1986), suggests that the decrease in the surface expression of both the fibronectin receptor and the TSP receptor could constitute one of the mechanisms involved in the release of differentiated erythrocytes from the interstitial matrix of the bone marrow into the blood stream. GP IV might also play an important role in the interaction of erythroid precursor cells with stromal cells. Since macrophages express GP IV as well as TSP, one might speculate that

erythroid GP IV could be involved in the formation of the erythroblastic island composed of one or two macrophages surrounded by maturing erythroblasts (Bessis & Breton-Gorius, 1962). In support of this hypothesis are the recent results obtained by Morris *et al.* (1988), who have reported that murine fetal liver macrophages bind developing erythroblasts by a bivalent-cation-dependent haemagglutinin.

Taken together, the present data establish the existence of a previously unidentified 78 kDa erythroblast cell-surface glycoprotein whose expression is developmentally regulated during erythroid differentiation and which is immunologically related to the platelet thrombospondin receptor. Our results thus support the idea that not only the fibronectin receptor, but also the TSP receptor, could play a functional role in erythroid-cell attachment to bone-marrow stromal cells. Further studies will be necessary to demonstrate the functional role of erythroid GP IV in TSP binding and cell adhesion to the micro-environment of the bone marrow.

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