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Characterization of Human Platelet Basic Protein, a Precursor Form of Low-Affinity Platelet Factor 4 and β-Thromboglobulin†

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ABSTRACT: Platelet basic protein (PBP) was purified from the supernatant of thrombin-stimulated, washed human platelets by ion-exchange, affinity, molecular sieve, and high-performance liquid chromatography (HPLC). The NH_2-terminal amino acid sequence was determined by automated Edman degradation, revealing 9 unique residues followed by 10 residues of the established low-affinity platelet factor 4/β-thromboglobulin (LA-PF_4/βTG) sequence. Among the nine were three basic residues, accounting for the high isoelectric point of PBP. Additional evidence for precursor status includes the immunological cross-reactivity of all three species and the ability of plasmin and trypsin to produce from PBP a species resembling βTG in charge, hydrophobicity and size. Tryptic peptide maps of PBP and LA-PF_4 obtained by reverse-phase HPLC were very similar, and from each protein, a peptide was isolated which showed the amino acid composition predicted for the COOH-terminal tryptic peptide of βTG. Normal platelets contained predominantly LA-PF_4, with PBP ranging from 10% to 30% of total βTG antigen. This was true even when fresh platelets were lysed with trichloroacetic acid in order to provide the most complete and rapid inhibition of proteolytic activity. βTG itself was never detected in this situation or in the release supernatant of stimulated platelets, and only rarely in unprotected lysates. In agreement with earlier results, crude preparations of PBP were mitogenic for 3T3 cells, but highly purified preparations of PBP and LA-PF_4 were free of this activity.

Upon stimulation, human platelets secrete a number of proteins from their α-storage granules. Several of these are platelet specific and bind reversibly to insolubilized heparin [for a review, see Holt & Niewiarowski (1985)]. Major platelet-specific proteins are β-thromboglobulin (βTG),[1] eluted from heparin-agarose at 0.5 M NaCl (Moore et al., 1975; Rucinski et al., 1979), and platelet factor 4 (PF_4), which is displaced only by 1.2-1.5 M NaCl [e.g., see Levine & Wohl (1976)]. Both βTG and PF_4 have been sequenced, yielding subunit molecular weights of 7800 (PF_4) and 8800 (βTG)

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[1] Abbreviations: PF_4, platelet factor 4; βTG, β-thromboglobulin; PBP, platelet basic protein; LA-PF_4, low-affinity platelet factor 4; PDGF, platelet-derived growth factor; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SDS, sodium dodecyl sulfate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; KIU, kallikrein inactivator unit(s); Cbz, carbobenzoxy.

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(Deuel et al., 1977; Hermodson et al., 1977; Walz et al., 1977; Morgan et al., 1979; Begg et al., 1978). The amino acid sequences show about 50% homology. Immunological cross-reactivity is, however, evident only in direct binding assays and not in competitive radioimmunoassays [e.g., see Ludlam et al. (1975) and Rucinski et al. (1979, 1983)].

Continuing studies on β-thromboglobulin revealed in the platelet release supernatant a species differing from the original protein by the presence of four additional NH_2-terminal residues (Rucinski et al., 1979). The larger protein was named low-affinity platelet factor 4 (LA-PF_4) and in both purified and unpurified systems could be converted to βTG by proteolysis (Niewiarowski et al., 1980; Holt & Niewiarowski, 1980). βTG and LA-PF_4 were immunologically identical (Varma et al., 1982) but could readily be distinguished by their different isoelectric points (Niewiarowski et al., 1980) and mobilities on native gels (Holt & Niewiarowski, 1980). When the unfractionated secreted proteins were analyzed by isoelectric focusing and radioimmunoassay, not only were βTG and LA-PF_4 distinguished (p*I* = 7 and 8, respectively) but also a small amount of the same antigen was detected at p*I* = 10.5 (Niewiarowski et al., 1980). This material, named "platelet basic protein" (PBP), was partially freed of ampholytes by dialysis and batch elution from heparin–agarose.

In the present work, we describe the isolation of platelet basic protein by ion-exchange, affinity, and size-exclusion chromatography. Assays of mitogenic activity during the purification of PBP suggest that this protein is not itself a mitogen. The mitogen activity present in the starting material, although not recovered in high yield, appeared to be separable from PBP. No mitogenic activity toward 3T3 cells was associated with LA-PF_4. We show by amino acid sequence studies and cleavage with proteolytic enzymes that PBP is a precursor of LA-PF_4 and βTG. Under all conditions examined, LA-PF_4 was, nevertheless, the major form of the protein contained in or secreted by platelets.

A preliminary account of some of this work has already been given (Holt et al., 1982).

Materials and Methods

Reagents. All chemicals were ACS reagent grade or equivalent. Sodium dodecyl sulfate was from Gallard-Schlesing (BDH Chemicals, specially purified grade) and dithiothreitol from Calbiochem. Ampholytes for isoelectric focusing (LKB Instruments, pH range 3–10) were used at a dilution of 1 to 25. Dialysis tubing with a molecular weight cutoff of 2000 or 3500 was "Spectrapor", obtained from Fisher Scientific. Heparin–agarose was prepared by attaching porcine mucosal heparin (Sigma Chemical Co.) to Sepharose 4B (Pharmacia Fine Chemicals) following the procedure of Thaler and Schmer (1975). Fetal calf serum (Flow Laboratories) was heated at 56 °C for 30 min before use in assays for mitogenic activity.

Platelets and Platelet Releasate. Human blood in acid citrate–dextrose was obtained from Interstate Blood Bank, Philadelphia, or from individuals who donated blood to the Thrombosis Research Center, Temple University, after giving informed consent. Washed platelets were prepared from platelet-rich plasma as described by Mustard et al. (1972) using apyrase prepared according to the procedure of Molnar and Lorand (1961). The recovery of platelets after washing averaged 50% ± 10% in five preparations. After final suspension in albumin-free buffer at pH 7.3 and 37 °C, the platelets [(1–4) × 10^9/mL] were stimulated with 5 units/mL human α-thrombin, provided by Dr. J. W. Fenton, New York State Department of Health, Albany, NY, or 50 nM Ca^{2+} ionophore A23187 (Eli Lilly Co.). Aggregated platelets were removed by centrifugation, and the supernatant, containing secreted proteins, was heated briefly at 100 °C to inactivate thrombin and secreted proteases. The supernatant was sometimes incubated with phenylmethanesulfonyl fluoride (PMSF, 2 mM) and soybean trypsin inhibitor (1 mg/mL) prior to, or in place of, heating. No systematic difference was observed between the various platelet stimuli and measures to prevent proteolysis. The proteins studied in this work were not cleaved by thrombin (see Results). The final, treated supernatant is referred to as platelet releasate. In accord with results previously reported from this laboratory (Rucinski et al., 1979), thrombin released 40–60% of the βTG antigen present in washed platelets. A platelet releasate obtained from 4 units (1600 mL) of whole blood thus contained 1.5–2.5 mg of βTG antigen.

Clinically unusable platelet concentrates were obtained through the generosity of the American Red Cross, New Jersey/Pennsylvania Regional Center.

Radioimmunoassay. Levels of LA-PF_4 and the completely cross-reacting antigens βTG and PBP were determined as described in detail previously (Rucinski et al., 1979). The antiserum currently available was used at a dilution of 1:10000 and provided a linear standard curve (log concentration vs. log % binding) from 2 to 50 ng/mL LA-PF_4. Maximum binding of ^{125}I-LA-PF_4 was not less than 70%.

Proteins and Antisera. Rabbit antiserum to LA-PF_4 was prepared in this laboratory by Dr. B. Rucinski (Rucinski et al., 1979). β-Thromboglobulin isolated from outdated platelets was a gift from Dr. D. S. Pepper, Scottish Regional Blood Transfusion Service, Edinburgh, Scotland. This material was previously found to be indistinguishable from a limited cleavage product of LA-PF_4 obtained with plasmin or trypsin (Holt & Niewiarowski, 1980). In the present work, we extended the demonstration of identical properties in the two βTG preparations to include reverse-phase HPLC.

An 18-residue peptide with the COOH-terminal sequence Pro^{64}-Asp^{81} of βTG was custom synthesized by Peninsula Laboratories, Belmont, CA. The crude peptide was purified by reverse-phase HPLC and yielded an amino acid composition in accord with the intended sequence.

Assay for Mitogenic Activity. The assay was based on the incorporation of [*methyl*-^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) in cells arrested in G_0/G_1 phase (Witte et al., 1978; Poggi et al., 1984). NIH 3T3 cells were generously provided by Dr. R. Baserga (Temple University Health Sciences Center, Philadelphia, PA). They were grown in Dulbecco's modified Eagle's medium (DME) in the presence of 10% calf serum, 4 mM L-glutamine, and 50 μg/mL gentamycin sulfate USP (Schering Corp., Kenilworth, NJ) in a humidified CO_2 (10%)–air (90%) incubator at 37 °C. Cells were transferred twice per week after detachment by brief incubation with trypsin (Flow Laboratories) in Hanks' balanced salt solution. They were routinely examined for contamination with bacteria or mycoplasma.

For the assay [cf. Witte et al. (1978)], cells in DME containing 10% calf serum were placed in the flat-bottomed wells of microtiter plates (Linbro, 24 wells per plate, well volume 3.5 mL, surface area 2 cm^2). Seeding density was 10^4 cells per well. After 24 h, the medium was removed and replaced with DME containing 1% calf serum. After a further 48 h, the test samples and a series of calf serum standards were added. The total volume placed in each well was 1 mL. Sixteen hours later, [*methyl*-^{3}H]thymidine (2.5 μCi per well) was added, and the cells were incubated a further 4 h. The

medium was removed, and the cells were washed twice with Hanks' solution, once with cold 5% (w/v) trichloroacetic acid, and once with ethanol and ether (2:1 v/v) and air-dried. They were then incubated 30 min at room temperature with 0.5 mL of 0.5 N NaOH, neutralized with acetic acid, and counted by liquid scintillation. Results are expressed as the concentration of heat-inactivated fetal calf serum producing the same extent of incorporation in the same assay. The response of the cells was usually linear from 0.5% to 5% (v/v) fetal calf serum.

Gel Electrophoresis in Sodium Dodecyl Sulfate. To obtain good resolution of low molecular weight proteins, a gel containing 20% polyacrylamide (cross-linker 2.6% w/w of total acrylamide) was used. The buffer was 0.1 M Tris, 0.1 M Bicine, pH 8.2, and 0.1% sodium dodecyl sulfate, in both the gel and the reservoirs. No stacking gel was present. All samples were reduced by heating for 2 min at 100 °C with 2% SDS–20 mM dithiothreitol. Molecular weight markers were prepared by cleaving sperm whale myoglobin (Sigma Chemical Co.) with CNBr (Edmundson, 1963).

Completed gels were stained with silver after mandatory fixation of protein with glutaraldehyde (Morrissey, 1981) or were transferred to nitrocellulose paper (Schleicher & Schuell) for staining with specific antibody. Transfer was performed electrophoretically (6 V/cm for 5–15 h at 4 °C) in an apparatus intended for gel destaining (Pharmacia). The buffer was 25 mM Tris, 192 mM glycine, pH 8.2, and 20% v/v methanol (Towbin et al., 1979).

Antibody Staining of Gel Transfers. Completed transfers were incubated at 20 °C on a rocker (Ames aliquot mixer, Miles Laboratories) as follows: 5 min in 0.15 M NaCl–0.01 M Tris–glycine, pH 8.3, which was the buffer used throughout; 5 min in 0.15% gelatin (Fisons, Loughborough, U.K.) dissolved in buffer by heating at 100 °C for 20 min; 1–2 h in gelatin containing 30–60 μL of antiserum; 20 min in several changes of buffer to remove excess first antibody; 1 h in 30 μL of peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical, Cappel-Worthington Division); 20 min in several changes of buffer to remove second antibody; 5 min in substrate solution (10 mg of 4-chloro-1-naphthol dissolved in 3 mL of methanol and added to 47 mL of buffer containing 50 μL of 30% H_2O_2). All volumes were 5–10 mL, the minimum to ensure uniform wetting of the paper. The titer of the antiserum was such that it could be used at 1:2500 dilution or more in radioimmunoassay.

Nonequilibrium Isoelectric Focusing in Polyacrylamide Gel. The procedure is described in detail elsewhere (Holt & Niewiarowski, 1986). Briefly, samples in carrier cytochrome *c* were applied at the top, anodal end of a vertical 6% slab gel, 1.6 mm thick. A constant current of 3 mA was maintained by allowing the applied voltage to rise from 25 to 400 V during the separation. The run was terminated when the visible cytochrome *c* was 1 cm from the gel bottom (3 h for a 4-cm gel, 7 h for a 10-cm gel). Transfer to nitrocellulose paper was by capillary action for 18 h at 20 °C (Reinhart & Malamud, 1982) since native βTG antigen did not bind in an electric field.

High-Performance Liquid Chromatography. Two systems were used, each with two pumps (Waters Associates or Milton Roy), a sample injector (Waters Associates), and a UV monitor, either with a fixed wavelength of 206 nm (Uvicord S, LKB Instruments) or with a fully variable wavelength (Waters Associates). Water and acetonitrile were Baker analyzed reagents (Fisher Scientific), and trifluoroacetic acid (TFA) was from Pierce Chemical Co. The column (4.6 × 250 mm) used for reverse-phase separations of protein contained Vydac TP RP (The Separations Group) and was obtained from Chrompack (Bridgewater, NJ). No guard column was used. The column was equilibrated at 20 °C in 0.1% trifluoroacetic acid–22% acetonitrile and was developed at 1.5 mL/min with a linear gradient of 22–32% acetonitrile in 0.1% TFA over 40 min. For fractionation of tryptic peptides, a column (4 × 250 mm) containing Lichrosorb RP-18 (5-μm particles) was obtained from Merck (Hibar EC RT). Equilibration was in 0.1% TFA at 1.5 mL/min. Bound peptides were eluted with a linear gradient of 0–70% acetonitrile over 45 min.

NH_2-Terminal Sequencing. The Edman degradation method in the sequenator version was employed (Edman & Begg, 1967; Edman & Henschen, 1975). A prototype spinning-cup sequenator was used. The phenylthiohydantoin derivatives of the amino acids were identified by HPLC using a solvent system which separates all components isocratically (Lottspeich, 1980).

Peptide Mapping. PBP (10 nmol), LA-PF_4 (45 nmol), and the peptide P18A obtained after reverse-phase HPLC were reduced, S-methylated, and cleaved with TPCK–trypsin (Cooper Biomedical, Cappel-Worthington Division) in parallel. The proteins, in 0.25 M ammonium acetate, $7 < pH < 8$, were made 5% v/v in 2-mercaptoethanol and incubated 2 h at 37 °C. Methyl iodide was then added to a final concentration of 7% v/v and the mixture kept 15 min at 20 °C in the dark with frequent shaking. The supernatant, combined with one aqueous wash of the oily residue, was evaporated to dryness, washed with water, and dried again to a volume of 0.1 mL. Protein was dissolved by addition of 0.5 mL of 0.04% TFA and the solution adjusted to $7 < pH < 8$ with dilute ammonia in the presence of 0.15 M ammonium acetate. Trypsin (1 mg/mL in 1 mM HCl) was added to the extent of 1:30 w/w of the protein substrate. Digestion was allowed to proceed 3 h at 37 °C before acidification with TFA and analysis by HPLC.

Amino Acid Analysis. Salt-free samples (1–5 nmol) were hydrolyzed in vacuo with 6 N HCl for 24 h at 110 °C. They were then dried over NaOH pellets in a vacuum desiccator, dissolved in 100 μL of 0.2 M sodium citrate buffer, pH 2.2 (Pierce Chemical Co., Rockford, IL), filtered (0.2 μm), and injected onto the column of a Glenco MM-70 amino acid analyzer.

Proteolytic Cleavage of PBP. Digestion mixtures contained 0.1–0.3 mg/mL PBP in 0.1 M NaCl–0.01 M Tris-HCl, pH 7.5. Plasmin (final concentration 1.6 IU/mL) was a glycerol-activated preparation which serves as the first international reference preparation for plasmin activity (National Institute for Biological Standards and Control, London, U.K.). TPCK–trypsin (final concentration 2% by weight of protein substrate) was obtained from Cooper Biomedical, Cappel-Worthington Division. Proteolysis was allowed to proceed at 37 °C for 10 min (trypsin) or 60 min (plasmin), after which time Trasylol (Sigma Chemical Co.) was added to a final concentration of 500 KIU/mL. When this amount of Trasylol was added simultaneously with plasmin or trypsin at zero time, no cleavage was observed.

RESULTS

Purification of Platelet Basic Protein. Pilot experiments showed that a satisfactory separation of LA-PF_4 and PBP could be obtained by using CM-Sepharose or CM-Sephadex under a variety of ionic conditions. The conditions finally chosen were the same as those used by several laboratories as the first step in purification of PDGF. Figure 1 [lower curve (○)] shows the elution profile of a CM-Sephadex column to which whole platelet releasate had been applied. Radioim-

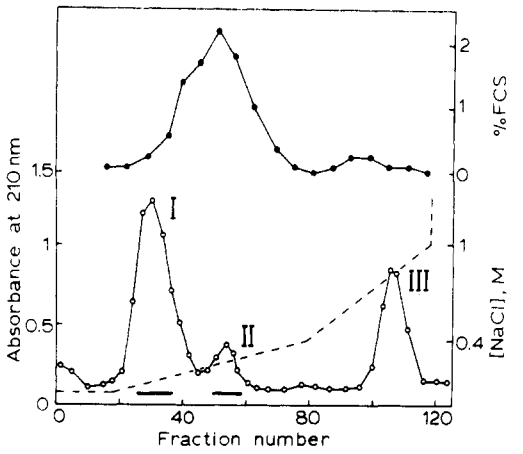

FIGURE 1: Fractionation of platelet release supernatant on CM-Sephadex. The platelet releasate from 8 units of fresh, washed platelets (see Materials and Methods) was applied at 40 mL/h to a column (1.9 × 8 cm) of CM-Sephadex C-50 equilibrated in 0.08 M sodium chloride–0.01 M sodium phosphate, pH 7.4. The conductivity and pH of the sample (ca. 120 mL) were adjusted to match those of the buffer. The column was washed with 100 mL of buffer and bound material eluted with two linear gradients, the first composed of 125 mL each of starting buffer and 0.38 M sodium chloride–0.01 M sodium phosphate, pH 7.4, and the second, 60 mL each of 0.4 and 1.0 M sodium chloride, both containing 0.01 M sodium phosphate, pH 7.4. The gradient is represented in the figure by a dashed line. Fractions of 3 mL were collected. The upper curve (●) shows the mitogenic activity of six-fraction pools, expressed as the concentration of fetal calf serum with the same activity in the same assay. The lower curve (○) shows the absorbance at 210 nm as a measure of total protein.

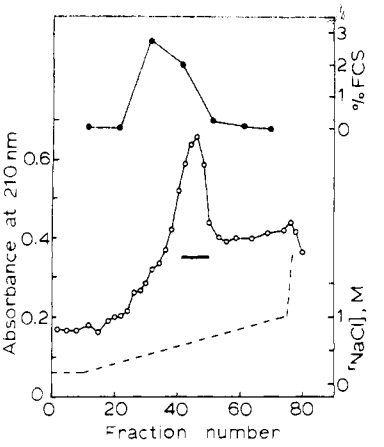

FIGURE 2: The PBP fraction from CM-Sephadex (see Figure 1) was diluted with water containing 0.1 mg/ml albumin (crystallized, fatty acid free; Sigma Chemical Co.) to an ionic strength of 0.15 M and applied at 40 mL/h to a column (1.6 × 10 cm) of heparin–agarose equilibrated in 0.15 M sodium chloride–10 mM sodium phosphate, pH 7.4. The column was washed with 50–100 mL of buffer before application of a linear gradient consisting of 60 mL each of starting buffer and 1 M sodium chloride–0.01 M sodium phosphate, pH 7.4. As in Figure 1, the upper curve (●) shows the mitogenic activity of eight-fraction pools and the lower curve (○) the absorbance at 210 nm. The fraction size was 1.8 mL. Fractions indicated by the bar were pooled, desalted by dialysis against 0.04% TFA, and freeze-dried.

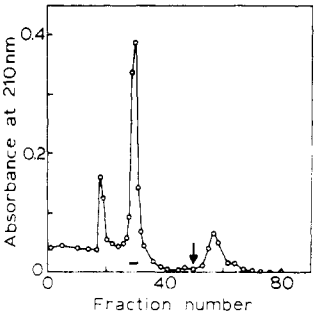

FIGURE 3: Chromatography on Sephadex G-75. PBP from heparin–agarose was dissolved in 2 mL of 0.1% trifluoroacetic acid and applied to a column (85 × 1.6 cm) of Sephadex G-75 equilibrated in the same solvent. The column was developed at 7 mL/h, and 2.3-mL fractions were collected. Similar results were obtained when the same column was equilibrated in 1 M acetic acid, with absorbance monitored at 236 nm (the lowest wavelength at which measurements could be made in this highly UV-absorbing solvent). The arrow indicates the position at which mitogenic activity was detected in two of six experiments analyzed. In the remaining experiments, no activity was detected in 10-fold-concentrated eight-fraction pools.

munoassay showed that all βTG-related antigen present in the starting material was bound to the column and that peaks I and II both contained this antigen; equilibrium isoelectric focusing (not shown) identified the first peak as LA-PF_4 (pI = 8) and the second as PBP (pI = 10.5). The cation-exchange column was equilibrated at pH 7.4 so that βTG (pI = 7) would be expected in the unbound fraction. That no unbound antigen was detected is consistent with the absence of βTG from fresh platelet releasates or lysates analyzed by gel isoelectric focusing (see below). Peak III in Figure 1 (lower curve) was identified immunologically and by its high affinity for heparin as PF_4. The interaction of PF_4, LA-PF_4, and PBP with CM-Sephadex and heparin–agarose was therefore very similar. With either matrix, PBP bound slightly more strongly than LA-PF_4, while PF_4 bound much more strongly than the other two proteins.

Peaks I and II were pooled as indicated by the bars, diluted with water containing 1 mg/mL albumin as carrier to an ionic strength of 0.15, and applied separately to a column of heparin–agarose. Elution of bound PBP with a gradient of NaCl is shown in Figure 2 [lower curve (○)]. Fractions were pooled as indicated, desalted by dialysis against 0.04% trifluoroacetic acid, and freeze-dried. After dissolution in 2 mL of 0.1% trifluoroacetic acid, the material was applied to a column of Sephadex G-75 equilibrated in the same solvent (Figure 3). For purposes other than sequence analysis, the major peak was considered pure PBP. Purified LA-PF_4 was obtained in the same way from peak I of Figure 1. After three steps of purification, the yield from 4 units of blood (1600 mL) was approximately 1 mg of LA-PF_4 and 0.1 mg of PBP. This represented 45–75% recovery of the βTG-related antigen present in the releasate. At each step, the total recovery was >85%, implying >60% overall recovery for three steps. Values lower than this resulted from rejection of CM-Sephadex fractions that were heterogeneous on isoelectric focusing gels.

Separation of Mitogenic Activity from PBP. Initial preparations of PBP obtained after the heparin–agarose step were relatively low in activity compared with the material originally described (Paul et al., 1980) or with PDGF. We therefore analyzed the whole elution profile obtained in this and the preceding CM-Sephadex step for mitogenic activity toward 3T3 cells.

On CM-Sephadex [Figure 1, upper curve (●)], a broad peak of activity was found at approximately the ionic strength reported for PDGF [e.g., see Westermark & Wasteson (1976)]. Much of this activity was contained in the pooled fractions corresponding to PBP. When the elution of PBP from heparin–agarose was analyzed [Figure 2, upper curve (●)], peak activity was detected at an ionic strength of 0.4–0.5 M, again similar to that reported for PDGF (Raines & Ross, 1982), while the protein peak appeared slightly later, at 0.6–0.7 M NaCl. This step was therefore effective in partially separating mitogenic activity from PBP. In the third step, chromatography on Sephadex G-75 in 0.1% trifluoroacetic acid, no peak of activity was reproducibly detected. In two independent experiments, however, some activity was detected slightly

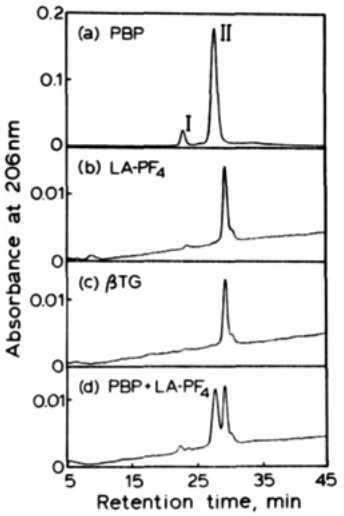

FIGURE 4: Reverse-phase HPLC. Samples of PBP and LA-PF_4 obtained after the three steps illustrated in Figures 1–3 were injected onto a column (250 × 4.6 mm) of Vydac TP RP (see Materials and Methods) equilibrated in 0.1% TFA–22% acetonitrile at a flow rate of 1.5 mL/min. After the column was washed for 3 min, a 40-min gradient of 22–32% acetonitrile was programmed. The column effluent was monitored for protein at 206 nm, and fractions were collected manually where appropriate. Panel a, PBP, 180 μg; panel b, LA-PF_4, 10 μg; panel c, βTG, 10 μg, prepared by limited cleavage of LA-PF_4 [the retention time of authentic βTG (see Materials and Methods and Table III) was the same within 0.1 min]; panel d, a deliberate mixture of LA-PF_4 and PBP, 10 μg each, to demonstrate the true separation of the two species, which was 1.3 min under these conditions.

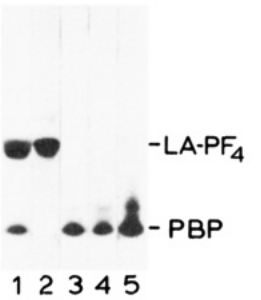

FIGURE 5: Nonequilibrium isoelectric focusing in polyacrylamide gel, with detection by immunoblotting with anti-LA-PF_4 serum. As described under Materials and Methods, a 6% polyacrylamide slab gel was run, and protein bands were transferred to nitrocellulose paper by capillary action for 16 h at 20 °C. The paper was saturated with gelatin and incubated first in anti-LA-PF_4 serum and then in peroxidase-conjugated goat anti-rabbit IgG. Antigen-containing bands were detected by soaking the paper in 0.15 M NaCl–0.01 M Tris–glycine, pH 8.2, containing 0.2 g/L 4-chloro-1-naphthol and 0.03% v/v hydrogen peroxide. Samples analyzed were (1) thrombin release supernatant of washed platelets, (2) purified LA-PF_4, (3) purified PBP, and (4 and 5) peaks I and II, respectively, from the reverse-phase HPLC analysis of PBP in Figure 4a. Loads varied from 50 ng of PBP in sample 1 to 250 ng of PBP in sample 5; in particular, we see only a quantitative and not a qualitative difference between samples 4 and 5.

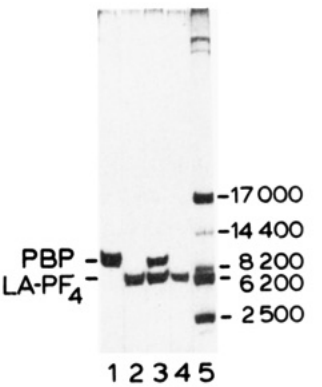

FIGURE 6: Analysis of purified materials on a 20% polyacrylamide gel containing SDS. Reduced samples applied to the gel were (1) PBP, 500 ng; (2) LA-PF_4, 200 ng; (3) a deliberate mixture of PBP and LA-PF_4, 200 ng each; (4) βTG prepared by limited cleavage of LA-PF_4, 150 ng; and (5) CNBr fragments of myoglobin, 800 ng, augmented with uncleaved myoglobin, 200 ng.

before the included volume of the Sephadex column (arrow in Figure 3). The identity of this material could not be established because it was not reproducibly present.

For a number of preparations of PBP, an inverse specific activity was determined at each stage of purification. This was defined as the amount of PBP required to produce the same mitogenic activity as 1% fetal calf serum. The mean values ± the standard deviation (with the number of determinations in parentheses) obtained were as follows: CM-Sephadex pool, 150 ± 90 ng/mL (6); heparin–agarose pool, 700 ± 650 ng/mL (5); Sephadex G-75 pool, >3200 ± 1200 ng/mL (4). That the recovery of PBP was high (70% or more after each stage), and the recovery of activity low, is consistent with the separate existence of PBP and a mitogenic factor which were progressively separated from one another during the purification of PBP.

When LA-PF_4 was purified by the same procedure, the mitogenic activity toward 3T3 cells detected in the final product was extremely low. In the most active of five preparations assayed, 50 μg of LA-PF_4 was required to produce the same effect as 1% fetal calf serum. Consistent with this low activity, there was essentially no overlap between the peak of LA-PF_4 protein and the broad peak of mitogenic activity in the elution profile of the CM-Sephadex column (Figure 1).

HPLC Analysis of PBP, LA-PF_4, and βTG. For chemical studies on PBP, reverse-phase HPLC was employed as a further purification step. Figure 4 shows the elution of PBP, LA-PF_4, and βTG from a C-18 reverse-phase column with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The deliberate mixture is shown to confirm that the apparent separation of PBP and LA-PF_4, when analyzed alone, was real. Several PBP preparations were resolved into variable amounts of two distinct components in this system (peak I, 0–40% w/w; peak II, 60–100% w/w), but the basis for the separation was not established. Components I and II had the same NH_2-terminal sequence (five residues compared), isoelectric properties (Figure 5), and apparent molecular weights (results not shown). βTG isolated from outdated platelets or βTG derived from LA-PF_4 by cleavage with trypsin (Holt & Niewiarowski, 1980) could not be distinguished from each other or from LA-PF_4 in this system. The particular samples examined, however, showed the expected charge difference both before and after HPLC.

Physicochemical Properties of PBP. As described above, PBP was identified by its antigenic properties and its high isoelectric point. Figure 5 shows samples analyzed by nonequilibrium gel isoelectric focusing as described under Materials and Methods. The resolving power of the gels was high and revealed heterogeneity not apparent on a sucrose density gradient focusing column (Niewiarowski et al., 1980). Purified PBP showing a single band corresponding to that present in fresh, unfractionated releasate or lysate was not stable. During storage and handling, one or two additional bands became evident, anodal to true PBP as defined by the species in the least processed material. We did not determine whether these resulted from cleavage or from side-chain modification.

The purity of isolated PBP is demonstrated in the dodecyl sulfate gels of Figure 6. The size difference between PBP

```
1                5                  10                  15
Ser-Ser-Thr-Lys-Gly-Gln-Thr-Lys-Arg-Asn-Leu-Ala-Lys-Gly-Lys-Glu-Glu-Ser-Leu-   PBP[1]

                                    Asn-Leu-Ala-Lys-Gly-Lys-Glu-Glu-Ser-Leu-   LA-PF4[2]

                                                Gly-Lys-Glu-Glu-Ser-Leu-   βTG[3]
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FIGURE 7: NH_2-terminal amino acid sequence of PBP compared with the established sequences of LA-PF_4 and βTG. Superscripts 1, 2, and 3 indicate this work, Rucinski et al. (1979), and Begg et al. (1978), respectively.

Table I: PTH-Amino Acid Yields during NH_2-Terminal Sequencing of PBP (3 nmol)

step	residue	yield (nmol)	step	residue	yield (nmol)	step	residue	yield (nmol)
1	Ser	0.63	8	Lys	1.21	15	Lys	0.45
2	Ser	0.53	9	Arg	1.45	16	Glu	0.92
3	Thr	0.76	10	Asn	1.38	17	Glu	0.92
4	Lys	2.02	11	Leu	1.60	18	Ser	0.32
5	Gly	2.68	12	Ala	1.87	19	Leu	0.98
6	Gln	1.29	13	Lys	0.52			
7	Thr	0.46	14	Gly	1.51			

and LA-PF_4 can be seen clearly on these gels and is emphasized by the deliberate mixture (see legend). βTG (M_r 8400) could not be distinguished from LA-PF_4 (M_r 8850) in this system. The molecular weight markers are included mainly to demonstrate the resolving power of the gels. The estimates of molecular weight based on their migration are clearly not reliable for the βTG-related proteins studied in this work.

Relationship of PBP to LA-PF_4 and βTG. (A) NH_2-Terminal Amino Acid Sequence. The greater apparent molecular weight of PBP compared to LA-PF_4, which had also been documented by gel filtration in 6 M guanidinium chloride (Varma et al., 1982), together with immunological cross-reactivity, suggested that PBP might be the precursor of LA-PF_4. For this reason, NH_2-terminal sequencing of HPLC-purified PBP was undertaken. As shown in Figure 7, a novel nine-residue sequence was found, followed by 10 residues of the established LA-PF_4/βTG sequence. Recoveries at each step are detailed in Table I. The purity of the PBP preparation was confirmed by the absence of significant contaminating sequences.

(B) HPLC Mapping of Tryptic Peptides. To extend our comparison from the NH_2-terminal residues to the whole polypeptide chain, PBP and LA-PF_4 were digested with trypsin, and the resulting peptides fractionated by reverse-phase HPLC. Both the retention times and the relative proportions of the peaks detected were very similar (Figure 8). The parallels observed between the tryptic peptide maps of PBP and LA-PF_4 provide further support for the contention that PBP is the precursor of LA-PF_4.

(C) COOH-Terminal Tryptic Peptides. To compare the COOH-terminal sequence of PBP with those of LA-PF_4 and βTG, we recovered the separated tryptic peptides shown in Figure 8 and sought to identify the COOH-terminal peptide by amino acid analysis. Table II shows the amino acid composition of two peptides (arrows in Figure 8) isolated from the digest of LA-PF_4 (peptide 1) and PBP (peptide 2). Peptide 3 was isolated at the same retention time as peptides 1 and 2 from the tryptic digest of a synthetic 18-residue peptide with the COOH-terminal sequence of βTG (Pro^{64}-Asp^{81}). Peptide 4 is the composition of the COOH-terminal peptide expected from the sequence of βTG. The composition of each of the three isolated peptides agreed well with that predicted from the βTG sequence. The presence of one lysine residue in the "synthetic" COOH-terminal peptide as well as in PBP and LA-PF_4 peptides suggests that tryptic cleavage occurred between Lys-67 and Lys-68 of βTG.

(D) Enzymatic Cleavage of Native PBP. As a third approach, we attempted to generate by in vitro proteolytic

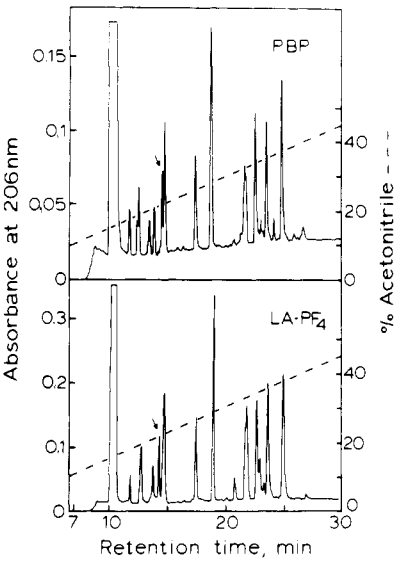

FIGURE 8: Reverse-phase HPLC comparison of tryptic peptides obtained from PBP and LA-PF_4. The two proteins were reduced, S-methylated, and cleaved with trypsin under identical conditions (see Materials and Methods). The digests obtained from 10 nmol of PBP (upper panel) or 45 nmol of LA-PF_4 (lower panel) were injected in 120 or 500 μL onto a column (4.6 × 250 mm) of Lichrosorb RP18 (Hibar RT, Merck) in 0.1% TFA. After the column was washed, bound peptides were eluted with a linear gradient of 0–70% acetonitrile over 45 min. The flow rate was 1.5 mL/min throughout.

Table II: Amino Acid Composition of COOH-Terminal Tryptic Peptides Purified from (1) a Synthetic Peptide Homologue of βTG, (2) LA-PF_4, and (3) PBP and (4) the Composition Deduced from the Sequence of βTG[a]

amino acid	peptide 1	peptide 2	peptide 3	peptide 4
Lys	1.2	1.2	1.0	1
Asx	1.8	1.7	2.0	2
Ser	0.5	0.8	0.5	1
Glx	1.4	1.3	1.2	1
Gly	0.9	1.1	1.0	1
Ala	1.8	1.7	1.8	2
Leu	1.1	1.1	0.9	1

[a] Experimental results are expressed as residues per mole of peptide with the composition of the established βTG sequence represented as peptide 4. For each peptide, results of two hydrolyses (for 24 h at 110 °C) were averaged. Residues not listed were found at 0.2 mol/mol of peptide or less. Low recovery of Ser is expected after acid hydrolysis.

cleavage of native PBP species which we could identify as LA-PF_4 or βTG. The action of trypsin, plasmin, and thrombin was investigated, as in previous work in which βTG was identified as a limited cleavage product of purified LA-PF_4

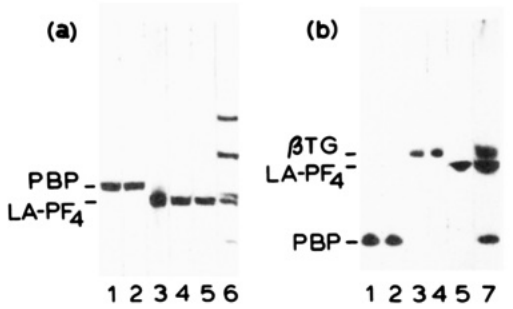

FIGURE 9: Comparison of proteolytically cleaved PBP with LA-PF_4 and βTG. Panel a, SDS–polyacrylamide gel electrophoresis as in Figure 6; panel b, nonequilibrium isoelectric focusing gel with detection by staining with a specific antibody as in Figure 5. In each panel, the samples were as follows: (1) untreated PBP; (2) PBP + thrombin; (3) PBP + plasmin; (4) PBP + trypsin; (5) untreated LA-PF_4; (6) myoglobin and its CNBr fragments; (7) a deliberate mixture of βTG, LA-PF_4, and PBP. Digestion conditions are given under Materials and Methods.

Table III: Comparison of Proteolytically Cleaved PBP with LA-PF_4 and βTG: Retention Times on a C-18 Reverse-Phase HPLC Column[a]

sample	retention time (min)
PBP	
+buffer	17.03
+plasmin	19.21
+trypsin	19.23
+thrombin	17.08
LA-PF_4	19.28
βTG (authentic)	19.23
βTG (from in vitro cleavage of LA-PF_4)	19.30

[a] Cleavage of PBP was carried out as described under Materials and Methods. When bovine lung trypsin inhibitor (Trasylol) was added in advance of trypsin or plasmin, no cleavage occurred by electrophoretic criteria, and the retention time was unaltered. The preparations of PBP used here contained less than 10% of peak I material (see Figure 4). Authentic βTG, isolated from clinically outdated platelets, was a gift from Dr. D. S. Pepper. Cleavage of LA-PF_4 to yield βTG was carried out as described previously (Holt & Niewiarowski, 1980). Conditions for HPLC were as in Figure 4, except that the column was equilibrated in 25% acetonitrile at 1 mL/min and a gradient to 32% acetonitrile was programmed over 30 min.

(Holt & Niewiarowski, 1980). The species present after incubation of native PBP with each protease as described under Materials and Methods was characterized with respect to charge (by isoelectric focusing), hydrophobicity (by reverse-phase HPLC), and size (by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels) with the results shown in Figure 9 and Table III. By each criterion, the major product of cleavage with trypsin or plasmin resembled βTG. Thrombin, on the other hand, did not cleave PBP since all three properties were unchanged.

Occurrence of PBP. With mounting evidence that PBP was the precursor of LA-PF_4 and βTG, efforts were made to determine whether PBP might in fact be the only species present in platelets and the smaller forms a consequence of proteolysis associated with cell stimulation or lysis. Our approach was therefore to lyse platelets in the presence of a variety of protease inhibitors and identify the form of the βTG-related antigen. Liquid column isoelectric focusing and radioimmunoassay were too time consuming for analysis of multiple samples, so an alternative procedure was developed as described under Materials and Methods. This consisted of nonequilibrium polyacrylamide gel isoelectric focusing, followed by transfer of the resolved protein bands to nitrocellulose paper and detection with specific antibody. All antibodies to βTG or LA-PF_4 so far tested recognize all forms of the antigen described in the present study.

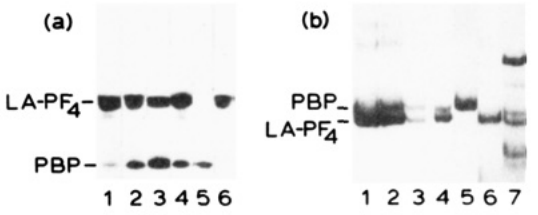

FIGURE 10: Platelet releasates and lysates analyzed on antibody-stained transfers of (a) isoelectric focusing gels and (b) SDS–polyacrylamide gels. Samples were as follows: (1–3) soluble extract from trichloroacetic acid lysed platelets from three normal donors, showing low, average, and high ratios of PBP to LA-PF_4, respectively; (4) thrombin release supernatant from washed platelets of the same donor as sample 2; (5) PBP marker; (6) LA-PF_4 marker; (7) myoglobin and fragments as in Figure 7. Of 26 samples analyzed, 2 are typified by sample 1, 16 by sample 2, and 8 by sample 3.

Samples were initially analyzed qualitatively, since we sought a major change in the PBP:LA-PF_4 ratio when the crucial inhibitor was present. Normal human platelets (pelleted from PRP in 5 mM EDTA–1 mg/mL theophylline or washed by gel filtration or repeated centrifugation and resuspension) were lysed in combinations of the following inhibitors: 2 mM phenylmethanesulfonyl fluoride, 10 mM benzamidine, 15 mM Cbz-Glu-Tyr, 100 μM leupeptin, 0.2 mg/mL soybean and pancreatic trypsin inhibitors. In three experiments, washed platelets were stimulated with the Ca^{2+} ionophore A23187 (50 nM) and within 3 min treated with 1 mM diisopropyl fluorophosphate (DFP) prior to separation of a release supernatant by centrifugation. All these samples contained mostly LA-PF_4 and only small amounts of PBP. There was no visible departure from the pattern established by a large number of platelet releasates, obtained over a period of several years without rigorous control of proteolysis. These releasates had been fractionated on CM-Sephadex (cf. Figure 1), and so the percentage of PBP could be estimated from the absorbance of the eluted fractions. The value found was 13.5% ± 4.0% by mass (average with SD for 16 multidonor pools of washed platelets). In summary, LA-PF_4 was the major antigenic species in both lysates and release supernatants, whether or not protease inhibitors were present before lysis or added immediately after secretion.

Negative results in the attempt to demonstrate high levels of supposed precursor in the presence of protease inhibitors are readily open to the criticism that the crucial protease was never successfully inhibited. We therefore looked for the most complete abolition of biological activity that could be achieved. Accordingly, platelets pelleted after addition of 5 mM EDTA, 3 μg/mL prostaglandin E_1, and 1 mg/mL theophylline (final concentrations) were resuspended in 0.15 M NaCl and immediately brought to a final 9% (w/v) trichloroacetic acid or 0.06 N perchloric acid at 0 °C. The precipitate formed was collected by centrifugation and resuspended with sonication in 0.1% trifluoroacetic acid–0.01% Triton X-100. The antigenic species present in the extract of the trichloroacetic acid pellet showed exactly the same isoelectric properties as the secreted, native proteins. In 11 such samples fractionated on CM-Sephadex, PBP was still the minor component (21.0% ± 5.7% by mass of βTG-antigen). Selected samples are shown in the focusing gel of Figure 10a and the sodium dodecyl sulfate gel of Figure 10b. (Although the sodium dodecyl sulfate gel did not provide the resolution and clarity of the focusing gel, it was important to confirm that the species identified on the native, nondissociating gel were indeed intact chains.) βTG was very rarely detected; it was present in 1 of 17 lysates obtained without protease inhibitors and absent from all lysates with protease inhibitors (n = 20) and all release

supernatants (n = 15) which were analyzed.

Discussion

The experimental results presented in this report provide strong evidence that platelet basic protein is the precursor form of low-affinity platelet factor 4 and β-thromboglobulin. Most compelling is the NH_2-terminal and COOH-terminal sequence information, the similarity of the tryptic peptides fractionated by HPLC, and the conversion of native PBP by plasmin and trypsin to species indistinguishable from LA-PF_4 and βTG. The apparent molecular weights and complete immunological cross-reactivity of the three proteins are also in accord with this conclusion (Varma et al., 1982). Finally, the NH_2-terminal sequences are themselves sufficient to explain the differences in the isoelectric points of the three species. The nine residues unique to PBP (pI = 10.5) and absent in LA-PF_4 (pI = 8.0) include two lysines and one arginine. The amino acid compositions of PBP and LA-PF_4 also differed only by the residues contained within the additional NH_2-terminal sequence of PBP (results not shown). In summary, all evidence, both direct and indirect, points toward a precursor–product relationship between PBP, LA-PF_4, and βTG.

Since the NH_2-terminal nonapeptide unique to PBP is a previously unreported sequence, we searched the protein sequence data base maintained by the National Biomedical Research Foundation, Washington, DC, updated to July 13, 1984. No significant homologies were found. The rest of the polypeptide chain is already included in the data base as the βTG sequence (Begg et al., 1978).

Purified PBP is not mitogenic for 3T3 cells. The progressive decrease in specific mitogen activity accompanying purification of PBP with good recovery implies the separate existence of PBP and one or more mitogenic factors in the starting material. One such factor expected in the platelet releasate is PDGF, and indeed the activity detected behaved on CM-Sephadex and heparin–agarose as has been described for this mitogen (Wasteson & Westermark, 1976; Raines & Ross, 1982). It is also possible that low molecular weight growth factors (Childs et al., 1982; Clemmons et al., 1983; Oka & Orth, 1983; Assoian et al., 1984) were present in crude PBP preparations. All of these factors occur at very low levels so that we could not expect significant recovery from the small quantities of blood used in the present work.

The physiological significance of the PBP/LA-PF_4/βTG group of proteins has yet to be established with certainty. Both chemotactic (Senior et al., 1983) and mitogenic activities have been reported. Connective tissue activating peptide III (CTAP III), with a sequence identical with that of LA-PF_4, has consistently been found by one group of investigators to be mitogenic for synovial cells (Castor et al., 1979, 1983). In contrast, βTG, isolated in another laboratory, was inactive. The first preparations of LA-PF_4 tested were mitogenic for 3T3 cells (Paul et al., 1980; Niewiarowski & Paul, 1981), but the highly purified material obtained in the present work was inactive (compare protein and activity profiles in Figure 1). This demonstrates that since biological activity may be expressed at the picogram level, the purity of the tested material is a crucial issue.

A novel suggestion as to function, which is free of the uncertainty over extent of purification, has recently been made based on studies of amino acid sequence homology. Both PBP/LA-PF_4/βTG and PF_4 share significant homology with a protein synthesized in the cultured cell line U937, a lymphoma with monocytic properties, in response to induction with interferon γ (Luster et al., 1985). Since this protein was produced rapidly and specifically in the induced cells, it may form, with PF_4 and βTG antigen, a family of proteins with involvement in inflammation and immune cell regulation. In a separate study, the NH_2-terminal tetrapeptide that distinguishes LA-PF_4 from βTG was found to occur with some specificity in proteins synthesized in response to cell transformation (Davis et al., 1985).

Platelets appear to contain mostly LA-PF_4 accompanied by small amounts of PBP. Under no conditions of proteolytic inhibition, including cell lysis with trichloroacetic acid, was the larger PBP found to be the major component. βTG was rarely seen, even when platelets were lysed in the absence of proteolytic inhibitors. Since all of these species are antigenic, the values measured by radioimmunoassay are not of a unique antigen. We therefore suggest that, when samples are not further analyzed, such values be described as βTG antigen or βTG-related antigen, rather than βTG. The latter is a defined chemical species which will not usually be the species present.

Most releasates used for preparative purposes were obtained from multidonor pooled platelets, thus obscuring any variations in the LA-PF_4 to PBP ratio which might occur between individuals. The economy of the gel focusing procedure with respect to both time and material required, however, allowed us to analyze single-donor samples. As shown in Figure 10, some variation between normal individuals was observed, but in no case did the amount of PBP exceed that of LA-PF_4. The low and high PBP to LA-PF_4 ratios shown were in fact unusual and were included only to show the range in normal platelets against which any departures would have to be measured, e.g., in megakaryocytes. The majority of normal donors are represented by samples 2 and 4 of Figure 10, which are consistent with the average proportions of LA-PF_4 and PBP obtained in preparative fractionations (see Results).

The studies presented in this report allow the protein used in continuing explorations of biological activity to be defined with precision. A degraded molecule may lack the activity expressed by a larger species. Moreover, since LA-PF_4 and βTG appear to be cleavage products of PBP, it is presumably PBP that is synthesized in megakaryocytes. The predominance of LA-PF_4 in platelets even after cell lysis with trichloroacetic acid suggests that cleavage of PBP may occur in megakaryocytes. Preliminary results (Holt et al., 1986) do indeed show approximately twice as much PBP in megakaroycytes as in platelets from the same donors; i.e., precursor cells contain more precursor protein. If cleavage of PBP is a specific event, the unique NH_2-terminal sequence of PBP could be a useful marker of megakaryocyte development and maturation. With the knowledge of these proteins gained in the present work, and the methods established for their identification, we are now in a position to study the biosynthesis of a platelet-specific protein in purified or cultured megakaryocytes and their stem cell progenitors.

Acknowledgments

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Registry No. PF_4, 37270-94-3.

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