Purification of Two Heparin-Binding Proteins From Porcine Platelets and Their Homology With Human Secreted Platelet Proteins

By Boguslaw Rucinski, Andreina Poggi, Pranee James, John C. Holt, and Stefan Niewiarowski

Two heparin-neutralizing proteins secreted by thrombin-stimulated platelets were purified to homogeneity by means of heparin-agarose affinity chromatography. These proteins, termed porcine platelet basic protein (PBP) and porcine platelet factor 4 (PF4), were eluted from a heparin-agarose column at 0.6-0.9 M NaCl and at 1-1.4 M NaCl. respectively. The molecular weight of porcine platelet basic protein was 7,000-7,700 daltons, as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and amino acid analysis. The isoelectric point of this protein was at pH 9.0. The amino acid composition of porcine platelet basic protein resembled that of human low affinity platelet factor 4 (LA-PF4), except that the porcine protein did not contain tyrosine. The molecular weight of porcine platelet factor 4 ranged from 10,000 (estimated from amino acid analysis) to 14,000 (estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis). The amino acid compositions of human platelet factor 4 and of porcine platelet factor 4 were similar. Monospecific antibodies against porcine platelet factor 4 and porcine platelet basic protein were raised in rabbits. Competitive radioimmunooassay demonstrated a low but significant immunologic cross-reactivity between human and porcine platelet factor 4, and between porcine platelet basic protein and a group of human secreted platelet proteins that bind to heparin with low affinity (β-thromboglobulin [βTG] and low affinity platelet factor 4). Experiments with direct immunoprecipitation of 3H-labeled antigens suggested that all four proteins investigated (human platelet factor 4, porcine platelet factor 4, human low affinity platelet factor 4 or human β-thromboglobulin, and porcine platelet basic protein) share common antigenic determinants. However, there was a higher degree of immunologic cross-reactivity between heterologous antigens with similar heparin binding affinity (human platelet factor 4 and porcine platelet factor 4) than between heterologous antigens with different binding affinity (human platelet factor 4 and porcine platelet basic protein). In conclusion, our finding suggests a significant structural homology among the four proteins.

STIMULATED BLOOD platelets secrete α-granule proteins with various biologic functions. A number of these proteins bind to heparin and can be fractionated using heparin-agarose chromatography. Heparin-binding proteins derived from human platelets, such as platelet factor 4 (PF4) and β-thromboglobulin (βTG), appear to be immunologically specific for platelets and are considered to be indicators of platelet activation. Radioimmunooassays of these proteins in biologic fluids have been used to evaluate platelet stimulation in vivo in various conditions.

Human PF4 binds to heparin-agarose with high affinity and is eluted at 1.2-1.5 M NaCl.5,6 Human βTG, low affinity platelet factor 4 (LA-PF4), and platelet basic protein (PBP) bind to heparin-agarose with lower affinity.7 These three low molecular weight proteins are immunologically identical with available antisera, but they show different physicochemical properties; the isoelectric points of PBP, LA-PF4, and βTG are at pH 10.5, 8.0, and 7.0, respectively.8,9

Radioimmunooassays developed for measurement of human specific α-granule proteins cannot be applied to biologic material derived from mammalian nonprimate species because there are significant immunologic differences between α-granule proteins in various species. A protein homologous with human platelet factor 4 has been purified to homogeneity from rabbit10 and bovine platelets.11 However, proteins corresponding to β-thromboglobulin or related antigens have not been demonstrated so far in mammalian nonprimate species.

The pig model is an important animal model for research of vascular diseases, atherosclerosis, diabetes, and congenital bleeding disorders such as von Willebrand's disease.12,14 The availability of well characterized porcine platelet proteins and their radioimmunoassay would be of great significance for investigators working on the role of platelet abnormalities in these diseases.

This article reports the isolation and characterization of two porcine platelet secreted proteins that are termed porcine platelet factor 4 and porcine platelet basic protein. As the terminology implies, our results suggest homology of these proteins with human platelet factor 4 and a group of human proteins binding to heparin with low affinity (platelet basic protein, low affinity platelet factor 4, β-thromboglobulin).
MATERIALS AND METHODS

Washing of the Platelets and Platelet Release Reaction

Porcine blood was collected in the slaughterhouse on acid-citrate-dextrose (1 part for 8 parts of blood) and allowed to sediment at room temperature for 1–2 hr. Supernatant was then collected and spun for 15 min at 150 g to obtain platelet-rich plasma (PRP) free of red cells. Platelets were separated from plasma by centrifuging at 600 g for 10 min and then washed twice by alternating centrifugation (1,500 g, 20 min) and resuspension in magnesium-free Tyrode buffer containing 0.01 M sodium citrate or 30 mM HEPES in 0.15 M NaCl, pH 6.5. Washed platelets were finally resuspended in Tyrode buffer or 30 mM HEPES-saline at pH 7.4 (1/10 of the original fluid volume; 5-7 x 10^9 platelets/ml) and aggregated by purified human thrombin (kindly provided by Dr. Fenton, Albany, NY) at the concentration of 5 U/ml in the presence of 5 mM CaCl_2. The mixture of aggregated platelets was spun at 2,200 g for 15 min. The supernatant was heated at 56°C for 30 min and stored frozen at -20°C.

Fractionation of Secreted Platelet Proteins by Heparin-Agarose Chromatography

Two procedures were applied to fractionate secreted porcine platelet proteins. In procedure 1, heparin-agarose slurry in 0.15 M NaCl was added to the supernate after platelet-release reaction (in a proportion of 40 ml of heparin-agarose per 200 ml of supernatant), gently shaken for a few minutes, and left overnight at 4°C. Then a column (1.5 x 30 cm) was packed with heparin-agarose containing adsorbed proteins. After washing with 200 ml of 0.15 M NaCl-0.05 M Tris buffer, pH 8.0, heparin-binding proteins were eluted with 170 ml of 1.5 M NaCl-0.05 M Tris, pH 8.0. The eluate was dialyzed against 0.1 M ammonium bicarbonate, lyophilized, dissolved in a small volume of 0.1 M NaCl-0.05 M Tris, and applied on a second heparin-agarose column (1.5 x 30 cm). The proteins were eluted using a gradient of 0.1 M – 2 M NaCl in 0.05 M Tris, pH 8.0, at 4°C, and the eluted fractions were monitored at 280 nm and 230 nm in a spectrophotometer.

In procedure 2, ZnSO_4 was added to the supernatant after release reaction to a final concentration of 0.04 M and the mixture was incubated for 30 min at 4°C. The ZnSO_4 precipitate was collected, dissolved in 1 M NaCl, and passed through a Sephacryl S200 column in 0.15 M NaCl-0.05 M Tris, pH 7.0. The eluate was monitored at 280 nm. The material was divided into high molecular weight fractions (above 40,000 daltons) and low molecular weight fractions (below 40,000). The low molecular weight fraction amounted to 40% of the total protein, approximately. The low molecular weight fraction (in 0.15 M NaCl-0.05 M Tris, pH 7.0) was applied to a heparin-agarose column, and the proteins were eluted using increasing salt gradient and monitored as in procedure 1.

Human Platelet Proteins

Human PF_4, and LA-PF_4, were obtained as previously described. Preparations of LA-PF_4/βTG contained approximately 76% LA-PF_4, 21% βTG, and less than 3% PBP.

Protein Determination

Protein determination was performed by the method of Lowry et al. and the results were calculated from the standard dilution curve of human serum albumin.

SDS-PAGE

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Weber and Osborn, with modifications described previously.

Amino Acid Analysis

Protein samples were hydrolyzed with 6 N HCl in sealed evacuated vials. Methionine and half cystine were estimated after hydrolysis of proteins oxidized with performic acid. The amino acids were analyzed on a single column with a Beckman 119 automatic amino acid analyzer equipped with an Infotronics integrator (Columbia Scientific Industries, Austin, TX).

Antiheparin Activity

The antiheparin activity of porcine PF_4 was tested according to the modified technique of Poplawski and Niewiarowski. Human platelet-poor plasma (PPP, pool of four volunteers) was used as a substrate plasma. Topical bovine thrombin (Parke Davis, Detroit, MI) and heparin sodium (Sigma, St. Louis, MO) were used in a buffer containing 0.05 M Tris, pH 7.0, 0.5 M NaCl. Heparin-thrombin time was measured in a test system consisting of 0.2 ml of PPP, 0.05 ml of heparin or buffer, 0.05 ml of buffer or protamine sulfate or sample, and 0.1 ml of thromboplastin. Concentration of thrombin (25 U/ml, approximately) was selected to give a thrombin time of 13–15 sec. Heparin was used at a concentration of 1.6–2.5 U/ml, which gave a heparin-thrombin time of 45–55 sec. A standard curve was prepared, using different concentrations of protamine sulfate (Sigma) (0.4–25 μg/ml). Samples of human and porcine PF_4 at concentrations ranging from 16 to 25 μg/ml were tested in the heparin-thrombin time as described above. The antiheparin activity was expressed in units of protamine sulfate equivalents per milligram protein.

Preparation of Antisera

Anti-human PF_4 and anti-human LA-PF_4/βTG sera were raised in rabbits as described previously. Antibodies against porcine platelet proteins were obtained according to Vaitukaitis et al. Anti-human platelet-poor plasma (PPP, pool of four volunteers) was used as a substrate plasma. Topical bovine thrombin (Parke Davis, Detroit, MI) and heparin sodium (Sigma, St. Louis, MO) were used in a buffer containing 0.05 M Tris, pH 7.0, 0.5 M NaCl. Heparin-thrombin time was measured in a test system consisting of 0.2 ml of PPP, 0.05 ml of heparin or buffer, 0.05 ml of buffer or protamine sulfate or sample, and 0.1 ml of thromboplastin. Concentration of thrombin (25 U/ml, approximately) was selected to give a thrombin time of 13–15 sec. Heparin was used at a concentration of 1.6–2.5 U/ml, which gave a heparin-thrombin time of 45–55 sec. A standard curve was prepared, using different concentrations of protamine sulfate (Sigma) (0.4–25 μg/ml). Samples of human and porcine PF_4 at concentrations ranging from 16 to 25 μg/ml were tested in the heparin-thrombin time as described above. The antiheparin activity was expressed in units of protamine sulfate equivalents per milligram protein.

Radioimmunoassay

Human LA-PF_4/βTG and PF_4 antigens were radioiodinated by the chloramine-T method of Greenwood et al. The detailed procedure was described previously. Porcine PF_4 was also iodinated with chloramine-T under the conditions established for human PF_4. Porcine PBP was labeled with 125I using Bolton-Hunter reagent and separated from unbound iodine by gel filtration on a column of Sephadex G-25. Specific radioactivities of labeled proteins were as follows: human LA-PF_4/βTG, 11,700 cpm/ng; human PF_4, 3,500 cpm/ng; porcine PF_4, 16,000 cpm/ng; porcine PBP, 2,600 cpm/ng. For each assay, 100 μl of 125I-protein (10,000–20,000 cpm) was used. Radioimmunoassays of human LA-PF_4/βTG and human PF_4 were performed as described previously. Radioimmunoassay of porcine PF_4 and porcine PBP was carried out by a similar procedure. Ammonium sulfate at 35–40% saturation was used to separate free antigens from antigen–antibody complexes. In this assay, the non-
specific binding of $^{125}I$-human LA-PF$_4$/βTG, $^{125}I$-human PF$_{a}$, and $^{125}I$-porcine PF$_4$ amounted to 9%, 20%, and 10%–25%, respectively. The maximal binding of all three antigens with a high concentration of homologous antibodies was approximately 95%. The radioimmunoassay of porcine PF$_4$ was sensitive in the range of 0.1–25 ng protein/ml. The $^{125}I$-PBP was precipitated only partially by 35% ammonium sulfate, with nonspecific binding amounting to 30% and maximal binding amounting to 80%. Ammonium sulfate was used for immunoprecipitation studies with $^{125}I$-PBP; however, the radioimmunoassay of porcine PBP was developed using a second antibody, sheep anti-rabbit serum (Arnel Products Co., Brooklyn, NY). The incubation mixture included 100 μl of $^{125}I$-porcine PBP, 100 μl of standard unlabeled protein or tested sample, 100 μl phosphate-saline buffer, 100 μl of anti-porcine PBP serum (1:50), and 500 μl of buffer. The phosphate-saline buffer was composed of 1% bovine serum albumin, 0.05 M sodium phosphate buffer, 0.8 M NaCl, pH 7.4. After overnight incubation at 37°C, 100 μl of phosphate-saline buffer and 100 μl of sheep anti-rabbit γ-globulin (1:4 dilution) were added. Samples were incubated again for 16 hr at 4°C. The precipitates and supernates were separated by centrifugation and counted in the Intertechnique gamma counter. The nonspecific binding in this method amounted to 5% of total, the maximal separation by heparin-agarose chromatography was 23% of the starting material. The protein content recovered in procedure 1 showed variability. They contained about 4%–15% contaminants in both reduced and nonreduced systems. In a typical preparative procedure, 10$^{12}$ platelets were obtained from 32 liters of porcine blood. The material released by thrombin (250 ml) contained 2.6 mg protein/ml. The total recovery of protein after the second heparin-agarose column amounted to 23% of the starting material. The protein content recovered in fraction 2 was 1.9 mg and in fraction 3–6.6 mg. The combination of ZnSO$_4$ precipitation and heparin-agarose chromatography increased further the purity of the isolated proteins, and contaminants were less than 1% (Fig. 2). In all further biochemical and immunologic studies, fractions obtained by either procedure but containing less than 5% contaminants were used.

Comparison of migration of molecular weight markers and protein of fraction 2 on SDS-PAGE indicates that its apparent molecular weight was 7,000 daltons.

RESULTS

Material released by porcine platelets has been fractionated on several occasions by procedure 1 or 2 as described in Materials and Methods. Figure 1 shows separation by heparin-agarose chromatography of secreted porcine platelet proteins that had been previously adsorbed to heparin-agarose slurry and eluted with 1.5 M NaCl. The eluted fractions were monitored at 280 nm and 230 nm. Three protein fractions were separated. In five different experiments, fraction 1 was eluted at NaCl concentration range of 0.2–0.5 M, fraction 2 at 0.6–0.95 M NaCl, and fraction 3 at 1.1–1.4 M NaCl. Since fraction 2 absorbed ultraviolet light at 230 nm but not at 280 nm, it apparently contained no aromatic amino acid residues. All other fractions absorbed ultraviolet light at both wavelengths. Material precipitated by ZnSO$_4$, filtered through Sephacryl S200, and applied on heparin-agarose column, yielded four fractions eluted at 0.15–0.25 M, 0.3–0.5 M, 0.6–0.75 M, and 1.0–1.1 M NaCl. Fractions eluted at 0.6–0.75 M NaCl corresponded to fraction 2 of procedure 1 and fractions eluted at 1.0–1.1 M NaCl corresponded to fraction 3 of the procedure 1 when evaluated by SDS-PAGE.

The fractions eluted from the heparin-agarose column were dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and applied on SDS-PAGE. Fraction 1 of procedure 1, eluted at lower ionic strength, was heterogeneous and it was discarded. The purity of preparations of fractions 2 and 3 obtained by procedure 1 showed variability. They contained about 4%–15% contaminants in both reduced and nonreduced systems. In a typical preparative procedure, 10$^{12}$ platelets were obtained from 32 liters of porcine blood. The material released by thrombin (250 ml) contained 2.6 mg protein/ml. The total recovery of protein after the second heparin-agarose column amounted to 23% of the starting material. The protein content recovered in fraction 2 was 1.9 mg and in fraction 3–6.6 mg. The combination of ZnSO$_4$ precipitation and heparin-agarose chromatography increased further the purity of the isolated proteins, and contaminants were less than 1% (Fig. 2). In all further biochemical and immunologic studies, fractions obtained by either procedure but containing less than 5% contaminants were used.
Fig. 2. SDS polyacrylamide gel electrophoresis (10%) of porcine platelet proteins. Lane 1 contains the following molecular weight standards: phosphorylase b, 94,000; bovine serum albumin 68,000; ovalbumin, 43,000; carbonic anhydrase 30,000; soybean trypsin inhibitor 21,000; lysozyme, 14,300; human platelet factor 4, 7,800. The position of migration of platelet factor 4 that gave a very faint band on this photograph is marked with an arrow. Lane 2 contains porcine fraction 2 (PBP) nonreduced, lane 3 porcine fraction 2 (PBP) reduced, lane 4 porcine fraction 3 (PF4) nonreduced, and lane 5 porcine fraction 3 (PF4) reduced. Anode is at the bottom. Each sample contained 15-20 μg protein.

in a reduced system. The apparent molecular weight of fraction 3 protein was about 14,000 in a reduced system (Fig. 2). Subsequently, we determined the amino acid composition of porcine fractions 2 and 3 and compared them with amino acid compositions of human LA-PF4 and human PF4. The amino acid compositions of human proteins were deduced from their amino acid sequences. The results are summarized in Table 1. The amino acid compositions of porcine fraction 3 and human PF4 showed many similarities. Both proteins lack methionine and phenylalanine and show similar contents of lysine, alanine, cysteine, valine, isoleucine, and leucine. The main difference between the two proteins is the higher concentrations of proline in porcine fraction 3 as compared to human PF4. Since porcine fraction 3 and human PF4 are eluted from heparin-agarose at a similar ionic strength and contain similar proportions of basic and hydrophobic amino acids, we postulate that porcine fraction 3 is similar to human platelet factor 4. The minimum molecular weight of porcine platelet factor 4 calculated on the basis of amino acid composition with one tyrosine residue per molecule amounted to 10,000 daltons. The amino acid composition of fraction 2 protein (termed porcine platelet basic protein, PBP, for the reasons to be presented below) and human LA-PF4/βTG showed significant similarities, the main difference being the absence of tyrosine in porcine PBP. The minimum molecular weight of porcine PBP calculated on the basis of amino acid composition with one methionine residue per molecule amounted to 7,700.

Heparin-neutralizing activity of human PF4, porcine PF4, and porcine PBP, measured on three different occasions, amounted to 290–670, 200–600, and 50–220 μg protamine sulfate equivalents/mg protein, respectively. These values reflect a great variability of the assay. However, the heparin-neutralizing activity of porcine PBP was consistently lower than that of human and porcine PF4.

Double immunodiffusion studies showed that porcine PBP and porcine PF4 gave distinct precipitation lines with homologous antibodies. In this system, no precipitation was observed for heterologous reactions. On immunoelectrophoresis at pH 8.6, both porcine PF4 and porcine PBP gave single precipitin lines with homologous antibodies. Porcine PBP migrated to the cathode and porcine PF4 to the anode, suggesting that porcine PBP is a more basic protein than porcine PF4.

Figure 3 shows the results of the separation of
Table 1. Amino Acid Analysis of Porcine and Human Platelet Proteins

<table>
<thead>
<tr>
<th></th>
<th>Porcine PF₄</th>
<th>Residues/100 Amino Acids</th>
<th>Human PF₄</th>
<th>Porcine PBP</th>
<th>Human LA-PF₄</th>
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<tr>
<td>Lys</td>
<td>11</td>
<td>11.42</td>
<td>9.96</td>
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<tr>
<td>His</td>
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<td>2.86</td>
<td>3.94</td>
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<td>4.29</td>
<td>5.10</td>
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<td>9.32</td>
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<tr>
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<td>6.48</td>
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<td>3.26</td>
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<tr>
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<td>4.29</td>
<td>7.66</td>
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<tr>
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<td>7.14</td>
<td>11.00</td>
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<tr>
<td>Val</td>
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<td>4.29</td>
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<td>0.00</td>
<td>1.40</td>
<td>1.08</td>
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<tr>
<td>Iso</td>
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<td>8.57</td>
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<tr>
<td>Tyr</td>
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<td>0</td>
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<td>Phe</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>Trp</td>
<td>ND</td>
<td>0</td>
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The amino acid composition of porcine PF₄ was calculated on the basis of determinations of two different preparations. If porcine PF₄ contained one tyrosine residue per molecule, the total number of residues would be 91 and minimum molecular weight 10,000. The amino acid composition of porcine PBP was calculated on the basis of determinations of four different preparations. The absence of Trp in PBP is inferred from the fact that this protein does not absorb ultraviolet light at 280 nm. If porcine PBP contained one residue of methionine per molecule, the total number of residues per molecule would be 72 and minimum molecular weight 7,700. The composition of human PF₄ and human LA-PF₄ was calculated from amino acid sequencing data.

Porcine PBP on the isoelectric focusing column. The levels of specific antigen and pH were determined in each fraction eluted from the column. The antigen was focused predominantly in four fractions (tubes 66–69, pH 8.83–9.03). Another small fraction with PBP antigenic activity was focused at pH 8.5. We accepted that the isoelectric point of porcine PBP is at pH 9.0.

Figure 4A shows typical results of binding experiments in which labeled human LA-PF₄/βTG was incubated with various antisera to human and porcine proteins and direct immunoprecipitation was measured. The highest affinity was observed for homologous reactions, e.g., between ¹²⁵I-human LA-PF₄/βTG and anti-human LA-PF₄/βTG. There was, however, significant binding of the labeled antigen by antisera heterologous with respect to both species and antigen. In Fig. 4A, anti-porcine PBP, anti-human-PF₄ and anti-porcine PF₄ all bound ¹²⁵I-LA-PF₄/βTG at concentrations from 2 to 3 orders of magnitude higher than that required for the homologous reaction. That the binding was specific was shown by the inability of anti-albumin antiserum to bind any of the labeled antigens (Fig. 4A).

Fig. 3. Isoelectric focusing of purified porcine PBP. The amount of protein applied on the column was 400 μg. The recovery was 41.6%.
lowing Prager and Wilson. Each curve is characterized by the antiserum dilution, $S$, corresponding to half-maximal binding. For a pair of antigens, ID is calculated from the ratio of the antiserum dilutions at half-maximal binding for homologous and heterologous reactions as:

$$\text{ID} = 100 \times \log_{10} \left( \frac{S_{\text{antiserum homologous}}}{S_{\text{antiserum heterologous}}} \right)$$

According to this equation, higher immunologic distance (ID) indicates lower degree of cross-reactivity between two antigens. Since a ratio is used, the titer of a particular serum does not influence the calculated immunologic distance.

The results of this calculation are presented in Table 2. As implied by inspection of the binding curves (Fig. 4A and B), the average values of immunologic distance between all couples of heterologous antigens were comparable, with the exception of one anti-LA-PF$_4$/βTG antiserum tested with porcine 125I-PBP (Fig. 4B). One anti-LA-PF$_4$/βTG antiserum showed a very high affinity (ID = 73) toward 125I-PBP; however, three other anti-LA-PF$_4$/βTG antisera tested showed lower affinity (average ID = 173) toward the same labeled antigen. Table 3 also shows a certain correlation between immunologic cross-reactivity of heterologous antigens and their binding affinity to insolubilized heparin. Two heterologous antigens that have similar heparin-binding affinity (for instance, human PF$_4$ and porcine PF$_4$) showed a higher degree of immunologic cross-reactivity (that is lower ID) than two heterologous antigens with different heparin-binding affinity (like human PF$_4$ and porcine PBP).

The existence of immunologic cross-reactivity
Table 2. Indices of Immunologic Distance

<table>
<thead>
<tr>
<th>Antigen X</th>
<th>Antigen Y</th>
<th>Anti-X Versus Y</th>
<th>Anti-Y Versus X</th>
<th>Average</th>
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<tr>
<td>p PBP</td>
<td>h LA-PF₄/βTG</td>
<td>200†</td>
<td>173†</td>
<td>186†</td>
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<tr>
<td>p PBP</td>
<td>h PF₄</td>
<td>315</td>
<td>365</td>
<td>340</td>
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<td>p PF₄</td>
<td>h PF₄</td>
<td>178</td>
<td>200</td>
<td>189</td>
</tr>
<tr>
<td>p LA-PF₄/βTG</td>
<td>h PF₄</td>
<td>323</td>
<td>289</td>
<td>306</td>
</tr>
<tr>
<td>h LA-PF₄/βTG</td>
<td>h PF₄</td>
<td>150</td>
<td>280</td>
<td>215</td>
</tr>
<tr>
<td>p PBP</td>
<td>p PF₄</td>
<td>216</td>
<td>265</td>
<td>240</td>
</tr>
</tbody>
</table>

p, Porcine; h, human.

*Immunologic distance (ID) was calculated by the method of Prager and Wilson29 (see text for explanation). The concentration of the antiserum causing half-maximal precipitation of the labeled antigen was taken as that producing binding midway between maximum and nonspecific binding.

†The average data of experiments with four anti-human LA-PF₄/βTG antisera and two anti-porcine PBP antisera. Each antiserum was obtained from a different animal. Numbers in parentheses indicate immunologic distances calculated from single experiments presented in Fig. 4, A and B.

between porcine and human PF₄ and between porcine PBP and human LA-PF₄/βTG was also confirmed by means of a competitive radioimmunoassay (Table 3). These data show that there is about 0.05%-0.1% of cross-reactivity between human and porcine PF₄ and 0.05%-0.3% between human LA-PF₄/βTG and porcine PBP.

Preliminary measurements by radioimmunoassay on eight samples of porcine platelet-rich plasma indicate that PF₄ and PBP amounts to 10.6 μg (±5.1 SD)/10⁹ platelets and 1.1 μg (±0.59 SD)/10⁹ platelets, respectively. In human platelets, the figures for the homologous proteins are 12.4 μg and 24.2 μg, respectively.5

**DISCUSSION**

Our results demonstrate that porcine platelets stimulated by thrombin secrete two low molecular weight heparin-binding proteins which, by analogy with human platelet secreted proteins, were named porcine platelet factor 4 and porcine platelet basic protein. There is good evidence that the porcine PF₄ is the analog of human PF₄. Both proteins bind to heparin-agarose with similar affinity and show similar heparin-neutralizing activity. Their amino acid composition is similar (Table 1). Both proteins show low but significant immunologic cross-reactivity in competitive binding radioimmunoassay and in direct precipitation immunoassay (Fig. 4, Table 3). Porcine PF₄ appeared to be immunologically closer to human PF₄ than to human LA-PF₄ as shown by the calculation of immunologic distance. Molecular weight of human PF₄, established by means of amino acid sequence analysis, amounts to 7,800 daltons.26-28 Molecular weight of porcine PF₄ ranges between 10,000 (calculated on the basis of amino acid composition) and 14,000 (estimated on the basis of SDS polyacrylamide gel electrophoresis). The discrepancy between these two values may reflect anomalous behavior of porcine PF₄ in SDS-PAGE. The electrophoretic mobility may correlate poorly with the molecular weight of proteins below 15,000 mol wt.30 Human PF₄ is released from platelets as a high molecular weight complex with proteoglycan carrier.31 The same is probably true for porcine platelets since Nath et al.32 observed that the anti-heparin activity released by thrombin-stimulated porcine platelets is eluted in the void volume during Sephadex G-200 gel filtration. It is of interest that reduction of porcine PF₄ and PBP resulted in the increase of their mobility on SDS-PAGE (Fig. 2). The same characteristic has been reported for human PF₄ and LA-PF₄.5

Although porcine platelet basic protein shows slightly higher affinity for heparin (and heparin-neutralizing activity) than human low affinity heparin-binding proteins, immunologic studies (Fig. 4, Tables 2 and 3) demonstrate significant cross-reactivity between porcine PBP and human LA-PF₄/βTG antigen. Immune precipitation curves (Fig. 4) suggested that porcine PBP is closer to human LA-
PF<sub>4</sub>/βTG than is PF<sub>4</sub>. The amino acid compositions of porcine PBP and human LA-PF<sub>4</sub> are similar (Table 1), especially if acidic, basic, and hydrophobic residues are grouped together. The molecular weights of the two proteins are also similar. The human low affinity heparin-binding proteins βTG (isoelectric point at pH 7.0) and LA-PF<sub>4</sub> (isoelectric point at pH 8.0) are derived from human PBP (isoelectric point at pH 10.5) by limited proteolysis. This occurs in whole platelets as well as in vitro when purified proteins are incubated with plasmin or trypsin<sup>33</sup> (Holt, unpublished results).

At present, there is no evidence that limited proteolysis of porcine platelet low affinity anti-heparin proteins does occur.

Characterization of secreted platelet proteins from species other than man has so far been limited to PF<sub>4</sub>, which was identified by its high affinity for heparin. Ginsberg et al.<sup>10</sup> demonstrated by competitive radioimmunoassay that the level of cross-reactivity between human and rabbit PF<sub>4</sub> is at the level of 1%. Similar results were obtained in our study when human and porcine PF<sub>4</sub> were compared (Table 3).

The minimum molecular weights of bovine platelet factor 4 and of rabbit platelet factor 4 calculated from amino acid analysis amount to 9,000 daltons<sup>34</sup> and to 8,900 daltons,<sup>35</sup> respectively. The apparent molecular weight of guinea pig platelet factor 4 is 18,000 daltons. It does appear that the molecular weight of platelet factor 4 derived from nonprimate mammalian platelets is higher than that obtained from human platelets. This may result from a mutation during evolution or from posttranslational modification, i.e., proteolysis of the human protein.

Recently, Muggli et al.<sup>35</sup> isolated from the material released by thrombin-stimulated rabbit platelets a cationic protein (isoelectric point above pH 9.5) that was bound to heparin-agarose and eluted at a salt concentration of about 0.9 M NaCl. This protein, termed “platelet-specific protein 2” (PSP-2), migrated in SDS-PAGE as a single band with a molecular weight below 12,000 daltons. Our preliminary data indicate low level of immunologic cross-reactivity between PSP-2, human PF<sub>4</sub>, human LA-PF<sub>4</sub>/βTG, and porcine PBP (unpublished observations).

It is generally accepted that the surface residues contain almost completely the antigenic determinants of any protein. In a homologous series of proteins, the overall conformation appears to be preserved despite extensive changes in primary structure. The surface residues are more subjected to changes than the interior residues responsible for conformational stability. Since antigenicity is a protein surface phenomenon, it follows that antigenic variability is a close reflection of protein evolution.<sup>36</sup> Prager and Wilson<sup>37</sup> and Ibrahimie et al.<sup>38</sup> observed a reverse correlation between immunologic distance and percentage of amino acid sequence homology in lysozymes purified from egg whites of ten different birds. Proteins differing from each other by 40% or more in sequence exhibited no immunologic cross-reactivity in microcomplement fixation tests. The observed dependence of immunologic cross-reactivity measured by immunoprecipitation and by complement fixation on sequence resemblance is also valid for a wide variety of other proteins.<sup>37,39</sup> It is accepted on the basis of extensive studies on synthetic polypeptides that each antigenic determinant on a protein comprises about five amino acids. When protein sequences reach differences of about 30%, the exposed parts of the molecules can be expected to differ by an even larger percentage than the molecules as a whole.<sup>36</sup> The levels of immunologic distance found in our experiments indicate that there is a significant cross-reactivity between human and porcine low and high affinity heparin-binding proteins. On the basis of our data, we should also predict a significant homology of amino acid sequence of these proteins.

In addition, our data provide further evidence for the existence of common antigenic determinants in human PF<sub>4</sub> and LA-PF<sub>4</sub>/βTG antigens. Partial immunologic cross-reactivity between these proteins was demonstrated previously by various investigators on the basis of competitive radioimmunoassay studies.<sup>40,41</sup> However, the possibility of cross-contamination in the material used for immunization of animals has not been excluded. Since we detected cross-reactivity between antigens obtained from two different species, our study provides stronger evidence for the existence of a genuine immunologic cross-reactivity between human PF<sub>4</sub> and human βTG or related antigens such as LA-PF<sub>4</sub> or PBP. Although human βTG and PF<sub>4</sub> proteins show only 50% sequence homology,<sup>28</sup> they have indeed been found to have very similar secondary structure. The proportions and location in the sequence of α-helix, β-sheet, and β-turns appear to be almost identical.<sup>42,43</sup> The residues that are not identical are at least conservatively replaced. A similar spatial distribution of the antigenic sites on both molecules can explain the observed immunologic cross-reactivity and a significant similarity between their secondary structure.

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Purification of two heparin-binding proteins from porcine platelets and their homology with human secreted platelet proteins

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