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Cell Biology

Synthesis of analogs of human platelet membrane glycoprotein IIb–IIIa complex by chicken peripheral blood thrombocytes

(phylogeny)

THOMAS J. KUNICKI* AND PETER J. NEWMAN

The Blood Center of Southeastern Wisconsin, Milwaukee, WI 53233

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ABSTRACT **Human platelets and their phylogenetic counterparts, avian thrombocytes, play a key role in primary hemostasis. Based upon extensive studies in mammals, platelet cohesion resulting in the formation of the "hemostatic plug" is known to be mediated by the mammalian platelet glycoprotein IIb–IIIa complex in concert with fibrinogen and calcium. The immunological and biochemical technology already developed for the analyses of mammalian platelet glycoproteins has never been applied to avian thrombocytes. By indirect immunofluorescence, we now show that a polyclonal rabbit antibody specific for human glycoproteins IIb plus IIIa and the well-characterized murine monoclonal anti-IIb–IIIa complex antibody, AP2, both crossreact with IIb and IIIa analogs on intact chicken thrombocytes. By two-dimensional polyacrylamide gel electrophoresis, we also demonstrate that chicken thrombocytes will incorporate [^{35}S]methionine into several proteins, including the glycoprotein IIb and IIIa analogs during short-term (4 hr) incubation *in vitro*. This finding indicates that peripheral blood nucleated thrombocytes of the chicken, unlike their mammalian counterparts, retain the capacity to synthesize protein. The significance of these findings is 2-fold. First, we provide biochemical and immunological evidence that those proteins responsible for platelet cohesion in humans are structurally conserved in cells of analogous function in chickens despite the fact that these species have diverged from a common ancestor more than 200–250 million years ago. Second, we identify chicken thrombocytes as a readily available source of messenger RNA encoding numerous proteins analogous to those already characterized in human platelets, including glycoproteins IIb and IIIa.**

Mammalian platelets function as major components of the hemostatic system by adhering to exposed subendothelium in the immediate area of vascular damage and by forming a cohesive plug, which then can serve as a surface for the binding of plasma coagulation factors. Platelet cohesion, a central feature of the hemostatic process, is now known to be mediated by plasma membrane glycoproteins (GPs) IIb and IIIa in concert with fibrinogen, a requisite cofactor in this event (for a review, see ref. 1). GPIIb and GPIIIa have been shown to exist in a calcium-dependent heterodimer complex (2–4) that is present in nonactivated, intact platelets in the presence of physiologic levels of calcium (5–7). Several agonists, including adenosine diphosphate, thrombin, and epinephrine, are known to induce the expression of an active fibrinogen receptor (8–10), which is currently thought to represent an altered conformation of preexisting GPIIb–IIIa complexes (5–7).

Peripheral blood thrombocytes of nonmammalian vertebrates perform a hemostatic role analogous to that of mammalian platelets. Avian thrombocytes have been reported to aggregate *in vitro* in response to thrombin, collagen, or serotonin, but not adenosine diphosphate (11–13), to undergo shape change (13), and to take up and store serotonin in organelles functionally analogous to mammalian platelet dense bodies (14, 15). In view of the common mechanism operative in mammalian platelet cohesion, one might expect that avian thrombocyte cohesion is subject to similar requirements—i.e., that the cohesive mechanism of the thrombocyte/platelet phylogenetic lineage has been conserved. Intrigued by this possibility and the obvious implication that nucleated avian thrombocytes would then be likely to contain translatable mRNA encoding various analogs of human platelet proteins or GPs, we initiated studies to determine the GP composition of chicken thrombocytes relative to human platelets and the capacity of chicken thrombocytes for membrane protein synthesis.

MATERIALS AND METHODS

Mammalian Platelets. Human, baboon, or rat blood (6 vol) was mixed with 1 vol of acid/citrate/dextrose (ACD) anticoagulant (National Institutes of Health formula A). Dog blood (9 vol) was anticoagulated by addition of 1 vol of 3.8% (wt/vol) citrate. Platelet-rich plasma was then obtained from whole blood by differential centrifugation (200 × *g* for 10 min). Prostaglandin E_1 (final concentration 20 ng/ml) was added to the platelet-rich plasma, and the mixture was allowed to stand at ambient temperature for 15 min. Platelets were pelleted and freed of plasma proteins by three successive washes (1500 × *g* for 12 min) in Ringer's citrate/dextrose, pH 6.5, containing prostaglandin E_1 at 20 ng/ml (16). Washed platelets were freed of residual erythrocyte or leukocyte contamination by intermittent, slow-speed (200 × *g* for 5 min) centrifugation during the platelet washing procedure. The final ratio of leukocytes or erythrocytes to platelets was <1 per 2000 as determined by phase microscopy. Platelet concentration was determined by phase microscopy.

Chicken Thrombocytes. Chicken peripheral blood cells (PBC; this term will henceforth be used to designate nucleated, nonerythroid blood cells, including thrombocytes, lymphocytes, granulocytes, and monocytes) were isolated from whole blood anticoagulated in either citrate (1 vol of 3.8% citrate plus nine vol of whole blood) or ACD anticoagulant (1 vol of ACD anticoagulant plus 6 vol of whole blood) by Ficoll/Hypaque flotation (density, 1.076) essentially as described by Boyum (17). In some experiments, thrombocytes were enriched by virtue of their capacity to adhere to plastic surfaces as described by Ries *et al.* (18). Briefly, heparinized-blood (1000 units/ml) was poured into polystyrene flasks (10 ml) and incubated with gentle agitation at 39°C for 2 hr. The supernatant was decanted, and the flasks were rinsed thor-

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Abbreviations: GP, glycoprotein; CBR, Coomassie blue-R; PBC, peripheral blood cell(s).

*To whom reprint requests should be addressed.

oughly with Hanks' balanced salt solution. To detach adherent cells, each flask was filled with ice-cold buffered Ca^{2+}/Mg^{2+}-freeEDTA solution (EDTA buffer as in ref. 19). After 2 hr those cells not yet freed were removed with the aid of a rubber policeman. PBC concentration was determined by phase-contrast microscopy. PBC were washed three times in EDTA buffer.

Indirect Immunofluorescence. PBC isolated from ACD-anticoagulated blood were pelleted by centrifugation at 400 × *g* for 10 min, washed three times in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.145 M NaCl and 0.1% NaN_3, and resuspended to 10^8 cells per ml in the same buffer. Washed PBC (100 μl; 10^7 cells) were incubated with platelet-specific murine monoclonal IgG (100 μg) or rabbit polyclonal IgG (1 mg) for 90 min at ambient temperature, washed three times in phosphate/NaCl/NaN_3 buffer, and then incubated in an ice bath (4°C) with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (Tago, Burlingame, CA) for 30 min. After three additional washes in sodium phosphate/NaCl/NaN_3 at ambient temperature, PBC were resuspended in 50 μl of 0.04 M glycine/0.07 M NaCl, pH 8.6, containing 60% (vol/vol) glycerol and analyzed by fluorescence phase-contrast microscopy.

Radioiodination. Washed, intact human platelets were resuspended in 0.015 M Tris·HCl/0.001 M EDTA/0.145 M NaCl, pH 7.4 (THEN buffer) to a final concentration of 10^9 per ml and were labeled with ^{125}I by the lactoperoxidase method of Phillips and Poh Agin (20). Washed intact thrombocytes were resuspended in EDTA buffer (19) to a final concentration of 10^8 per ml and labeled with ^{125}I as described by Felsted and Gupta (21). After radioiodination, cells were washed three times in the respective buffers and resuspended in THEN buffer (pH 7.0) containing 0.01 M *N*-ethylmaleimide. For mammalian platelets, the final cell concentration was nominally 2–4 × 10^9 per ml; for chicken thrombocytes, 2 × 10^8 per ml. $NaDodSO_4$ was added to the cell suspensions at a final concentration of 1% (wt/vol), and the mixtures, which immediately became transparent, were incubated at 37°C for 1 hr. If not used immediately, the lysates were frozen at −80°C. The protein concentration of lysates was determined by the method of Markwell *et al.* (22). Because DNA was not digested or removed prior to solubilization, chicken thrombocyte samples usually gelled at room temperature. To facilitate handling, these samples were routinely incubated at 56°C just prior to use.

$NaDodSO_4$/PAGE. The protein composition of mammalian platelet and chicken thrombocyte lysates was analyzed by two-dimensional nonreduced–reduced $NaDodSO_4$/PAGE by the method of Phillips and Poh Agin (20) as described by Kunicki *et al.* (23), with the discontinuous buffer system of Laemmli (24) used in both dimensions. First-dimension cylinder gels (nonreduced) were composed of a 3.0% (wt/vol) acrylamide stacking gel and a 5.0% acrylamide resolving gel; second-dimension slab gels (reduced) were composed of a 3.0% acrylamide stacking gel and a 7.0% acrylamide resolving gel. After electrophoresis, gels were fixed and stained with Coomassie blue-R (CBR) in isopropanol/acetic acid/water, 25:10:65 (vol/vol), destained, photographed, and then dried *in vacuo* (23). Autoradiography was performed as described (23).

Incorporation of [^{35}S]Methionine into Membrane Proteins of Intact Chicken Thrombocytes. Freshly prepared chicken PBC were washed three times in Hanks' balanced salt solution, incubated for 4 hr at 37°C in methionine-free RPMI 1640 medium containing 125 μCi (800 Ci/mmol; 1 Ci = 37 GBq) of [^{35}S]methionine and 10% dialyzed fetal calf serum, then washed three times in Hanks' balanced salt solution containing nonradiolabeled methionine at 1 mg/ml. Labeled cells were then resuspended to a final concentration of 1 × 10^8/ml in THEN buffer, pH 7.0, containing 0.01 M *N*-ethylmaleimide, lysed by addition of 1% $NaDodSO_4$, incubated for 1 hr at 56°C, and analyzed by two-dimensional $NaDodSO_4$/PAGE. After electrophoresis, gels were processed as described above, except that after photography, gels were soaked in Enlightening (New England Nuclear) for 30 min, dried, and subjected to fluorography at −80°C.

RESULTS

Thrombocyte Preparation. The PBC fraction prepared by Ficoll/Hypaque flotation contains 50–70% thrombocytes, 20–40% lymphocytes, and 10% monocytes/granulocytes (25, 26), whereas the thrombocyte-enriched fraction prepared by adhesion to plastic (18) contains 90% thrombocytes and 10% monocytes/granulocytes. In preliminary studies, no significant differences were observed in the relative protein composition of the $NaDodSO_4$/PAGE profiles of the PBC fraction versus the thrombocyte-enriched fraction. This is not surprising in view of the modest enrichment for thrombocytes in the latter preparation. Therefore, we compared total chicken PBC to mammalian platelet preparations.

Indirect Immunofluorescence. In chicken PBC suspensions (Fig. 1), those cells that fit the morphologic criteria of thrombocytes (18, 25, 26) were the same cells that were labeled by antibodies specific for human platelet GPIIb and/or IIIa, such as the murine monoclonal antibody AP-2 (7) (Fig. 1 *A* and *B*), or a polyclonal rabbit antibody raised against a mixture of purified human platelet GPIIb and GPIIIa (Fig. 1 *C* and *D*). Under identical conditions, negative results were obtained with nonimmune murine monoclonal IgG (Fig. 1 *E* and *F*) or nonimmune rabbit IgG (not shown). Identical results were obtained in five separate experiments using several different sources of blood. Of interest is the fact that those cells designated "spindle cells" and previously thought

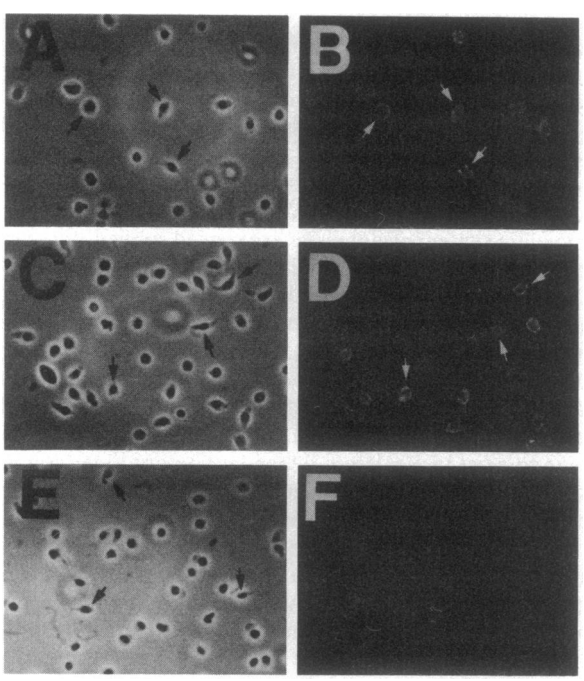

FIG. 1. Indirect immunofluorescence. Washed chicken PBC (10^7 cells) were treated with 100 μg of AP-2 (*A* and *B*), 1 mg of rabbit polyclonal anti-human platelet GPIIb and GPIIIa antibodies (*C* and *D*), or nonimmune murine monoclonal IgG (*E* and *F*) and then with the appropriate fluorescein isothiocyanate-conjugated second antibody. (*A*, *C*, and *E*) Phase-contrast images. (*B*, *D*, and *F*) Respective fluorescence images. Arrows in each panel indicate thrombocytes and can be used to orient the corresponding phase-contrast and fluorescence images.

to represent modified thrombocytes by various investigators (18, 25, 26) were consistently labeled by the antibodies specific for human platelet GPIIb and/or IIIa used in these studies.

Two-Dimensional NaDodSO$_4$/PAGE of Human Platelets: A Reference Point. The CBR-stained gel depicted in Fig. 2 shows the protein distribution typical of nonactivated human platelets. In this two-dimensional nonreduced–reduced gel system, proteins whose mobility decreases upon reduction are located above the diagonal, while proteins whose mobility increases upon reduction are located below the diagonal. The latter effect obviously can result from separation of disulfide-bonded subunits; the former effect is thought to result from increases in the effective size of proteins caused by reduction of intrachain disulfide bonds. Based upon several hundred such analyses and comparable studies with purified human platelet proteins, the positions of bands corresponding to several platelet proteins have been determined. These proteins, indicated in Fig. 2, include: actin, actin-binding protein, albumin, fibrinogen (Aα, Bβ, and γ_A subunits), myosin, the cytoskeletal component P235, thrombospondin, and selected major membrane glycoproteins that can be unambiguously identified in CBR-stained gels—namely, GPIb, GPIIb, and GPIIIa. Note that GPIb and GPIIb are both composed of disulfide-bonded larger α and smaller β subunits, each of which can be identified in CBR-stained gels. The apparent molecular weights of these platelet proteins, based upon their relative mobilities in this gel system, are listed in Table 1.

All of the major human platelet proteins that are surface-labeled with ^{125}I by the lactoperoxidase method of Philips and Poh Agin (20) were subsequently identified as GPs. Fig. 3 shows an autoradiograph of the typical human platelet GP profile that is derived by this method. In addition to those GPs already cited above, one can also detect major GPs designated Ia, IIa, and IIIb. The smaller β subunit of GPIIb is clearly visible. However, the β subunit of GPIb is poorly labeled by this method and usually not visible on such autoradiographs (20).

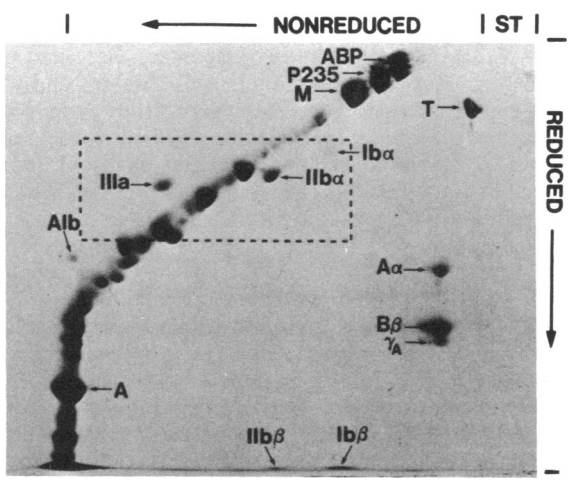

FIG. 2. Two-dimensional nonreduced–reduced NaDodSO$_4$/PAGE of human platelets. Two hundred micrograms of protein derived from ^{125}I-labeled human platelets was separated by NaDodSO$_4$/PAGE as described in the text. The gel was stained for protein with CBR. The direction of electrophoresis in both dimensions is indicated. ST represents the region encompassed by the stacking gel of the first dimension. The proteins indicated in the figure, as well as their apparent molecular weights, are listed in Table 1. A, actin; ABP, actin-binding protein; Alb, albumin; M, myosin; P235, cytoskeletal component P235; T, thrombospondin; Aα, Bβ, and γ_A, subunits of fibrinogen; Ibα, Ibβ, IIbβ, IIIa, GPs.

Table 1. Electrophoretic characteristics of selected human platelet proteins

Protein	Apparent $M_r \times 10^{-3}$* Nonreduced	Reduced
Thrombospondin	450	190
Fibrinogen	340	—
Subunit Aα	—	71
Bβ	—	58
γ_A	—	54
Actin-binding protein	260	260
Protein 235	235	235
Myosin (heavy chain)	200	200
GP		
Ib	170	—
Ibα	—	145
Ibβ	—	25
IIb	145	—
IIbα	—	130
IIbβ	—	20
IIIa	100	115
Albumin	65	74
Actin	45	45

*Calculated in the NaDodSO$_4$/PAGE system used in this study with reference to exogenous molecular weight standards.

Comparative GPIIb–IIIa Content of Platelets from Various Species. The two-dimensional gel sections containing GPIbα, GPIIbα, and GPIIIa derived from platelets of different species are shown in Fig. 4. It is readily apparent in these CBR-stained gels that structural analogs of GPIIb and GPIIIa can be identified unambiguously (arrows) in platelets from baboons, dogs, and rats and in thrombocytes from chickens. It should be pointed out that, in our hands, the presence of these GPs is a characteristic feature only of the platelet/thrombocyte lineage. By indirect immunofluorescence of intact cells with GP-specific monoclonal antibodies or by two-dimensional gel electrophoresis of metabolically labeled protein, structural analogs of these GPs have not been found in the murine plasma cell line P3x63Ag8.653, three cloned human T-lymphocyte cell lines (provided by David D. Eckels, Milwaukee, WI), or human peripheral blood monocytes.

When surface proteins of chicken thrombocytes were labeled with ^{125}I and analyzed in the two-dimensional gel system (Fig. 5), it was apparent that those proteins thought to be structurally analogous to GPIIb and GPIIIa were indeed exposed on the surface of thrombocytes and intensely labeled, relative to the remaining thrombocyte proteins. Al-

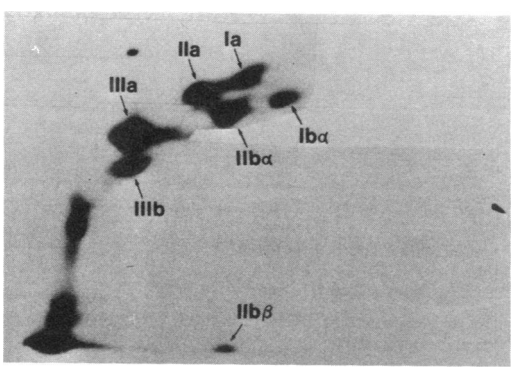

FIG. 3. Autoradiograph of NaDodSO$_4$/PAGE gel depicted in Fig. 1. Radiolabeled spots corresponding to major membrane GPs are indicated. Note that the β subunit of GPIb is not radiolabeled by this method.

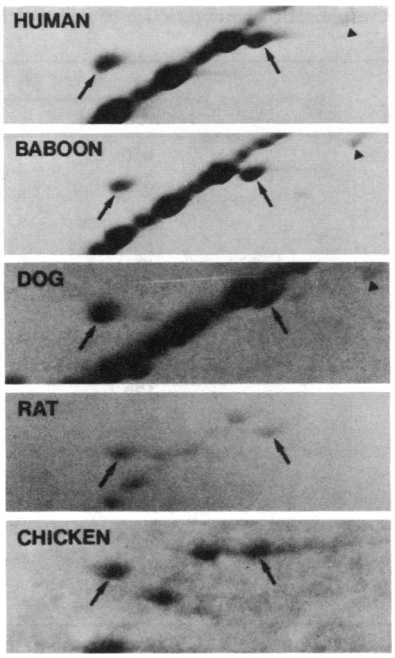

FIG. 4. NaDodSO$_4$/PAGE of platelet/thrombocyte proteins from various species. The region of the gel encompassing GPs Ibα, IIbα, and IIIa (see area surrounded by dashed lines in Fig. 1) is depicted in the figure for each species. Arrowhead, GPIbα; right arrow, GPIIbα; left arrow, GPIIIa. The grainy texture of the gel containing chicken proteins is due to the fact that this gel was photographed after it was dried onto filter paper. The remaining gels were photographed prior to drying.

though faint, a spot corresponding to the β subunit of GPIIb was also discernible in autoradiographs of chicken thrombocyte protein. The results depicted in Fig. 5 are typical of three independent experiments.

In mammalian platelets, the ratio of ^{125}I incorporated into GPIIbα to that incorporated into GPIIIa is $\approx$1:3 as determined by direct determination of the radioactivity of gel sections encompassing the respective protein spots. In the case of chicken thrombocytes, the ratio was found to be 3:1, indicating that chicken thrombocyte GPIIbα has a greater number of exposed iodinatable residues relative to GPIIIa or that there is an increased amount of GPIIbα relative to GPIIIa in chicken thrombocytes.

Synthesis of GPIIb–IIIa by Chicken Thrombocytes. After a

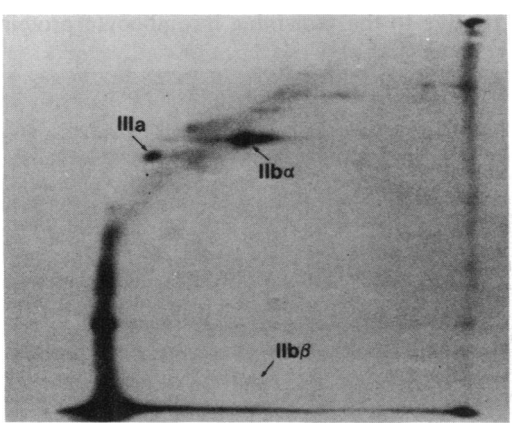

FIG. 5. NaDodSO$_4$/PAGE of ^{125}I-labeled chicken thrombocyte proteins. Peripheral blood thombocytes were labeled with ^{125}I prior to NaDodSO$_4$/PAGE. The resultant autoradiograph is shown. Those spots representing GPs IIbα, IIbβ, and IIIa are indicated.

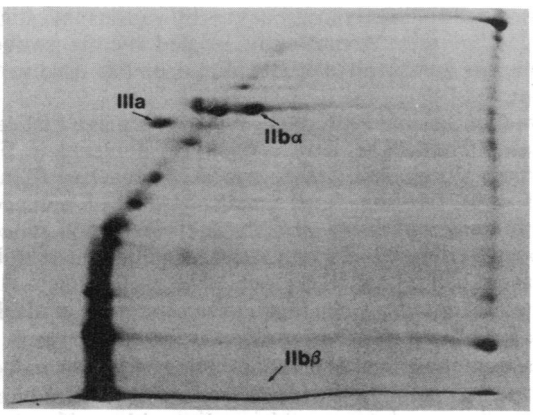

FIG. 6. NaDodSO$_4$/PAGE of metabolically labeled chicken thrombocyte proteins. Thrombocyte proteins were labeled by incubation of cells in the presence of [^{35}S]methionine prior to NaDodSO$_4$/PAGE. The resultant autoradiograph is shown. Those spots corresponding to GPs IIbα, IIbβ, and IIIa are indicated.

4-hr pulse-labeling with [^{35}S]methionine, a significant number of thrombocyte proteins had incorporated radiolabeled amino acid (Fig. 6). Prominent radiolabeled proteins included analogs of GPIIbα and GPIIIa. Once again, the β subunit of GPIIb was also discernible. The results depicted in Fig. 6 are typical of thrombocyte preparations radiolabeled on two separate occasions.

DISCUSSION

Like their mammalian counterpart, avian thrombocytes play an important role in hemostasis (25). The normal peripheral blood thrombocyte concentration (30,000–60,000 per mm^3) is lower than that of mammalian platelets, but unlike mammalian platelets, avian thrombocytes are nucleated cells similar in size to lymphocytes (26). They can be distinguished morphologically from monocytes and lymphocytes by electron microscopy and, in fresh preparations, by phase-contrast microscopy. Given differences in experimental conditions (e.g., the choice of anticoagulant or the method of thrombocyte enrichment) as well as the morphological and functional diversity of avian thrombocytes, there had been some controversy regarding their cell lineage and their relationship to lymphocytes. However, the more recent identification of thrombocyte-specific antigens not shared by lymphoid or erythroid cells is regarded as proof for the existence of a genuine thrombocyte system in chickens (18, 26). In addition, PBC selected for their positive reaction with antisera directed against these markers (i.e., thrombocytes) do not react with lymphocyte-specific probes, such as anti-Ig and anti-Ia or the lymphocyte-specific antibody anti-B1 and are not stimulated by lymphocyte mitogens such as pokeweed mitogen, concanavalin A, or phytohemagglutinin (26). In support of this hypothesis, the results of immunofluorescence assays using AP-2 and rabbit anti-human GPIIb and GPIIIa antibodies reported here provide unambiguous proof for the existence of a population of chicken PBC that express these thrombocyte/platelet-specific markers.

In this report, we have applied biochemical analyses routinely used for the characterization of mammalian platelet GPs to the study of avian nucleated thrombocytes. Not unexpectedly, the protein composition of these avian PBC bears a striking resemblance to that of their mammalian counterparts. Both contain GPIIb and GPIIIa, which are known to comprise the human platelet receptor complex that binds to fibrinogen and mediates platelet cohesion. Nurden *et al.* (27) previously demonstrated that GPIIb and GPIIIa are

highly conserved, from a structural standpoint, within numerous mammalian species. Our findings confirm and extend the observation of Nurden *et al.* (27), showing that GPIIb and GPIIIa analogs can also be distinguished in nonmammalian vertebrates—e.g., birds. One implication of these findings is that the protein receptor complex, which is functionally the most characteristic component of human platelets—i.e., GPIIb–IIIa, has withstood over 200 million years of natural selection. These results are consistent with the contention that natural selection is the driving force for gene sequence fixation and that functionally analogous polypeptides demonstrate markedly restrained sequence divergence (28).

Several investigators have searched for actively translating mRNA in human platelets (29, 30), but these studies clearly showed that human platelets contain only vestigial remnants of megakaryocyte RNA that do not produce detectable translation products. Recently, Burckhardt *et al.* (31) and Gogstad *et al.* (32) reported that human monocytes express surface proteins immunologically crossreactive with human platelet GPIIb–IIIa. As noted in the results of this paper, we have found that human monocytes do not synthesize analogs of GPIIb or GPIIIa, thus confirming the observation of Levene and Rabellino (33) that cultured human monocytes can passively absorb but do not synthesize either of these GPs.

More recently, a number of investigators have been more successful in the isolation of mRNA species that encode human platelet GPIIb and GPIIIa. Silver *et al.* (34) showed that the gene for GPIIIa is expressed after induction of K562 cells with the phorbol ester, TPA (phorbol 12-myristate 13-acetate), since *in vitro* mRNA translation resulted in the synthesis of an M_r 85,000 polypeptide that reacts with a polyclonal rabbit anti-human GPIIIa antibody. In a preliminary report, Bray *et al.* (35) isolated mRNA from the human cell line HEL and were able to translate precursors of both GPIIb and GPIIIa *in vitro*. Moreover, in the presence of dog pancreas microsomes, signal sequence removal and core glycosylation of GPIIb was demonstrated. In addition to the preceding studies using transformed cell lines, Rabellino *et al.* (36), Thiagarajan *et al.* (37), and Gastineau *et al.* (38) have reported preliminary results indicating synthesis of GPIIb and GPIIIa by human megakaryocytes *in vitro*. Cell-free translation of megakaryocyte mRNA has not yet been accomplished, probably because the isolation of adequate numbers of megakaryocytes for this purpose is prohibitive.

The discovery that chicken peripheral blood thrombocytes synthesize numerous proteins structurally analogous to human platelet proteins, including GPIIb and GPIIIa, opens yet another avenue to the study of the molecular biology of platelet/thrombocyte protein receptors. Studies are currently in progress to isolate polyadenylylated mRNA from thrombocytes and to establish a thrombocyte cDNA library, with the ultimate goal of isolating probes specific for the genes encoding thrombocyte/platelet GPIIb and GPIIIa.

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