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Structural and functional characterization of major platelet membrane components derived by limited proteolysis of glycoprotein IIIa

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The authors isolated a product of proteolytic degradation of glycoprotein IIIa (GPIIIa) which is formed on the surface of human platelets during incubation with chymotrypsin and which was previously described as the 66 kDa platelet membrane component. This component migrated with an apparent M_r 62400 in a non-reduced system of sodium dodecyl sulfate polyacrylamide gel electrophoresis. In a reduced system it yielded two major subunits migrating with apparent M_r 14000–17000 and 65000. The low-molecular weight component began with the NH_2-terminal sequence of GPIIIa (GPNICTTR...) and the larger component with residue 348 of GPIIIa (GKIRSKKA...) as deduced from a cDNA clone of this glycoprotein. The two subunits appeared to be linked by one or more S-S bridges supporting the contention that GPIIIa is a highly folded molecule on the platelet membrane. In contrast to GPIIIa, the '66 kDa component' did not bind to GRGDSPK-agarose, to fibrinogen-agarose nor to insolubilized monoclonal antibody recognizing the GPIIb/IIIa complex. The exposure of fibrinogen receptors during the course of incubation of platelets with chymotrypsin preceded the formation of the '66 kDa component' characterized in this study. An intermediate product of GPIIIa proteolysis migrating with an apparent M_r 120000 in a non-reduced system and M_r 80000 in a reduced system was identified as a precursor of the '66 kDa component'. The '120 kDa component' was not retained on GRGDSPK-agarose or on fibrinogen-agarose but it was retained on insolubilized antibody recognizing the GPIIb/IIIa complex. Incubation of platelets with porcine pancreatic elastase or human granulocytic elastase resulted in the formation of similar proteolytic degradation fragments.

Introduction

It is well established that the binding of fibrinogen to specific receptors is essential for platelet aggregation. These receptors are associated with the glycoprotein IIb/IIIa (GPIIb/IIIa) complex [1,2]. Fibrinogen receptors remain cryptic on resting platelets and therefore unstimulated platelets do not bind fibrinogen and do not aggregate in the circulation. When platelets are stimulated by physiological agonists such as ADP [3–5] or thrombin [6,7], fibrinogen receptors become exposed on the platelet surface, resulting in subsequent fibrinogen binding and platelet aggregation. Incubation of platelet suspensions with proteolytic enzymes such as chymotrypsin, pronase, porcine pancreatic elastase and human granulocytic elastase [8–12] also can expose fibrinogen binding sites on the platelet surface.

In 1983, we described the appearance of a component on the surface of chymotrypsin or pronase treated platelets migrating with an apparent M_r of 60000 in a non-reduced system and 66000 in a reduced system [13]. This component, radiolabeled with ^{125}I, was precipitated by several anti-platelet membrane polyclonal antibodies. As demonstrated by immunoprecipitation and immunoblotting techniques, the PLA_1 alloantigen was found to reside on this '66 kDa component' providing evidence that it was derived from GPIIIa [14]. On the basis of preliminary data we suggested that this component may play a role in fibrinogen binding and

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fibrinogen-induced platelet aggregation [13]. McGregor et al. [15] presented data supporting this suggestion while Peerschke and Coller [16] reported that in the course of chymotrypsin digestion of human platelet suspension, the exposure of fibrinogen receptors preceded the formation of the 66 kDa component.

In the present study we isolated the '66 kDa component'. We studied the time course of its formation from GPIIIa and its structural, immunological and functional properties. Finally, we attempted to align the NH_2-terminal sequences of the '66 kDa component' with the GPIIIa sequence deduced from a cDNA clone [17,18]. We also identified an intermediate product of GPIIIa proteolysis migrating with an apparent M_r 120000 in a non-reduced system and M_r 80000 in a reduced system. This study was presented in part during the 30th Annual Meeting of the American Society of Hematology [19].

Materials and Methods

Reagents. Human granulocyte elastase [12] and porcine pancreatic elastase [11] were purified as previously described. Some preparations of human granulocyte elastase, prepared from human purulent sputum, were purchased from Elastin Products Co, Inc. (Pacific, MO). Elastase preparations showed a single protein band on SDS-polyacrylamide gel electrophoresis migrating with an apparent molecular weight of 25000. Highly purified human alpha thrombin was supplied by Dr. J.W. Fenton III (Albany, NY). Human fibrinogen was purchased from Kabi, (Stockholm, Sweden). The peptides RGDS and GRGDSPK were provided by Peninsula Laboratory, Inc. (Belmont, CA). The purity of peptides was higher than 95% as evaluated by thin-layer chromatography, electrophoresis and amino acid analysis. Chymotrypsin (grade IS) and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Collection of blood. Blood was obtained from healthy individuals with the approval of the Institutional Human Experimentation Committee at Temple University School of Medicine, Philadelphia, PA.

Human washed platelets. Platelets from freshly collected blood in the anticoagulant acid citrate-dextrose were washed in the presence of apyrase and heparin by the method of Mustard et al. [20]. Preparations of enzymatically-treated platelets were obtained as described previously [9,12,13]. For preparative purposes, we used outdated platelet concentrates supplied by the American Red Cross, Philadelphia, PA. These platelet suspensions were washed with a solution containing 9 parts of 0.15 M NaCl and one part of 3.8% trisodium citrate (pH 6.5) and then with Tyrode's solution (pH 7.35).

Platelet aggregation. Platelet aggregation was studied using a Payton aggregometer (Scarborough, Ont, Canada). The results were expressed in arbitrary light transmission units as described in our previous publications. Platelets were counted electronically (Coulter Channelyzer, Hialeah, FL).

Fibrinogen binding to platelets. The labelling of fibrinogen with ^{125}I and measurements of ^{125}I-fibrinogen binding to platelets were studied as previously described with minor modifications [9,12]. The number of fibrinogen binding sites and fibrinogen binding constants were calculated by means of Scatchard analysis as described [12]. Since high concentrations of fibrinogen were used in these experiments, the number of high affinity sites was not determined.

Platelet membrane glycoproteins. Preparations of GPIIb, GPIIIa and the '66 kDa component' were obtained from outdated platelet suspensions. A portion of the platelet suspension was left intact while the remainder was incubated with chymotrypsin (100 $\mu g/10^9$ platelets) for 45 min at 37°C in order to generate the GPIIIa proteolytic products on the platelet surface. Then, chymotrypsin was inhibited with phenylmethylsulfonyl fluoride (PMSF) and soy bean trypsin inhibitor as described previously [9]. Finally, platelets were washed and resuspended in Tyrode albumin-free buffer. Two methods were used for the purification of GPIIb/GPIIIa and the '66 kDa component'. In the first method, the GPIIb/IIIa complex and the '66 kDa component' were extracted from platelet membrane suspensions [21] by phase separation in the presence of 1% Triton X-114 at 37°C [22] and further isolated by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [23]. Alternatively, the GPIIb/IIIa and its degradation products were isolated by the modified method of Fitzgerald et al. [24]. Platelets solubilized in 1% Triton X-100 were adsorbed on Concanavalin A-agarose; material containing GPIIb/IIIa and its degradation products was eluted with 100 mM methyl mannoside and further chromatographed on a HPLC size exclusion column (TSK G4000, LKB) equilibrated with a 6 mM octyl glucoside, 100 mM Tris/100 mM NaCl buffer (pH 7.25). In some experiments, material containing GPIIb/IIIa and the '66 kDa component' was adsorbed from detergent solubilized platelets on CNBr-Sepharose coupled to a monoclonal anti-GPIIIa antibody (SSA6) and eluted from this column [25,26]. The immunopurified components were subsequently separated by preparative SDS-polyacrylamide gel electrophoresis. We did not observe the formation of GPIIIa dimers either in platelet lysates or in purified preparations. A partially purified preparation of GPIIIa and the '120 kDa component' was obtained from chymotrypsin-treated platelets, solubilized with Triton X-100, by means of concanavalin A-agarose chromatography, followed by preparative SDS-polyacrylamide gel electrophoresis.

Polyclonal and monoclonal antisera. Anti-GPIIb, anti-66 kDa component and anti-GPIIIa antisera were raised in rabbits injected with 50 μg of antigen emulsified in complete Freund's adjuvant per animal. The same dose of antigen, suspended in incomplete Freund's adjuvant, was given during booster injections made every two weeks until a high titer of antibody was detected by enzyme linked immunoassay. The purity of the antigen before injection was evaluated by SDS-polyacrylamide gel electrophoresis. The murine monoclonal antibodies AP2 [27] and A2A6 [26] were donated by Dr. T. Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee, WI) and by Dr. J.S. Bennett (University of Pennsylvania, Philadelphia, PA), respectively. These antibodies were characterized previously and shown to inhibit platelet aggregation and react with GPIIb/IIIa. AP3 [28] and SSA6 [25] were kindly provided by Dr. P. Newman (Milwaukee, WI) and by Dr. J.S. Bennett (Philadelphia, PA), respectively. The purity and titer of these antibodies were characterized by enzyme-linked immunoassay [29], radioimmunoassay [30] and immunoblotting [31].

Absorption of GPIIb and GPIIIa on insolubilized antibodies. The monoclonal antibodies SSA6 (anti-GPIIIa) and AP3 (anti-GPIIIa) were each coupled to CNBr-agarose. Platelet lysate or Con A-agarose eluate from intact or chymotrypsin-treated platelets was applied to these columns equilibrated with a buffer containing 100 mM Tris-HCl, 10 μM leupeptin and 1 μM PMSF (pH 7.4). Elution was accomplished using a buffer containing 0.05 M diethylamine at pH 11.5 [26]. The eluate was neutralized immediately to pH 7.4 with Tris-HCl buffer containing 1% Triton X-100.

Adsorption of GPIIb and GPIIIa on insolubilized GRGDSPK. Chymotrypsin-treated or intact platelets were lysed in a phosphate saline buffer (pH 7.3) containing 3 mM PMSF and 50 mM octyl glucoside. After incubation on ice for 1 h, the platelet lysate was centrifuged at $100000 \times g$ for 15 min and the cytoskeleton pellet discarded. Platelet lysate or eluate from Con A-agarose was applied on a GRGDSPK-agarose column equilibrated with 25 mM octyl glucoside and 1 mM PMSF. Elution of the GPIIb/IIIa complex was accomplished by a modified method of Pytela et al. [32] using a buffer containing 25 mM octyl glucoside and 1 mg/ml RGDS.

Adsorption of GPIIb / IIIa on insolubilized fibrinogen. Platelets were lysed in a buffer composed of 20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 10 μM leupeptin and 60 mM octyl glucoside (pH 7.4). A fibrinogen-Sepharose column was equilibrated with a buffer containing 20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 6 mM octyl glucoside, 10 μM leupeptin and 1 μM PMSF. Material adsorbed on fibrinogen-agarose was eluted with the same buffer supplemented with 0.5 M NaCl.

Protein determination was carried out in most cases by the method of Bradford [33] after adjusting concentrations of the Triton X-100 or SDS below 0.1%.

SDS-polyacrylamide gel electrophoresis and Western blotting. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [34] using a 5% polyacrylamide stacking gel and either a 7.5%, 10% or 12.5% polyacrylamide running gel. Samples were reduced by heating for 2 min at 100 °C with 2% SDS and 1.5% β-mercaptoethanol. Molecular weight standards of myosin (200 K), β-galactosidase (116 K), phosphorylase B (97.4 K), bovine serum albumin (66 K), and ovalbumin (42 K) were run on each gel for molecular weight calculation of unknown samples. Completed gels were stained either with Coomassie brilliant blue or silver reagent [35] or transferred to nitrocellulose paper for staining with specific antibodies by the method of Towbin et al. [31]. The sensitivity of the Western blots was approx. 20–50 ng of protein per sample. The nonreduced '66 kDa component' band was excised from 10% polyacrylamide preparative gels and subjected to electroelution. A portion of the purified non-reduced 66 kDa preparation was saved for amino acid sequencing while the remainder was reduced into its components for subsequent sequencing. Reduction was accomplished by the addition of 5 mM dithiothreitol and boiling at 100 °C for 2 min. The subunits of the '66 kDa component' were separated on a Superose 6 size exclusion HPLC column using an SDS buffer supplemented with 5 mM DTT to maintain reducing conditions. Each subunit was processed for amino acid sequencing as described below.

Amino acid sequence. The sodium dodecyl sulfate and other reagents which contaminated the proteins eluted from gels or the HPLC column were eliminated by precipitating protein with methanol according to Wessel and Flügge [36]. The procedure was repeated twice and the precipitate resuspended in undiluted trifluoroacetic acid, and transferred quantitatively to a glass fiber sample support of the sequencer. The sample was then subjected to NH_2-terminal sequencing performed on a gas phase sequencer (Applied Biosystems Model 470) with an on line PTH analyzer (Applied Biosystems Model 120). Standard protocols from manufacturers were followed with regard to Edman degradation and separation of PTH amino acids by high performance liquid chromatography.

Results

1. Immunological and structural characterization of the purified '66 kDa platelet membrane component'

Highly purified preparations of the '66 kDa component' were obtained from chymotrypsin-treated platelets. All methods of purification followed by preparative SDS-polyacrylamide gel electrophoresis yielded preparations of adequate purity; typical recovery was about

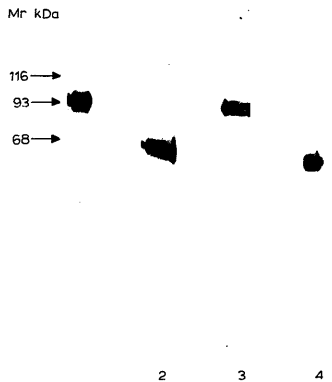

Fig. 1. Immunological crossreactivity between GPIIIa and the '66 kDa component'. Purified GPIIIa (lanes 1,3) and purified '66 kDa component' (lanes 2,4) were subjected to SDS-polyacrylamide gel electrophoresis in a non-reduced system (10.0% gel); the nitrocellulose transfers were stained with monoclonal antibody SSA6 diluted 1:1000 (1.8 mg IgG/ml) (lanes 1, 2) or with polyclonal antibody anti-66 kDa (dilution of serum 1:1000) (lanes 3, 4). The molecular weight standards are indicated with arrows.

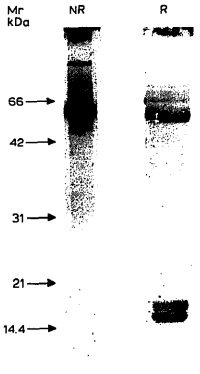

Fig. 2. SDS-polyacrylamide gel electrophoresis of immunoaffinity purified '66 kDa component' in a reduced and in a non-reduced system. The electrophoresis was performed in 12.5% gels according to Laemmli [30]. Gels were stained with silver. Lane 1: non-reduced protein (one major band); lane 2: reduced protein showing a high-molecular weight (approx. 65000) and a low-molecular weight subunit migrating as two bands with apparent molecular weights of 14000 and 17000. The molecular weight standards are indicated with arrows. In three experiments the average apparent molecular weight of the '66 kDa component' in a non-reduced system was 62400 and in a reduced system the apparent molecular weight of the high-molecular weight subunit was 65100. The multiple bands (lane 1) migrating more slowly than the '66 kDa component' constituted less than 10% of the total protein.

5–10%. Fig. 1 demonstrates immunological crossreactivity between GPIIIa and the '66 kDa component' in a Western blot (non-reduced system) using both a monoclonal-antibody (SSA6) and a polyclonal antibody raised in rabbits against purified '66 kDa component'. Monoclonal anti-GPIIIa antibody obtained from another laboratory (AP3) also recognized the non-reduced '66 kDa component'. Reduced '66 kDa component' showed very low crossreactivity with polyclonal antibodies and no reactivity with the monoclonal antibodies and no reactivity with the monoclonal antibodies (AP3 and SSA6) using the Western blot technique (data not shown). Studies using radioimmunoassay [30] and enzyme-linked immunoassay (data not shown) confirmed the complete immunological crossreactivity between GPIIIa and the non-reduced '66 kDa component' using these antibodies.

To compare reduced and non-reduced preparations of the '66 kDa component', we used SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 2). In agreement with our previous studies using autoradiography of radiolabeled membrane glycoproteins [13], we found that the isolated preparation migrated with an apparent molecular weight of 62.4 kDa in a non-reduced system and 65.1 kDa in a reduced system. The

TABLE I

NH_2-terminal sequences in the 66 kDa component

Position at which residue is not determined or quantitated is identified by an *X*. Glycine could not be quantitated due to the presence of residual glycine from the electroelution buffers, despite two precipitations with methanol. Cysteine was not detected and the recovery of serine and threonine was low, as expected after Edman degradation. Three independent preparations were sequenced.

G^1 *X*	G^{348} *X*
P 234 pmoles	K 230 pmoles
N 266	I 262
I 213	R 156
C *X*	S 150
T 70	K 181
T 90	V 185
R 121	E 235
G *X*	L 231
V 160	E 198

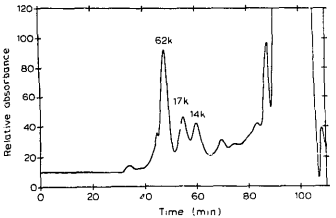

Fig. 3. Separation of the 66 kDa subunits by means of size exclusion HPLC. A Superose 6 size exclusion HPLC column, using a buffer maintaining reducing conditions adequately resolved the 65 kDa, 17 kDa and 14 kDa subunits of the reduced '66 kDa component'. Absorbance was monitored at 206 nm. Fractions collected after 60 min contained no proteins detected by SDS-polyacrylamide gel electrophoresis. Apparent molecular weights were assigned after SDS-PAGE and silver staining.

reduced preparation yielded a heterogenous low molecular weight fragment migrating as two bands with apparent molecular weight of 14000 and 17000. These fragments were absent in the non-reduced gel. Edman degradation analysis of non-reduced preparations of the '66 kDa component' revealed two NH_2 terminal sequences. The first sequence corresponded to the NH_2-terminal sequence of GPIIIa, the second one began at residue 348 of GPIIIa as deduced from a cDNA clone [17,18]. The two sequences occurred in approximately equal proportions (Table I). Size exclusion HPLC on a Superose 6 column adequately distinguished the subunits of the reduced 66 kDa component (Fig. 3). Sequence analysis of each major component revealed that the larger 65 kDa subunit began at the 348th residue of GPIIIa (Table II). The smaller 14 kDa subunit contains the NH_2 terminal of GP IIa and should contain about 120 amino acids as suggested by its apparent molecular weight. These data correlate highly with that obtained from non-reduced preparations. The 17 kDa band was not sequenced. Since the non-reduced preparations revealed only two NH_2 terminal sequences, we assume that the 17 kDa component also may represent an NH_2-terminal fragment of GPIIIa retaining additional C-terminal amino acids which are deleted from the 14 kDa band.

2. Formation of the '66 kDa component' and its putative precursor on the platelet membranes in relation to the exposure of fibrinogen receptors

Western blots with a polyclonal antibody against the '66 kDa component' (Fig. 4) show that this component does not appear following platelet stimulation with ADP or thrombin but it does appear following incubation with enzymes exposing fibrinogen receptors: human granulocytic elastase, chymotrypsin and porcine pancreatic elastase. Incubation of platelets with these enzymes also resulted in the formation of another immunoreactive component migrating with an apparent molecular weight of 120 kDa in a non-reduced system. Similar results were obtained using anti-GPIIIa monoclonal antibodies (SSA6 and AP3) in Western blot experiments (data not shown). Fig. 5 shows the effect of incubation time of human platelets with chymotrypsin on the formation of the '120 kDa component' and '66 kDa component' along with the exposure of fibrinogen

TABLE II

Alignment of NH_2-terminal of the fragments of the 66 kDa component and GPIIIa sequence deduced from cDNA clone

X identifies a position at which the residue was not determined or quantitated. Glycine could not be quantitated due to residual glycine from the electroelution buffers and cysteine was not detected.

	1	5	10
GPIIIa	G P N I C T T R G V . . .		
14 kDa	X P N I X T T R X V		
	345	350	355
GPIIIa	D A Y G K I R S K V E . . .		
65 kDa	X K I R S K V E . . .		

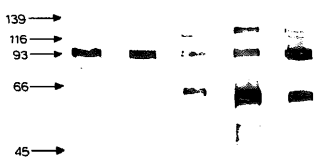

Fig. 4. Detection of the '66 kDa component' on human platelets stimulated with ADP or proteolytic enzymes. Suspensions of human platelets were treated with 10 μM ADP for 5 min, thrombin (0.5 units/ml; for 5 min), human granulocyte elastase (20 μg/10^9 platelets at 37°C for 45 min), chymotrypsin (100 μg/10^9 platelets at 37°C for 45 min) or porcine pancreatic elastase (20 units/10^9 platelets at 37°C for 45 min), in lanes 1–5, respectively. After incubation with ADP, thrombin or enzymes, platelet pellets were separated by centrifugation, solubilized in electrophoresis sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The figure represents nitrocellulose transfers stained with anti-66 kDa antibody diluted 1:1000. Molecular weight markers are indicated by arrows.

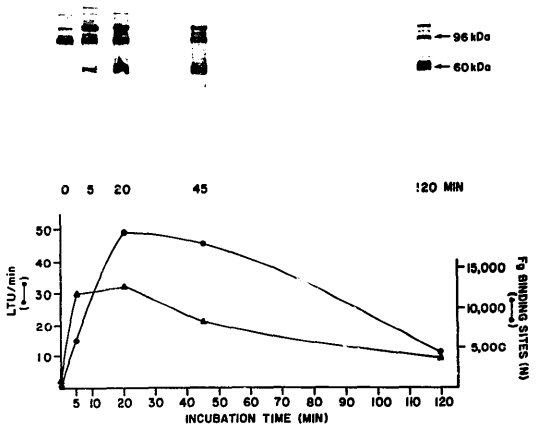

Fig. 5. Correlation between proteolytic degradation of GPIIIa, exposure of fibrinogen receptors and spontaneous platelet aggregation upon the addition of fibrinogen during a time course of platelet incubation with chymotrypsin. A suspension of washed human platelets was incubated with chymotrypsin (100 $\mu g/10^9$ platelets) and the incubation was stopped at various time intervals by the addition of PMSF and soy bean trypsin inhibitor [9,12]. Aliquots of platelet suspensions were centrifuged and the pellets were subjected to SDS-polyacrylamide gel electrophoresis (10% gels) and analyzed by means of Western blotting with anti-66 kDa antibody diluted 1:1000. Another aliquot of platelets was washed once and resuspended in Tyrode-albumin buffer. Aggregation was studied by the addition of 50 μl 0.1% fibrinogen per 450 μl platelet suspension ($3 \cdot 10^8$ platelets per ml) and expressed in arbitrary light transmission units (LTU). Binding of ^{125}I-fibrinogen was studied as described in Materials and Methods and analyzed by the method of Scatchard. The affinity constants of fibrinogen binding to platelets remained unchanged during the entire process of proteolytic degradation. The K_d values were between $2 \cdot 10^{-7}$ M and $4 \cdot 10^{-7}$ M. The number of fibrinogen binding sites on ADP (50 μM) stimulated platelets before proteolysis amounted to 34200 ± 13900 ($K_d = 4{,}2 \cdot 10^{-7}$ M). Titration by means of immunoblotting demonstrated that the percentages of the '66 kDa component' after 5, 20 and 40 min of digestion corresponded to 5%, 13% and 25% of the total GPIIIa antigen.

receptors and the resultant spontaneous aggregation of platelets upon the addition of fibrinogen.

In 10 experiments the number of fibrinogen binding sites on ADP-stimulated platelets amounted to 34200 $\pm$ 13900 sites per platelet (mean $\pm$ S.D.). The number of fibrinogen binding sites on platelets incubated with chymotrypsin for 20 min corresponded to $10700 \pm$ 6400 sites per platelet and the number of binding sites on chymotrypsin-treated platelets stimulated further with ADP (50 μM) amounted to 18600 ± 11800 sites per platelet. The K_d values in all experiments were $(2\text{–}4) \cdot 10^{-7}$ M.

We found that the formation of the '120 kDa component' seen after 5–20 min correlated better with the exposure of fibrinogen receptors than did the formation of the '66 kDa component'. The '66 kDa component' accumulated maximally during the later stages of enzymatic digestion after 120 min when the platelets were much less sensitive to fibrinogen addition. The '120 kDa component' band was most intense during the early stages of digestion; it decreased after 20 min suggesting that this component may represent an intermediate product of GPIIIa proteolysis. According, the '120 kDa component' migrated with an apparent molecular weight of 80 kDa in a reduced system (Fig. 6) of SDS-polyacrylamide gel electrophoresis.

3. Functional characteristics of the '66 kDa component' and its putative precursor

In order to assess the functional characteristics of the '66 kDa component', we compared the adsorption of this protein and that of GPIIIa on insolubilized fibrinogen, insolubilized GRGDSPK and insolubilized monoclonal (AP2) anti-GPIIb/IIIa antibody (Table III). This antibody recognizes an epitope specific for the GPIIb/IIIa complex and blocks fibrinogen binding to platelets. GPIIb (not shown) and GPIIIa were adsorbed on all columns. Experiments presented in Figs. 7 and 8

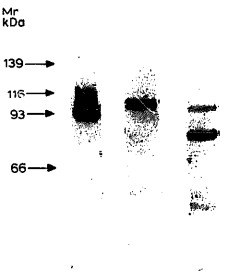

Fig. 6. Immunoblots of GPIIIa and the intermediate product of its proteolysis. Lane 1: GPIIIa (0.4 μg), non-reduced system; lane 2: GPIIIa (0.8 μg), reduced system; lane 3: mixture of partially purified GPIIIa and '120 kDa component' (1.5 μg) in a reduced system. Proteins were reduced by boiling in 1.5% β-mercaptoethanol (v/v) for 2 min. All lanes were incubated with rabbit anti-human 66 kDa protein antiserum (1:1000 dilution). Molecular weight markers are indicated by solid arrows.

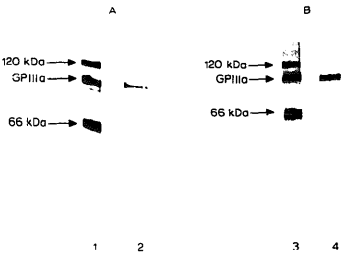

Fig. 7. Characterization of GPIIIa, the '66 kDa component', and the '120 kDa component' in detergent solubilized chymotrypsin-treated platelets by means of adsorption on fibrinogen-agarose or GRGDSPK-agarose. Chymotrypsin-treated platelets were lysed in octyl glucoside buffer, applied on columns and eluted as described in Materials and Methods. Material obtained from $2 \cdot 10^{11}$ platelets was applied on a 3.5 ml column of fibrinogen-agarose (A) material obtained from $5 \cdot 10^{10}$ platelets was applied on a 1.0 ml column of GRGDSPK-agarose (B). The eluates were subjected to SDS-polyacrylamide gel electrophoresis in a non-reduced system (10%); the nitrocellulose transfers were stained with polyclonal antibody anti-66 kDa in the dilution 1:1000. Lanes 1 and 3 correspond to the material applied onto the columns or not bound to the columns; lanes 2 and 4 correspond to material specifically eluted from the columns. The band migrating with an apparent molecular weight of 120000 likely represents an early degradation product of GPIIIa (see text).

show that in contrast to GPIIIa, the '66 kDa component' was not adsorbed on any of these columns. Moreover, experiments in which various concentrations of material eluted from columns were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting showed that less than 1% of 'the 66 kDa component' and at least 50% of the GPIIIa were retained on GRGDSPK-column. Control experiments (Fig. 8) show adsorption of the '66 kDa component' on a column containing insolubilized monoclonal antibody directed against GPIIIa epitopes (SSA6). The '120 kDa component' was not retained on GRGDSPK-agarose or on fibrinogen-agarose but it was retained on AP2-agarose and on SSA6-agarose (Figs. 7 and 8). The results of Figs. 7 and 8 are presented in Table III.

TABLE III

Adsorption of GPIIIa and its proteolytic fragments on various affinity columns

AP2 is a monoclonal antibody reacting with a specific epitope of the GPIIb/IIIa complex. SSA6 is a monoclonal antibody specific for purified GPIIIa.

	GPIIIa	120 kDa	66 kDa
Fbg-agarose	+	−	−
GRGDSPK-agarose	+	−	−
AP2-agarose	+	+	−
SSA6-agarose	+	+	+

These data suggest that the '66 kDa component' is not involved in fibrinogen binding to platelets. Consistent with this observation, a polyclonal antibody raised against the '66 kDa component' did not inhibit fibrinogen binding to ADP-stimulated or to chymotrypsin-treated platelets (data not shown).

Discussion

In conclusion, our data suggest that the '66 kDa component' is composed of a short NH_2-terminal fragment of GPIIIa and a large C-terminal fragment of this molecule. This is consistent with the work of Beer and Coller [37] who demonstrated that the '66 kDa component' is recognized by a polyclonal antibody directed against a synthetic NH_2-terminal peptide of GPIIIa [1–13]. Furthermore, the '66 kDa component' also is immunoreactive with a polyclonal antibody against a synthetic C-terminal peptide of GPIIIa (D'Souza and Plow, personal communication). Both fragments appear to be linked by one or more S-S bridges. The putative structure of the '66 kDa component' is compatible with

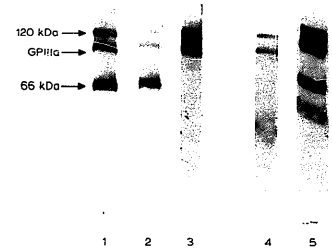

Fig. 8. Characterization of GPIIIa and the '66 kDa component' and the '120 kDa component' adsorption on insolubilized monoclonal antibodies recognizing in the GPIIb/IIIa complex or GPIIIa. Chymotrypsin-treated platelets were solubilized in detergent and fractionated by means of a Con A-agarose column as described in Materials and Methods. Material eluted from the Con A-agarose column by means of 6 mM octyl glucoside and 100 mM methyl mannoside was applied on an AP2-agarose column or SSA6-agarose column. The eluate from the Con A-agarose column and the eluates from immunoaffinity columns were subjected to SDS-polyacrylamide gel electrophoresis (10% gels) and the nitrocellulose transfers were stained with the 'anti-66 kDa component' antiserum diluted 1:1000. Lane 1: original material eluted from Con A-agarose column. Lane 2: material not adsorbed on AP2-agarose. Lane 3: material adsorbed on AP2-agarose and eluted with 0.05 M diethylamine (pH 11.5). Lane 4: material not adsorbed on SSA6-agarose. Lane 5: material adsorbed on SSA6-agarose and eluted with 0.05 M diethylamine (pH 11.5).

a model of the GPIIb/IIIa complex recently proposed by Phillips et al. [2] on the basis of electron microscopic studies previously published by this group of investigators [38]. The linkage of a short NH_2-terminal fragment of GPIIIa to a rather remote portion of this molecule

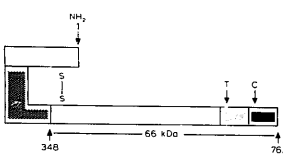

Fig. 9. Putative structure of GPIIIa and its 66 kDa domain. It is proposed that the '66 kDa component' is comprised of a light subunit near the N- terminal (white area) and a heavy subunit near the C-terminal containing an extracellular region (white area), the transmembrane region (light stippled area) and the cytoplasmic region (solid bar). The dark stippled area represents the region deleted from GPIIIa in the formation of 66 kDa. It is unknown how many S-S bridges are involved in maintaining the structure of the '66 kDa component'.

suggests that this protein is highly folded on the platelet surface (Fig. 9). The essential chymotryptic cleavage of GPIIIa resulting in the formation of the '66 kDa component' is between Tyr-347 and Gly-348. Proteolysis of GPIIIa on the platelet surface with chymotrypsin, human granulocyte elastase, porcine pancreatic elastase (Fig. 4), pronase [10] or trypsin (unpublished data) results in the formation of a major degradation product which crossreacts immunologically with GPIIIa and migrates with an apparent molecular weight of 60000 in a non-reduced system of SDS-polyacrylamide gel electrophoresis. Thus, it is conceivable that this region of GPIIIa is exposed on the platelet surface to the action of proteolytic enzymes with specificities different than chymotrypsin.

On the basis of N-terminal sequencing and SDS-polyacrylamide gel electrophoresis, we estimate that the proteolytic conversion of GPIIIa to the '66 kDa component' results in the deletion of a domain which spans from the 130th (approximately) to the 347th amino acid of the NH_2-terminal of the molecule. We assume that RGD binding sites are deleted from the 66 kDa molecule. Most recently, two groups of investigators attempted to localize, by means of chemical or photoaffinity crosslinking, an RGD recognition site on the GPIIIa molecule isolated either from platelets [39] or human placenta [40]. According to D'Souza et al. [39], this region corresponds to residues 109–171 of platelet GPIIIa. On the other hand, Smith and Cheresh [40] provided evidence that amino acid residues 61–203 are proximal to the RGD binding domain of the vitronectin receptor (placental GPIIIa). If the molecular weight of the low molecular weight subunit of the 66 kDa is correct, this would further narrow the crosslinking site, but would not move it from the regions identified by chemical or photoaffinity crosslinking.

Our data suggest that the component migrating with an apparent molecular weight of 120000 in a non-reduced system and 80000 in a reduced system (Fig. 6) is an intermediate product in the conversion of the GPIIIa to the '66 kDa component' (Fig. 5). This is consistent with the work of Beer and Coller [37] who reported the formation of a 110 kDa component during the early stages of chymotryptic proteolysis and a 66 kDa component after longer periods of digestion. The appearance of the '120 kDa component' correlated well with the exposure of fibrinogen binding sites in the course of proteolytic digestion. However, in the presence of GPIIb the 120 kDa component was adsorbed on insolubilized antibody directed against the GPIIb/IIIa complex but not on GRGDSPK-agarose or fibrinogen-agarose (Figs. 7 and 8). For this reason, we consider that the RGD binding site is not localized on this component. We propose that the '120 kDa component' may represent an unfolded conformation of the GPIIIa molecule formed during the early stages of proteolytic digestion.

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