

$\alpha_{IIb}\beta_3$ Integrin Dissociation Induced by EDTA Results in Morphological Changes of the Platelet Surface-connected Canalicular System with Differential Location of the Two Separate Subunits

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Abstract. Treatment of human platelets by EDTA (5 mM at 37°C and pH 7.4 for 30 min) induces ultrastructural morphological changes of the surface-connected canalicular system (SCCS). The first consists in dilations of some portions of the channels, whereas the second is represented by collapse of parts of the canaliculi. The collapsed elements of the EDTA treated SCCS are made up of two parallel limiting membranes and a central striated zone. Some of the EDTA treated platelets form microaggregates, the cohesion of which is apparently due to the appearance of pentalaminar interplatelet structures. EDTA treatment is known to induce an irreversible loss of platelet aggregability which is due to irreversible dissociation of the membrane GPIIb-IIIa complexes. In the present study, we looked for involvement of GPIIb-IIIa in the formation of these pentalaminar structures, and were able to demonstrate that the morphological changes described are in fact directly dependent on the EDTA induced dissociation of GPIIb-IIIa complexes. Indeed, we observed that these changes (a) cannot be induced in type I Glanzmann's thrombasthenia, where GPIIb-IIIa complexes are absent, (b) do not appear when human platelets are preincubated with monoclonal anti-GPIIb-IIIa complex-dependent (CD41a) antibodies,

which protect the complex from EDTA induced dissociation, (c) appear only at alkaline pH and at 37°C, which corresponds to the range of pH and temperature where EDTA can dissociate GPIIb-IIIa complexes, (d) are accompanied by the disappearance in fluorescence flow cytometry of the heterodimer complex-dependent epitopes, when using anti-CD41a antibodies and (e) do not appear in rat platelets, where GPIIb-IIIa does not dissociate after EDTA treatment. Furthermore, using gold-labeled mAbs concomitantly with the addition of EDTA, we observed that almost only GPIIb was present in the collapsed regions of the canaliculi. Using double labeling studies with polyclonal anti-GPIIb antibodies coupled to 10 nm gold particles and polyclonal anti-GPIIIa antibodies coupled to 20 nm gold particles, we observed that while both 10 and 20 nm particles were present in the dilated portions of the canaliculi almost only the small particles, coupled to the anti-GPIIb antibodies, labeled the collapsed portions of the SCCS. On Lowicryl thin sections, polyclonal antibodies against GPIIb labeled the central striated zone while both GPIIb and GPIIIa were found in the dilated portions of the SCCS. All these observations lead us to suggest that homopolymers of GPIIb could be responsible for "zipping" of the SCCS.

SINCE the early observations by Zucker and Borelli (28) and by White (25), it is well known that the divalent cation chelator EDTA, which is used as an anticoagulant, is responsible for changes in platelet morphology. Indeed, platelets incubated with EDTA at 37°C, either in plasma or in physiological media, become irregularly swollen with multiple pseudopods and loss of granule content. Moreover, at the ultrastructural level, White (25) noticed that EDTA induces modifications of the platelet membrane surfaces in contact with the extracellular medium, i.e., both the membranes forming the cell wall and those lin-

ing the surface-connected canalicular system (SCCS)¹. The latter is the site of two types of modification: the first consists in massive dilation of the SCCS channels whereas the second is represented by collapse of some portions of the SCCS, which become narrow and elongated, following serpentine courses through the cytoplasm. The hypothesis put forward by White (25) to explain the occurrence of these SCCS modifications was that dilation of some parts of the channels

1. *Abbreviations used in this paper:* CIE, crossed immunoelectrophoresis; GP, glycoprotein; SCCS, surface-connected canalicular system.

causes tension in others, resulting in their collapsed appearance.

On the other hand, when human platelets are incubated for 30 min at 37°C with 5 mM EDTA and then resuspended in a calcium containing medium, they lose their ability to bind fibrinogen and to aggregate in response to ADP stimulation (29). This effect of EDTA is irreversible. It has been shown by crossed immunoelectrophoresis (CIE) (20), binding of mAb (15) and sucrose density gradient centrifugation (12), that under these conditions, EDTA dissociates the platelet membrane associated specific integrin $\alpha_{IIb}\beta_3$. This integrin, also called glycoprotein (GP) IIb-IIIa, is a calcium-dependent glycoprotein heterodimer complex. It is essential for platelet aggregation, as it serves as an inducible receptor for the adhesive proteins fibrinogen, fibronectin, vitronectin and von Willebrand factor upon platelet stimulation (18). These adhesive proteins circulate in plasma and are contained in platelet α granules. They all contain RGD (Arg-Gly-Asp) sequences and bind to the activated GPIIb-IIIa complex in an RGD dependent manner (10, 18). In patients with Glanzmann's thrombasthenia, an inherited autosomal hemorrhagic disorder, the GP IIb-IIIa complex is either absent (type I), reduced or abnormal (variants). Their platelets are therefore unable to bind fibrinogen when activated by an agonist and consequently do not aggregate (7).

The purpose of our study was to re-examine the ultrastructural modifications induced by EDTA in the SCCS and to look for possible involvement of the $\alpha_{IIb}\beta_3$ integrin in the genesis of these changes.

Materials and Methods

Monoclonal and Polyclonal Antibodies

Two different murine mAbs anti-CD41a (24), detecting specifically GPIIb-IIIa complex-dependent epitopes, were used: IOP41a (IgG1) from Immunotech (Marseille, France) and AP-2 (IgG1) kindly supplied by Dr. T. J. Kunicki (The Blood Center of Southeastern Wisconsin, Milwaukee, WI) (19). The following murine mAbs were used to identify the two GPIIb-IIIa subunits: D33 (IgG1) kindly supplied by Dr. G. Marguerie (Centre d'Etudes Nucléaires, INSERM U.217, Grenoble, France) which detect the GPIIb subunit and IOP61 (IgG1) from Immunotech, which identify the GPIIIa subunit. A polyclonal antiserum anti-GPIIIa was provided by Dr. B. Steiner (Hoffmann-La-Roche and Co., Basel, Switzerland) and a purified

polyclonal anti-GPIIb antibody was obtained from Dr. J. Sixma (University Hospital Utrecht, Department of Haematology, Utrecht, The Netherlands). For isotype controls, an irrelevant mouse IgG1 mAb anti-rat kappa chain (Immunotech) was employed. An FITC-conjugated goat anti-mouse antibody (Becton Dickinson, Mountain View, CA) was used for the immunofluorescent labeling procedures.

Gold-labeled antibodies were prepared as already described (8). Briefly, gold particles were produced according to the method of Frens (5). The concentration of immunoglobulin necessary to stabilize the colloidal gold particles was estimated as previously described by Faulk and Taylor (4). According to these results, the mAb IOP41a (anti-GPIIb-IIIa), D33 (anti-GPIIb) and IOP61 (anti-GPIIIa) were adsorbed to 10-nm gold particles, whereas the polyclonal anti-GPIIb and anti-GPIIIa antisera were conjugated to 10 and 20 nm gold particles, respectively. Tween 20 was added as stabilizer. Gold-labeled (10 nm) secondary antibodies (BioCell Research Laboratories, Cardiff, UK) used for immunocytochemical procedures are listed in Table I.

The specificity and origin of the polyclonal antibodies employed in immunocytochemical procedures on Lowicryl sections are described in Table I.

Preparation of Washed Human Platelets

Blood was obtained from healthy human donors and from a patient with type I Glanzmann's thrombasthenia, who denied taking any drugs for at least 8 d before blood collection. Platelets were isolated from acid-citrate-dextrose anticoagulated blood by differential centrifugation and washed twice at 37°C according to a modification (3) of the method of Mustard (16). Unless otherwise stated, platelets were washed in Tyrode's buffer, pH 7.3, 295 mOsmol, containing 5 mM Hepes (Sigma Chemical Co, St. Louis, MO) (Tyrode's-Hepes buffer), 0.35% purified human serum albumin (CRTS, Strasbourg, France) and 1 μ M prostaglandin I₂ (PGI₂) (Sigma Chemical Co.). Platelets were finally suspended in Tyrode's-Hepes buffer containing 0.35% human serum albumin and 2 μ g/ml apyrase and adjusted to \sim 500,000 platelets/mm³.

EDTA Treatment of Human Platelets

For EDTA treatment a solution of 0.1 M EDTA (Sigma Chemical Co.), previously warmed to 37°C, was added to the washed human platelets in the second washing fluid, the final concentration of EDTA being 5 mM. Care was taken to maintain pH 7.3 and temperature 37°C when not otherwise stated. Control platelets were similarly incubated with the same volume of Tyrode's buffer containing neither Ca²⁺ nor Mg²⁺. After 30 min, control and EDTA treated platelets were fixed for EM studies.

Preparation and EDTA Treatment of Washed Rat Platelets

Wistar rat blood (5 vol) was collected from the aorta under ether anesthesia and anticoagulated with acid-citrate-dextrose (1 vol). Platelets were isolated by differential centrifugation and washed twice as already described (6).

Table I. List of the Antibodies Used for Immunocytochemical Procedures

Antibody to human	Primary polyclonal antibodies			Gold-labeled secondary antibodies
	Host animal	Dilution	Origin	
GP IIb	Rabbit	1:250	DR Phillips, San Francisco, CA	
GP IIIa	Rabbit	1:250	Cor Therapeutics, San Francisco, CA	
Fibrinogen	Rabbit	1:2,500	CRTS, Strasbourg, France	
von Willebrand factor	Rabbit	1:1,000	CRTS, Strasbourg, France	Goat anti-rabbit IgG
Vitronectin	Rabbit	1:500	Calbiochem, Corp., La Jolla, CA	
Thrombospondin	Rabbit	1:500	Diagnostica Stago, Asnières, France	
Fibronectin	Sheep	1:1,000	The Binding Site, Birmingham, UK	Donkey anti-sheep IgG

They were finally suspended in Tyrode's-Hepes buffer at pH 7.4 or 8.5. In some experiments, EDTA was added to the second washing fluid (final concentration of EDTA 5 mM) for 30 min, the platelets then being fixed for EM studies. Particular care was taken to maintain the pH constant at 7.4 or 8.5. Controls were performed by adding only Tyrode's-Hepes buffer to the platelets in suspension, pH again being maintained at 7.4 or 8.5.

Incubation with Antibodies

Washed human platelet suspensions were incubated with the mouse mAb IOP41a or AP-2, detecting specifically GPIIb-IIIa complex-dependent epitopes, or with the irrelevant mouse mAb anti-rat kappa chain before (first wash) and during (second wash) EDTA treatment (0.1 mg/ml final concentration of antibody). After 30 min incubation in the presence of EDTA, platelet suspensions were processed for EM or for CIE. In the same way, untreated platelets were processed for EM or CIE. In another set of experiments, gold labeled antibodies against the GPIIb-IIIa complex or its subunits GPIIb and GPIIIa, were added to the platelet suspensions at the time of EDTA treatment. After 30 min incubation at 37°C platelets were fixed for EM.

Crossed Immunoelectrophoresis

Untreated and EDTA treated platelets (preincubated or not with a mAb anti-GPIIb-IIIa or an irrelevant mAb) were sedimented and washed in Tyrode's-Hepes buffer containing no albumin, to remove excess albumin. After centrifugation, the pellet was resuspended in 0.1 M glycine, 38 mM Tris, pH 8.7 (Tris-glycine), prechilled to 4°C. Platelet solubilization was performed by incubating the platelets for 30 min at 4°C with 1% (vol/vol) Triton X-100 in Tris-glycine. Triton insoluble material was removed by ultracentrifugation at 100,000 g for 10 min and the supernatant stored at -80°C until use. CIE was carried out on platelet extracts as previously described (6). Briefly, 70 µg of solubilized platelets was first electrophoresed at 10 V/cm for 1 h into 1% (wt/vol) agarose containing 0.5% (vol/vol) Triton X-100 in Tris-glycine. Second dimension electrophoresis was performed at 2 V/cm for 18 h into a biphasic gel system. An intermediate 1% agarose, 0.5% Triton X-100 gel was present between the first dimension and the upper gel containing precipitating concentrations of polyspecific rabbit antiplatelet antibodies. Immunoprecipitates were located by Coomassie blue staining (CB-R-250, Bio-Rad Labs, Richmond, CA).

Flow Cytometry

Control and EDTA treated platelets, washed once in Tyrode's-Hepes buffer, were fixed with 2% paraformaldehyde in Tyrode's-Hepes buffer for 30 min at 37°C and washed again in Tyrode's-Hepes buffer. 2 µg of the primary mAb anti-GPIIb-IIIa or the irrelevant mAb anti-rat kappa chain was then added to 5-µl aliquots (5×10^5 platelets/µl) diluted in 50 µl Tyrode's-Hepes buffer, for 30 min at room temperature. After washing the platelets with Tyrode's-Hepes buffer, 10 µg of FITC-labeled goat anti-mouse IgG was added, for 30 min in the dark at room temperature, to 50-µl aliquots of the platelet suspensions. Samples were again washed with Tyrode's-Hepes buffer and then diluted in 1 ml Isoton III (Coultertronics, Paris, France). 5,000 platelets were analyzed for each sample using a Facscan flow cytometer (Becton-Dickinson) gated to exclude nucleated cells.

Preparation of Platelets for Transmission Electron Microscopy

Washed platelets (control and EDTA treated) were processed either for Epon or for Lowicryl embedding. For Epon embedding, washed platelets (1 ml) maintained at 37°C were fixed by adding an equal volume of fixative solution, previously warmed to 37°C, composed of 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer containing 1% sucrose, pH 7.3. After 5 min the mixture was centrifuged, the supernatant discarded and the platelet pellet resuspended and further fixed for 1 h at 37°C in the same fixative solution. Following an additional centrifugation, the samples were incubated for 1 h at room temperature with 1% tannic acid in 0.05 M Na cacodylate buffer, pH 7.0. They were then postfixed for 1 h at 4°C with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3. Platelets were washed once more in 0.1 M Na cacodylate buffer, pH 7.3, for 10 min at room temperature before being stained for 1 h at 4°C with a 1% aqueous solution of uranyl acetate and dehydrated in successively increasing (50, 70, 80, 95, and 100%) ethanol concentrations. Finally, the samples were incubated overnight in Epon-absolute alcohol (1:1, vol/vol) and embedded in Epon. Ultrathin sections, stained with lead citrate, were examined under a Siemens Elmiskop 102

electron microscope (60kV). For Lowicryl embedding, samples of washed platelets (1 ml) were fixed in two different ways. Firstly, they were incubated at 37°C for 10 min with an equal volume of 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.3. This mixture was then replaced by the same fixative and the platelets further fixed for 1 h at 20°C. Secondly, 1 ml of 5% paraformaldehyde, 0.2% glutaraldehyde in 0.2 M Na cacodylate buffer (pH 7.3) was added to the platelet samples maintained at 37°C. After 10 min incubation, the mixture was replaced by 2.5% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3) and the samples further fixed for 1 h at 20°C. Thereafter, the platelets fixed in either of the two ways were washed once in 0.1 M Na cacodylate buffer, concentrated in agar (27) and further washed overnight at 4°C in 0.1 M Na cacodylate buffer. After 30 min staining at 4°C with 2% uranyl acetate diluted in Michaelis buffer, dehydration and Lowicryl K4M embedding procedures were carried out as previously described by Whitehouse et al. (26).

Immunocytochemical Procedures

Thin sections of Lowicryl embedded samples were collected on 200 mesh Formvar-carbon coated nickel grids (Agar, Stansted, UK). These sections were pretreated for 1 h at room temperature with PBS (pH 7.2) containing 1% serum of the same origin as the second antibody. They were then incubated for 16 h at room temperature with the primary antibody (dilutions of the various primary antibodies are given in Table I). Thereafter, the sections were washed three times in PBS/1% serum and further incubated for 1.5 h at room temperature with the gold labeled secondary antibody used at 1:20 dilution in PBS/1% serum (the nature of the secondary antibody varies according to the primary antibody as detailed in Table I). After two washes in PBS/0.5% serum and two washes with PBS, the sections were postfixed for 30 min at room temperature with 2.5% glutaraldehyde in PBS. Finally, the sections were washed with distilled water and coated with 1.8% uranyl acetate/0.2% methylcellulose (22). They were examined under a Siemens Elmiskop 102 electron microscope (80 kV). Controls were performed by substituting non immune rabbit or sheep IgG for the primary antibody.

Results

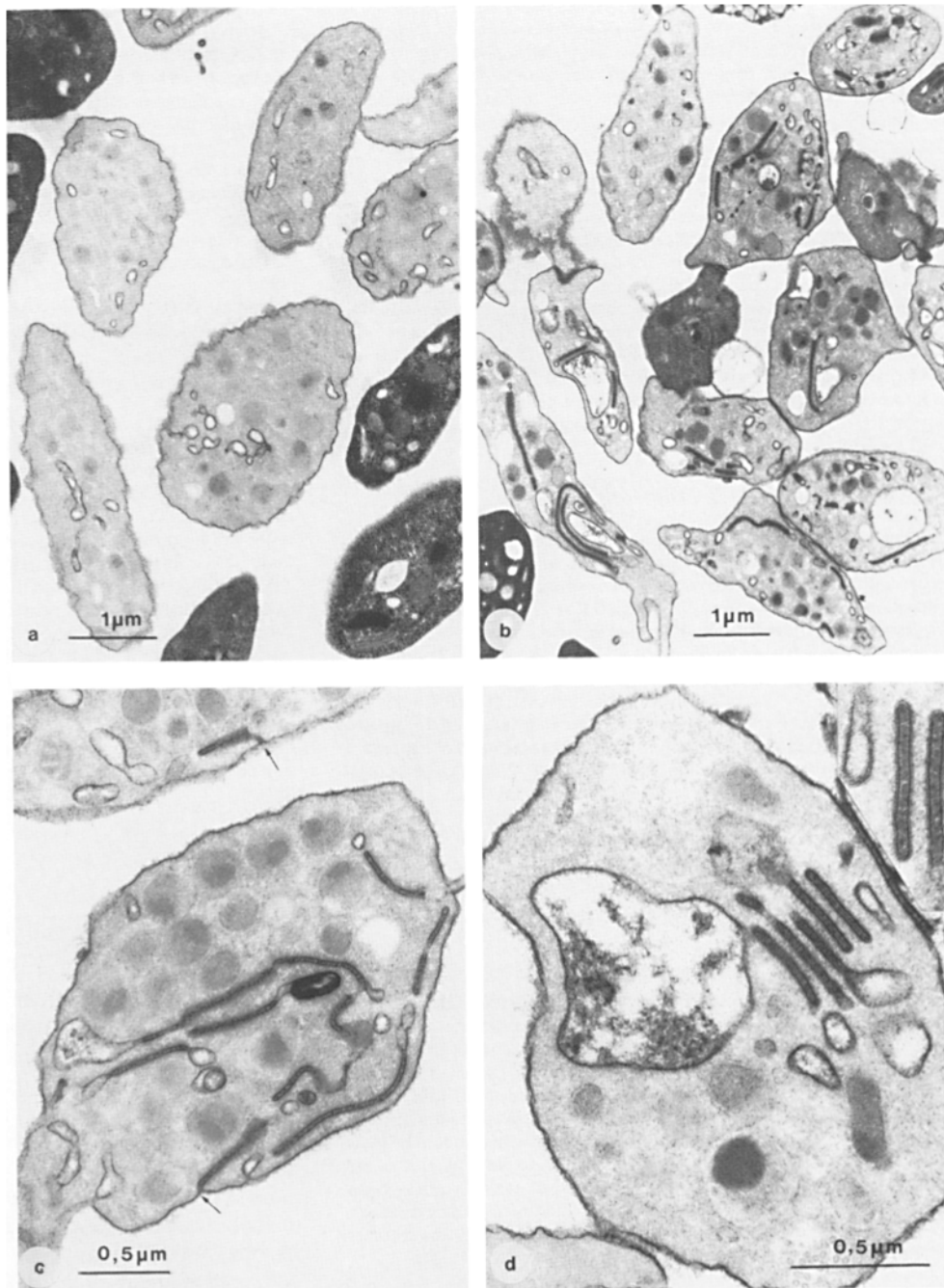
Ultrastructural Effects of EDTA on Normal Human Platelets

Treatment of normal human platelets by EDTA induced the previously described morphological changes, i.e., increase in platelet volume, shape change from disc to sphere and degranulation. At the ultrastructural level the SCCS, which normally appears as a mass of unconnected short tubules and small vacuoles (Fig. 1 a), presented the dilated and collapsed portions already mentioned (Fig. 1 b) (25). The SCCS remained in continuity with the cell surface (Fig. 1 c) and extended through the cytoplasm, describing over variable lengths straight or more or less winding trajectories. The collapsed elements of the EDTA treated SCCS were made up of two parallel limiting membranes and a central irregular striated zone (Fig. 1 d), formed by the juxtaposition of small lumps with rounded or angular contours (Fig. 1 e). Some of the EDTA-treated platelets formed microaggregates (Fig. 1 f). The cohesion of the platelets in these microaggregates seemed to be due to the appearance of pentalamellar interplatelet structures.

Ultrastructural Effect of EDTA on Platelets from a Type I Glanzmann's Thrombasthenia Patient

Washed platelets from a type I Glanzmann's thrombasthenia patient, i.e., platelets lacking the GPIIb-IIIa complex, were incubated with EDTA 5 mM for 30 min at 37°C and then fixed for EM. Fig. 2 shows that these platelets did not display the typical features observed after EDTA treatment of normal platelets. Indeed, despite dilation of some portions of the

Figure 1. Ultrastructural effects of EDTA on the SCCS of normal human platelets. Tannic acid enhances the contrast of the channels of the SCCS which appear, on thin sections, in untreated human blood platelets (a) as unconnected, more or less dilated vacuoles or canaliculi and after EDTA treatment (b-e) as composed of dilated zones and zones of collapse. The EDTA treated SCCS remained in continuity (c) with the cell surface (arrows). Continuity is also clearly visible between the dilated and collapsed portions of the EDTA treated SCCS (c, d), which can take a "piles of plates" arrangement (d). A higher magnification of these elements of the SCCS shows (d, inset) that they are made up of two parallel limiting membranes and a central irregular striated zone. On a section parallel to the plane of an element of the SCCS (e), this striation appears to be formed by the juxtaposition of small lumps with rounded or angular contours. Similar striation is also visible at a zone of contact between two adjacent platelets (arrows), in an EDTA treated human platelet microaggregate (f).

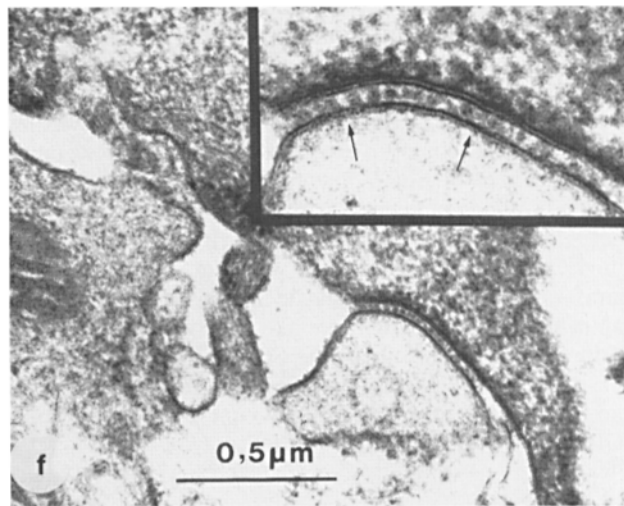


SCCS, no collapsed regions were detected, suggesting that the GPIIb-IIIa complex is essential in the genesis of this phenomenon. Likewise, no platelet microaggregates were observed when type I Glanzmann's thrombasthenia platelets were subjected to EDTA treatment.

The Pentalaminar Structures Induced by EDTA Appear Only when the GP IIb-IIIa Complex is Dissociated

When human platelets were incubated with EDTA 5 mM, either at pH 7.3 and room temperature or at 37°C and pH < 7, the typical pentalaminar structures induced by incubating platelets with EDTA at 37°C and pH 7.3 did not appear. Flow cytometry performed using the mAb IOP41a, detecting

specifically GPIIb-IIIa complex-dependent epitopes, revealed that at room temperature (pH 7.3) the GPIIb-IIIa complex did not dissociate. Similarly, at pH < 7 (37°C) platelets were still labeled with the complex specific antibody (Fig. 3). These results are closely related to the observations of Zucker and Grant (29) and of Pidard et al. (20), who noted the temperature and pH dependence of EDTA induced loss of aggregability due to GPIIb-IIIa dissociation (20). Thus, dissociation of the GPIIb-IIIa complex seems to be necessary for induction of pentalaminar structures in the SCCS. This conclusion was confirmed by further experiments where washed human platelets were incubated with anti-GPIIb-IIIa complex-dependent mAb (AP-2, IOP41a) before and during EDTA treatment (5 mM, 37°C, pH 7.3). Under these conditions, despite dilation of some regions of



the SCCS, no pentalaminar structure formation could be observed in the EDTA treated platelets, whereas addition of an irrelevant mAb did not prevent the appearance of SCCS pentalaminar structures. CIE (Fig. 4) revealed that preincubating the platelets with anti-GPIIb-IIIa complex-dependent mAb protected the complex from EDTA induced dissociation (Fig. 4 D), while addition of an irrelevant mAb did not prevent this dissociation (Fig. 4 C). Thus, we deduced once again that by preventing GPIIb-IIIa dissociation, one could preclude the formation of the SCCS pentalaminar structures. Our conclusion was reinforced by additional data. We have previously shown (6) that rat platelets are less sensitive to the effects of EDTA than human platelets and that the GPIIb-

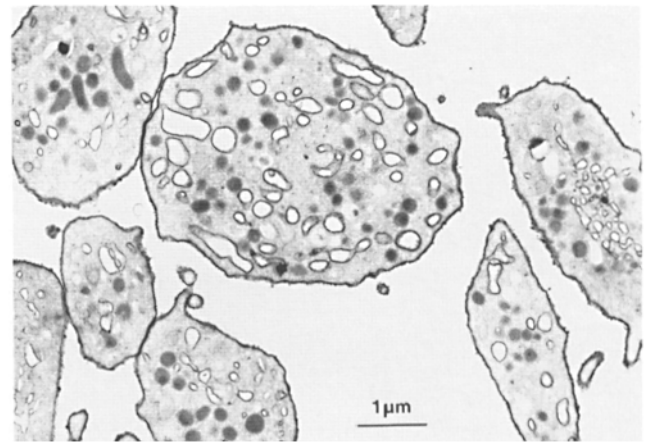


Figure 2. Ultrastructural effects of EDTA on the platelet SCCS of a type I Glanzmann's thrombasthenia patient. Despite dilation of some portions of the SCCS, no collapsed regions are visible.

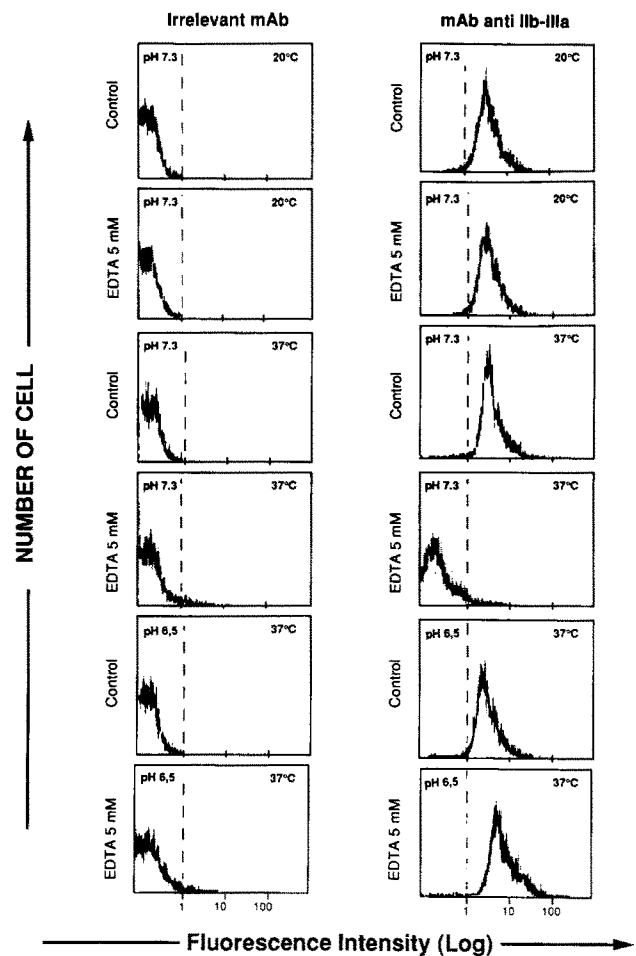


Figure 3. Temperature and pH dependence of EDTA induced dissociation of the GPIIb-IIIa complex. Flow cytometric profiles using the mAb IOP41a, detecting GPIIb-IIIa complex-dependent epitopes, are displayed in the right column, whereas profiles obtained with an irrelevant mAb are indicated on the left. The complex specific epitopes did not disappear when platelets were incubated with EDTA at 37°C and pH < 7 or at pH 7.3 and at room temperature. Loss of the epitopes visualized by a decrease in fluorescence intensity, occurred at 37°C and pH 7.3, the well known conditions in which GPIIb-IIIa dissociates under EDTA treatment.

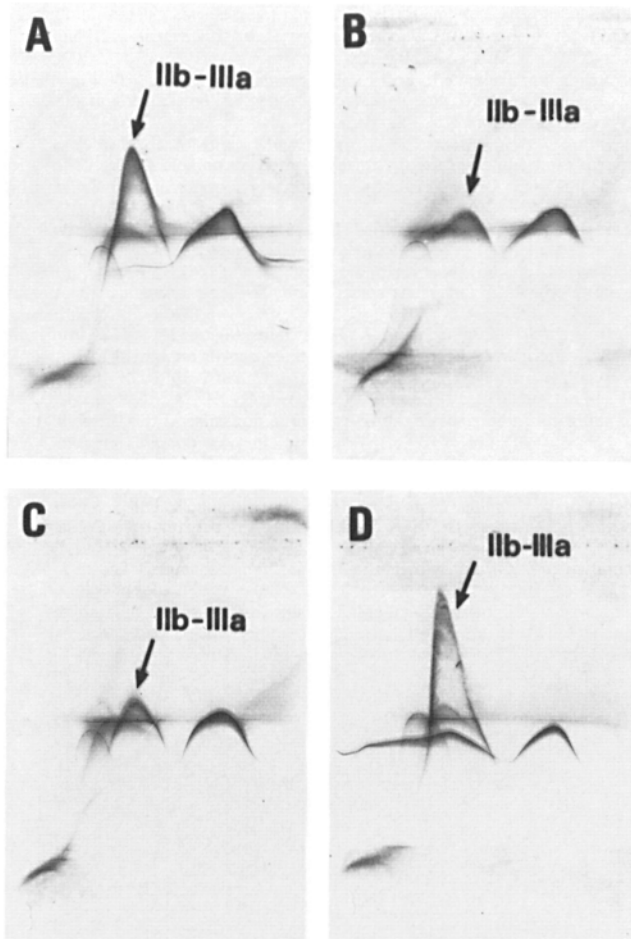


Figure 4. Monoclonal anti-GPIIb-IIIa complex antibodies protect GPIIb-IIIa complex from EDTA induced dissociation. Human platelets, treated or not with 5 mM EDTA at 37°C and pH 7.4, in the presence or not of mAb AP2 or an irrelevant mAb, as described in the Methods section, were solubilized in Triton X-100 and submitted to crossed immunoelectrophoresis. The gels were stained with Coomassie blue. (A) control platelets, (B) EDTA treated platelets, (C) EDTA treated platelets incubated with an irrelevant mAb, (D) EDTA treated platelets incubated with AP2. Under conditions (A) and (D), the GPIIb-IIIa complex did not dissociate.

IIIa complex of rat platelets does not dissociate under conditions (37°C, pH 7.4, 30 min) where the human complex is almost completely dissociated. When EDTA treated rat platelets (37°C, pH 7.4, 30 min) were examined by EM, we did not observe pentalaminar structures (Fig. 5 *a*) along the SCCS. However, when rat platelets were incubated with EDTA 5 mM at 37°C and pH 8.5, few but typical pentalaminar structures appeared (Fig. 5, *b* and *c*). Under these conditions, 20% of the rat GPIIb-IIIa complexes are dissociated (6).

Nature of the Glycoproteins Involved in the Pentalaminary Structures

As dissociation of the $\alpha_{\text{IIb}}\beta_3$ integrin leads to the appearance of monomeric subunits, i.e., free GPIIb and GPIIIa, as well as homo and/or heteroglycoprotein polymers (2, 17, 21), we attempted to identify the glycoprotein(s) involved in the EDTA induced “zipping” of the SCCS. In a first set of ex-

periments (Fig. 6 *A*), we incubated control platelets with gold-labeled antibodies against free GPIIb (the polyclonal antiserum coupled to 10 nm gold particles) and free GPIIIa (the polyclonal antiserum coupled to 20 nm gold particles) at 37°C, pH 7.4 and for 30 min. Under the EM gold particles of both sizes were observed within the short tubules and small vacuoles of the SCCS, while there was only sparse labeling of the plasma membrane. These results are closely related to the observations of Isenberg et al. (11), who noted that incubation of platelets at 37°C in the presence of gold-labeled anti-GPIIb and anti-GPIIIa mAb induces internalization and a surface membrane clearing of GPIIb-IIIa. In a second set of experiments, these two types of antibodies were added to the platelet suspensions concomitantly with the addition of EDTA. We then observed that while anti-GPIIIa and anti-GPIIb antibodies were both present in the dilated portions of the SCCS, almost only the anti-GPIIb antibody was present in the collapsed portions of the canaliculi (Fig. 6, *B* and *C*). These results reproduced those we obtained using either gold labeled mAb anti-GPIIb-IIIa or anti-GPIIb or anti-GPIIIa concomitantly with the addition of EDTA: while all the gold labeled mAb were present in the dilated portions of the SCCS, almost only the mAb anti-GPIIb was present in the collapsed portions of the canaliculi.

To identify a possible ligand, which could be located in the striated zone and participate through “ligand-receptor” interactions in collapse of the SCCS, we incubated Lowicryl thin sections successively with (*a*) polyclonal antibodies against the major platelet adhesion molecules (fibrinogen, fibronectin, vitronectin, von Willebrand factor and thrombospondin) and (*b*) a gold-labeled secondary reagent. In control platelets, all these antibodies stained the α granules, where these adhesion molecules are normally located. When EDTA treated platelets were examined, α granule staining was less pronounced, as could be expected by taking into account a certain degree of EDTA induced granule secretion. However, no staining of the pentalaminary structures could be detected (not shown). It was essentially with the polyclonal antibody to GPIIb that we observed (Fig. 7, *a* and *b*), on paraformaldehyde/glutaraldehyde fixed platelets (23), labeling of the limiting membranes and also of the striated zone of the collapsed regions of the SCCS. Less often, we observed labeling with the polyclonal anti-GPIIIa antibody and rarely with the anti-GPIIb-IIIa complex-dependent mAb, IOP4 Ia.

Discussion

In this study, we show that the previously described EDTA induced morphological modifications of the platelet SCCS are directly dependent on the presence and dissociation of the platelet specific $\alpha_{\text{IIb}}\beta_3$ integrin. The prominent feature we have observed was the appearance (Fig. 1) of pentalaminary structures made up of two parallel limiting membranes with a central striated zone. Although mainly located in the SCCS, pentalaminary structures were also found between the EDTA treated platelets, where they seemed to be responsible for the cohesion of small platelet aggregates. This is in agreement with the findings of White (25) who pointed out that the effect of EDTA was “limited to membranes exposed to plasma since previous work has shown that EDTA does not enter the living cells”. The necessity for the presence of

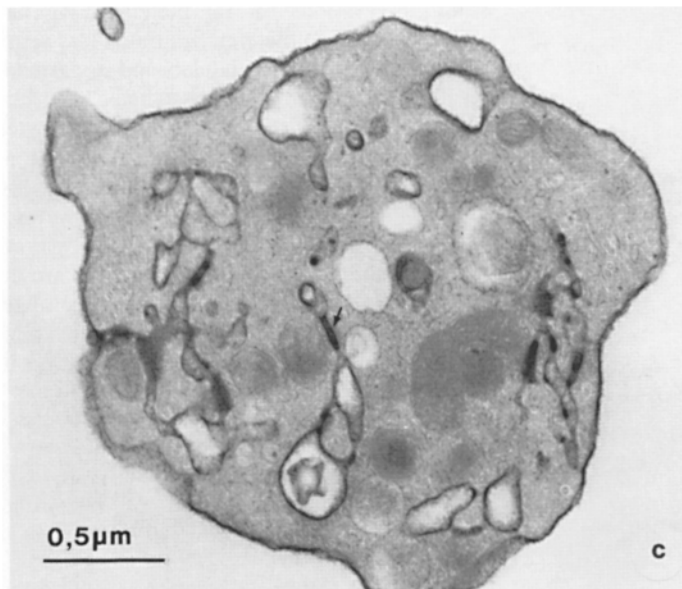
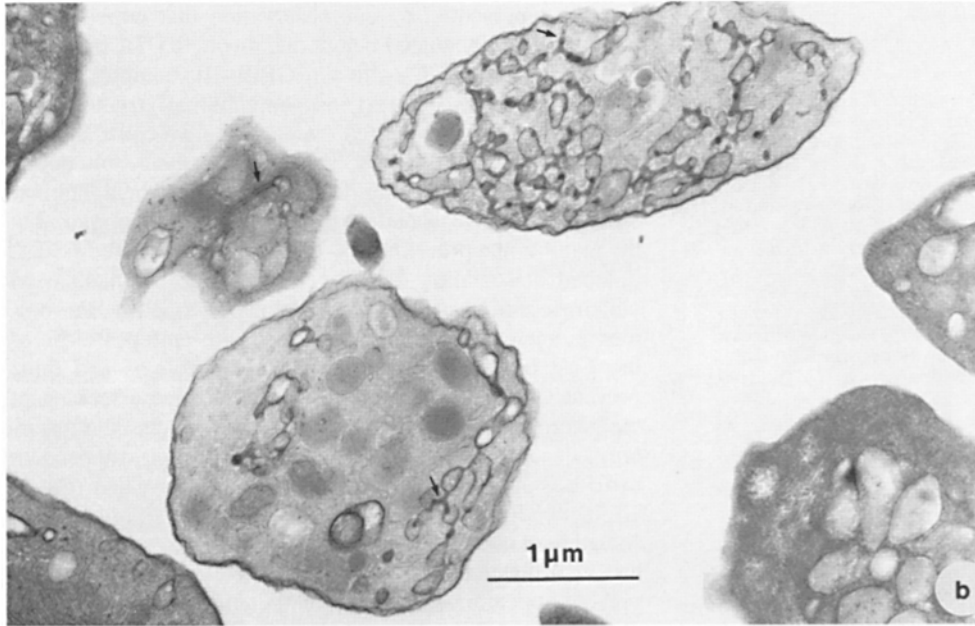
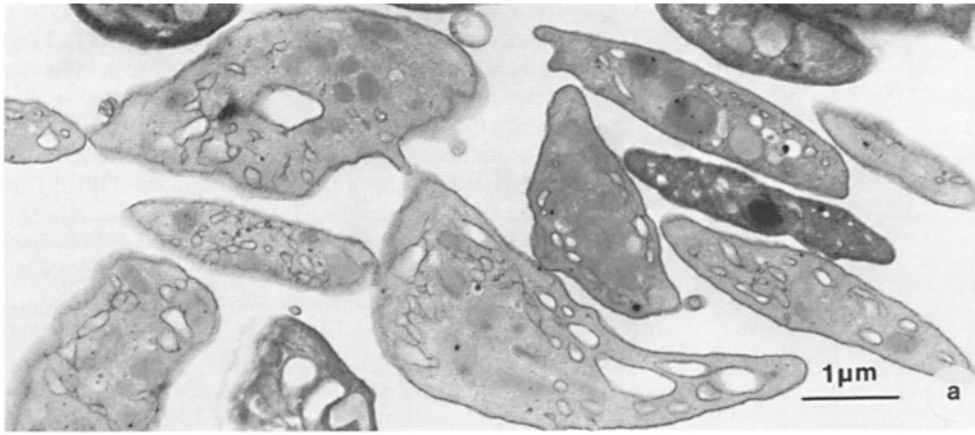
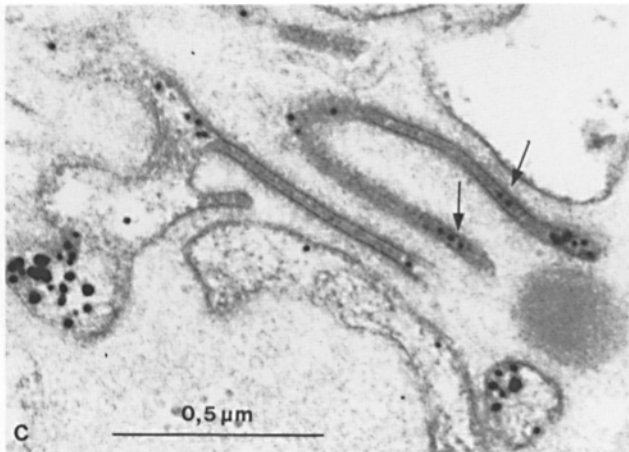
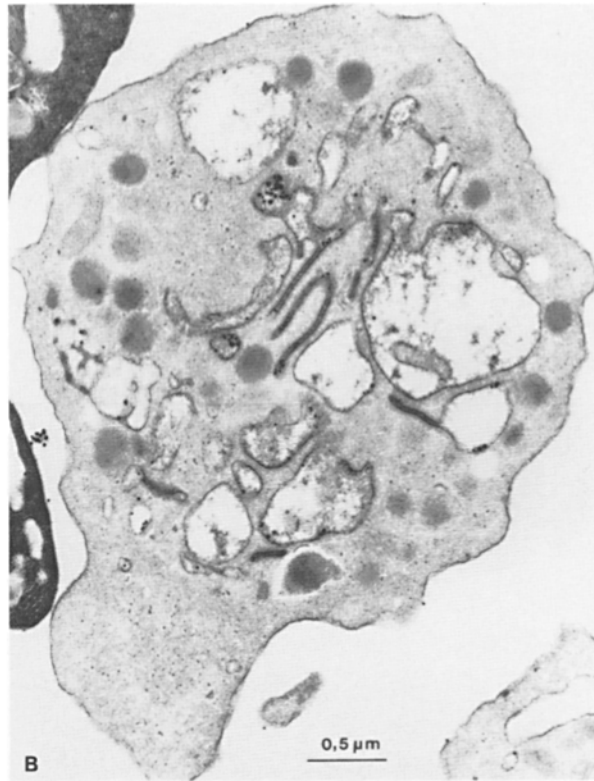
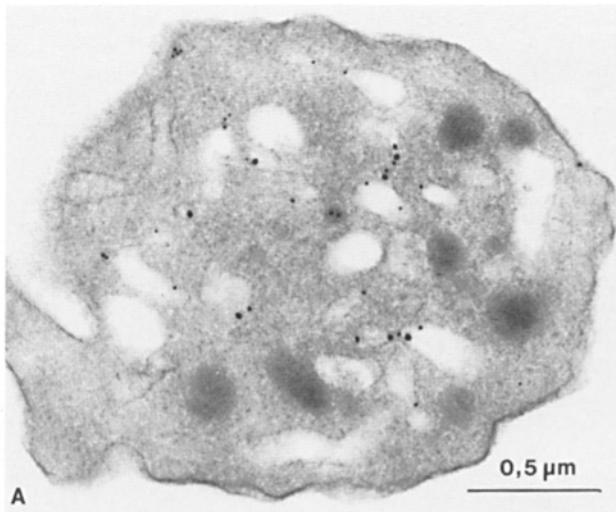


Figure 5. pH dependence of EDTA induced collapse of rat platelet SCCS. Incubation of rat platelets with 5 mM EDTA at 37°C and pH 7.4 (a) did not induce the appearance of collapsed regions along the SCCS. Under the same conditions but at pH 8.5 (b, c), typical pentalaminar structures appeared (arrows) in some parts of the SCCS.



GPIIb-IIIa was deduced from the observation that in type I Glanzmann's thrombasthenia platelets, i.e., platelets lacking almost completely the GPIIb-IIIa complex, these pentalaminar elements did not appear after EDTA treatment (Fig. 2). The necessity for GPIIb-IIIa to dissociate was supported by a series of arguments. First, by the observation that these elements appear only under conditions where the GPIIb-IIIa complex can be dissociated by EDTA, namely, when incubation is performed for 30 min at 37°C and pH 7.4. It is well known that at acidic pH (< 7) and/or at temperatures below 37°C, the GPIIb-IIIa complex is not dissociated in intact platelets exposed to 5 mM EDTA (2, 20). We showed that under these conditions (pH < 7, T° < 37°C) the pentalaminar structures did not appear and that simultaneously the complex-dependent epitopes of GPIIb-IIIa remained present, as visualized by flow cytometry (Fig. 3). A second argument is represented by our observation that when human platelets were incubated before and during EDTA treatment (5 mM, 30 min, 37°C) with anti-GPIIb-IIIa complex-dependent mAb (AP-2, IOP41a), no pentalaminar structures appeared in the EDTA treated platelets. As we could demonstrate using CIE (Fig. 4), this was not the result of the antibodies impairing the formation of the pentalaminar structures after dissociation of the GPIIb-IIIa complex, but the result of the prevention by these antibodies of the EDTA-induced dissociation of the complex. The mechanism by which these mAb prevent dissociation of the GPIIb-IIIa complex is unclear. They could interact with epitopes close to the Ca²⁺ binding domains of the glycoproteins and thus, prevent Ca²⁺ chelation, or they might induce a locking of the heterodimer which renders it resistant to dissociation after Ca²⁺ chelation. This effect of the complex-dependent mAb was specific. Indeed, as CIE studies revealed (Fig. 4 C), incubation with irrelevant antibodies did not prevent dissociation of the GPIIb-IIIa complex and, consequently, incubation of platelets in the presence of irrelevant mAb did not prevent appearance of SCCS pentalaminar structures after appropriate EDTA treatment. A final argument is drawn from observations on rat platelets. We have previously shown (6) that rat platelets are much less sensitive to EDTA treatment than human platelets and in particular that the rat GPIIb-IIIa complex is not dissociated after long exposure to 5 mM EDTA at 37°C and pH 7.4. Under these conditions, we found that EDTA treated rat platelets did not contain the typical structures described in human platelets and that they did not form aggregates (Fig. 5 a). However, under drastic conditions, i.e., 5 mM EDTA, 37°C and pH 8.5, where ~20% of the rat GPIIb-IIIa complexes are dissociated (6), we observed that some portions of the rat platelet SCCS displayed the characteristic pentalaminar features (Fig. 5, b and c). Thus, it was demonstrated that the occurrence of the

Figure 6. Colocalization of gold labeled anti-free GPIIb (10 nm) and anti-free GPIIIa (20 nm) polyclonal antibodies in the SCCS. In control untreated platelets (A) gold particles of both sizes are visible in the short tubules and the small vacuoles of the SCCS. When the two types of antibodies were added concomitantly with EDTA (B), both anti-free GPIIb and anti-free GPIIIa were located in the dilated portions of the SCCS, while only anti-free GPIIb localized (arrows) in the collapsed regions of the SCCS. C is a higher magnification of B.

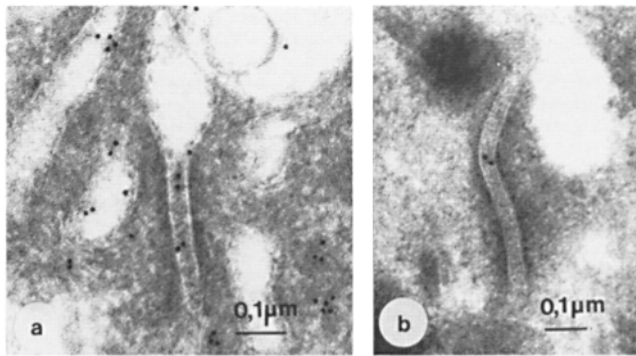


Figure 7 (a and b) GPIIb localizes in the collapsed regions of the SCCS. EDTA treated platelets embedded in Lowicryl K4M were incubated successively with (i) a rabbit polyclonal anti-GPIIb antibody and (ii) gold-labeled goat anti-rabbit IgG. Gold particles label both the limiting membranes and the central striated zone of the collapsed SCCS.

SCCS pentalaminar structures is directly dependent on dissociation of the $\alpha_{IIb}\beta_3$ integrin.

Because GPIIb-IIIa is the receptor for fibrinogen and other platelet adhesion molecules such as fibronectin, vitronectin and von Willebrand factor, we looked for the presence of one or more of these ligands in the SCCS pentalaminar structures. Our hypothesis was that some of these adhesion molecules could participate, via receptor-ligand interactions, in the membrane bonding of the SCCS. We therefore incubated Lowicryl thin sections successively with polyclonal antibodies raised against the major platelet adhesion molecules and with the appropriate gold-labeled secondary antibodies. As expected, we were able to label the α granules of control platelets, where these adhesion molecules are normally located. The α granules of EDTA-treated platelets were also labeled, although this labeling was less pronounced when compared to untreated platelets, indicating that α granule secretion occurred under EDTA treatment. However, we did not find any staining of the pentalaminar structures of EDTA treated platelets with any of the antiadhesion molecule antibodies tested. These results were in fact not surprising, as we have observed (data not shown) that incubation of human platelets before and during EDTA treatment with the RGDS peptide, which is known to interfere competitively with binding of RGD containing adhesion molecules to the GPIIb-IIIa complex, did not prevent the appearance of pentalaminar structures. Likewise, we observed the formation of typical pentalaminar structures in platelets from the recently described variant of Glanzmann's thrombasthenia (14), the GPIIb-IIIa of which is not able to support fibrinogen binding (data not shown). Although this does not exclude definitively the possibility that an adhesive protein is involved in the bonding of the pentalaminar structures, it was essentially with a polyclonal antibody to free GPIIb subunit that we observed a labeling of both the limiting membranes and the striated zone of the SCCS collapsed regions (Fig. 7). Altogether these data suggest that the mechanism which leads to zipping of the SCCS does not result from receptor-ligand interactions, but rather from the appearance of homopolymers of free GPIIb, "bridging" the limiting membranes of the channels. Homopolymers of free glycoprotein subunits have been described, after dissociation of the GPIIb-IIIa complex, in purified systems (2, 17). Moreover,

it has been reported that GPIIb could form covalently linked homopolymers (disulphide bonded) after 10 min exposure to EDTA at 37°C (21). Strikingly, our observations are that the kinetics of appearance of the collapsed portions of the SCCS correlates well with this report, because it is only after 10 min incubation with EDTA that the first pentalaminar structures do appear in several platelets. It has been reported very recently that activated platelets release a protein disulfide isomerase activity (13). Because EDTA induces the release of the platelet granule content, it is possible that such an enzyme may participate in the formation of homopolymers of GPIIb. A low level of labeling was also found on Lowicryl thin sections using polyclonal anti-GPIIIa and monoclonal anti-GPIIb-IIIa complex-dependent antibodies. Whether this labeling resulted only from the presence of remaining non dissociated GPIIb-IIIa complexes or also from retention of free dissociated GPIIIa in the SCCS is not clear. Nevertheless, when gold-labeled polyclonal antibodies against free GPIIb and free GPIIIa were added simultaneously with EDTA, almost only the anti-GPIIb antibodies were present in the collapsed portions of the SCCS (Fig. 6, B and C), clearly indicating a differential location of the two separate subunits along the SCCS after EDTA induced dissociation of the GPIIb-IIIa heterodimer complex. These results suggest that GPIIb subunits assemble to "bridge" the canaliculi, leaving most of the free GPIIIa outside the bridge, essentially in the dilated portions of the SCCS. The mechanism by which dilation of the EDTA treated SCCS occurs remains unknown, but seems not to be related to the presence of the GPIIb-IIIa complex since in each case studied dilation occurred even when collapse did not.

Striking similarities have been reported between the platelet pentalaminar structures induced by EDTA and the typical pentalaminar cytoplasmic organelle of epidermal Langerhans cells, the so-called "Birbeck granule" (1, 9). The mechanisms leading to the appearance of Birbeck granules in Langerhans cells remain unknown. However, recent data have shown that exposure of Langerhans cells to 25 mM EDTA induces unzipping of the Birbeck granules, i.e., gradual transformation of the rodlike Birbeck granules to blown-up vesicles (1). This suggests that divalent cations might be important for the interlinking of the Birbeck granule's membranes. Thus, EDTA appears to have an opposite effect, according to whether it is incubated with human platelets ("zipping" of the SCCS) or with human Langerhans cells ("unzipping" of the Birbeck granules).

In conclusion, we have demonstrated that (a) the ultrastructural changes induced by EDTA in the human platelet plasma membrane and SCCS, i.e., the appearance of pentalaminar structures, are directly dependent on dissociation of the $\alpha_{IIb}\beta_3$ integrin and (b) at least GPIIb participates in bonding of the limiting membranes. Because we found no platelet adhesion molecule in these structures so far, we suggest that homopolymers of GPIIb may at least participate in the bridging of adjacent SCCS membranes and induce their collapse.

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