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DEMONSTRATION OF A NEW GLYCOPROTEIN Ib-RELATED COMPONENT IN PLATELET EXTRACTS PREPARED IN THE PRESENCE OF LEUPEPTIN

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The water-soluble protein glycocalicin is generated during platelet lysis by a proteolytic attack on the integral membrane glycoprotein GP Ib. However, only small amounts of glycocalicin are formed when platelets are solubilized by 1% Triton X-100. Crossed immunoelectrophoresis of such extracts using an antiserum to glycocalicin, shows a continuous immunoprecipitate consisting of two peaks, one representing glycocalicin and the other GP Ib. When leupeptin was present during solubilization, subsequent immunoelectrophoresis revealed yet another GP Ib-related component represented by a third, slow-migrating peak of the immunoprecipitate. During incubation of platelets with dibucaine followed by solubilization in the presence of leupeptin, a gradual transformation of this new form of GP Ib into the previously defined one took place prior to the formation of glycocalicin. An increase followed by a decrease in the agglutination response of the platelets to bovine von Willebrand factor occurred concomitant with these transformations. SDS-polyacrylamide gel electrophoresis of Triton X-100 extracts of platelets did not reveal any difference in the size of GP Ib whether or not leupeptin had been present during the solubilization.

Introduction

The water-soluble protein glycocalicin is formed during lysis of platelets concomitant with the disappearance of GP Ib from the membrane [1,2]. This process is inhibited by agents such as EDTA, *N*-ethylmaleimide, iodoacetamide and leupeptin, which has led us to believe that glycocalicin is derived from GP Ib by proteolysis [1,2] after activation of a thiol-containing, calcium-dependent protease present in platelets [3–6]. The observation by Berndt and Phillips [7] that extensively purified calcium-dependent protease added exogenously to intact platelets catalyzed the same conversion, strongly supports this assumption. GP Ib is considered to consist of two disulfide-linked polypeptide chains termed GP Ibα and GP Ibβ [8], and considerations of molecular weights lead to the conclusion that glycocalicin represents almost the full length of the GP Ibα-chain [1,2]. Recent studies by Clemetson et al. [9] comparing tryptic peptides of glycocalicin and the GP Ibα-chain strongly support this idea.

In all instances studied so far, splitting of GP Ib to glycocalicin has been accompanied by a deterioration and loss of the ability of human platelets to become agglutinated by bovine factor VIII-related protein [1,2,7,10,11,12]. We have also observed that incubation of platelets in a regular buffer for a week at 4°C led to an increase fol-

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The nomenclature for platelet membrane glycoproteins (GP) in this paper is adapted from Phillips and Poh Agin [8] with the exception that GP III is referred to as GP IIIa.

lowed by a decrease in the agglutination response [10]. Coller [12] recently made the same observation on incubation of platelets with the local anesthetic dibucaine for a short period of time. During this process GP Ib was lost from the platelet membrane [12]. The increase in agglutination is unexplained at present. Therefore, incubation of platelets with dibucaine followed by analyses of the protein pattern would be of interest. The crossed immunoelectrophoresis technique could be used for this. It requires, however, that any further alterations which might occur during the subsequent solubilization of the platelets have to be prevented. Previous studies of platelet extracts prepared by solubilization in buffers containing 1% Triton X-100 have shown that glycocalicin is present in only small amounts [1,2]. This means that GP Ib is protected from extensive degradation by the use of the detergent. We wanted to test this concept and, further, to see if a more specific inhibitor of the calcium-dependent protease, such as leupeptin, would be even more effective than Triton X-100 in preventing transformations during the solubilization of platelets. The present paper describes such studies, and explains how the information obtained has been used to demonstrate a previously unrecognized GP Ib-related component present in platelet extracts prepared in the presence of relevant protease inhibitors and its transformation into the previously known GP Ib and glycocalicin during incubation of the platelets with dibucaine.

Materials and Methods

Commercial materials. Triton X-100 was purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.; leupeptin either from Sigma or from Protein Research Foundation, Japan; dibucaine (cinchocaine hydrochloride) from CIBA, Horsham, U.K.; agarose type HSA from Litex, Glostrup, Denmark; CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals, Uppsala, Sweden; and heparin from Nyegaard & Co., Oslo, Norway. Dibucaine at 10 mM was prepared by addition of the relevant amount of dibucaine to the standard Tris-buffered saline, dissolution of the dibucaine by addition of the minimal amount of HCl required, and readjustment with NaOH to neutral pH. One-tenth dilution in the same buffer gave pH 7.4.

Washing solution and standard buffers. These contained 148 mM NaCl, 5 mM glucose, 0.6 mM EDTA and 20 mM Tris-HCl (pH 7.4, 280 mosM) as washing solution for platelets, 148 mM NaCl and 20 mM Tris-HCl (pH 7.4) as Tris-buffered saline for resuspension of washed platelets, and 38 mM Tris and 100 mM glycine (pH 8.7) plus Triton X-100 as standard extraction buffer. Unless stated otherwise, the concentration of Triton X-100 was 1% (v/v).

Human platelets. Human platelets were obtained and isolated as previously described [1,13]. Platelets from platelet concentrates were most often stored in washing solution overnight at 4°C and thereafter subjected to an extra wash in the Tris-buffered saline prior to resuspension in the same buffer to avoid contamination with EDTA from the washing solution. Platelets were counted in a Thrombocounter model C.

Antisera. Rabbit antiserum to purified human glycocalicin was obtained and adsorbed as described previously [1] except for one important point which accidentally was omitted from that description. During purification of glycocalicin, an overnight dialysis of the KCl-extract against 0.2 mM EDTA and 15.3 mM NaN_3 was followed immediately by a dialysis against distilled water at 4°C for another 3–4 h. The glycocalicin-containing precipitate is formed after this shift of dialysis fluid. Antibodies to whole platelet proteins were obtained as previously described [14].

Bovine factor VIII-related protein (bovine von Willebrand factor). The protein was purified as described [10]. In the present agglutination studies protein at an intermediate stage of purification was used at a concentration corresponding to 1 μg/ml of the highly purified protein.

Thrombin-Sepharose 4B. This was prepared with highly purified bovine thrombin as previously described [15] with 10 mg thrombin coupled per ml of wet gel.

Heparin-Sepharose 4B. This was prepared from CNBr-activated Sepharose 4B according to the manufacturer's instructions using 25 000 IU heparin per g activated Sepharose powder.

Crossed immunoelectrophoresis. Crossed-immunoelectrophoresis in the presence of Triton

X-100 was performed as described elsewhere [1,14].

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed as described by Laurell [16] and modified by Weeke [17].

SDS-polyacrylamide gel electrophoresis. Electrophoresis using gels containing urea and EDTA was performed as described in detail elsewhere [18].

Agglutination of platelets. Agglutination was followed in a Payton aggregometer equipped with a W + W recorder. Calibration was such that undiluted and one-half-diluted platelet supensions represented a difference in absorbance corresponding to 50 chart divisions or 5 mV on the recorder. Platelet suspensions for agglutination experiments (450 μl) normally contained 300 000–500 000 platelets/μl. Agglutination was induced by addition of bovine factor VIII-related protein (50 μl) and the response was calculated as chart divisions during the first 2.5 min of agglutination, and presented as percentage of that of a control.

Total protein. Total protein was determined by the Bio-Rad protein assay essentially as described by Bradford [19].

Affinity chromatography of GP Ib. Affinity chromatography from platelet extracts was performed according to conventional procedures. GP Ib adsorbed to thrombin-Sepharose was eluted by 0.5 M arginine in the regular Triton/Tris/glycine buffer, and the eluate was passed through heparin-Sepharose. Binding of GP Ib to thrombin has been described previously [20,21].

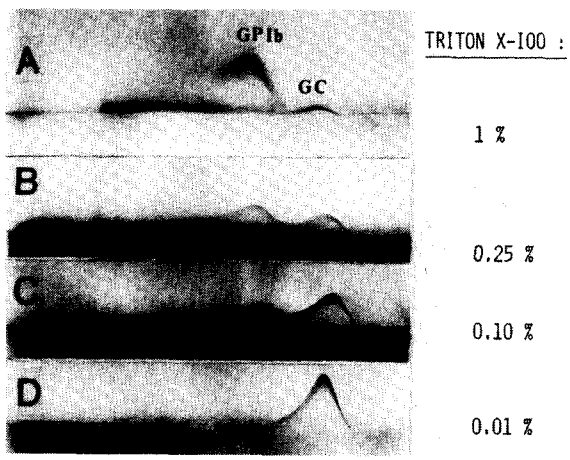


Fig. 1. Effect of variation in the concentration of Triton X-100 used for extraction of platelets as to the relative amounts of GP Ib and glycocalicin in the extracts. 200 μl Triton X-100-containing standard Tris-glycine buffer (pH 8.7) were added to each of four aliquots of sedimented, washed platelets ($1.75 \cdot 10^9$ cells per aliquot). The concentrations were 0.01, 0.10, 0.25 and 1.0%, respectively. The platelets were suspended and shaken at 20°C for 30 min followed by centrifugation at 8000 × g for 4 min. The supernatants were subjected to crossed immunoelectrophoresis using antiserum to glycocalicin. Migration of glycocalicin (GC) in the first dimension was 3.8 cm in all electrophoreses.

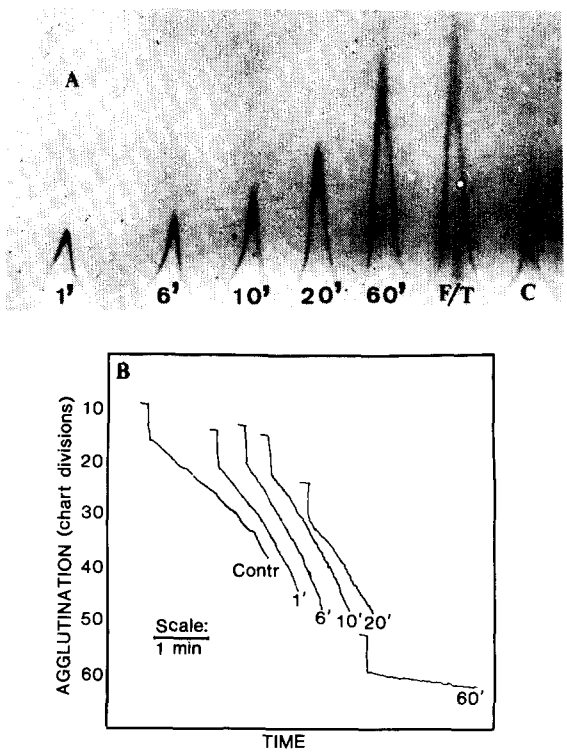
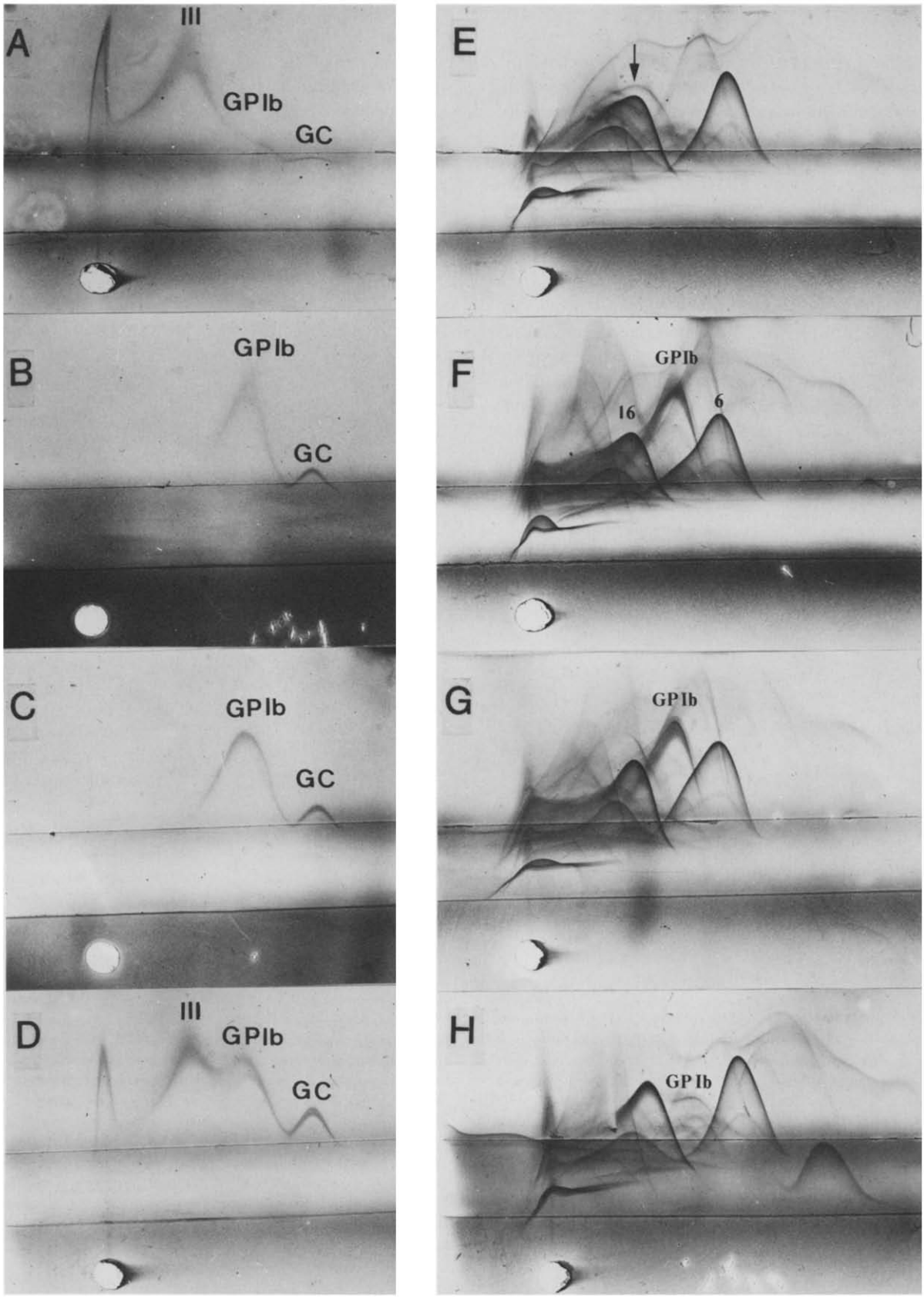


Fig. 2. Effect of dibucaine on (A) the production of extracellular glycocalicin, and (B) the agglutination of the platelets by bovine factor VIII-related protein. Washed platelets were suspended in Tris-buffered saline (pH 7.4) and dibucaine was added in the same buffer (final concentration 1 mM) followed by incubation at 37°C (approx. $3 \cdot 10^9$ cells/ml). After 1, 6, 10, 20 and 60 min, two aliquots were removed. (A) One of the aliquots was centrifuged immediately at 8000 × g for 2 min and the supernatant used for 'rocket'-immunoelectrophoresis against anti-glycocalicin antiserum. (B) The other aliquot was diluted with eight volumes of the Tris-buffered saline and tested for agglutination by a standard dose of bovine factor VIII-related protein. In Fig. 2A the rocket termed C represents the supernatant of untreated control platelets, whereas F/T represents the supernatant of an aliquot of such platelets after these have been lysed by freezing and thawing. F/T is considered to represent the maximal amount of glycocalicin which could be produced.


A
III
GPIb
GC
B
GPIb
GC
C
GPIb
GC
D
III
GPIb
GC
E
F
GPIb
16
6
G
GPIb
H
GPIb

Results

Crossed immunoelectrophoresis of extracts of platelets solubilized by Triton X-100 at the conventional concentration of 1% using an antiserum against glycocolicin, shows a continuous immunoprecipitate forming two peaks (Fig. 1A). These represent glycocalicin and GP Ib, respectively [1,2]. Fig. 1 demonstrates that the degree of transformation of GP Ib into glycocalicin during such extractions was strongly dependent on the concentration of the detergent. At very low concentrations of Triton X-100, practically all of the extracted GP Ib was present in the form of glycocalicin (Fig. 1D). At higher concentrations, the transformation was inhibited (Fig. 1B–C) and nearly prevented at the concentration of 1% (Fig. 1A).

Incubation of human platelets with dibucaine at 37°C led to the formation of glycocalicin in the extracellular medium (Fig. 2A). This incubation also induced an immediate increase in the agglutination response of the platelets towards bovine factor VIII-related protein (Fig. 2B). However, upon prolonged incubation this response decreased and was finally lost (Fig. 2B). This was accompanied by a shift in the aggregometer base line (Fig. 2B) reflecting shape change and swelling of the platelets. Comparisons of Fig. 2A and Fig. 2B showed that an agglutination response at least as strong as the normal one could be observed even after a significant amount of glycocalicin was produced (i.e., 20 min of incubation). However, agglutination was not observed after the maximal production of glycocalicin was obtained (60 min or more).

Solubilization of platelets in a buffer which contained leupeptin as an inhibitor of the calcium-dependent protease in addition to 1% Triton X-100, led to crossed immunoelectrophoresis patterns significantly different from those obtained with extracts prepared with Triton X-100 alone (Fig. 3A compared to 3B). A third GP Ib-related component of the platelet extract was clearly observed (peak III of Fig. 3A). The corresponding immunoprecipitate demonstrated an identity with the faster-migrating GP Ib component (Fig. 3A). At the same time, a 'rocket-shaped' immunoprecipitate originating from the application well was also seen (Fig. 3A). Such an effect on the immunoprecipitate patterns was also seen with iodoacetamide, *N*-ethylmaleimide, EDTA and EGTA but not with *N*-carbobenzoxy-L-glutamyl-L-tyrosine or phenylmethylsulfonylfluoride used instead of leupeptin (data not shown). Addition of leupeptin to already prepared extracts had no effect (data not shown).

In the crossed immunoelectrophoresis pattern of extracts prepared in the absence of inhibitor using antibodies to whole platelet proteins, the immunoprecipitate representing GP Ib is located midway between that of the GP IIb/IIIa-complex (number 16) and albumin (number 6) (Fig. 3F). After platelet solubilization in the presence of leupeptin, this immunoprecipitate could not be recognized (Fig. 3E). In some experiments, a new immunoprecipitate could be seen above and slightly to the left of the GP IIb/IIIa-precipitate (marked by an arrow in Fig. 3E). This position corresponds to that of the most slowly migrating component of Fig. 3A.

Incubation of platelets with dibucaine was then

Fig. 3. Effects of leupeptin during solubilization of platelets in 1% Triton X-100, and of a 20 min incubation of platelets with dibucaine, on the GP-Ib-related components of platelet extracts. Crossed immunoelectrophoresis of extracts using (A–D) antiserum to glycocalicin or (E–H) antibodies to whole platelet proteins. (A and E) Platelets incubated only with buffer (control platelets), leupeptin present during solubilization. (B and F) Control platelets, leupeptin absent. (C and G) Dibucaine-incubated platelets, leupeptin absent. (D and H) Dibucaine-incubated platelets, leupeptin present. Washed platelets suspended in Tris-buffered saline (pH 7.4) were divided into two portions. One portion was diluted with buffer containing dibucaine (final concentration 1 mM), and the other with buffer only. Both were incubated at 37°C. After 20 min, two aliquots were removed from each portion and centrifuged at $8000 \times g$ for 2 min. The sedimented platelets of one of the aliquots were then solubilized in Tris-glycine buffer (pH 8.7) which contained both 1% Triton X-100 and 4.2 mM leupeptin, whereas the platelets of the other aliquot were solubilized in the same buffer containing Triton X-100 only ($9 \cdot 10^9$ cells/ml). Solubilization was performed by stirring at 20°C and centrifugation at $8000 \times g$ for 2 min. Crossed immunoelectrophoresis of the extracts was then performed according to the scheme above (A–H). For technical reasons, the extracts used in B and C were diluted 1/3 as compared to the other extracts prior to electrophoresis. GC, glycocalicin; 6, albumin, and 16, GP IIb/IIIa complex. Agglutination response of the dibucaine-incubated platelets was checked as in Fig. 2.

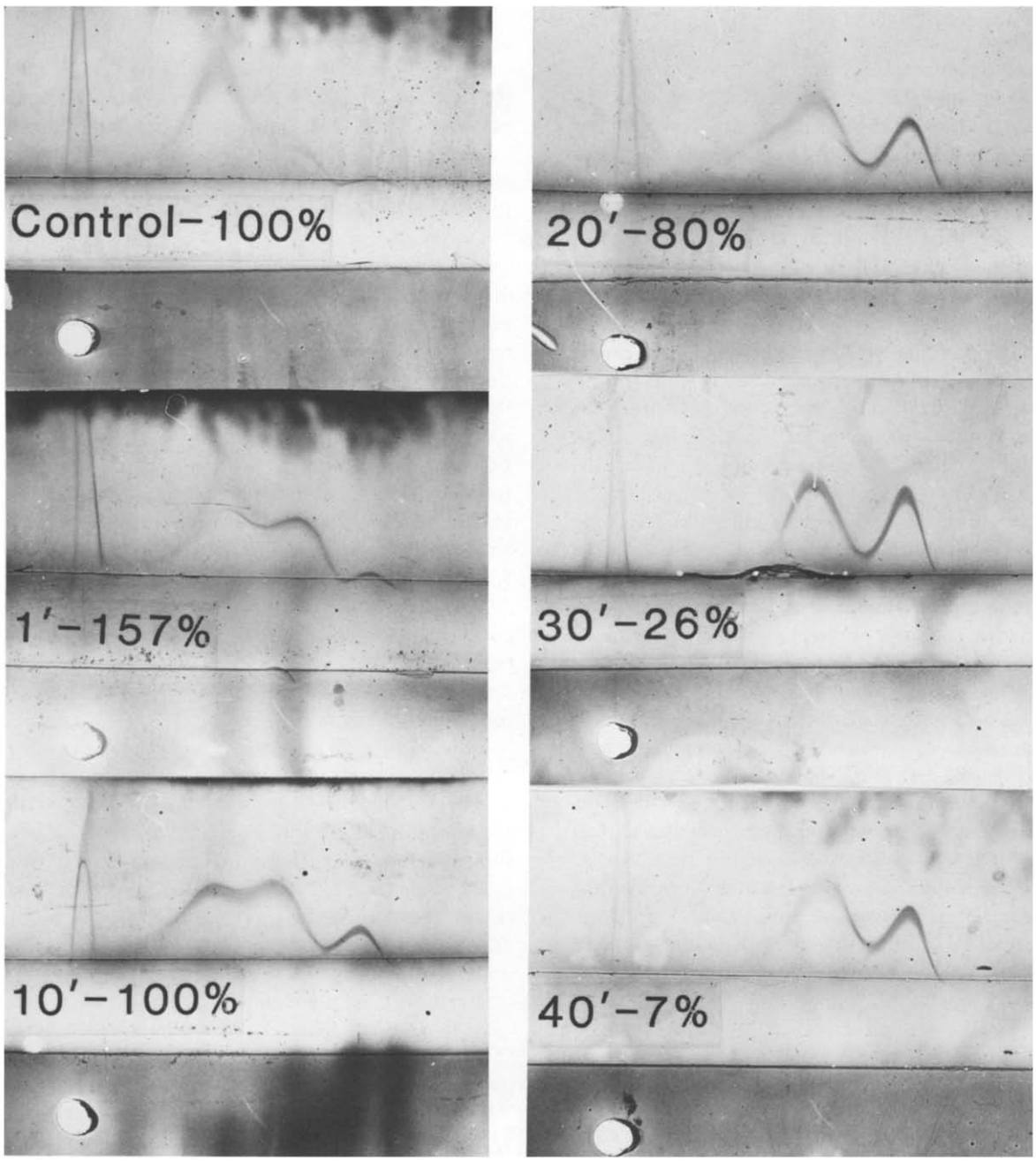


Fig. 4. Effect of dibucaine-incubation of platelets. The transformation of the GP-Ib-related components as a function of incubation time. The numbers stated on each plate represent incubation time in min, and agglutination response as percentage of that of control platelets, respectively Washed platelets were suspended in Tris-buffered saline (pH 7.4) and dibucaine was added to a final concentration of 1 mM followed by incubation at 37°C ($4.5 \cdot 10^9$ cells/ml). After 1, 10, 20, 30 and 40 min of incubation, respectively, two aliquots were removed. One aliquot was centrifuged at $8000 \times g$ for 2 min, the sedimented platelets solubilized by 1% Triton X-100 in Tris-glycine buffer pH 8.7 which also contained 4.2 mM leupeptin ($1.35 \cdot 10^{10}$ cells/ml) and the extract used for crossed immunoelectrophoresis against antiserum to glycocalicin. The other aliquot was diluted in Tris-buffered saline (pH 7.4) to $5 \cdot 10^8$ cells/ml, and tested for its agglutination response to a standard dose of bovine factor VIII-related protein. Washed platelets suspended in the Tris-buffered saline alone, served as control. Aliquots from this suspension were treated as described for the dibucaine-incubated platelets.

studied using a fixed incubation time chosen such that the agglutination response was not significantly reduced (i.e., 20 min, see Fig. 2). The results are shown in Fig. 3. The use of leupeptin during solubilization of the platelets revealed that a considerable amount of material corresponding to the regular GP Ib peak had formed during the incubation (Fig. 3D compared to 3A, and Fig. 3H compared to 3E). If leupeptin were absent during the solubilization, the immunoprecipitate patterns were the same whether the platelets had been incubated with dibucaine or not (Fig. 3C compared to 3B; and Fig. 3G compared to 3F). These observations can be explained by the following hypothesis. Dibucaine induces a transformation of the GP Ib-related material in the direction from the most slow-moving to the more fast-moving components, and the same process occurs during lysis in the absence of specific inhibitors of the calcium-dependent protease. Further, 1% Triton X-100 alone stops this process only between GP Ib and glycocalicin, whereas leupeptin, in addition to the Triton X-100, stops the process also between the most slowly moving components.

Fig. 4 demonstrates that incubation of platelets with dibucaine prior to solubilization, induced transformations of the GP Ib-related components in a continuous and time-dependent manner accompanied by variations in the agglutination response (see also Fig. 2). Dibucaine had no effect if this were added to the leupeptin-containing Triton X-100 solution used for solubilization and thus was present only at the solubilization step (data not shown). This means that dibucaine acts prior to the extraction, which is also evident from Fig. 2A where the extracellular amount of glycocalicin was measured.

When Triton X-100 extracts were analyzed by SDS-polyacrylamide gel electrophoresis, GP Ib showed the same electrophoretic mobility whether the extracts had been prepared in the presence or in the absence of leupeptin (Fig. 5A and Fig. 5B). Further, this mobility was the same as that of GP Ib from platelets dissolved directly in SDS (data not shown). Also, GP Ib which had been separated from the main platelet glycoproteins by affinity chromatography, showed the same electrophoretic mobility when the Triton X-100 extracts from which it originated had been prepared in the presence or absence of leupeptin (Fig. 5B, b and c). The only clear-cut difference concerning platelet glycoproteins observed in this system relates to the α-granule glycoprotein thrombospondin. Electrophoresis of a mixture of such extracts revealed a doublet representing thrombospondin (Fig. 5B, a). Separate experiments showed that the upper thrombospondin band, which stemmed from the leupeptin-containing extract, corresponded to that observed when platelets were dissolved directly in SDS, whereas the lower one represented thrombospondin of the extract prepared in the absence of leupeptin (data not shown).

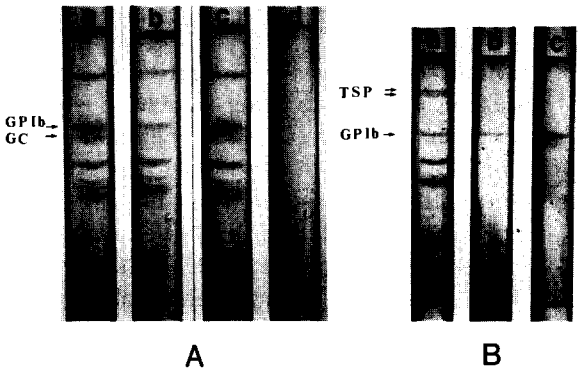


Fig. 5. SDS-polyacrylamide gel electrophoresis of platelet extracts prepared with 1% Triton X-100 in the presence or in the absence of leupeptin. (A) The gels represent extracts with (a) leupeptin absent during extraction, (b) leupeptin present during extraction and (c) leupeptin added after extraction, (d) represents leupeptin as such. Two aliquots of sedimented, washed platelets were solubilized by 1% Triton X-100 (10^{10} cells/ml) in Tris-glycine buffer (pH 8.7); one aliquot in the presence and the other in the absence of leupeptin (4.2 mM). After extraction, leupeptin (4.2 mM) was also added to a portion of the extract which was prepared in the absence of leupeptin. All three solutions (extracts) were then analysed by SDS-polyacrylamide gel electrophoresis as unreduced samples. Total protein applied to each gel was 113 μg. A sample of leupeptin (0.1 μmol) was applied to a fourth gel (same amount as applied with the extracts). After electrophoresis, the gels were stained with the periodic acid-Schiff reagent for glycoproteins. (B) The gels represent (a) a 1 : 1 mixture of platelet extracts prepared in the presence or absence of leupeptin, (b) GP Ib obtained by affinity chromatography on batches of thrombin-Sepharose and heparin-Sepharose, from an extract prepared in the presence of leupeptin, and (c) GP Ib obtained as in (b) but from an extract prepared in the absence of leupeptin. Experimental conditions as in (A). GC, glycocalicin; TSP, thrombospondin.

Discussion

Previous studies strongly indicate that platelet glycocalicin represents a water-soluble split product of the α-chain of the integral membrane glycoprotein GP Ib [1,2,7,9], and that this splitting results from the activation of a calcium-dependent protease [3] activated during platelet lysis [1,2]. The present study shows that such a transformation also takes place during lysis of platelets by Triton X-100 at low concentrations of the detergent, but only to a very limited extent at a concentration of 1%. The reason for this preventive effect of Triton X-100 at this concentration is not known, but it may be suggested that the enzymatic conversion is largely dependent on organized structures disassociated by the detergent. Endogenous inhibitors may also be involved [5]. Rapid denaturation of the enzyme in the presence of Ca^{2+} may play a role as well [6].

Further, the present paper shows that if leupeptin, or other inhibitors of the calcium-dependent protease, is present during solubilization, a third GP Ib-related component is observed. We have also confirmed the observation by Coller [12] that incubation of platelets with dibucaine leads to the formation of glycocalicin extracellularly, and, like Coller [12], we interpret this by the assumption that dibucaine initiates an activation of the calcium-dependent protease. Our data show that a transformation between the three GP Ib-related components takes place in the direction from the most slowly moving to the more quickly moving ones when platelets are incubated with dibucaine. Further, it shows that the presence of leupeptin during extraction will stop this process at any stage.

The true nature of the new GP Ib-related component (the peak III material of Fig. 3A) is not known at present. However, the observation that SDS-polyacrylamide gel electrophoresis did not reveal any difference in molecular weight of GP Ib whether the extracts were prepared in the presence or in the absence of leupeptin, is relevant to this problem. Alternative explanations may be considered. First, peak III may represent the 'true' GP Ib and the previously defined GP Ib peak a proteolytic degradation product. However, if so, the difference in molecular size would be too small to be detected in our SDS-electrophoresis system and, at the same time, the difference in electric charge should be great enough to explain the significant difference in electrophoretic mobility seen on crossed immunoelectrophoresis. Alternatively, the alteration in electrophoretic mobility might represent an aggregation of the hypothetical 'unproteolyzed' GP Ib molecules. For various reasons, such as the defined peak form of the immunoprecipitate, this is considered less likely, however.

Another explanation would be that peak III represents a noncovalent complex between GP Ib and some other material, and that dissociation of the complex occurs as a result of a proteolytic cleavage of this other material. As a non-covalently linked complex, this would also dissociate in SDS. If this is correct, the complex may be formed during the actual process of extraction, or, even more interesting, it may reflect an association of GP Ib to some structure in the intact platelet which is preferentially attacked by the protease. Such an association might be of physiological significance. The possibility that GP Ib may be associated to cytoskeletal structures in the intact platelet is of special interest. In this respect it should be noted that White [4] and Truglia and Stracher [5] have reported that calcium-dependent platelet protease is able to cleave the actin-binding protein of platelets. This may be related to an irreversible disruption of microfilament assembly in the platelet cytoskeleton [5]. The problem is being studied along these lines.

Much evidence indicates that the presence of GP Ib on the surface is a prerequisite for the agglutination of intact platelets or platelet membranes by bovine factor VIII-related protein (bovine von Willebrand factor) [23]. If GP Ib functions as receptor for this protein [2,22], one may ask which of the GP Ib-related components it is that reflects the receptor function. As a water-soluble split product, glycocalicin is of little interest in this respect. As to the other two forms of the GP Ib-related material observed here, it is to be noted that the immediate increase in agglutination response upon incubation of platelets with dibucaine is accompanied by some transformations of the peak III material to the previously defined GP Ib. However, a considerable amount of this latter GP Ib is seen even after the agglutination

response is almost lost. One possible explanation would be that the peak III material most closely reflects the receptor form of GP Ib, but that some reduction in the number of receptors would be favourable for the agglutination. In this respect it should be kept in mind that the agglutination by itself does no represent a physiological reaction, even if it reflects a binding of the von Willebrand factor to its receptor. It should also be noted that the number of GP Ib molecules per platelet, which can be calculated from the amount of glycocalicin produced during platelet lysis [1], is much higher than the number of receptors, which can be calculated from kinetic data [24,25].

The protease assumed to be responsible for the transformations described here has not been identified. Sakon et al. [6] have claimed that platelets contain at least two different calcium-activated proteases with different sensitivities towards Ca^{2+}. The concentration of leupeptin used throughout these studies (4.2 mM) was chosen arbitrarily. Later experiments have shown that this is much in excess of the minimal concentration required to observe the peak III component, as an effect was observed even at 0.042 mM.

One would not expect that solubilization of platelets in other commonly used detergents would conserve the peak III form of GP Ib unless mild non-ionic detergents and effective inhibitors of the protease are applied. It is therefore not surprising that the existence of this form of GP Ib has not been observed previously.

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