Biochimica et Biophysica Acta, 1070 (1991) 20–26

BBAMEM 75396

Platelet glycoprotein IIb–IIIa is associated with 21-kDa GTP-binding protein

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(Received 31 May 1991)

Key words: Platelet; Concanavalin A receptor; GP IIb–IIIa; G-protein; Signal transduction

Platelet membrane glycoprotein IIb–IIIa has been widely studied in the last years because of its role as an activation-dependent, adhesive protein receptor. Recently we demonstrated that occupancy of glycoprotein IIb–IIIa-receptor sites by specific ligands exerts an inhibitory effect on platelet responses induced by mild stimulation, leading us to suppose that this event may interact with activation pathways. Although the mechanisms of signal transduction in human platelets are not completely elucidated, the hypothesis that GTP-binding proteins are involved is generally accepted. Our results demonstrate that platelet ConA receptors, known to be located mainly on GP IIb–IIIa, are able to bind [^{35}S]GTPγS; the GTP-binding activity is specific and is due to the association with the receptors of two G-proteins, with apparent molecular masses of 25 and 21 kDa, respectively. After the purification of GP IIb–IIIa, a glycoprotein complex electrophoretically pure was obtained that was still associated with a GTP-binding activity, migrating in SDS-polyacrylamide gel electrophoresis as a narrow band of about 21 kDa.

Introduction

It is widely accepted that platelet membrane glycoprotein (GP) IIb–IIIa plays a crucial role in platelet function [1]. This glycoprotein complex is an activation-dependent receptor for adhesive proteins with specific binding sites able to recognize the RGD sequence in fibrinogen, fibronectin and von Willebrand factor [2,3]. RGD-containing peptides inhibit binding of adhesive proteins to activated platelets, with concomitant inhibition of platelet function, mainly aggregation [4–6]; in fact, thrombastenic platelets, whose molecular defect is due to the absence of glycoprotein IIb–IIIa in their plasma membrane or to the exposition of an unfunctional glycoprotein complex [7], fail to bind fibrinogen [8], fibronectin and von Willebrand factor [9,10], and show dramatically impaired aggregation. There is also evidence to suggest that this membrane component actively participates in platelet activation, in fact, the binding of RGD containing ligands, including fibrinogen, determines: (i) structural modification of the receptor [11], (ii) expression of a neoantigenic site on the cell surface [12], (iii) clustering of platelet associated GP IIb–IIIa, [13] and (iv) Na^+/H^+ exchange after epinephrine stimulation [14]. In addition, the GP IIb–IIIa complex represents a high-affinity binding site for calcium ions on the platelet surface [15] and much evidence suggests that it is implicated in Ca^{2+} movements after activation: purified GP IIb–IIIa, incorporated onto the surface of liposomes, displays Ca^{2+}-channel properties when its Ca^{2+}-dependent integrity is maintained [16]. Moreover agonist-induced Ca^{2+} flux is significantly modified in the presence of specific ligands for the GP IIb–IIIa complex [17,18]. Recently, we confirmed these observations [19], and demonstrated that both fibrinogen and fibronectin have an inhibitory effect on protein phosphorylation and affect cAMP content in mildly activated platelets [20,21]. We also observed that fibrinogen, as well as an anti-GP IIb–IIIa monoclonal antibody and GRGDS peptide, inhibit inositol phosphates production in low-thrombin-activated platelets. These results suggest that

Abbreviations: GP, glycoprotein; RGD, Arg-Gly-Asp; cAMP, adenosine 3',5'-cyclic monophosphate; GRGDS, Gly-Arg-Gly-Asp-Ser; GTP, guanosine 5'-triphosphate; ConA, concanavalin A; WGA, wheat germ agglutinin; GTPγS, guanosine 5'-[γ-thio]triphosphate; BCA, bicinchoninic acid; PMSF, phenylmethylsulphonylfluoride; DTT, dithiothreitol.

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the occupancy of GP IIb–IIIa represents a signal which may interact with the pathways of signal transduction in activated platelets. However, the mechanisms by which GP IIb–IIIa displays these effects are far from being elucidated. It is well known that the fibrinogen receptor associates with cytoskeletal components during platelet aggregation [22], suggesting that new functional connections are present in activated platelets with respect to resting cells. It is generally accepted that a number of regulatory GTP-binding proteins function as transducers in signal transduction pathways, from the agonist receptors to second messenger generating systems [23,24], and it is well known that adenylate cyclase activity is mediated in human platelets, as well as in other cell types, by distinct trimeric G-proteins [25,26]. In addition, it has been hypothesized that the receptor-linked hydrolysis of phosphoinositides by activation of phospholipase C (PLC) is mediated by a GTP-binding protein termed "Gp" [30]. Another group of GTP-binding proteins, with molecular masses ranging from 20 to 30 kDa, has been described in different mammalian cells including platelets [27–29]. The physiological role of these proteins is unknown, even if it has been recently indicated that some "small" G proteins are involved in activation and inactivation of platelets. Other cellular events in which G-proteins may be involved in platelets are ion channel regulation [24], secretion [31] and phospholipase A_2 activation [32]. Considering the possible involvement of GP IIb–IIIa in the mechanisms of signal transduction, we investigated whether G-proteins are associated with GP IIb–IIIa.

Materials and Methods

Reagents

Sephacryl S200, Sephacryl S300, heparin-Sepharose and ConA-Sepharose were from Pharmacia-LKB; leupeptin, GTPγS, AMP, CTP, ADP and the lectins WGA and ConA conjugated with peroxidase were from Sigma; BCA and Lubrol PX were obtained from Pierce. The liquid scintillation mixture used was Riatron, Kontron. [α-^{32}P]GTP (110 TBq/mmol) was purchased from Amersham International; [^{35}S]GTPγS (50 TBq/mmol) was from Du Pont.

Purification of platelet ConA-receptor(s) from human platelets

Fresh human platelet concentrates were obtained from a local blood bank. Platelets were collected by centrifuging 10–15 concentrates at $1200 \times g$ for 10 min. Cells were washed three times with Tris-HCl buffer, pH 7.4, (20 mM Tris, 150 mM NaCl) containing 5 mM glucose and 3.3 mM EDTA, and finally resuspended in modified Hepes-Tyrode buffer, pH 7.4, (10 mM Hepes, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$) (buffer A). The final platelet count was adjusted to 10^9 cells/ml with the same buffer Platelet lysis was obtained by adding 1 vol. of buffer A containing 0.2 mM PMSF, 20 μM leupeptin, 2 mM $CaCl_2$ and 2% Lubrol PX. The mixture was shaken in ice for 10 min, then unsolubilized particles were pelleted by centrifuging at $10000 \times g$ for 10 min. Lubrol PX-lysed platelets ($100 \cdot 10^9$) were loaded on a 10-ml ConA-Sepharose affinity column equilibrated with Tris-HCl buffer, pH 7,4 (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3) containing 0.1% Lubrol PX (buffer B). The sample was eluted with the same buffer at a flow rate of 1 ml/min. After extensive washing with buffer B, the ConA-bound glycoproteins were eluted with the same buffer containing 0.1 M α-methyl-D-mannopyranoside at a flow rate of 0.5 ml/min, and 1-ml fractions collected. α-Methyl-D-mannopyranoside-eluted fractions were pooled, dialyzed against ten-fold diluted buffer B and finally, concentrated using PEG 20000. The protein concentration was determined by the BCA method [34].

Purification of platelet GP IIb–IIIa

GP IIb–IIIa was purified from Lubrol-PX-lysed platelets by subsequent chromatographies on ConA-Sepharose, Heparin-Sepharose and Sephacryl S300, essentially as described by Fitzgerald et al. [35] using buffer B as eluent. Protein concentration in the collected fractions was determined by BCA assay at every purification step [34] and the GP IIb–IIIa distribution in the fractions eluted from Sephacryl S300 was determined by SDS-PAGE and silver staining.

GTP γS-binding assay

Binding of [^{35}S]GTPγS to ConA-retained glycoproteins was assessed essentially as described by Northup et al. [36] with some modification. ConA-retained material (60–100 μg of proteins) was incubated with [^{35}S]GTPγS (6×10^5 cpm) at 30 °C for 5 h in 0.5 ml Tris-HCl buffer, pH 8.0 (20 mM Tris, 100 mM NaCl), containing 1 mM EDTA, 1 mM DTT, 30 mM $MgCl_2$, and 0.1% Lubrol PX (buffer C). In some experiments, 0.5 mM GTPγS, ATP, CTP or AMP were also present. Samples were then applied to a 14-mm YM10-Amicon membrane (cut-off 1×10^4 Da) and quickly filtered using an Amicon Micropartition System MPS1. Filters were rinsed twice with 0.5 ml of the same buffer and then dried; the associated radioactivity was counted in 10 ml scintillation mixture using a Kontron Betamatic V scintillation counter. This procedure routinely yielded blank values (no protein or denaturated protein) of less then 0.5% of the total radioactivity applied on the membrane.

Electrophoresis and Western blotting procedures

In order to obtain a good resolution of membrane glycoproteins or G-proteins, SDS-PAGE was per-

formed in either 5–15% or 10–20% polyacrylamide gradient gels, respectively, according to Laemmli [37]. Proteins were stained with Coomassie brilliant blue or with silver using the method of Merril et al. [38]. In some experiments electrophoresed proteins were transferred to a nitrocellulose membrane in a Bio-Rad apparatus, at 150 mA for 1 h using a 20 mM Tris, 192 mM glycine buffer containing 20% methanol. For the detection of [α-^{32}P]GTP binding to transferred proteins, the nitrocellulose membranes were washed three times for 1 h in buffer C containing 0.2% Tween 20 (not Lubrol PX) and then incubated overnight at room temperature with [α-^{32}P]GTP ($1.6 \cdot 10^5$ cpm/ml) in the same buffer. After extensive washing, the membranes were dried and then exposed to X-ray film (Hyperfilm MP, Amersham) at $-70\,^{\circ}$C. After development of the autoradiograms, the proteins were visualized by staining with Amido black (0.02% in methanol/water/acetic acid, 9:9:2).

Resolution of the GTP-binding polypeptides associated with platelet ConA-receptors

Resolution of GTP-binding polypeptides was carried out as described by Wang et al. [39]. ConA-retained material or purified GP IIb–IIIa complex (0.3–0.4 mg of protein) was incubated with [^{35}S]GTPγS ($6 \cdot 10^5$ cpm) at $30\,^{\circ}$C for 5 h in buffer C containing 1 μM GTPγS and then applied to a Sephacryl S200 column (1.6 × 90 cm) equilibrated with the same buffer containing 0.02% NaN_3. Elution was carried out at constant flow rate (60 ml/h) collecting 2-ml fractions. Protein content in the eluent was monitored at 280 nm and radioactivity was counted in each fraction, diluting 0.2-ml aliquots with 5 ml of scintillation fluid.

Results

Physical association between ConA receptor(s) and GTP-binding protein(s)

When Lubrol-PX-solubilized human platelets were applied to a ConA-Sepharose affinity column, about 7–8% of total platelet proteins where found to bind to the gel and eluted with the haptenic sugar α-methyl-mannoside. SDS-PAGE, performed under reducing and non-reducing conditions, revealed that GP IIb–IIIa was the main constituent (50% of total proteins) of the ConA-bound fraction. Fibrinogen, thrombospondin and GP IV were also present, together with some minor unidentified contaminant proteins (data not shown).

The ConA-bound fraction was tested for its ability to bind GTP using the non-hydrolyzable substrate analog [^{35}S]GTPγS (Fig. 1). The ConA-retained material effectively bound labelled GTPγS, and the binding activity was specific in that it was totally inhibited by 0.5 mM GTPγS, as previously shown by Northup et al. [35]; equal amounts of AMP, CTP or ATP did not display any significant effect (Fig. 1). When the crude ConA-bound fraction was submitted to SDS-PAGE, transfer onto nitrocellulose paper and incubation with [α-^{32}P]GTP, two [α-^{32}P]GTP-binding polypeptides, with apparent molecular masses of 25 and 21 kDa, respectively, were observed after autoradiography (Fig. 2). This experiment confirmed the results reported above, and also showed that GTP-binding activity was due to the presence of two 'small' G-proteins physically associated to platelet ConA receptor(s).

This technique is unsuitable for revealing the presence of trimeric G-proteins, since these proteins loose

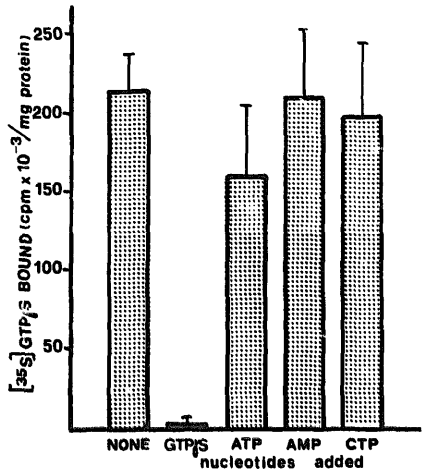

Fig. 1. [^{35}S]GTPγS-binding to platelet ConA receptor(s). The binding of [^{35}S]GTPγS to Lubrol-PX-solubilized ConA receptor(s) was evaluated both in the absence and presence of 0.5 mM GTPγS, ATP, AMP or CTP, as described in the text. Results are mean ± S.D. of three experiments.

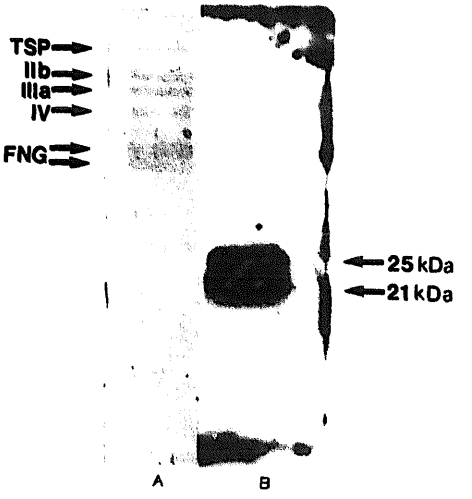

Fig. 2. [α-^{32}P]GTP binding to platelet ConA receptor(s). Proteins contained in ConA-bound material were separated by SDS-PAGE on a 10–20% polyacrylamide-gradient gel, transferred to nitrocellulose paper and then incubated with [α-^{32}P]GTP (for details see text). The figure shows the nitrocellulose paper stained with amido-black (A) and its autoradiography (B). These results are representative of four different experiments.

their ability to bind GTP under denaturing conditions [23]. To verify if some ($\alpha\beta\gamma$)G-proteins are associated with platelet ConA receptor(s), the Con-A-bound fraction from Lubrol-PX-solubilized human platelets was incubated with 1 μM [^{35}S]GTPγS and then analyzed by gel-filtration on a Sephacryl S200 column, as described by Wang et al. [38]. Two main peaks of radioactivity were recovered, eluted with approximate molecular masses of 110–130 kDa (peak A) and 20–25 kDa (peak B) (Fig. 3). About 75% of total bound radioactivity was recovered in peak A; peak B accounted for the remnant bound radioactivity. The volume corresponding to a molecular mass of 40 kDa, in which Gα-GTP should be eluted, was free of radioactivity, showing that no trimeric G-protein is associated with platelet ConA receptor(s).

Spectrophotometric analysis at 280 nm showed the presence of four peaks: the second corresponded to peak A of radioactivity, while only a weak absorbance at 280 nm was detected in peak B. Electrophoretic analysis and densitometric scanning of silver-stained gels revealed that in the fractions corresponding to peak A about 85% of the eluted IIb and IIIa glycoproteins were present, together with fibrinogen and some other minor unidentified components (Fig. 3). Among the other fractions, only that corresponding to the void volume of the column contained traces of the two glycoproteins (data not shown).

Some low mass, unidentified proteins were present in the other fractions. Western blotting of electrophoresed samples and ConA/WGA-peroxidase staining of nitrocellulose membrane confirmed the presence of most glycoproteins IIb and IIIa in peak A. To better characterize the molecular mass of GTP-binding activities present in both radioactive peaks eluted from Sephacryl S200, aliquots, containing the same amounts of proteins, evaluated by the BCA method, were electrophoresed onto a 10–20% polyacrylamide gel, blotted onto a nitrocellulose membrane and then incubated with [α-^{32}P]GTP. These experi-

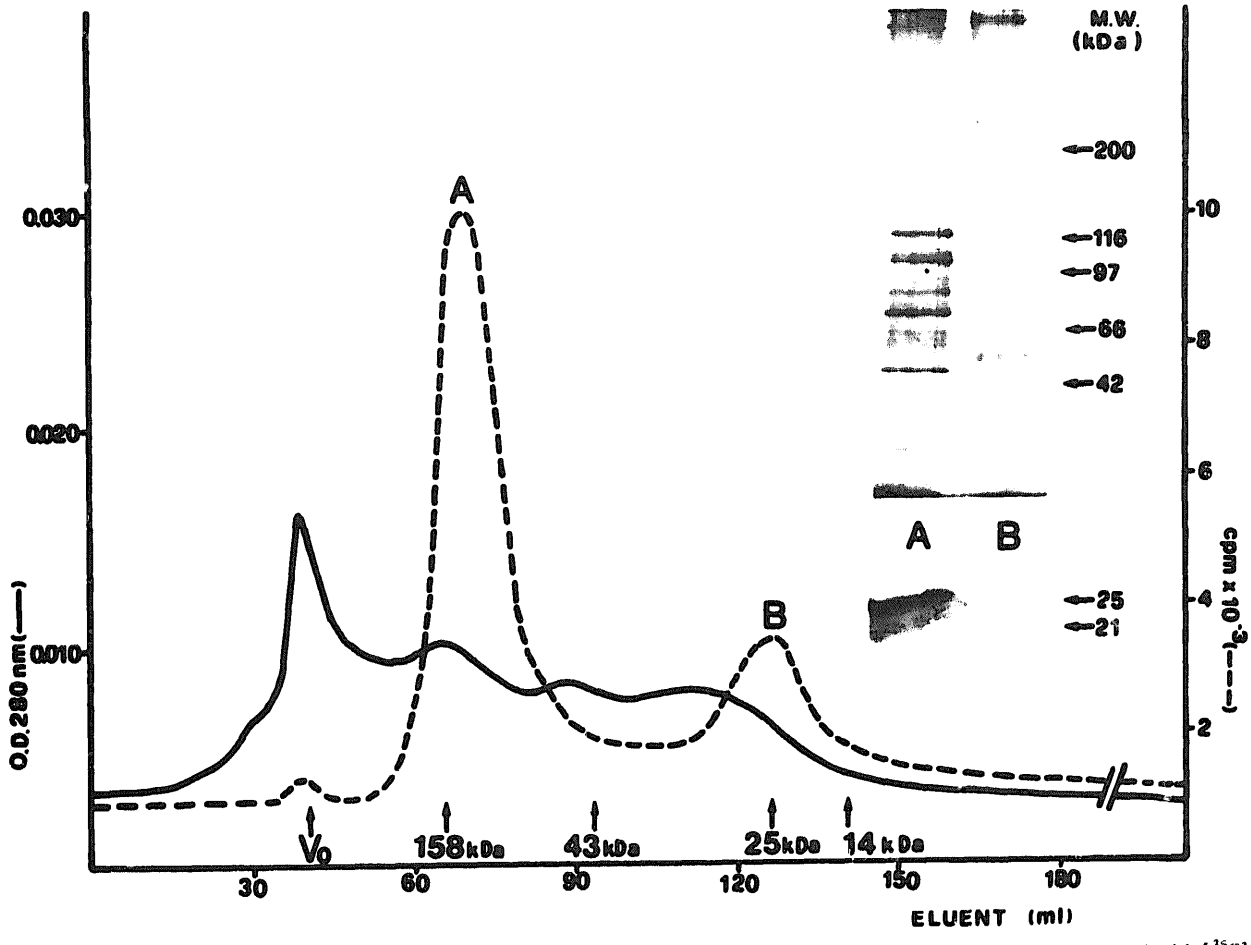

Fig. 3. Resolution of GTP-binding polypeptides associated with platelet ConA receptors. ConA-retained material preincubated with [^{35}S]GTPγS was chromatographed on Sephacryl S200 (for details see text). Figure shows the absorbance at 280 nm (——) and the radioactivity content (- - - - -) of the eluted fractions. The inset shows the silver-stained protein profile (upper part) and the autoradiography after blotting and [α-^{32}P]GTP incubation (lower part) of the two radioactive peaks A and B recovered from Sephacryl S200. These results are representative of three experiments performed under identical experimental conditions.

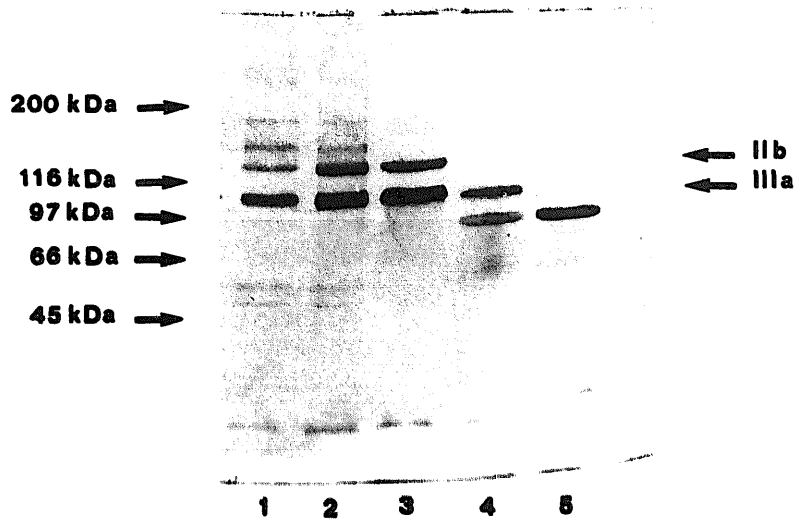

Fig. 4. SDS-PAGE of the fractions eluted from Sephacryl S300. Fractions eluted from Sephacryl S300 as reported in Material and Methods, were loaded on a 5–15% gradient gel under reducing conditions. The gel was stained with silver. Similar amount of proteins, as determined by the BCA method, were loaded on the gel. The reported experiment is representative of five identical experiments.

ments showed the same two GTP-binding proteins, of apparent molecular masses 25 and 21 kDa, respectively, associated with ConA-retained material to be present in peak A (Fig. 3). It is noteworthy that these GTP-binding proteins did not dissociate or only partly dissociated from the receptors after incubation with [^{35}S]GTPγS. No GTP-binding activity was detectable after blotting of the other fractions eluted from the column. Peak B, eluted with a volume corresponding to a mass of 20–25 kDa, also lost its ability to bind [α-^{32}P]GTP after blotting (Fig. 3); this peak could be due to the same 21-kDa G-protein that lost its ability to bind [α-^{32}P]GTP after dissociation from ConA receptors, or to some other GTP-binding polypeptide unable to renature after SDS-PAGE.

Physical association between platelet membrane GP IIb–IIIa and 21 kDa GTP-binding protein

GP IIb–IIIa was purified as described in Materials and Methods from Lubrol-PX-solubilized platelets, as reported previously [35]. This technique allowed 5 fractions to be obtained which were analyzed for their protein content by SDS-PAGE and silver staining. Fractions 2 and 3 showed the presence of GP IIb–IIIa in about 1:1 ratio and accounted for 75–80% of the total eluted GP IIb–IIIa. These fractions contained similar amounts of the glycoprotein complex; however, only fraction 3 did not contain any detectable contaminant (Fig. 4). All the other fractions contained low amounts of both glycoproteins in different ratios, together with relatively high amounts of contaminants.

Both fractions 2 and 3 were tested for their ability to bind [^{35}S]GTPγS: these experiments showed a significantly higher [^{35}S]GTPγS-binding activity in fraction 2 where some contaminants were detected (Table I). Western blotting of fraction 3, containing electrophoretically pure GP IIb–IIIa, followed by incubation with [α-^{32}P]GTP revealed that only one narrow band of GTP-binding activity was present, with an apparent molecular mass of 21 kDa (Fig. 5). GTP-binding activity associated with purified GP IIb–IIIa was further characterized, filtering fraction 3 on Sephacryl S200 after incubation with [^{35}S]GTPγS. The radioactivity elution profile was similar to that obtained with crude ConA receptors: most radioactivity eluted in a narrow peak with an apparent mass of 110–130 kDa. A minor radioactive peak was also present and eluted with an apparent mass of 20–25 kDa. Both peaks of radioactivity eluted from Sephacryl S200 were analyzed

TABLE I

Glycoprotein IIb–IIIa content and [^{35}S]GTPγS-binding activity of fractions eluted from Sephacryl S300

The GP IIb–IIIa content of fractions 2 and 3 from Sephacryl S300, containing the two glycoproteins in about 1:1 ratio is reported, expressed as arbitrary optical units obtained by densitometric scanning of 5–15% polyacrylamide-gradient gels stained with silver (see Fig. 4). The percentage of GP IIb–IIIa with respect to the total stained material in the two fractions is also reported. [^{35}S]GTPγS-binding activity of fractions 2 and 3 evaluated as described in Materials and Methods, is expressed as cpm/mg protein, evaluated by the BCA method. Results are representative of three similar experiments.

Fraction from Sephacryl S300	Glycoprotein IIb–IIIa (Arbitrary optical units)	Glycoprotein IIb–IIIa (% content)	[^{35}S]GTPγS bound (cpm/mg protein)
2	284173	87	142200
3	282110	98	90400

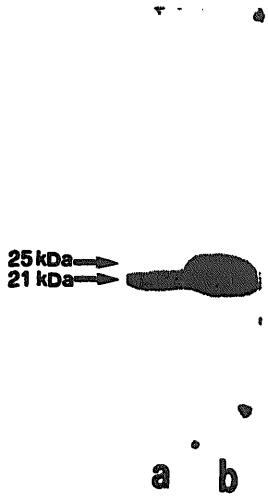

Fig. 5. Association of a GTP-binding activity with purified GP IIb–IIIa. Purified platelet membrane GP IIb–IIIa (a) and crude ConA receptors (b) were electrophoresed on a 10–20% gradient gel under reducing conditions. Similar amounts of proteins were loaded on the two lanes; proteins were then transferred to nitrocellulose membrane and incubated with [α-^{32}P]GTP. Autoradiography of blotted platelet membrane GP IIb–IIIa (a) and crude ConA receptor(s) (b) is shown. The results are representative of three different experiments.

for their [α-^{32}P]GTP-binding activity. The 110–130 kDa peak showed the presence of the same 21 kDa GTP-binding activity found associated with purified GP IIb–IIIa (data not shown). Likewise, it was observed in experiments performed with ConA-retained material that the [α-^{32}P]GTP-binding activity eluted from Sephacryl S200 with an apparent mass of 20–25 kDa was unable to bind [α-^{32}P]GTP after SDS-PAGE and blotting onto nitrocellulose paper.

Discussion

Platelet membrane glycoprotein GP IIb–IIIa has been widely studied in recent years: since it is the fibrinogen receptor in activated platelets, it supports platelet aggregation and allows correct haemostasis. To date, most research has focused on the characterization of its structure and binding properties to adhesive proteins. Little is known about the mechanisms by which GP IIb–IIIa regulates its own functions and it is not clear whether it has an influence on the activation pathways involved in the biochemical events of signal transduction. At present it is generally accepted that the transduction of signals from membrane receptors to second messenger-generating systems is provided by GTP-binding proteins. ConA receptors, known to be located mainly on GP IIb–IIIa [33], were purified from Lubrol-PX-solubilized platelets, and it was proved that they are able to bind [^{35}S]GTPγS. The GTP-binding activity was found to be specific and due to the association of the receptors with two G-proteins with apparent molecular masses of 25 and 21 kDa, respectively, as demonstrated by [α-^{32}P]GTP binding to ConA-retained material after SDS-PAGE and Western-blotting. After incubation with [^{35}S]GTPγS and filtration on Sephacryl S200, no trimeric G-protein was found in the ConA-retained material using the method described by Wang at al. [39], although it is possible that interaction with ConA during the purification procedure could induce the dissociation of some G-protein from platelet ConA receptors. Even if GP IIb–IIIa is the main ConA receptor on the platelet surface [33] in our experimental conditions the complex represented no more then 50% of total proteins retained by the ConA-Sepharose column. In fact, electrophoretic analysis of ConA-retained material showed the presence of GP IV, fibrinogen and thrombospondin together with GP IIb–IIIa. The main target of this research was to demonstrate the association between platelet membrane GP IIb–IIIa and GTP-binding signal transducers. GP IIb–IIIa complex was purified from the ConA-retained fraction, as described by Fitzgerald et al. [35], obtaining a fraction that contained pure GP IIb–IIIa, as judged by SDS-PAGE and silver staining. Incubation with [α-^{32}P]GTP after SDS-PAGE and Western blotting revealed that purified GP IIb–IIIa was still associated with the 21 kDa GTP-binding activity. Although GTP-binding activities are also probably linked to some contaminants eluted in fraction 2 from Sephacryl S300, it still appears that a GTP-binding activity copurifies with the apparently pure GP IIb–IIIa present in fraction 3. Electrophoretic resolution of GTP-binding polypeptides showed that GTP-binding proteins, which did not dissociate either from the ConA receptors or from purified GP IIb–IIIa after [^{35}S]-GTPγS incubation and filtration on Sephacryl S200, were still able to bind [α-^{32}P]GTP, whereas the GTP-binding activities eluted with apparent mass ranging from 20 to 25 kDa, which were presumably dissociated from the receptors, could not. This result might be due to the presence of G-proteins associated with ConA-retained material and purified GP IIb–IIIa, with similar masses but different behaviour in the experimental conditions used, although the hypothesis that it could be the same protein cannot be excluded. The presence of 21 and 25 kDa G-proteins in human platelets has been previously demonstrated [27–29,40], but at present their biological role is far from being elucidated. Our previous research demonstrated that the occupancy of GP IIb–IIIa with specific ligands influences the transduction mechanisms of the activation signal [20,21]. Fibrinogen, an anti GP IIb–IIIa monoclonal antibody and a synthetic peptide containing the RGD sequence, all cause inhibition of the processes following activation with low doses of thrombin. These results suggest that binding of adhesive proteins to GP IIb–IIIa is not only a fundamental event in processes of adhesion and aggregation, but also a mechanism

that reduces platelet responsiveness to low-intensity stimulation. How the binding of specific ligands to GP IIb–IIIa influences the events that follow platelet activation is still obscure. At present the role of the 21 kDa G-protein associated with platelet fibrinogen receptor can be only a matter of speculation, although if one considers the regulatory role of GP IIb–IIIa ligands [20,21], it is possible that the GP IIb–IIIa complex is involved in mechanisms of signal transduction.

Acknowledgements

This work was supported by grants from the Ministero Pubblica Istruzione and the C.N.R, Italy.

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