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PURIFICATION AND CHARACTERIZATION OF THE HUMAN PLATELET FIBRINOGEN RECEPTOR, GpIIb/IIIa COMPLEX

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Abstract

Platelet GpIIb/IIIa is a member of the integrin family of structurally related adhesion receptors, and adhesive proteins, such as fibrinogen. Ligand binding requires platelet activation by an agonist to convert the receptor to a state where it is capable to bind the ligand. In addition to this a modification, induction of the surface expression and ligand-binding function of an additional pool of GpIIb/IIIa derived from α-granuler membranes and/or surface-connecting open canalicular system are established. Fibrinogen binding to platelets appears to be important for platelet aggregation. Platelet aggregation is essential for normal hemostasis and also plays a role in thrombosis.

In this study, we purified GpIIb/IIIa on a single step from normal human platelets by GRGDSP-affinity chromatography. In addition, studies on the characterization of the binding properties to GIIb/IIIa of the specific ligands were carried out with the purified GpIIb/IIIa complex.

Key words: *GpIIb/IIIa, affinity chromatography, platelet fibrinogen receptor.*

İnsan Trombosit Fibrinojen Reseptörü GpIIb/IIIa Kompleksinin Saflaştırılması ve Tanımlanması

İntegrin ailesinin bir üyesi olan GpIIb/IIIa, yapısal olarak fibrinojen gibi adeziv proteinlerle ilişkili trombosit adezyon reseptörüdür. Ligand bağlamak için bir agoniste ihtiyaç duyar ve yapısında konformasyonel bir değişikliğe uğrar. Bu modifikasyona ek olarak, trombosit α-granüllerinden ve yüzey bağlantılı açık kanalikül̈er sistemden GpIIb/IIIa havuzuna katkı sağlanarak, ligand-bağlama fonksiyonu ve yüzey expresyonu artar. Trombositlere fibrinojen bağlanması trombosit agregasyonu için önemlidir. Trombosit agregasyonu normal hemostas için temeldir ve trombozda da rol oynamaktadır..

Bu çalışmada, sağlıklı insan trombositlerinden GRGDSP-afinite kromatografisi ile GpIIb/IIIa tek adımda saflaştırıldı. Saflaştırılmış GpIIb/IIIa kompleksi ile, GpIIb/IIIa'ya spesifik ligandlarla bağlanma özelliklerinin çalışmaları yapıldı.

Anahtar Kelimeler: *GpIIb/IIIa, afinite kromatografisi, trombosit fibrinojen reseptörü.*

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INTRODUCTION

Human glycoprotein (Gp)IIb/IIIa is a member of the integrin family of cell adhesion receptor (1) and the most abundant membrane protein on the platelet surface (2). The integrin family consists of heterodimeric molecules composed of a series of α- and β-subunits. Specific combinations of these subunits form receptors with unique specificities for various ligands (3) (Fig.1.).

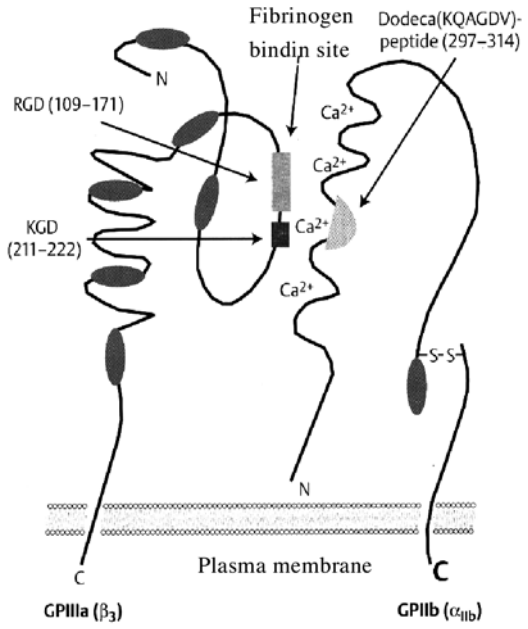


Figure 1. Structure of GpIIb/IIIa (4).

On nonstimulated platelets, GpIIb/IIIa is incapable of binding most of its soluble macromolecular ligands (5) but after exposure of the cells to appropriate agonists, the receptor undergoes a conformational change (6), as a consequence of inside-outside signal transmission (7). This makes platelets to interact with several plasma protein ligands, including fibrinogen, fibronectin, von Willebrand factor and vitronectin which are essential for platelet aggregation (8). Recent evidence has demonstrated a direct and specific interaction between this glycoprotein and fibrinogen (3).

Glanzmann's thrombasthenia helped to elucidate the role of GpIIb/IIIa receptor in platelet aggregation (9). This inherited disease, characterized by recurrent bleeding, arises from genetic defects that result in an absence of or a large decrease in functional GpIIb/IIIa receptors (9).

The recognition specificity of the GpIIb/IIIa receptor is defined by two peptide sequences (10). Arg-Gly-Asp (RGD) sequence is identified in fibrinogen, von Willebrand factor (vWF), fibronectin and vitronectin (11).

The activation of GpIIb/IIIa is associated with stimulation of several metabolic pathways, changes in the shape of platelets, activation of the GpIIb/IIIa, and induction of platelet coagulant

activity (3). GpIIb/IIIa is essential for platelet aggregation that leads to hemostatic plug formation and pathological thrombus formation (12). The clinical significance of GpIIb/IIIa is further highlighted by the fact that platelet aggregation, mediated by this receptor, may be primarily responsible for life-threatening re-occlusion in coronary vessels initially cleared of an infarct by the application of thrombolytic therapy or surgical intervention (13). The biological linkage between GpIIb/IIIa and platelet adhesion and aggregation can be at least partially understood based on the highly regulating binding activity of this integrin and its ligand specificity (14). Purified GpIIb/IIIa is fundamental to understand the molecular events that lead to induction of GpIIb/IIIa binding activity in activated platelets, the molecular basis for GpIIb/IIIa binding specificity and seconder events triggered by ligand binding.

Several groups have reported on specific purification methods of GpIIb/IIIa (12,15-17), there is, however, considerable disagreement about the properties of purified GpIIb/IIIa bioligical activity. It was therefore the aim of this study to purify and characterize of GpIIb/IIIa from human platelets.

In this study, the platelet receptor GpIIb/IIIa complex was purificated by affinity chromatography on the heptapeptide Gly-Arg-Gly-Asp-Ser-Pro coupled to Sepharose (GRGDSP-Sepharose). An extract of human platelets was applied to this affinity matrix. Specific proteins were eluted and eluted fractions were migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). GpIIb/IIIa showed a mobility corresponding to a molecular weight of 116 kilo Dalton (kDa). As controls in flow cytometry activation and ligand binding specificity of the GpIIb/IIIa complex were detected with either monoclonal antibody (MoAb) CD61-fluorescence isothyocyanate (FITC) specific to GpIIIa or fibrinogen-FITC specific to GpIIb/IIIa complex.

EXPERIMENTAL

Chemicals

H-GlyArg-Gly-Ser-Pro-OH (Chalbiochem), Tyrode's buffer, CNBr-Activated Sepharose (Pharmacia), bovine serum albumin (BSA), fetal calf serum (FCS), phenylmethylsulfonyl fluoride (PMSF), phosphate buffered saline (PBS) (Sigma), CD61-FITC (Becton-Dickinson), fibrinogen (Fraction I, Sigma) were used and all other chemicals were reagent grade from Sigma.

Apparatus

Clinical centrifuge (Hettich Universal/K2S), Vortex (Maximix II), pH meter (Orion Research, ionanalyzer/Model 47A), Spectrophotometer (Shimadzu UV-120-02), Ultracentrifuge (Sigma 3K30, Germany), Colon (1x7cm, Pharmacia), Vacuum pomp (KNF), Peristaltic pomp (Pharmacia), Electrophoresis supply (EC250-90), flow cytometry (Becton Dickinson FACs).

Preparation of crude extract from platelets

Three units (3U) of isolated platelets from thromboapheresis were washed three times with Tyrode's buffer containing 1 mM $CaCl_2$. After centrifugation at 800x g (Clinical centrifuge, Hettich Universal/K2S), the pellet (5 ml) was suspended in the cold solution of 10 mM phosphate, 150 mM NaCl, 1mM $CaCl_2$, 1 mM $MgCl_2$ phosphate-buffered saline (PBS), pH 7.3 containing 50 mM octylthioglucoside and 3 mM phenylmethylsulfonyl fluoride (PMSF). The suspension

was incubated for 15 min at 4°C and centrifuged at 30,000x g (Ultracentrifuge, Sigma 3K30, Germany) for 20 min at 4°C. The supernatant contained the crude platelet extract.

Preparation of affinity matrix

a. 3.5 g of CNBr-Activated Sepharose (Pharmacia-Biotech) was washed with 1 mM HCl for 15 min using gooch crucible.
b. 100 mg GRGDSP-peptide was dissolved in 50 ml of 0.1 M $NaHCO_3$+ 0.5 M NaCl buffer, pH 8.3, and incubated and mixed gently overnight at 4°C (12).
c. a and b materials were further incubated overnight at 4°C.
d. The suspension was washed with 0.1 M $NaHCO_3$+0.5 M NaCl buffer, pH 8.3.
e. 50 ml of 0.1 M Tris-HCl, pH 8, was added to above suspension and incubated for two hours at room temperature.
f. This suspension was washed with three different buffers as follows:
   1. 0.5 M NaCl+0.1 M $NaCH_3COO$ pH 4,5,
   2. 0.5 M NaCl+ 0.1 M Tris-HCl pH 8,5
   3. 0.5 M NaCl+0.1 M $NaCH_3COO$ pH 4,5

PBS which contains 150 mM NaCl, 1mM $MgCl_2$, 9.5 mM Na_2HPO4, 9.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 1 mM PMSF was added to the affinity matrix and this mixture was incubated with crude platelet extract overnight at 4°C (Pharmacia-Biotech).

Purification of GpIIb/IIIa

After overnight incubation at 4°C the affinity material was packed into a column (1x17 cm, Pharmacia) and washed with 50 ml of equilibrium buffer (PBS containing 1 mM PMSF and 25 mM octylthioglucoside) at 4°C. The proteins bound to the matrix were eluted with 20 ml of the equilibrium buffer containing 1 mg/ml GRGDSP-peptide. Fractions (1ml) were collected, at a flow rate of 0.96 ml/min (15). Protein concentrations were determined by the method of Lowry (18).

Electrophoresis

SDS-PAGE was performed in 10% polyacrylamide slab gels, containing 15% (w/v) glycerol. Buffer used contained 10% SDS and 1.5 M Tris-HCl , pH 8.8. Samples were prepared in 0.4 ml of β-mercaptoethanol under reducing condition. Protein bands were made visible by staining with Coomassie Brillant Blue (14). Molecular weight markers used were; myosin, (205 kDa); β-galactosidase, (116 kDa); phosphorylase b, (97 kDa); bovine serum albumin, (66 kDa); ovalbumin, (45 kDa); and carbonic anhydrase, (29 kDa).

Flow cytometry

A Becton Dickinson FACS-Scan flow cytometry analyzer was used to quantify fluorescence (excitation wavelenght:488 nm and emission wavelenght:530 nm) at the single-cell level, and data were analyzed using Cell quest version 3.3 (Becton Dickinson) software.

Binding specificity of purified GpIIb/IIIa

Washed polystyrene micro beads were added to purified GpIIb/IIIa and incubated with slight mixing overnight at 4°C to establish the coating with polystyrene micro bead (19). Fluorescence isothyocyanate (FITC) labeled BSA and monoclonal antibody (MoAb) CD61-FITC (specific ligand for GpIIIa) were used as negative and positive controls, respectively. Fibrinogen-FITC (Fg-FITC, specific ligand for GpIIb/IIIa complex) was used. GpIIb/IIIa coated micro beads were incubated with FITC-labeled proteins in various concentrations and binding were evaluated by flow cytometry (20).

RESULTS AND DISCUSSION

The predominant membrane protein in human platelets, is the GpIIb/IIIa complex. This receptor has apparent molecular masses of 142 kDa for GpIIb and 95-115 kDa for GpIIIa, respectively (16,21). Upon reduction with β-mercaptoethanol the molecular mass of GpIIIa raises by 4-10 kDa. This identification is substantially supported by binding studies with two antibodies that have been shown to bind to 114-kDa protein (17).

In this study, the platelet receptor was identified by affinity chromatography on the heptapeptide Gly-Arg-Gly-Asp-Ser-Pro coupled to Sephearose (GRGDSP-Sepharose). The crude extract of washed human platelets was applied to this affinity matrix, and specifically bound components were eluted with a solution of the GRGDSP peptide. As shown in Fig.2, GpIIb/IIIa complex migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a mobility corresponding to the molecular weight of 116 kDa.

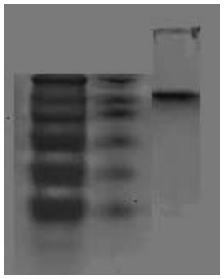
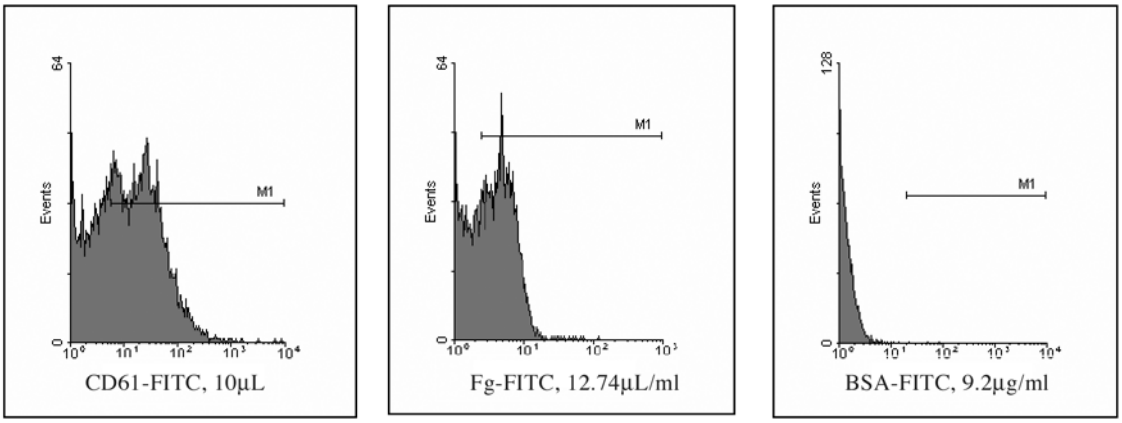


Figure 2. Electrophoretic analysis of GpIIb/IIIa by SDS-PAGE. a: molecular weight markers: myosin, (205 kDa); β-galactosidase, (116 kDa); phosphorylase b, (97 kDa); bovine serum albumin, (66 kDa); ovalbumin, (45 kDa); and carbonic anhydrase, (29 kDa). b: crude platelet extract before affinity chromatography**.** c. GpIIb/IIIa complex obtained after affinity chromatography.

To analyze the binding properties of the receptor, the platelet GpIIb/IIIa complex obtained by affinity chromatography on the Arg-Gly-Asp- (RGD) containing heptapeptide was analyzed for its binding to different FITC-labeled ligands. For binding experiments of GpIIb/IIIa complex coated micro beads and bound FITC labeled ligands were measured by flow cytometry (Fig.3).



A B C

Figure 3. FACS analysis of MoAb CD61-FITC, Fg-FITC and BSA-FITC binding to GpIIb/IIIa complex. A. MoAb CD61-FITC binding to GpIIb/IIIa complex coated micro beads B. Fg-FITC binding to GpIIb/IIIa complex coated micro beads. C. BSA-FITC binding to GpIIb/IIIa complex coated micro beads was not performed.

In addition to provide information about receptor specificity, the affinity chromatography procedure represented in Fig.3 provides a convenient way to purify the receptor, with this procedure high degree of purity and reasonable yield were achieved in a single step and that a total extract of platelets can be used as the starting material. Moreover, the purified receptor retains its biological activity with regard to binding to specific ligands.

We could show that the 116 kDa band recognized by an antibody against GpIIIa, MoAb CD61-FITC, FITC-labeled fibrinogen and FITC-labeled BSA as were used positive and negative controls, respectively. This binding was specific, because FITC-labeled fibrinogen and MoAb CD61-FITC showed binding activities but BSA-FITC did not. Therefore our purified receptor was specific for its ligands.

A growing understanding of the role of platelets in cardiovascular disease has led to the development of a new class of drugs to control platelet function. Inhibition of the final common pathway of platelet aggregation, with the GpIIb/IIIa receptor as a target, is a particularly logical strategy in drug targeting.

CONCLUSION

This purification method gives important advantages to the other purification methods described in the literature. The advantages are:

1. In a single step, highly purified GpIIb/IIIa could be obtained.
2. Sustainable and stable biological activity are established.
3. Low concentration of platelets could be used as the starting material.

4. High concentration of GpIIb/IIIa could be obtained.
5. Matrix regeneration allows the matrix to be used repeatedly.
6. Purified platelet GpIIb/IIIa complex activity in different diseases could give important results to the place of GpIIb/IIIa complex in mechanism of diseases, in stimulation of platelets and it platelet aggregation. Also drug effects and drug targeting studies could be performed easily with this purification procedure.

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