Mechanisms of platelet activation by a stimulatory antibody: cross-linking of a novel platelet receptor for monoclonal antibody F11 with the FcγRII receptor

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The mechanisms by which a stimulatory monoclonal antibody (mAb), called mAb F11, induces granular secretion and aggregation in human platelets have been characterized. Fab fragments of mAb F11, as well as an mAb directed against the platelet FcγRII receptor (mAb IV.3) were found to inhibit mAb F11-induced platelet secretion and aggregation, indicating that the mAb F11 IgG molecule interacts with the FcγRII receptor through its Fc domain and with its own antigen through its Fab domain. The mAb F11 recognized two platelet proteins of 32 and 35 kDa on the platelet membrane surface, as identified by Western blot analysis. We purified both proteins from human platelet membranes using DEAE-Sepharose chromatography followed by mAb F11 affinity chromatography. When added to platelet-rich plasma, the purified proteins dose-dependently inhibited mAb F11-induced platelet aggregation. The purified protein preparation also competitively inhibited the binding of ^{125}I-labelled mAb F11 to intact platelets. The N-terminal 26 amino acid sequences of both the 32 and 35 kDa proteins were identical and contained a single unblocked serine in the N-terminal position. When digested with *N*-glycanase, the 32 and 35 kDa proteins were converted into a single ~ 29 kDa protein, indicating that these two proteins are derived from the same core protein but differ in their degree of glycosylation. Internal amino acid sequence analysis of the F11 antigen provided information concerning 68 amino acids and suggested two consensus phosphorylation sites for protein kinase C (PKC). The phosphorylation by PKC of the isolated F11 antigen was observed following stimulation by phorbol 12-myristate 13-acetate. Databank analysis of the N-terminal and internal amino acid sequences of the F11 antigen indicated that the N-terminal sequence exhibited the highest degree of similarity to the variable region of the α-chain of human T-cell receptors (TCR). In contrast, the F11 internal sequences did not exhibit any similarity to the TCR. Our results demonstrate that the F11 antigen is a novel platelet membrane surface glycoprotein which becomes cross-linked with the FcγRII receptor when platelets are activated by the stimulatory mAb F11. These mechanisms may be relevant to the production of immune thrombocytopenia by platelet-activating antibodies.

INTRODUCTION

A number of agonists activate platelets by interacting with specific receptors on the platelet surface. Several monoclonal antibodies (mAbs) have been identified that specifically bind to platelet membrane protein components and stimulate platelet secretion and aggregation [1–4]. Of these antibodies, most are directed against GPIIb/IIIa, GPIV (CD36) and the CD9 antigen [3,5–9]. The fibrinogen receptor, GPIIb/GPIIIa, has been well characterized and cloned [10,11]. Platelet CD36 has been purified and cloned [12,13]. P-24, a platelet membrane receptor for CD9 mAbs has also been purified [14] and its cDNA has been cloned [15,16]. Antibodies of other specificities which activate platelets have also been described. Scott et al. [17] have described an mAb which stimulates platelet secretion and aggregation and is directed against a platelet membrane glycoprotein of 67 kDa. Yanabu et al. [18] have detected an auto-antibody in a patient with immunothrombocytopenia, which activates normal platelets by interacting with a 36 kDa platelet surface protein.

Several anti-CD9 mAbs and certain anti-GPIIb/IIIa mAbs activate platelets via an FcγRII (CD32) receptor-mediated process [8,9,19–21]. In contrast, several mAbs stimulate platelets as $F(ab')_2$ fragments without the necessity of an intact IgG molecule [4,22,23]. Among those antibodies that stimulate platelets through the Fab domain are the well-described anti-LIBS-1 and LIBS-2 [24] and D3GP3 [25,26]. However, these antibodies induce exposure of fibrinogen binding sites and platelet aggregation without inducing signal transduction pathway (stimulus–response coupling) nor granular secretion. Presently, the involvement of the FcγRII receptor in antibody-stimulated platelet activation is not completely clear. Of all the stimulatory antibodies described to date, most are directed against one of three known major platelet glycoproteins namely, CD42 (GPIIb/IIIa), CD36 and CD9.

We have previously reported that an mAb called mAb F11 recognizes two membrane proteins of 32 and 35 kDa, termed the F11 antigen, and induces vesicular secretion and aggregation in human platelets [27]. Platelet activation induced by mAb F11 involves the phosphorylation of intracellular 40 and 20 kDa proteins and an increase in intracellular calcium levels. We now demonstrate that intraplatelet cross-linking of the F11 antigen with the FcγRII receptor is an essential mechanism which is responsible for the intracellular signal transduction process induced by mAb F11. Furthermore, we report the isolation, characterization and partial structural analysis of the novel 32 and 35 kDa platelet membrane proteins recognized by mAb F11.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; PGE_1, prostaglandin E_1; CNBr, cyanogen bromide; mAb, monoclonal antibody; PKC, protein kinase C; GP, glycoprotein; PVDF, poly(vinylidene difluoride); PVP-40, polyvinylpyrrolidone 40; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell receptor; NP-40, Nonidet P.40; PTH, phenylthiohydantoin.

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EXPERIMENTAL PROCEDURES

Materials

Trisma base, phenylmethanesulphonyl fluoride (PMSF), leupeptin, aprotinin, iodoacetamide, benzamidine/HCl, soybean trypsin inhibitor, BSA, apyrase grade V, Nonidet P.40 (NP-40), prostaglandin E_1, phorbol 12-myristate 13-acetate (PMA) and Me_2SO were purchased from Sigma Chemical Co. CNBr-activated Sepharose CL-4B and DEAE-Sepharose were purchased from Pharmacia. Sequencing grade endoproteinase Glu-C, endoproteinase Lys-C and trypsin were obtained from Boehringer Mannheim. Immobilized papain, Iodo beads, Protein A–agarose, immunopure binding buffer and immunopure elution buffer were purchased from Pierce. Luciferin–luciferase reagent was purchased from Chronolog. ^{125}I-Iodine was obtained from Amersham. Reagents for SDS/PAGE were obtained from BioRad. All other reagents used were of analytical grade.

Monoclonal antibodies

mAb F11 (IgG_1) Fab fragments were produced and purified as previously described [27]. CD32 antibody IV.3 (IgG_{2b}) was purchased from Mederax, Inc.

Blood collection

Blood was obtained from individuals who were free of any medication for at least 2 weeks before experimentation. All volunteers signed an informed consent form, approved by the Committee on Human Research of the State University of New York Health Science Center, Brooklyn, NY, U.S.A. Venous blood was collected into tri-sodium citrate (3.8%). Platelet-rich plasma was prepared by centrifugation at 200 *g* for 10 min at 24 °C.

Platelet aggregation

The platelet aggregation experiments were carried out in a Chronolog lumi-aggregometer (Chronolog Corp., Havertown, PA, U.S.A.) as previously described [27]. Platelet-rich plasma (0.45 ml) was placed in a siliconized cuvette and aggregation was initiated by the addition of mAb F11 (10 μg) with constant stirring at 1200 rev. min^{-1} at 37 °C.

Platelet isolation

Platelets were isolated from outdated platelet concentrates by centrifugation at 1200 *g* for 10 min at 24 °C. Platelets were then washed three times using Tyrode's/BSA (0.35%) solution buffered with 20 mM Hepes, 2 mM calcium chloride, 11.9 mM sodium bicarbonate, 0.36 mM sodium dihydrogen phosphate, 5.5 mM glucose, 1 mM magnesium chloride, 1 μM PGE_1, 1 unit/ml apyrase and 2 units/ml of heparin [28,29]. Washed platelets were stored frozen at −70 °C until use.

Membrane preparation and solubilization

Washed platelets (2×10^{12}) were resuspended in 200 ml of 20 mM Tris-HCl buffer, pH 8.0, containing PMSF (2 mM), leupeptin (10 μg/ml), aprotinin (10 μg/ml), iodoacetamide (20 mM), benzamidine-HCl (5 mM) and soybean trypsin inhibitor (10 μg/ml) and subjected to pressure homogenization using the N_2-cavitation technique [30]. The crude homogenate was centrifuged at 10000 *g* for 10 min at 4 °C to remove cell debris and unbroken platelets and then at 100000 *g* for 90 min at 4 °C to sediment the membrane fraction. The crude membrane preparations were solubilized in 1% NP-40 as described previously. The membrane suspensions in homogenization buffer were made 1% with NP-40 and solubilized by stirring overnight at 4 °C. Insoluble material was removed by centrifugation at 100000 *g* for 30 min.

F11 antigen purification

Following ultracentrifugation, the NP-40 extract was diluted 5-fold with 20 mM Tris-HCl buffer (pH 8.0) containing protease inhibitors and then passed through a DEAE-Sepharose column pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 0.1% NP-40. The flow-through material which contained about 90% of the F11 antigen, as determined by immunoblotting, was concentrated by ultrafiltration using Amicon P-30 filters to its original volume and then applied to a Sepharose CL-4B column to remove the non-specifically bound proteins. The flow-through material was then applied to an mAb F11 affinity column (5 ml) obtained by coupling the mAb F11 (5 mg/ml) to CNBr-activated Sepharose CL-4B. After extensive washing of the affinity column with 1000 ml of Tris-HCl buffer (pH 8.0) containing 1 M NaCl, followed by Tris-HCl buffer (pH 8.0) containing 0.5% sodium deoxycholate to remove non-specific bound proteins, the bound F11 antigen was eluted at a flow rate of 2 ml/min by use of 50 mM diethylamine (pH 11.5 in 0.5% sodium deoxycholate). Fractions were collected into 1 M Tris-HCl buffer (pH 8.0) and immediately dialysed against 10 mM Tris-HCl buffer (pH 7.4) containing 0.1% sodium deoxycholate. The purification was monitored by Western blot analysis using mAb F11. The protein profile was determined by silver staining of the SDS/PAGE gels [31].

Amino acid sequencing

The affinity purified F11 antigen proteins were separated on SDS/5–15%-polyacrylamide gels and transferred to poly-(vinylidene difluoride) (PVDF) membranes [32]. The proteins were stained by 0.1% Ponceau S in 1% acetic acid. The F11 receptor bands corresponding to 32 and 35 kDa were excised and sequenced in the Protein Sequencing Center of the Department of Biochemistry at the SUNY/HSC at Brooklyn, NY, U.S.A. Edman degradations and phenylthiohydantoin (PTH)-amino acid analyses were carried out on a Model 470A gas-phase protein sequencer/model 900A controller/data processor connected on-line to a microbore HPLC PTH-amino acid analyser (Model 120A), both from Applied Biosystems, Foster City, CA, U.S.A. Major modifications to the standard sequencing cycle involved five rather than three coupling reactions and the use of ethyl acetate rather than butyl chloride to extract PTH-amino acids. N-terminal sequence data on the 32 kDa protein were obtained with a yield of 19, 22, 11, 18 and 13 pmol per amino acid residue for cycles one to five respectively.

The protein bands eluted from the PVDF membrane were also proteolytically digested with endoproteinase Glu-C, trypsin and endoproteinase Lys-C as described [33]. These procedures were performed at the Rockefeller Sequencing Center, NY, U.S.A. Ponceau S-stained bands (5 μg of protein) were destained by 0.5 ml of 200 mM NaOH in 20% acetonitrile for 1 min. The remaining non-specific binding sites were blocked with 0.2% PVP-40 (polyvinylpyrrolidone; average molecular mass 40000) in methanol. Samples were digested in the presence of 1% hydrogenated Triton X-100 in 10% acetonitrile in 100 mM Tris-HCl, pH 8.0, by trypsin (0.1 μg of enzyme/μg of substrate protein), endoproteinase Glu-C (0.1 μg of enzyme/μg of substrate) or endoproteinase Lys-C (0.0075 i.u./μg substrate).

Samples were sonicated for 5 min and then centrifuged. The supernatants containing the digested peptides were analysed by HPLC. The endoproteinase Lys-C failed to digest the protein, but endoproteinase Glu-C and trypsin gave distinctive peptide profiles. The endoproteinase Glu-C and trypsin-derived peptides were separated on a 1090 M microbore HPLC (Hewlett-Packard) using a Vydac C_{18}-reverse phase column (2.1 × 250 mm) with a flow rate of 150 μl/min. Peptide elution was monitored at 220 nm. Fractions of 75 μl were collected and used for sequence analysis.

Iodination of antibodies and antibody binding to platelets

Purified mAbs were radiolabelled by using the method of Iodo beads as described previously [34]. The specific radioactivities were approximately 2×10^7 c.p.m./μg when 50 μg/ml of mAbs were radiolabelled by this method. Binding of radiolabelled antibody to platelet-rich plasma or to washed platelets was performed over a 200 μl cushion of 20% sucrose. The incubation mixture consisted of a 90 μl platelet aliquot [$(2–5) \times 10^8$ platelets/ml] and radiolabelled monoclonal antibodies in a total volume of 100 μl.

Phosphorylation of F11 antigen

Partially purified F11 antigen (5 μg), obtained by single-step affinity-column chromatography, was incubated with [γ-^{32}P]ATP (10 μCi/reaction, 0.1 μM) in 100 μl of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 100 μM Ca^{2+} and 1 mM Mg^{2+}, in the presence or absence of 10 μg/ml mAb F11 or in the presence or absence of 100 nM PMA and 20 μg/ml phosphatidylserine. The reactions were carried out at 37 °C for 10 min. At the end of the incubation period the reactions were terminated by adding 2 × Laemmli stop buffer and boiling for 3 min.

SDS/PAGE

SDS/PAGE was performed in 3% stacking gels and in 5–15% gradient separation polyacrylamide slab gels [35]. The gels were stained for proteins with Coomassie Brilliant Blue, destained in 10% acetic acid/20% methanol, dried *in vacuo* and exposed to Kodak X-Omat AR film with Dupont-Cronex Lightning Plus intensifying screens for approximately 1–2 h at −70 °C and developed in a Kodak X-Omat developer.

Western blot analysis

The proteins separated by SDS/PAGE were electrophoretically transferred to nitrocellulose membrane [36]. The nitrocellulose membranes were then blocked using Tris-buffered saline containing 5% non-fat dairy milk for 1 h followed by incubation with mAb F11 (10 μg/ml) overnight at 4 °C. The blots were then thoroughly washed with blocking solution containing 0.1% Tween 20. The blots were developed using goat anti-mouse–(alkaline phosphatase) conjugate as described previously [34].

RESULTS

Platelet activation by mAb F11 and inhibition by antibody to FcγRII

The induction of platelet aggregation and secretion by mAb F11 was found to be dependent on mAb F11 concentration in two ways, i.e. the time required for the onset of aggregation (latency) and the initial velocity of aggregation were both concentration-dependent events. An increase in the mAb F11 concentration resulted in shortened latency and enhanced initial velocity of platelet aggregation. In the experiment shown in Figure 1, maximal aggregation was achieved at a concentration of 20 μg/ml, however the absolute value of antibody concentration which produced maximal effects varied from donor to donor. Both Fab and Fc fragments of mAb F11, generated by proteolytic digestion, failed to stimulate platelets as shown previously [27],

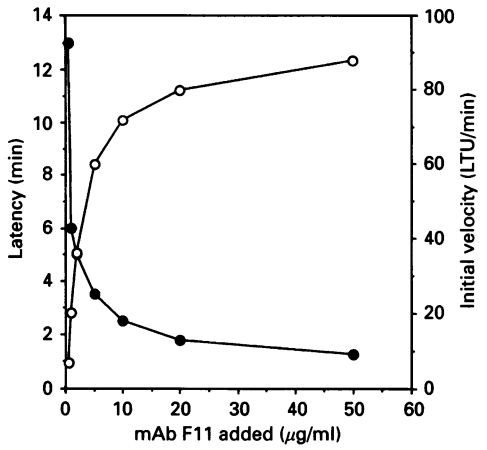

Figure 1 Effect of increasing concentrations of mAb F11 on the latency of platelet aggregation

Platelet suspensions (0.45 ml) were incubated with various concentrations of mAb F11. The aggregation response was monitored using a Chronolog lumi-aggregometer. ●, Latency, the time elapsed from the addition of mAb F11 to the onset of aggregation; ○, initial velocity of aggregation (LTU, light transmission unit). Data are representative of five separate experiments.

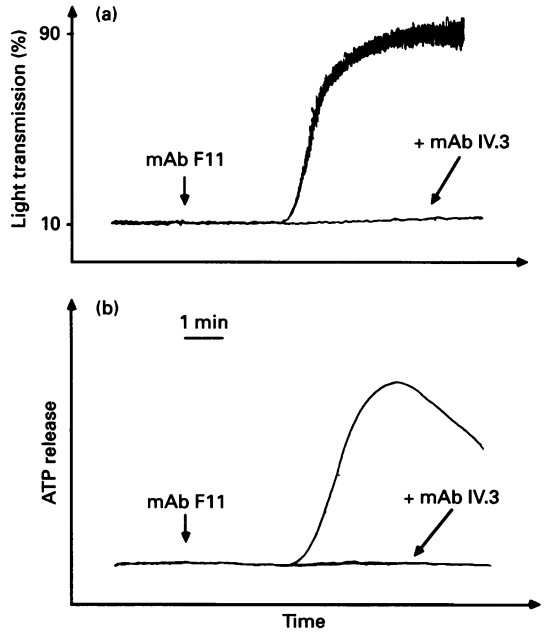

Figure 2 Inhibition of platelet aggregation and secretion induced by mAb F11 by anti-FcγRII antibody (mAb IV.3)

Aliquots (0.45 ml) of washed platelets were incubated for 1–2 min at 37 °C with 50 μl of a luciferin/luciferase reagent solution in a lumi-aggregometer. Aggregation (**a**) and secretion of ATP (**b**) were initiated by the addition of mAb F11 (10 μg/ml). mAb IV.3 (10 μg/ml) was added 2 min before addition of mAb F11.

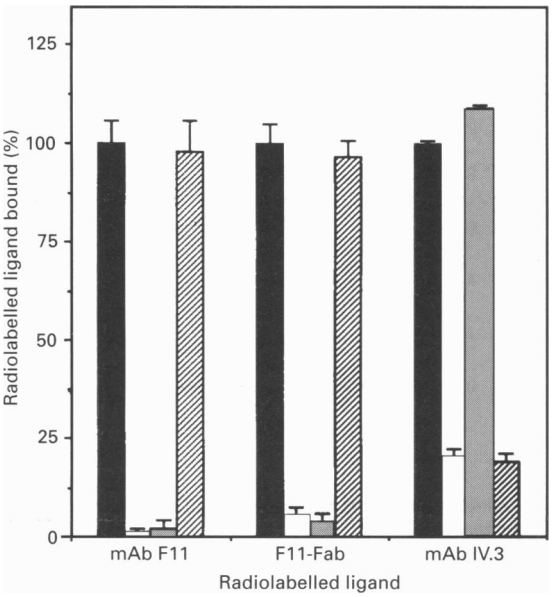

Figure 3 Specific binding of ^{125}I-mAb F11 to washed intact platelets

Fresh human platelet-rich plasma was incubated for 30 min at 37 °C with the ^{125}I-labelled ligands mAb F11, F11-Fab or mAb IV.3. Displacement of binding of each ligand was studied by addition of either unlabelled mAb F11 IgG (200 μg/ml; □), unlabelled mAb F11-Fab (▨), or unlabelled mAb IV.3 (10 μg/ml; ▨). No competitor added (■).

Table 1 Purification of the platelet F11 antigen recognized by mAb F11

Purification steps	Total protein	Protein recovery (%)	F11 antigen recovery (%)†	Fold purification
Platelet (2×10^{12})	–	–	–	–
Homogenate	11.9 g	100	100	1
NP-40 extract	2.495 g	20.97	97	4.6
DEAE flow-through	960 mg	8.07	90	11.2
Affinity eluate	24.8 μg*	2.0×10^{-4}	82	4.1×10^{5}

* Protein estimation by silver staining.
† Estimated from the immunoblot by densitometry.

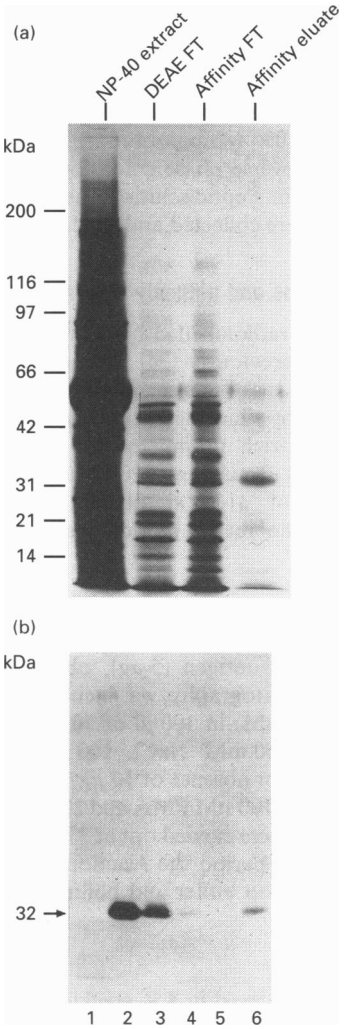

Figure 4 (a) Purification pattern of F11 receptor protein and (b) immunoblot of samples taken at each step of purification

(**a**) Silver staining of the proteins separated by 5–15% gradient SDS/PAGE under non-reducing conditions. NP-40 extract, 500 μg; DEAE flow-through (FT), 20 μg; mAb F11 affinity column FT, 20 μg; and mAb F11 affinity eluate, 200 ng, were applied to the gel. (**b**) Immunoblot of the proteins separated by SDS/PAGE under non-reducing conditions. Lane 1, 200 μg of cytosol; lane 2, 20 ng of mAb F11 affinity eluate; lane 3, 100 μg of DEAE flow-through; lane 4, 100 μg of affinity column flow-through material; lane 5, 200 μg DEAE-bound material; and lane 6, 500 μg of NP-40 extract. The arrow indicates the position of the F11 antigen.

however, only Fab fragments inhibited mAb F11-induced platelet aggregation. The concentration of Fab which produced half-maximal inhibition of binding of ^{125}I-mAb F11 was approximately 9.5 μg/ml; complete inhibition occurred between 50 and 100 μg/ml. In addition, F(ab')$_2$ fragments (11 μg/ml) completely blocked mAb F11-induced platelet aggregation, similar to that observed with Fab fragments.

Figure 2 shows the effect of the FcRγII antibody, mAb IV.3, on mAb F11-induced platelet aggregation and granular secretion. In the presence of mAb IV.3, the mAb F11-induced platelet aggregation (Figure 2a) and ATP secretion (Figure 2b) were completely blocked. A concentration of approximately 150 ng/ml doubled the time for induction of aggregation by mAb F11. The addition of a concentration of 500 ng/ml of mAb IV.3 resulted in complete blockage of mAb F11-induced platelet aggregation (even after a 12 h period following the addition of mAb F11; results not shown).

Although the binding of ^{125}I-mAb F11 to platelets was specifically inhibited by the addition of non-labelled mAb F11 or its Fab fragments, the binding of ^{125}I-mAb F11 was not inhibited by mAb IV.3 (Figure 3). Similarly, the binding of the ^{125}I-Fab fragment of mAb F11 was inhibited by mAb F11 and its Fab fragments, but such binding was not inhibited by mAb IV.3 (Figure 3). These results indicate that mAb F11 (through its Fab domain) and mAb IV.3 bind to two distinct receptors on the platelet surface. On the other hand, we found that the binding of ^{125}I-mAb IV.3 was inhibited by mAb F11 IgG molecule (Figure 3). This finding demonstrates that the Fc domain of the intact mAb F11 IgG molecule interacts with the platelet Fc receptor and competes with the binding of mAb IV.3 to the FcγRII receptor.

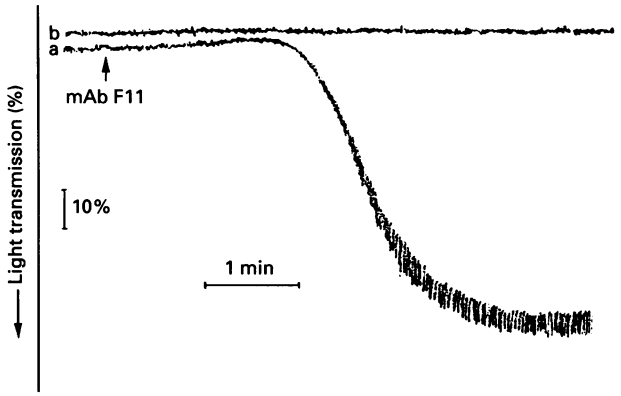

Figure 5 Inhibition of platelet aggregation by purified F11 antigen

Washed platelet suspensions [(2–3) × 10^8 platelets/ml] were preincubated for 2 min with buffer (trace a); or with the purified F11 antigen (1 μg/ml) (trace b). Aggregation was initiated by addition of mAb F11 (5 μg/ml), indicated by the arrow.

Purification of platelet F11 antigen

From our ^{125}I-mAb F11 binding studies, we have determined that washed, outdated platelets obtained from the blood bank can serve as an excellent source of starting material for the purification of the F11 antigen [dissociation constant $K_d = (1.74 \pm 0.79) \times 10^{-9}$, number of binding sites $B_{max.} = (13411 \pm 3316)$, $n = 5$, in outdated platelets; $K_d = 3.04 \pm 1.17 \times 10^{-8}$, $B_{max.} = 12801 \pm 5545$, $n = 6$, for freshly-prepared platelets (B. Walkowiak, U. P. Naik and E. Kornecki, unpublished work]. By immunoblotting we observed that the 32 kDa protein was the major form of the F11 antigen present in outdated platelets, whereas the 35 kDa protein was the minor form. Freshly-prepared platelet preparations exhibited nearly equal intensities of these two forms of the F11 antigen. The purification steps are shown in Table 1. Platelet membrane and cytosol proteins were prepared from 2×10^{12} platelets in the presence of protease inhibitors. Immunoblotting with mAb F11 showed that 100% of the F11 antigen was associated with the membrane fraction and not with the cytosolic fraction. The membrane NP-40 detergent extract contained 21% of the total platelet proteins and exhibited 100% of the detectable F11 antigen (see Table 1, Figure 4a, lane NP-40 extract and Figure 4b, lane 6). The NP-40 extract was subjected to DEAE ion-exchange chromatography. A salt concentration of 150 mM NaCl and a pH of 8.0 for the equilibrating buffer were selected to obtain maximum elution of the F11 antigen; the DEAE flow-through material contained the F11 antigen. Albumin, actin, myosin, GPIIb and GPIIIa remained bound to the DEAE column (Figure 4a, lane 2) and were eluted using 0.5 M NaCl. This step of purification resulted in 90% recovery of the F11 antigen, as determined by immunoblotting (Figure 4b, lane 3), with only 8% of total platelet proteins (Table 1). The final step of purification involved passage through an mAb F11-coupled CNBr-activated Sepharose column. We obtained a total of 24.8 μg of the F11 antigen with a yield of 82% (Table 1, Figure 4a, and Figure 4b, lane 2); a 4.1×10^5 fold purification of the F11 antigen was achieved. The purified F11 antigen preparation contained the 32 kDa protein as a predominant form of this receptor, with smaller amounts of the 35 kDa protein. In order to confirm further that the purified protein was the active F11 antigen, we performed direct functional tests using a platelet lumiaggregometer. We found that the preincubation of platelet suspensions with the purified F11 receptor (1 μg/ml) resulted in the complete inhibition of platelet aggregation induced by mAb F11 (Figure 5, trace b).

Amino acid sequencing of the N-terminus of the F11 antigen

The N-terminal amino acid sequences of the 35 and 32 kDa forms of the F11 antigen were determined by using solid-phase amino acid sequencing. The N-terminal amino acid sequence of the 32 kDa protein consisted of the following 26 amino acids: SVTVHSSEPEVRIPENNPVKLSXAYS (Table 2). This sequence was recognized as a highly hydrophilic sequence according to Kyte–Doolittle hydropathy analysis [37]. For determination of potential evolutionary relationships between this sequence and other known protein sequences, computations were performed at the National Center for Biotechnology Information using the BLAST network service in July and August, 1994.

Among 105060 sequences, the highest-scoring identities were found to the α-chain of the T-cell receptor (TCR); over 50% of the 24 highest-scoring sequences were TCR sequences. Overall, about 47% identity was found within amino acids 12–45 of the variable region of the human TCR α-chain (Figure 6). It is interesting that the highest degree of identity occurred around the cysteine residue involved in intramolecular disulphide bonding in TCR and immunoglobulins. Our inability to identify the amino acid residue at position 23 may be due to the presence of a cysteine residue at this position.

We also determined that the N-terminal sequence of the platelet F11 35 kDa protein was identical with that of the 32 kDa

Table 2 Amino acid sequence of the peptides derived from F11 receptor protein

Peptide	Amino acid sequence	Phosphorylation site
	(P)	
N-terminal	SVTVHSSEPEVRIPENNPVKLSXAYS	Casein kinase II
Glu-C digestion		
(1)	FDKDXTIYLNXY	
(2)	KFKLIVLV	
	(P)	
(3)	DRVTFLPTGITFKSVTRE	PKC
	(P) (P)	
(4)	WKFDQGDTTRLVEYNNKITASYEDRVTFLPTGITFKSVTRED	PKC
Trypsin digestion	(P)	
(1)	VTFLPTGITFK	PKC
(2)	LTDXGQ	

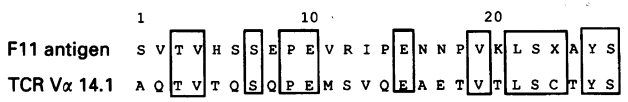

Figure 6 Similarity of the amino acid sequence of the N-terminal region of the F11 receptor protein to the variable region of the α-chain of human T-cell receptor aligned to maximize amino acid identity

X denotes the unassigned residue for the F11 protein at position 23 (possibly a cysteine residue).

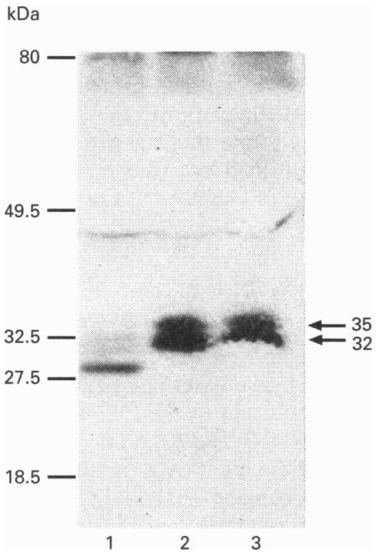

Figure 7 Post-translational modification of the F11 receptor proteins

Partially purified F11 receptor proteins (32 and 35 kDa) were digested overnight at 37 °C with *N*-glycanase (lane 1), with *O*-glycanase (lane 3), or without enzyme (control, lane 2). The F11 receptor proteins were separated by SDS/PAGE under non-reducing conditions and immunoblotted using mAb F11 and goat anti-mouse–(alkaline phosphatase) conjugate as second antibody.

protein, suggesting that differences in their electrophoretic mobility were due to post-translational modifications of the core F11 protein.

Post-translational modification of the F11 antigen

In order to determine the type of post-translational modification resulting in differences in the electrophoretic mobility of the F11 antigen, we digested the partially-purified F11 proteins with *N*-glycanase and *O*-glycanase. The digested proteins were analysed by immunoblotting using mAb F11. As shown in Figure 7, lane 2, the undigested proteins were recognized by mAb F11 as a protein duplex of 32 and 35 kDa. Treatment with *O*-glycanase had no effect on these proteins (Figure 7, lane 3). Treatment with *N*-glycanase converted the two proteins into a single protein band of ~ 29 kDa. As pointed out by Scott et al. [17], the molecular mass of a single N-linked sugar moiety is generally of the order of 3–4 kDa; in this case it is possible that the 32 kDa polypeptide has one N-linked sugar, whereas the 35 kDa polypeptide has two such sugar groups. These results demonstrate that the F11 antigen is composed of 32 and 35 kDa glycoproteins derived from a single core protein by differential N-glycosylation.

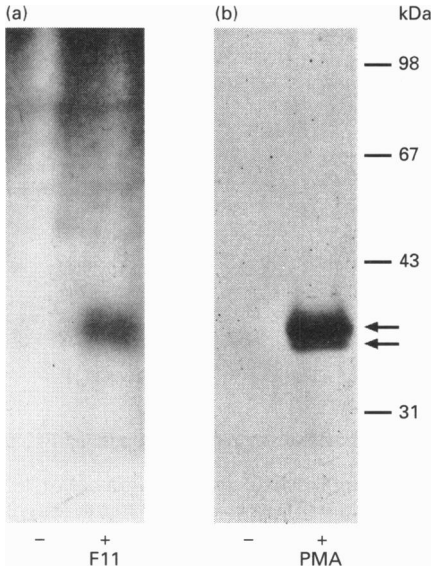

Figure 8 Phosphorylation of the F11 receptor proteins induced by mAb F11 and PMA

Partially purified F11 antigen preparations were incubated for 10 min at 25 °C with [γ-^{32}P]ATP in the absence (−) or presence (+) of mAb F11 (5 μg/ml) (as shown in **a**) or in the absence (−) or presence (+) of PMA (100 nM) (as shown in **b**) as described in the Experimental procedures section. The F11 receptor proteins were separated by SDS/PAGE under reducing conditions and analysed by autoradiography. The arrows indicate the positions of the 35 and 32 kDa proteins.

Internal amino acid sequencing of the F11 antigen

In order to obtain the internal amino acid sequences of the F11 antigen, the purified F11 antigen, corresponding to the 32 kDa protein on PVDF membranes, was digested using endoproteinase Glu-C, endoproteinase Lys-C or trypsin. The resultant peptides were eluted from the membrane and separated using C_{18} reverse-phase HPLC. Table 2 provides the amino acid sequences of four peptides obtained using Glu-C digestion and two peptides obtained using trypsin digestion. The endoproteinase Lys-C completely failed to digest the F11 antigen. Glu-C digestion produced two peptides which overlapped by 18 amino acids, and trypsin digestion yielded a peptide which showed an 11 amino-acid overlapping sequence. Interestingly, when all of these peptides were analysed by the Prosite computer program for consensus sequences for phosphorylation sites, the N-terminal peptide indicated a putative phosphorylation site for casein kinase II, whereas the internal digest peptides obtained by trypsin or Glu-C digestion indicated two putative phosphorylation sites for protein kinase C (PKC) (Table 2).

Peptide sequence databases were examined for possible identical sequences between the internal sequences of the F11 antigen and other protein sequences. This analysis did not reveal any regions of similarity to platelet proteins, to the TCR, nor to any other protein in the database.

Phosphorylation of the F11 receptor

To determine whether the F11 antigen is phosphorylated by endogenous protein kinases, we incubated the mAb F11 affinity-purified F11 antigen under phosphorylating conditions with [γ-^{32}P]ATP, Ca^{2+} and Mg^{2+}. For these experiments, the F11 antigen was isolated by mAb F11 chromatography but not subjected to

DEAE ion-exchange chromatography, since endogenous protein kinases associated with the receptor complex would be removed. When this preparation was incubated under phosphorylating conditions there was very little incorporation of [^{32}P]phosphate into proteins (Figure 8, lanes marked −), however, when the reaction mixture included aliquots of the mAb F11 (Figure 8a) or phosphatidyl serine and PMA (Figure 8b), there was significant phosphorylation of the F11 antigen. These results indicate that the phosphorylation shown in Figure 8 is carried out by an agonist-activated protein kinase and by a PKC, as would be expected for a receptor protein. The results shown in Figure 8 also point out that the electrophoretic mobility of the F11 antigen is sensitive to changes in conformation. Under reducing conditions, the mobility decreases and the protein migrates with an apparent molecular mass of 36–39 kDa (Figure 8) as compared with 32–35 kDa under non-reducing conditions. It should be mentioned however, that phosphorylation of the F11 receptor protein does not change its mobility, either under reducing or non-reducing conditions.

DISCUSSION

mAb F11 specifically recognizes two unique platelet membrane proteins of 32 and 35 kDa [27]. The mechanism by which mAb F11 activates human platelets involves both the Fab and Fc domains of the intact IgG molecule. The specific binding of radiolabelled ^{125}I-mAb F11 to its receptor occurs through the Fab domain, since the binding of the intact IgG molecule is completely inhibited by isolated Fab but not by Fc fragments. The Fab and F(ab′)$_2$ fragments alone do not stimulate platelet aggregation but inhibit the mAb F11-induced activation of platelets. The evidence that the Fc domain is also needed for the activation of platelets by mAb F11 was obtained from experiments using the anti-FcγRII receptor antibody, IV.3. The mAb F11-induced activation of platelets is completely blocked by preincubation of platelets with mAb IV.3. MAb IV.3 also blocks granular secretion induced by mAb F11, as measured by inhibition of ATP release. These findings directly demonstrate the involvement of the FcγRII receptor in the pathway of signal transduction triggered by the binding of mAb F11 to its own surface receptor. Variation among donors, observed in the extent of platelet activation by mAb F11, may reflect, in part, the heterogeneity of FcγRII expression on platelets [6,38].

Interaction of the IV.3 antibody with the platelet FcγRII receptor is not sufficient by itself to induce cellular activation. The platelet FcγRII receptor, a 40 kDa protein, has been shown to transduce signals leading to platelet activation only when cross-linked by immune complexes or aggregated IgG [39,40]. Platelet stimulation induced by FcγRII receptor antibodies has been shown to occur also in those experiments in which two Fab fragments of mAb IV.3 were cross-linked by (Fab′)$_2$ fragments of a secondary antibody [19,41]. Although the biochemical mechanism by which FcγRII transduces a signal across the platelet plasma membrane is not fully understood, these experiments indicate that cross-linking of two identical antigen molecules recognized by mAb IV.3 on the platelet surface appears to trigger the signal transduction process. The results of our study reported here suggest another mechanism by which a stimulatory mAb induces platelet secretion and aggregation. Our data demonstrate that platelet activation by mAb F11 requires the simultaneous interaction of the mAb F11 molecule with both the antigen recognized by its own Fab domain (the F11 receptor) as well as with the FcγRII receptor. The intact IgG molecule of mAb F11 therefore produces a heterodimeric cross-link between the F11-receptor recognized by the Fab domain of mAb F11 and the FcγRII protein interacting with the Fc domain of mAb F11. This cross-linking is essential for platelet activation by this stimulatory antibody. We propose that the F11 antigen represents a new receptor on the platelet surface with potential involvement in platelet function under normal and pathophysiological conditions.

To date, the purification of three major platelet glycoproteins (CD9, CD36, CD42) recognized by various stimulatory mAbs has been reported [10,13,14]. The F11 receptor protein is a fourth platelet surface glycoprotein involved in platelet activation induced by the stimulatory antibody mAb F11 [27]. The distinguishing characteristics of F11 antigen are its occurrence as two proteins of 32 and 35 kDa in platelets, which increases by ~ 4 kDa on reduction, and its resistance to chymotrypsin and endoproteinase Lys-C. The gel electrophoretic data obtained under reducing and non-reducing conditions, and the finding of a single unblocked serine in the N-terminal position of both the 32 and 35 kDa F11 proteins suggest that these proteins consist of a single-chain structure. The two F11 antigen proteins are differentially N-glycosylated forms of the same protein as determined by the N-terminal amino acid sequence of the two proteins and their ability to be converted into a single ~ 29 kDa protein upon digestion with *N*-glycanase. The inability of *O*-glycanase to alter the molecular size of these proteins indicates the absence of *O*-glycosylation.

The sequence analysis of the N-terminal 26 amino acids of F11 indicates that this sequence is highly hydrophilic and provides a long extracellular domain for the F11 antigen. Of approximately 100000 peptide sequences analysed for structural similarity, the highest scoring sequences were of the TCR; about 47% amino acid identity was found between the F11 N-terminal sequence and the N-terminal sequence of the variable region of the α-chain. Our inability to identify the amino acid at position 23 may indicate the presence of a cysteine residue, which is conserved in that position in the TCR. The T-cell antigen receptor has a characteristic primary and secondary structure made up of a series of intrachain disulphide-bonded domains [42,43]. The platelet F11 receptor also shows the presence of intrachain disulphide bonds, as observed from its increase in mobility following reduction of the protein with 2-mercaptoethanol. However, in contrast to the TCR, the platelet F11 receptor consists of a single polypeptide chain.

In contrast to the N-terminal sequence of the F11 receptor, the internal amino acid sequences of the F11 receptor obtained to date did not exhibit any similarity to the TCR nor to any known protein. Based on these results we conclude that the F11 antigen may indeed represent a novel platelet receptor protein. The presence of putative phosphorylation sites for PKC and the finding that the phosphorylation of the F11 receptor can be stimulated by the addition of mAb F11 as well as by PMA, suggest that the sensitivity of this receptor and its down-regulation are regulated by protein phosphorylation [44].

The detailed elucidation of the biochemical pathways mediating signal transduction by mAb F11 and the regulation of its receptor function are expected to contribute to our understanding of processes underlying the production of immune thrombocytopenia by antibodies that activate platelets in the circulation.

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