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Two Regions of the Human Platelet F11-Receptor (F11R) Are Critical for Platelet Aggregation, Potentiation and Adhesion*

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Keywords

Human platelet F11 receptor, F11R, junctional adhesion molecule, JAM

Summary

The F11 receptor (F11R) was first identified on the surface of human platelets as a target for a stimulatory monoclonal antibody (M.Ab.F11) that induces secretion, followed by exposure of fibrinogen receptors and aggregation. Cloning of the gene of F11R has revealed that this protein is a cell adhesion molecule (CAM), a member of the Ig superfamily and an ortholog of the murine protein called junctional adhesion molecule (JAM). The present study has identified two domains through which M.Ab.F11 triggers a platelet response culminating with aggregation. M.Ab.F11-mediated platelet adhesion, and the potentiation of collagen and ADP-induced platelet aggregation by M.Ab.F11, were found to involve the same two domains. A F11R recombinant protein (sF11R) completely inhibited platelet aggregation, adhesion and potentiation induced by M.Ab.F11, indicative that the active conformation of the external domain of F11R is present in the soluble, secreted recombinant protein. Furthermore, a specific peptide containing the sequence of the N-terminal amino acids S-1 to C-23 of F11R, and a peptide with the sequence of K-70 to C-82 in the 1st immunoglobulin-like (Ig) fold of F11R, both inhibited M.Ab.F11-induced aggregation, adhesion and potentiation of the aggregation of human platelets. Modeling of the 3D structure of the extracellular domain of the human platelet F11R suggests that these two regions form an active site within the conformation of this CAM. The sequence of these functional domains of F11R (in the N-terminus and 1st Ig-fold) provide the basis for new drug development in the treatment of certain types of thrombocytopenia and inflammatory thrombosis.

Introduction

The human platelet F11 receptor (F11R) is a surface glycoprotein (32–35 kDa), member of the immunoglobulin superfamily. F11R was first discovered as the target of a potent stimulatory monoclonal antibody that induces platelet secretion followed by aggregation (1–8). Sig-

nal transduction mechanisms for platelet secretion and aggregation induced by M.Ab.F11 following its initial binding to F11R include: crosslinking of the F11R to the Fc γ RII (3), activation and translocation of specific PKC isozymes (4), phosphorylation of the F11R through activation of PKC (3, 4), phosphorylation of the F11R following induction of platelet aggregation by the physiological agonists thrombin and collagen and by M.Ab.F11 (6–8), and phosphorylation of myosin light chain and plectrin, leading to shape change and granular secretion, respectively (2). Following secretion, this signal transduction pathway culminates in the activation of latent fibrinogen receptors and platelet aggregation (1). Partial amino acid sequences representing 30% of the length of purified F11R were reported by us in 1995 (3). Cloning of the full-length cDNA for the platelet F11R has revealed that it is a cell adhesion molecule (CAM), a member of the immunoglobulin superfamily (6–8). As a CAM, the F11R participates in mechanisms underlying adhesion of platelets, endothelial cells, and epithelial cells (8, 9).

The conclusion that in addition to its role as a receptor that triggers signal transduction leading to secretion, the F11R also serves as a CAM involved in platelet adhesion was supported by the high degree of sequence similarity found between the human platelet F11R and an adhesion protein called Junctional Adhesion Molecule (JAM), a protein found in murine endothelial cells (9). Comparison of the murine JAM sequence to the previously-reported sequences of human-platelet F11R (3) revealed over 70% homology of JAM to the N-terminus (23 a.a.) of F11R and to two digested products of F11R. In addition, both the human platelet F11R core protein and the murine JAM protein were found to contain a single transmembrane domain and two pairs of cysteine residues in their extracellular domains that allow formation of intramolecular disulfide bridges forming characteristic Ig-like folds. It is now well established that the protein referred to as JAM (9–14) is the murine ortholog of the human F11R (1–8). JAM was localized at intercellular junctions of mouse endothelial and epithelial cells (9), and we found that the platelet antibody M.Ab.F11 recognizes F11R molecules present at intercellular junctions of cultured human umbilical vein endothelial cells (Sobocka et al., manuscript in preparation).

Despite the recognition of multiple physiological functions of F11R/JAM, the functional domains of the molecule that participate in triggering each of its activities in platelets have not yet been identified. The present report initiates this investigation by identifying the domains of the F11R involved in platelet activation.

Material and Methods

All chemicals and biochemicals were of analytical grade quality.

Human platelets. Whole blood was collected into the anticoagulant ACD (pH 4.6), as detailed (2). Platelets were washed and isolated by differential cen-

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trifugation, and resuspended in a Tyrode-albumin (0.35%) solution buffered with 11.9 mM sodium bicarbonate (pH 7.35) in the presence of apyrase, heparin, and PGE₁ (2). Final platelet suspensions did not contain any inhibitors. Platelet aggregation was measured in a Chronolog Whole Blood Lumi-Aggregometer (Chronolog Corp., Havertown, PA). Potentiation of platelet aggregation was measured by adding a mixture of two platelet agonists, each at a sub-threshold concentration that did not induce any platelet aggregation. The lowest concentration of each agonist which caused platelet aggregation was determined in these experiments for each donor on the day of blood collection.

Immunoblotting. Polyacrylamide gel electrophoresis, immunoblotting of transferred proteins onto nitrocellulose strips, and detection using ECL chemiluminescence were performed as detailed (2).

Antibodies. Monoclonal antibody M.Ab.F11 (IgG1 isotype) was affinity-purified as described (2). Histidine antibody was obtained from InVitrogen (Carlsbad, CA).

Construction of the plasmid pcDNA3.1/F11R. A 726-base-pair fragment (nucleotide -6 till +720) was amplified by PCR using a human F11 receptor cDNA as a template (6-8) utilizing the forward primer [GC^GGGGATCCATCGC-GATGGGGACAAAGGCG], and the reverse primer [CCGACCTC-GAGCGGCATTCCGCTCCACAGCTTCC] (bases in bold represent BamHI and XhoI sites), respectively. This PCR fragment encodes amino acids ser-1 to asn-208 of F11R, and excludes the C-terminal transmembrane and cytoplasmic domains. The 726-base-pair PCR-product was subcloned into plasmid pcDNA3.1/Myc-His (+) C (Invitrogen, Carlsbad, CA) using BamHI and XhoI to yield pcDNA3.1/F11R. Transcription of the F11R in this plasmid is under the control of CMV immediate-early promoter. The construct pcDNA3.1/F11R was verified by sequencing and fine restriction mapping prior to its use in expression studies in COS-7 cells.

Recombinant DNA methods. *E. coli* transformation, plasmid DNA isolation, restriction analysis, extraction of DNA from agarose gels and ligation of insert into pcDNA3.1/myc-His(+)-C vector were carried out as described (15). Plasmids were isolated from *E. coli* DH5 α (Life Technologies, Grand Island, NY) using Qiaprep columns (Qiagen, Valencia, CA). DNA restriction fragments were separated by agarose gel electrophoresis and isolated with the QIAquick Gel extraction kit (Qiagen). PCR was performed using the Perkin Elmer Gene Amp 2400PCR System. DNA sequencing was performed by PCR-cycle sequencing using ABI PRISM Dye Terminator Cycle Sequencing Kit from Perkin Elmer (Foster City, CA) and the ABI Prism 377 DNA Sequencer. Computer analysis of sequence data was performed with the Biology WorkBench, release 3.2.

Transfection of COS-7 cells. COS-7 cells were grown in DMEM/10%FBS (Cellgro Mediatech, Inc.) and 1% antimycotic (Life Technologies), at 37° C/5% CO₂. Cells (about 50% confluence), plated in a 75 mm flask, were used for transfection one day later. The plasmid pcDNA3.1/F11R (10 μ g) was transfected into cells using 30 μ l of FuGENE-6 (Roche Diagnostics). Cells were maintained at 37° C/5% CO₂ in 7 ml complete medium. Cells were also treated with FuGENE-6 alone as controls. Total RNA was isolated (RNasey Mini Kit, Qiagen) and used for subsequent RT-PCR. RNA (2 μ g) was used for reverse transcription (Omniscript Reverse Transcriptase, Omniscript RT Kit, Qiagen). Half of the reaction mixture was used to amplify F11R in a 35-cycle PCR using the specific F11R primers as detailed previously (6-8). PCR cycling was as follows: 94° C for 5 min followed by 35 cycles of 94° C for 45 sec, 55° C for 45 sec, 72° C for 2 min and 94° C for 2 min. A 726-bp fragment was obtained only from pcDNA3.1/F11R transfected cells. Conditioned media, collected 72 h posttransfection, were pooled and passed twice over a M.Ab.F11-immunoaffinity column. After washing of the affinity column, the bound sF11R was eluted by use of 50 mM diethylamine (pH 11.5), fractions were collected into 1 M Tris-HCl buffer (pH 8.0) and immediately dialysed against 10 mM Tris-HCl buffer (pH 7.4). The sF11R solution was concentrated 5 \times by Centricon YM-10 (Bedford, MA), and stored frozen at -20° C.

Synthesis of F11R-peptides. Five F11R peptides (95% pure) were synthesized (New England Peptides, Inc., Fitchburg, MA). The sequence of amino acids in these peptides and their location within the F11R molecule are shown in Table 1 and Fig. 1. Their mass was determined by MALDI-TOF DE mass spectrometry.

Platelet adhesion to an immobilized matrix. An adhesion assay, based on the determination of cell-derived protein using Bicinchoninic Acid (BCA) protein assay (16), was used for platelet adhesion to immobilized M.Ab.F11. Wells of a 96-well plate (Nunc-Immuno™ Plate, MaxiSorp™ Surface, flat bottomed) were incubated overnight at 4° C with 150 μ l of a 1 mg/ml solution of M.Ab.F11. Wells were aspirated, washed, treated with TBS/1%BSA for 1 h at 37° C, and washed with TBS/0.1 mM MnCl₂/0.1 mM CaCl₂. Isolated platelet suspensions (100 μ l) (3 \times 10⁸/ml) were added and plates were incubated at 37° C for 90 min. Total platelet-associated protein was determined by dissolving the attached platelets directly with 100 μ l BCA. Plates were incubated at 37° C for 2 h, and absorbance (595 nm) determined (Dynatech Laboratories, Chantilly, VA).

3D-Structure of human sF11R. The crystal structure of the external domain of mouse recombinant JAM (17) was used as a template to generate a 3D model of the human recombinant sF11R based on the sequence (8) of the mature human platelet F11R (GenBank accession #AF207907).

Results

Preparation and use of Recombinant sF11R. A schematic model of the external domain of the F11R protein constructed on the basis of its sequence (8) is illustrated in Fig. 1. We prepared in COS-7 cells a secreted, recombinant F11R protein (sF11R) which contained only the extracellular portion (amino acids ser-1 to asn-208) of the mature F11R molecule. The transcription of the recombinant sF11R in COS-7 cells was determined by RT-PCR. A 726-base-pair fragment was detected only in pcDNA3.1/F11R transfected cells. Lanes 4 and 5 in Fig. 2A identify the identical 726 bp insert (see arrow) obtained from two separate sF11R clones. This fragment was not present in cells transfected with an empty vector or mock-transfected cells. To determine the expression of sF11R in COS-7 cells, the conditioned media obtained from transfected cells were examined by immunoblotting using both a polyclonal anti-F11R antibody and the monoclonal M.Ab.F11. The sF11R polypeptide was detected in the F11R COS-7 conditioned media obtained from these two separate F11R secreting clones, as shown in lanes 1 and 2 of panels B and C in Fig. 2. COS-7 cells which were treated with only Fugene 6 (mock-transfected), or COS-7 cells transfected with a plasmid lacking F11R DNA, did not secrete sF11R (Fig. 2, panels B and C, lanes 3 and 4). The sF11R protein was engineered to contain a Histidine tag sequence, and indeed, it was recognized by an anti-His antibody, as shown in panel D, of Fig. 2. The use of a control protein (Positope, 53 kD, obtained from InVitrogen) that contains the His tag, shown in lane 1 (panel D of Fig. 2), confirmed this identification. Finally, we purified sF11R from COS-7 cell media using M.Ab.F11 immunoaffinity chromatography. As shown in Panel E of Fig. 2, the purified sF11R was recognized by both the platelet stimulatory monoclonal antibody, M.Ab.F11, and by a polyclonal F11R antibody, directed against the N-terminal amino acids ser-1 to cys-23. The results detailed above demonstrate that transfected COS-7 cells not only synthesize but also secrete sF11R.

Inhibitory effects of sF11R on platelet aggregation. The effects of the recombinant sF11R on platelet aggregation induced by M.Ab.F11 are shown in Fig. 3. Panel A shows that the addition of aliquots of sF11R culture media, collected from transfected COS-7 cells, completely inhibited platelet aggregation induced by M.Ab.F11. In contrast, no inhibition of M.Ab.F11-induced platelet aggregation occurred in the presence of equivalent volumes of COS-7 cell media collected from control, nontransfected cells. Similarly, as shown in Panel B of Fig. 3, the addition of purified sF11R completely inhibited aggregation induced by M.Ab.F11. A 100% inhibition was found with concentrations as low as 100 ng/ml sF11R. In separate experiments, recombinant

Table 1

Peptide name	PEPTIDE SEQUENCE
F11R-peptide 1	*SVTVHSSEPEVRIPEENNPKLSC 1-----23
F11R-peptide 2	SYEDRVTFLPTGIFTKSVTRED 55-----76
F11R-peptide 3	WKFDQGDTTRLVEYNNKITASY 35-----56
F11R-peptide 4	KSVTREDTGTYTC 70-----82
F11R-peptide 5	EQDGSPPSEYTWFKD 128-----142

The amino acid numbers refer to the sequence of the mature platelet cell surface F11 receptor and of the recombinant protein, sF11R, which does not include the leader peptide sequence. * In the present study, ser-1 is the first amino acid that follows the 27 amino acid leader peptide sequence of the nascent protein (8).

sF11R (250 ng/ml) produced a prolonged delay (about 1 h) in the onset of M.Ab.F11-induced platelet aggregation, and when aggregation finally was observed, there was about a 60% decrease in the extent of M.Ab.F11-induced platelet aggregation (data not shown). These results indicate that the recombinant F11R is secreted with a correct functional conformation that can bind to M.Ab.F11 and thereby prevent the activation of platelets by this antibody. We conclude that the soluble recombinant protein sF11R contains an epitope recognized by the conformationally-sensitive monoclonal antibody, M.Ab.F11 (2).

Inhibitory effects of specific F11R-peptides. In addition to recombinant protein, we prepared in this study synthetic peptides according to published sequences of F11R (3) (see also Table 1) and used them for the identification of domains involved in M.Ab.F11-induced platelet aggregation. As shown in Fig. 4A, peptide 1 (50 µM) completely inhibited M.Ab.F11-induced platelet aggregation, and aggregation did not ensue when examined even after a 12 h period. In contrast, the addition of 50–500 nM of peptides 2, 3, or 5 (shown in panel C), derived from the F11R sequence (see Table 1 and Fig. 1), had no inhibitory effects. On the other hand, in addition to peptide 1, peptide 4 (50 µM), was able to completely block M.Ab.F11-induced platelet aggregation (Fig. 4, panel B).

Potentiation of agonist-induced platelet aggregation by F11R. We have found that the presence of low, non-aggregating (sub-threshold) concentrations of the physiological agonist ADP, collagen or thrombin can cause platelet aggregation when added together with non-aggregat-

ing concentrations of M.Ab.F11 (6, 7, 18). Such potentiating effects of M.Ab.F11 on agonist-induced aggregation are depicted in Fig. 5. When subthreshold concentrations of ADP (0.5 mM) or M.Ab.F11 (0.3 mg/ml), respectively, were added separately to platelet suspensions, there was no aggregation (panels A and B). However, when subthreshold concentrations of ADP were added together with subthreshold concentrations of M.Ab.F11, a pronounced aggregation response was observed, reflecting the potentiating effect (see “M.Ab.F11 plus ADP” tracings in each panel). Similar potentiating effects were observed with thrombin (data not shown). As shown in Panels C and D of Fig. 5, the addition of 50 µM of F11R peptide 1 or peptide 4 completely inhibited the potentiation by M.Ab.F11 of ADP-induced platelet aggregation. In contrast to these two peptides, peptide 3 (as shown in Panel E), did not inhibit the potentiation of ADP-induced platelet aggregation by sub-threshold concentrations of M.Ab.F11. The lack of inhibition by F11R-peptides 2 and 5 was the same as shown in Fig. 5E for peptide 3.

F11R-peptides inhibit the potentiation of collagen-induced platelet aggregation. The effect of F11R peptides on M.Ab.F11-induced potentiation of platelet aggregation induced by nonaggregating concentrations of collagen (0.5 µg/ml) also was examined. These results are shown in Fig. 6. Subthreshold concentrations of collagen and of M.Ab.F11 used here were determined separately for each blood donor. Panel A of Fig. 6 demonstrates that the selected concentrations of each of the agonists were not able to induce platelet aggregation when added alone. However, the addition of collagen just after adding M.Ab.F11 readily triggered a full-blown platelet aggregation. The potentiation by M.Ab.F11 of collagen-induced platelet aggregation could be completely blocked by F11R-peptide 1, as shown in Panel B, as well as by peptide 4, as shown in Panel C, but not with peptide 5 (also shown in Panel C) nor with peptide 3 (shown in panel D). The addition of sF11R (1 µg/ml) to platelet suspensions completely blocked the potentiation by M.Ab.F11 of both ADP- and collagen-induced platelet aggregation, and the results were the same as shown for peptides 1 and 4 in Figs. 5 and 6.

Recombinant sF11R inhibits platelet adhesion to M.Ab.F11. We concluded the present study by examining the effects of recombinant sF11R and F11R-peptides on the adhesion of intact platelets to immobilized M.Ab.F11 (8). The preincubation of platelet suspensions with sF11R (0.5 µg/ml) resulted in 50% inhibition of platelet adherence to M.Ab.F11 (Fig. 7). Inhibition of about 75–85% was observed with 1.0–2.5 µg/ml sF11R. We conclude that at least 80% of the observed adhesion is mediated by specific interactions between the active site of M.Ab.F11 and its epitope in F11R.

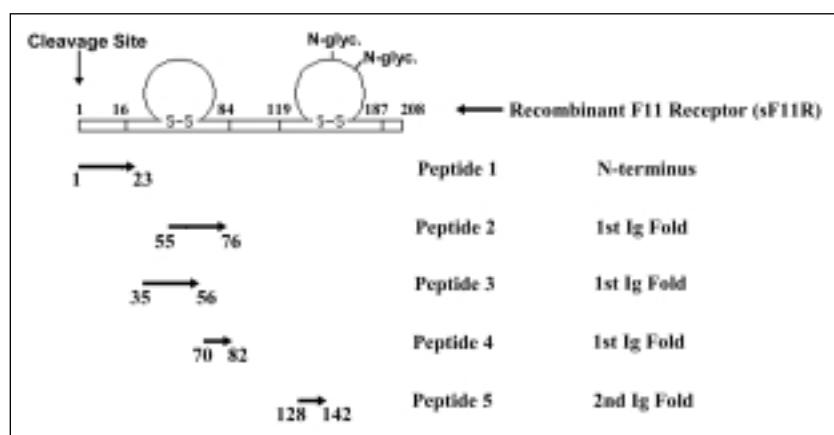


Fig. 1 Structural features of recombinant sF11R molecule. Amino acids of the secreted recombinant sF11R molecule are numbered 1 to 208, with ser-1, the 1st amino acid following the cleavage of the 27-amino-acid signal peptide. Two putative glycosylation sites are found in the 2nd Ig fold. The locations of sequences representing the five F11R peptides (listed in Table 1) are noted

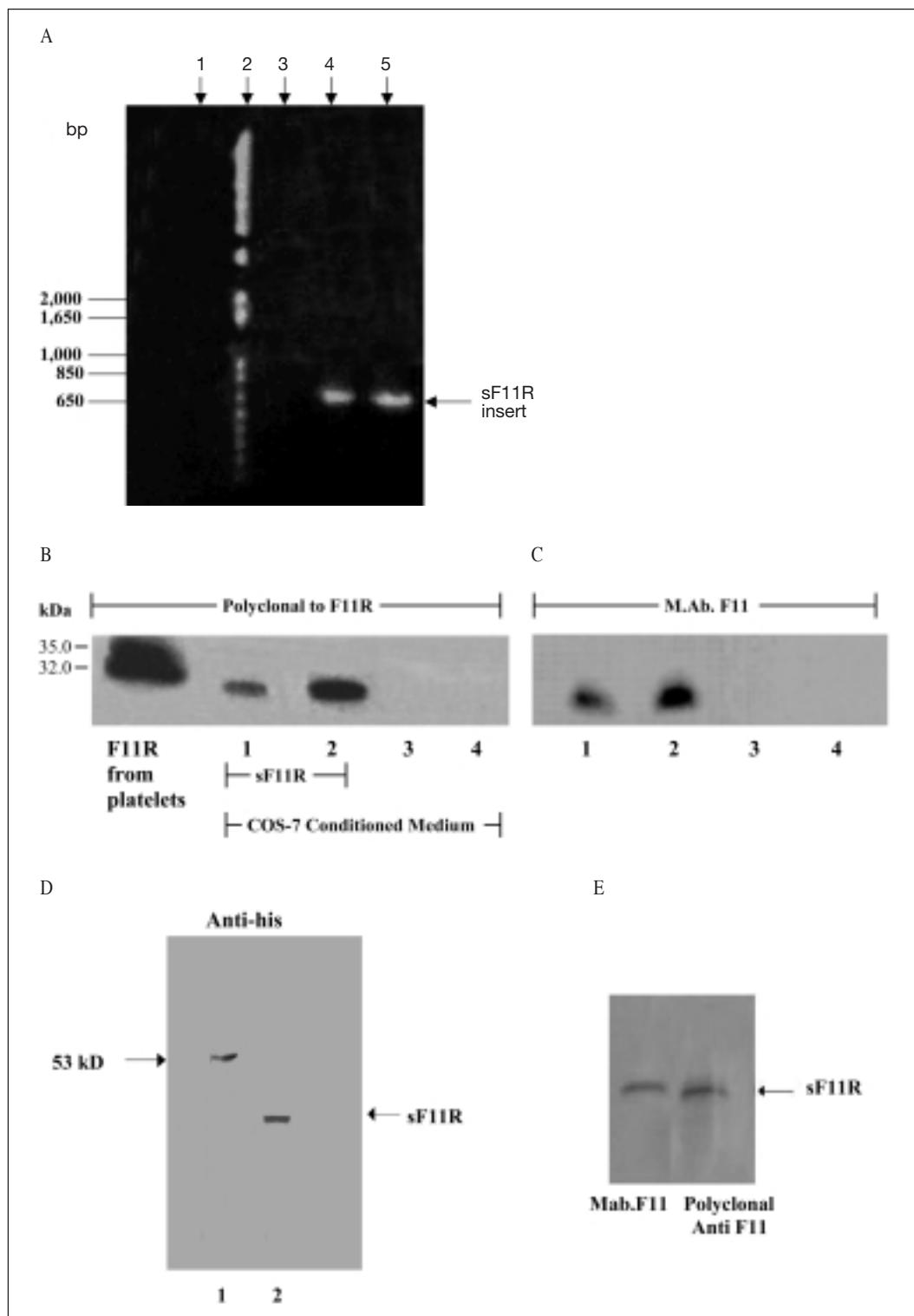


Fig. 2 Panel A) Presence of the sF11R transcript in F11R transfected COS-7 cells. Total RNA was isolated from 2×10^6 cells transfected with either plasmid pcDNA3.1/F11R or a vector lacking F11R, or from non-transfected cells. RNA (2 μ g) was used for reverse transcription reactions (Omniscript Reverse Transcriptase, Omniscript RT Kit, Qiagen), with half used to amplify F11R in a 35-cycle PCR using specific F11R primers. Lane 1: Vector lacking sF11R insert; Lane 2: 1 Kb DNA molecular weight marker; Lane 3: Nontransfected cells; Lane 4: Cells transfected with pcDNA3.1/F11R; and Lane 5: Cells transfected with pcDNA3.1/F11R, obtained from a separate F11R positive clone yielding the same results. The arrow points to the F11R insert. Panel B) Recognition of sF11R in conditioned media of COS-7 cells by a polyclonal F11R antibody to the N-terminal 23 amino acids. Immunoblot: Lane "F11R from platelets" depicts recognition of 32/35 kDa F11R (arrows) in human platelet detergent extracts by antibody. Lanes 1 and 2 depict two separate clones secreting sF11R. Lane 3, controls lacking F11R cDNA Lane 4, mock-transfected cells. Panel C) Monoclonal antibody F11 recognizes sF11R. Immunoblot: The same samples were applied to SDS-gels as detailed in Panel B, and the recombinant protein was probed with M.AbF11. Panel D) Recognition of sF11R by His antibody. Lane 1 depicts recognition of a control polypeptide (Positope 53 kD), engineered to contain the His tag, by His antibody. Lane 2 depicts recognition of the His tag of the purified recombinant sF11R protein by His antibody. Panel E) Purified sF11R is recognized by F11R antibodies. Affinity-purified sF11R (1 μ g/well) was separated by SDS-PAGE and blotted with F11R antibodies. Arrow points to the eluted sF11R protein.

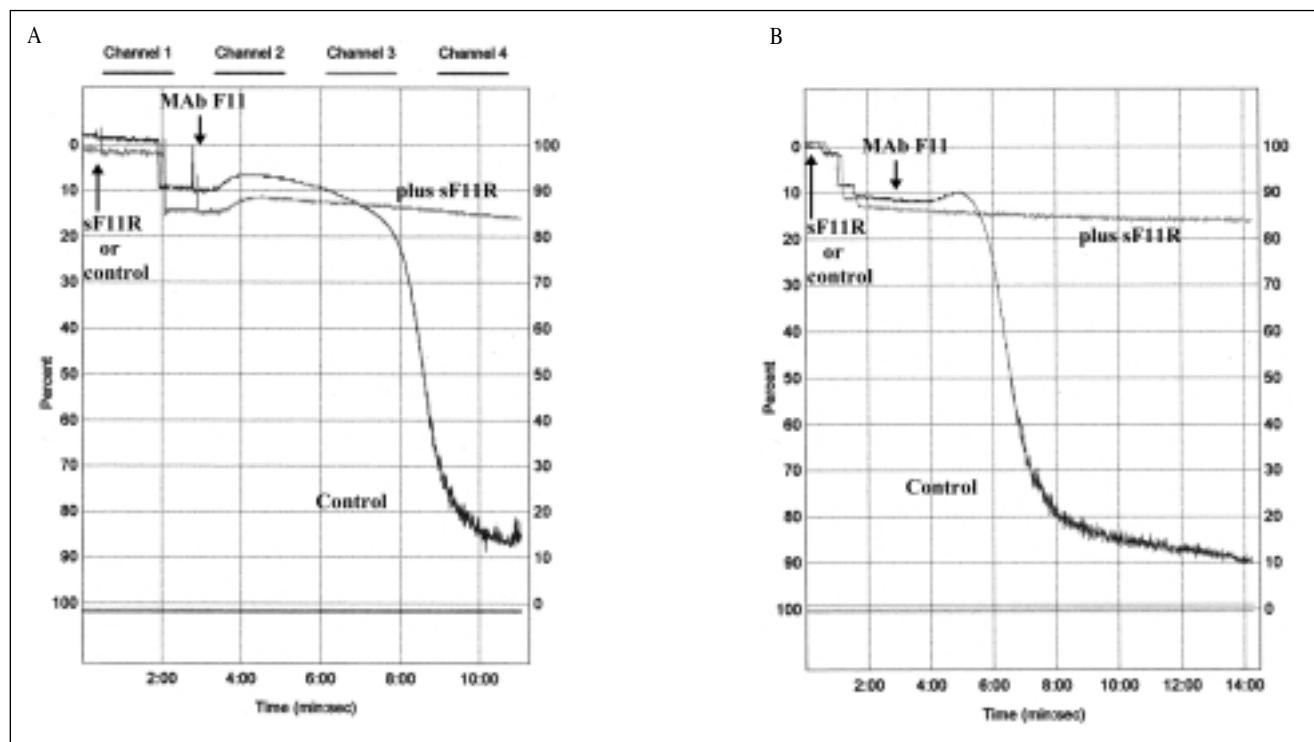


Fig. 3 Recombinant sF11R inhibits M.Ab.F11-induced platelet aggregation. Panel A) Inhibition of M.Ab.F11 (2.8 µg/ml)-induced aggregation by sF11R present in the conditioned media (100 µl) of transfected COS-7 cells. Panel B) Inhibition of M.Ab.F11 (2.8 µg/ml)-induced platelet aggregation by purified sF11R (1 µg/ml). The results shown in Panels A and B were obtained with different donors. Differences in the sensitivity of donors to M.Ab.F11 (latency following the addition of M.Ab.F11) were observed, however, the inhibitory effect shown here for sF11R supernatants, and for purified sF11R are representative of experiments with 5 separate donors each. Control media (100 µl), obtained from nontransfected cells or from cells lacking F11R, did not inhibit aggregation under any conditions

Two specific F11R-peptides inhibit platelet adhesion to M.Ab.F11. The five peptides with sequences shown in Table 1 were tested also for their effects on the adhesion of platelets to immobilized M.Ab.F11 (150 ng/well). The left bar of Fig. 8 demonstrates the control adhesion measured without added peptide. Peptide 1, added at 50 µM caused about 60% inhibition of the adhesion of platelets to immobilized M.Ab.F11, and with 500 µM of peptide 1, about 70% inhibition was observed. Peptide 4, at 50 µM, produced very little (10%) inhibition compared to peptide 1 at similar concentrations. However, 500 µM of peptide 4 produced approximately 70% inhibition in the adhesion of platelets to M.Ab.F11, similar to that observed with peptide 1 at the same concentration. On the other hand, the addition of 500 µM (or higher) of peptides 2, 3 or 5 did not cause significant inhibition of platelet adhesion to M.Ab.F11.

Three-dimensional structure of the recombinant human platelet F11R. A 3-dimensional structural model of the human platelet F11R, highlighting the two domains that are occupied by sequences of the platelet inhibitory peptides 1 and 4, are depicted in Fig. 9. From this modeling it appears that the N-terminal portion of the molecule (containing the sequence of peptide 1), forms a loop around the 1st Ig-fold (containing the sequence of peptide 4) to form an “active pocket” containing the sequences of both peptides 1 and 4, arranged in an anti-parallel orientation. Our data demonstrates that these two domains contain critical sites of the molecule that are responsible, at least in part, for triggering platelet aggregation, potentiation and adhesion by and to this immunologic agonist. This “active pocket” may thus constitute the stereospecific binding site for M.Ab.F11 (2), and for homophilic interactions of F11R molecules (19).

Discussion

Studies using flow cytometry (8) demonstrated directly that M.Ab.F11 binds to the surface membrane of intact platelets, but the domain(s) and binding sites of the F11 receptor involved in these interactions were not identified. In the present study we have extended this investigation by using a recombinant protein as well as specifically synthesized peptides for identifying regions of the F11R involved in the three platelet responses triggered by M.Ab.F11. The recombinant sF11R protein was recognized by M.Ab.F11 as shown by immunoblotting, indicating the presence of the activation epitope(s) within its structure. This demonstration has particular importance since our previous studies have determined that the binding of M.Ab.F11 to isolated F11R requires the maintenance of the native conformation of this receptor protein, and that even mild, reversible denaturation prevents this binding (2). Further confirmation of the presence of the activation epitope in the structure of sF11R was provided by the finding that sF11R completely inhibited M.Ab.F11-induced platelet aggregation, and completely inhibited the adhesion of platelets to an immobilized matrix of M.Ab.F11. The observation that all responses to M.Ab.F11 are inhibited by sF11R is of special importance since their activation by M.Ab.F11 is not triggered by the same initiating event. While platelet secretion leading to aggregation, induced by M.Ab.F11, was found to be triggered by crosslinking of F11R molecules to the platelet Fcγ RIIA (3), the spreading of platelets induced by adhesion to immobilized M.Ab.F11 was not dependent on this crosslinking (8). Finally, sF11R was observed to block the M.Ab.F11-induced potentiation process that sensitizes platelets to subthreshold concentrations of ADP or collagen.

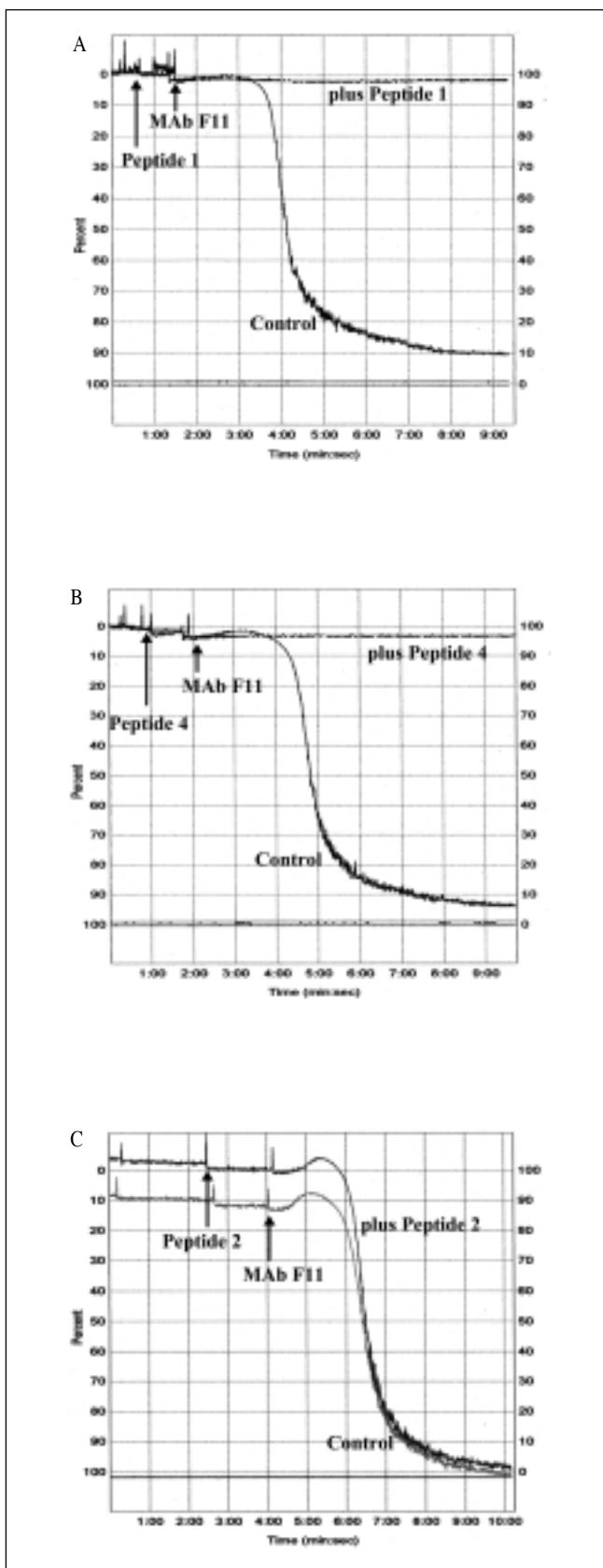


Fig. 4 Inhibition of M.Ab.F11-induced platelet aggregation by F11R peptides. Panel A) Inhibition of M.Ab.F11 (2.45 μ g/ml)-induced platelet aggregation by 50 μ M F11R peptide 1, and by 50 μ M F11R peptide 4 (Panel B). Control: Panel C shows the lack of inhibition of M.Ab.F11 (2.45 μ g/ml)-induced platelet aggregation by F11R peptide 2 (500 μ M). Likewise, no inhibition occurred with peptides 3 and 5

These results prove that the recombinant F11R molecule, secreted by COS-7 cells as a soluble protein called sF11R, contains binding sites for M.Ab.F11 which have the active conformation of the external portion of the F11R molecule residing on the surface of intact human platelets.

In a complementary approach, we used F11R peptides and tested their effects under the different experimental conditions used to observe each of the three platelet functional responses to M.Ab.F11. Among several such peptides, we found two that completely inhibited platelet aggregation induced by M.Ab.F11, the peptides F11R-1 and F11R-4. We observed that these two peptides completely inhibited the M.Ab.F11-induced potentiation of platelet aggregation induced by ADP and collagen, and the adhesion of platelets to immobilized M.Ab.F11, that results in platelet spreading (8). These results have thus identified two specific regions of the F11R responsible for the activation of platelets by the stimulatory antibody M.Ab.F11: one region (contained within the sequence of peptide 1), which encompasses the 23 amino acids beginning with ser-1 of the N-terminus of mature F11R, and a second region, (contained within the sequence of peptide 4), encompassing 13 amino acids spanning from K-70 to C-82 of the proximal half of the first Ig-like domain (Ig fold) of mature F11R.

The finding that the F11R-mediated platelet activation by a stimulatory monoclonal antibody involves two separate regions in the F11R molecule suggests that these two regions combine to form a sterically active site within the conformation of the F11R molecule. This possibility is supported by the modeling that provided the 3D-structure shown in Fig. 9. In addition, it is possible that a second site in the sequence of F11R that is not recognized directly by M.Ab.F11, operates in transducing signals through the F11R molecule following binding of the activating antibody to the first site. The interaction of a synthetic peptide with this second site could interfere with the transmission of signals initiated by the binding of M.Ab.F11 to the first site. Thus, a peptide could inhibit platelet responses triggered by F11R even when M.Ab.F11 binding to its activation epitope is not impaired. Further studies are required to determine the exact mechanism of F11R-mediated platelet activation. Nonetheless, the results reported here already allow for the important conclusion that the sequence of peptide 1, as well as the sequence of peptide 4, both represent the potential for development of new drugs based on the functions of F11R, as discussed below.

The platelet responses triggered by activation of F11R may represent a physiological process. It is possible that a naturally circulating peptide or another molecule that has not been identified yet acts as a soluble, natural ligand for the F11 receptor. Such a natural agonist has not been identified yet. In addition, we have already determined that the membrane-bound form of F11R can serve as a stimulator of another F11R molecule. This stimulation can occur through two separate processes: first, the homophilic interaction (*trans*) of F11R molecules on the surface of separate cells (19). This homophilic interaction can be homologous, between platelets, or heterologous, e.g. between platelets and endothelial cells. Second, lateral interactions (*cis*) between F11R molecules on the surface of the same cell can occur, resulting in dimerization and clustering (Sobocka M, Sobocki T, Babinska A, Hartwig J, Muller W, Ehrlich YH, Kornecki E, manuscript in preparation). Such dimerization can be triggered by trans-homophilic interactions of F11R molecules, and following platelet stimulation by physiological platelet agonists that can induce phosphorylation of F11R in human platelets (8). The dimerization and clustering of N-CAM molecules on the cell surface was shown to trigger outside-in signaling pathways (20). Similarly, lateral dimerization of F11R on the surface of platelets may trigger a signal transduction pathway which acts synergistically

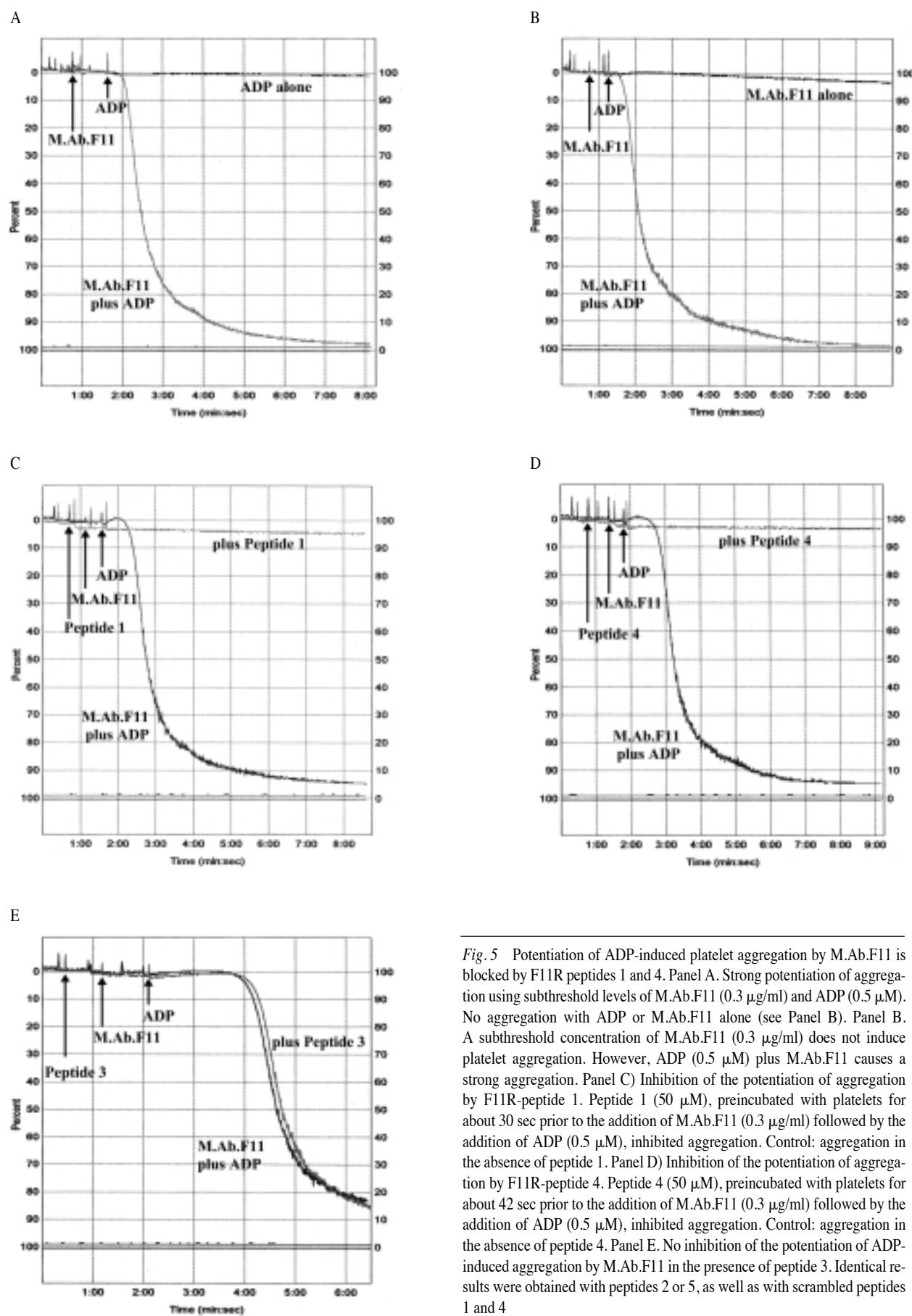


Fig. 5 Potentiation of ADP-induced platelet aggregation by M.Ab.F11 is blocked by F11R peptides 1 and 4. Panel A. Strong potentiation of aggregation using subthreshold levels of M.Ab.F11 (0.3 µg/ml) and ADP (0.5 µM). No aggregation with ADP or M.Ab.F11 alone (see Panel B). Panel B. A subthreshold concentration of M.Ab.F11 (0.3 µg/ml) does not induce platelet aggregation. However, ADP (0.5 µM) plus M.Ab.F11 causes a strong aggregation. Panel C) Inhibition of the potentiation of aggregation by F11R-peptide 1. Peptide 1 (50 µM), preincubated with platelets for about 30 sec prior to the addition of M.Ab.F11 (0.3 µg/ml) followed by the addition of ADP (0.5 µM), inhibited aggregation. Control: aggregation in the absence of peptide 1. Panel D) Inhibition of the potentiation of aggregation by F11R-peptide 4. Peptide 4 (50 µM), preincubated with platelets for about 42 sec prior to the addition of M.Ab.F11 (0.3 µg/ml) followed by the addition of ADP (0.5 µM), inhibited aggregation. Control: aggregation in the absence of peptide 4. Panel E) No inhibition of the potentiation of ADP-induced aggregation by M.Ab.F11 in the presence of peptide 3. Identical results were obtained with peptides 2 or 5, as well as with scrambled peptides 1 and 4

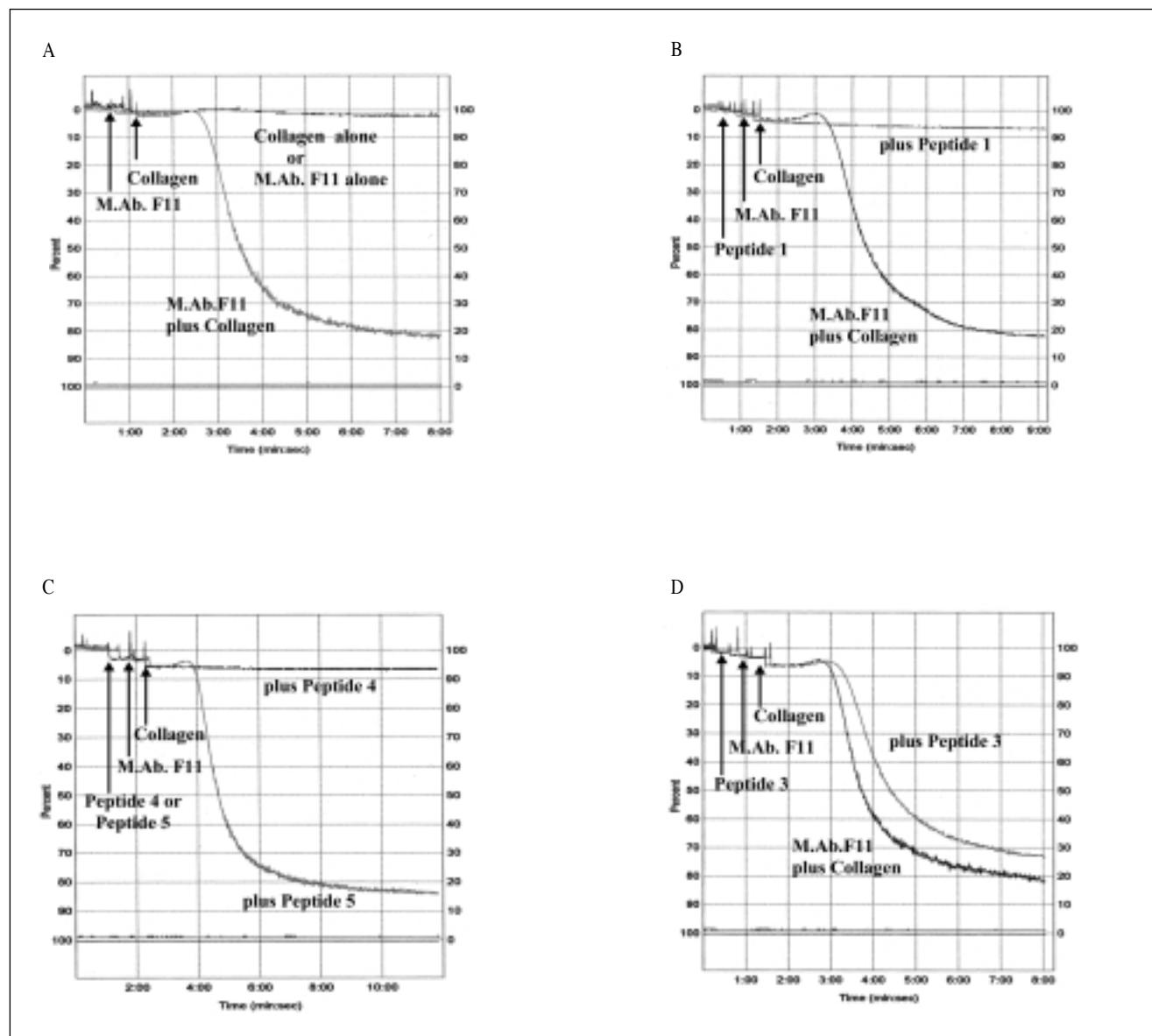


Fig. 6 Potentiation of collagen-induced platelet aggregation by M.Ab.F11 is inhibited by F11R peptides 1 and 4. Panel A) No aggregation was observed to collagen alone (0.5 μ g/ml) or M.Ab.F11 alone (1.2 μ g/ml); however, aggregation was observed in the presence of both agonists (M.Ab.F11 plus collagen). Peptide 1 (50 μ M) (Panel B) or peptide 4 (50 μ M) (Panel C) produced complete inhibition of aggregation. Control peptide 5 did not inhibit aggregation. Controls: Neither peptide 3 (50 μ M) (Panel D) nor peptide 2 (50 μ M) produced inhibition. The addition of recombinant F11R (1 μ g/ml) gave the same complete inhibition of platelet aggregation as shown here for either peptide 1 or peptide 4.

with pathways activated by physiological agonists. We propose that these synergistic forces could play an important role in the rapid formation of a platelet aggregate required to stop bleeding from an injured vessel. The F11R-mediated potentiation of platelet activity would enable sub-threshold concentrations of circulating agonists to trigger aggregation, which is of particular importance under conditions of rapid blood flow. In addition, the homologous/homophilic interactions between F11R molecules may contribute to the stabilization of irreversible aggregates formed under these conditions. Through these mechanisms, F11R together with other adhesion molecules could contribute to the physiological process of wound healing operating in hemostasis.

Two recent findings indicate that F11R studies also have pathophysiological relevance with important clinical implications. First, in a

double-blind study of 10 patients, we detected the presence of antibodies recognizing F11R in the plasma of thrombocytopenic patients (8). Second, studies of the murine homolog of the F11 receptor, JAM, have determined that the proinflammatory cytokines TNF- α and IFN- γ can cause a redistribution of this adhesion molecule in endothelial cells, with increased expression in the nonjunctional, luminal surface of the cells (10). Indeed, we observed that cytokine-induced increase in the expression of F11R proteins on the luminal surface of endothelial cells resulted in enhanced platelet adhesion to HUVEC (19, 21; Babinska A, Kedeas M, Athar H, Ehrlich YH, Hussain M, Batuman O, Kornecki E, manuscript in preparation). This adhesion, mediated by homophilic interactions between F11R molecules of platelets and endothelial cells, could cause the pathological formation of platelet plaques on a nondenuded vessel wall. Following platelet adhesion to the endothelium, the

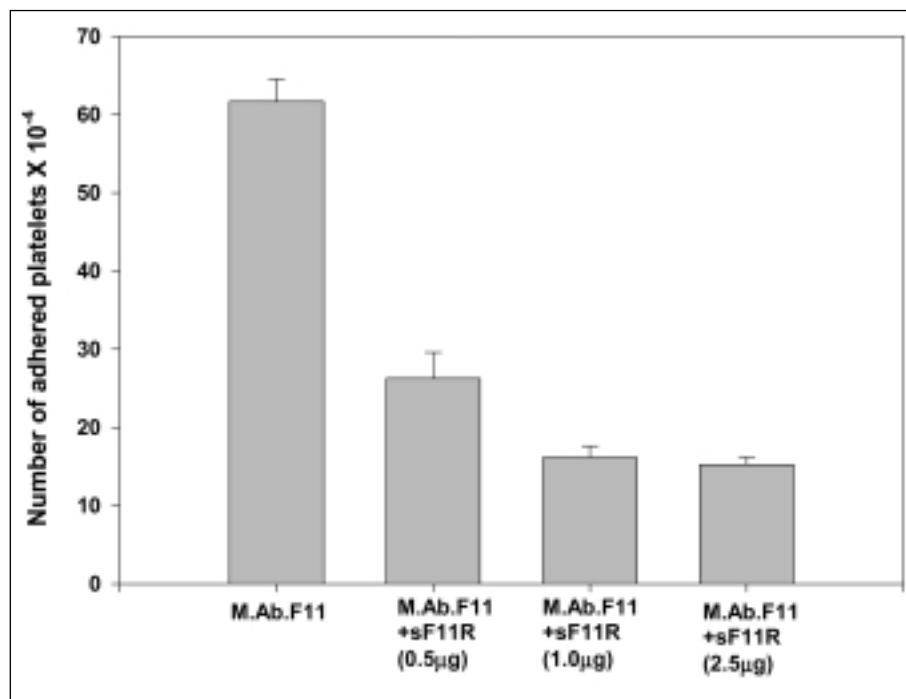


Fig. 7 Platelets adhere to immobilized M.Ab.F11: inhibition of adhesion by sF11R. M.Ab.F11 (150 ng) was immobilized in wells of a 96-well plate, as detailed in Material and Methods. Aliquots (100 µl) of platelet suspensions ($3 \times 10^8/\text{ml}$) containing sF11R (0.2–2.5 µg/ml) were applied, and adhered platelets were measured by the BCA method (16). Values represent the mean + SEM of three separate determinations

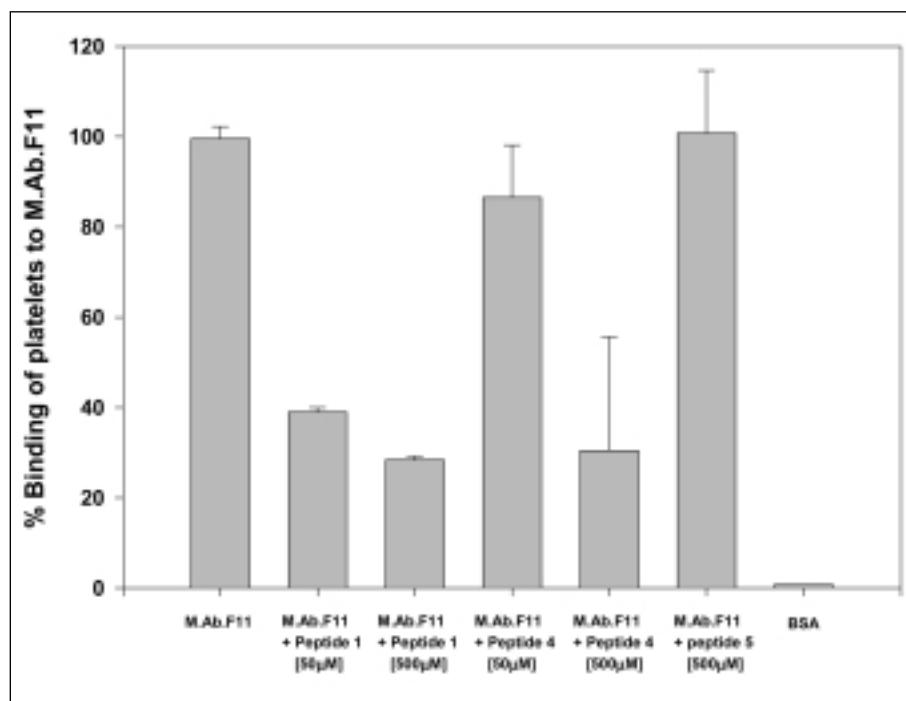


Fig. 8 Platelets adhere to immobilized M.Ab.F11: inhibition of adhesion by F11R peptides 1 and 4. M.Ab.F11 (150 ng) was immobilized in wells of a 96-well microtiter plate. Individual peptides (peptide 1 at 50 µM or 500 µM; peptide 4 at 50 µM or 500 µM; or peptide 5 at 50 µM or 500 µM) were added to platelet suspensions ($10^8/\text{ml}$), and aliquots of this mixture (100 µl) were applied to each well. Following a 90 min incubation period at 37°C, platelet adhesion was measured (16). Control wells were coated with BSA (1 µg), and this background value was subtracted from total values. Numbers represent the mean + SE of 3 separate determinations. No inhibition of adhesion was observed with peptides 2, 3, and 5

dimerization of F11R on the platelet surface may trigger a signaling pathway that primes the adhered platelets to become activated by low levels of circulating agonists. Through this mechanism, F11R and other CAMs could participate not only in the adhesion process itself, but also contribute to the initial formation of a platelet aggregate, as well as to the slow growth of platelet plaques underlying the pathophysiology of thrombosis associated with inflammatory processes.

Since F11R peptide 1 as well as peptide 4 can inhibit F11R-mediated platelet aggregation, adhesion, and potentiation, the sequence of these two peptides provides the basis for designing novel therapeutic

agents for the treatment and prevention of certain types of thrombocytopenia, as well as that of thrombosis.

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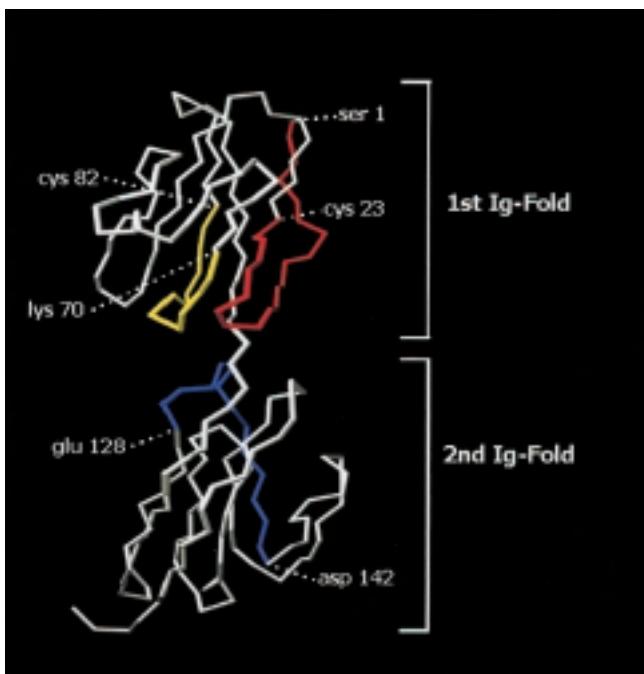


Fig. 9 The 3-D structure of the external domain of the mature human platelet F11R. The two Ig-like folds of the human recombinant F11R protein (sF11R) are shown here as a backbone structure based on the template of the mouse JAM (17) which shares approximately 70% homology to that of human F11R (6-8). The red color shows the position of the N-terminal 23 amino acids from ser-1 of the mature protein to cys-23 (sequence of peptide 1), and the yellow color represents the amino acid sequence lys-70 to cys-82 (sequence of peptide 4). The blue color represents the amino acid sequence of the non-inhibitory peptide 5 (glu-128 to asp-142) located in the 2nd Ig fold.

Addendum

E. Kornecki, Y. H. Ehrlich and A. Babinska were involved in the design and planning of all experiments; M. H. Kedeess, H. Athar, T. Sobocki and M. M. Hussain designed and prepared the recombinant sF11R in transfected COS-7 cells; M. B. Sobocka initiated and participated in experiments of platelet potentiation by M.Ab.F11; and T. Ahmed used the template to generate the 3D structural model of the human sF11R.

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