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Signaling Pathways of the F11 Receptor (F11R; a.k.a. JAM-1, JAM-A) in Human Platelets: F11R Dimerization, Phosphorylation and Complex Formation with the Integrin GPIIIa

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ABSTRACT

The F11 receptor (F11R) (a.k.a. Junctional Adhesion Molecule, JAM) was first identified in human platelets as a 32/35 kDa protein duplex that serves as receptor for a functional monoclonal antibody that activates platelets. We have sequenced and cloned the F11R and determined that it is a member of the immunoglobulin (Ig) superfamily of cell adhesion molecules. The signaling pathways involved in F11R-induced platelet activation were examined in this investigation. The binding of M.Ab.F11 to the platelet F11R resulted in granule secretion and aggregation.

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These processes were found to be dependent on the crosslinking of F11R with the FcγRII by M.Ab.F11. This crosslinking induced actin filament assembly with the conversion of discoidal platelets to activated shapes, leading to the formation of platelet aggregates. We demonstrate that platelet secretion and aggregation through the F11R involves actin filament assembly that is dependent on phosphoinositide-3 kinase activation, and inhibitable by wortmannin. Furthermore, such activation results in an increase in the level of free intracellular calcium, phosphorylation of the 32 and 35 kDa forms of the F11R, F11R dimerization coincident with a decrease in monomeric F11R, and association of the F11R with the integrin GPIIIa and with CD9. On the other hand, F11R-mediated events resulting from the binding of platelets to an immobilized surface of M.Ab.F11 lead to platelet adhesion and spreading through the development of filopodia and lammelipodia. These adhesive processes are induced directly by interaction of M.Ab.F11 with the platelet F11R and are not dependent on the FcγRII. We also report here that the stimulation of the F11R in the presence of nonaggregating (subthreshold) concentrations of the physiological agonists thrombin and collagen, results in supersensitivity of platelets to natural agonists by a F11R-mediated process independent of the FcγRII. The delineation of the two separate F11R-mediated pathways is anticipated to reveal significant information on the role of this cell adhesion molecule in platelet adhesion, aggregation and secretion, and F11R-dependent potentiation of agonist-induced platelet aggregation. The participation of F11R in the formation and growth of platelet aggregates and plaques in cardiovascular disorders, resulting in enhanced platelet adhesiveness and hyperaggregability, may serve in the generation of novel therapies in the treatment of inflammatory thrombosis, heart attack and stroke, and other cardiovascular disorders.

Key Words: F11 receptor; F11R; Junctional adhesion molecule; JAM; JAM-1; JAM-A; PI-3 kinase; Calcium ions; Platelet aggregation; Cell adhesion molecule (CAM); F11 receptor dimerization, integrin GPIIIa.

INTRODUCTION

The F11 receptor (F11R) is a cell adhesion molecule (CAM), member of the immunoglobulin superfamily described first in human platelets in 1990 (1–6). In 1998, amino acid sequences homologous to the F11R were found in a protein present within tight junctions of endothelial cells (7) where the molecule was called Junctional Adhesion Molecule (JAM). The Human Genome Nomenclature committee of the NIH (HGN) has designated F11R as the official name for the human protein (LocusID:50848) with this sequence, with F11r (LocusID:16456) the designated name for the murine protein, (www.ncbi.nlm.nih.gov/LocusLink/list.cgi). Aliases of F11R are listed by the HGN as JAM-1/JAM-A, with sequence similarities found in related cell adhesion molecules identified as JAM-2 and JAM-3, (a.k.a. JAM-B, JAM-C).

Monoclonal antibody F11 (M.Ab.F11) was demonstrated to recognize a 32 and 35 kDa protein duplex on the membrane surface of unstimulated, intact human platelets (1). The 32 kDa and 35 kDa proteins are glycosylated forms of the same core protein with molecular weight of 29 kDa (1,2). Activation of the F11R by the

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addition of M.Ab.F11 was shown to result in platelet shape change, secretion of granular contents, followed by the exposure of fibrinogen receptors and culminating with platelet aggregation (1). A biochemical pathway that underlies F11R-mediated platelet secretion and fibrinogen-dependent aggregation is triggered by the binding of M.Ab.F11 to an activation epitope within the external domain of the F11R (6), with subsequent coupling by M.Ab.F11 of the F11R to the platelet Fc receptor, FcγRII (2). This pathway involves the activation of PKC, specifically the platelet PKC isozymes α, β, δ, ζ, n (n'), and θ (4). PKC activation is followed by a time-dependent translocation, from the cytoplasm to the plasma membrane, of PKC isozymes α and ζ, which occurs in a transient, reversible manner. The translocation of PKC isozymes δ, β, n, and θ from the cytosol to the plasma membrane occurs in an irreversible manner (4), allowing for the phosphorylation of membrane constituents within the cytoplasmic domain. Platelet aggregation and secretion induced by M.Ab.F11 results in the rapid phosphorylation of platelet plecstrin (p40) and the light chain of myosin (p20), followed by their dephosphorylation (1). Components of activation epitopes within the external domain of the F11R recognized by M.Ab.F11 have been identified recently (6) and found to reside within two regions of close steric proximity to each other, the 23 amino acid stretch starting at the N-terminus and a thirteen amino acid region found within the first Ig-fold of the F11R molecule.

Unlike platelet aggregation, the adhesion of platelets through the F11 receptor was found to be independent of the FcγRII (6). Platelet binding to immobilized M.Ab.F11, and its continued adhesion and spreading by the development of lamellopodia and filopodial extensions, were shown to occur in the presence of the FcγRII blocker, M.Ab.IV.3, placing this mechanism as an FcγRII-independent process. In the present study, the potentiation of platelet aggregation through activation of the F11R was shown by the enhanced sensitivity of platelets to natural agonists due to the ligation of the F11R by M.Ab.F11. We determined that this process is FcγRII-independent, functioning through direct activation of the F11R. Through this process, the F11R can contribute significantly to the adhesion of platelets to endothelial cells (6), and may play an important role in the initiation of inflammatory thrombosis (8).

MATERIALS AND METHODS

Reagents

Sepharose CL4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) Electrophoresis-pure reagents, including acrylamide, ammonium persulfate, nitrocellulose, SDS, and TEMED were purchased from BioRad (Hercules, CA). Wortmannin was obtained from Sigma (St. Louis, MO) and dissolved in DMSO (J.T. Baker Chemical Co., Phillipsburg, NJ). All other reagents were obtained from Sigma (St. Louis, MO) and were of the highest reagent grade.

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Antibodies

Monoclonal antibody F11, MAb.F11, directed against the F11R, was prepared as described previously (1). FcγRII antibody, M.Ab.IV.3, was purchased from Medarex (Annandale, NJ). The characteristics of an anti-GPIIIa antibody (M.Ab.G10) have been detailed previously (1,3). The CD9 antibody was obtained from a panel of characterized CD9 antibodies (3).

Washing of Platelets

Whole blood was collected into the anticoagulant acid–citrate dextrose (ratio of 1:6). Platelets were isolated following the Mustard washing procedure in the presence of the platelet aggregation inhibitors apyrase, heparin, and PGE_1 as detailed previously (1). The final platelet pellet was resuspended gently in the absence of inhibitors in Tyrode's solution, pH 7.4 as detailed in our previous studies (1).

Aggregation of Human Platelets

Platelet aggregations were carried-out at 37°C under stirring conditions in a Chronolog Lumi-Aggregometer (Chronolog Corp., Havertown, PA) as previously described (1). Aggregation tracings were plotted on a dual channel recorder as changes in optical transmission following the addition of various agonists to washed platelet suspensions (3×10^8/mL) supplemented with fibrinogen (0.1 mg/mL).

Sensitization of Agonist-Induced Platelet Aggregation by M.Ab.F11

The potentiation of agonist-induced platelet aggregation through the F11R by M.Ab.F11 was examined in the following manner: washed platelet suspensions (3×10^8/mL) were preincubated for 2 min with FcγRII monoclonal antibody M.Ab.IV.3 (at a final concentration of 20 µg/mL). In the presence of this concentration of M.Ab.IV.3, platelet aggregation did not occur in response to M.Ab.F11 (4 µg/mL). Subthreshold concentrations of ADP (1 nM), thrombin (0.002 U/mL), or collagen (3 µg/mL), which alone did not induce any aggregation, were added to platelet suspensions in the presence of a combination of M.Ab.F11 and M.Ab.IV.3. Quantitation of the extent of M.Ab.F11-induced platelet aggregation was determined by measuring arbitrary light transmission units (LTU) on a chart recorder.

Measurement of Intracellular Free Calcium Levels

Aliquots of isolated, washed human platelets were loaded with the photoprotein, aequorin, in the presence of DMSO as described previously (9).

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Intracellular Protein Phosphorylation and Immunoprecipitation

Isolated, washed platelet pellets were resuspended gently in a phosphate-free Tyrode's solution (pH 7.4) containing PGE_1 (1 nM), and incubated for 1 h at 37°C in the presence of 1 mCi [^{32}Pi]. Following this incubation period, the platelets were centrifuged and the platelet pellet was resuspended in Tyrode's solution (pH 7.4). The radiolabeled platelets were treated with M.Ab F11 (10 μg/mL) at 37°C under stirring conditions for various periods of time. At each time point, aliquots of the suspension were removed and lysed in the presence of 1% NP-40 buffer A [50 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, leupeptin (10 μg/mL). To immunoprecipitate the F11R complex, aliquots of these detergent extracts were centrifuged and supernatants were incubated with M.Ab F11 coupled-Sepharose Cl-4B or Sepharose Cl-4B (used as a control)] overnight at 4°C, centrifuged and the immunoprecipitated proteins were washed with 0.1% NP-40-buffer A and 0.1% NP 40, 1 M NaCl-buffer A. The immunoprecipitated F11 receptor protein was analyzed by SDS-PAGE followed by autoradiography. Quantitation of the incorporated [^{32}Pi] was performed by a laser densitometric scanning (Ultrascan XL, LKB) of autoradiograms.

Measurement of F-Actin Content

The F-actin content of resting and activated platelets was determined as previously detailed (10), by quantitation of FITC-phalloidin binding to formaldehyde fixed platelets after permeabilization with Triton X-100 (11,12). Platelets were fixed with an equal volume of 3.6% formaldehyde at various times after addition of M.Ab.F11 at 37°C for a minimum of 30 min. Fixed cells were permeabilized with 0.1 vol. of 1% Triton X-100 containing 10 μM FITC-phalloidin at 25°C for a minimum of 60 min. Labeled platelets were gated by forward- and site- scattering, and the mean fluorescence of 10,000 cells quantitated in a flow cytometer using the image II software (Becton-Dickinson FACScan, Mansfield, PA). The results are expressed as the percentage of F-actin, using the well-established value of 40% as the amount of F actin in resting cells (13). Each value represents a minimum of 6 measurements obtained from two separate experiments.

Immunoprecipitation

The platelets were isolated following the washing procedure detailed above. The platelet pellets were resuspended at 37°C in Tyrode's solution (pH 7.4) at a concentration of 10^{10} platelets/mL and treated with M.Ab.F11 (8 μg/mL) in the presence of fibrinogen (400 μg/mL). Aliquots (100 μl) containing 10^9 platelets were removed at different time points. Platelet activation was terminated by the addition of 6X concentrated lysis buffer [1X lysis buffer containing the following: 1% NP-40, 50 mM Tris HCl pH 7.5,150 mM NaCl, 5 mM NaF,1 mM Na_3VO_4, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, and leupeptin (10 μg/mL)]. Platelet lysates were centrifuged for 10 min, and the supernatants were incubated with Sepharose Cl-4B

conjugated with M.Ab.F11, or with control, Sepharose Cl-4B (not conjugated with M.Ab.F11) for 16 h at 4°C. The samples were centrifuged and the pellets were washed with lysis buffer containing 0.1% NP-40 followed by lysis buffer containing 0.1%NP 40 and 1 M NaCl, to remove nonspecifically-bound proteins. The immunoprecipitated proteins were subjected to SDS-PAGE and analyzed by immunoblotting using various antibodies, the F11R antibody, M.Ab.F11 (1,5), anti-β3 integrin, M.Ab.G10 (1,5), and a CD9 antibody (3).

RESULTS AND DISCUSSION

F11 Receptor (F11R) Stimulation Induces Changes in the Morphology of Human Platelets

Activation of the F11R by the direct application of M.Ab.F11 to washed, isolated platelet suspensions, resulted in changes in platelet morphology as depicted in Fig. 1. Platelets photographed exactly at the time of addition of M.Ab.F11 (at

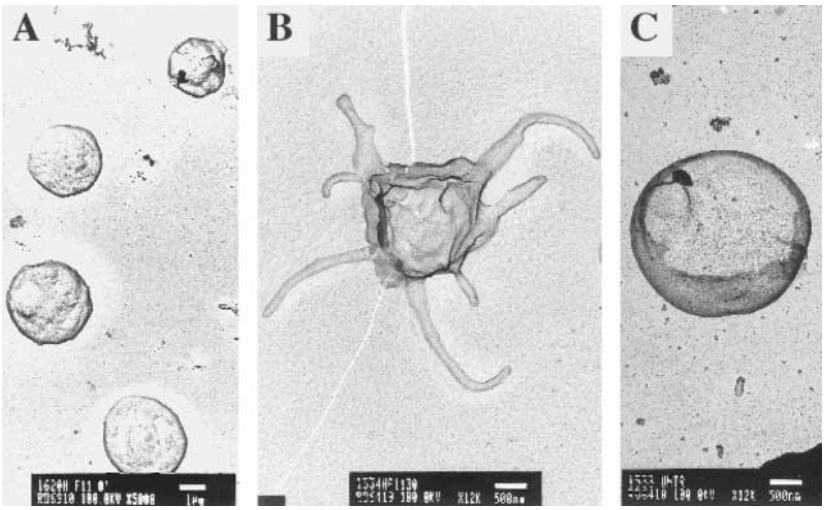

Figure 1. Changes in the morphology of platelets activated by M.Ab.F11. Washed platelets were prepared as described in the Materials and Methods section. Following procedures detailed by Hartwig (10) for morphological studies by light microscopy, untreated, control platelets or M.Ab.F11-treated platelets were fixed by the addition of 1 vol of 4% paraformaldehyde and visualized using TRITC-phalloidin after permeabilization with Triton X-100. Panel A. The morphology of platelets was examined at zero time, by fixing immediately following the addition of M.Ab.F11 (10 µg/mL). A scale of 1 µm is indicated on the bottom of the panel. Panel B. Changes in platelet morphology were observed following a 30 min incubation period with M.Ab.F11. A scale of 500 nm is indicated at the bottom of the panel. (C) Control, untreated platelets were fixed following a 30 min period of incubation under the same conditions, as in Panel B, but without antibody. The bar on the bottom of this panel represents a scale of 500 nm.

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zero time) (Fig. 1A) or intact, nonstimulated platelets not exposed to M.Ab.F11 (Fig. 1C), exhibited a flat discoidal configuration characteristic of unactivated, resting platelets. On the other hand, following the addition of M.Ab.F11, the platelets acquired a rounded, spherical appearance with filopodial extensions viewed as protrusions from the platelet surface (Fig. 1B).

The morphological changes observed above were accompanied by significant rearrangements in the platelet cytoskeleton, following the activation of platelets by M.Ab.F11. As depicted in Fig. 2A (at low magnification) and Fig. 2B (at higher magnification), the stimulation of the F11R by M.Ab.F11 resulted in the development of well-formed, intricate platelet cytoskeletal lattice networks that were assembled along dense, centralized core structures. The morphological changes seen in Fig. 1, as well as the underlying cytoskeletal rearrangements seen in Fig. 2, that are induced in platelets by activating the F11R, are very similar, in fact appear to be identical, to those induced by the activation of receptors for the naturally-occurring strong agonists of platelets, collagen, and thrombin.

The examination of the distinguishing molecular events associated with F11R activation of platelets was investigated in the subsequent series of experiments.

F11R Stimulation Induces Changes in the F-Actin Content of Platelets

To determine the biochemical processes operating in inducing the cytoskeletal changes that occur in platelets following F11R stimulation by M.Ab.F11, we measured the level of F-actin assembly as a function of time following F11R induced platelet activation. As shown in Fig. 3, the percentage of F-actin (%) formed in platelets following the addition of M.Ab.F11 increased from a basal level of 40% in resting platelets to 54% within a 5 min period. This value was determined to differ significantly from the basal F-actin level. A ratio value of 1.35 of F-actin (F-actin in the activated state/F-actin at rest) was obtained at the peak of M.Ab.F11-induced platelet activation at this time and the ratio value of about 1.35 remained constant for the entire extent of platelet activation. Similar changes in levels of F-actin were determined following activation of platelets by phorbol myristate acetate and other agonists (14).

The assembly of an F-actin lattice in platelets undergoing shape-change is the common biochemical mechanism for stimulation by thrombin and collagen (15). In addition to the critical role of actin in the conversion of platelet discs into spherical shapes, a large number of other platelet proteins are also involved in this morphological conversion, including α-actinin, adducin, Arp2/3 protein complex, gelsolin, GPIb-IX-V, GPIIb-IIIa, myosin II, spectin, and tubulin, as well as fyn kinase, lyn kinase, PI-3 kinase, pleckstrin, and trimeric G-proteins (15). Shape-change induced by the stimulation of the F11R would appear to reach the same final molecular events responsible for cytoskeletal rearrangements as those that are induced by potent physiological agonists.

The preincubation of platelets with a PI-3 kinase inhibitor resulted in the complete inhibition of the M.Ab.F11-induced F-actin assembly (Fig. 3). At each time point examined, wortmannin (at a final concentration of 100 nM) interfered with the induction by M.Ab.F11 of F-actin assembly. These results indicate that PI-3

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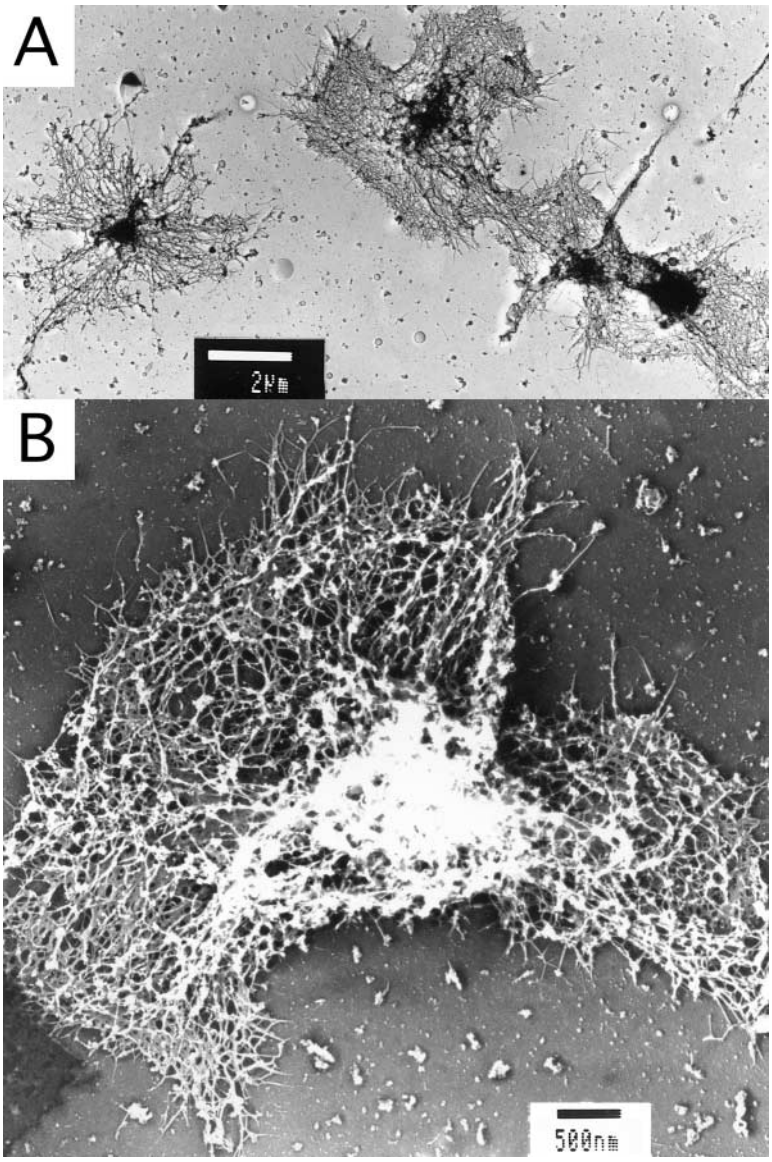

Figure 2. Structure of the isolated cytoskeleton following stimulation of platelets with M.Ab.F11. Following procedures described by Hartwig (10) for morphological studies examined by electron microscopy, washed platelets were incubated with M.Ab.F11 for 10 min and platelet cytoskeletons were prepared as detailed (10) for the Rapid Freezing and Freeze-drying of permeabilized platelets. Panel A. Isolated platelet cytoskeletons derived from M.Ab.F11 (10 μg/mL)-activated platelets. The bar indicates a scale of 2 μm. Time of incubation with M.Ab.F11 was 10 min. Panel B. Magnified view of the M.Ab.F11 (10 μg/mL)-activated platelet cytoskeleton. The bar indicates a scale of 500 nm.

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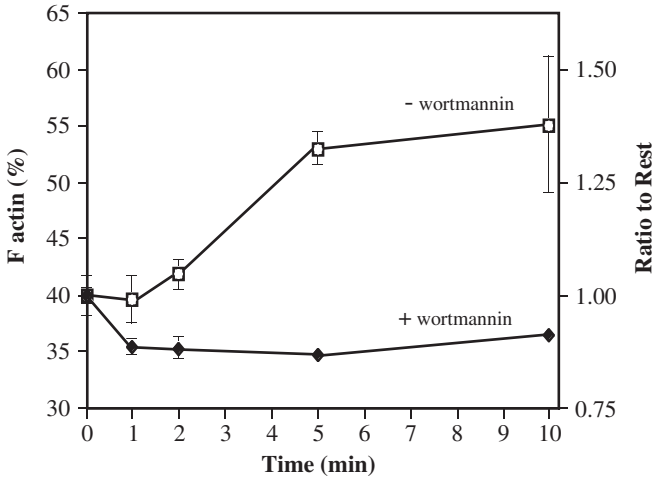

Figure 3. Assembly of actin induced by stimulation of platelets by M.Ab.F11: inhibition by the PI-3 kinase inhibitor, wortmannin. M.Ab.F11 (10 μg/mL) was added to resting, gel-filtered platelets in the presence of 100 nM wortmannin (**circles**) or in the absence of wortmannin (**squares**). At various time points examined during platelet aggregation, the percentage of the F-actin content in M.Ab.F11-activated platelets was compared to the % F-actin content found in resting platelets (Ref. 10–14). The ratio of the F-actin content in activated versus resting platelets (**Ratio to Rest**), determined in the presence and absence of wortmannin, is indicated on the right side of this figure.

kinase activation is a necessary step in F11R induced, actin-dependent platelet shape change. Interestingly, the presence of wortmannin (100 nM) caused an immediate drop in the level of F-actin, and inhibition was maintained throughout the entire period of exposure to M.Ab.F11.

Platelet Activation by M.Ab.F11 Is Blocked by Inhibitors of PI-3 Kinase and Protein Kinase C

In addition to F-actin assembly, the involvement of PI-3 kinase in platelet activation through F11R was studied by a functional test: examination of the effects of wortmannin on M.Ab.F11-induced platelet aggregation. As shown in Fig. 4A, wortmannin, at concentrations of 50 nM to 100 nM, completely inhibited M.Ab.F11-induced platelet aggregation as evidenced by the findings that even after a long latency period of 30 min, no aggregation could be observed. Lower concentrations of wortmannin (5 nM to 25 nM), significantly delayed the onset of platelet aggregation by a four to five-fold extension of the latency period.

The role of protein kinase activity in M.Ab.F11-induced platelet aggregation was examined by using the inhibitor staurosporine. Fig. 4B shows that, at 100 nM, staurosporine completely blocked the M.Ab.F11-induced platelet aggregation as evidenced by the prolonged latency of over 30 min. Concentrations of staurosporine

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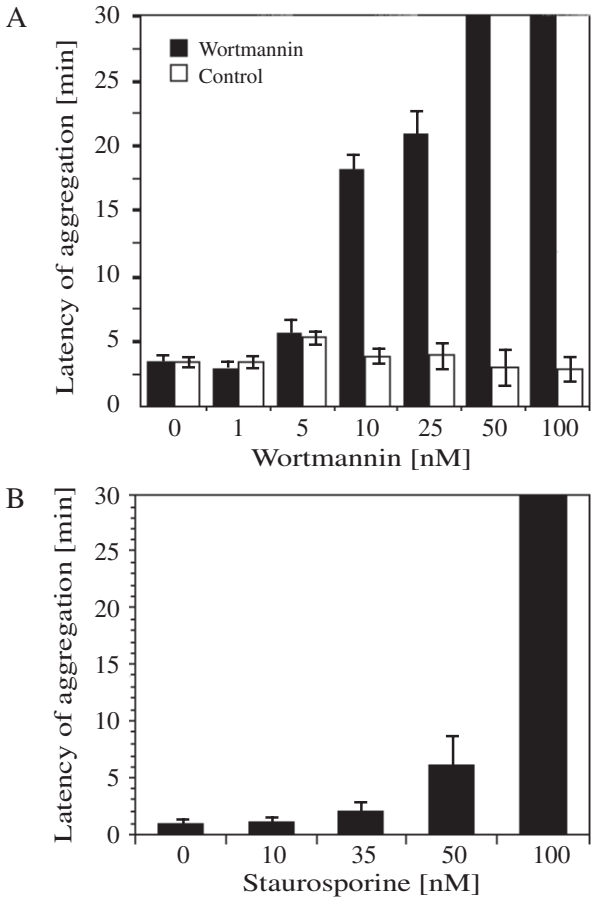

Figure 4. Inhibition of M.Ab.F11-induced platelet aggregation by the PI-3 kinase inhibitor, wortmannin, and by the PKC inhibitor, staurosporine. (A) Inhibition by wortmannin. Washed platelets were incubated for 5 min at 37°C with wortmannin, at the concentrations indicated above (nM) (dark histograms), or with equivalent volumes of DMSO as control samples (open histograms), and then stimulated with M.Ab.F11 (8 μg/mL). At 50 nM, complete inhibition of platelet aggregation was observed which lasted for hours. The values represent the mean ± SD of three experiments. (B) Inhibition by staurosporine. Washed platelets were preincubated for 1 min at 37°C with staurosporine, at the concentrations indicated in the figure (nM), and the M.Ab.F11 (8 μg/mL) was added to initiate platelet aggregation. The latency period is indicated as the time-period required for the detection of a change in optical transmission following the addition of M.Ab.F11. At the concentration of 100 nM, complete inhibition of M.Ab.F11-induced platelet aggregation was observed which lasted for hours. The values represent the mean ± SD of three experiments.

of more than 50 nM significantly prolonged the latency period and delayed the onset of platelet aggregation. Among the protein kinases known to be inhibited by nanomolar concentrations of staurosporine, are PKC (IC_{50} value of 2.7 nM) (16), cAMP-dependent protein kinase (IC_{50} value of 8 nM) and $p60^{v\text{-}src}$ tyrosine protein

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kinase (IC_{50} of 6 nM) (17,18). Furthermore, in intact platelets, staurosporine (at 1 μM) was shown to inhibit thrombin-induced phosphorylation of both the 47 kDa protein, a PKC substrate, as well as the phosphorylation of the 20 kDa substrate for myosin light chain kinase (19).

Phosphorylation of the F11R Protein During Platelet Aggregation

The sequencing of the cloned F11R (5) has identified several sites with consensus sequences for phosphorylation within the cytoplasmic tail domain of the F11R protein. Fig. 5 demonstrates that M.Ab.F11-induced platelet aggregation is associated with the phosphorylation of the F11R protein itself. A tracing demonstrating the actual time-course of platelet aggregation induced by the application of M.Ab.F11, is shown in Fig. 5A. Concurrently, we removed aliquots at various time points from these aggregating platelets, and examined the phosphorylation status of the F11R at each point. We observed a time-dependent phosphorylation of the F11R, both in the 32 and 35 kDa protein components of the F11R, as shown in Fig. 5B. Phosphorylation of the 32 and 35 kDa proteins was observed within 30 s, the shortest time point measured following the addition of M.Ab.F11 (see Fig. 5, lane #2). The phosphorylation of both the 32 and 35 kDa proteins of the F11R increased to a maximal level within a 4-min period following the induction of platelet aggregation by M.Ab.F11 (Fig. 5B, lane #4). This time point was observed to coincide with the achievement of a maximal M.Ab.F11-induced platelet aggregation (Fig. 4A, see arrow at position 4).

The phosphorylation of sites within the intracellular cytoplasmic tail region of a receptor protein (that would be measured when intact cells are labeled with ^{32}Pi, as performed here) is known to play a role in the signaling pathway triggered by the stimulation of this receptor. The results presented in Fig. 5 indicate that this molecular event occurs also in platelet activation through the F11R.

The first study which demonstrated that the intracellular phosphorylation of a receptor protein plays an important role in its regulation focused on the beta-adrenergic receptor (20). We have reported that the F11R becomes phosphorylated specifically by PKC in vitro, in a mixture consisting of affinity-purified F11R, purified brain PKC and the PKC activator, phorbol 12-myristate 13-acetate in the presence of phosphatidyl serine (2). The cytoplasmic tail of the F11R was found to become phosphorylated following activation of the [^{32}Pi]-loaded platelets by addition of the physiological agonists thrombin and collagen, and by M.Ab.F11 itself (5). Ozaki and colleagues (21) have confirmed our earlier findings showing the incorporation of phosphate into F11R following platelet activation, and presented evidence demonstrating that following the stimulation of platelets with thrombin, the amino acid within the cytoplasmic domain of F11R that becomes phosphorylated is Ser 284. In the present study, both the 32 kDa and 35 kDa proteins of the F11R were phosphorylated upon platelet activation indicating that phosphorylation of these proteins occurred intracellularly, at sites within the cytoplasmic tail of F11R (5).

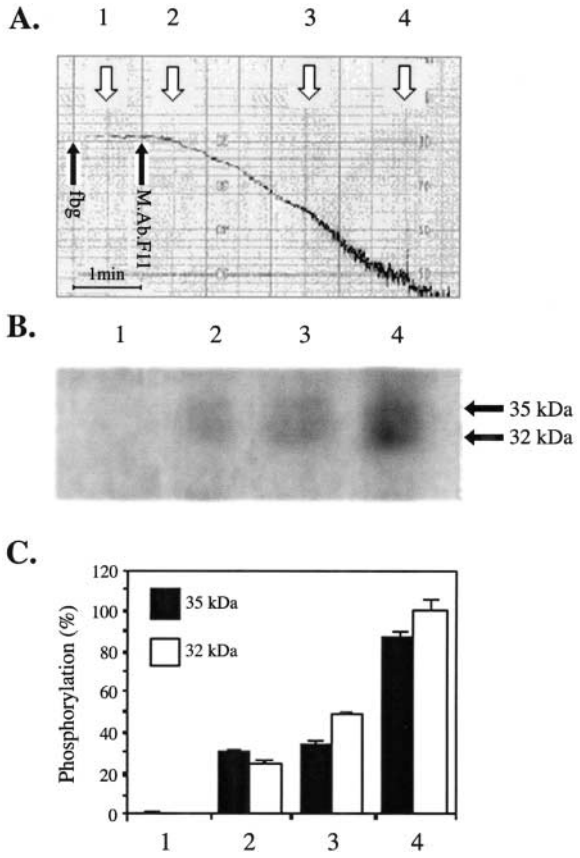

Figure 5. Phosphorylation of the platelet F11 receptor (F11R) following platelet activation by M.Ab.F11. (A) Platelet aggregation by M.Ab.F11. Washed human platelets (10^{10}/mL) were loaded with inorganic ^{32}Pi for 1 h at 37°C, as described in Materials and Methods. These platelets were induced to aggregate, in the presence of fibrinogen (400 µg/mL) at 37°C under stirring conditions, by adding M.Ab.F11 (8 µg/mL), as shown in the representative tracing. (B) Autoradiogram. Lane 1 depicts the basal state of phosphorylation of the F11R, 30 s prior to the addition of M.Ab.F11 (arrow 1 in 5A). Lane 2 depicts the phosphorylation of the F11R at 30 s following the addition of M.Ab.F11 (arrow 2 in 5A). Lane 3 depicts the phosphorylation of the F11R 2.5 min following the addition of M.Ab.F11 (arrow 3 in 5A). Lane 4 depicts the phosphorylation state of the F11R 4 min following the addition of M.Ab.F11 (arrow 4 in 5A). The arrow points to the 35 and 32 kDa protein bands of the F11R. Aliquots of the activated platelets were removed at four time points (shown by the arrows numbered 1–4 in Fig. 4A). Platelet proteins were solubilized in NP40 detergent containing buffer, and the F11R was immunoprecipitated using a M.Ab.F11-coupled to Sepharose 4B. The F11R-immunoprecipitates were washed extensively, proteins separated by SDS-PAGE and the gels processed for autoradiography. (C) Quantitation. The level of phosphorylation of the 32 and 35 kDa components of the F11R were quantitated by densitometric scanning of the autoradiogram. The highest degree of ^{32}Pi incorporation was observed in the 32 kDa component of F11R. For quantitative purposes, the densitometric reading, obtained for the phosphorylated 32 kDa protein at position 4, was set at 100%, and used for comparative purposes. Values represent the mean ± SD of four experiments.

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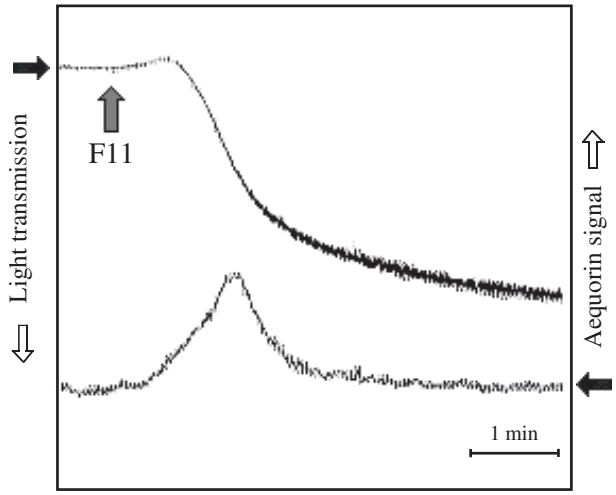

Figure 6. Increase in intracellular free calcium levels by M.Ab.F11. The *upper tracing* depicted in this panel demonstrates platelet aggregation following the addition of M.Ab.F11 (4 μg/mL) (at arrow), associated with an increase in light transmission. The *lower tracing* shows the changes in intracellular free calcium levels, measured as an increase in the generation of a light signal in aequorin-loaded platelets following the addition of M.Ab.F11. The figure shown here is representative of five separate experiments.

Increase in the Level of Intracellular Free Calcium by M.Ab.F11

Similar to the determination of F11R phosphorylation in real time, the levels of intracellular free calcium were measured simultaneously along with the induction of platelet aggregation induced by M.Ab.F11. As shown in Fig. 6 (in the lower tracing), using platelets preloaded with the calcium probe aequorin, we determined that an increase of intracellular free calcium levels had occurred within 30 s following the addition of M.Ab.F11. This transient increase in the level of intracellular free calcium reached a maximal level within 2 min, coincident with the attainment of a maximal extent of platelet aggregation.

Dimerization of the F11R and the Association of GPIIIa with F11R During Platelet Aggregation

During the process of platelet aggregation induced by M.Ab.F11, aliquots of stirred platelet suspensions were removed from the cuvettes, and platelets were lysed in the presence of protease inhibitors. The lysates were immunoprecipitated with M.Ab.F11, as detailed in the Materials and Methods section, and subjected to SDS-PAGE followed by immunoblotting with either one of three antibodies: first, M.Ab.F11, that recognizes the F11R (1,5), second, M.Ab.G10 that recognizes specifically GPIIIa (1,5), and third, an antibody that recognizes specifically CD9. The resulting immunoblots are shown in Fig. 7. Panel A of Fig. 7 shows the

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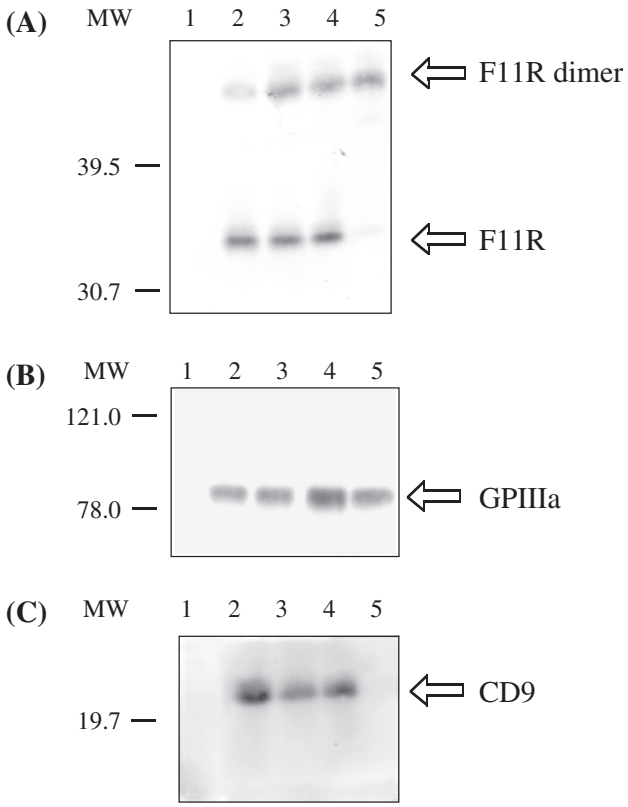

Figure 7. Dimerization of F11R and association of F11R with GPIIIa and CD9. Washed platelets were simulated with M.Ab.F11 (8 μg/mL). Aliquots were removed at the time points indicated below for panel B and processed for immunoprecipitation of the F11R by M.Ab.F11, followed by immunoblotting with specific antibodies. Panel A. Immunoblotting of F11R immunoprecipitates using M.Ab.F11. The same experiments, as detailed above in Panel A, were performed, expect that the F11R immunoprecipitates were probed using M.Ab.F11. The lower arrow at about 32–35 kDa points to the F11R, and the higher arrow points to a protein band of approximately 60 kDa, called the F11R dimer. Panel B. Immunoblotting of F11R immunoprecipitates using anti-GPIIIa antibody. *Lane 1*, Control. Unstimulated platelets were "immunoprecipitated" using uncoupled Sepharose. *Lane 2*, Control. Unstimulated platelets were immunoprecipitated using M.Ab.F11-Sepharose. *Lane 3*. Immunoblot of F11R immunoprecipitates obtained from M.Ab.F11-stimulated platelets (30 s following the addition of M.Ab.F11), using M.Ab.F11-Sepharose. *Lane 4*. Immunoblot of F11R immunoprecipitates obtained from M.Ab.F11-stimulated platelets (90 s following the addition of M.Ab.F11), using M.Ab.F11-Sepharose. *Lane 5*. Immunoblot of F11R immunoprecipitates obtained from M.Ab.F11-stimulated platelets (120 s following the addition of M.Ab.F11). Maximal platelet aggregation by M.Ab.F11 was observed at this time point. The arrow points to the recognition of GPIIIa by the anti-GPIIIa antibody, M.Ab.G10. Panel C. Immunoblotting of F11R immunoprecipitates using CD9 antibody. The same experiments as detailed above in Panel B, were performed, except that the F11R immunoprecipitates were probed using CD9 antibody. The arrow points to a protein band of approximately 24 kDa. The results shown in Panels A,B,C are representative of five experiments.

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immunostaining pattern of blots immunostained with M.Ab.F11. The panel contains two arrows, with the higher arrow pointing to a 66 kDa band identified as an F11R dimer, and the lower arrow (labeled F11R) points at the monomeric state of the F11R. Lanes depicted by the numbers 3, 4, and 5 indicate the time of platelet activation, following the addition of M.Ab.F11, of 30 s, 90 s, and 120 s, respectively. We observed that as a function of time following platelet activation by M.Ab.F11 there was a significant increase in the formation of a 66 kDa protein that was recognized by M.Ab.F11 (and quantitated) as a F11R dimer (see Fig. 8B). Concurrently, the level of the F11R monomer significantly decreased with the time of platelet activation (see Fig. 8A), and dropped to 40–50% of basal levels. These results clearly demonstrate that like other receptors of the Ig superfamily (e.g., insulin, PDGF-R) (22,23), receptor dimerization is a required molecular event in the activation of platelets via F11R-stimulation. Current studies in our laboratory investigate whether, like F11 receptor phosphorylation, also F11R receptor dimerization, is involved in platelet activation by natural agonists, collagen, and thrombin.

The separate immunostaining of F11R-immunoprecipitates with the anti-GPIIIa antibody, M.Ab.G10, is shown in the immunoblot depicted in Fig. 7B. The arrow identifies GPIIIa as a protein present within the immunoprecipitate obtained with M.Ab.F11. Interestingly, already under basal, resting conditions, GPIIIa is observed to be associated with the F11R (see Fig. 7B, lane 2), as determined by its presence in the F11R immunoprecipitate obtained from intact platelets.

During the time-course of platelet activation by M.Ab.F11, a significant increase in the level of GPIIIa was observed (see Figs. 7B and 8C) indicating association of F11R with the integrin GPIIb/IIIa ($\alpha_2\beta_3$). Similarly, recent work by Naik et al., (24) has found the F11R to be associated with the integrin $\alpha_v\beta_3$ in endothelial cells. Along with the presence of GPIIIa as an F11R-associated protein, we detected the presence of CD9 in the F11R-immunoprecipitates. However, in contrast to an enhancement during platelet activation by M.Ab.F11, we observed that CD9 was associated with the F11R complex, under resting conditions (see Fig. 7, lane 2). With an increase in the duration of platelet activation by M.Ab.F11, the association of CD9 with F11R decreased significantly, and eventually CD9 levels dropped to 40–50% of basal levels (see Fig. 7C lane 5, and Fig. 8D).

Silver staining of a PAGE gel of the F11R protein purified by immunoaffinity chromatography with M.Ab.F11 (2) revealed, in addition to the predominant 32 and 35 kDa protein duplex, the presence of four minor protein components. We have interpreted these results to mean that a naturally-occurring F11R protein complex exists within the platelet plasma membrane, which is not dissociated by the mild detergent used (NP40), and the proteins within this complex are co-precipitated with F11R by M.Ab.F11. The results depicted in Figs.7 and 8 identify two of the proteins associated with this F11R complex as GPIIIa and CD9. The change with time in the level of these proteins following stimulation of platelets by M.Ab.F11 suggests that dynamic alterations, association/dissociations of components within this complex, are involved in platelet activation.

Previous studies have demonstrated that GPIIIa is associated with CD9 (25–27). We now report the F11R is physically associated with both GPIIIa and CD9 already in the resting state of the platelet. Following platelet activation, a further increase in

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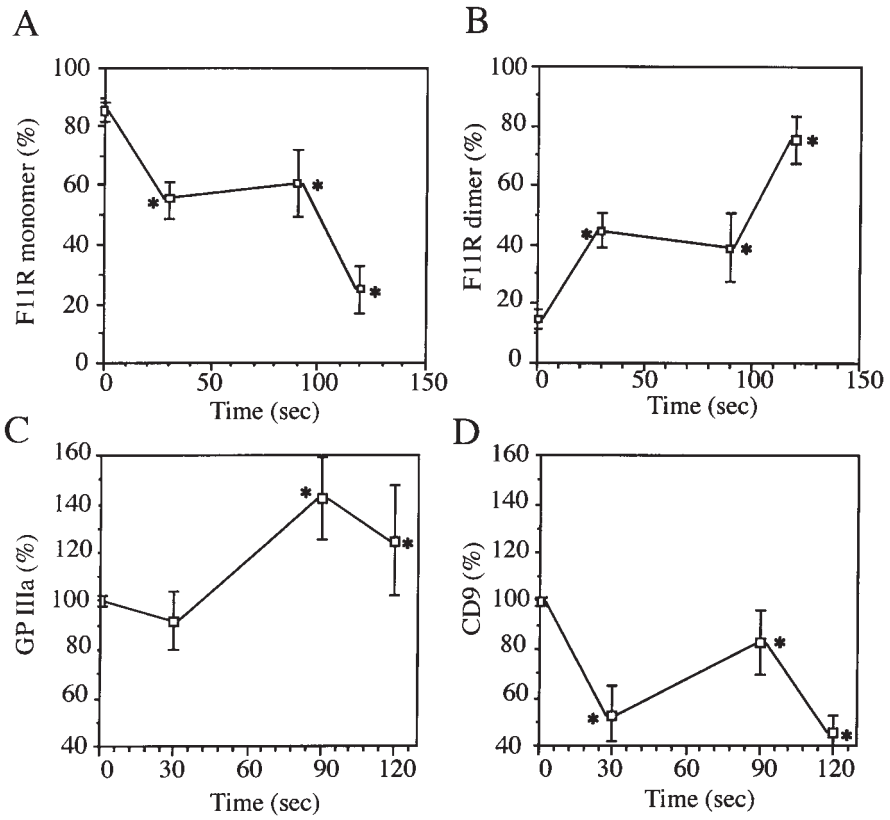

Figure 8. Quantitation of F11R dimerization and F11R-associated proteins, GPIIIa and CD9, following platelet activation. Panel A. The immunoblotted protein bands, identified in Fig. 7, were quantitated. Quantitation of F11R monomer (32 kDa protein band) as a function of time following platelet aggregation by M.Ab.F11 using NIH Image 1.61 for Macintosh Computers. Panel B. Quantitation of the F11R dimer (66 kDa protein band recognized by M.Ab.F11). Panel C. Quantitation of F11R-associated GPIIIa following platelet activation by M.Ab.F11. Panel D. Quantitation of F11R-associated CD9 following platelet activation by M.Ab.F11. Values represent the mean $\pm$ SD obtained in these experiments at each time point. An asterick at a designated time point, post-activation, indicates a significant difference ($p < 0.05$) from the baseline values obtained at zero time.

the association of the F11R with GPIIIa occurrs with time following platelet aggregation. On the other hand, the association of F11R with CD9 decreases with time following platelet activation.

Sensitization of Platelet Responses to Natural Agonists by Stimulation of F11R

We have reported that platelet aggregation and secretion induced by M.Ab.F11 require the crosslinking of the F11R with FcγRII (2). In our present study we

investigated whether another mechanism that operates following the activation of the F11R, the mechanism of platelet sensitization, also involves this pathway.

To examine the involvement of FcγRII in the activation of platelets through the F11R, we utilized the anti-FcγRII antibody M.Ab.IV.3 at concentrations that completely block M.Ab.F11-induced platelet aggregation. As depicted in every panel (panels A, B, and C) of Fig. 9 (see arrow pointing to the tracing depicting the addition of M.Ab.F11 + M.Ab.IV.3), the presence of M.Ab.IV.3 (20 μg/mL) inhibited M.Ab.F11 (4 μg/mL)-induced platelet aggregation for hours, and assured that binding of the Fc domain of M.Ab.F11 IgG to the platelet FcγRII was completely blocked. Under such FcR inhibitory conditions, the sensitization of platelets to their stimulation by low concentrations of natural agonists was examined. Fig. 9 (panel A) demonstrates that a nonaggregating, subthreshold concentration of ADP (1 nM), in the presence of M.Ab.F11 (plus the FcR inhibitor, IV.3), was able to induce a full-blown platelet aggregation and secretion (see M.Ab.IV.3 + M.Ab.F11 + ADP arrow), whereas, the addition of ADP alone (1 nM) produced only a minimal aggregation (see ADP arrow).

Similar platelet sensitization through engagement of the F11R, to low concentrations of thrombin could be measured by incubations of platelets with M.Ab.F11, in the presence of the anti-FcγRII, M.Ab.IV.3. As shown in Fig. 9B, subthreshold concentrations of thrombin (0.002 U/mL), in the presence of M.Ab.F11 (plus M.Ab.IV.3), induced strong platelet aggregation and secretion which was not observed when thrombin was added alone. In another series of experiments, we examined whether platelet aggregation induced by collagen could be potentiated by the presence of M.Ab.F11. We observed that collagen-induced platelet aggregation indeed, was sensitized to a large extent by the presence of M.Ab.F11 (plus M.Ab.IV.3) (see Fig. 9C). Interestingly, we observed that the anti-FcγRII antibody, M.Ab.IV.3, itself was capable of inhibiting collagen-induced platelet aggregation (Fig. 9C, see M.Ab.IV.3 + collagen). In spite of such strong, direct inhibitory effects on collagen-induced aggregation produced by M.Ab.IV.3 alone, the activation of the F11R by M.Ab.F11 resulted in a substantial degree of potentiation of the platelet aggregation induced by collagen. A possibility exists that the addition of low concentrations of agonists could overcome a partial FcγRII blockade by M.Ab.IV.3, with some signaling still occurring through the FcγRII pathway. However, this possibility is not very likely because experiments were conducted with excess amounts of M.Ab.IV.3, and even doubling the amount of M.Ab.IV.3 did not interfere with the potentiation of platelet aggregation in the presence of M.Ab.F11. The potentiation of platelet aggregation to substimulatory concentrations of ADP, thrombin and collagen by subthreshold concentrations of M.Ab.F11, as seen in Fig. 9, could be observed also in the absence of M.Ab.IV.3. Potentiation of agonist-induced aggregation by M.Ab.F11 in the presence of M.Ab.IV.3 demonstrates that the mechanism of crosslinking of F11R to FcγRII appears not to be involved in this process.

These results demonstrate that activation of the F11R can trigger two separate pathways leading to different cellular responses: one that leads directly to platelet activation and is triggered by the coupling of F11R to the FcγRII. This pathway may contribute to the development of immune-thrombocytopenia in individuals with circulating antibodies to the F11R (5). In a second pathway, independent of FcγRII,

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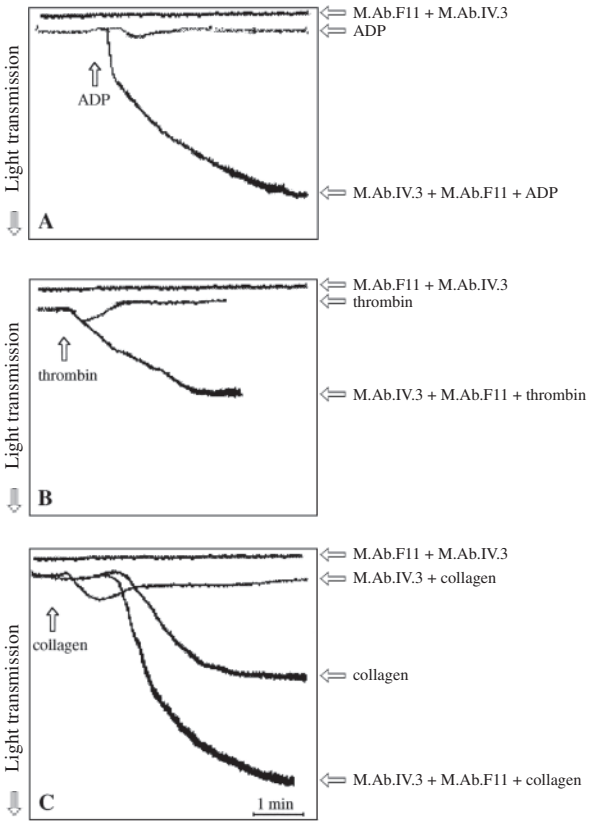

Figure 9. F11R-induced potentiation of platelet aggregation by subthreshold concentrations of agonists. Washed, isolated platelet suspensions were examined for platelet potentiation responses using subthreshold concentrations of agonists, as detailed below. Panel A. *Combined M.Ab.IV.3 + M.Ab.F11 + ADP treatment*. Platelet suspensions were preincubated for 1 min with M.Ab.IV.3 (20 μg/mL). This concentration of M.Ab.IV.3 and incubation period were tested for each donor and found to completely block platelet aggregation induced by M.Ab.F11 for over 1 h. Next, M.Ab.F11 (4 μg/mL) was added to platelet suspensions, and no aggregation was observed, as expected. Following a 1 min period, a concentration of ADP (1 nM) was added to the platelet suspension. This concentration of ADP was deemed to be a suboptimal, subthreshold concentration since, when added alone, it produced a minimal degree of platelet aggregation. *The ADP tracing*. This aggregation tracing demonstrates the minimal degree of platelet aggregation induced by a subthreshold concentration of ADP (1 nM), whether alone, or measured in the presence of M.Ab.IV.3. *M.Ab.F11 + M.Ab.IV.3*. The anti-FcγRII antibody, M.Ab.IV.3 (20 μg/mL) was added 1 min prior to the addition of M.Ab.F11 and completely blocked the aggregation. Panel B. *Combined M.Ab.IV.3 + M.Ab.F11 + thrombin treatment*. Similar experiments to those detailed above in Panel A were performed, except that a subthreshold concentration of thrombin (0.002 units/mL) was used. Comparisons in platelet aggregation were made in the presence of M.Ab.F11 + M.Ab.IV.3, thrombin alone, and to thrombin in the presence of M.Ab.IV.3. Panel C. *Combined M.Ab.IV.3 + M.Ab.F11 + collagen treatment*. Similar experiments to those detailed above in Panels A and B were performed, except that a subthreshold concentration of collagen (3 μg/mL) was used. Comparisons of platelet aggregation were made in response to *M.Ab.F11 + M.Ab.IV.3, collagen added alone* and to *collagen added in the presence of M.Ab.IV.3*. All figures are representative of five separate experiments.

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stimulation of the F11R leads to the sensitization of platelets to subthreshold concentrations of natural agonists in the circulation. Such sensitization may be caused by F11R-mediated adhesion of platelets to an inflamed endothelium (8). In this situation, the sensitized receptors of adhered platelets will be activated by low levels of circulating agonists, contributing to the slow growth of platelet plaques characteristic of inflammatory thrombosis. Accordingly, we propose that inhibition of the action of F11R would interfere with both the initial platelet plaque formation as well as with subsequent thrombi-growth and therefore drugs designed to inhibit F11R action could be useful for the prevention and treatment of thrombosis, atherosclerosis, heart attacks, and stroke.

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REFERENCES

1. Kornecki E, Walkowiak B, Naik UP, Ehrlich YH. Activation of human platelets by a stimulatory monoclonal antibody. J Biol Chem 1990; 265:10042–10048.
2. Naik UP, Ehrlich YH, Kornecki E. Mechanisms of platelet activation by a stimulatory antibody: cross-linking of a novel platelet receptor for M.Ab.F11 with the FcγRII receptor. Biochem J 1995; 311:155–162.
3. Kornecki E, Naik UP, Sobocka M, Ehrlich YH. Mechanisms of stimulation and inhibition of platelet activation by monoclonal antibodies. In: Schlossman SF, et al., ed. Leukocyte Typing V. Oxford University Press, 1995: 1241–1243.
4. Wang F, Naik UP, Ehrlich YH, Osada SI, Ohno S, Kornecki E. Stimulatory antibody-induced activation and selective translocation of protein kinase C isoenzymes in human platelets. Biochem J 1995; 311:401–406.
5. Sobocka MB, Sobocki T, Banerjee P, Weiss C, Rushbrook JI, Norin AJ, Hartwig J, Salifu M, Markell MS, Babinska A, Ehrlich YH, Kornecki E. Cloning of the human platelet F11 receptor: a cell adhesion molecule member of the immunoglobulin superfamily involved in platelet aggregation. Blood 2000; 95:2600–2609.
6. Babinska A, Kedees MH, Athar H, Sobocki T, Sobocka MB, Ahmed T, Ehrlich YH, Hussain M, Kornecki E. Two regions of human platelet receptor F11R critical for platelet aggregation, potentiation and adhesion. Thromb Haemost 2002; 87:712–721.

7. Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 1998; 142:117–127.
8. Babinska A, Kedees MH, Athar H, Ahmed T, Batuman O, Ehrlich YH, Hussain MM, Kornecki E. F11-receptor (F11R/JAM) mediates platelet adhesion to endothelial cells: role in inflammatory thrombosis. Thromb Haemost 2002, 88(5):843–850.
9. Yamaguchi A, Suzuki H, Tanoue K, Yamazaki H. Simple method of aequorin loading into platelet using dimethyl sulfoxide. Thromb Res 1986; 44:165.
10. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. J Cell Biol 1992, 118(6):1421–1442.
11. Howard T, Oresajo C. The kinetics of chemotactic peptide-induced change in F-actin content, distribution, and the shape of neutrophils. J Cell Biol 1985; 101:1078–1085.
12. Howard T, Oresajo C. A method for quantifying F-actin in chemotactic peptide activated neutrophils: Study of the effect of tBOC peptide. Cell Motil 1985; 5:545–557.
13. Nachmias VT, Yoshida K. The cytoskeleton of the blood platelet: a dynamic structure. Adv Cell Biol 1988; 2:181–211.
14. Hartwig JH, Kung S, Kovacsovics T, Janmey PA, Cantley LC, Stossel P, Toker A. D3 phosphoinositides and outside-in signaling by glycoprotein IIb–IIIa mediate platelet actin assembly and filopodial extension induced by phorbot 12-myristate 13-acetate. J Biol Chem 1996; 271:32986–32993.
15. Hartwig JH. Platelet morphology and shape change. In: Loscalzo J, Schafer AI, eds. Thrombosis and Hemorrhage. Third Edition. Lippincott Williams & Wilkens, 2003:140–160.
16. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F. Staurosporine, a potent inhibitor of phospholipids/Ca++ dependent protein kinase. Biochem Biophys Res Commun 1986; 135:397–402.
17. Nakano H, Kobayashi E, Takahashi I, Tamaoki T, Iba H. Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J Antibiot 40:706–708.
18. Ruegg UT, Burgess GM. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol Sci 1989; 10:218–220.
19. Watson SP, McNally J, Shipman LJ, Godfrey PP. The action of the protein kinase C inhibitor, staurosporine, on human platelets. Evidence against a regulatory role for protein kinase C in the formation of inositol trisphosphate by thrombin. Biochem J 1988; 249:345–350.
20. Sibley DR, Benovic JL, Caron MG, Lefkowitz RJ. Molecular mechanisms of β-adrenergic receptor desensitization. In: Ehrlich YH, Lenox RH, Kornecki E, Berry WO, eds. Molecular Mechanisms of Neuronal Responsiveness. Advances in Experimental Medicine and Biology. Plenum Press, 1987; 221: 253–273.

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21. Ozaki H, Ishii K, Arai H, Horiuchi H, Kawamoto T, Suzuki H, Kita T. Junctional adhesion molecule (JAM) is phosphorylated by protein kinase C upon platelet activation. Biochem Biophys Res Commun 2000; 276:873–878.
22. Pang DT, Lewis SD, Sharma BR, Shafer JA. Relationship between the subunit structure of insulin receptor and its competence to bind insulin and undergo phosphorylation. Arch Biochem Biophys 1984, 234(2):629–638.
23. Heldin CH, Ernlund A, Rorsman C, Ronnstrand L. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. J Biol Chem 1989, 264(15):8905–8912.
24. Naik MU, Parkos CA, Mousa SA, Naik UP. Signaling through JAM-1 and alpha v beta 3 is required for the angiogenic action of bFGF: dissociation of the JAM-1 and alpha v beta 3 complex. Blood 2003; 102:2108–2114.
25. Slupsky JR, Seehafer JG, Tang SC, Masellis-Smith A, Shaw AR. Evidence that monoclonal antibodies against CD9 antigen induce specific association between CD9 and the platelet glycoprotein IIb–IIIa complex. J Biol Chem 1989; 264:12289–12293.
26. Slupsky JR, Kamiguti AS, Rhodes NP, Cawley JC, Shaw ARE, Zuzel M. The platelet antigens CD9, CD42 and integrin $\alpha_{IIb}\beta_{IIIa}$ can be topographically associated and transduce functionally similar signals. Eur J Biochem 1997; 244:168–175.
27. Indig FE, Diaz-Gonzalez F, Ginsberg MH. Analysis of the tetraspanin CD9-integrin $\alpha_{IIb}\beta_{IIIa}$ (GPIIb–IIIa) complex in platelet membranes and transfected cells. Biochem J 1997; 327:291–298.

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