

Thrombin Receptors on Human Platelets

INITIAL LOCALIZATION AND SUBSEQUENT REDISTRIBUTION DURING PLATELET ACTIVATION*

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Platelet responses to thrombin are at least partly mediated by a G-protein-coupled receptor whose NH₂ terminus is a substrate for thrombin. In the present studies we have examined the location of thrombin receptors in resting platelets and followed their redistribution during platelet activation. The results reveal several new aspects of thrombin receptor biology. 1) On resting platelets, approximately two-thirds of the receptors were located in the plasma membrane. The remainder were present in the membranes of the surface connecting system. 2) When platelets were activated by ADP or a thromboxane analog, thrombin receptors that were initially in the surface connecting system were exposed on the platelet surface, increasing the number of detectable receptors by 40% and presumably making them available for subsequent activation by thrombin. 3) Platelet activation by thrombin rapidly abolished the binding of the antibodies whose epitopes are sensitive to receptor cleavage and left the platelets in a state refractory to both thrombin and the agonist peptide, SFLLRN. This was accompanied by a 60% decrease in the binding of receptor antibodies directed COOH-terminal to the cleavage site irrespective of whether the receptors were activated proteolytically by thrombin or nonproteolytically by SFLLRN. 4) The loss of antibody binding sites caused by thrombin was due in part to receptor internalization and in part to the shedding of thrombin receptors into membrane microparticles, especially under conditions in which aggregation was allowed to occur. However, at least 40% of the cleaved receptors remained on the platelet surface. 5) Lacking the ability to synthesize new receptors and lacking an intracellular reserve of preformed receptors comparable to that found in endothelial cells, platelets were unable to repopulate their surface with intact receptors following exposure to thrombin. This difference underlies the ability of endothelial cells to recover responsiveness to thrombin rap-

idly while platelets do not, despite the presence on both of the same receptor for thrombin.

In its proteolytically active form, thrombin evokes responses from a number of cells located in and around the vascular space, including endothelial cells, vascular smooth muscle, and platelets. Although the existence of an unknown second thrombin receptor in mouse platelets has recently been inferred from knockout studies (1), many of the responses of human platelets to thrombin are thought to be mediated by a previously identified G-protein-coupled receptor whose NH₂ terminus is a substrate for thrombin (2). The evidence that this receptor is responsible in part for thrombin responses in human platelets is compelling: RNA encoding the receptor is present in platelets and in megakaryoblastic cell lines (2); antibody binding studies show that it is expressed on the platelet surface (3, 4); and peptide agonists based on the receptor's tethered ligand domain are able to activate human platelets, mimicking many of the effects of thrombin (e.g. 2, 5–8). This is in contrast to mouse platelets, which respond to thrombin but not to the agonist peptides (9, 10).

One of the properties that sets thrombin receptors apart from most other G-protein-coupled receptors is their inability to be activated by thrombin more than once. This is thought to be due in part to receptor phosphorylation and in part to the apparent inability of thrombin to reactivate cleaved receptors (11–14). Therefore, for cells to recover responsiveness to thrombin, cleaved thrombin receptors have to be replaced with intact receptors, a process that is usually preceded by the clearance of at least some of the cleaved receptors from the cell surface (15). Recent studies in cells other than platelets have demonstrated two general mechanisms for accomplishing this. In the megakaryoblastic HEL and CHR-288 cell lines, where >90% of the cleaved thrombin receptors are internalized within 5 min, recovery is a slow process dependent on the synthesis of new receptors (13, 14, 16). Endothelial cells and fibroblasts, on the other hand, contain a large intracellular reserve of preformed receptors (17–19). These receptors can quickly repopulate the cell surface after the addition of thrombin, allowing recovery to occur more rapidly than it can when dependent upon receptor synthesis alone.

Platelets normally need to respond to thrombin only once, in contrast to endothelial cells, which may encounter thrombin repeatedly. We were interested in determining whether this difference between platelets and endothelial cells would be reflected in differences in thrombin receptor biology between the two cell types. We were particularly interested in determining the distribution of thrombin receptors on resting platelets, identifying any intracellular pools of receptors which

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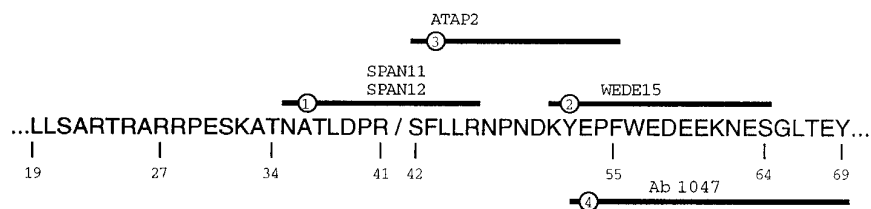


FIG. 1. Antibodies directed against defined epitopes within the thrombin receptor NH_2 terminus. The figure shows the sequence of the human thrombin receptor between residues 19 and 69, including the site of cleavage between Arg⁴¹ and Ser⁴² (2). The numbered lines indicate the sequences of the peptides used to prepare the antibodies, and except for the polyclonal antibody (1047), the labels mark the approximate location of the antibody epitopes.

might exist and tracing the movements of thrombin receptors when platelets are activated, either by thrombin or by agonists that activate platelets via receptors other than the thrombin receptor. Information about the distribution of thrombin receptors on human platelets is limited, in part because of the small size of platelets and the small number of receptors per cell. Based upon antibody binding, resting platelets express 1,500–2,000 copies of the thrombin receptor per cell on the cell surface (3, 4). Data on the redistribution of thrombin receptors following platelet activation is limited to a study in which Norton and co-workers (4) found no decrease in the binding to cleaved receptors of at least one antithrombin receptor antibody, leading them to propose that in contrast to endothelial cells and the megakaryoblastic cell lines, cleaved thrombin receptors on platelets remain on the platelet surface.

In the present studies we have used electron microscopy and flow cytometry to examine the initial distribution of thrombin receptors on and within human platelets and follow the redistribution that occurs when platelets are activated. Since thrombin can interact with more than one protein on the platelet surface (20–22), peptide-directed monoclonal and polyclonal antibodies were used to detect the receptor. The results show that in resting platelets only two-thirds of the total number of thrombin receptors are located on the plasma membrane. The remainder are initially present in the membranes of the intracellular surface connecting system, a structure contiguous with the platelet plasma membrane that is exposed during platelet activation. In the case of platelet activation by ADP and the thromboxane A_2 analog U46619, this leads to a net increase in the number of cell surface thrombin receptors by exposing receptors that were initially in the surface connecting system. Platelet activation by thrombin or the peptide agonist SFLLRN, on the other hand, caused a net decrease in the number of receptors that could be detected by all of the antibodies that were tested. When platelet aggregation occurred, this decrease appeared to be at least partly due to receptor internalization and the shedding of thrombin receptors into membrane microparticles. We found no evidence, however, for an intracellular reserve of thrombin receptors in platelets comparable to that found in endothelial cells and no evidence that platelets could mobilize intact receptors from sites other than the surface connecting system. This accounts for the observed inability of platelets to recover responsiveness to thrombin or SFLLRN following an initial exposure to thrombin.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies SPAN11, SPAN12, ATAP2, and WEDE15 were prepared against peptides corresponding to overlapping regions of the human thrombin receptor NH_2 terminus (Fig. 1) (3, 14, 16). Antibody 1047 is the purified IgG fraction from a polyclonal antibody prepared in rabbits immunized with the peptide YEPFWEDEEKNESGLTEYC conjugated via the cysteine to keyhole limpet hemocyanin (23). Monoclonal antibody A2A9 is directed against the platelet glycoprotein IIb-IIIa (integrin $\alpha_{\text{IIb}}\beta_3$) complex (24). EH1, an IgG1 antibody reactive with the human immunodeficiency virus type I nef protein, was used as an isotype-matched control in the flow cytom-

etry experiments (14). When indicated, the antibodies were purified using a protein A column (Bio-Rad).

Flow Cytometry—Blood (60 ml) was obtained from healthy volunteers and anticoagulated with acid-citrate-dextrose (8.6 ml). Platelet-rich plasma was prepared by centrifugation for 15 min at $169 \times g$ with the centrifuge brake off and incubated with prostaglandin E_1 ($1 \mu\text{M}$) for 5 min at room temperature. Afterwards, the platelets were sedimented at $1,200 \times g$ for 15 min, resuspended in 10 ml of HEPES-Tyrode buffer (129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4 , 8.9 mM Na_2HCO_3 , 0.8 mM MgCl_2 , 5.6 mM glucose, 10 mM HEPES, pH 7.4) supplemented with $1 \mu\text{M}$ prostaglandin E_1 and 1 mM EGTA, sedimented a second time at $1,200 \times g$ for 15 min, and then resuspended at 2×10^8 platelets/ml in HEPES-Tyrode buffer. RGDS (200 μM) was added to inhibit platelet aggregation. When indicated, the platelets were incubated with thrombin, SFLLRN, ADP, or U46619 for 15 min at room temperature, after which the desired monoclonal antibody was added (10 $\mu\text{g}/\text{ml}$ of purified protein), and the platelets were incubated for a further 15 min at 4°C . Afterwards, 1 ml of HEPES-Tyrode buffer containing 2 mM EGTA and 2 mM EDTA was added, and the platelets were sedimented ($1,200 \times g$ for 5 min) and resuspended in 50 μl of fetal calf serum plus 50 μl of a 1:40 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG (BioSource International, Camarillo, CA). After a 15-min incubation with the secondary antibody at 4°C , the platelets were diluted with 500 μl of HEPES-Tyrode buffer containing 2 mM EGTA and 2 mM EDTA, sedimented at $1,200 \times g$ for 5 min, resuspended in 500 μl of HEPES-Tyrode buffer, and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Electron Microscopy—For the studies on resting platelets, 10 ml of human blood was dripped directly from intravenous tubing into a beaker containing 90 ml of 4% paraformaldehyde in McLean and Nakane's buffer for 1 h (25). The mixture was spun at $150 \times g$ for 20 min at 20°C (26). The supernatant was then spun at $1,000 \times g$ for 10 min, producing a cell pellet that was embedded in 2.1 M sucrose, frozen, and stored in liquid nitrogen. Frozen thin sections were processed as described previously (27–29). The primary antibody (1047) was used at a dilution of 1:100. This was followed by the gold label, protein A-10, obtained from the Department of Cell Biology, University of Utrecht (Utrecht, the Netherlands) at 1:50 dilution. The grids were then stained with uranyl acetate and embedded in methyl cellulose. As a control, normal rabbit serum was also tested. For the studies of activated platelets, washed platelets were prepared from human blood anticoagulated with citrate as described previously (30) then activated with thrombin (1 unit/ml) and processed in the same way as the resting platelets.

Cytosolic Calcium—Washed platelets were resuspended at $3 \times 10^8/\text{ml}$ in HEPES-Tyrode buffer with $1 \mu\text{M}$ prostaglandin E_1 and incubated with 5 μM Fura-2/AM for 20 min at 37°C . Afterwards, 2 mM EGTA and $1 \mu\text{M}$ prostaglandin E_1 were added, and the platelets were sedimented and resuspended in HEPES-Tyrode buffer containing 1 mM CaCl_2 at $1 \times 10^8/\text{ml}$. Changes in the cytosolic free Ca^{2+} concentration were measured using a SLM/Aminco AB-2 spectrophotometer (31).

Microparticle Studies—Washed platelets were resuspended at $2 \times 10^8/\text{ml}$ in the presence of 200 μM RGDS and stimulated for 15 min at room temperature with either 0.25 unit/ml thrombin or 100 μM SFLLRN without stirring. Platelets were sedimented by centrifugation at $1,200 \times g$ for 15 min. The supernatants were then centrifuged at $27,000 \times g$ for 30 min. Both fractions were resuspended and processed as follows. Samples were stained for 15 min at 4°C with either the thrombin receptor antibodies or EH1, washed with HEPES-Tyrode buffer containing 2 mM EGTA and 2 mM EDTA, resuspended in fetal calf serum, and stained with a 1:40 dilution of a fluorescein isothiocyanate-labeled anti-mouse secondary antibody for 15 min at 4°C . After a further wash samples were incubated under the same conditions with

FIG. 2. Electron microscopy of resting platelets. Transmission electron microscopy of human platelets illustrates the presence of the thrombin receptor on the plasma membrane and in the surface connecting canalicular system (SC). The α -granules and other organelles are only rarely labeled, consistent with background (magnification, $\times 50,000$).



biotinylated AP-1, a monoclonal antibody that recognizes glycoprotein Ib $_{\alpha}$ (a kind gift from T. Kunicki, Scripps Research Institute, La Jolla, CA), washed again, incubated with a 1:400 dilution of phycoerythrin-labeled streptavidin, washed, and analyzed by flow cytometry. Microparticles were gated as AP-1-positive events (red fluorescence) smaller than platelets and analyzed for the binding of thrombin receptor antibodies (green fluorescence). In the studies in which aggregation was allowed to occur, RGDS was omitted, and the platelets were stirred and activated in the presence of 2 mM CaCl $_2$. Samples were then immediately stained with fluorescein isothiocyanate-labeled AP-1 and the number of microparticles determined without washing.

Other Methods and Materials—Human α -thrombin (3,000 units/mg) was provided by J. Fenton (New York State Department of Health, Albany, NY). ADP and U46619 were obtained from Sigma.

RESULTS

Distribution of Thrombin Receptors on Resting Platelets—We have shown previously that monoclonal antibodies directed against the thrombin receptor NH $_2$ terminus can be used as probes to detect thrombin receptor cleavage and internalization and to discriminate between receptor recycling and receptor replacement (14, 16–18, 32, 33). Four such antibodies were used in the present studies. Two, designated SPAN11 and SPAN12, are directed at the site of cleavage and were selected for their ability to recognize intact receptors but not cleaved receptors (Fig. 1). A third antibody, ATAP2, is directed against an epitope within the tethered ligand domain and has been shown to recognize cleaved as well as intact thrombin receptors, as does antibody WEDE15, which is directed against the domain of the receptor NH $_2$ terminus thought to interact with the thrombin anion-binding exosite. The fifth antibody shown in Fig. 1, 1047, is a rabbit polyclonal antibody that was raised against a peptide that partially overlaps the peptide used to prepare antibody WEDE15 (23).

Fig. 2 is an electron micrograph of human platelets that were fixed and then stained with polyclonal antibody 1047. The results show that on resting platelets thrombin receptors are present on the both the plasma membrane and the membranes of the surface connecting system (SC in the figure). Receptors were not detectable in α -granule membranes, nor was there a detectable intracellular pool of thrombin receptors within a

self-contained organelle such as is present in endothelial cells and transfected fibroblasts (17, 18). Previous studies using radioiodinated monoclonal antibodies have shown that there are 1,500–2,000 copies of the thrombin receptor on the surface of resting human platelets (3, 4), a number similar to the number of moderate affinity 125 I-thrombin binding sites (34, 35). Data presented below suggest that the exposure of receptors within the surface connecting system can increase this number by as much as 40% during platelet activation, which means that in resting platelets approximately two-thirds of the thrombin receptors are on the plasma membrane and one-third are in the surface connecting membrane system.

Changes in Receptor Distribution When Platelets Are Activated—The effect of platelet activation on the distribution of thrombin receptors was studied with platelets incubated with thrombin, SFLLRN, ADP, or the thromboxane analog U46619. In the initial studies, platelet aggregation was inhibited by removing Ca $^{2+}$ and adding RGDS to inhibit fibrinogen binding to the α _{IIB} β ₃ integrin (glycoprotein IIb-IIIa). Antibody binding to the platelet surface was detected by flow cytometry. Incubation with thrombin caused a >90% loss of binding sites for the cleavage-sensitive antibodies, SPAN11 and SPAN12, and a 60% decrease in the binding of the cleavage-insensitive antibodies, WEDE15 (Figs. 3 and 4) and ATAP2 (see Fig. 8). The decrease in SPAN12 binding was maximal within 5 min of the addition of thrombin. The decrease in WEDE15 binding was maximal within 15 min (Fig. 3). These results suggest that thrombin rapidly cleaves all of its receptors on the platelet surface and causes a net decrease in the number of detectable receptors by as much as 60% (see below). Notably, incubating the platelets with SFLLRN caused a 60% decrease in the binding of both the cleavage-sensitive and the cleavage-insensitive antibodies (Fig. 4). Although it has been proposed that thrombin receptor cleavage can decrease the binding of antireceptor antibodies by allowing the insertion of the tethered ligand domain into a protected environment (4), this explanation alone would not readily account for the decrease in antibody binding seen when the receptors were activated by SFLLRN.

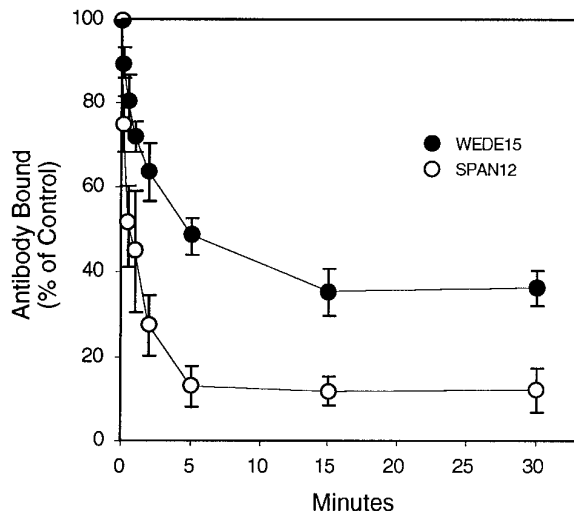


FIG. 3. Time course of the loss of antibody binding sites from platelets incubated with thrombin. Platelets were incubated with thrombin (0.25 unit/ml) at room temperature without stirring for the times indicated after which hirudin (2 units/ml) was added, and the samples were placed on ice. Antibody binding was determined by flow cytometry. The results shown are the mean \pm S.E. for three studies and are expressed as a percent of the mean fluorescence intensity in the absence of thrombin.

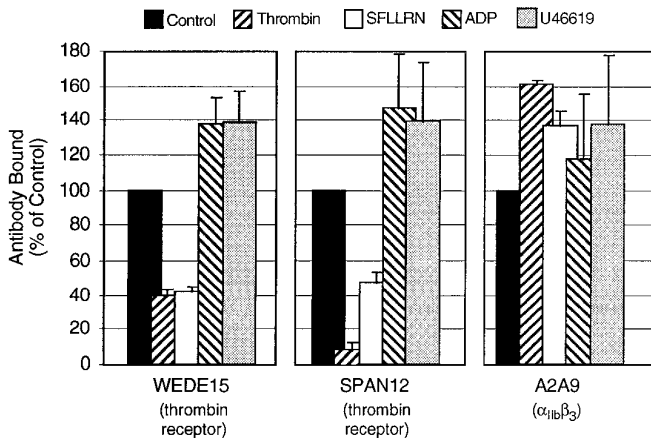


FIG. 4. Binding of thrombin receptor antibodies to human platelets. Washed human platelets were incubated without stirring for 10 min at room temperature with the agonists shown. Antibody binding was measured by flow cytometry. Antibodies WEDE15 and SPAN12 are directed against the thrombin receptor NH₂ terminus (see Fig. 1). Antibody A2A9 binds to the integrin $\alpha_{IIb}\beta_3$ complex. The final agonist concentrations were: thrombin (0.25 unit/ml), SFLLRN (100 μ M), ADP (10 μ M), and U46619 (10 μ M). The results shown are the mean \pm S.E. of three studies and are expressed as a percent of control after subtracting EH1 fluorescence. Mean fluorescence intensity values were: 99 for WEDE15, 51 for SPAN12, 255 for A2A9, and 6 for EH1.

A different result was obtained when the platelets were activated with ADP or U46619 rather than thrombin. Both of these agonists caused an increase, rather than a decrease, in thrombin receptor antibody binding (Fig. 4). This increase averaged approximately 40% and was similar in magnitude to the increase observed in the binding of an antibody, A2A9, which recognizes the integrin $\alpha_{IIb}\beta_3$ (Fig. 4). $\alpha_{IIb}\beta_3$ complexes, like thrombin receptors, are initially present in both the platelet plasma membrane and the surface connecting system. Previous studies have shown that platelet activation increases the amount of $\alpha_{IIb}\beta_3$ on the platelet surface in part by exposing complexes that were initially within the surface connecting system (36–38) (for review, see Ref. 39). By extension, this suggests that the increase in WEDE15 and SPAN12 binding seen in Fig. 4 following the addition of ADP and U46619 is due

to the exposure of thrombin receptors that were initially in the surface connecting system. It also suggests that the net decrease in WEDE15 and ATAP2 binding caused by thrombin and SFLLRN occurs despite the exposure of additional receptors from the surface connecting system. If so, then the near complete loss of binding sites for SPAN12 further suggests that the newly exposed receptors must also be cleaved by thrombin.¹

Fate of the Activated Thrombin Receptors—We have shown previously that thrombin receptor activation on endothelial cells, fibroblasts, and several megakaryoblastic cell lines is followed by receptor internalization. This process was shown by colocalization studies and electron microscopy in the megakaryoblastic cell lines to involve coated vesicles and endosomes (16). Coated vesicles have been observed in platelets (40, 41) and could provide a mechanism for thrombin receptor internalization in platelets, but other alternatives were considered as well.

One such alternative is that activated thrombin receptors are shed from the platelet surface in membrane microvesicles. Several studies have shown that membrane microparticles are formed when platelets are activated, particularly if they are activated by strong agonists such as thrombin or collagen under conditions in which aggregation is allowed to occur (e.g. 42–45). These microparticles include not only platelet membrane lipids, but also integral membrane proteins such as glycoprotein Ib. To test whether loss of thrombin receptors into microparticles could account in part for the observed decrease in antibody binding sites on activated platelets, platelets were incubated with thrombin or SFLLRN under the same conditions in which both agonists caused a decrease in antibody binding to the platelets. Microparticles were detected by flow cytometry and defined by size and by the presence of glycoprotein Ib using the glycoprotein Ib _{α} antibody, AP-1. Thrombin receptors were detected with antibody WEDE15. In the initial experiments, RGDS was included to prevent platelet aggregation and limit microparticle formation (45). The results show that thrombin receptors are present in the membrane microparticles and that the addition of thrombin under these conditions causes a decrease in WEDE15 binding to platelets but has no effect on the binding of the antibody to microparticles (Fig. 5A).

This suggests that thrombin receptors are present in microparticles, but since there was no apparent change in the number of receptors per microparticle, this would not account for the decrease in receptor number on activated platelets unless there were also an increase in microparticle number under the conditions in which the platelets were activated. To determine if this is the case, platelets were activated with thrombin or SFLLRN, and the number of microparticles was counted. Under conditions in which aggregation was prevented, microparticles represented less than 5% of total particles counted and increased little in number upon platelet activation (Fig. 5B). On the other hand, when platelet aggregation was allowed to occur, both thrombin and SFLLRN caused a substantial increase in microparticle number (Fig. 5C). These results suggest that microparticle formation could account for part of a decrease in receptor number when platelets are activated by thrombin or SFLLRN, but only if aggregation is allowed to occur.

¹ This conclusion derives from the following calculation. If there were originally 100 thrombin receptors on the platelet surface and an additional 40 in the surface connecting system, then activated platelets would have (transiently) a total of 140, as was observed with ADP and U46619. If all of the original surface receptors were cleaved, but the newly exposed receptors were not, then 40 intact receptors would remain on the platelet surface, which would leave 40% of the original number of SPAN12 binding sites, not the 10% that was observed.

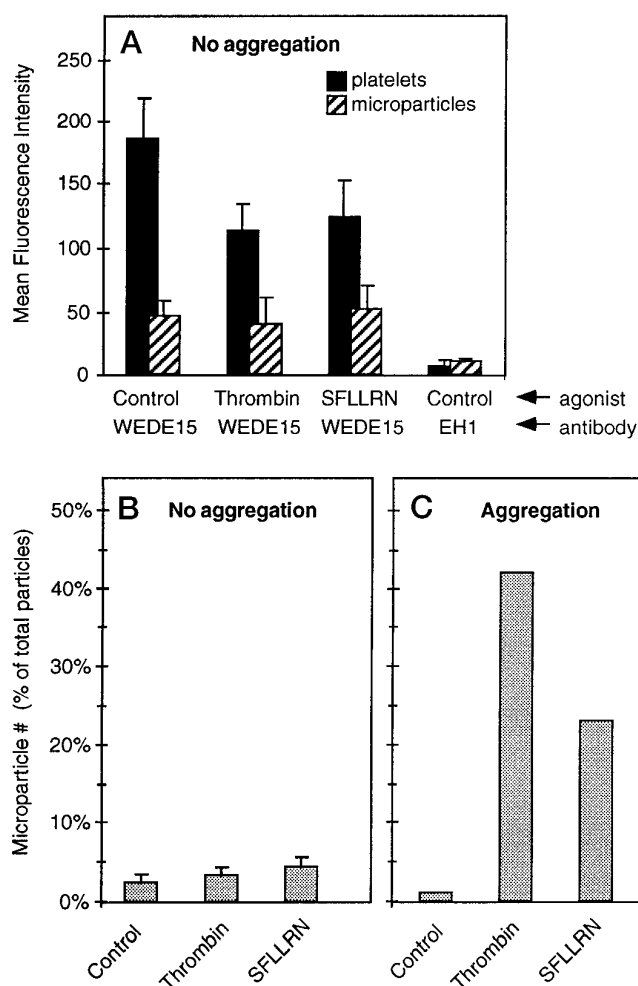


FIG. 5. Flow cytometry analysis of thrombin receptors on microparticles. *Panel A*, platelets were incubated with thrombin (0.25 unit/ml) or the agonist peptide SFLLRN (100 μ M) 15 min at room temperature without stirring in the presence of 200 μ M RGDS. Afterwards, platelet and membrane microparticles were separated and stained with the thrombin receptor antibody WEDE15, as described under "Experimental Procedures." The results shown are the mean \pm S.E. for four studies. *Panel B*, platelets were stimulated with thrombin or SFLLRN without aggregation and stained with the glycoprotein Ib $_{\alpha}$ antibody, AP-1. The number of microparticles present in the platelet suspension was determined and is expressed as a percent of total "particles" counted (mean \pm S.E., $n = 6$). *Panel C*, platelets and microparticles were prepared and analyzed as in *panel B*, except that aggregation was allowed to occur by omitting RGDS, adding Ca^{2+} , and stirring the samples (average of two studies).

Electron Microscopy of Thrombin-activated Platelets—To examine directly the distribution of thrombin receptors in activated platelets, platelets were incubated with thrombin and allowed to aggregate before being studied by immunoelectron microscopy using the cleavage-insensitive polyclonal antibody 1047. The results are shown in Fig. 6. Compared with the resting platelet shown in Fig. 2, there was a marked decrease in the staining of the plasma membrane (*pm* in the figure), presumably in part because of loss of thrombin receptors into microparticles under these conditions. The surface connecting system, which evaginates during platelet activation by thrombin, was no longer detectable. Instead, large vacuoles were present which have been shown previously to contain the contents of fused α -granules (30). In addition, occasional smaller intracellular vesicles were seen which were stained sparsely with the thrombin receptor antibody. These vesicles were not present in resting platelets, and no gold labeling was seen when normal rabbit serum was substituted for the thrombin

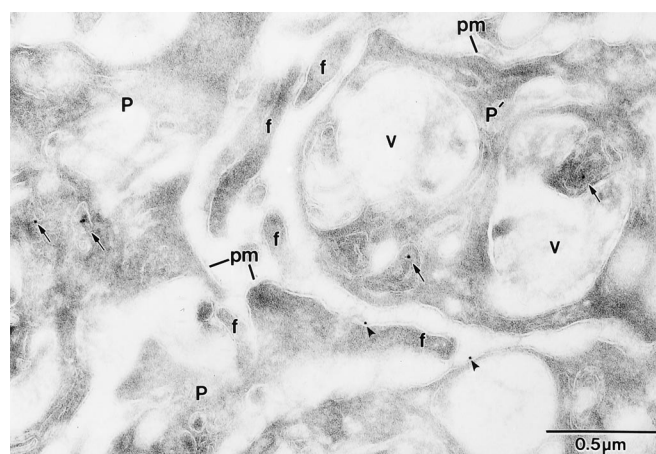


FIG. 6. Electron microscopy of platelets activated with thrombin. Shown is an aggregate of platelets from a sample stimulated with thrombin (1 unit/ml) and then immunolabeled as in Fig. 2. The platelets (*P*) have degranulated and formed long interdigitating extensions or filopodia (*f*). Most remarkable is the marked decrease in gold labeled on the plasma membrane (*pm*) compared with the resting platelets shown in Fig. 2, as well as the presence of particles within intracellular vesicles (*arrows*) of varying sizes. A few gold particles are also present on the plasma membrane (*arrowheads*). The large intracellular vacuoles (*v*) seen in platelet *P'* have been shown previously to contain the contents of fused α -granules, some of which are in contact with the extracellular space (30). Magnification, $\times 65,000$.

receptor antibody as a control.

Loss and Recovery of Thrombin Receptor Function—Thrombin receptor activation typically precludes a subsequent response to either thrombin or SFLLRN unless resensitization or replacement of the receptors occurs in the interim. However, recent studies have shown that the extent of thrombin receptor desensitization varies among cell types, as does the rate of recovery. In the studies shown in Fig. 7, human platelets were loaded with Fura-2, and changes in the cytosolic free Ca^{2+} concentration were measured. Thrombin and SFLLRN both caused a transient rise in cytosolic Ca^{2+} and, as was seen with the megakaryoblastic cell lines, a subsequent addition of either agonist elicited little or no response when added within a few minutes of the first stimulus (Fig. 7, *A* and *B*), although responses to other agonists such as U46619 did occur (not shown). To determine whether recovery would occur after the passage of a longer period of time, platelet responses to SFLLRN were measured 4 h after briefly incubating them with thrombin. Despite the passage of time, control platelets showed a full response to SFLLRN. Thrombin-treated platelets, on the other hand, continued to show no response. By comparison, HEL cells and endothelial cells recover 40–60% of their initial response to SFLLRN over the same time period, due, respectively, to receptor recycling (14) and receptor mobilization (18). Thus, in human platelets receptor activation by either thrombin or SFLLRN is followed by desensitization to both which lasts at least 4 h. Antibody binding studies with ATAP2 and SPAN11 performed under the same conditions show that there was a continued gradual loss of cleaved receptors from the platelet surface during this period (Fig. 8). There was no evidence for either the appearance of new intact receptors (causing a parallel increase in ATAP2 and SPAN11 binding) or recycled receptors (causing an increase in ATAP2 binding without a corresponding increase in SPAN11 binding). These results suggest that the platelets fail to recover their ability to respond to thrombin because they are unable to replace cleaved receptors with intact ones.

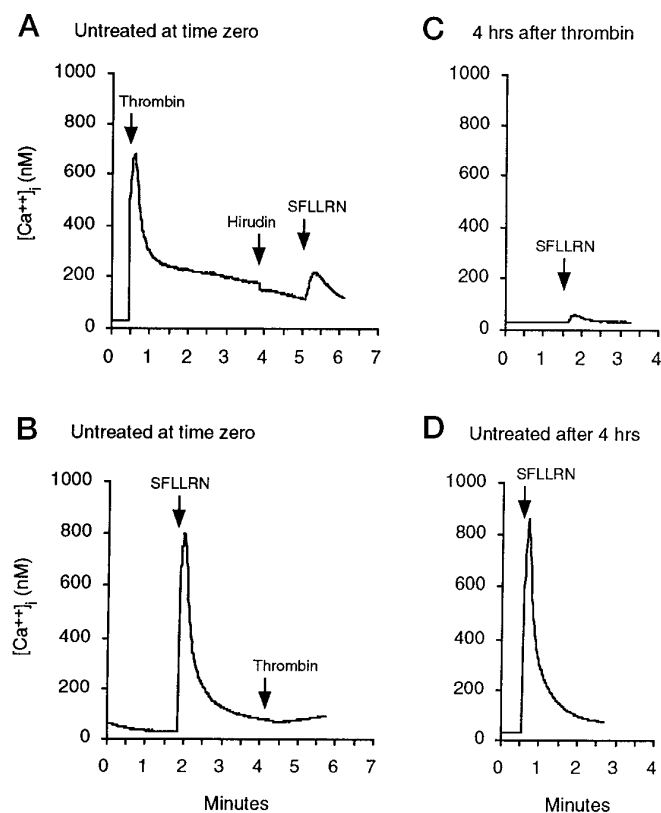


FIG. 7. Thrombin receptor response and desensitization in human platelets. The tracings show the changes in cytosolic Ca^{2+} which occurred following the addition of 1 unit/ml thrombin, 50 μM SFLLRN, or 5 units/ml hirudin at room temperature without stirring. *Panels A and B*, previously untreated platelets at time zero. *Panel C*, platelets were incubated with thrombin for 10 min, after which hirudin was added. The tracing shows the response when SFLLRN was added 4 h later. *Panel D*, the response of previously untreated platelets to SFLLRN 4 h after the start of the experiment. The results are typical of those obtained in five such studies.

DISCUSSION

In contrast to most G-protein-coupled receptors, thrombin receptor activation by proteases involves an irreversible proteolytic event. This means that to engender a new round of thrombin-responsiveness, cells must bring new receptors to their surface while clearing the old ones. Several strategies appear to have evolved to accomplish this. In megakaryoblastic HEL and CHR-288 cells, essentially all of the cleaved receptors are rapidly removed and then gradually replaced over a period of hours by new receptors as they are synthesized (14). In human umbilical vein endothelial cells and transfected fibroblasts, receptor replacement initially occurs via a pool of preformed receptors, and only later do freshly synthesized receptors emerge (17, 18).

The present studies examine the distribution of thrombin receptors on resting platelets and the redistribution that occurs when platelets are activated. In several respects the results are different from those obtained with other types of cells. In resting platelets, thrombin receptors were detectable in the plasma membrane and the membranes of the surface connecting system, but not in the membranes of the α -granules. In contrast to endothelial cells and fibroblasts, there was no evidence for the existence of an intracellular pool of thrombin receptors. However, the number of thrombin receptors initially within the surface connecting system appears to be substantial since the addition of ADP and U46619, which expose the surface connecting system, increased the number of binding sites for thrombin receptor antibodies by 40%. Using the average of

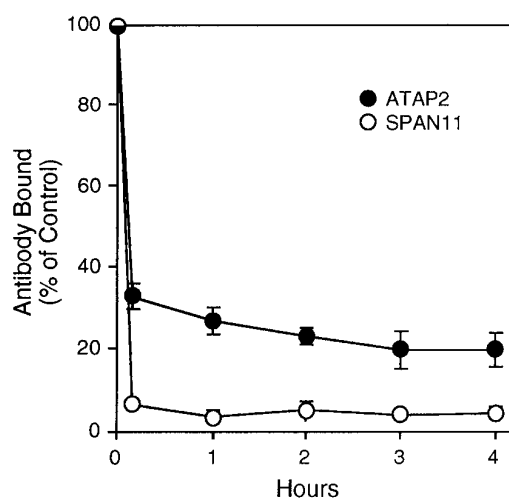


FIG. 8. Lack of recovery of thrombin receptors on the surface of platelets activated with thrombin. Platelets were incubated with 0.25 unit/ml thrombin for 5 min after which 1 unit/ml hirudin was added. The binding of antibodies ATAP2 and SPAN11 was measured by flow cytometry at each of the times shown and is expressed as a percent of antibody binding to untreated platelets (mean \pm S.E., $n = 3$).

1,500–2,000 plasma membrane thrombin receptors per platelet previously determined with radioiodinated antibodies (3), this suggests that resting platelets have 600–800 thrombin receptors per cell in the surface connecting system. Receptors in the surface connecting system are not accessible to antibodies before platelet activation, but they are also not in a self-contained organelle since the surface connecting system opens on to the plasma membrane even in resting platelets. The exposure of the surface connecting system by agonists other than thrombin provides a mechanism by which such agonists may increase the number of activable thrombin receptors present on the platelet surface. Since thrombin receptor responses are directly related to the number of thrombin receptors activated (32), this may be partially responsible for the synergism between ADP and thrombin reported 30 years ago by Niewiarowski and Thomas (46).

Following the addition of thrombin, there was a >90% loss of binding sites for two antibodies whose epitopes are removed when thrombin cleaves the receptor. There was also a 60% loss of binding sites for two monoclonal antibodies whose epitopes are located COOH-terminal to the thrombin cleavage site. The first of these observations suggests that thrombin rapidly cleaves all of its available receptors on platelets, just as it does on other cells. It also suggests that the newly exposed receptors that were originally in the surface connecting system have also been cleaved. The net loss of receptors requires further explanation. We have shown previously in cells other than platelets that the loss of binding sites for antibodies that bind COOH-terminal to the thrombin receptor cleavage site can be due to receptor internalization. However, although coated vesicles have been observed in platelets, they have never been seen in great numbers (40, 41), and the study by Norton and co-workers (4) which was cited earlier concluded that cleaved thrombin receptors remain on the platelet surface. In that study, the insertion of the tethered ligand domain into a protected environment following receptor cleavage was offered as the explanation for the observed decrease in the binding of some thrombin receptors antibodies when platelets are activated by thrombin.

The present studies suggest that the fate of activated thrombin receptors on human platelets is at least as complex as it is in other cells. The fact that nonproteolytic activation of the receptors with SFLLRN caused the same 60% decrease in

antibody binding to the platelet surface as did activation of the receptors with thrombin argues against occlusion of the tethered ligand domain as the sole cause of the reduction in antibody binding. Nevertheless, at least 40% of the receptors clearly remain on the platelet surface after activation. Of the remainder, some appear to be internalized, and some appear to be shed into microparticles along with other integral membrane proteins, at least under aggregating conditions. As was suggested previously (4), some of the others may remain in the plasma membrane but be inaccessible to any of the antibodies. Whether the 40% of receptors which remain on the platelet surface following exposure to thrombin are primarily those that were originally within the surface connecting system cannot be determined from the present data, but it seems unlikely since these receptors do become cleaved as they emerge.

In cells other than platelets, thrombin receptor function is restored over time following an initial exposure to thrombin. In HEL and CHRF-288 cells this is accomplished by synthesizing new receptors that are then moved to the cell surface. On endothelial cells and fibroblasts, cleaved receptors are initially replaced from a large intracellular store that is eventually replenished by synthesizing additional receptors. In either case, the return of receptor function parallels the return to the cell surface of intact receptors. Unlike the megakaryocytes from which they arise, platelets have only a limited capacity to synthesize proteins, and we were unable to detect an intracellular receptor reserve other than the surface connecting system, which would be exposed to thrombin too rapidly to provide a useful reserve for future use. This leaves platelets without evident means of restoring thrombin responsiveness. Conceptually, this makes sense. Platelets apparently need to respond to thrombin only once, after which they are incorporated into a growing hemostatic plug. The structure of the one currently known thrombin receptor appears to be the same in all cells. However, in platelets strategies have been adopted which maximize responses to thrombin while minimizing the cost to the cell of maintaining pathways to produce fresh, but perhaps unneeded, receptors on the cell surface.

Finally, the existence of additional types of thrombin receptors has recently been inferred from knockout studies in mice (1, 47). Those receptors may or may not ultimately prove to exist on human platelets. However, their mechanism of activation is likely to involve proteolysis by thrombin. Whether their location preceding activation and their redistribution following activation are the same as for the one thrombin receptor that has been identified remains to be determined, but many of the same limitations and issues inherent in a "single-shot" receptor that is activated by an irreversible event are likely to apply to them as well on whatever cell they are expressed.

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**Thrombin Receptors on Human Platelets: INITIAL LOCALIZATION AND
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