Stimulation of Platelet Activation and Aggregation by a Carboxyl-terminal Peptide from Thrombospondin Binding to the Integrin-associated Protein Receptor*

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Thrombospondin, a major secretory product of the α-granules of activated platelets, is a large trimeric glycoprotein that plays an important role in platelet aggregation. On resting platelets, thrombospondin binds to a single receptor in a cation-independent manner, but upon platelet activation it binds to at least two further, distinct receptors that are both dependent upon divalent cations. Each of these receptors on the platelet surface binds to different regions of the thrombospondin molecule, and such binding may be responsible for the multifunctional role of thrombospondin in aggregation. We show here that a peptide from the carboxyl terminus of thrombospondin, RFYVWMK, directly and specifically induces activation and aggregation of washed human platelets from different donors at concentrations of 5–25 μM. At lower concentrations the peptide synergizes with suboptimal concentrations of ADP to induce aggregation. Peptide affinity chromatography and immunoprecipitation with a monoclonal antibody were used to identify the receptor for the carboxyl-terminal peptide as the integrin-associated protein. The integrin-associated protein remained bound to the RFYVWMK-containing peptide column when washed with a scrambled peptide in the presence of 5 mM EDTA, indicating a divalent cation-independent association. It is suggested that integrin-associated protein is the primary receptor for thrombospondin on the surface of resting platelets and is implicated in potentiating the platelet aggregation response.

Platelet aggregation and clot formation in vivo are initiated when platelets are activated by soluble physiological activators such as thrombin, or directly by binding to components of the subendothelial matrix, such as von Willebrand factor or fibrinectin. This event activates the platelets in a process that induces platelet shape change and secretion of the contents of the platelet dense granules and α-granules. It also induces a conformational change in the integrin αIIbβ3 (GPIIIaIIIa), enabling this cell surface molecule to function as a receptor for fibrinogen. Platelet aggregation proceeds when fibrinogen, present both in the plasma and secreted from the α-granules, is bound by the αIIbβ3 receptor in a process that cross-links the platelets (reviewed in Ref. 1).

It has been recognized for some time that thrombospondin 1 (TSP)1 plays an essential accessory role in platelet activation and aggregation (2, 3). The concentration of TSP in plasma is very low (4, 5), but it is the most abundant protein component of platelet α-granules, representing some 25% of the total protein secreted upon platelet activation, after which a proportion of TSP becomes bound to the surface of the activated platelet (6) and also becomes incorporated into the fibrin clot (7). TSP has also been reported to promote platelet aggregation of both non-stimulated platelets and platelets stimulated with thrombin or ADP (8). In addition, some anti-TSP antibodies can inhibit platelet aggregation (2, 9, 10). Surface-bound TSP also interacts with fibrinogen bound to the surface of the platelet via αIIbβ3 (11). Leung (2) has suggested that TSP serves to stabilize fibrinogen binding to the activated platelet surface reinforcing the strength of interplatelet interactions and thereby determining the size and reversibility of platelet aggregates; this in turn serves to regulate clot formation.

The TSP molecule is large and complex and can be depicted as comprising a series of modules, each exhibiting discrete functions. Proteolytic digestion of TSP, together with the expression of individual modules as recombinant proteins or synthetic peptides, has enabled the identification of several cell-binding domains within the TSP molecule (reviewed in Refs. 3 and 12). Platelets exhibit at least four known putative TSP receptors: a proteoglycan that binds predominantly to the heparin-binding domain in the amino-terminal region of TSP (10); CD36, which binds to a sequence CSVTCG present in the type I repeats (13); the integrins αIIbβ3 (GPIIIaIIIa) and αβ3, which can bind the RGD sequence contained within the last of the type III repeats (14, 15); and an unknown receptor that binds to the carboxy terminus of TSP (9). In addition, TSP can bind platelet sulfatides (16) and to fibrinogen (11), interactions which may modulate TSP binding to platelets. This plethora of receptors for TSP on platelets has confounded studies seeking to identify the role of individual receptors, and it is apparent that there exists some redundancy in the system; for example, platelets deficient in CD36 expression bind TSP normally (17), as do platelets deficient in αIIbβ3 and fibrinogen binding (18, 19).

Gartner and Dockter (20) found that TSP bound to platelet (and erythrocyte) membranes in the absence of divalent cations, apparently contradicting the work of Phillips et al. (6) who had demonstrated that this association was cation dependent. These findings were resolved in a detailed study by Wolff et al.1

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1The abbreviations used are: TSP, thrombospondin 1; IAP, integrin-associated protein; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis.
Peptide Synthesis—Peptides were assembled on an Applied Biosystems monomeric TSP peptide, RFYVVMWK, even in the presence of neutralizing antibodies, which was partially blocked by an mAb to IAP synergizes in this aggregation response. Prostaglandin E1 was added to 1 μM, and platelets were gently pelleted by further centrifugation at 120 × g for 10 min. Finally, platelets were carefully resuspended using a plastic Pasteur pipette from the soft pellet into buffer A (138 mM NaCl, 2.9 mM KCl, 0.5 mM MgCl₂, 1 mM NaHCO₃, 0.3 mM NaH₂PO₄, 5.5 mM glucose, 10 mM HEPES, pH 7.4). Platelet Aggregation—Platelet aggregation was monitored on a dual-channel platelet aggregometer (Chrono-Lag, Havertown, PA). 450- or 250-μl platelet suspensions (1 × 10⁷/ml) were treated with thrombin, ADP, or a range of monoclonal antibodies or challenged with defined peptides. Appropriate vehicle blanks were processed in parallel. The platelet aggregation response was monitored under conditions of constant stirring at 1000 rpm at 37°C.

Isolation of Platelet Membranes—Platelet-rich plasma was isolated as described above from fresh whole blood samples collected into acid citrate dextrose anticoagulant containing fibrinolysin, and the pH of the plasma was adjusted to 6.6 with 1 M citric acid as required. Platelets were recovered by centrifugation as described above. Pelleted platelets were washed twice with COSS buffer (120 mM NaCl, 13 mM trisodium citrate, 30 mM glucose, pH 7.4) followed by two washes with HES buffer (10 mM HEPES, pH 7.4, 5 mM EDTA, 150 mM NaCl). Washed platelets were resuspended into HES buffer and layered onto a 0–40% glycerol (in HES buffer) gradient. Samples were spun at 1500 × g for 40 min and then 5000 × g for 10 min at room temperature. The platelet pellet was resuspended into HES buffer containing 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM iodoacetamide. The suspension was sonicated by 4 × 15-s pulses at maximal power using a 6-mm probe (MSE Soniprep 150). The sonicated suspension was centrifuged at 7900 × g for 30 min at 4°C, and platelet membranes were recovered from the supernatant by further centrifugation at 100,000 × g for 60 min and again at 4°C. Pelleted membranes were resuspended into HES buffer containing the protease inhibitors described above and maintained at 4°C.

Affinity Column Chromatography of Platelet Membrane Suspensions—Following resuspension into HES buffer, platelet membrane preparations were homogenized by 15 strokes in a tight fitting Wheaton siliconized glass homogenizer. This and subsequent procedures were performed at 4°C. Membrane suspensions were then applied to an RFYVVMWKQVTQSYWDTNK¢-Aff-501 column equilibrated with HES buffer containing protease inhibitors (all solutions used for affinity chromatography contained protease inhibitors unless stated otherwise). The homogenate was allowed to run into the gel matrix and stand within the column bed for 30 min before washing the column bed with five column volumes of fresh HES buffer. After washing, one column volume of 5 mg/ml of scrambled peptide (VFRKYVM) solubilized in a minimal volume of Me₂SO and made up to volume with HES buffer was run into the column bed. Again, the solution was kept within the column for 30 min before allowing the column to void with any eluted protein(s). The column was re-washed with five column volumes of HES buffer. To elute specific surface receptor(s) for the TSP carboxyl-terminal peptide, a 5 mg/ml solution of RFYVVMWK (prepared in the same manner as the scrambled version) was applied to the column and processed as described for the randomly scrambled peptide. In one experiment protease inhibitors were omitted from all column solutions to exaggerate any proteolytic products.

Iodination of Eluted Proteins—Iodogen reagent (1,3,4,6-tetrachloro-3a,4a-diphenylglycouril), 5.2 μg, was solubilized in CHCl₃ and coated onto the surface of a glass tube by evaporation of the vehicle under a stream of N₂. Sample protein from the peptide column was added to the
FIG. 1. TSP carboxyl-terminal peptide (K)RFYVVMWK(K) induces platelet aggregation and activation. A, dose-response aggregation of resting platelets induced by the TSP carboxyl-terminal peptide (K)RFYVVMWK(K). Resting, washed human blood platelets were stimulated with a range of concentrations of peptide (5–200 μM), and aggregation was monitored optically using a Chrono-Log aggregometer as described under “Experimental Procedures.” Addition of peptide is indicated by winged arrowhead. The data represent a typical response in several experiments was found to be between 11 and 25 μM RFYVVMWK, 45 μM scrambled peptide, or left untreated. Aggregation with thrombin or thrombin and RFYVVMWK was allowed to proceed with mixing until aggregation was maximal as determined by aggregometer trace. The scrambled sample was treated for the same time period as used for the unscrambled TSP peptide. All samples were lysed with an equal volume of 2% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA plus protease inhibitors as described before. Insoluble material was removed by centrifugation at 15000 g for 15 min at 4°C. Equivalent protein concentrations from the soluble lysate of each sample were analyzed by SDS-PAGE and gels autoradiographed overnight at –70°C.

RESULTS AND DISCUSSION

Peptide RFYVVMWK Specifically Induces Activation and Aggregation of Washed Human Platelets—Over a range of concentrations the peptide RFYVVMWK induced platelet aggregation; the lowest concentration to induce such aggregation in several experiments was found to be between 11 and 25 μM with peak aggregation commonly occurring at 45 μM concentration, although this varied with platelets from different donors (data not shown). This peptide did not appear to be completely soluble after addition of water to the Me2SO carrier (see “Experimental Procedures”). We therefore prepared a more soluble form of this peptide by lyophilization and stored this form as a lyophilized powder.

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water soluble version of the peptide by the addition of charged (lysine) residues to each end of the peptide. In aggregation assays this modified peptide (KRFYVVMWK) gave almost identical results, except that the lower aggregation threshold was 5 μM (Fig. 1A). Therefore, in subsequent experiments described below, the RFYVVMWK peptide was used. Aggregation was rapid after addition of either peptide, and the aggregates formed were large as seen both microscopically (data not shown) and from the amplitude of the trace (Fig. 1A). A scrambled version of the peptide (VFRWKYVM) did not cause any platelet aggregation, and it did not influence aggregation induced by the subsequent addition of the RFYVVMWK peptide (Fig. 1B). To demonstrate that the peptide was inducing activation and aggregation of the platelets rather than agglutination, platelets were loaded with 32P and treated with scrambled peptide (VFRWKYVM) or stimulated with RFYVVMWK peptide or with thrombin. The platelet lysate was examined for the appearance of phosphoproteins as a measure of activation (36). As expected, the RFYVVMWK peptide and thrombin treatments induced the appearance of a phosphoprotein band at 47 kDa, which was not seen in platelets treated with the scrambled peptide (Fig. 1C). This band is likely to be plextrin, which is the major protein phosphorylated upon platelet activation (37).

Effects of Cell Binding Peptides from Other Domains of Thrombospondin upon RFYVVMWK-mediated Platelet Aggregation—Gartner et al. (10) reported that a polyclonal antibody to the heparin-binding domain of thrombospondin inhibited platelet aggregation induced by ADP, collagen, or thrombin. A peptide from this region of thrombospondin that binds to the cellular proteoglycan receptor in endothelial cells was recently identified (38). In addition, Tuszynski and colleagues (39) reported that the CSVTCG peptide from the type 1 repeats of thrombospondin, which binds CD36 (13), inhibited ADP-induced platelet aggregation in a dose-dependent manner. We therefore tested these peptides for their effect upon RFYVVMWK-induced aggregation of platelets. Neither the heparin-binding domain peptide (ELTGAARKGSRRILVKGPD) nor the CSVTCG peptide themselves induced any platelet aggregation when tested over a range of concentrations (data not shown). The heparin-binding domain peptide however inhibited the extent of platelet aggregation induced by RFYVVMWK in a dose-dependent manner (Fig. 2A), although it did not alter the shape change or initial peptide-induced aggregation (see below). In contrast, pre-treatment of the platelets with

![Fig. 2. Effect of cell-binding peptides from TSP on the platelet aggregation response induced by TSP carboxyl-terminal peptide RFYVVMWK. A, effect of heparin-binding domain peptide (ELTGAARKGSRRILVKGPD) on the aggregation of resting platelets induced by the TSP carboxyl-terminal peptide RFYVVMWK. Resting washed platelets were stimulated with carboxyl-terminal peptide alone (trace i, 45 μM) or exposed to heparin-binding domain peptide (trace ii, 100 μM; trace iii, 200 μM) for 60 s prior to stimulation with carboxyl-terminal peptide (45 μM). Aggregation was monitored optically using a Chrono-Log aggregometer as described under “Experimental Procedures.” Winged arrowheads indicate addition of RFYVVMWK; triangular arrowheads indicate addition of heparin-binding domain peptide. The data represent a typical response derived from at least three individual donors. B, effect of CD36-binding peptide KCSVTCG on platelet aggregation induced by RFYVVMWK. Resting washed platelets were stimulated with KCSVTCG alone (100 μM) (trace ii) or KCSVTCG (100 μM) 60 s prior to addition of RFYVVMWK (45 μM) (trace i). Aggregation response was monitored as described in A. Winged arrowheads indicate addition of RFYVVMWK; triangular arrowheads indicate addition of KCSVTCG peptide. C, effect of RGDS peptide on the aggregation response of resting platelets induced by the TSP carboxyl-terminal peptide RFYVVMWK. Resting washed platelets were stimulated with carboxyl-terminal peptide alone (trace i, 45 μM) or treated with increasing concentrations of RGDS peptide (trace ii, 0.3 mM; trace iii, 0.6 mM; trace iv, 1.2 mM) 60 s prior to stimulation with RFYVVMWK peptide (45 μM). Aggregation was monitored as in A. Winged arrowheads indicate addition of RFYVVMWK peptide; triangular arrowheads indicate addition of RGDS. The data represent a typical response derived from three individual donors.](https://example.com/image-url)
CSVTcg peptide prior to stimulation with RFYVVMWK peptide had no effect on the aggregation response (Fig. 2B).

Contained within the last type III repeat of thrombospondin is the RGD motif that serves as a recognition sequence for a number of integrins including αIIbβ3 (GPIIIa) of platelets and is well characterized as inhibiting platelet aggregation induced by a number of agonists (13). As expected, upon testing in the RFYVVMWK-induced platelet aggregation assay, the RGDS peptide inhibited aggregation in a dose-dependent manner (Fig. 2C). This result indicates direct involvement of the αIIbβ3 integrin in the peptide-induced aggregation response.

Suboptimal Concentrations of the RFYVVMWK Peptide Synergize with ADP to Induce Platelet Aggregation—The decrease in the extent of aggregation caused by the heparin-binding domain peptide (Fig. 2A) is unlikely to be caused by any antagonistic effect on the RFYVVMWK peptide but is most likely the result of inhibition of the platelet cross-linking caused by secreted endogenous platelet thrombospondin that results in stabilization of formed platelet aggregates as propounded by Leung (2). Taken together, these data may account for reports demonstrating that a monoclonal antibody against the carboxyl region of thrombospondin (9), and also a polyclonal antibody to the amino-terminal heparin-binding domain (10) of thrombospondin, can both inhibit platelet aggregation. From our data based on the peptide from the carboxyl-terminal region of thrombospondin, it appears that this region is involved in an activation step rather than in the thrombospondin-mediated stabilization of platelet aggregation (2). Since the plasma levels of TSP are low (4, 5), the major source of TSP accessible to resting platelets is likely to be that secreted from the α-granule store of adjacent, activated platelets or other cells (40, 41). Such secretion from activated platelets would be accompanied by the release of ADP, itself a weak platelet agonist (42). If the carboxyl terminus of TSP indeed plays a role in initiating platelet aggregation, it might therefore be expected to synergize with ADP. To test this concept, platelets were treated with suboptimal doses of the weak agonist ADP or with suboptimal concentrations of the RFYVVMWK peptide, or with both together. As shown in Fig. 3A, the peptide and ADP synergized to induce rapid and complete platelet aggregation. No aggregation was seen with suboptimal concentrations of ADP together with the scrambled peptide (Fig. 3A).

RFYVVMWK Peptide Binds to IAP from Platelets—Gao et al. (25) have recently identified IAP as a cellular receptor for the carboxyl-terminal domain of thrombospondin. Since Brown and associates (26) have produced antibodies to IAP that can influence cellular function, we tested one of these antibodies for its effect on platelet aggregation. The antibody, B6H12, has been reported as a functional antibody (for example, endothelial cell migration is blocked (26–31)). When tested for its effects upon platelets, this antibody induced direct platelet aggregation of the platelets from some subjects (Fig. 3B) but not others. Monoclonal antibodies directed against other platelet glycoproteins that are able to induce aggregation (anti-CD9 (43), anti-CD36 (19), and anti-PTA1 (34)) all require involvement of the Fc receptor (44). Because of a polymorphism of the human Fc receptor, FeyRIII, only platelets from some individuals (about 50%) can bind the Fc of mouse monoclonal antibodies, and only platelets from these “responder” subjects are activated by the antibodies; “non-responder” platelets bind antibody only through antigen recognition and are not induced to aggregate (45). We therefore tested platelets from a number of subjects.
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**Fig. 4. IAP of platelets binds to the carboxyl-terminal peptide RFYVVMWK.** A, integrin-associated protein is expressed on the surface of resting platelets. Proteins on the surface of resting platelets were labeled with biotin, the platelets were lysed, and immunoprecipitation was performed on the soluble lysate with antibodies to IAP (B6H12), β3 (AP-3), CD36 (IE8), and a platelet irrelevant antibody (OKT-4). Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and biotinylated protein(s) detected by ECL. Molecular mass markers in kDa are shown at left. B, silver stain of SDS-PAGE analysis of protein(s) from platelet membrane suspensions bound to an RFYVVMWK-containing peptide affinity column in the presence of EDTA. Resting platelet membrane protein(s) eluted in EDTA-containing buffer known to be “responders” or “non-responders” to the anti-PTA1 antibody (34, 44). The B6H12 antibody to IAP aggregated platelets only from the responder group suggesting that, as with other activating antibodies, Fc receptor engagement is required for this response (data not shown). Also in common with other platelet-activating antibodies (19, 43, 34), B6H12 antibody-induced platelet aggregation was preceded by a prolonged lag phase (Fig. 3B), the length of which correlated with the concentration of antibody used (data not shown). This lag phase was greatly shortened when the platelets were treated with suboptimal concentrations of ADP (data not shown). In addition, the B6H12 antibody also synergized with suboptimal concentrations of the RFYVVMWK peptide to induce platelet aggregation (Fig. 3B).

These results suggest that IAP may function as a platelet receptor for this carboxyl-terminal peptide from thrombospondin. To confirm that platelets express IAP on their surface, immunoprecipitation experiments were carried out from surface-labeled platelets. As shown in Fig. 4A, A, a band in the position of IAP was immunoprecipitated specifically with the B6H12 antibody. IAP on the cell surface labels very poorly (26), and without carrying out binding analysis it cannot be stated whether or not the faint nature of this band is the result of low copy numbers of IAP receptors on the cell surface. We then sought to determine whether the RFYVVMWK peptide bound IAP. Gao and Frazier (24) had reported that in a cross-linking study the RFYVVMWK peptide bound to a 52-kDa protein when cross-linked to the surface of K562 cells, but binding was substantially reduced after the cells had been lysed in Triton X-100-containing buffers. We attempted to affinity purify RFYVVMWK-binding proteins using detergent-lysed platelet membranes and obtained no specific binding proteins (data not shown). This result substantiates the suggestion of Gao and Frazier (24) that the receptor for this peptide requires a specific membrane conformation to bind its ligand and that this is disrupted by Triton X-100. We therefore prepared disrupted platelet membranes in the absence of detergent (see “Experimental Procedures”). These were passed over an affinity column of an RFYVVMWK-containing peptide, which was then extensively washed with buffer containing EDTA, and bound materials subsequently eluted with the scrambled peptide (VFRWKYVM) in the continued presence of EDTA followed by specific elution with RFYVVMWK peptide. Material eluted at each step was concentrated and analyzed by SDS-PAGE and silver staining (Fig. 4B). The only bands seen from the specific eluate were proteins of ~45 and 35 kDa (Fig. 4B). In different experiments the proportions of the 45- and 35-kDa proteins varied, and in the absence of protease inhibitors the 35-kDa protein became more prominent (Fig. 4B). These results are in good agreement with the RFYVVMWK peptide cross-linking data of Gao and Frazier (24) who suggest that the 55-kDa band represented a major degradation product of the 52-kDa protein from the peptide affinity column in the absence of protease inhibitors were analyzed by SDS-PAGE and silver staining. Homogenate, total material loaded to column; Scrambled (VFRWKYVM), proteins eluted by the scrambled peptide (5 mg/ml); column wash, post-scrambled elution; RFYVVMWK, proteins eluted by the TSP carboxyl-terminal peptide (5 mg/ml). Proteins eluted by the carboxyl-terminal peptide are arrowed at right. Molecular weight markers are shown at left in kDa. C, molecular weight markers are shown at left in kDa. C, immunoprecipitation identifies integrin-associated protein as binding the TSP carboxyl-terminal peptide RFYVVMWK. Proteins eluted by the scrambled and specific peptides with buffer containing EDTA and protease inhibitors were iodinated and immunoprecipitated with a range of antibodies to platelet surface proteins: IAP (B6H12), β3 (AP-3), α2β1 (AK-7), GPIb-IX (AK-2), CD4 (OKT-4), and CD36 (IE8). Precipitated material was analyzed by SDS-PAGE, and labeled protein was detected by autoradiography. Molecular mass markers are shown at left in kDa.
from K562 cells. Next, we sought to identify the 45-kDa band by immunoprecipitation. The peptide affinity chromatography experiments were repeated, and the proteins eluted by the scrambled peptide and the specific peptide were detergent treated to disrupt the membranes and then labeled with 

\[ ^{125} \text{I} \]. The labeled proteins were then immunoprecipitated with antibodies to the platelet membrane receptors: IAP, the integrin \( \beta \) subunit of \( \alpha I \beta \beta \), the \( \alpha \) subunit of the integrin \( \alpha 2 \beta 1 \), the \( \beta \) subunit of GPIb-IX, CD36, or with an antibody irrelevant to platelets (CD4). IAP protein was seen to be precipitated from the specific eluate but not from the scrambled eluate (Fig. 4C). This same band appeared also to be coprecipitated by the antibody against \( \beta 3 \), an expected result since Brown and colleagues have shown previously that IAP from platelets associates with the integrin \( \beta 3 \) subunit (26) and also that IAP on K562 cells cross-linked to the RFYVMWK peptide is coprecipitated with antibodies to \( \beta 3 \) (24). The IAP band was not precipitated with antibodies to CD36 or CD4, but a trace was seen in the GPIb-IX precipitant. A band apparently co-incident with IAP was also seen in the GPIb-IX precipitant. A band apparently co-incident with IAP was also seen in the \( \alpha 2 \beta 1 \) precipitant (Fig. 4C). Brown et al. (26) have reported that IAP associates with integrins other than \( \beta 3 \), and it may be that in platelets this receptor preferentially associates with \( \alpha 2 \beta 1 \). However, the relatively strong labeling of this band in the anti-\( \alpha 2 \beta 1 \) immunoprecipitant compared with the IAP immunoprecipitant together with its slightly less diffuse appearance does not allow this conclusion without further analysis.

Further work will be required to place these results in a physiological context. The platelets used in our study were washed since the peptide did not induce aggregation of platelets contained in plasma. Preliminary studies have shown that this is the result of the peptide binding to an unidentified component of plasma (also present in murine ascites fluid) rather than the status of the platelets; by every criterion measured the washed platelets used for the aggregation studies were resting. As such, the data suggest that the IAP receptor is functionally expressed on the surface of resting platelets. Extrapolating from this, it is likely that IAP is the cation-independent receptor for TSP on resting platelets identified by Wolff et al. (21). These authors carried out TSP binding studies to demonstrate that resting platelets bound 3100 ± 1000 TSP molecules/platelet with a \( K_d \) of 50 nM. This binding was not disrupted by the presence of 5 mM EDTA, and the same group went on to establish that TSP binding to resting platelets was not influenced by \( \alpha I \beta \beta \) (GPIIbIIa) or fibrinogen binding (18). Our affinity chromatography was carried out in 5 mM EDTA, confirming that the binding of RFYVMWK peptide to IAP is not inhibited by the presence of EDTA (24), and it will be instructive to measure the numbers of IAP molecules expressed by resting platelets; although Brown and associates (26) have shown that platelets bind less anti-IAP antibody than erythrocytes, which, in a later study (44), were demonstrated to display some 10,000 antibody binding sites per erythrocyte. The relatively high concentrations of peptide required to induce platelet activation is unlikely to be a measure of the true binding affinity of TSP for IAP since the peptide is removed from its natural conformation and the trimeric nature of TSP could alter the avidity of binding. Based primarily upon blocking studies with anti-TSP and anti-receptor antibodies, it has been recognized for some time that TSP plays an important role in platelet aggregation (2, 3, 8–10). While there has been some disagreement about the relative importance of the different receptors for TSP identified on platelets, it is generally conceded that TSP is involved in determining the size and reversibility of platelet aggregates (2). As evidenced by TSP binding to thrombosthenic platelets, TSP binding in the absence of a functional \( \alpha I \beta \beta \) receptor is insufficient to promote aggregation (18). However, the intriguing experiments described by Aiken et al. (19) suggest that TSP alone may support the aggregation of stimulated normal platelets in the virtual absence of platelet binding to other adhesive proteins, including fibrinogen. Our results with a carboxy-terminal peptide from TSP substantiate and extend the observations made by Tuszyński et al. (8), that TSP is also able to potentiate the reversible first phase of ADP-induced platelet aggregation and to drive the platelets through to the irreversible second phase. Platelets binding to immobilized ligands such as fibronectin or collagen spread out and become activated in the absence of soluble agonists (45). These ligands would be well expressed upon damage to endothelial cells; in this event TSP secreted by the substrate-bond, activated platelets could play a substantive role in recruiting resting platelets into the forming clot.

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REFERENCES

1. Plow, E. F., and Ginsberg, M. H. (1989) Prog. Hemostasis Thromb. 9, 117–156
2. Leung, L. L. K. (1984) J. Clin. Invest. 74, 1764–1772
3. Lawler, J. (1986) Blood 67, 1197–1209
4. Saglio, S. D., and Slattery, H. S. (1982) Blood 59, 162–166
5. Dawes, J., Clemenston, J. K., Gogstad, G. O., McGregor, J., Clezerdin, P., Prowde, C. V., and Pepper, D. S. (1983) Thromb. Res. 29, 569–581
6. Phillips, D. R., Jennings, L. K., and Prassanna, H. B. (1980) J. Biol. Chem. 255, 11629–11632
7. Bale, M. D., Westrick, L. G., and Mosher, D. F. (1985) J. Biol. Chem. 260, 7503–7508
8. Tuszyński, G. P., Rothman, V. L., Murphy, A., Siegel, K., and Knudsen, K. A. (1988) Blood 72, 110–115
9. Dixit, V. M., Haverstick, D. M., O’Rourke, K. M., Hennessey, S. W., Grant, G. A., Santoro, S. A., and Frazier, W. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3472–3476
10. Gao, A.-G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1994) J. Biol. Chem. 269, 23747–23753
11. Asch, A. S., Silbiger, S., Heimer, E., and Nachman, R. L. (1992) J. Clin. Invest. 89, 1000–1007
12. Aiken, M. L., Ginsberg, M. H., Byers-Ward, V., and Plow, E. F. (1986) J. Clin. Invest. 78, 1713–1716
13. Aiken, M. L., Ginsberg, M. H., Byers-Ward, V., and Plow, E. F. (1990) Blood 76, 2501–2509
14. Gartner, W. K., and Dockter, M. E. (1983) Thromb. Res. 33, 19–30
15. Wolff, R., Plow, E. F., and Ginsberg, M. H. (1986) J. Biol. Chem. 261, 6840–6846
16. Hynes, R. O. (1977) Cell 48, 549–554
17. Wold, P. F., and Frazier, W. A. (1993) J. Biol. Chem. 268, 8808–8814
18. Gao, A.-G., and Frazier, W. A. (1994) J. Biol. Chem. 269, 29650–29657
19. Gao, A.-G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1996) J. Biol. Chem. 271, 21–24
20. Brown, J. E., Hoofer, L. H., and Gresham, H. D. (1990) J. Cell Biol. 111, 2785–2784
21. Lindberg, F. F., Gresham, H. D., and Brown, E. J. (1993) J. Biol. Chem. 268, 485–496
22. Schwartz, M. A., Brown, E. J., and Fazeli, B. (1993) J. Biol. Chem. 268, 19931–19934
23. Lindberg, F. P., Lublin, D. M., Telen, M. J., Veilé, R. A., Miller, Y. E., and Burns, G. F. (1989) J. Biol. Chem. 264, 13475–13482
24. Senior, R. M., Gresham, H. D., Griffin, G. L., Brown, E. J., and Chung, A. E. (1992) J. Clin. Invest. 90, 2253–2257
25. Shugar, D. S., and Brown, E. J. (1993) J. Exp. Med. 178, 1165–1174
26. Tsao, P. W., and Mousa, S. A. (1995) J. Biol. Chem. 270, 23747–23753
27. Berndt, M. C., Mazurov, A. V., Vinogradov, D. V., Burns, G. F., and Chesterman, C. N. (1993) Thromb. Res. 4, 190–196
28. Scott, J. L., Dunn, S. M., Jin, B., Hiliam, A. J., Walton, S., Berndt, M. C., Murray, A. W., Krissansen, G. W., and Burns, G. F. (1989) J. Biol. Chem. 264, 13475–13482
35. Schnölzer, M., and Kent, S. B. H. (1992) Science 256, 221–225
36. Huang, E. M., and Detwiler, T. C. (1986) in Biochemistry of Platelets (Phillips, D. R., and Schuman, M. A., eds.) pp. 1–68, Academic Press, New York
37. Craig, K. L., and Harley, C. B. (1996) Biochem. J. 314, 937–942
38. Murphy-Ullrich, J. E., Gurusiddappa, S., Frazier, W. A., and Hook, M. (1993) Biochem. J. 288, 26784–26789
39. Tuszynski, G. P., Rothman, V. L., Deutsch A. H., Hamilton, B. K., and Eyal, J. (1992) J. Cell Biol. 116, 209–217
40. McPherson, J., Sage, H., and Bernstein, P. (1981) J. Biol. Chem. 256, 11330–11336
41. Jaffe, E. A., Ruggerio, J. T., Leung, L. K., Doyle, M. J., McKeown, M. J., and Mosher, D. F. (1982) Nature 295, 246–248
42. Jennings, L. K., Fox, C. F., Kouns, W. C., McKay, C. P., Ballou, L. R., and Schultz, H. E. (1990) J. Biol. Chem. 265, 3815–3822
43. Mazurov, A. V., Vinogradov, D. V., Vlasik, T. K., Burns, G. F., and Berndt, M. C. (1992) Platelets 3, 181–188
44. Rosales, C., Gresham, H. D., and Brown, E. J. (1992) J. Immunol. 149, 2759–2764
45. Haimovich, B., Lipfert, L., Brugge, J. S., and Shattil, S. J. (1993) J. Biol. Chem. 268, 15868–15877

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