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Fibronectin Potentiates Actin Polymerization In Thrombin-Activated Platelets

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The effect of fibronectin on the polymerization state of actin was studied. Triton X-100-insoluble cytoskeleton was prepared from thrombin-activated platelets, and the conversion of G-actin into F-actin was monitored by an assay involving DNase I inhibition by G-actin. It was found that fibronectin bound to membrane receptors decreased the level of platelet G-actin. This observation suggests that in the presence of fibronectin a larger amount of F-actin becomes incorporated into the Triton X-100-insoluble cytoskeleton. At the same molar concentration, fibrinogen only slightly increased actin polymerization, whereas bovine serum albumin at a much higher concentration caused a small inhibition of actin immobilization. Our data show that fibronectin, through interaction with the platelet actomyosin fibrillar system, facilitates actin polymerization into the cytoskeleton.

Key words: platelet cytoskeleton, fibronectin, actin polymerization

Activation of blood platelets with thrombin or other aggregating agents results in a rapid shape change from discoidal form to spiny sphere possessing many filopodia. Electron microscopic studies of activated platelets have shown that the filopodia contain bundled actin filaments oriented in such a way that membrane-associated proteins appear to have a function similar to the Z-line of striated muscles [1–4]. This indicates that platelet shape change is associated with the polymerization of actin.

Thrombin activation of platelets causes not only an increased association of actin, actin-binding protein, and myosin with platelet cytoskeleton but also causes the cells to acquire the ability to bind fibronectin to cell-surface receptors [5]. Radioimmunoassay analysis of the fibronectin-related antigen in cytoskeletons obtained from thrombin-activated platelets revealed that all of the platelet fibronectin is associated with the cytoskeleton [6]. Studies on fibroblasts indicate that fibronectin molecules associated with membranes communicate either directly or through some transmem-

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brane linkages with actin microfilaments [7–9]. Therefore, we decided to study the effect of fibronectin on the course of actin polymerization in platelets.

MATERIALS AND METHODS

Preparation Procedures

Platelet-poor plasma (PPP) was obtained by centrifugation of freshly drawn whole human blood at 1,000 rpm for 20 min, and protease activity was inhibited by addition of 10 mM diisopropylfluorophosphate (DPF). Fibronectin was isolated from PPP by affinity chromatography on a gelatin-Sepharose 4B column [10]. Fibronectin was concentrated to about 3 mg/ml and stored at −20°C. The purity of fibronectin was assessed by SDS-polyacrylamide gel electrophoresis [11] and radioimmunoassay analysis [12]. Contamination with fibrinogen was less than 1%.

Fibrinogen was isolated from freshly frozen human blood plasma by a cold ethanol precipitation and further purified by ammonium sulfate fractionation at 26% saturation and 4°C as previously described [13] To remove traces of fibronectin, the fibrinogen solution was passed through a gelatin-Sepharose 4B column. SDS-polyacrylamide gel electrophoresis showed that fibrinogen contained only intact $A\alpha$, $B\beta$, and γ chains.

Washed platelets were isolated from fresh human blood by differential centrifugation [14] and suspended in a buffer coantaining 140 mM NaCl, mM Hepes, 5 mM KCl, and 10 mM glucose, pH 7.4 (Hepes-Tyrode buffer) to a cell count giving 40–60% DNase inhibition. Platelet counts were approximately 8×10^8 cells/ml.

Cytoskeletons were prepared from isolated platelets activated with thrombin in the presence of Gly-Pro-Arg-Pro, an inhibitor of fibrin monomer polymerization, as described previously [15]. Platelets suspended in Hepes-Tyrode buffer were treated with different amounts of thrombin, usually for 1 min at 37°C. Activation was stopped by the addition of 10 μM DFP and heparin (10 units). Thrombin-activated platelets were then lysed with Triton X-100 added to a final concentration of 1%.

IgG specific to fibronectin was purified from antifibronectin antiserum by chromatography on a fibronectin affinity column. Usually, 14 mg of fibronectin was coupled to 1 gm of CNBr-activated Sepharose 4B. Two milliliters of antiserum was applied to the column equilibrated with 0.15 M NaCl and 0.02 M Tris HCl, pH 7.4. Nonspecifically bound proteins were eluted with the same buffer. After washing the column with 0.5 M NaCl buffered with 0.02 M Tris HCl, pH 7.4, bound IgG was eluted with 0.1 M glycine buffer, pH 2.7. The eluate was neutralized with 1 M Tris HCl, pH 7.4, and dialyzed against 0.15 M NaCl buffered with 0.05 M Tris HCl, pH 7.4. IgG from preimmune serum was purified by affinity chromatography on protein A-Sepharose [16].

Analytical Procedures

The actin polymerization assay was performed as described by Pribluda et al [17]. Briefly, platelets (resting or activated) were preincubated in the presence or absence of fibronectin, fibrinogen, or bovine serum albumin for 20 min at 37°C. Then platelet samples were lysed with 1% Triton X-100, vortexed for 10–20 sec, and centrifuged for 1 min at 12,000g. Twenty microliters of supernatant was added together with 20 μl DNase (0.1 mg/ml in 0.05 M Tris HCl, pH 7.4, containing 0.001 M PMSF and 0.1 mM $CaCl_2$) to 2 ml of a mixture containing DNA (40 μg/ml) in

0.1 M Tris HCl, pH 7.5, and 4 mM $MgSO_4$ and 18 mM $CaCl_2$. Hydrolysis of DNA was followed by measuring the hyperchromicity at 260 nm. The slope of the linear part of transmission changes was measured, and actin polymerization was calculated as a percentage of total actin content. Total actin was determined by scanning 7% SDS-polyacrylamide gels. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [11]. Changes in DNase-available actin are referred to throughout as *actin immobilization.*

Platelet secretion induced by thrombin was monitored by determination of β-thromboglobulin (β-TG) concentration in lysates [19]. This was performed by radioimmunoassay analysis in duplicate in a double antibody system [12]. The content of β-TG was estimated prior to and after activation of platelets with different concentration of thrombin.

The effect of cytochalasin B and antifibronectin antibodies was studied in the following system: Five hundred microliters of platelet suspension (8×10^8/ml) was preincubated with 50 μM cytochalasin B for 45 min at 37°C. A control platelet suspension without cytochalasin B was incubated under the same conditions. Samples of the cytochalasin B treated platelets and of control platelets were then mixed with different concentrations of fibronectin and after 5 min incubation at 37°C activated with thrombin (2 U) for 1 min. Activation was stopped by the addition of 10 μM DFP and heparin (10 U), and platelets were lysed with 1% Triton X-100. Similarly, 80 μl of the same platelet suspensions was preincubated with immunochemically purified antifibronectin IgG or preimmune IgG (100 μg/ml) for 20 min. at 37°C. Then, fibronectin (250 μg/ml) was added, and, after incubation for 5 min at 37°C, platelets were activated with thrombin (2 U) for 1 min. Thrombin-activated platlets were lysed with 1% Triton X-100. All data represent the mean ± SD of from five to ten determinations performed on blood taken on different occasions. Statistical analysis included paired and nonpaired t tests.

RESULTS AND DISCUSSION

Fibronectin associated with membrane receptors of thrombin-activated platelets was to a large extent recovered in the Triton X-100-insoluble cytoskeleton [6]. This observation suggested that fibronectin molecules, after binding with platelets, somehow communicate with actin microfilaments and might influence the course of polymerization of G-actin. Experimental data presented here support this concept. Conversion of G-actin into F-actin was measured by the degree of DNase I inhibition occuring in the presence of G-actin [19]. The degree of inhibition was estimated for 1 min after addition of DNase I in order to avoid DNase-mediated F-actin depolymerization [17,20]. The extent of platelet activation under such conditions was estimated on the basis of β-TG release (Fig. 1A) a reaction known to precede both actin polymerization and platelet aggregation [20]. As is seen in Figure 1B, fibronectin significantly potentiates the course of actin immobilization. This observation indicates that fibronectin decreases the amount of DNase I available G-actin in Triton X-100 extracts of thrombin-activated platelets. At an equimolar concentration, only fibrinogen slightly increased actin immobilization, whereas bovine serum albumin, at a much higher concentration, had a slight inhibitory effect on this reaction. None of these proteins, when added after platelets were lysed, had any effect on DNase I activity. This indicates that the increased immobilization of actin is not an artifact of

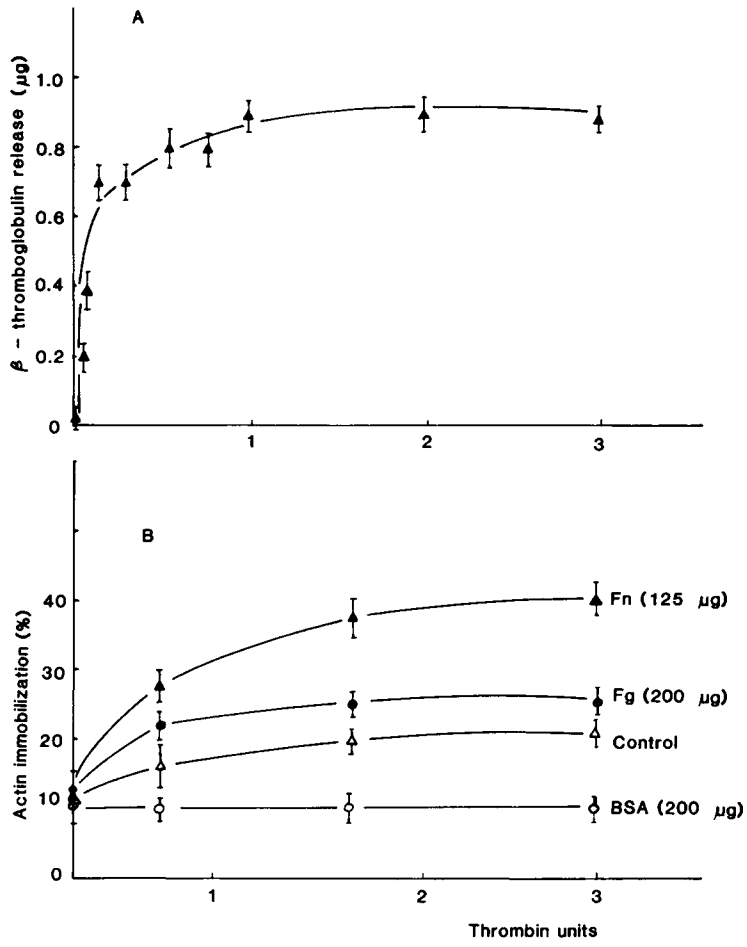

Fig. 1. Actin immobilization in platelets activated by thrombin in the presence of fibronectin (Fn), fibrinogen (Fg), and bovine serum albumin (BSA; B). The extent of platelet activation was estimated by β-TG release (A). Experiments were performed as described in Materials and Methods.

our experimental procedure but results from activation of G-actin conversion into F-actin caused by binding of fibronectin to its membrane receptor.

The effect of fibronectin on polymerization of actin was dependent both on thrombin and fibronectin concentration (Fig. 2). It is noteworthy that the potentiating effect reached a maximum at a fibronectin concentration of 125 μg per 10^8 platelets. This concentration corresponds to the physiological level of fibronectin in blood plasma, ie, $\sim 5 \times 10^{-7}$ M.

The specificity of fibronectin action on actin polymerization is demonstrated by the results presented in Figure 3. When samples of resting platelets were preincubated with 50 μM cytochalasin B (Fig. 3Ab), immunochemically purified antifibronectin antibodies (Fig. 3Ac), or preimmune IgG (Fig. 3Ad) there was no change in the amounts of G-actin compared to control platelets (Fig. 3Aa). Addition of thrombin to platelets preincubated under such conditions caused about 10% actin immobilization

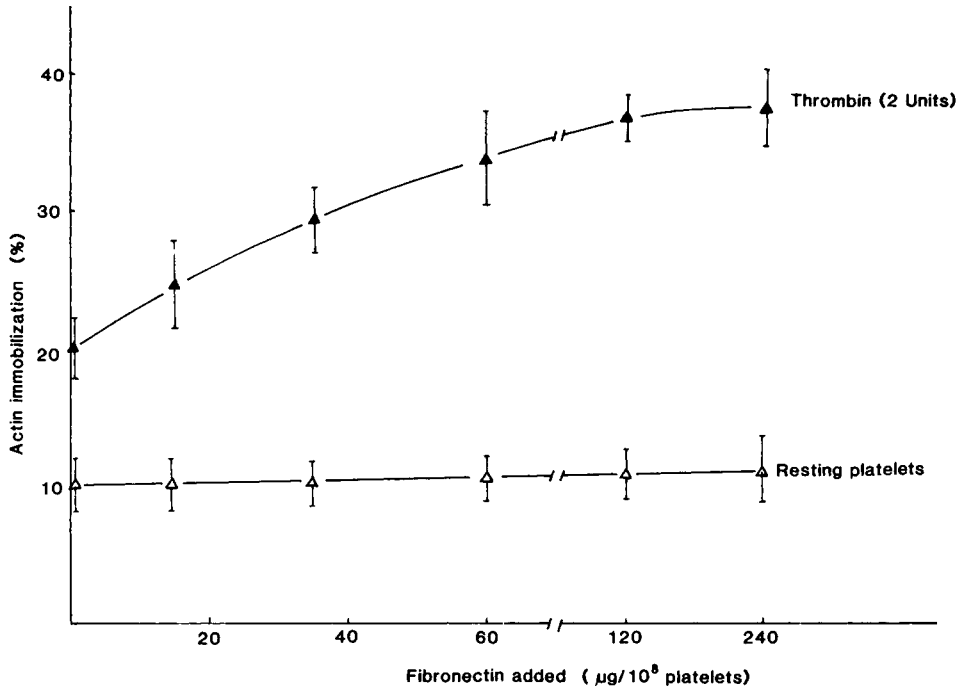

Fig. 2. Concentration dependence of fibronectin potentiating effect on actin immobilization in platelets activated with 2 U thrombin (compared to resting platelets).

regardless of the reagents to which platelets were exposed during preincubation (Fig. 3B). Activation of platelets by 2 U thrombin in the presence of 125 μg of fibronectin resulted in a significant potentiation of actin polymerization (Fig. 3Ca). This effect represented a shift of approximately 18% of the available G-actin to F-actin. Preincubation of platelets with preimmune IgG did not abolish the potentiating effect of fibronectin on actin immobilization in platelets activated by thrombin (Fig. 3Cd). Polyclonal antibodies specific for fibronectin added to platelet suspension prior to fibronectin neutralized the effect of fibronectin (Fig. 3Cc). However, this did not block actin immobilization caused by thrombin. Similarly, cytochalasin B neutralized the potentiating effect of fibronectin but did not block conversion of G-actin to F-actin stimulated by thrombin (Fig. 3Cb). Thus the potentiating effect of fibronectin is abolished by both cytochalasin B and immunochemically purified antifibronectin IgG. However, in our hands, cytochalasin B did not block actin polymerization induced by thrombin alone. Incubation of resting platelets with 50 μM cytochalasin B, immunochemically purified antifibronectin antibodies, or preimmune IgG did not change the level of immobilized actin. In control experiments, when cytochalasin B, antifibronectin antibodies, or preimmune IgG was added to Triton X-100-lysed platelets, there was no effect on DNase I activity (Fig. 3A).

The molecular mechanism of fibronectin's role in actin immobilization is difficult to interpret. Fibronectin is known to have a very close relationship with internal actin microfilaments in fibroblasts [8]. Such a transmembrane association between extracellular fibronectin fibers and intracellular fibers of vinculin and actin microfil-

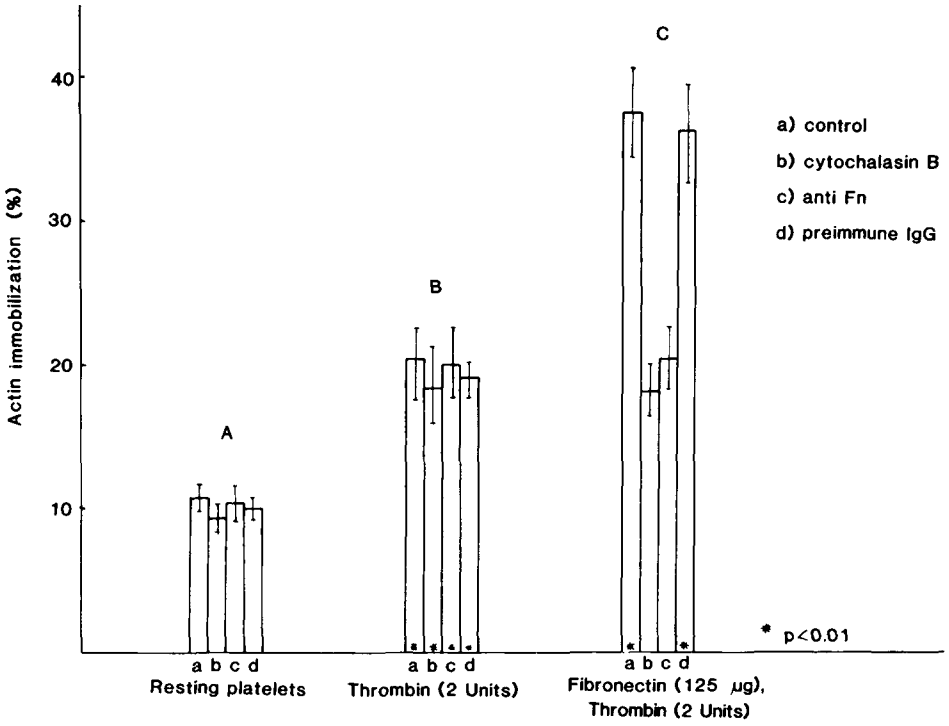

Fig. 3. Effect of cytochalasin B and antifibronectin IgG on actin immobilization in resting platelets and in platelets activated with 2 U thrombin. Samples of platelet suspension were incubated in the presence of Tyrode's buffer alone (Aa), 50 μM cytochalasine B (Ab), immunochemically purified antifibronectin antibodies (Ac), and preimmune IgG (Ad). Then aliquots of these samples were lysed with 1% Triton X-100, and level of actin immobilization was estimated during DNase I test. Aliquots of the same platelet suspensions prior to lysis with 1% Triton X-100, were activated for 5 min with 2 U thrombin (Ba–d). To analyze the effect of fibronectin on actin immobilization, control platelets (Ca) or platelets pretreated with cytochalasin B (Cb) were mixed with fibronectin and then activated with 2 U trombin. In parallel experiments, immunochemically purified antifibronectin antibodies (Cc) or preimmune IgG (Cd) were added prior to fibronectin and platelets activated with 2 U thrombin. Aliquots of relevant platelet suspensions were lysed with 1% Triton X-100, and level of G-actin was estimated by DNase test.

aments has been demonstrated by electron microscopy and is referred to as the *fibronexus* [9]. Beside actin and vinculin, fibronectin has a relatively high binding affinity towards other components of platelet actomyosin system, ie, gelsolin [21]. Finally, adhesive proteins such as fibrinogen and thrombospondin, found to be associated with cytoskeleton of activated platelets in substantial amounts [18], also can bind fibronectin with a relatively low dissociation constant, ie, 10^{-7} M [22]. It is possible that fibronectin facilitates actin polymerization through interaction with the platelet actomyosin fibrillar system and thus provides positive feedback during activation of platelets.

REFERENCES

1. Nachmias VT, Asch A: In Goldman R, Pollard T, Rosenbaum J (eds): "Cell Motility Book B." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1976, pp 771–783.

2. Crawford N: In Gordon JL (ed): "Platelets in Biology and Pathology." Amsterdam: North-Holland Publishing Co., 1976, pp 121–157.
3. Fox JEB, Philips DR: Semin Hematol 20:243, 1983.
4. Nachmias V, Sullender J, Asch A: Blood 50:39, 1977.
5. Plow EF, Ginsberg MH: J Biol Chem 256:9577, 1981.
6. Niewiarowska J, Cirniewski CS, Tuszynski GP: J Biol Chem 259:6181, 1984.
7. Singer II: J Cell Biol 92:398, 1982.
8. Singer II, Paradiso PR: Cell 24:481, 1981.
9. Singer II: Cell 16:675, 1979.
10. Engvall E, Ruoslahti E: Int J Cancer 20:1, 1977.
11. Laemmli UK: Nature 227:680, 1970.
12. Cierniewski CS, Babinska A, Niewiarowska J, Augustyniak W: Hoppe-Seyler Z Physiol Chem 364:515, 1983.
13. Plow EF, Edgington TS: Proc Natl Acad Sci USA 69:208, 1972.
14. Mustard JF, Perry DW, Ardlie NG, Packham MA: Br J Haematol 22: 193, 1972.
15. Tuszynski GP, Walsh PN, Piperno JR, Koshy A: J Biol Chem 257:4557, 1982.
16. Hjelm H, Hjelm K, Sjoquist J: FEBS Lett 28:73, 1972.
17. Pribluda V, Laub F, Rotman A: Eur J Biochem 116:293, 1981.
18. Cierniewski CS, Switalska H, Hershock D, Capitanio AM, Tuszynski GP, Niewiarowski S: Fed Proc 43:659, 1984.
19. Blikstad I, Markey F, Carlsson L, Persson T, Lindberg K: Cell 15:935, 1978.
20. Pribluda V, Rotman A: Biochemistry 21:2825, 1982.
21. Janney PA, Smith DB, Yin HL, Stossel IP: Blood 62:288a, 1983.
22. Lahav J, Schwartz MA, Hynes RO: Cell 31:253, 1982.