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Dynamics of Membrane–Cytoskeleton Interactions in Activated Blood Platelets†

Victor Pribluda and Avner Rotman*

ABSTRACT: The dynamics of actin polymerization, cytoskeleton formation, and interaction with membrane and cytoplasmic proteins as a result of platelet activation by temperature, ADP, or thrombin were studied. The polymerization of about 30% of platelet DNase I available actin to a nonavailable state is rapid and complete within 10 s after platelet activation with ADP and thrombin. This polymerization might be related to shape change rather than to aggregation or secretion. A similar value of actin polymerization is obtained when platelets are induced to change shape by cooling. This polymerization is partially reversible upon deactivation of the platelets by apyrase, hirudin, or rewarming. Cycles of temperature-mediated activation and deactivation show a cyclic variation in the state of actin, with a tendency to refractivity to further changes after a couple of cycles. No correlation is observed between microtubule integrity and actin polymerization when studies are performed with platelets pretreated with colchicine. Analysis of the Triton residue composition shows that the cytoskeleton of resting platelets is composed mainly of actin and myosin in a 4.5:1 ratio. Activation with ADP and thrombin leads to the association and incorporation of several other proteins (actin binding protein, 95 000 daltons, three to four proteins in the 35 000-dalton region, and two proteins in the 17 000-dalton region with the cytoskeleton). The incorporation of these proteins has a dynamic nature that depends on both the state of aggregation and the reversibility of the activation. Activation leads to a significant increase in the total cytoskeletal proteins, and although low temperature also induces such an increase, the cytoskeletal pattern of cooled platelets is not different from that of resting platelets. A complete reversibility in morphology and amount of protein was observed with temperature cycling. In light of these results, the dynamic nature of the state of actin in platelets is discussed.

The cytoskeletal structure of cells was the focus of many studies in recent years. Electron microscopy and immunofluorescent studies identified actin as the main component of the cytoskeleton (Edds, 1979; Haynes & Destree, 1978; Lazarides, 1976; Heuser & Kirschner, 1980; Nachmias, 1980; Tsukita et al., 1980; Wehland et al., 1979). Cell extracts are able to develop gel–sol transformation by the specific association of actin with certain proteins (Condeelis & Taylor, 1977; Ishiura & Okada, 1979; Koenig et al., 1981; Pollard, 1976a; Pollard & Ito, 1970; Stossel & Hartwig, 1976a). This well-defined microfilamentous structure may exist permanently, as in microvilli (Mooseker & Tilney, 1975; Mukherjee & Staehlin, 1971) or skeletal muscle (Peachey, 1966), or may develop from a soluble nonfilamentous actin precursor as a result of external stimuli (Badley et al., 1980; Nachmias, 1980; Tilney et al., 1973). On the other hand, sol–gel transformation has been suggested as the basis of ameboid movement or macrophage phagocytosis (Pollard, 1976b; Stossel & Hartwig, 1976b; Taylor, 1977; Taylor et al., 1973).

Microfilament bundles with a well-defined polarity have been described in platelet pseudopods (Nachmias & Asch, 1976; Crawford, 1976). These organized structures were probably formed from soluble, nonfilamentous actin during platelet activation. Two recent publications reported that a decrease in the DNase-available actin accompanied platelet activation (Carlsson et al., 1979; Pribluda et al., 1981). This decrease in DNase-available actin (actin polymerization) was fast and reached a maximum of about 30% of total actin within 10–15 s in the case of thrombin activation. In a recent publication, Phillips et al. (1980) showed that activation and aggregation of platelets by thrombin result in the specific association of the cytoskeleton with membrane proteins. Association of actin with platelet membrane was also shown by Taylor et al. (1975). In the present paper, we show that polymerization of actin is reversible to some extent and might be associated with the platelet shape change rather than release or aggregation. In addition, we give some evidence that the

† From the Department of Membrane Research, The Weizmann Institute of Science, 76100 Rehovot, Israel. *Received June 19, 1981.* The generous financial support of the Gatsby Foundation (London, England) is highly appreciated.

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cytoskeleton and its interaction with membrane and cytoplasmic proteins are not static but are rather of a dynamic nature.

Experimental Procedures

Materials. Thrombin (human), ADP, apyrase, hirudin, DNase I, DNA, and EGTA were purchased from Sigma, St. Louis, MO. All other chemicals used were of the highest purity available.

Washed platelets were prepared as described by Schmidt & Rasmussen (1979) from donors who did not take any medication at least 2 weeks before drawing of blood. The platelets were suspended in a buffer containing 108 mM NaCl, 3.9 mM KCl, 1.6 mM $CaCl_2$, 0.5% glucose, and 0.62% trisodium citrate. DNase activity was measured as previously described (Pribluda et al., 1981). Briefly, washed platelets (either resting or activated as indicated in each experiment) were lysed by the addition of 1% (final concentration) Triton X-100 and vortexed for 20 s. The lysate was diluted with buffer to a concentration giving 40–60% DNase inhibition (determined separately on resting platelets) and added together with DNase I in 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4) to a mixture containing 1.9 mM $MgCl_2$, 0.28 mM $CaCl_2$, 2.5 mM Tris-HCl (pH 8), 0.02 μg/mL DNA, and 0.1 μM ethidium bromide. The change in the enhanced fluorescence of ethidium bromide was determined by using an excitation light of 520 nm and an emission of 602 nm (Gitler et al., 1980). Actin polymerization was calculated as the percentage of the DNase-available actin in resting platelets which became DNase nonavailable after activation. No effect of hirudin or apyrase on the assay was observed. cytoskeleton was isolated as the Triton residue fraction essentially as described by Phillips et al. (1980): Briefly, one-fifth volume of a solution containing 5% Triton X-100 and 50 nM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added to the platelet suspension. After being vortexed for 10–15 s, the mixture was kept at room temperature for 30–60 min and centrifuged in a Beckman microfuge (12000*g*) for 4 min. The pellet obtained was washed twice with the suspension buffer (see above) containing 1% Triton X-100 and 10 mM EGTA.

Cycles of temperature-induced activations were done as follows: Resting platelets were incubated at 0 °C for 15 min; samples were removed for actin polymerization assay and for cytoskeleton preparation. The rest of the platelets were warmed to 20 °C and incubated at that temperature for 15 min, and two samples were removed. The rest of the platelet suspension was warmed to 37 °C and incubated at that temperature for 15 min, and two samples were removed. At each temperature, the morphology of platlets is determined as described above. Shape change was determined by the observation of "noise" in the base-line tracing of the aggregometer, by the disappearance of swirling of platelets, and by the observation of platelets (and counting) in a light microscope. Release was measured by preloading the platelets with [^{3}H]serotonin (Pribluda et al., 1981).

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was done according to Laemmli (1970). The gels were stained with Coomassie Brilliant Blue (de St. Groth et al., 1963), and their transparencies were scanned in a Gilford recording spectrophotometer at 550 nm. Aggregation was studied on a Chronolog aggregometer. Protein determinations were carried out according to Lowry et al. (1951).

Results

(*I*) *State of Actin.* (*a*) *Onset of Actin Polymerization.* The degree of DNase-available actin (defined here as actin polymerization) as a function of the thrombin or ADP concentration is shown in Figures 1 and 2, respectively. Actin polymerization reached its maximal level of ca. 30% with shape change and well before aggregation commenced, or release of serotonin was significant. (No release of [^{3}H]serotonin was observed at low doses of ADP or thrombin or in ADP activation without fibrinogen.) This result was obtained when platelets were activated with thrombin in the presence or absence of fibrinogen (Figure 1) or activated by ADP with or without fibrinogen (Figure 2). The same degrees of DNase inhibition values were obtained when the lysis buffer did not contain calcium (in the presence of 10 mM EDTA). When high doses of thrombin or ADP were used and aggregation was observed, the actin polymerization was measured at half the maximal aggregation. The same actin polymerization was observed when measured at different states of aggregation for a specified ADP or thrombin concentration (data not shown). DNase-available actin in resting platelets accounts for 80% of total actin [average of four experiments; see also Pribluda et al. (1981)]. High values (60–90%) of actin polymerization were consistently observed when a high dose (1 unit/mL) of thrombin was used in the presence of fibrinogen. The possibility that fibrin (known to interact with F-actin; Laki & Muszbek, 1974) might induce this effect was checked. No effect of polymerizing fibrin on the G-actin or on a mixture of (G + F)-actin was observed (using the DNase inhibition assay). Fibrin did not interfere with the complete lysis of platelets during the DNase inhibition assay as was verified by

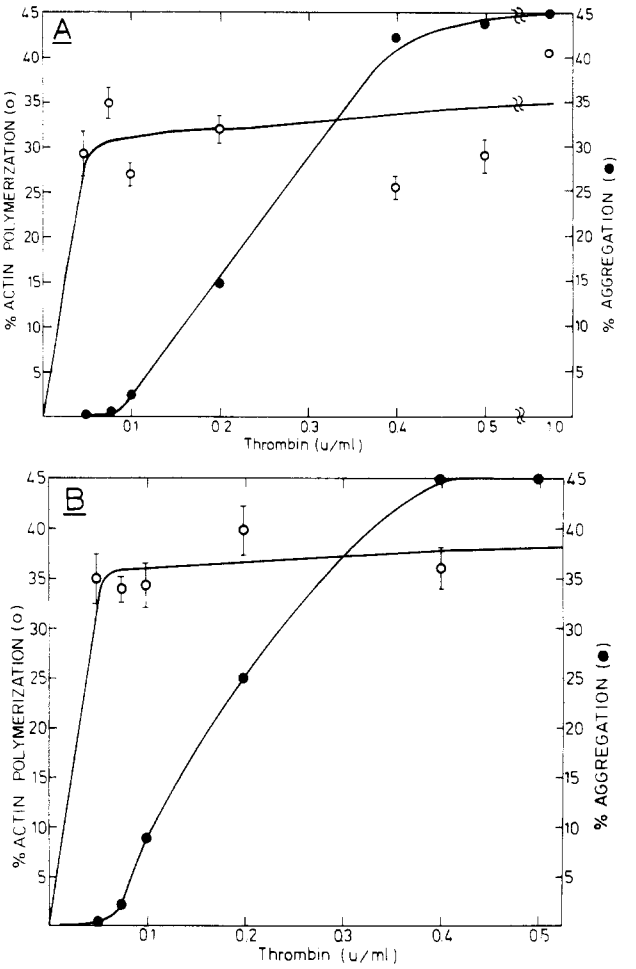

FIGURE 1: Actin polymerization during platelet activation with thrombin. (A) Without fibrinogen; (B) with fibrinogen (400 μg/mL). Four measurements were done for each lysis. Each measurement was studied over 1 min. All measurements were finished not later than 7 min after lysis.

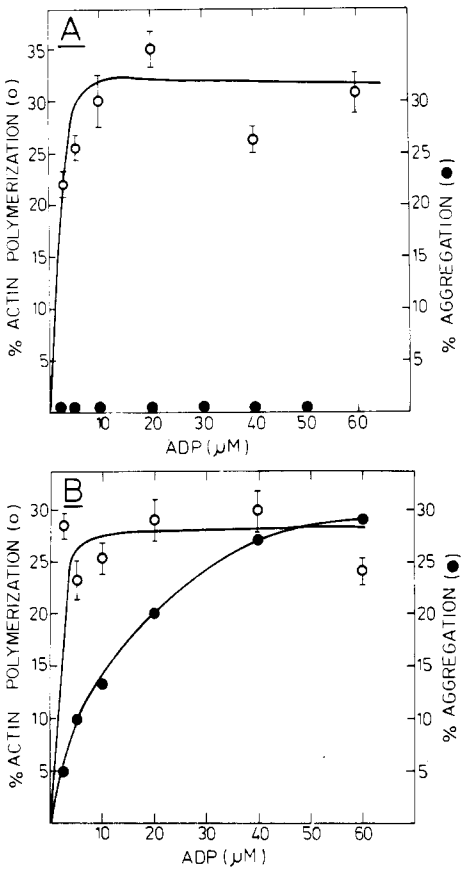

FIGURE 2: Actin polymerization during platelet activation with ADP. (A) Without fibrinogen; (B) with fibrinogen (400 μg/mL). Four measurements were done for each lysis. Each measurement was studied over 1 min. All measurements were finished not later than 7 min after lysis.

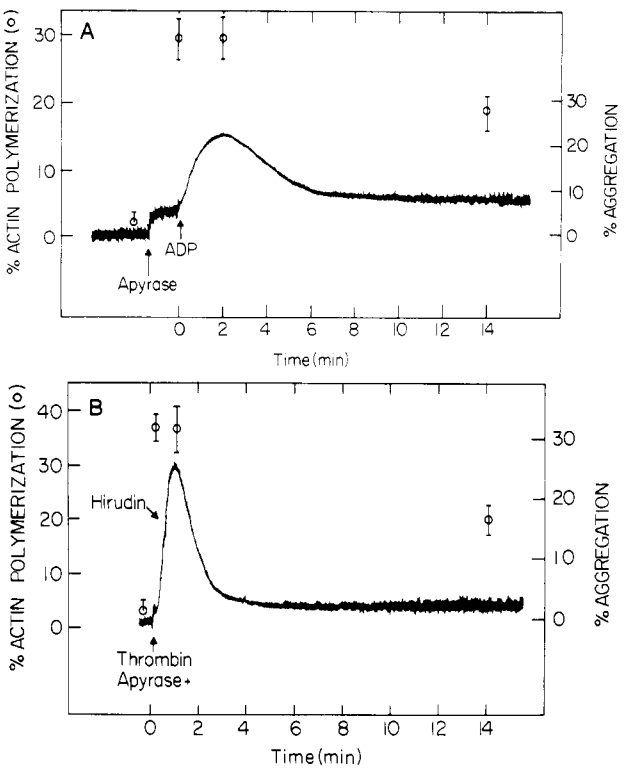

FIGURE 3: Actin polymerization during reversible aggregation of platelets (average of four experiments). (A) Activation by 2.5–10 μM (final concentration) ADP. The apyrase concentration was 160 μg/mL. Fibrinogen was added to a final concentration of 400 μg/mL. (B) Activation by 0.05–0.2 unit/mL (final concentration) thrombin. The apyrase concentration was 160 μg/mL. Hirudin concentration was 0.15–0.6 unit/mL. In some cases, fibrinogen was added to a final concentration of 400 μg/mL.

preloading the platelets with [^{3}H]serotonin. We have as yet no explanation for these extremely high values of actin polymerization.

The polymerization of actin as a result of temperature-induced shape change was also studied. Cooling of platelets to 0 °C resulted in the disappearance of the characteristic swirling of diskoid platelets and in 24 ± 4% of actin polymerization similar to the values obtained with ADP or thrombin activation.

When platelets were treated with 0.5 or 2 mM colchicine at 37 °C, an almost complete shape change was observed after 20–30 min. However, this colchicine-induced shape change was not accompanied by actin polymerization. On the contrary, a small (2–7%) increase in DNase-available actin was observed. When these colchicine-treated platelets were activated by low temperature or by thrombin, a normal actin polymerization was observed which was only slightly (3–6%) lower than the results obtained with nontreated control platelets. Complete inhibition of ADP-induced aggregation was observed when the platelets were pretreated with 0.5 mM colchicine for 90 min, and 60% inhibition was already obtained after 15 min. Incubation of platelets with 2 mM colchicine for 90 min caused 50% inhibition of thrombin-induced aggregation (the concentration of thrombin in this experiment was 1 unit/mL).

(*b*) *Reversibility of Actin Polymerization.* (*i*) *ADP and Thrombin Activation.* The state of actin in reversible platelet activation was studied. Figure 3 shows reversibility of actin polymerization during ADP and thrombin reversible aggregation. ADP-induced aggregation was reversed by the addition of apyrase together with the ADP. About 30% reversibility in actin polymerization was measured at the end of the reversible aggregation (Figure 3A). Thrombin-induced reversible aggregation was achieved by the addition of hirudin after thrombin activation (Figure 3B). For neutralization of any ADP released by the thrombin activation, apyrase was added together with thrombin. The same values were obtained whether thrombin-induced release was obtained or not. Similar values of actin mobilization were obtained when platelets were induced to undergo reversible shape change by ADP or thrombin.

(*ii*) *Temperature-Induced Activation.* Cycles of reversible shape change were performed by transfer of platelets periodically through 0, 20, and 37 °C, with the temperature of 20 °C as an intermediate point. The state of actin was measured at each temperature, and results are shown in Figure 4. It is clear that cooling platelets to 0 °C, accompanied by a shape change to the spiny sphere, results in a high actin polymerization similar to that obtained with ADP or thrombin (compare with Figures 1 and 2). After the first cycle, a recovery of approximately 50% is obtained, but a clear decay in the oscillations of actin polymerization is observed although the shape change observed is fully reversible. Moreover, a clear tendency toward a higher value of actin polymerization could be observed with repeated temperature cycles. When washed platelets were kept at room temperature throughout the experiment, a constant value of DNase-available actin was observed, while when platelets were kept at 37 °C, about 10–20% actin polymerization was observed after about 3 h.

(*II*) *Dynamics of Platelet Cytoskeleton.* (*a*) *Aggregation.* The content of the Triton residue of resting and activated platelets was analyzed with respect to the total amount of protein and the protein composition. NaDodSO$_4$–polyacryl-

Table I: Composition of Cytoskeleton of Thrombin-Aggregated Platelets[a]

aggregation (%)	rel amount of protein	actin binding protein	myosin	95 000 daltons	actin	35 000-dalton region
resting platelets	1	0–1	13.9 ± 3.6	0–1	75.0 ± 8.9	0–2
1–8	12 ± 1.5	0.2 ± 0.6	19.4 ± 5.3	2.8 ± 0.9	55.8 ± 4.9	22.3 ± 3.9
20–45	14 ± 1.5	4.1 ± 1.7	18.8 ± 6.8	3.1 ± 1.1	52.9 ± 3.3	21.8 ± 4.1
70–95	20 ± 2.2	5.3 ± 0.7	11.5 ± 3.7	10.0 ± 2.2	46.8 ± 6.1	16.9 ± 6.4
0 (EDTA)[b]	12 ± 1.1	0.4 ± 0.8	41.4 ± 8.5	0	40.2 ± 2.0	9.1 ± 4.4

[a] Concentration of thrombin was 1 unit/mL. Numbers represent means obtained from five to seven experiments with different platelet preparations. [b] Platelets were incubated with EDTA (5 mM) for 30 s before addition of ADP or thrombin, and incubation was continued for another 60 s until Triton X-100 was added.

Table II: Composition of Cytoskeleton of ADP-Aggregated Platelets[a]

aggregation (%)	rel amount of protein	actin binding protein	myosin	95 000 daltons	actin	35 000-dalton region
resting platelets	1	0–1	13.9 ± 3.6	0–1	75.0 ± 8.9	0–2
3–8	5 ± 0.5	1.6 ± 2.3	16.7 ± 7.3	2.1 ± 2.2	67.2 ± 5.0	3.4 ± 4.1
25–50	7 ± 0.8	2.7 ± 0.4	10.3 ± 2.1	4.9 ± 3.1	58.6 ± 8.6	8.9 ± 2.9
50–80	13 ± 1.5	6.7 ± 4.6	7.0 ± 1.3	7.8 ± 1.5	54.7 ± 2.0	10.4 ± 1.7
0 (EDTA)[b]	8 ± 0.9	0	27.4 ± 3.3	0	50.6 ± 6.9	4.6 ± 0.4

[a] Concentration of ADP was 20–40 μM. Numbers represent means from five to seven experiments with different platelet preparations. [b] Platelets were incubated with EDTA (5 mM) for 30 s before addition of ADP or thrombin, and incubation was continued for another 60 s until Triton X-100 was added.

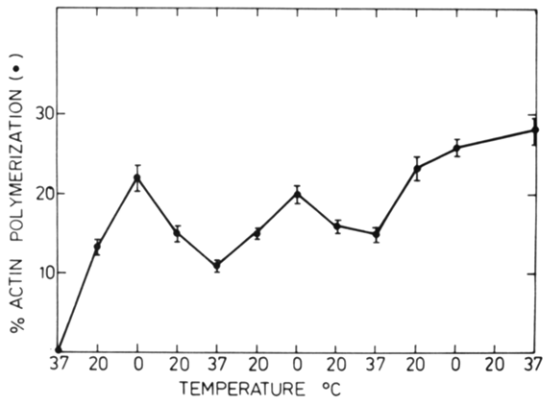

FIGURE 4: Actin polymerization during temperature-induced shape change. At each temperature, the platelets were incubated for 15 min.

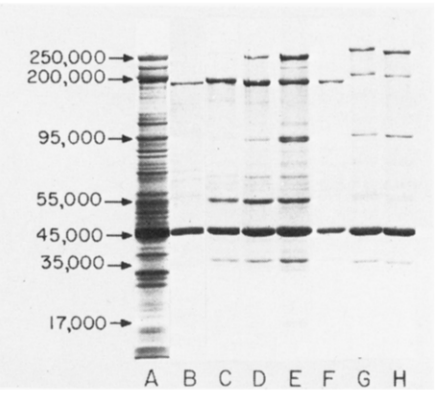

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of different platelet preparations (Coomassie brilliant blue staining). (A) Whole washed platelets; (B) Triton residue of resting platelets; (C) Triton residue of thrombin-activated platelets, 10 s after activation (beginning of aggregation); (D) Triton residue of thrombin-activated platelets (at half-maximal aggregation); (E) Triton residue of thrombin-activated platelets (at full aggregation); (F) Triton residue of ADP-activated platelets, 10 s after activation (beginning of aggregation); (G) Triton residue of ADP-activated platelets (at half the maximal aggregation); (H) Triton residue of ADP-activated platelets (at full aggregation). The concentration of thrombin was 1 unit/mL. The concentration of ADP was 40 μM (final concentration). All lanes are from the same experiment (one of those summarized in Tables I and II). Half-aggregation was achieved in 1–1.5 min. Aggregation was complete after 2–3 min from activation.

amide gel electrophoresis of resting diskoid platelets revealed two main bands in the Triton residue: actin and myosin (Figure 5, lane B). Activation of platelets either with ADP or with thrombin resulted in the appearance of a number of new bands in the Triton residue. These bands are actin binding protein (ABP) with a molecular weight of 250 000, a protein with a molecular weight of approximately 95 000, and a series of three to four proteins in the molecular weight range of 35 000. Two bands in the molecular weight range of 17 000 are sometimes observed (Figure 5, lanes C–H). The relative amount of each of these proteins was determined by scanning the transparencies of Coomassie Blue staining of their gels. The results of this quantitation are given in Table I for thrombin activation and Table II for ADP activation. Generally, an increase in the amount of Triton residue upon activation is observed both with ADP and with thrombin. Moreover, the residue obtained in thrombin aggregation is always about 50% greater than that obtained with ADP. It is quite evident from Tables I and II that the relative concentrations of ABP and the 95 000-dalton protein increase upon activation and aggregation of platelets with ADP or thrombin. The relative concentration of actin and the group of 35 000-dalton proteins is without significant change during the aggregation (i.e., from 1–2% aggregation to full aggregation).

The relative amount of the 35 000-dalton group in thrombin-aggregated platelets is about double that found in the case of ADP aggregation. Two additional bands are present in the Triton residue of thrombin-aggregated platelets (Figure 5, lanes C–E). These bands, having molecular weights of 56 000–58 000 and 70 000, were identified as products of fibrinogen. They coincide with authentic bands of ^{125}I-labeled fibrinogen added to the platelets (Figure 6, lanes A and C), and their intensity was reduced or sometimes they disappeared completely when hirudin was added together with the Triton solution (Figure 6, lane B) or before the Triton solution (Figure 6, lane D). A marked increase in the intensity of these bands was observed when fibrinogen was added during thrombin-induced aggregation. For this reason, these bands were not

Table III: Composition of the Cytoskeleton of Thrombin-Activated Platelets[a]

activation	rel amount of protein[b]	actin binding protein	myosin	95 000 daltons	actin	35 000-dalton region	17 000-dalton region
shape change	1.0	0–2	2–12	3–15	50–77	4–12	ND[c]
reversible shape change	0.5	0–5	1–15	4–16	58–83	7–12	ND
aggregation (25–30%)	5.0	2–4	7–13	3–6	53–72	13–18	4–6
reversible aggregation	2.0	0–2	3–7	3–16	69–89	7–8	0–2

[a] Numbers represent ranges obtained from three experiments with different platelet preparations. [b] The relative amount of protein content of the cytoskeleton is given in relation to that of shape-changed platelets. [c] ND refers to not detected.

Table IV: Composition of the Cytoskeleton of ADP-Activated Platelets[a]

activation	rel amount of protein[b]	actin binding protein	myosin	95 000 daltons	actin	35 000-dalton region	17 000-dalton region
shape change	1.0	1–2	2–14	4–21	69–77	5–9	1–2
reversible shape change	1.0	0–1	16–18	8–16	51–64	5–6	5–6
aggregation (25–30%)	3.5	3–5	3–13	5–8	59–71	9–14	1–2
reversible aggregation	2.0	0–2	13–19	5–15	45–64	5–7	3–5

[a] Numbers represent ranges obtained from three experiments with different platelet preparations. [b] The relative amount of protein content of the cytoskeleton is given in relation to that of shape-changed platelets.

included in the calculations described in Tables I–IV. Thus, the results obtained in the experiments without hirudin (when these bands appeared but were not included in calculations) or with hirudin (when these bands did not appear at all) were exactly the same. The presence of ethylenediaminetetraacetic acid (EDTA) during platelet activation caused an increase in the relative amount of myosin and a decrease of the ABP and the 95 000-dalton bands in the Triton residue. This increase in the myosin heavy chain band is due to the presence of EDTA in the lysate and is not a result of any EDTA effect on the platelets themselves (Figure 6, lane K). This effect of EDTA was observed in the case of both ADP and thrombin activation. A similar pattern to that shown in lane K of Figure 6 was obtained when EDTA was added to the platelet suspension after lysis or in the Triton solution with or without EGTA. An increase in the total amount of protein in the Triton residue was observed in the case of ADP (but not in the case of thrombin) activation in those experiments performed with EDTA.

(*b*) *Reversibility of Activation.* Results summarized in Tables III and IV and Figure 6 clearly demonstrate that there are some oscillations in a few proteins that constitute the Triton residue upon reversible activation of platelets (reversible shape change or reversible aggregation). Reversal of thrombin-induced shape change resulted in the same distribution of proteins in the Triton residue as was found during shape change (Table III; Figure 6, lanes A and D). Aggregation of platelets by thrombin resulted in an increase in the relative concentration of ABP and the 35 000-dalton group and a significant appearance of the 17 000-dalton group. Reversal of this aggregation by hirudin resulted in a significant decrease of ABP, myosin, the 35 000-dalton group, and the 17 000-dalton group (Table III and Figure 6, lanes G and H). A similar phenomenon of oscillation of proteins constituting the Triton residue was observed when platelets were activated by ADP (Table IV and Figure 6, lanes E, F, I, and J). Reversal of ADP-induced shape change caused a decrease in the relative amount of ABP and the 35 000-dalton group. A striking increase in myosin and the 17 000-dalton protein group was observed upon reversal of ADP-induced shape change. A similar picture was observed in the case of ADP-induced reversible aggregation: a profound increase in myosin and the 17 000-dalton group and a significant decrease in ABP and the 35 000-dalton group. Reversal of thrombin-induced activation (either shape change or aggregation) resulted in about 50% reduction in the total protein content of the Triton residue. A similar change in the total protein content was observed

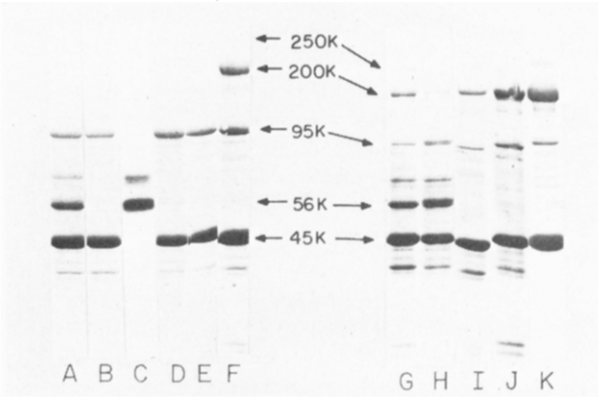

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of Triton residue of different platelet fractions. All lanes except lane C represent Coomassie blue staining. (A) Triton residue of thrombin-induced shape change. (B) Triton residue of thrombin-induced shape change. Hirudin was added together with the Triton solution. (C) Autoradiogram of Triton residue of thrombin-induced shape change where ^{125}I-labeled fibrinogen was added before activation. (D) Triton residue of thrombin-induced shape change. Hirudin was added to the spiny sphere platelets, and when they regained the diskoid shape, Triton solution was added. (E) Triton residue of ADP-induced shape change. (F) Triton residue of ADP-induced shape change. Apyrase was present during activation, and when the platelets regained their diskoid shape, Triton solution was added. (G) Triton residue of thrombin-induced aggregation (25–30% aggregation). (H) Triton residue of thrombin-induced reversible aggregation (25–30% aggregation). Hirudin was added, and when the platelets deaggregated and regained their diskoid shape, Triton solution was added. (I) Triton residue of ADP-induced aggregation (25–30% aggregation). (J) Triton residue of ADP-induced reversible aggregation (25–30%). Apyrase was present during activation, and when the platelets deaggregated and regained their diskoid shape, Triton solution was added. (K) Triton residue of ADP-activated platelets in the presence of EDTA. Thrombin concentration was 0.05–0.1 unit/mL in the shape-change experiments and 0.2–0.4 unit/mL in the aggregation experiments. ADP concentration was 2.5–5 μM (in the shape-change experiments) and 10–40 μM (in the aggregation experiments). Hirudin concentration was 3 times the respective thrombin concentration. Apyrase concentration was 160 μg/mL. Shape change (induced by ADP or thrombin) was usually observed 10–20 s after activation, and the reaction was stopped 0.5–1 min after activation. Aggregation was usualy completed after 2–3 min.

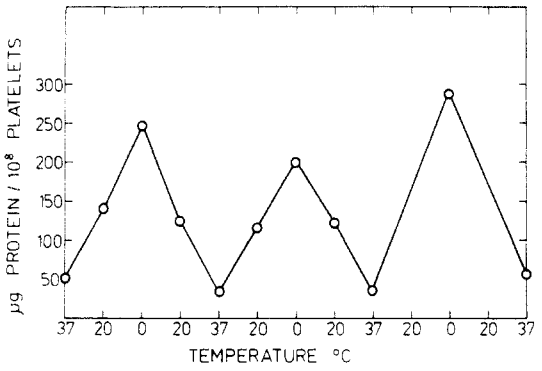

FIGURE 7: Protein content of the Triton residue during temperature cycles. Platelets were incubated for 15 min in each of the shown temperatures before the addition of the Triton solution (see Experimental Procedures). Each point is an average of three experiments. (The amount of protein in whole platelets is 1.6–1.8 mg/10^8 platelets.)

when platelets were aggregated by ADP and the aggregation was reversed. No change was obtained in the case of ADP-induced reversible shape change. When platelets were passed through cycles of temperature (0, 20, and 37 °C), shape change was observed at 0 °C and was always fully reversible when the platelets were rewarmed to 37 °C. During this cycle, the only proteins consistently detected in the Triton residue were actin and myosin in a ratio of (4.5 ± 0.5):1. The total amount of protein in the Triton residue oscillated in parallel to the temperature cycle and in parallel to the actin polymerization but was fully reversible (Figure 7).

Discussion

The results presented in this paper lead to the following conclusions: The size and composition of the platelet cytoskeleton and its interaction with membrane and with cytoplasmic proteins are of a dynamic nature, depending on the degree of activation. The polymerization of actin and its incorporation into the cytoskeleton are reversible to a certain extent.

Shape Change. The cytoskeleton of resting platelets contains some 3% of total platelet protein (calculated from data shown in Figure 7) and is composed of actin and myosin (with traces of actin binding protein and a few other proteins). In some cases, usually when the platelet concentration was relatively high (5×10^8 platelets/mL), the Triton X-100 residue of resting platelets also contained some of the 35 000-dalton group as well as the 95 000-dalton protein. This is probably due to some activation of the platelets during preparation. The cytoskeleton of platelets temperature activated to the shape of spiny spheres (termed by us as shape-changed platelets according to their morphology) is about 5 times bigger in terms of total protein amount but contains the same pattern of proteins (actin and myosin). Moreover, when platelets are transformed through cycles of temperature-induced shape change, the total protein content of the cytoskeleton cycles as well. Thus, the cytoskeleton of resting and temperature-induced shape-changed platelets switches reversibly between two limits: the lower one in resting platelets which contain about 3% of total platelet protein and the upper one in the shape-changed state containing 12–15% of total platelet protein. The proteins constituting this dynamic cytoskeleton throughout the temperature cycle are actin and myosin. The relative amount of each of these proteins in the cytoskeleton remains constant in spite of the reversible assembly and diassembly of the cytoskeletal structure: 82–85% actin and 15–18% myosin. Thus, it is clear that there is only minimal, if any, Triton-resistant interaction between the cytoskeleton of resting or temperature-induced shape-changed platelets with other cytoplasmic and membrane proteins. In addition, there is a rapid polymerization of actin with consequent enlargement of the cytoskeleton as a result of incorporation of actin and myosin into it while keeping the ratio between them constant. Although it is known that low temperature stabilizes G-actin (Condeelis & Taylor, 1977; Gordon et al., 1977; Ishiura & Okada, 1977; Pollard, 1967a), an opposite situation exists here, where cooling results in actin polymerization. This is also contrary to the effect of temperature on microtubules [both in vivo and in vitro (Crawford, 1976)]. This suggests that other cellular factors participate in this phenomenon. Recently, Bennett & Lynch (1980) reported low temperature induced phosphorylation of platelet myosin light chain as well as secretion. Although we did not observe any release upon cooling the platelets, such a phosphorylation of myosin light chain which will increase the interaction of the phosphorylated myosin with actin (Adelstein et al., 1975; Lebowitz & Cooke, 1978) might explain the larger cytoskeletal residue upon cooling. Whether the full reversibility of cytoskeletal proteins observed in the temperature cycles is related to the phosphorylation–dephosphorylation process of myosin or to the integrity of the microtubular structure remains to be studied.

Careful analysis of the state of actin during the early stages of activation by using the DNase inhibition assay clearly indicates that the actin polymerization already reached its maximum with the shape change, no matter whether the activation is performed with ADP or thrombin, with or without fibrinogen. At least part of this polymerized actin is incorporated into the cytoskeleton. When the activation step is reversed and the platelets change their shape back to the diskoid form, there is partial reversibility in the amount of proteins in the cytoskeleton in the case of thrombin but no reversibility at all in the case of ADP.

The decrease in total cytoskeletal proteins in the reversibility of thrombin-induced shape change (compared to the state of the spiny sphere platelets) is not accompanied by any change in protein pattern but is rather parallel to the decrease in actin polymerization (Figure 6). Thus, the reduction in the cytoskeletal size is at the expense of those proteins that constitute the organized structure of the Triton residue and a further depolymerization of F-actin to DNase-available actin. On the other hand, the lack of reversibility in the total protein content and the smaller transformation of F-actin → G-actin (as measured by the DNase assay) in the case of ADP activation are paralleled by a significant increase in the amount of myosin in the Triton residue. The formation of an actomyosin complex might explain these results although we have no explanations as to why the possibility of such a complex is favored in the case of ADP and not thrombin. The decay in the partial reversibility of actin observed during the cycles of temperature-induced activation suggests that the organized structure of actin (with possible weak interaction with other proteins) is in full equilibrium with the soluble F-actin; the latter is itself only in partial equilibrium with the DNase-available monomeric actin. The reason for the inability of actin to reverse completely to its initial state and the decay in reversibility with cumulative cycles is not yet clear. Does it represent an exhausted reversibility mechanism or a nonaccessible compartmentalization of the polymerized actin?

The ionic conditions and actin concentration existing in platelets favor its polymerization; nevertheless, most of the actin in resting platelets is not in the polymerized form but rather in the two monomeric form, partially as a complex with profilin (Harris & Weeds, 1978; Markey et al., 1978). Profilin

inhibits nucleation but would not reverse the already formed polymer (Grumet & Lin, 1980a). During the last few years, numerous proteins interacting and severing F-actin or actin filaments have been described. Among them, one can mention actin depolymerization factors (Bamburg et al., 1980; Harris & Gooch, 1981; Norberg et al., 1979), DNase (Hitchcock et al., 1976; Mannherz et al., 1980), plasticin (Isenberg et al., 1980), a platelet cytochalasin-like protein (Grumet & Lin, 1980b), villin (Craig & Powell, 1980; Glenney et al., 1980), and others. It might be that one or more similar proteins are responsible for modulation of the state of actin in platelets. Thus, because of the decay in reversibility of actin with temperature cycles, 30% of the total actin is polymerized after four to five such cycles although the platelets are morphologically diskoid. This is the maximal degree of actin polymerization obtained by us even with fully aggregated platelets. Therefore, it is clear that morphologically diskoid platelets are not necessarily resting, nonactivated, at least with respect to their state of actin. It was suggested that the diskoid shape of platelets is maintained by the microtubule ring (Crawford, 1976). Our results actually indicate no connection between the state of actin and the morphology of the platelets, and in order to study the possible effect of the microtubule ring on actin polymerization, we used colchicine. Colchicine is known to bind in vitro to tubulin and to induce microtubule disassembly (Dustin, 1978). Therefore, treatment of diskoid platelets with colchicine and disassembly of the peripheral microtubule ring combined by analysis of the state of actin could give us information about the possible effect of the microtubule on actin polymerization. The fact that no change in the state of actin was observed upon treatment of platelets with colchicine, in spite of the shape change, strenghthens our results that actin polymerization is not affected by the integrity of the microtubule ring and that the microtubule disassembly is not a triggering signal for actin polymerization. The normal actin polymerization obtained in colchicine-treated platelets after activation by thrombin or low temperature is another indication for the lack of correlation between microtubule integrity and the state of actin.

The dynamic characteristic of the cytoskeleton structure and its interaction with other cytoplasmic and membrane proteins as discussed exists in principle also during the first stages of aggregation. The process of platelet aggregation is reversible under certain conditions, and we have shown that the same degree of actin polymerization was obtained with shape change or with full aggregation. Platelets aggregated to the extent of 25–30% with ADP or thrombin and induced to reverse their aggregation with apyrase or hirudin, respectively, recovered about 30% and 50% of their polymerized actin, respectively (Figure 3). Thus, the polymerization of actin is maximal with shape change, no further polymerization during aggregation is observed, and the capability of this polymerized actin to partially depolymerize is maintained up to at least 25–30% aggregation (no experiments were carried out beyond 25–30% aggregation due to difficulties in obtaining full morphological reversibility at higher degrees of aggregation). In contrast to observations reported by Carlsson et al. (1979), we could not observe changes in actin polymerization (as compared with resting platelets) with or without Ca^{2+} in the lysis buffer.

However, although the DNase assay gave the same results in shape-changed and in aggregated platelets, different interactions of the cytoskeleton with membrane and other proteins could be observed in these two cases. According to Figure 5, it is clear that the Triton residue of platelets at the very early stages of aggregation (3–5% aggregation, 10-s activation) already shows different patterns from the cytoskeleton of resting platelets and that of shape-changed platelets.

The relative concentration of myosin in the Triton residue was quite variable, but the general tendency was toward a slight decrease of this protein with progress in aggregation (both with ADP and thrombin activation).

It is not yet clear if there is any connection between the changes in the presence of myosin heavy chain and the low molecular weight proteins that constitute the 35 000- and the 17 000-dalton groups on deactivation. The changes in the appearance of ABP upon aggregation and reversible aggregation are in accordance with results describing the role of this protein in the stabilization of actin fibers. The ABP–actin interaction is known to affect the organization of actin networks (Brotschi et al., 1978; Hartwig & Stossel, 1981; Hartwig et al., 1980) and is capable of sol–gel transformation by interaction with other proteins (Stendahl & Stossel, 1980; Yin & Stossel, 1979). Bundles of actin filaments are indeed seen in platelet pseudopods, and a sol–gel transformation would favor the consolidation of platelet–platelet interaction via the pseudopods in a similar mechanism to that proposed for macrophage phagocytosis (Stossel & Hartwig, 1976b). The reduction in the amount of ABP after recovery of the diskoid shape favors the role of this protein as a participant in the stabilization of actin bundles during aggregation. These results confirm those of Nachmias et al. (1979) in which lidocain-induced pseudopod suppression is accompanied by reduction in the amount of actin binding protein in the Triton residue.

The 95 000-dalton protein is a membrane protein (Rotman et al., 1982), barely detected in the Triton residue of resting platelets but appearing after activation with a tendency to increase in its concentration in the cytoskeleton during the progress of aggregation. It is not yet clear to us whether this protein is related to the process of activation or whether it is with the aggregation. This assumption is based on the fact that reversal of aggregation and recovery of the diskoid morphology did not affect the relative concentration of this protein in the cytoskeleton.

Effects of EDTA. Platelets are known to have two myosin-associated ATPase activities, an actin-activated Mg^{2+} and an activated K^{+}(EDTA) (Adelstein et al., 1975). The latter has been shown in other systems to be inhibited by actin, and in addition, actin–myosin binding is enhanced in the presence of EDTA, i.e., in the absence of divalent cations (Cooke & Franks, 1980; Krisanda & Murphy, 1980). This enhanced binding might explain the results obtained with EDTA, whenever it was added to the lysate (Figure 6, lane K). A similar effect was obtained by Phillips et al. (1980) when using activating or aggregating buffer. This enhanced actomyosin binding was also accompanied by an increase in the cytoskeletal protein content and a decrease in the relative amount of ABP and the 95 000- and 35 000-dalton regions as compared with shape change or the first steps of aggregation.

In light of the results obtained in this study, we think that the different rate of actin polymerization observed with ADP in platelet-rich plasma as reported previously (Pribluda et al., 1981) may be a consequence of the interaction of actin with other cytoskeletal proteins and the reported depolymerizing factor in plasma (Harris & Gooch, 1981; Norberg et al., 1979). As shown here, there is an aggregation-dependent increase in the amount of ABP, which by its interaction with actin may protect the latter from depolarizing. This effect is of course more evident in the later stages of aggregation than in the early stages or in the presence of EDTA. Another actin-depolymerizing protein, DNase I (in the absence of ATP), has been shown to interact differently with F-actin or

actomyosin (Hitchcock et al., 1976).

Added in Proof

Our recent results identify the 95 000-dalton protein as α-actinin.

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