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# ASSOCIATION OF ACTIN WITH THE PLATELET MEMBRANE

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**Human platelet membrane-actin associations were studied by means of differential extraction of purified membranes and low-shear viscometry of membrane-F-actin mixtures. As indicated by resistance to extraction with 0.6 M potassium iodide, a significant amount of platelet actin appears to be tightly associated with the membrane. When tested by falling-ball viscometry, both whole and KI-extracted membranes increased the low-shear viscosity of preformed rabbit skeletal muscle F-actin at physiologically reasonable pH and ionic conditions. This membrane-associated actin gelation activity was dependent upon low free calcium concentration ($10^{-8}$–$10^{-7}$ M). The results are consistent with specific associations between actin and platelet membranes and may be relevant to membrane-cytoskeletal interactions believed to occur in the intact cell.**

## Introduction

In blood platelets, as in other non-muscle contractile cells, interactions between membrane and cytoskeletal proteins are believed to have a major functional role [1,2]. In resting platelets, cytoskeletal proteins are probably in a disassembled state, as indicated by the amorphous appearance of the cytoplasm on electron microscopy and by the scant precipitate of cytoskeletal proteins formed by Triton X-100 extraction [3–5]. Upon platelet activation by contact with surfaces or with soluble aggregating agents, there is a rapid reorganization of cytoskeletal proteins and change in cell shape and membrane properties. Actin polymerization occurs, and the platelet cytoplasm becomes filled with an extensive network of cytoskeletal proteins, primarily actin-containing microfilaments [2,6]. It is presumed that platelet cytoskeletal proteins can associate with the inner surface of the membrane, thereby accounting for contractile properties of the cell, such as filopodia extension and clot retraction. This hypothesis is supported by ultrastructural studies showing submembrane actin-containing microfilaments apparently inserting into the inner membrane surface [7]. Also, platelet membranes isolated by all current methods contain significant amounts of actin [8–10]. There is additional evidence for platelet membrane-cytoskeletal associations from recent studies using isolation of cytoskeletal proteins by Triton X-100 extraction. By this method, integral membrane glycoproteins IIb and III appear to be associated with the cytoskeleton of thrombin-aggregated or concanavalin A-treated platelets

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Abbreviations: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

[11,12]. The specific protein interactions responsible for platelet membrane-cytoskeletal associations are not yet known.

To examine platelet membrane-actin associations, I have used differential extraction of membrane proteins, followed by low-shear viscometry of mixtures of membranes and preformed rabbit skeletal muscle F-actin. As rigorously demonstrated for red cells, differential extraction of membrane-associated proteins can provide valuable information on the nature of membrane-cytoskeletal interactions [13]. Also, as shown for red cell, *Dictyostelium discoideum*, and adrenal chromaffin granule membranes, low-shear viscometry appears to be a valid indication of the capacity of isolated membranes to form new associations with F-actin [14–17]. This paper presents an initial characterization of platelet membrane-actin associations by these two methods.

## Materials and Methods

*Materials*

24-h-old platelet concentrates in citrate/phosphate/dextrose anticoagulant were obtained from the American National Red Cross Blood Program, Northeast Region, Boston. Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt [18], as modified by Eisenberg and Kielley [19], and dialyzed for 3 days at 4°C against frequent changes of G-actin buffer (2 mM Tris-HCl/0.2 mM $CaCl_2$/0.2 mM ATP/0.5 mM dithiothreitol, pH 8.0). The G-actin was centrifuged at $100\,000 \times g$ for 90 min, adjusted to 4 mg/ml based on $\varepsilon_{290}$ of 0.63 $ml \cdot mg^{-1} \cdot cm^{-1}$ [20], and polymerized by addition of KCl and $MgCl_2$ to 100 mM and 2 mM, respectively.

*Preparation and characterization of platelet membranes*

Platelet concentrates were brought to pH 6.5 by addition of 2.2% trisodium citrate, 1.2% citric acid. The platelet suspension was centrifuged repeatedly at $2000 \times g$ (20 s each time) to remove all red cells, and platelets were collected by centrifugation at $2000 \times g$ for 20 min. Platelet membranes were prepared by washing, sonication, and centrifugation over a one-step 30% sucrose gradient, according to 'Method 2' of George [21]. Membrane pellets were stored at −20°C until use.

Lactate dehydrogenase specific activity was used as a measurement of contamination of membranes by cytoplasmic constituents [22]. Membranes or washed intact platelets were sonicated in 0.1 M potassium phosphate, pH 7.4, and mixed with one-ninth volume of 10% Triton X-100/0.1 M potassium phosphate, pH 7.4. After centrifugation at $39\,000 \times g$ for 20 min, lactate dehydrogenase activity in the supernatant solution was determined by the method of Wroblewski and LaDue [23]. The specific activity of membrane preparations was consistently low (1.4%, 1.1%, and 2.2% of the specific activity of intact platelets in three experiments).

At present there is no method for preparation of large amounts of homogeneous platelet surface membranes of well-defined orientation, analogous to inside- or right-side-out red cell membranes. However, in an attempt to estimate the fraction of purified platelet membranes in the inside-out orientation, binding of the membranes to agarose concanavalin A was measured. A membrane sample was washed twice with 140 mM NaCl/10 mM Tris-HCl, pH 7.4, and suspended in this buffer to a protein concentration of 1.3 mg/ml, as measured by Bio-Rad dye-binding assay. A 0.8 ml portion of membrane suspension was applied to a 2.8 ml column of agarose-concanavalin A (Pharmacia) previously washed with buffer. After 15 min at room temperature, elution was resumed, and cloudy fractions were collected. Assay of eluted material showed that 27% of applied protein passed through the column. Similar analysis of washed intact platelets showed that only 1.2% of platelets in a highly concentrated suspension ($1.1 \cdot 10^7$ cells/$\mu$l) passed through the column.

*Extraction of membranes*

In all steps, membranes were suspended by brief sonication. All materials were kept at 4°C. Membranes were first washed once by suspension in 140 mM NaCl/10 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol, pH 7.4 (buffer 1), and centrifugation at $200\,000 \times g$ for 35 min. The washed membranes were then extracted with the following solutions:

*KI solution.* Membranes were suspended in about 50 vol. of 0.6 M KI/10 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol, pH

7.4, for each volume of pellet. The suspension was constantly stirred for 1 h and centrifuged at 200 000 × *g* for 1 h. The pellet was washed once with buffer 1.

*Alkaline solutions.* Membranes were suspended in about 5 vol. of buffer 1, and 0.1 ml aliquots were distributed into glass tubes. Portions of water and 0.1 M NaOH were added to produce constant final volumes of 0.4 ml and pH values of 10.4–12.6, as measured by a pH meter calibrated with standard buffers (pHydrion) at pH 11 and 12. The suspensions were mixed several times over a 10 min or 60 min period and centrifuged at 200 000 × *g* for 1 h over 0.15 ml cushions of 2% sucrose in buffer 1.

*Triton X-100 solution.* Membranes were suspended in about 10 vol. of a buffer containing 20 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol/0.4 mM PMSF, pH 6.8. The suspension was mixed with an equal volume of 2% Triton X-100 in the buffer. After 15 min, the precipitate was collected by centrifugation at 39 000 × *g* for 20 min. Triton X-100 extraction of intact platelets was done in a similar manner, except that platelets washed three times with buffer 1 were used.

*SDS-polyacrylamide gel electrophoresis*

Membrane pellets were dissolved in a gel sample solution containing 3% SDS/0.25 M sucrose/50 mM Tris-HCl/2 mM EDTA/20 mM dithiothreitol/0.005% pyronin Y, pH 7.4. Supernatant solutions from KI extractions were dialyzed overnight against 50 mM Tris-HCl/2 mM EDTA, pH 7.4, and concentrated against sodium carboxymethyl cellulose (Aquacide II-A, Calbiochem-Behring). One-fourth volume of a five-fold concentrated gel sample solution was then added. Supernatant solutions from alkaline extractions were immediately mixed with concentrated gel sample solution.

Samples were analyzed on 3 mm thick 7.0% or 7.5% polyacrylamide slab gels by the method of Laemmli [24]. Gels were stained with Coomassie blue or with periodic acid Schiff reagent prepared according to Clemetson et al. [25]. Some gels were scanned using a Helena Laboratories densitometer with a 550 nm filter, and amounts of protein in specified bands were calculated from areas under the tracings.

*Low-shear viscometry*

Platelet membranes were washed twice with a buffer containing 10 mM Tris-HCl/1 mM dithiothreitol/0.1 mM PMSF, pH 7.4. The membranes were then suspended in 1 ml of this buffer/g wet weight of pellet. Protein concentration was measured by Bio-Rad dye-binding assay. In some cases, membrane organic phosphorus concentration in the suspension was measured by chloroform/methanol extraction [26] and phosphorus assay [27], with dipalmitoylphosphatidylcholine (Sigma) used as a standard.

Low-shear viscosity of membrane-F-actin mixtures was determined by the falling-ball method of MacLean-Fletcher and Pollard [28]. In a standard assay, membrane suspension, stock solutions, and F-actin were mixed to produce the following final concentrations in a volume of 60 μl: 0–6 mg/ml membrane protein/0.8 mg · ml$^{-1}$ F-actin/40 mM Pipes/20 mM KCl/0.23 mM $CaCl_2$/5 mM EGTA/1.2 mM $MgCl_2$/0.04 mM ATP/6 mM Tris/0.7 mM dithiothreitol/0.06 mM PMSF, pH 7.0. Where indicated, pH and concentrations of $CaCl_2$ and EGTA were varied. Each mixture was thoroughly vortexed and drawn into a 100 μl disposable micropipette. After incubation horizontally for 1 h at 30°C, the falling time of a 0.64 mm diameter stainless steel ball (Micro Ball Co., Peterborough, New Hampshire) over 2 cm was measured at an inclination of 20–80°. Apparent low-shear viscosity in centipoise was calculated from calibration curves prepared using 20–100% (w/w) glycerol standard solutions. Each assay was done in duplicate or triplicate, and the results were averaged. Free calcium concentrations were calculated from total concentrations of $CaCl_2$, $MgCl_2$, and EGTA, using the method of Portzehl et al. [29] and published apparent association constants at various pH values [30].

*Sucrose density gradient centrifugation*

Membranes were washed twice with 140 mM NaCl/10 mM Tris-HCl/0.1 mM dithiothreitol, pH 7.4 (buffer 2), and 0.5 ml portions of membrane suspension were placed over 5 ml linear gradients containing 10–50% sucrose in buffer 2. The gradients were centrifuged at 63 500 × *g* for 18 h. Protein content of the fractions was determined by dye-binding assay, and actin content was de-

termined by gel electrophoresis using skeletal muscle actin standards.

## Results

*Membrane extraction with KI and alkaline solutions*

First, platelet membrane-cytoskeletal protein associations were examined by extraction under conditions favoring actin depolymerization and removal of peripheral membrane proteins. The residual membrane material after extraction was analyzed by SDS-polyacrylamide gel electrophoresis. Platelet proteins were identified by apparent molecular weight, comparison to standards, and reference to published data [31,32]. Fig. 1 shows the composition of membranes before and after treatment with KI-containing and alkaline solutions. In unextracted membranes, proteins of molecular weight 260 000, 200 000, and 43 000 represent actin binding protein, myosin heavy chain, and actin, respectively. Extraction with buffered 0.6 M KI, an actin-depolymerizing solution, removed most of the membrane-associated myosin and a smaller amount of actin. Further extraction with pH 10.4 or pH 10.7 solutions removed nearly all of the actin and lesser amounts of actin binding protein and unidentified proteins. By densitometric scanning, 100% of myosin, 95% of actin, and 37% of actin binding protein were removed at pH 10.7 in this experiment. The extraction pattern of platelet membrane-associated proteins was further demonstrated by analysis of supernatant solutions after extraction (Fig. 1). As shown by the first pair of gels, a KI extract of membranes contained considerable amounts of myosin and actin, with lesser amounts of actin binding protein and 120 000, 104 000, 74 000, and 55 000 dalton proteins. The remaining gels show the results of vigorous alkaline treatment of the KI-extracted membranes. Treatment with a pH 11.2 solution for 10 min removed additional actin, actin binding protein, 120-kDa protein, and minor constituents. At pH 11.9, all but a trace of the actin was removed, and the residual membrane material contained mainly proteins of molecular weight 120 000 and 100 000, with lesser amounts of actin binding protein and a 130-kDa protein. At pH 12.6, there was evidence of protein degradation, with a reduction

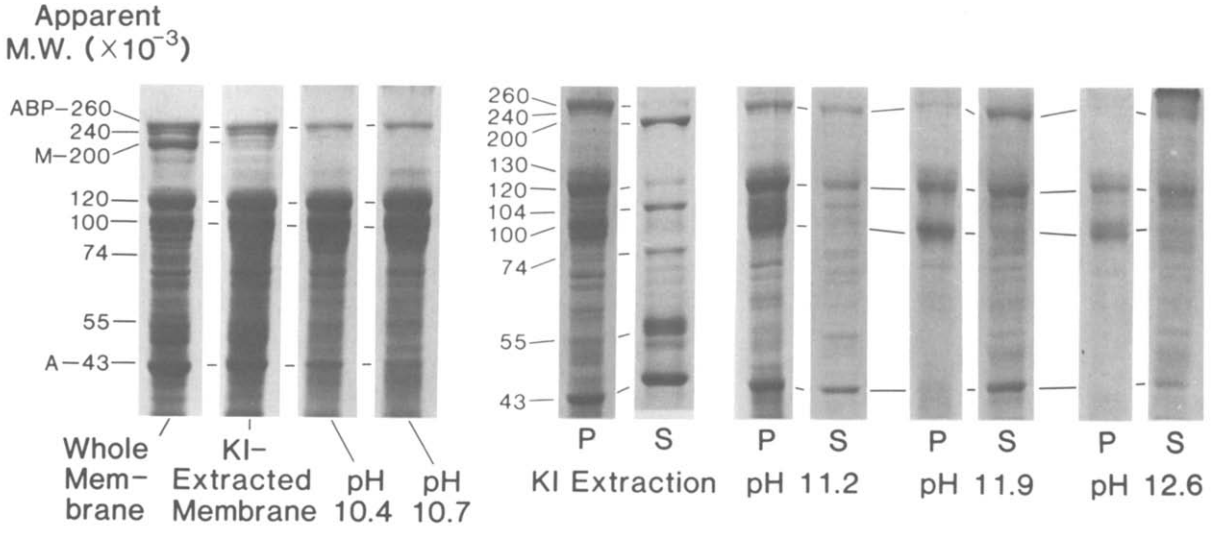


Fig. 1. Extraction of platelet membranes with KI and alkaline solutions. Whole membranes were extracted with buffered 0.6 M KI for 1 h, washed once, and extracted with alkaline solutions at the indicated pH, as described in Materials and Methods. Alkaline extraction was for 1 h (pH 10.4 and 10.7) or 10 min (pH 11.2, 11.9 and 12.6). Abbreviations: ABP, actin-binding protein; M, myosin heavy chain; A, actin; P, pellet; and S, supernatant solution. Each gel of supernatant solution represents 80% of starting membrane material. After correction for this factor, extraction at pH 11.2 and 11.9 was quantitative, as estimated by densitometry (data not shown).

TABLE I

EXTRACTION OF PLATELET MEMBRANES WITH KI AND ALKALINE SOLUTIONS

Membranes were extracted with buffered 0.6 M KI and then with alkaline solutions, as described in Fig. 1. Aliquots representing equal amounts of starting membrane material were analyzed by gel electrophoresis. Coomassie blue stained patterns were scanned, and the amounts of membrane-associated proteins were estimated from areas under the curves and expressed as fractions of the amounts in nonextracted membranes.

| Extraction solution | Fraction of remaining membrane-associated proteins (%) | | | |
|---|---|---|---|---|
| | Actin-binding protein | Myosin | 104-kDa protein | Actin |
| Buffered 0.6 M KI | 81 | 1 | 7 | 40 |
| Alkaline solutions | | | | |
| pH 11.2 | 72 | 0 | 3 | 26 |
| pH 11.9 | 17 | 0 | 0 | 1 |
| pH 12.6 [a] | 9 | 0 | 0 | 0 |

[a] Degradation of extracted proteins noted.

in intensity of bands in the supernatant fraction and the appearance of Coomassie blue staining material at the top of the gel.

Table I shows the results of quantification of the amounts of cytoskeletal proteins remaining after extraction with KI-containing, followed by alkaline solutions. These data, taken from the experiment shown in Fig. 1, indicate the relative resistance of actin binding protein to extraction, with about 17% remaining membrane-associated after extraction of 99% of the actin by exposure to a pH 11.9 solution. About 9% of the actin binding protein remained on the membrane even after extraction at pH 12.6, with protein degradation grossly visible in the gel pattern. The identity of the residual 260-kDa band as actin binding protein was confirmed by use of goat antiserum against guinea pig vas deferens actin binding protein (anti-filamin, gift of Dr. M.C. Willingham, National Cancer Institute, U.S.A.) using electroblotting on nitrocellulose sheets and immunoperoxidase staining (data not shown). It is not certain whether the residual actin-binding protein after alkaline extraction is specifically associated with integral membrane glycoproteins or is simply denatured or nonspecifically adherent to membrane lipids. The data also confirm that most of the 104-kDa protein, discussed below, is removed by KI extraction.

From studies of other cell types, the residual

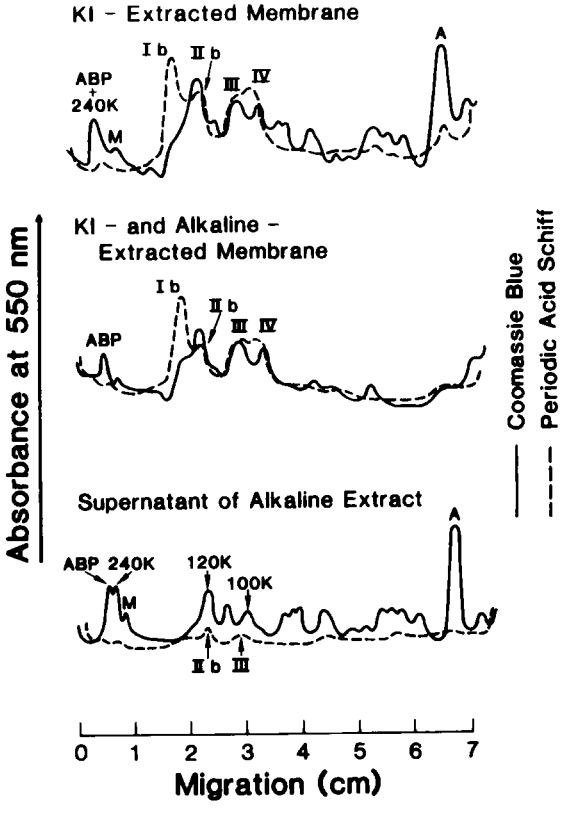


Fig. 2. Glycoprotein composition of extracted membranes. Membranes were extracted with buffered 0.6 M KI, followed by a pH 11.8 solution for 10 min. Material was analyzed by gel electrophoresis with Coomassie blue and periodic acid Schiff staining. Abbreviations are as in Fig. 1. Each tracing represents an equivalent amount of starting membrane material.

proteins after alkaline extraction of platelet membranes are expected to be primarily integral membrane glycoproteins. These glycoproteins were demonstrated by periodic acid Schiff staining of gels of alkaline-extracted material, followed by densitometric scanning. The glycoproteins were identified by apparent molecular weight and labeled according to the nomenclature of Phillips and Agin [31]. Fig. 2 shows the Coomassie blue and periodic acid Schiff staining proteins of KI-extracted membranes before and after treatment with a pH 11.8 solution that removed most of the actin. A comparison of the gel patterns shows that the residual 130-, 120- and 100-kDa Coomassie blue staining proteins after alkaline extraction are probably glycoproteins Ib, IIb, and III, respectively. Identical electrophoretic mobilities of the Coomassie blue and periodic acid Schiff staining proteins was confirmed by marking of glycoprotein bands with India ink, followed by staining of the same gel with Coomassie blue (data not shown). Glycoprotein IV did not correspond to a definite Coomassie blue band, presumably because of poor staining of this glycoprotein [31]. The gel pattern of the supernatant solution shows that only small amounts of glycoproteins IIb and III were removed by alkaline extraction. The supernatant solution also contained many other extractable proteins, as described previously, including 120- and 100-kDa proteins that have electrophoretic mobilities quite similar to those of glycoproteins IIb and III.

*Membrane extraction with Triton X-100*

In Fig. 3, the gel electrophoretic patterns of whole platelets and purified membranes is shown, with the corresponding patterns for the insoluble material after Triton extraction. The Triton precipitate of whole platelets has been well characterized and is known to contain actin, myosin, actin binding protein, and a 100–105-kDa protein [4,5,11,33]. Recently, this last protein has been identified as platelet α-actinin [34]. The Triton precipitate from membranes contained mainly actin and myosin, with small amounts of actin binding protein, the 104 kDa protein, and minor unidentified constituents. The results confirm the presence of cytoskeletal proteins in the membrane preparation and show that the 104 kDa protein is

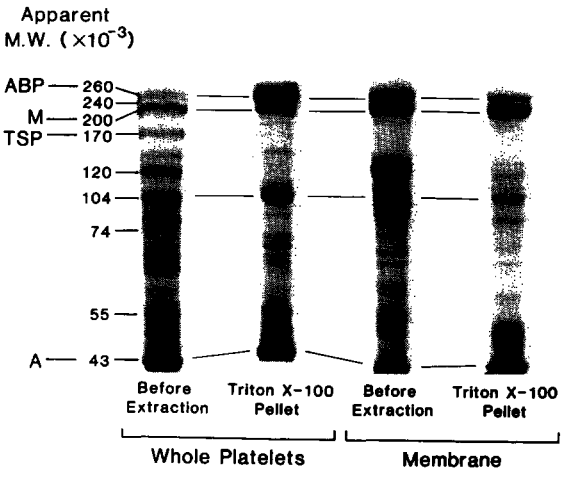


Fig. 3. Triton X-100 extraction of whole platelets and purified membranes. Materials were extracted with buffered 1% Triton X-100, as described in Materials and Methods, and the insoluble material was analyzed. Gel patterns of Triton-extracted material represent three times the starting whole platelet or membrane material. Abbreviations are as in Fig. 1, plus TSP, thrombospondin.

platelet α-actinin. Compositions of the membrane Triton precipitate (Fig. 3) and the supernatant solution after KI extraction (Fig. 1) were similar, again indicating that the membrane-associated cytoskeletal proteins are present as extractable (peripheral) proteins. The significant amounts of myosin in the Triton precipitates of both whole platelets and membranes probably indicates partial activation of platelets in concentrates used in this work, especially after washing and disruption for preparation of membranes. Previous studies have shown that there is only a small amount of myosin in Triton precipitates of whole, non-activated platelets, with a significant increase in myosin content after activation [4,5].

Triton X-100 treatment of KI-extracted membranes also yielded a precipitate containing smaller amounts of cytoskeletal proteins. Triton treatment of alkaline-extracted membranes yielded only a trace amount of precipitate, consistent with absence of nearly all cytoskeletal proteins from the alkaline-extracted membrane (data not shown).

*Extraction with other solutions*

Platelet membranes were also extracted with alkaline solutions without prior KI extraction. Un-

der these conditions, extraction of most proteins, including myosin and other cytoskeletal proteins, was incomplete in a pH range not associated with evidence of protein degradation (data not shown). Other extraction conditions included actin-depolymerizing solutions similar to those used by Luna et al. [15] in studies of *D. discoideum* membranes. The following solutions were tried: (a) 0.1 mM sodium phosphate/0.1 mM EDTA/0.1 mM dithiothreitol/0.1 mM PMSF, pH 8.0, (b) 0.5 mM Tris-HCl/1 mM $MgCl_2$/1 mM ATP/0.1 mM dithiothreitol/0.1 mM PMSF, pH 7.0, (c) 2 mM Tris-HCl/1 mM ATP/0.5 mM dithiothreitol/0.2 mM $CaCl_2$, pH 8.0, and (d) 1 M NaCl/3.5 M urea/50 mM glycine/10 mM EDTA/0.1 mM dithiothreitol, pH 7.2. Extraction of platelet membranes by overnight dialysis against the first three solutions removed variable amounts of actin and myosin, with poor reproducibility compared to KI extraction. Treatment of membranes for 1 h with the NaCl/urea solution yielded an extraction pattern closely resembling that obtained with a 0.6 M KI solution.

*Sucrose gradient sedimentation of membranes*

Since myosin can be fully removed from the membrane by KI extraction, it is unlikely that the apparent membrane association of actin is due simply to trapping within sealed vesicles. Also, the resistance of a significant fraction of actin to extraction under these strongly depolymerizing conditions suggests a tight association with the membrane, rather than nonspecific adherence or cosedimentation of F-actin. To provide additional evidence that membrane-associated cytoskeletal proteins are not simply cosedimented with membranes prepared by the sonication method, membranes were analyzed on sucrose gradients. As shown in Fig. 4, platelet membranes formed a band near the middle of the 10–50% sucrose gradient. There was a close correspondence between total protein and actin content of the fractions from the gradient. In contrast, purified skeletal muscle F-actin centrifuged in parallel with membrane samples was almost completely sedimented to the bottom of the tube, forming a visible pellet (data not shown). The wide separation between membrane-associated actin and purified F-actin indicates that cosedimentation cannot account for

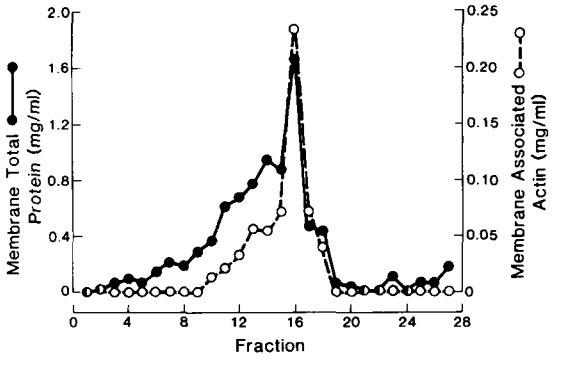


Fig. 4. Analysis of platelet membrane-associated actin by sucrose density gradient centrifugation. After centrifugation of membranes on a 10–50% sucrose gradient, total membrane protein and actin in the fractions were determined.

actin present in the membrane preparations.

Extracted membranes were also analyzed on sucrose gradients. The actin in KI-extracted membranes also cosedimented with the bulk of membrane protein. The gel electrophoretic pattern of whole or extracted membranes was not altered by fractionation on the sucrose gradient, indicating no significant loss of actin binding protein, myosin, or 104-kDa protein (data not shown).

*Low-shear viscometry of membrane-actin mixtures*

The F-actin gelation activity of platelet membranes was tested by low-shear, falling-ball viscometry of mixtures of membranes and preformed rabbit skeletal muscle F-actin. First, membranes were tested at pH 7.0 and low free calcium concentration ($1.05 \cdot 10^{-8}$ M). Fig. 5 shows that whole or KI-extracted membranes caused an increase in viscosity and, at high membrane concentrations, gelation of F-actin under these conditions. In six such tests, there was no decrease in gelation activity after KI extraction. In four of these six tests, the gelation activity of membranes appeared to be slightly greater after KI extraction, as in the experiment shown in Fig. 5. However, the semiquantitative nature of the falling-ball viscometric assay does not allow a conclusion to be drawn from these small differences [15]. Also, as shown in Fig. 5, alkaline-extracted membranes lacked gelation activity at all membrane concentrations tested. Attempts to demonstrate gelation activity in neutralized, concentrated super-

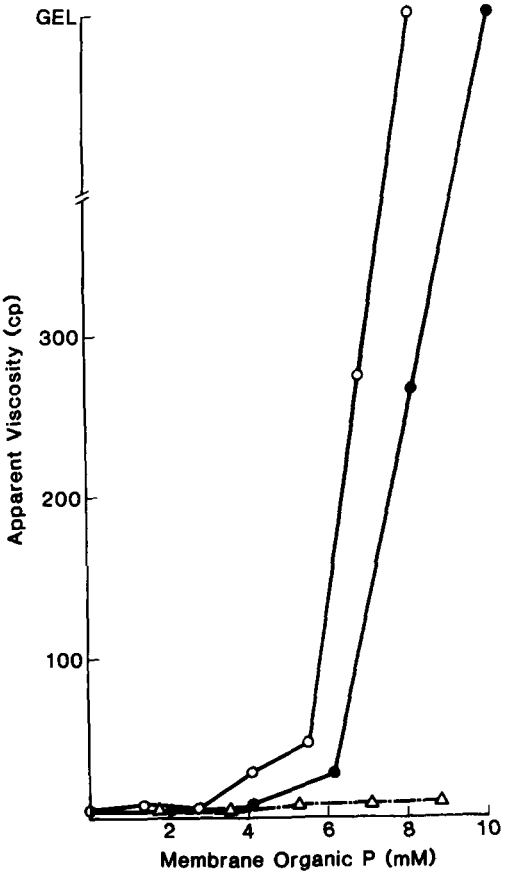


Fig. 5. Falling-ball viscometry of platelet membrane-F-actin mixtures. Whole or extracted membranes were mixed with 0.8 mg/ml rabbit skeletal muscle F-actin under the standard conditions described in Materials and Methods. After 1 h at 30°C, apparent low-shear viscosity was measured. The alkaline-extracted membrane sample was prepared by treatment of a KI-extracted sample for 10 min at pH 11.5. In order to compare the gelation activity of equivalent amounts of membrane material, concentration of membranes in the assays is expressed as organic phosphorus. The apparent viscosities of membrane suspensions without F-actin were less than 3 cp at all concentrations (data not shown). O———O, KI-extracted; ●———●, whole; △— · —△, alkaline-extracted.

natant solutions after alkaline extraction at various pH values were not successful (data not shown).

For additional evidence that a protein is responsible for membrane-associated actin gelation activity, heat- or trypsin-treated membranes were tested. Membranes suspended in 10 mM Tris-HCl/1 mM dithiothreitol/0.1 mM PMSF, pH 7.4, were heated for 5 min at 100°C. Also, membranes were suspended in 140 mM NaCl/10 mM sodium phosphate, pH 7.4, containing 0.1 mg/ml bovine pancreatic trypsin (Sigma, Type III) and left for 30 min at 37°C, Soybean trypsin inhibitor (Sigma, Type I-S) was then added to a final concentration of 0.2 mg/ml, and the membranes were washed twice. The heat or trypsin treatment abolished gelation activity of whole or KI-extracted membranes (data not shown).

*Dependence of membrane-associated gelation activity on ionic conditions*

An increase in cytoplasmic calcium concentration and pH is believed to occur during platelet activation [35,36]. Also, Fowler and Pollard [16]

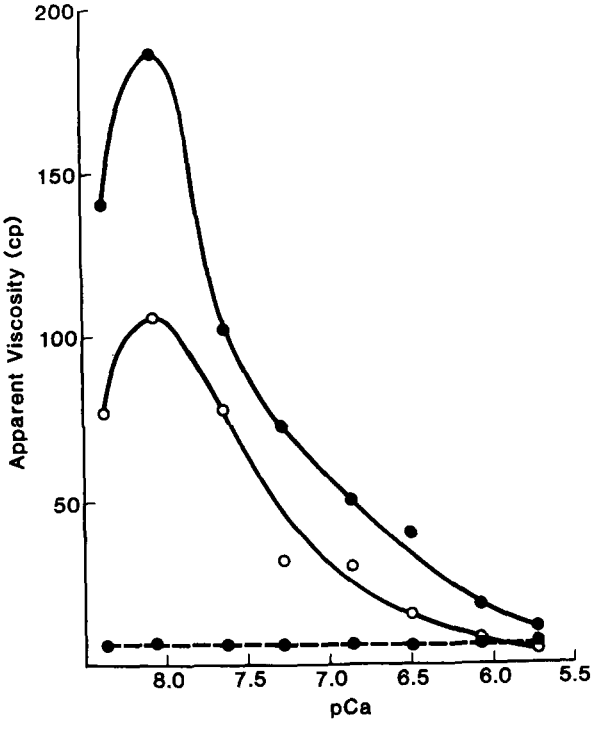


Fig. 6. Calcium dependence of membrane-associated actin gelation activity. The ratio of total $CaCl_2$ to total EGTA added was varied to produce the indicated free calcium concentration, expressed as pCa. Other conditions were as in Fig. 5, except that 0.1 mg/ml leupeptin (Boehringer Mannheim) was included as an inhibitor of the calcium-activated platelet protease [38]. The final concentration of membrane protein was constant at 3.4 mg/ml and 2.4 mg/ml for whole and KI-extracted membranes, respectively. ●———●, whole; O———O, KI-extracted; ●- - -●, F-actin alone.

found that the actin gelation activity of adrenal chromaffin granule membranes is markedly decreased by an increase in free calcium concentration from $10^{-8}$ to $10^{-6}$ M, suggesting a regulatory role of calcium in membrane-actin associations. These authors did not find a significant effect of pH in the physiological range.

To determine the calcium dependence of platelet membrane-associated gelation activity, the ratio of total calcium to EGTA in the membrane F-actin mixture was varied. As shown in Fig. 6, the gelation activity of both whole and KI-extracted membranes was optimal at about $10^{-8}$ M free calcium. Gelation activity was abolished by an increase in free calcium concentration to about $10^{-6}$ M. As indicated, the apparent viscosity of F-actin alone was low at these various calcium concentrations. These results may indicate that a local increase in platelet cytoplasmic calcium loosens membrane-cytoskeletal associations at that site. As for chromaffin granule membranes [16], the mechanism of calcium sensitivity of platelet membrane-associated gelation activity is speculative. It is possible that the responsible protein requires low calcium conditions to associate with F-actin. Also, platelets contain gelsolin, a calcium-sensitive F-actin severing protein [37]. Presence of small amounts of gelsolin in the platelet membrane preparations as a specific membrane-associated protein or as a contaminant could probably account for the observed dependence on calcium concentration. Calcium-activated proteolysis of the membrane-associated gelation factor is unlikely, since leupeptin was included in the assays as a specific inhibitor of the calcium-activated platelet protease [38], as described in Fig. 6. Also, SDS-polyacrylamide gel analysis of membranes before and after incubation under these conditions revealed no change in pattern, excluding gross proteolysis (data not shown).

Fig. 7 shows the effect of pH on platelet membrane-associated gelation activity, with the free calcium concentration kept constant by appropriate adjustment of the total calcium-EGTA ratio at each pH tested. There was an increase in apparent viscosity of whole membrane-actin mixtures with increasing pH. However, this effect was

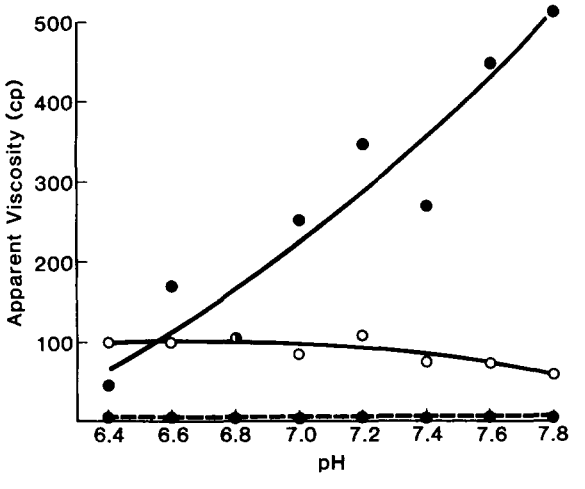


Fig. 7. Dependence of membrane-associated actin gelation activity on pH. Pipes buffer stock solutions of various pH values were used to produce the indicated pH in the membrane-F-actin mixture. The ratio of total $CaCl_2$ to total EGTA added was adjusted to yield a constant final free calcium concentration of $1.05 \cdot 10^{-8}$ M. Other conditions were as in Fig. 5. The final concentration of membrane protein was constant at 3.6 mg/ml and 4.5 mg/ml for whole and KI-extracted membranes, respectively. For definition of symbols see Fig. 6.

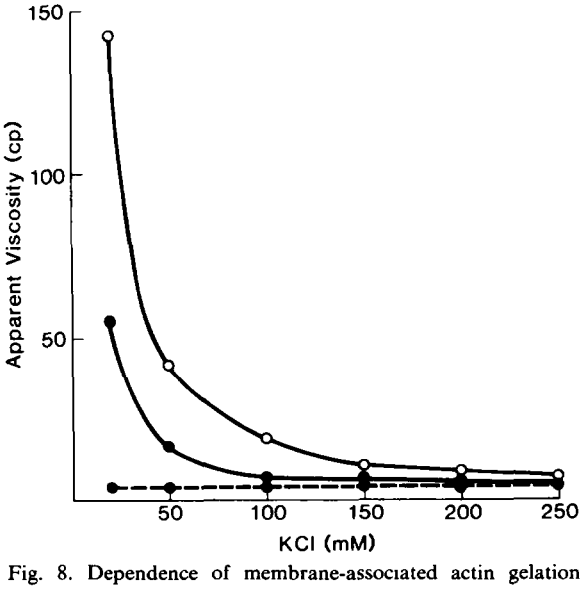


Fig. 8. Dependence of membrane-associated actin gelation activity on KCl concentration. The final concentration of KCl in the membrane-F-actin mixture was varied over the indicated range. The lowest concentration of KCl was 20 mM, since this concentration is contributed by the addition of F-actin polymerized with 100 mM KCl and 2 mM $MgCl_2$. Other conditions were as in Fig. 5. The final concentration of membrane protein was constant at 4.5 mg/ml for both whole and KI-extracted membranes. For definition of symbols see Fig. 6.

small in the range of pH change measured in intact platelets, in which the pH probably increases from about 7.0 to 7.3 during thrombin activation [36]. After KI extraction, there was little effect of pH on the apparent viscosity of the membrane-actin mixtures.

The effect of ionic strength on platelet membrane-associated gelation activity was tested by addition of KCl or potassium glutamate to the membrane-F-actin mixtures at low calcium concentrations. A calculation based on the dissociation constants of glutamic acid demonstrates that equimolar concentrations of these salts produce very similar ionic strengths at neutral pH (data not shown). As shown in Fig. 8, an increase in KCl concentration caused a marked decrease in apparent viscosity with both whole and KI-extracted membranes. As shown in Fig. 9, the effect of high potassium glutamate concentrations on apparent viscosity was much less pronounced. Also, there was an apparent optimal glutamate concentration of about 100 mM in the experiment with whole membranes. The curves depicted in Figs. 8 and 9 were reproducible on repeated testing (data not shown). As shown in these figures, the apparent viscosity of F-actin alone was not significantly influenced by concentration of KCl or potassium glutamate. The results are generally similar to those of Fowler and Pollard [17], who found that the gelation activity of chromaffin granule membranes was decreased by high KCl concentrations but scarcely affected by high concentrations of potassium glutamate. Since the intracellular concentration of chloride is low in most cells, these findings may be an important indication that the platelet membrane F-actin interactions demonstrated by the falling-ball viscometric method are possible in the environment of the intact cell where anions other than chloride predominate.

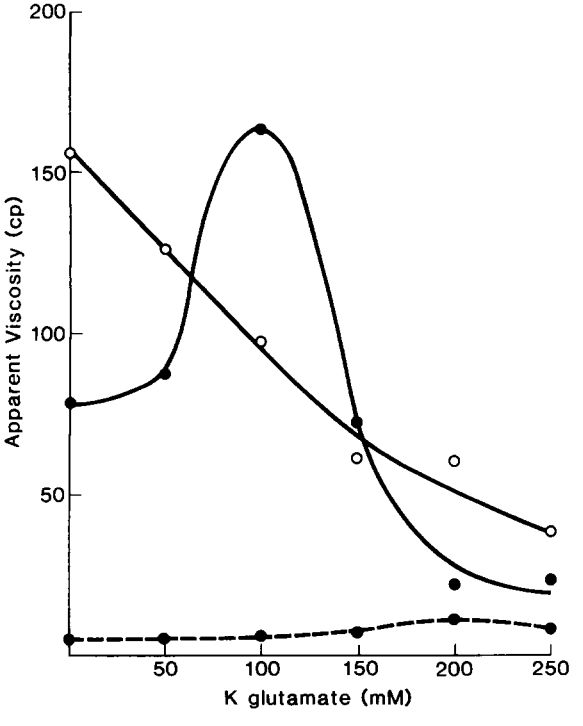


Fig. 9. Dependence of membrane-associated actin gelation activity on potassium glutamate concentration. The final concentration of potassium glutamate in the membrane-F-actin mixture was varied over the indicated range. Other conditions were as in Fig. 5. The final concentration of membrane protein was constant at 4.2 mg/ml and 3.6 mg/ml for whole and KI-extracted membranes, respectively. For definition of symbols see Fig. 6.

## Discussion

The preceding results show that a significant fraction of platelet actin is tightly associated with the membrane. Also, as determined by low-shear viscometry, both whole and KI-extracted membranes can interact with skeletal muscle F-actin in a calcium-dependent manner and under physiologically reasonable pH and ionic conditions. It is reasonable to assume that similar membrane-actin associations may occur in the intact cell.

The proteins responsible for membrane-actin associations have not been definitely identified in the platelet or in any other non-muscle contractile cell. However, the results presented here allow certain tentative conclusions concerning the possible role of several platelet proteins known to bind actin. By analogy with the association of actin filaments with $\alpha$-actinin in skeletal muscle, a role for platelet $\alpha$-actinin in membrane-cytoskeletal attachment has been suggested. Since platelet $\alpha$-actinin associates with F-actin in a calcium-dependent manner [34], this protein could perhaps also account for the calcium sensitivity of membrane-associated gelation activity. Preliminary evidence in this work is not consistent with involvement of $\alpha$-actinin, since most of this protein can be re-

moved from the membrane by KI extraction with retention of considerable actin. Also, the KI-extracted membranes retain gelation activity. Using differential extraction of HeLa cell membranes, Burridge and McCullough [39] also concluded that α-actinin was not involved in membrane-actin association in this cell type. Platelet actin binding protein, a potent F-actin gelation protein, is another potential contributing factor in membrane-actin interactions. The role of this protein is not clear, since alkaline-extracted membranes with some residual actin binding protein did not have gelation activity, perhaps because of denaturation of the protein. Also, the interaction of purified platelet actin binding protein with F-actin does not appear to depend on calcium concentration [33]. In platelets and other cells, actin binding protein is believed to function as an F-actin cross-linker throughout the cytoplasm [40]. Although it is conceivable that a fraction of total cellular actin binding protein functions as a membrane-actin connection, there is no direct evidence to support this possibility.

There is considerable previous evidence that membrane-cytoskeletal interactions are important in platelet function. Triton X-100 extraction of activated platelets has been used to show presumptive interactions between platelet cytoskeletal proteins and the integral membrane glycoproteins, IIb and III. Phillips et al. [11] showed that these glycoproteins remained associated with cytoskeletal proteins after Triton extraction of thrombin-aggregated platelets. This cosedimentation of membrane glycoproteins with the cytoskeleton was not observed with platelets activated with thrombin in the presence of EDTA to prevent aggregation. In a reexamination of this work, Zucker and Masiello [41] found that the Triton-insoluble material after extraction of thrombin-aggregated platelets contained considerable nonextracted membrane, as indicated by electron microscopy and organic phosphorus analysis. Thus, it is doubtful that the apparent glycoprotein association with the Triton precipitate of aggregated platelets is a valid indication of membrane-cytoskeletal interactions. However, using the same method, Painter et al. [12] found that activation with concanavalin A also induced an association between cytoskeletal proteins and glycoproteins IIb and III. This association was not dependent on platelet aggregation and could be disrupted by treatment of isolated cytoskeletons with pancreatic deoxyribonuclease I, an actin-depolymerizing agent. Recently, Rotman et al. [42] have shown that membrane glycoprotein Ia is associated with Triton-insoluble cytoskeletal proteins of resting or activated platelets. The evidence for membrane-cytoskeletal interactions in platelets is consistent with numerous studies in other cell types demonstrating interactions between cell surface receptors and underlying cytoskeletal proteins, as reviewed by Condeelis [43].

The molecular mechanism for membrane-actin association is well-defined only for the red cell, in which actin is bound to a high molecular weight tetrameric actin binding protein termed spectrin. Red cell spectrin is in turn linked to band 3, an integral membrane glycoprotein, by means of a linkage protein termed ankyrin [44]. Using immunological methods, several workers have demonstrated membrane-associated spectrin-like proteins in many cell types [45–49]. There is no published information on presence of a spectrin-like protein in platelets. However, using a competitive radioimmunoassay, Bennett [50] detected small amounts of a platelet protein related to red cell ankyrin, suggesting that a spectrin-like protein is likely to be present in platelets. From numerous studies on platelet cytoskeletal proteins, previously cited, it is probable that the amount of a platelet spectrin-like protein, if present, is very small. In preliminary work, Davies and Cohen (unpublished data) have found that rabbit anti-human red cell spectrin antiserum reacts with a single 240-kDa component in whole platelets, Triton X-100 precipitates, or purified membranes. This trace immunoreactive platelet protein comigrates with the α-chain of red cell spectrin in SDS-polyacrylamide gels. It is not yet known whether this spectrin-like protein contributes to the platelet membrane-associated gelation activity described in this paper.

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