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PLATELET CYTOSKELETON - MEMBRANE INTERACTIONS

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The order of attachment of the purified platelet cytoskeletal proteins, F-actin, α-actinin and Actin Binding Protein (ABP) to the isolated platelet membrane has been investigated. Of the three proteins, only F-actin would directly interact with a membrane preparation that had been extensively washed to remove associated cytoskeletal proteins. α-Actinin would only add to the membrane to which F-actin had been re-attached and ABP only if both F-actin and α-actinin were present on the membrane. These studies provide some insight into the nature of the attachment of the platelet cytoskeleton to the cytoplasmic side of the membrane.

The addition of a 1%-Triton-10mM EGTA - 40mM KCl solution to a platelet suspension immediately results in the appearance of a flocculent precipitate which when centrifuged at low speed (6,000 rpm) and examined by SDS-PAGE consists essentially of 3 proteins which have been identified as an actin-binding protein (ABP), α-actinin and F-actin (1,2). This precipitate we have termed the platelet cytoskeleton and an investigation into its interaction with the platelet membrane (PM) has been initiated. A number of laboratories have carried out similar studies on other membrane-cytoskeleton systems, the most widely studied being that of the erythrocyte (3). In all such instances it is thought that the association of the cytoskeleton with the plasma membrane has two main purposes, to confer strength and shape to the membrane and to act as a means of transmembrane communication whereby the binding of an external molecule to surface recepter results in the movement of the cytoskeleton on the cytoplasmic side of the membrane.

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ABBREVIATIONS: ABP, actin binding proteins; SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide, gel electrophoresis; EDTA, Ethylene diamine tetraacetic acid; EGTA, Ethylene Glycol bis (2-Amino ethyl ether) N,N'-tetraacetic acid; PIPES, piperazine-N,N'-bis (2-ethanesulfonic acid).

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Because in our laboratory we have purified the major protein components of the platelet cytoskeleton we have attempted to re-associate these proteins in a sequential manner to the isolated platelet membrane in order to delineate the order of attachment as well as the nature of interaction of these proteins, thus the cytoskeleton, to the platelet membrane. This report describes these studies.

Materials and Methods

Preparation of platelets and cytoskeletal proteins: Human platelet concentrates were obtained fresh from the Greater New York Blood Center and were freed from contaminating erythrocytes and leukocytes and sequentially washed according to the procedures described by Rosenberg et. al. (1,2). Cytoskeleton isolation and the purification of ABP, actin and α-actinin was carried out as described previously (1,2).

Preparation of platelet membranes: Platelets prepared as described above were resuspended in a buffer consisting of 50 mM Tris - 10 mM EGTA - 2.5 mM DTT at pH 7.5 in a ratio of 4 ml. of buffer/gm of platelet and placed in a Paar bomb and subjected to a pressure of 1200 psi of N_2 for 30 minutes at 4°C. The ruptured platelets were then centrifuged at 15,000 rpm for 25 minutes. The pellet, containing the crude membrane, was then fractionated by the aqueous two phase polymer system described by Brunette and Till (4). The purified membrane pellet, which contained considerable amounts of actin, was then washed 4-5 times in G-buffer consisting of 2mM Tris, 0.5 mM BME, 0.2 mM Ca^{++}-ATP, pH 8.0 at a ratio of 40 ml G-buffer/ml of membrane pellet. This procedure resulted in the removal of most of the actin and other cytoskeletal proteins found associated with the membrane. This membrane pellet could be used directly or be stored in 50% glycerol-Assembly buffer (40 mM KCl 10 mM PIPES, 1 mM EGTA, 1mM NaN_3) and was stable for several months.

SDS-PAGE: Was carried out by the procedure used routinely in this laboratory and described by Rosenberg et. al (1).

Association of washed platelet membranes and cytoskeletal proteins: 0.2 ml of stored membrane suspension in G-buffer (equivalent to 5 ul of pure membrane) was centrifuged at 6,000 rpm for 10 minutes. The membrane pellet was incubated with 0.06 ml of about 0.3 mg/ml of F-actin in 0.1 M KCl-10 mM PIPES - 1 mM EGTA, pH 6.8; or 0.06 ml of about 0.1 mg/ml of ABP or α-actinin in the same buffer at room temperature for one hour. After incubation the suspension was centrifuged at 6,000 rpm for 10 min. and both the pellet and supernatant were prepared for SDS-PAGE, or the pellet could be reincubated with another cytoskeletal protein and the above procedure repeated.

Results and Discussion

As seen in Fig. 1, lane A, extensive washing of the platelet membrane results in the removal of substantial amounts of protein. Little, if any, of the cytoskeletal proteins, ABP, α-actinin or myosin are visible in the washed sample. Small amounts of actin remain firmly attached to the membrane and cannot be removed even by further washes.

Other studies have pointed to the fact that while F-actin may bind to the plasma membranes of certain cells (5,6), there may be other binding proteins in the cytoskeleton which are intermediate between actin and the membrane. For example, in the erythrocyte membrane spectrin binds actin to the plasma membrane (7) whereas vinculin has been

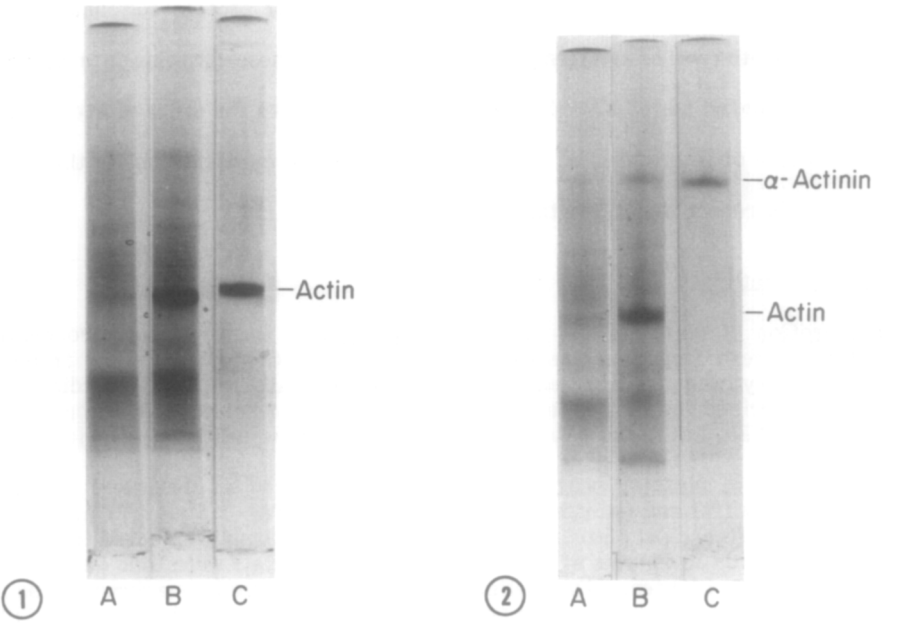

Figure 1. Association of F-actin with washed platelet membrane. See Methods for details. SDS-polyacrylamide gels stained with Coomassie Blue. Lane A) total washed platelet membranes; Lane B) pellet from 6,000 r.p.m. 10 min. centrifugation after incubation of washed membrane and F-actin at room temperature for 1 hour. Lane C) Supernatant from (B).

Figure 2. Association of α-actinin with washed platelet membrane to which F-actin has been previously added. See Methods for details. SDS-polyacrylamide gels stained with Coomassie Blue. Lane A) total washed platelet membrane; Lane B) pellet obtained as in Figure 1 after incubation of α-actinin with membrane to which F-actin had been previously re-attached; lane C) Supernatant from (B).

implicated in the attachment of actin to the plasma membrane in brush border and smooth muscle cells (8).

It was therefore of interest to ascertain whether any of the cytoskeletal-structural proteins would re-associate with this protein depleted (washed) membrane preparation. Of the four cytoskeletal proteins only F-actin would reattach to the washed platelet membrane (Fig. 1). Myosin (not shown), ABP (Fig. 3) and α-actinin (not shown) would not, by themselves, re-associate with the membrane.

Further studies with ABP (Fig. 3) and α-actinin with the actin enriched membrane show that only the α-actinin would associate (Fig. 2). ABP would only reassociate with the membrane after F-actin and α-actinin had first been re-attached (Fig. 4).

It is clear from the results presented here that only one of the platelet cytoskeletal proteins, F-actin, is capable of directly interacting with the inner surface of the

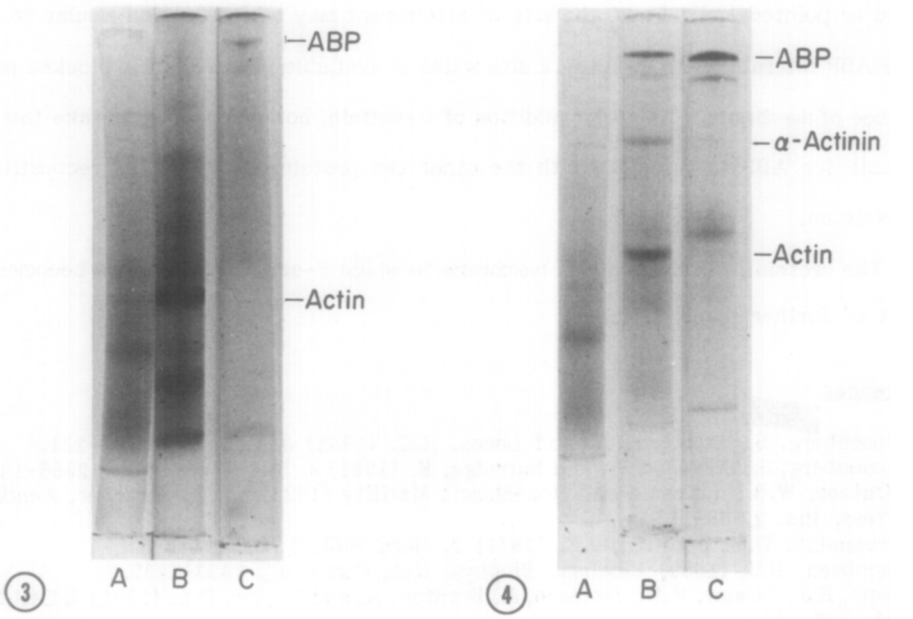

Figure 3. Association of ABP with washed platelet membrane. See Methods for details. SDS-polyacrylamide gels stained with Coomassie Blue. Lane A) total washed platelet membrane; Lane B) pellet obtained as in Figure 1 after incubating ABP with washed platelet membrane to which F-actin had been previously re-attached; Lane C) supernatant from lane (B).

Figure 4. Association of ABP with washed platelet membrane to which both F-actin and α-actinin has been previously re-attached. See Methods for details. SDS-polyacrylamide gels stained with Coomassie Blue. Lane A) total washed platelet membrane; Lane B) pellet obtained as in Figure 1 after incubation of ABP with washed platelet membrane to which both F-actin and α-actinin had been previously re-attached; Lane C) supernatant from lane (B).

membrane. The attachment of α-actinin to the membrane occurs only because of its association with F-actin. As we had shown previously, this interaction is inhibited by Ca^{++} and no doubt accounts for the failure to isolate platelet cytoskeleton in the presence of Ca^{++} (2).

The inability of ABP to interact with the membrane was rather surprising in view of the studies in which spectrin has been shown to interact directly with the erythrocyte membrane (9). Of further interest was ABP's failure to interact with the F-actin enriched membrane because of our previous studies showing the reformation of platelet cytoskeleton, in vitro, with ABP and F-actin (1). Since cytochalasin B did not prevent the re-association of F-actin with the membrane (this study, data not shown) a lateral attachment of the molecule with the membrane was suggested rather than through either the

barbed or pointed end. Thus, the site of attachment may be in a region similar to that where ABP interacts with F-actin, a site which is available in vitro but is blocked in the presence of membrane. The prior addition of α-actinin, however, may now make this site available for ABP to associate with the other two proteins and form the reconstituted cytoskeleton.

The protein(s) in the platelet membrane to which F-actin attaches now becomes the object of further investigation.

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