Spectrin Is Associated With Membrane-Bound Actin Filaments in Platelets and Is Hydrolyzed by the Ca\(^{2+}\)-Dependent Protease During Platelet Activation

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We recently showed that platelets contain submembranous actin filaments that are linked to glycoprotein (GP) Ib on the plasma membrane. In the present study, experiments were performed to determine whether spectrin was associated with these filaments. The membrane-bound filaments were isolated from Triton X-100 (Sigma, St Louis) lysates of unstimulated platelets by differential centrifugation. Platelet spectrin was detected immunologically by using antibodies against human brain and RBC spectrin. Immunoblots showed that platelet spectrin consisted of two polypeptides (mol wt 240,000 and 235,000) that were similar in apparent mol wt to those of the \(\alpha\) and \(\beta\) chains of brain spectrin but differed slightly from those of RBC spectrin (mol wt 240,000 and 220,000). Immunoprecipitation experiments identified platelet spectrin as two minor polypeptides migrating on sodium dodecyl sulfate (SDS)-polyacrylamide gels between actin-binding protein (mol wt 250,000) and the platelet polypeptide P235 (mol wt 236,000). Immunoblots of fractions isolated from Triton X-100-lysed platelets revealed that the \(\alpha\) and \(\beta\) chains of platelet spectrin were associated almost entirely with the actin filaments that were linked to the plasma membrane. Little spectrin was recovered in the Triton X-100-soluble fraction or with the actin filaments that were not membrane bound. During activation of platelets with thrombin or ionophore A23187, the \(\alpha\) and \(\beta\) chains of spectrin were hydrolyzed, generating a major degradation product of mol wt 160,000 and a minor one of mol wt 170,000. These two hydrolytic products were also generated in Triton X-100 lysates incubated in the presence of Ca\(^{2+}\) but were not produced when lysates were treated with leupeptin, ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(N,N,N',N'\)-tetraacetic acid (EGTA), or N-ethylmaleimide, known inhibitors of the Ca\(^{2+}\)-dependent protease. These experiments show that spectrin is a previously unidentified component of the membrane-bound actin filament network and that hydrolysis of spectrin by the Ca\(^{2+}\)-dependent protease may regulate the interactions of the filaments during platelet activation.

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PLATELETS HAVE a characteristic discoid shape that is rapidly lost when physiological agonists activate the platelets. The factors responsible for maintaining the discoid shape or directing the activation-induced changes are not well understood. Microtubules have been thought to be critical in regulating platelet shape,\(^1,2\) but the finding that agonists induce a change in shape even when microtubules are stabilized with taxol\(^3\) suggests that additional factors are involved.

We recently showed that platelets contain a peripheral layer of actin filaments that is linked by actin-binding protein to glycoprotein (GP) Ib on the plasma membrane.\(^4,5\) This membrane-bound layer of actin filaments is distinct from the rest of the cytoskeleton and might function as a membrane skeleton, stabilizing the shape of the unstimulated platelet.\(^4\)

The only other cell known to contain a membrane skeleton that regulates the shape of the cell is the RBC (for review, see refs 6 and 7). In this cell, the major component of the membrane skeleton is spectrin, which consists of elongated heterodimers comprising an \(\alpha\) chain (mol wt 240,000) and a \(\beta\) chain (mol wt 220,000). The heterodimers are associated head to head, forming tetramers. The tail regions of the spectrin tetramers bind to short actin filaments, cross-linking these filaments into networks. Spectrin also links the filamentous network to the RBC plasma membrane through ankyrin, which connects spectrin to the membrane glycoprotein known as band 3. An additional linkage to the membrane may be provided by protein 4.1, which binds to spectrin and is also thought to be associated with the membrane glycoprotein glycoporphin.\(^4\)

Proteins immunologically similar to RBC spectrin,\(^9,14\) ankyrin,\(^15,16\) and protein 4.1\(^15,21\) have been described in many nonerythroid cells, including platelets.\(^15,19,21\) This raised the question of whether these proteins might be associated with the membrane-bound actin filaments in platelets. In the present report, we (a) identify platelet spectrin as two polypeptides of mol wt 240,000 and 235,000, (b) show that spectrin is selectively associated with the membrane-bound actin filaments, and (c) demonstrate that spectrin is hydrolyzed by the Ca\(^{2+}\)-dependent protease during platelet activation, when the cell undergoes dramatic changes in shape.

MATERIALS AND METHODS

Preparation of platelet suspensions. Venous blood was drawn from healthy adult donors, and platelets were isolated from it by centrifugation as described previously.\(^4,22\) When the platelet pellet was removed from the tube after each centrifugation, care was taken not to include any RBCs that were sometimes found tightly packed below the platelets. The washed platelets were finally resuspended at about 1 x 10\(^8\) platelets/mL in Tyrode’s buffer containing 138 mmol/L of sodium chloride, 2.9 mmol/L of potassium chloride, 12 mmol/L of sodium bicarbonate, 0.36 mmol/L of sodium phosphate, 5.5 mmol/L of glucose, 1.8 mmol/L of calcium chloride, 0.4 mmol/L of magnesium chloride, and 10 ng of prostacyclin/mL, pH 7.4. When platelets were to be subsequently activated by platelet agonists, prostacyclin was omitted from the Tyrode’s buffer. Microscopic examination showed that no contaminating RBCs were present.

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To obtain platelets in which the surface glycoproteins were 1H-labeled, washed platelets were labeled by the sodium metaperiodate/sodium \(^{3}H\)borohydride method, as described previously. Platelet counts were made with a Coulter counter.

Incubations. For studies of intact platelets, platelet suspensions were stirred with 0.1 National Institutes of Health (NIH) U of thrombin/mL (a generous gift from Dr John W. Fenton II of the New York Department of Health, Albany) or with 1.0 mmol/L of ionophore A23187 (Calbiochem, San Diego) as described previously.

For studies of lysed platelets, lysates were prepared by adding to platelet suspensions an equal volume of buffer containing 2% Triton X-100 (Sigma, St Louis), 100 mmol/L of Tris-HCl, 100 mmol/L of benzamide, and 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF) (Sigma), pH 7.4. When indicated, leupeptin (Vega Biotechnologies, Tucson, AZ), ethylene glycol bis(\(\beta\)-aminoethyl)ether)-\(N,N,N',N'\)-tetraacetic acid (EGTA), or N-ethylmaleimide (Sigma) was included in the buffer to inhibit activity of the Ca\(^{2+}\)-dependent protease. Lysates were incubated at 25 °C, and incubations were terminated by the addition of sodium dodecyl sulfate (SDS)-containing buffer.

Isolation of subcellular fractions. Platelets (500-\(\mu\)L portions) were lysed by addition of an equal volume of ice-cold buffer containing 2% Triton X-100, 10 mmol/L of EGTA, and 100 mmol/L of Tris-HCl, pH 7.4. In some experiments, as indicated in the text, actin filament depolymerization was induced by omitting EGTA from the lysis buffer or by omitting EGTA and including Dnase I (2 mg/mL, Boehringer-Mannheim, Indianapolis). When EGTA was omitted, leupeptin (2 mg/mL) was included to inhibit activity of the Ca\(^{2+}\)-dependent protease. When Dnase I was included, 2 mmol/L of PMSF and 100 mmol/L of benzamide were added to the lysis buffer to inhibit any protease activity present in the Dnase I. Lysates were centrifuged at 100,000 g at 4 °C for 3 hours to obtain the Triton X-100-insoluble actin filaments and the Triton X-100-soluble fraction. To separate the membrane-bound filamentous components from the rest of the actin filaments, lysates were first centrifuged at 15,600 g at 4 °C for 4 minutes. The bulk of the actin filaments sedimented at these g forces. The membrane-bound filaments remained in the supernatant, however, and were subsequently isolated by centrifugation at 100,000 g at 4 °C for 3 hours.

SDS-polyacrylamide gel electrophoresis (PAGE). For analysis on SDS-polyacrylamide gels, 3 vol of sample were solubilized by addition of 1 vol of a buffer consisting of 8% SDS (wt/vol), 20% 2-mercaptoethanol (vol/vol), 40% glycerol (vol/vol), 0.008% bromphenol blue (wt/vol), 250 mmol/L of Tris-HCl, 20 mmol/L of EGTA, and 4 mg of leupeptin/mL, pH 6.8. Insoluble fractions were solubilized in the same buffer diluted four times with distilled water. All samples were incubated at 60 °C for 5 minutes to facilitate solubilization. Protein was electrophoresed through slab gels according to the method of Laemmli, using 3% acrylamide in the stacking gel and either 5% or 7.5% acrylamide in the resolving gel. For detection of total protein, gels were stained with Comassie brilliant blue or by a silver staining method. For detection of \(^{3}H\)-labeled glycoproteins by fluorography, gels were treated with EN3HANCE (New England Nuclear, Boston), dried, and exposed to x-ray film as described previously. Spectrin was detected on immunoblots as described below.

Antibody production and characterization. RBC ghosts were isolated and washed as described. Spectrin was extracted from ghosts at 37 °C in a low-ionic-strength buffer and was further purified by gel filtration on Sepharose 4B as described by Tyler and colleagues. Purified spectrin was electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide. The gel was stained briefly (~5 minutes) with Comassie brilliant blue, and the two major bands of mol wt 240,000 and 220,000 (corresponding to the \(\alpha\) and \(\beta\) chains of spectrin) were cut out of the gel in a narrow strip. The gel strip was washed, homogenized, and injected into rabbits as described previously. Antiserum was isolated by centrifugation of clotted blood for 20 minutes at 30,000 g. The immunoglobulin G (IgG) fraction was precipitated two times at 4 °C with 40% ammonium sulfate, dialyzed into a buffer containing 100 mmol/L of sodium chloride, 0.02% sodium azide, and 10 mmol/L of sodium phosphate, pH 7.4, and stored at 4 °C.

Antibodies against human brain spectrin were raised as described. Monoclonal antibody against the \(\beta\) chain of RBC spectrin was kindly provided by Dr Stanley Schrier of Stanford University. Antibodies against actin-binding protein and glycocalcin (the water-soluble hydrolytic product of GP Ib) were raised as described previously.

Antibody specificities were characterized on immunoblots as described previously. The specificities of the affinity-purified actin-binding protein antibodies and the glycocalcin antibodies have been described. Antibody concentrations were determined by their absorbance at 280 nm using an \(\varepsilon_{280}\) value of 14.0.

Immunoprecipitation of proteins from platelet lysates. Platelet suspensions (1 to 2 \(\times\) 10\(^8\) platelets/mL) were lysed by the addition of an equal volume of ice-cold buffer containing 2% Triton X-100, 10 mmol/L of EGTA, 2 mg of leupeptin/mL, and 100 mmol/L of Tris-HCl, pH 7.4. In some experiments, DNase I (1 mg/mL) was included in the lysis buffer to depolymerize actin filaments and release filament-associated proteins into the Triton X-100-soluble fraction. When DNase I was present, PMSF (2 mmol/L) and benzamidine (100 mmol/L) were included in the lysis buffer and the leupeptin concentration was increased to 4 mg/mL. Filamentous actin remaining in the Triton X-100 lysates was removed by centrifugation of the lysates at 100,000 g at 4 °C for 3 hours. Proteins were immunoprecipitated from 1-mL portions of the Triton X-100-soluble fraction as described previously. Immunoprecipitated protein was solubilized with 70 \(\mu\)L of an SDS-containing buffer and analyzed on SDS-polyacrylamide gels.

RESULTS

Characterization of spectrin antibodies. RBC ghosts and washed platelet suspensions were each solubilized in SDS-containing buffer and electrophoresed on SDS-polyacrylamide gels containing 7.5% acrylamide. The polypeptides were transferred electrophoretically to nitrocellulose paper, which was then incubated with antibodies against spectrin. Figure 1 shows that antibodies against both RBC spectrin and brain spectrin reacted with the \(\alpha\) chain of mol wt 240,000 and the \(\beta\) chain of mol wt 220,000 of RBC spectrin (Fig 1A and B, lane 1). The antibodies also reacted with several minor polypeptides of lower mol wt present in the RBC ghosts, an observation made previously by others. Antibodies against RBC spectrin were raised in three different rabbits. Two of the antisera did not react with any platelet polypeptide on immunoblots. As shown in Fig 1, however, one antiserum reacted with a closely spaced doublet in platelets (Fig 1A, lane 2). The doublet had a mol wt of 240,000, as indicated by its comigration with the \(\alpha\) chain of mol wt 240,000 of spectrin in RBC ghosts. A doublet of similar mol wt was also recognized in platelets by the antibodies against brain spectrin (Fig 1B, lane 2). There was no indication of a polypeptide in platelets that comigrated with the \(\beta\) chain of mol wt 220,000 of RBC spectrin. A monoclonal antibody against the \(\beta\) chain of RBC spectrin did not react with any platelet polypeptide (Fig 1C, lane 2).
PLATELET SPECTRIN

Fig 1. Characterization of spectrin antibodies. RBC ghosts (~25 μg of protein) (lane 1 in each panel) and washed platelet suspensions (~75 μg of protein) (lane 2 in each panel) were each solubilized in sodium dodecyl sulfate (SDS)-buffer and electrophoresed on SDS-polyacrylamide gels containing 7.5% acrylamide. The polypeptides were transferred to nitrocellulose paper, which was incubated with antibodies against (A) RBC spectrin (final concentration, 210 μg/mL), (B) brain spectrin (final concentration, 120 μg/mL), and (C) a monoclonal antibody against the β chain of RBC spectrin (final concentrations, 110 μg/mL for RBC polypeptides and 220 μg/mL for platelet polypeptides). Antigen–antibody complexes were detected by autoradiography after incubation of the nitrocellulose paper with 125I-labeled Protein A.

To resolve the α and β chains of platelet spectrin, platelet polypeptides were electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide. Figure 2A shows that the α chain of platelet spectrin comigrated with the α chain of mol wt 240,000 of RBC spectrin, whereas the β chain migrated to a point between the α and β chains of RBC spectrin, with a mol wt of 235,000. These were the only two polypeptides that reacted specifically with the spectrin antibodies. Although a few additional minor bands were apparent (Fig 2A), these represented nonspecific binding of the antibody, as indicated by their presence in control blots as well (Fig 2B).

Immunoprecipitation of platelet spectrin. Antibodies against both RBC spectrin and brain spectrin caused immunoprecipitation of two polypeptides of spectrin from platelet lysates (Fig 3A, lanes 3 and 4). The two polypeptides were precipitated from platelet lysates even by batches of RBC spectrin antibodies that did not interact with platelet spectrin on immunoblots. The polypeptides were present in very small amounts and were barely detectable over the background of other proteins that were recovered nonspecifically in the immunoprecipitates. Their identification as platelet spectrin was indicated by their absence from immunoprecipitates obtained with normal rabbit IgG (Fig 3A, lane 2), however, and their identification was confirmed on immunoblots showing that the two immunoprecipitated proteins reacted with antibodies against brain spectrin (Fig 3B). The α chain of platelet spectrin comigrated with the α chain of mol wt 240,000 of RBC spectrin, whereas the β chain had a mol wt that was higher than that of both the β chain of RBC spectrin and the platelet polypeptide of mol wt 235,000 (P235) (Fig 3A, lanes 3 and 4 as compared with lanes 5 and 6).

To identify which of the polypeptides in whole platelets represented spectrin, platelets were solubilized in SDS-buffer and then electrophoresed through SDS-polyacrylamide gels containing 5% acrylamide. Most one-dimensional gel systems resolve the two major high-mol-wt proteins actin-binding protein (mol wt 250,000) and P235 (mol wt 235,000), but not additional polypeptides in this mol-wt region. By using 5% acrylamide and running the gels until the dye front was well off the bottom of the gel, we were able to resolve two additional polypeptides that were visible on Coomassie brilliant blue-stained gels at points between actin-binding protein and P235 (Fig 3, lane 1). These polypeptides migrated to the same position on the gels as the two polypeptides precipitated by the spectrin antibodies. They were not present in platelet lysates from which spectrin had been immunoprecipitated (data not shown). We conclude that these two additional polypeptides represent platelet spectrin. The two polypeptides of platelet spectrin were present in approximately equal amounts and represented very minor components as compared with actin-binding protein and P235.

Association of spectrin with actin filaments in platelets. To determine whether spectrin was associated with actin filaments, platelets were lysed with Triton X-100 and...
the actin filaments were sedimented at g forces known to sediment all of the filamentous actin (100,000 g for 3 hours). Analysis of the Triton X-100-insoluble and -soluble fractions showed that spectrin was primarily recovered along with the Triton X-100-insoluble actin filaments (Fig 4A, lane 2 compared with lane 3). Because several other platelet proteins in addition to actin filaments are inherently insoluble in Triton X-100, the effect of depolymerization of the actin filaments on the Triton X-100 insolubility of spectrin was examined. Two depolymerizing agents were used: Ca\(^{2+}\) and DNase I. The addition of Ca\(^{2+}\) causes depolymerization of most of the actin filaments in platelet lysates, but not of the small pool of filaments that is membrane bound. Figure 4B shows that Ca\(^{2+}\) had little effect on the recovery of spectrin in the Triton X-100-insoluble fraction (lane 2 compared with lane 1). In contrast to Ca\(^{2+}\), DNase I depolymerizes both cytoplasmic filaments and membrane-bound filaments. When DNase I was present at concentrations shown previously to depolymerize ~40% of the total actin filament content and release ~20% of GP Ib from the Triton X-100-insoluble fraction, the recovery of spectrin in the Triton X-100-insoluble fraction was also reduced (Fig 4B, lane 3 compared with lane 1). The differential effect of Ca\(^{2+}\) and DNase I on the recovery of spectrin in the Triton X-100-insoluble fraction suggests that spectrin is selectively associated with the membrane-bound actin filaments in platelets.

An additional characteristic of the membrane-bound filaments is that they can be separated from other platelet actin filaments by differential centrifugation of platelet lysates. Figure 5A (lane 2) shows that many of the actin filaments in platelets can be sedimented from Triton X-100 lysates by low-speed centrifugation. Little GP Ib is associated with these filaments (Fig 5B, lane 2). The membrane-bound actin filaments require higher speed centrifugation to be sedi-
Fig 5. Recovery of spectrin with the membrane-bound actin filaments. Platelets, surface labeled by the sodium metaperiodate/sodium \(^{3}H\)borohydride method, were suspended in Tyrode’s buffer and solubilized directly into sodium dodecyl sulfate (SDS)-containing buffer (lane 1 in each panel) or lysed by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, and 100 mmol/L of Tris-HCl, pH 7.4. Lysates were centrifuged at 15,600 g at 4 °C for 4 minutes, and the Triton X-100 supernatant was centrifuged at 100,000 g for a further 3 hours. The sediments obtained at low speed (lane 2 in each panel) and high speed (lane 3 in each panel) and the supernatant obtained at high speed (lane 4 in each panel) were analyzed on SDS-polyacrylamide gels containing 7.5% acrylamide (A) or 5% acrylamide (B and C). Polypeptides were detected with Coomassie brilliant blue stain (A). \(^{3}H\)-labeled glycoproteins were detected by fluorography (B), and spectrin was detected on immunoblots using antibodies against brain spectrin (C). The protein analyzed in each lane originated from 30 μL of whole platelets. ABP, actin-binding protein; GP Ib\(_\alpha\), glycoprotein Ib\(_\alpha\).

Hydrolysis of spectrin during platelet activation. It has been shown that actin-binding protein links actin filaments and actin-binding protein (Fig 5A, lane 3) and GP Ib (Fig 5B, lane 3). This property of the membrane-associated actin filaments was used to provide further evidence that spectrin was selectively associated with these filaments. Figure 5C shows that, like GP Ib, little spectrin was associated with the actin filaments that could be sedimented from Triton X-100 lysates at low g forces (lane 2). Spectrin, like GP Ib, was primarily associated with the actin filaments requiring high-speed centrifugation to be sedimented (Fig 5C, lane 3).

It has been shown that actin-binding protein links actin filaments and GP Ib on the plasma membranes of platelets. To test whether spectrin was a component of this actin-binding protein-GP Ib linkage, actin filaments in platelet lysates were depolymerized with DNase I. The lysates were centrifuged at 100,000 g for 3 hours, and the resulting supernatant, which was free of actin filaments and contained proteins released from filaments during depolymerization, was used in immunoprecipitation experiments. Figure 6 shows that the actin-binding protein–GP Ib complex was precipitated from the high-speed supernatant by actin-binding protein antibodies (Fig 6A and 6B, lane 1) and by GP Ib antibodies (Fig 6A and 6B, lane 2). Spectrin antibodies precipitated spectrin (Fig 6A, lane 4) but not actin-binding protein (Fig 6A, lane 4) or GP Ib (Fig 6B, lane 4), indicating that spectrin is not a component of the actin-binding protein–GP Ib complex.

Hydrolysis of spectrin during platelet activation. Figure 7 shows that activation of platelets with the divalent cation ionophore A23187 resulted in hydrolysis of spectrin. The major degradation product was a polypeptide of mol wt 160,000. A minor degradation product of mol wt 170,000 was also apparent. The same degradation products were generated when platelets aggregated in response to thrombin (Fig 8). The major degradation product was apparent within seconds of addition of 0.1 NIH U of thrombin per milliliter. The amount of hydrolysis increased with time after thrombin was added (Fig 8, lanes 1 through 10). Hydrolysis was dependent on aggregation of platelets; it was not detected if platelets were prevented from aggregating by failure to stir the suspensions (data not shown).

Hydrolysis of spectrin by the \(Ca^{2+}\)-dependent protease in platelet lysates. It has been shown that the \(Ca^{2+}\)-dependent protease within platelets is activated when platelets aggregate after stimulation by thrombin, collagen, or ionophore A23187. To determine whether hydrolysis of spectrin within aggregated platelets resulted from activity of the \(Ca^{2+}\)-dependent protease, platelets were lysed with Triton X-100 under conditions in which the \(Ca^{2+}\)-dependent protease was active. The degradation products produced in lysates were compared with those produced in intact cells. Figure 9A shows that spectrin was rapidly hydrolyzed. The major degradation product had a mol wt of 160,000. Products with a mol wt of 210,000 and 170,000 were also generated. These three new polypeptides arose from activity of the \(Ca^{2+}\)-dependent protease, as indicated by the inhibition of their generation by leupeptin and EGTA (Fig 9A, lanes 11 and 12). N-Ethylmaleimide, another inhibitor of the \(Ca^{2+}\)-dependent protease, also inhibited hydrolysis of spectrin (Fig 9A, lane 13). This inhibition, however, even at a concentration of 5 mmol/L of N-ethylmaleimide, was not as complete as with the other two inhibitors. Silver staining of SDS-gels showed that actin-binding protein and P235, the two major substrates of the platelet \(Ca^{2+}\)-dependent protease...
A. B.

ABP-*1 Spectrin

GP

Ib

1234

Fig 6. Demonstration that spectrin is not a component of the actin-binding protein–GP Ib complex. Platelet suspensions were lysed with an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, 100 mmol/L of Tris-HCl, leupeptin 4 mg/mL, DNase I (1 mg/mL), 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L of benzamidine, pH 7.4. Triton X-100–insoluble actin filaments were removed from the lysates by centrifugation at 100,000 g at 4 °C for 3 hours.

Precipitates obtained from immunoprecipitation of 1 mL of the Triton X-100–soluble fraction with 4.5 μg of actin-binding protein antibodies/mL (lane 1 in each panel), 1.3 mg of glycocalcin antibodies/mL (lane 2 in each panel), 1.3 mg of normal rabbit IgG/mL (lane 3 in each panel), and 1.3 mg of RBC spectrin antibodies/mL (lane 4 in each panel) were each solubilized in sodium dodecyl sulfate (SDS)-containing buffer. Solubilized proteins were electrophoresed on gels containing 5% acrylamide. Immunoprecipitated actin-binding protein and spectrin were detected with Coomassie brilliant blue stain (A); GP lb was detected on immunoblots using antibodies against GP lb (B). ABP, actin-binding protein; GP lb, glycoprotein lb.

Identified previously,20 were hydrolyzed at a rate similar to that of spectrin.

**DISCUSSION**

Proteins immunologically and functionally similar to RBC spectrin have been described in many nonerythroid tissues.9–14 The best characterized of these proteins, that found in brain, is referred to as fodrin, calspectrin, or brain spectrin. Brain spectrin, like RBC spectrin, is a tetramer in which αβ heterodimers associate head to head and the tail regions bind to actin filaments.9,33 Despite the similarities in structure and function, the brain and RBC proteins do differ, as indicated by their peptide maps9,10 and the open weak reactivity of RBC spectrin antibodies against brain spectrin.10 The α chains, despite their similar molecular weights (~240,000), show different peptide maps; the β chains differ in both peptide maps and mol wt (~235,000 for brain spectrin as compared with ~220,000 for RBC spectrin). In general, the spectrinlike proteins in mammalian nonerythroid cells appear to have mol wts similar to those of brain spectrin. It is becoming increasingly clear, however, that more than one isoform of nonerythroid spectrin can exist, even within the same tissue.34,35

Early attempts, using antibodies against RBC spectrin, failed to detect spectrin in platelets.36 More recent experiments detected a single polypeptide that comigrated on SDS-gels with the α chain of mol wt 240,000 of RBC spectrin.37 Because only one chain was detected in the latter study, it was not clear whether platelets contained the RBC form of spectrin or a form more closely related to brain spectrin. Because of the often low reactivity of RBC spectrin antibodies against spectrin in other tissues, we used brain spectrin antibodies to probe for spectrin in platelets. These antibodies detected two spectrin polypeptides both on SDS–gels of immunoprecipitated proteins and on immunoblots of total platelet proteins. The α chain of platelet spectrin comigrated with the α chain of mol wt 240,000 of RBC spectrin, whereas the β chain had a mol wt of 235,000. Platelets did not contain a polypeptide that comigrated with...
Spectrin chains of platelet spectrin are similar to those of brain spectrin.

In RBCs, spectrin is the primary component of the membrane skeleton, a structure beneath the plasma membrane that regulates the shape of the cell.6,7 The membrane skeleton
is composed of short actin filaments that are cross-linked into networks by spectrin, which also links the networks to the plasma membrane. Immunofluorescence studies of several cell types, including platelets, have indicated that spectrin lies just under the plasma membrane in these cells too, raising the possibility that nonerythroid spectrin functions in the same way as RBC spectrin. We recently reported that a small pool of the filamentous actin in platelets is associated with glycoproteins on the plasma membrane. We suggested that the membrane-associated filaments may function like the membrane skeleton of RBCs in that they may play a role in regulating the shape of the platelet. Because the filaments that are attached to the membrane glycoproteins can be separated from other actin filaments within platelets, it was possible to test whether spectrin was associated with the membrane-bound filaments. The results reported here show that spectrin is indeed associated with the membrane-bound filaments in platelets.

A previous study has shown that one of the major components of the membrane-bound filamentous network in platelets is actin-binding protein. Like spectrin, it associates head to head, and the free tails of the polypeptide chains bind to actin filaments. Like spectrin in RBCs, actin-binding protein in platelets connects the cross-linked filaments to glycoproteins on the plasma membrane. The present study shows that although spectrin is an additional component of the membrane-bound actin filament network in platelets, it is present in very small amounts as compared with actin-binding protein, which is one of the major platelet proteins. These observations suggest that the membrane-bound filamentous network in platelets differs from RBC membrane skeleton in that in platelets, actin-binding protein provides the major linkage between actin filaments and the plasma membrane, whereas in RBCs, spectrin is the major component of this linkage. If spectrin functions in platelets in the same way it does in RBCs, it would be expected to link actin filaments to a plasma membrane glycoprotein. Although spectrin does not appear to be involved in the linkage between actin-binding protein and GP Ib, it may provide an additional linkage between actin and an as-yet-unidentified component of the plasma membrane. Alternatively, spectrin in nonerythroid cells may function differently from its counterpart in RBCs. There is some evidence, for example, that spectrin may associate with microtubules. The potential role of platelet spectrin in linking the membrane-associated filaments to the plasma membrane glycoproteins, microtubules, or other actin filaments awaits further study.

One of the initial responses of platelets to stimulation is their transformation from discs into irregular spheres. Later, platelets undergo dramatic changes in shape as they spread on surfaces or extend long filopodia during the process of clot retraction. By analogy to the RBC, in which alterations in the membrane skeleton produce changes in cell shape, the early changes in shape that platelets undergo in response to activation could result from reversible modifications of components of the membrane-bound filamentous network. The later changes in the shape of the platelet membranes are so dramatic that the membrane-bound network would probably have to be disrupted for these changes to occur. Such a disruption has been shown to result from activation of the Ca\(^{2+}\)-dependent protease within platelets. This protease hydrolyzes actin-binding protein, resulting in release of the actin filaments from their attachment to GP Ib on the plasma membranes. The results presented here show that platelet spectrin is also a substrate for the Ca\(^{2+}\)-dependent protease and is hydrolyzed during platelet activation with a time course similar to that reported for hydrolysis of actin-binding protein and P235. We reported that the Ca\(^{2+}\)-dependent protease is only activated in stimulated platelets if the platelets are allowed to aggregate. A similar aggregation dependency for the hydrolysis of spectrin within platelets was detected in this study. In platelet lysates, hydrolysis of spectrin by the Ca\(^{2+}\)-dependent protease resulted in generation of three degradation products (mol wt 210,000, 170,000, and 160,000). In intact platelets, in which hydrolysis was more limited, the mol wt 160,000 product was the most readily detected. The mol wt of this major degradation product is similar to the mol wt 150,000 reported by Siman and co-workers for the product of hydrolysis of brain spectrin by purified Ca\(^{2+}\)-dependent protease.

Many of the proteins that are considered candidates for linkage proteins between actin filaments and plasma membranes in non-muscle cells (for example, actin-binding protein, vinculin, a-actinin, protein 4.1, and P235) are substrates for the Ca\(^{2+}\)-dependent protease. Furthermore, it was recently shown that the Ca\(^{2+}\)-dependent protease, previously thought to be a cytosolic enzyme, can bind to RBC membrane in an interaction that activates the enzyme. These observations, together with the finding that activation of the Ca\(^{2+}\)-dependent protease during platelet activation results in hydrolysis of actin-binding protein and disruption of the interaction between actin filaments and GP Ib, emphasize the importance of the Ca\(^{2+}\)-dependent protease in regulating the interaction of actin filaments with plasma membranes. The finding that spectrin is an additional component of the membrane-bound filamentous network in platelets and that it is hydrolyzed by the Ca\(^{2+}\)-dependent protease during platelet activation suggests still another mechanism for regulation of the membrane-bound actin filaments during platelet activation.

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