Activation of Calpain I and Hydrolysis of Calpain Substrates (Actin-Binding Protein, Glycoprotein Ib, and Talin) Are Not a Function of Thrombin-Induced Platelet Aggregation

June D. Wencel-Drake, Janice R. Okita, Douglas S. Annis, and Thomas J. Kunicki

Calcium-activated neutral proteinase (calpain) has been shown to cleave proteins involved in the maintenance of cell structure. In human platelets, substrates of calpain include glycoprotein Ib (GPIb), actin-binding protein (ABP), and talin. GPIb–ABP complexes can be isolated in detergent extracts and are thought to represent membrane–cytoskeleton attachment sites. It has been hypothesized that the hydrolysis of GPIb–ABP by calpain is regulated by the extent of binding of this proteinase to the plasma membrane–cytoskeleton interface with platelet activation. Recently, another calpain substrate (talin) has been shown to redistribute from the cytoplasm to the plasma membrane–cytoskeleton interface as the result of thrombin stimulation. To investigate the intracellular distribution of calpain I, we employed the monoclonal antibody B27D8, specific for the heavy chain (catalytic subunit) of calpain I. Indirect immunofluorescent staining of resting human platelets revealed undetectable surface antigen. Permeabilization with Triton X-100, however, revealed a diffuse intracellular antigen consistent with a cytosolic distribution. To determine whether this antigen distribution reflected the proenzyme or the activated form of calpain I and to assess the degree of hydrolysis of ABP, GPIb, and talin, we employed B27D8 and murine monoclonal antibodies against ABP (1B3 and 3D1), GPIb (LJIib10), and rabbit polyclonal antibodies against talin (A2 and B11) in a quantitative immunotransblot assay. Examination of resting platelets revealed that calpain I existed as the 85-kd proenzyme form and that ABP, GPIb, and talin existed in their native intact forms. When platelets were aggregated with thrombin, autoproteolysis of calpain I occurred within the 30 seconds required to completely solubilize platelet aggregates in sodium dodecyl sulfate–containing buffer and not as a direct result of thrombin-induced activation. When EDTA was added to platelet samples before the addition of sodium dodecyl sulfate lysis buffer, negligible proteolysis of calpain I, ABP, or talin occurred. When platelets were lysed with sodium dodecyl sulfate in the absence of EDTA, autoproteolysis of calpain I and cleavage of ABP and talin occurred. GPIb is not proteolyzed under either condition. In parallel immunofluorescent studies, examination of thrombin-stimulated platelets demonstrated minimal surface staining. Permeabilization revealed a redistribution of the calpain I epitope to an intracellular circumferential ring pattern. We conclude that with thrombin-induced platelet activation, calpain I redistributes intracellularly in the absence of activation of the 85-kd proenzyme form or significant hydrolysis of three important calpain substrates, ABP, GPIb, and talin. Failure to chelate divalent cations at the time of platelet lysis in sodium dodecyl sulfate enables the redistributed calpain I in thrombin-activated platelets to efficiently proteolyze ABP and talin. This proteolysis is initiated at the time of solubilization and is unrelated to thrombin aggregation per se. These findings raise further doubts concerning the role of calpain I in the events that initiate and maintain thrombin-induced platelet aggregation. (Arteriosclerosis and Thrombosis 1991;11:882–891)
It is well documented that human platelets contain calcium-activated neutral proteinase (calpain, EC 3.4.22.17). Calpain activity is evident in platelet lysates created by sonication or by solubilization in Triton X-100 (Tex) in the absence of calpain inhibitors and in the presence of free calcium ions (Ca\(^{2+}\)). In such lysates, calpain catalyzes the limited proteolysis of actin-binding protein (ABP), talin/P235, glycoprotein Ib (GPIb), fibrinogen, and von Willebrand factor, as evidenced by the requirement for Ca\(^{2+}\) and inhibition by leupeptin, antipain, N-ethylmaleimide, or mersalyl.

Fox and coworkers reported the hydrolysis of ABP and talin on treatment of human platelets with thrombin, collagen, or the Ca\(^{2+}\) ionophore A23187. Hydrolysis products of the two proteins were identified and appeared to be identical to the cleavage products found when platelets are lysed in the absence of calpain inhibitors (EDTA or leupeptin). Thus, it was concluded that on stimulation of platelets, calpain is activated and hydrolysis of at least two of its substrates (ABP and talin) occurs.

Complexes containing GPIb and ABP can be isolated in detergent extracts of platelets. Such complexes are thought to represent attachment sites between the plasma membrane and the cytoskeleton that may be involved in maintaining the structure of platelets and in the shape change that occurs on platelet activation. Because GPIb, ABP, and talin are substrates for calpain and hydrolysis of ABP and talin has been reported to occur during platelet aggregation, it is thought that calpain hydrolysis of GPIb-ABP complexes and/or talin may be involved in platelet activation.

Two calpains requiring either low (calpain I) or high (calpain II) concentrations of Ca\(^{2+}\) have been described in numerous tissues, including platelets. Muscle calpains I and II undergo limited autoproteolysis, resulting in a decreased requirement for Ca\(^{2+}\) and an enhanced proteolytic activity. Recently, Samis and coworkers have reported that intracellular platelet calpain I is not active until it is converted to a 76–78-kd form. Autoproteolysis of calpain is thus an indicator of the state of activation of the enzyme. In the present study, we investigated whether calpain I undergoes autoproteolysis during thrombin-induced platelet aggregation, whether this activation of the proteinase correlates with its redistribution to the site of GPIb-ABP interaction, and whether such redistribution initiates hydrolysis of the substrates ABP, GPIb, and talin.

**Methods**

**Chemicals**

The following were purchased from Sigma Chemical Co., St. Louis, Mo.: bovine serum albumin (re-crystallized once, essentially globulin free) and poly-L-lysine. Paraformaldehyde was obtained from Polysciences, Warrington, Pa. Sepharose 2B, protein A-Sepharose, and Sephadex G-100 superfine were obtained from Pharmacia, Inc., Piscataway, N.J. Tx was purchased from J.T. Baker Chemicals, Phillipsburg, N.J. All other chemicals were reagent grade. α-Thrombin was the generous gift of John Fenton, New York State Department of Health, Albany, N.Y., and Richard A. Marlar, Blood Center of Southeastern Wisconsin, Milwaukee, Wis.

**Platelet Preparation**

Platelet-rich plasma was prepared from acid/citrate/dextrose-anticoagulated whole blood obtained from healthy aspirin-free volunteers as described. Washed platelets were subsequently prepared by centrifugation and gel filtration on Sepharose 2B as described. In activation studies platelets were stimulated with α-thrombin at concentrations of 0.2–1 unit/ml at 37°C for 0.5, 1, 2, 3, or 5 minutes, at which time the cells were either fixed with 2% paraformaldehyde and processed for immunofluorescence studies or solubilized in sample buffer (25 mM tris[hydroxymethyl]aminomethane [Tris] HCl, 25% glycerol, 1% sodium dodecyl sulfate [SDS], pH 6.8, with or without 0.25 mM EDTA), vortexed immediately, and processed for immunontransblot studies.

**Antibodies**

John S. Elce (Queen's University, Kingston, Canada) kindly provided the cell line (B27D8 sc E11) that produces a mouse monoclonal immunoglobulin (Ig) G1 (B27D8) specific for the heavy chain (catalytic subunit) of calpain I. The specificity of B27D8 was confirmed by quantitative immunontransblot of a platelet cytosome fraction as described. Although B27D8 also reacts with calpain II, the cross-reactivity is extremely low and negligible in these experiments. The cell line B27D8 sc E11 was used to produce ascites fluid in BALB/c BYJ mice (Jackson Labs, Bar Harbor, Me.), and IgG was purified by combining and modifying the rivanol and caprylic acid procedures. For immunofluorescence stud-
FIGURE 1. Photomicrographs showing immunofluorescent localization of calpain I in resting platelets. Intact or detergent-permeabilized fixed platelets were stained with B27D8 (a monoclonal anti-calpain I antibody) and rabbit anti-actin and counterstained with rhodaminated goat anti-mouse immunoglobulin G (IgG) and fluoresceinated goat anti-rabbit IgG. Intact resting cells demonstrated minimal surface staining for both calpain (panel a) and actin (panel b). Permeabilization revealed extensive immunofluorescent staining for both proteins, which occupied the cell interior (panels c and d). Cells doubly labeled with irrelevant ascites control (panel e) and rabbit pre-immune serum (panel f) did not demonstrate significant labeling. Tx, Triton X-100. ¥1,200.

Immunofluorescence Microscopy

Immunofluorescent staining of platelets was performed as previously described. Briefly, resting or thrombin-stimulated platelets were fixed with 2% paraformaldehyde on ice for 1 hour. Unreacted ies the IgG fraction was concentrated fivefold by use of a Centricon 30 microconcentrator (Amicon, Danvers, Mass.). The murine monoclonal anti-GPIb antibody LJ1b10 was a gift of Zaverio Ruggeri, Scripps Clinic and Research Foundation, La Jolla, Calif. Two monoclonal antibodies, 1B3 and 3D1, directed against ABP and its major proteolytic fragments at 190, 100, and 90 kd were kindly provided by Robert Ezzell, Whitehead Institute for Biomedical Research, Cambridge, Mass. Rabbit polyclonal antibodies A2 and B11 raised against avian smooth muscle talin and human platelet talin, respectively, were generously provided by Mary Beckerle, University of Utah, Salt Lake City, Utah.

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Immunofluorescence Microscopy

Immunofluorescent staining of platelets was performed as previously described. Briefly, resting or thrombin-stimulated platelets were fixed with 2% paraformaldehyde on ice for 1 hour. Unreacted
FIGURE 3. Photomicrographs showing time course of redistribution of calpain I. Platelets were stimulated with 1 unit/ml thrombin and stained as in Figure 2. Examination of additional timepoints at 1 (panels a and b), 2 (panels c and d), 3 (panels e and f), and 5 (panels g and h) minutes (min.) revealed that redistribution of calpain I to a peripheral ring pattern is maintained over time. ×1,200.

aldehyde was blocked with NH₄Cl-Tris-buffered saline, pH 7.4, and the cells were permitted to settle on poly-L-lysine-coated glass coverslips. In some cases, the cells were treated with 0.1% TX for 3 minutes to render them permeable to antibody before staining. The permeable or intact cells were then rinsed with Tris-buffered saline containing 0.1% bovine serum albumin and incubated for 20 minutes with either the primary antibody (B27D8), an irrelevant ascites, or preimmune serum control. After the cells were rinsed they were stained with rhodaminated goat F(ab')₂ anti-mouse IgG (Organon-Technika, Cappel Division, Malvern, Pa.). In double-label experiments, cells were additionally stained with rabbit anti-actin (ICN Biomedicals, Inc., Costa Mesa, Calif.) and counterstained with a fluoresceinated goat anti-rabbit IgG (Organon-Technika).

The platelets were viewed with a Zeiss standard 16 microscope equipped with an HBO 50-W mercury lamp, an IVF1 epifluorescence condenser, BP 485 and 546 excitation filters, and BP 520-560 and LP 590 barrier filters and were photographed with Kodak Tri-X panchromatic film (Eastman Kodak, Rochester, N.Y.).

Quantitative Immunotransblot Assay for Calpain I

Calpain I heavy-chain antigen was quantified by immunotransblot assay as described. Briefly, solubilized platelet proteins were reduced with 5% β-mer-
FIGURE 5. Photograph of immunotransblot assay of calpain I in thrombin-stimulated platelets. Standards (lane 1) and composite transblots of thrombin-stimulated platelets were prepared as in Figure 4. Sample treatments were 1 unit/ml thrombin (lane 2), 0.05 unit/ml thrombin (lane 3), and 1 unit/ml thrombin, with 0.25 mM EDTA added before lysis (lane 4). Time of stimulation for all samples was 1 minute.  

captoethanol and were resolved by 5% SDS–polyacrylamide gel electrophoresis (PAGE) to allow resolution of 85-, 81-, and 78-kd bands. The proteins were electrophoretically transferred to nitrocellulose, and calpain I heavy chain was detected with B27D8 followed by iodine-125–labeled rabbit anti-mouse Ig. The labeled bands were cut out using the autoradiograph as a guide, and the amount of 125I was quantified. Additionally, parallel samples were transblotted with LJIb10 (to detect GPIb and its proteolytic product glycocalicin), with 1B3 and 3D1 (to detect ABP and its major proteolytic fragments at 190, 100, and 90 kd), and with A2 and B11 (to detect talin).

To verify the separation of calpain I antigen into three bands on each gel, a standard was prepared as follows. A platelet cytosolic fraction was incubated at 4°C for 1–5 minutes in the presence of low concentrations of Ca2+ (0.2 mM). Under these conditions, calpain I heavy chain undergoes limited autoproteolysis. Aliquots were chosen that contained significant amounts of the three forms of calpain I (85, 81, and 78 kd). These standard preparations were run on SDS-PAGE gels along with test samples to verify the separation of calpain I into three distinct bands.

Results
Immunofluorescent Localization of Calpain I in Resting and Thrombin-Stimulated Platelets

Intact resting platelets doubly labeled with B27D8 and rabbit anti-actin demonstrated minimal surface staining (Figures 1a and 1b). Cells made permeable with Tx demonstrated extensive intracellular staining for both antigens (Figures 1c and 1d), while irrelevant ascites (Figure 1e) and rabbit preimmune controls (Figure 1f) remained negative. Intact platelets that had been stimulated with 1 unit/ml thrombin for 30 seconds also demonstrated minimal surface labeling (Figures 2a and 2b). These cells, subsequently stained in the permeable state, revealed a centralized clearing or an apparent redistribution of the B27D8 antigen to a peripheral fluorescent ring pattern (Figure 2c). That this pattern represents an internal redistribution is evidenced by the lack of labeling when cells were stained in the intact state.

Examination of multiple time points revealed that the peripheral distribution of calpain I is maintained while the cells subsequently aggregate (Figure 3). Based on the redistribution of calpain I antigen seen by immunofluorescence microscopy, we were compelled to evaluate the state of activation of this protease under the same conditions. To do so, we took advantage of the fact that once activated, calpain I undergoes autoproteolysis and the apparent molecular weight of its catalytic subunit decreases from 85 to 81 and 78 kd.

Calpain I Catalytic Subunit in Resting Platelets

By immunotransblot assay, the majority of resting gel-filtered platelet preparations contained calpain I as a single 85-kd band (Figure 4, lanes 2 and 3). However, some preparations demonstrated significant amounts of the 81-kd band (Figure 4, lanes 4 and 5). To determine the effect of external Ca2+ on this apparent variability, gel-filtered platelets were incubated at 37°C for 30 minutes in the presence or absence of 2 mM external Ca2+. In the absence of external Ca2+, no detectable amounts of the 81-kd form of calpain I were observed (Figure 4, lanes 6 and 7). In contrast, the 81-kd band was consistently observed in cells incubated in the presence of external Ca2+ (Figure 4, lanes 8 and 9). The amount of the 85- or 81-kd forms of calpain I in resting platelets was...
not affected by the presence or absence of EDTA in the solubilization buffer.

**Calpain I Catalytic Subunit in Thrombin-Stimulated Platelets**

After stimulation of gel-filtered platelets with 1 unit/ml thrombin, significant proteolysis of calpain I was observed. As shown in Figure 5, lane 2, 50–80% of the total calpain I antigen was hydrolyzed to lower-molecular-weight forms (81 and 78 kd) within 1 minute after addition of thrombin. Under these conditions, total calpain I antigen was conserved (±10% assay variability) over a 10-minute time course. In addition, the 81-kd form was still detected even when the thrombin dose was lowered to 0.05 unit/ml (Figure 5, lane 3). At all thrombin doses tested (0.02–1.0 unit/ml), hydrolysis occurred as early as 30 seconds and temporally preceded any detectable macroaggregation, as viewed with an aggregometer. Maximum autoproteolysis was detected by 3 minutes.

To differentiate between autoproteolysis occurring as a result of thrombin stimulation from that potentially resulting from solubilization procedures, 0.25 mM EDTA was added to thrombin-stimulated platelet aggregates before addition of solubilization buffer. As shown in Figure 5, lane 4, this treatment inhibited detectable autoproteolysis.

With the assumption that autoproteolysis is a true indicator of calpain I activation, these results suggest that redistribution of calpain I and autoproteolysis/activation are independent events. Because proteolysis of ABP and talin by calpain has been reported to occur after thrombin stimulation and GPIb forms a complex with ABP, we sought to determine the status of ABP, GPIb, and talin under the same conditions by the immunoblot approach.

**Proteolysis of Calpain I, Glycoprotein Ib, Actin-Binding Protein, and Talin in Resting and Thrombin-Stimulated Platelets**

Nonactivated platelets lysed in the presence or absence of EDTA demonstrated the 85-kd form of calpain (Figure 6, lanes 1 and 3). To assess the effect of thrombin-induced aggregation, platelets were incubated with thrombin for 5 minutes at 37°C with stirring, at which time the platelets were irreversibly macroaggregated (Figure 7). Thrombin-aggregated platelets lysed in the absence of EDTA demon-
Table 1. Quantification of Thrombin-Induced Proteolysis of Calpain I, Actin-Binding Protein, and Talin

<table>
<thead>
<tr>
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<th>Without thrombin or EDTA</th>
<th>With thrombin, without EDTA</th>
<th>Without thrombin, with EDTA</th>
<th>With thrombin and EDTA</th>
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<tr>
<td>Calpain (B27D8)</td>
<td></td>
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<td>85 kd</td>
<td>1,163±163 (90±4)</td>
<td>1,021±264 (67±16)</td>
<td>1,115±147 (92±3)</td>
<td>1,214±26 (89±4)</td>
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<td>81 kd</td>
<td>69±26 (5±2)</td>
<td>371±149 (24±9)</td>
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<td>78 kd</td>
<td>54±26 (4±2)</td>
<td>146±138 (9±8)</td>
<td>35±17 (3±2)</td>
<td>57±23 (4±2)</td>
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<td>ABP (1B3)</td>
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<td>Parent</td>
<td>1,616±117 (98±1)</td>
<td>1,856±641 (97±2)</td>
<td>1,726±133 (98±1)</td>
<td>1,850±163 (98±2)</td>
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<td>190 kd</td>
<td>28±14 (2±1)</td>
<td>59±72 (3±2)</td>
<td>28±14 (2±1)</td>
<td>47±43 (2±2)</td>
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<td>Total</td>
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<td>1,916</td>
<td>1,754</td>
<td>1,898</td>
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<td>ABP (3D1)</td>
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<tr>
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<td>1,655±185 (99±1)</td>
<td>1,517±60 (93±2)</td>
<td>1,675±165 (96±2)</td>
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<td>100 kd</td>
<td>12±20 (1±1)</td>
<td>80±32 (5±2)</td>
<td>39±30 (2±2)</td>
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<tr>
<td>90 kd</td>
<td>5±7 (0,±1)</td>
<td>80±32 (5±2)</td>
<td>39±30 (2±2)</td>
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<td>Talin (A2)</td>
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<td></td>
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</tr>
<tr>
<td>223 kd</td>
<td>773±139 (90±8)</td>
<td>633±103 (78±14)</td>
<td>736±49 (93±6)</td>
<td>741±96 (91±4)</td>
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<td>200 kd</td>
<td>93±98 (10±8)</td>
<td>190±150 (22±14)</td>
<td>54±53 (7±6)</td>
<td>78±32 (10±4)</td>
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<td>Total</td>
<td>886</td>
<td>823</td>
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<td>Talin (B11)</td>
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<td>223 kd</td>
<td>4,825±703 (95±2)</td>
<td>4,010±660 (70±7)</td>
<td>4,775±738 (94±4)</td>
<td>4,671±675 (91±3)</td>
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Values are counts per minute obtained from corresponding gel slices and represent mean±SD of four determinations. Percentage of total counts per minute is in parentheses. ABP, actin-binding protein.

strated significant proteolysis with the appearance of the 81- and 78-kd forms of calpain (Figure 6, lane 2). Inclusion of EDTA to such aggregates before lysis (Figure 6, lane 4) resulted in no detectable 81- or 78-kd forms. Parallel studies run on the same lysates blotted with a monoclonal anti-GPIb antibody, LJIb10, indicated that GPIb remained intact under all conditions tested (Figure 6, lanes 6–9).

To evaluate activation of calpain I and hydrolysis of ABP and talin, platelet lysates from four individuals were blotted with a panel of monoclonal and polyclonal antibodies. The results obtained with each of the four individuals were nearly identical, and mean values are listed in Table 1. Figure 8 depicts a typical set of results from one individual. Lysates blotted with B27D8 confirmed that autoproteolysis of calpain I occurred only when EDTA was omitted from thrombin-stimulated samples before lysis (Figure 8, lanes 1–4; and Table 1).

Parallel blots prepared with 3D1 and 1B3, monoclonal anti-ABP antibodies that recognize the 190- and 90–100-kd proteolytic fragments of ABP, respectively, are shown in Figure 8. When 3D1 was used as the primary antibody, 190-kd fragments of ABP were detected in platelets aggregated by thrombin (Figure 8, lanes 5–8). 1B3 blots revealed detectable 100- and 90-kd fragments in identical samples (Figure 8, lanes 9–12). The percentage of degraded ABP, estimated by excising the bands identified by autoradiography and determining the counts per minute of 125I-labeled second antibody, is shown in Table 1.

These data suggest that a low level of proteolysis (3–7%) of ABP may occur as a result of platelet lysis in the absence of EDTA after thrombin activation. However, when EDTA was added to the thrombin aggregates before lysis, the counts per minute associated with the 90-, 100-, and 190-kd bands were essentially eliminated with no evidence of ABP digestion (Table 1). Blots prepared with A2, specific for avian smooth muscle talin (Figure 8, lanes 13–16), and B11, specific for human platelet talin (Figure 8, lanes 17–20), yielded similar results (Table 1). In the case of talin, the 200-kd fragment represents 9–10% of the total antigen when thrombin-aggregated platelets are lysed in the presence of EDTA. This compares with a value of 5–7% when the same platelets are lysed in the presence of EDTA but not treated first with thrombin. On the other hand, the same fragment represents 22–29% of antigen when platelets are aggregated by thrombin but lysed in the absence of EDTA.

Discussion

In the resting platelet, the calpain I catalytic subunit displays a homogeneous cytosolic distribution. This observation is in agreement with previous
subcellular fractionation studies that indicate that 95% of the total calpain I antigen as well as more than 95% of the total calpain proteolytic activity are found in the cytosolic fraction.\(^1,16\)

When platelets were activated by thrombin, we observed a redistribution of the calpain I antigen into a circumferential ring. This redistribution occurred within 30 seconds after addition of thrombin to gel-filtered platelets. Whether this reflects directed movement of calpain I antigen or is a passive event resulting from cytoskeletal reorganization and granule centralization remains to be determined. The requirement for permeabilization before visualization of the calpain I antigen by immunofluorescence microscopy suggests that the epitope defined by B27D8 is not accessible on the platelet surface, either in the resting state or after thrombin stimulation. The presumptive localization of calpain I to the cytoplasmic face of the plasma membrane and the fact that calpain is thought to exert its proteolytic effect on components of the plasma membrane–cytoskeleton interface\(^1-4,7\) would be consistent with the argument that redistribution of calpain I is involved in modulation of membrane–cytoskeleton attachment sites during platelet activation.

To determine whether redistributed calpain I was present in its proenzyme or activated form, we employed a quantitative immunotransblot assay.\(^16\) Resting gel-filtered platelets incubated in the absence of external Ca\(^2+\) or in the presence of prostaglandin E\(_1\) plus EDTA\(^16\) demonstrated the 85-kd form of calpain I. In contrast, platelets incubated in the presence of external Ca\(^2+\) exhibited both the 85- and the 81-kd forms. These results suggest that in the resting cell, calpain I exists as the 85-kd form and that a variable amount of proteolysis occurs during the process of gel filtration. Similarly, when platelets were stimulated with thrombin, we observed that autoproteolysis of calpain I occurred within the 30-second time frame required to completely solubilize platelet aggregates in SDS-containing buffer. Addition of EDTA to the aggregate before solubilization completely inhibited detectable autoproteolysis. With the assumption that autoproteolysis is a true reflection of calpain activation, these results suggest that calpain I is not activated as a result of thrombin-induced platelet aggregation, in agreement with the recent reports by Samis et al\(^13\) and Fox and Elce.\(^23\)

Given the magnitude of the previously reported proteolysis of ABP and talin by calpain after thrombin stimulation\(^7\) and the known association of GPIb with ABP\(^6,21,22\) we sought to determine the status of ABP, GPIb, and talin under the same conditions with the immunoblot method. GPIb remained intact un-
under all conditions tested. In contrast, the 90-, 100-, and 190-kd proteolytic fragments of ABP were detected in platelets aggregated by thrombin. After elimination of artificial proteolysis of ABP associated with lysis in the absence of EDTA, it is apparent that the degree of degradation of ABP is considerably smaller than previous estimates and is essentially negligible. These results question the significance of ABP proteolysis during thrombin-induced aggregation. While low-level degradation of ABP localized to specialized regions of the cytoskeleton during thrombin-induced aggregation may be important, more precise quantification of that degree of hydrolysis is required to document this point. To date, none of the approaches that have been used to quantify ABP hydrolysis have had the necessary degree of sensitivity. The quantitative immunoblot assay employed in this study is more sensitive than previously applied techniques yet still fails to detect significant hydrolysis of ABP as a result of thrombin-induced aggregation. Similarly, quantitative immunoblots with antibodies against talin revealed a lack of significant proteolysis occurring as a result of thrombin stimulation.

Our results seriously question a role for calpain I in the events that initiate and complete irreversible platelet aggregation. In addition, our results indicate that autoproteolysis of calpain I does not coincide with and is probably not required for its peripheral redistribution as a result of thrombin activation (aggregation). Thus, calpain migrates to the platelet periphery in an inactivated form. If autoactivation via proteolysis is then required for proteolysis of ABP or talin, then this, too, must occur at the point of subsequent platelet solubilization. A most intriguing observation that follows from our data is that platelet aggregation brings platelet calpain and certain of its substrates (e.g., talin) in close physical proximity, thereby predisposing them to subsequent proteolysis. The exact nature of this physical proximity and the role, if any, of subsequent proteolysis in the events that follow activation remain interesting questions that beg further investigation.

Acknowledgments

The authors gratefully acknowledge the generous gift of the murine hybridoma cell line of B27/D8 sc E11 from John S. Elce, Queen’s University, Kingston, Canada. LJIb10, a monoclonal anti-GP Ib antibody, was a gift of Zaverio Ruggeri, Scripps Clinic and Research Foundation, La Jolla, Calif. Two monoclonal antibodies designated 1B3 and 3D1 directed against ABP were kindly provided by Robert Ezzell, Whitehead Institute for Biomedical Research, Cambridge, Mass. Additionally, rabbit polyclonal antibodies A2 and B11 raised against avian smooth muscle talin and human platelet talin, respectively, were generously provided by Mary Beckerle, University of Utah, Salt Lake City, Utah. Finally, we would like to acknowledge the technical expertise and photography of Michael G. Dieter.

References


KEY WORDS • immunofluorescence • quantitative immunotransblot assay • calpain I
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