Role of Calcium and Calpain in Complement-Induced Vesiculation of the Platelet Plasma Membrane and in the Exposure of the Platelet Factor Va Receptor†

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ABSTRACT: The role of calcium and intracellular calpains in the expression of platelet prothrombinase activity was investigated. Incubation of gel-filtered platelets with complement proteins C5b-9 resulted in α-granule and dense granule secretion and exposure of membrane binding sites for coagulation factors Va and Xa. This was accompanied by the release of microparticles from the cell surface that incorporated plasma membrane glycoproteins GP Ib, IIb, and IIIa and the α-granule membrane protein GMP-140. Generation of these membrane microparticles was dependent on the presence of extracellular calcium and was accompanied by proteolytic degradation of the cytoskeletal proteins, actin binding protein (ABP), talin, and myosin heavy chain. Microparticle formation was also detected when unstirred platelets were activated by thrombin plus collagen, although proteolysis of ABP, talin, or myosin was not observed. Preincorporation of the calpain inhibitor leupeptin into the platelet cytosol completely blocked C5b-9-induced proteolysis of ABP, talin, and myosin. However, inhibition of this calpain-mediated proteolysis had no effect on platelet secretion, the generation of microparticles, the exposure of membrane sites for factors Va and Xa, or the expression of prothrombinase activity. Furthermore, the microparticles that formed in the presence of leupeptin contained intact ABP, talin, and myosin heavy chain. Prior depletion of ATP with metabolic inhibitors eliminated all platelet responses to thrombin plus collagen, but did not affect C5b-9-induced microparticle formation or exposure of binding sites for factor Va on the microparticles. These data indicate that the formation of microparticles and the expression of platelet prothrombinase activity in response to C5b-9 are dependent upon an influx of calcium into the platelet cytosol, but do not require metabolic energy or calpain-mediated proteolysis of cytoskeletal proteins.

Complement proteins C5b-9 are known to activate intracellular protein kinases and to stimulate the secretory degranulation of human platelets and endothelial cells (Wiedmer et al., 1987; Hattori et al., 1989; Sims, 1989). Activation of platelets by C5b-9 is accompanied by vesiculation from the cell surface of small membrane vesicles that contain cell surface glycoproteins Ib, IIb, and IIIa and GMP-140,[1] a glycoprotein derived from the membrane of α-granules (Sims et al., 1988). These vesicles, or "microparticles", have been shown to express a high density of binding sites for coagulation factor Va and to provide a catalytic surface for assembly of the prothrombinase enzyme complex (factors Va and Xa). In addition to exposing membrane sites for prothrombinase assembly and thereby accelerating blood clotting, the generation of these microparticles plays an integral role in protecting platelets from the cytolytic consequences of complement activation, by sequestering and removing functional C5b-9 pores from the plasma membrane (Sims & Wiedmer, 1986; Sims et al., 1988).

The C5b-9-induced microparticles contain C9 antigen at high density, indicating that they are selectively shed from the platelet surface near the site of the inserted C5b-9 proteins (Sims & Wiedmer, 1986; Sims et al., 1988). These vesicles also incorporate GP Ib and other integral membrane glycoproteins that are normally anchored to the platelet cytoskeleton. This suggests that the C5b-9 proteins may initiate blebbing and vesiculation of the plasma membrane by liberating GP Ib and other transmembrane proteins from their associations with the cytoskeleton. Release of GP Ib from its attachment to actin binding protein (ABP) has been demonstrated to occur in platelets stimulated to aggregate by thrombin, collagen, or calcium ionophore, and this process has been related to the proteolytic cleavage of ABP by one or more calcium-dependent cellular proteases (calpains; Phillips & Jakabova, 1977; Fox et al., 1983, 1985; Fox, 1985a,b). Calpain-mediated proteolysis of ABP has also been observed to correlate with the expression of platelet prothrombinase activity, implying a role for intracellular calpain in the exposure of the plasma membrane receptor for factor Va (Verhallen et al., 1987, 1988). In this paper, we have used platelets

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[1] Abbreviations: GMP, granule membrane protein; GP, glycoprotein; FITC, fluorescein 5-isothiocyanate; PIPES, 1,4-piperazinediethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ABP, actin binding protein.

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induced to vesiculate by the C5b-9 proteins to investigate the role of platelet calpains in the release of GP Ib containing membrane microparticles and in the exposure of membrane receptors for factor Va.

Experimental Procedures

Materials. Bovine serum albumin (globulin and fatty acid free), prostaglandin E1, apyrase, and *N*-hydroxysuccinimide biotin ester were obtained from Sigma; A23187 and leupeptin were from Behring Diagnostics; (*p*-amidinophenyl)methanesulfonyl fluoride was from Med Cal; fluorescein 5-isothiocyanate–Celite was from Calbiochem; phycoerythrin–streptavidin conjugate was from Southern Biotechnology Associates (Birmingham, AL), and Spectrozyme TH was from American Diagnostica (Greenwich, CT). Murine monoclonal antibody S12, specific for the platelet α-granule membrane glycoprotein, GMP-140, was a gift from Dr. Rodger P. McEver (Oklahoma Medical Research Foundation, Oklahoma City) (McEver & Martin, 1984). Murine monoclonal antibody AP1, specific for membrane glycoprotein, GP Ib, was from Dr. Thomas J. Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee). Murine monoclonal antibody V237 recognizes an epitope in the light chain of factor Va (human or bovine) and binds to both factor V and factor Va (Sims et al., 1988). Murine monoclonal antibody PAC-1 recognizes an activation-associated neoepitope in the platelet glycoprotein IIb–IIIa complex (Shattil et al., 1985). Affinity-purified rabbit antibody against actin binding protein was a gift from Dr. Joan E. B. Fox (Gladstone Foundation, San Francisco, CA). Bovine factors Va and Xa, prothrombin, thrombin, and the light chain of factor Va were gifts from Dr. Charles T. Esmon (Oklahoma Medical Research Foundation). All other chemicals were of reagent or analytical grade.

Solutions. Solution I: 145 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.1% (w/v) glucose, 0.1% (w/v) bovine serum albumin, and 5 mM PIPES, pH 6.8. Solution II: 137 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.1% glucose (w/v), 0.1 bovine serum albumin, and 20 mM HEPES, pH 7.4.

Fluorescence Labeling and Radiolabeling of Proteins. All monoclonal antibodies were conjugated with FITC, except antibody AP1, which was conjugated with *N*-hydroxysuccinimide biotin ester as described previously (Sims et al., 1988). Factor Xa and antibody S12 were labeled with $Na^{125}I$ using IODO-GEN, and unincorporated label was removed by gel filtration followed by dialysis. Specific activities are given in figure legends.

Protein Concentrations. Concentrations of unlabeled proteins were estimated assuming the following extinction coefficients ($E^{1\%}_{280}$): murine IgG, 15; factor Va, 15.1; factor Va light chain, 18.7; factor Xa, 12.4; prothrombin, 15.5; C5b6, 10; C7, 9.9; C8, 15.1; C9, 9.6. The concentrations of FITC-labeled proteins were determined by the dye binding assay (Bio-Rad), using the respective unlabeled protein as standard. FITC concentration was determined assuming a molar extinction (492 nm) of 68 000.

Activation of Platelets by C5b-9 and Other Agonists. Gel-filtered human platelets were prepared and collected into solution I at (1–2) $\times 10^9$ mL as described previously (Wiedmer et al., 1987). Human complement proteins C5b6, C7, C8, and C9 were purified and analyzed for functional activity according to methods previously described (Wiedmer & Sims, 1985). To assemble membrane-bound C5b67 complexes, gel-filtered platelets (10^9/mL) were incubated for 3 min at 37 °C with C5b6 (5–15 $\mu g/10^8$ platelets, equivalent to 17–50 pmol/10^8 platelets) and C7 (1–2.5 $\mu g/10^8$ cells, equivalent to 8–21 pmol/10^8 platelets). To assemble membrane-bound C5b-9 complexes, C5b67 platelets were generally suspended to 10^8 platelets/mL in solution II containing 2.5 mM $CaCl_2$. Then C8 (4 $\mu g/10^8$ cells, equivalent to 27 pmol/10^8 cells) and C9 (10 $\mu g/10^8$ cells, equivalent to 140 pmol/10^8 cells) were added, and the cells were incubated at 37 °C without stirring for 10 min. In all experiments, comparison was made to identical matched-pair controls (platelets incubated in the absence of the C5b-9 proteins). In certain experiments, comparison was also made to gel-filtered platelets exposed to thrombin (0.1–0.5 unit/mL) and collagen (10 μg/mL). In these cases, cells were incubated with agonists (10 min, or as indicated) at 37 °C without stirring.

Incorporation of Leupeptin into Platelet Cytosol. Because of the slow and variable influx of leupeptin into the cytosol of untreated platelets (Tsujinaka et al., 1988; our unpublished data), the following protocol was employed to preload leupeptin into C5b-9 platelets: C5b67 platelets were suspended in solution II containing 0.1 mM EGTA and incubated at 37 °C with C8, C9, and leupeptin (0–2 mM). After 2 min, platelet activation was initiated by addition of 2.5 mM $CaCl_2$. As previously described, assembly of the C5b-9 complex in 0.1 mM EGTA results in permeabilization of the platelet plasma membrane while maintaining low cytosolic calcium, thereby preventing activation of protein kinases and granule secretion until the subsequent addition of calcium to the medium (Wiedmer et al., 1987).

Metabolic Inhibition. Gel-filtered platelets (C5b67 or controls) were suspended at 10^8/mL in solution II (omitting glucose) containing 16.7 mM 2-deoxyglucose, 19 μM antimycin A, and 2.5 mM $CaCl_2$. After 10-min incubation at 37 °C, platelet activation was initiated by addition of C8 and C9, or other agonists, as indicated.

Secretion Assays. Dense granule secretion was measured by the release of [^{14}C]serotonin (Valdorf-Hansen & Zucker, 1971). α-Granule secretion was monitored by the surface expression of GMP-140, detected by the binding of either radiolabeled or FITC-labeled monoclonal antibody S12 (McEver & Martin, 1985; Wiedmer et al., 1987; Sims et al., 1988).

Prothrombinase Assay. Platelet prothrombinase activity was measured by modification of methods previously described, using the chromogenic substrate Spectrozyme TH (Wiedmer et al., 1986a). After activation, platelets were diluted (final concentration, 4×10^6/mL) in solution II containing 1% (w/v) albumin, 2.5 mM $CaCl_2$, 2 nM factor Va, and 2.7 μM prothrombin, and incubated at 37 °C. Prothrombin conversion was initiated by addition of factor Xa (2 nM final). When leupeptin-treated samples were assayed, non-leupeptin controls were adjusted to the same final leupeptin concentration (20 μM) immediately before addition of factor Xa (see Discussion). The reaction was stopped at 0, 30, and 60 s by transfer of 1 volume of sample into 9 volumes of ice-cold buffer containing 1% albumin and 10 mM EDTA, and thrombin was assayed as described previously (Wiedmer et al., 1986a). The binding of factor Xa to gel-filtered platelets was measured from the specific binding of the radiolabeled protein (Wiedmer et al., 1986b).

Preparation of Platelets for Flow Cytometry. A total of 5×10^6 C5b-9-treated or control platelets were incubated in the dark in a total volume of 60 μL for 10 min at 23 °C in the presence of biotin–AP1 (1 μg/mL; 6.7 nM IgG) and a saturating concentration of one of the following fluorescein-conjugated antibodies: FITC–PAC1 (30 μg/mL; 30 nM IgM), FITC–S12 (10 μg/mL; 67 nM IgG), or FITC–V237

(20 μg/mL; 134 nM IgG). In certain experiments, cells were first incubated for 10 min at 23 °C with factor Va light chain (2 μg/mL; 25 nM) before addition of FITC–V237. Following incubation with the labeled antibodies, phycoerythrin–streptavidin was added (5 μL of a 1:10 dilution), and the cells were incubated an additional 10 min. Then 0.5-mL aliquots of solution II were added and the samples analyzed by flow cytometry. All analyses were complete within 30 min.

Flow Cytometry. Samples were analyzed in a Becton Dickinson FACStar flow cytometer formatted for two-color analysis as previously described (Shattil et al., 1987; Sims et al., 1988). The light scatter and fluorescence channels were set at logarithmic gain. In order to resolve platelet-derived microparticles from background light scatter, acquisition was gated so as to include only those particles distinctly positive for biotin–AP1 (detected as phycoerythrin fluorescence), using a fluorescence lower limit threshold on the 585-nm channel that excluded background scatter. Thus, only those cells (and microparticles) expressing the platelet-specific membrane glycoprotein, GP Ib, were included for analysis. Ten thousand phycoerythrin-positive particles from each sample were analyzed for forward and right-angle light scatter and for FITC and phycoerythrin fluorescence intensities. All fluorescence data were corrected for platelet or microparticle autofluorescence (generally, eight arbitrary fluorescence units per particle). Fluorescence and light scatter were calibrated using 2-μm Calibrite beads by gating on forward light scatter (Becton-Dickinson).

Isolation of Platelet-Derived Microparticles. After platelet activation, cell suspensions were made 0.5 mM in leupeptin and centrifuged for 20 min at 800*g* to remove platelets, and the microparticle-containing supernatants were recovered. These centrifugation conditions removed >99.9% of the platelets from the microparticle-containing supernatant (Sims et al., 1988). For analysis of cytoskeletal proteins, microparticles were then concentrated by centrifugation (30 min; 12000*g*) and analyzed by gel electrophoresis (below).

Analysis of Cytoskeletal Proteins. Proteolytic breakdown of cytoskeletal proteins in platelets or concentrated microparticles was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis performed under reducing conditions. Samples were denatured by incubation at 100 °C for 3 min in 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, 1 mM EDTA, 4 mM EGTA, and 71 mM Tris, pH 8.0. Polyacrylamide gel electrophoresis was performed by using 7.5% homogeneous gels (PHAST System, Pharmacia) and developed by silver stain (Bio-Rad), or by using 7% homogeneous gels (Hoeffer Scientific) developed by Coomassie stain. ABP, talin, and myosin heavy chain, and the proteolytic fragments of these proteins generated by calpain cleavage, were identified as described (Phillips & Jakabova, 1977; White, 1980; Truglia & Stracher, 1981; Fox et al., 1983; Verhallen et al., 1987). Immunoblotting of actin binding protein and its proteolytic fragments was performed essentially as described by Fox et al. (1985). Following 7% SDS–PAGE and transfer to nitrocellulose, Western blotting was performed by overnight incubation with 2 μg/mL affinity-purified antibody to actin binding protein, and developed with ^{125}I protein A. Radioactive bands were then visualized and quantitated (AMBIS Radioanalytic Imaging System).

Results

C5b-9-Induced Microparticle Formation Is Accompanied by the Proteolytic Degradation of Cytoskeletal Proteins. We have shown previously that assembly of the terminal complement proteins on gel-filtered platelets results in the release of vesicles from the plasma membrane that are approximately 0.1 μm in diameter (Sims & Wiedmer, 1986; Sims et al., 1988). These vesicles, or "microparticles", are highly enriched in binding sites for coagulation factors Va and Xa and express prothrombinase activity. In addition, they contain the membrane-inserted C5b-9 proteins, the plasma membrane glycoproteins GP Ib, IIb, and IIIa, and GMP-140, a protein derived from the membrane of platelet α-granules. Generation of these microparticles does not require stirring and is not accompanied by platelet lysis, suggesting that they are formed by exocytic budding of plasma membrane (Sims & Wiedmer, 1986; Sims et al., 1988).

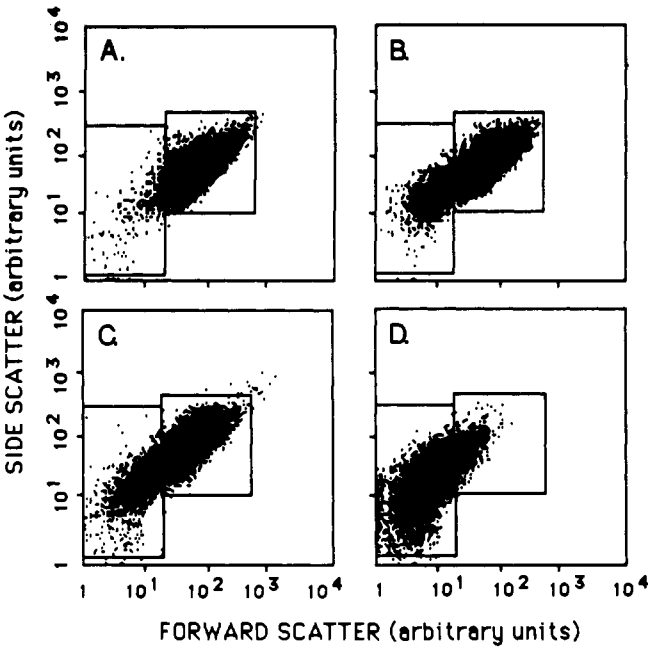

FIGURE 1: Release of GP Ib containing microparticles by activated platelets. Gel-filtered platelets were exposed to various agonists while suspended in solution II containing 2.5 mM $CaCl_2$. After incubation without stirring for 10 min at 37 °C, the cells were stained with monoclonal antibody AP1 (directed against GP Ib) and analyzed by flow cytometry. The red fluorescence threshold was set so that only GP Ib positive particles were analyzed (see Experimental Procedures). Two rectangles are drawn on each dot plot to denote analysis gates of forward (abscissa) and side (ordinate) angle light scatter used to discriminate platelets (right gate) and microparticles (left gate). (Panel A) Untreated control. Microparticles represented 6% of all particles analyzed. (Panel B) C5b67 platelets incubated with 4 μg of C8 plus 10 μg of $C9/10^8$ cells. Microparticles represented 27% of all particles analyzed. (Panel C) Platelets activated by thrombin (0.5 unit/mL) plus collagen (10 μg/mL). Microparticles represented 32% of all particles analyzed. (Panel D) Platelets activated by A23187 (1 μM). Microparticles represented 62% of all particles analyzed. Data of a single experiment, representative of six similar experiments performed on different days.

In order to monitor the release of these membrane vesicles from C5b-9-treated platelets, fluorescence-gated flow cytometry was employed using a labeled monoclonal antibody against GP Ib to resolve both platelets and platelet-derived microparticles (Shattil et al., 1987; Sims et al., 1988; Experimental Procedures). By these methods, small plasma membrane-derived vesicles (approximately 0.1-μm diameter) containing the integral membrane protein GP Ib can be differentiated from electronic noise and other light-scattering particles. As shown in Figure 1, C5b-9 assembly resulted in the generation of GP Ib containing microparticles. No microparticles formed when C8 and C9 were added to control platelets free of C5b67 (data not shown). These microparticles also formed when platelets were incubated without stirring with a combination of thrombin (0.5 unit/mL) plus collagen (10

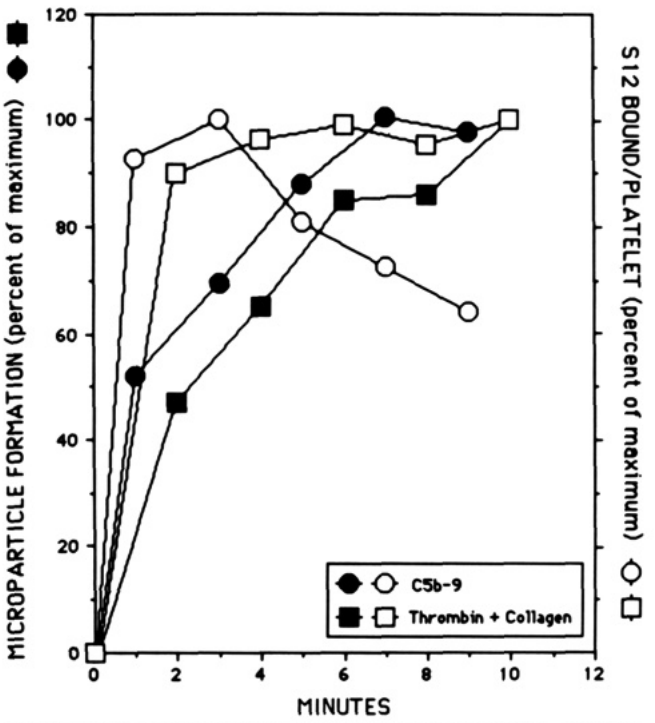

FIGURE 2: Time course of microparticle release and platelet α-granule secretion. Platelets were exposed to C5b-9 (open and closed circles) or to thrombin plus collagen (open and closed squares) under the conditions described for Figure 1 and then analyzed at the times indicated on the abscissa for activation-dependent microparticle release (left ordinate) and α-granule secretion (right ordinate). Platelet activation and microparticle formation were arrested at each time point by addition of 3 mM EDTA. Microparticle formation (closed symbols) was monitored by forward and side-angle light scatter, using the left-hand analysis gate depicted in dot plots of Figure 1. α-Granule secretion (open symbols) was monitored by surface expression of GMP-140, as measured by binding of FITC-S12 (see Experimental Procedures). All data were corrected for background microparticles and FITC-S12 binding of untreated controls and are expressed as the percent of the maximally stimulated responses. Data of a single experiment, representative of three similar experiments performed on different days.

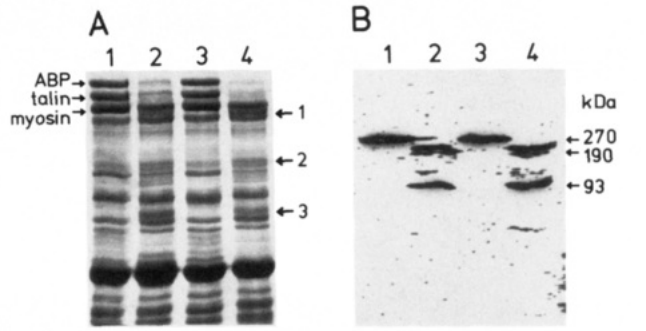

FIGURE 3: C5b-9-induced proteolysis of platelet cytoskeletal proteins. (Panel A) Platelets were suspended (5×10^8/mL) at 37 °C in solution II containing 2.5 mM $CaCl_2$ and activated by either C5b-9 (lane 2), 0.5 unit/mL thrombin plus 10 μg/mL collagen (lane 3), or 1 μM A23187 (lane 4). Following incubation with each agonist without stirring for 10 min at 37 °C, gel electrophoresis was performed, and bands were visualized by Coomassie stain. Untreated controls are shown in lane 1. (Panel B) After transfer to nitrocellulose, ABP and its proteolytic fragments were detected with anti-ABP followed by ^{125}I protein A and analyzed with the AMBIS Radioanalytic imaging system. Lanes 1 and 3 showed no reactivity (above background) at 190 or 93 kDa. Arrows denote ABP (270 kDa) and its proteolytic fragments, 190 kDa (fragment 1) and 93 kDa (fragment 3); talin (235 kDa) and its 190-kDa fragment (fragment 1); and the heavy chain of myosin (200 kDa) and its 135-kDa fragment (fragment 2). Data of a single experiment, representative of six similar experiments performed on different days.

μg/mL) or with the calcium ionophore A23187 (1 μM) (Figure 1). The formation of microparticles was detected within 1 min of the addition of C8 plus C9 to C5b67 platelets and was maximal by 5 min. This time course was similar to that observed for microparticle formation when control platelets were activated by thrombin plus collagen (Figure 2). The process of microparticle formation was somewhat slower than the process of platelet α-granule secretion in that surface expression of the α-granule membrane protein GMP-140 (measured by platelet binding of antibody S12) was maximal by 1–3 min (Figure 2). For platelets stimulated by C5b-9, surface-expressed GMP-140 was observed to decrease during incubations >3 min (open circles, Figure 2). Because the surface density of GMP-140 on the shed microparticles is enriched relative to the platelet plasma membrane, this loss of GMP-140 from the cell surface is likely to reflect its sequestration and removal in newly formed microparticles, subsequent to fusion of α-granules with the plasma membrane (Sims et al., 1988).

As illustrated in Figure 3, activation of platelets by the C5b-9 proteins was accompanied by proteolytic degradation of the cytoskeletal proteins, actin binding protein (native ABP = 270 kDa; proteolytic fragments, 190 and 93 kDa), talin (native = 235 kDa; proteolytic fragment = 190 kDa), and the heavy chain of myosin (native = 200 kDa; proteolytic fragment = 135 kDa) (Phillips & Jakabova, 1977; White, 1980; Truglia & Stracher, 1981; Fox et al., 1983; Verhallen et al., 1987). The identity of ABP and its proteolytic fragments was confirmed by immunoblotting with a monospecific antibody against the protein (Figure 3B). The extent of proteolysis of these three cytoskeletal proteins in complement-treated platelets was observed to vary considerably from one experiment to another, with increased proteolysis generally observed with increased input of the C5b-9 proteins (data not shown). Proteolysis of these cytoskeletal proteins was also observed for platelets treated with the calcium ionophore A23187. By contrast, under the conditions of these experiments (suspension at 37 °C without stirring), we were unable to detect significant proteolytic degradation of ABP, talin, or myosin heavy chain upon platelet activation by thrombin (0.5–1 unit/mL), collagen (10 μg/mL), or thrombin plus collagen (Figure 3; see Discussion).

The proteins depicted in Figure 3 necessarily include cytoskeletal components associated with both platelets and microparticles. In order to examine the cytoskeletal proteins specifically associated with the microparticles shed from C5b-9-treated platelets, a microparticle-enriched suspension was prepared by differential centrifugation. Examination of the cytoskeletal proteins associated with the shed microparticles revealed that only the proteolytically degraded forms of ABP, talin, and myosin heavy chain were present (Figure 4).

Requirement for Calcium in C5b-9-Induced Microparticle Formation and Cytoskeletal Proteolysis. We have shown previously that C5b-9 assembly results in an elevation of $[Ca^{2+}]$ in the platelet cytoplasm due to influx of the ion across the plasma membrane and that this occurs without liberation of inositol polyphosphates or the release of intracellular calcium stores (Wiedmer et al., 1987). This rise in cytosolic calcium concentration is abolished when the complement pore is assembled in calcium-free media, and under these conditions, the C5b-9-induced activation of cellular protein kinases and storage granule secretion do not occur (Wiedmer et al., 1987). As shown in Figure 5, generation of microparticles by the C5b-9 proteins was found to depend upon the concentration of extracellular calcium, implying that this process is activated

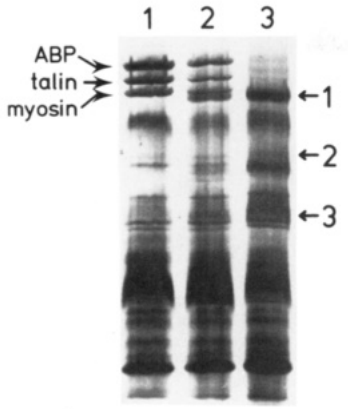

FIGURE 4: Analysis of cytoskeletal proteins associated with shed microparticles. 5×10^8 C5b67 platelets were suspended in 1 mL of solution II containing 2.5 mM $CaCl_2$, and C5b-9 assembly was initiated by addition of C8 and C9. After incubation without stirring for 10 min at 37 °C, microparticles were isolated by differential centrifugation and samples analyzed by gel electrophoresis (see Experimental Procedures). Lane 1, untreated platelet suspension; lane 2, C5b-9-treated platelet suspension (before centrifugation to isolate microparticles); lane 3, platelet-free microparticles isolated from C5b-9-treated suspension. Protein bands are identified as indicated in legend to Figure 3. Data of a single experiment, representative of three identical experiments performed on different days.

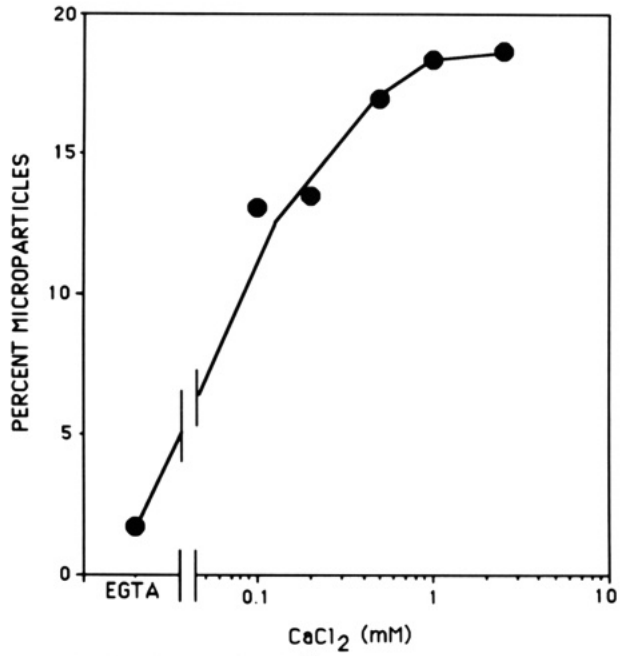

FIGURE 5: Requirement for Ca^{2+} in C5b-9-induced microparticle formation. C5b67 platelets (1×10^8/mL) were incubated (10 min, 37 °C) with C8 and C9 in solution II containing $CaCl_2$ at concentrations indicated on the abscissa. Microparticle formation was quantitated as described in the legend of Figure 1. EGTA denotes solution II containing 0.1 mM EGTA with no added calcium. Data shown are corrected for 11% background microparticles of complement-free controls incubated in 2.5 mM $CaCl_2$. Data of a single experiment representative of two so-performed.

as the result of Ca^{2+} influx across the plasma membrane. Microparticle formation was maximal at calcium concentrations ≥1mM. When platelets were suspended in 0.1 mM EGTA, microparticle formation was inhibited by >90%, but it could be restored by subsequent addition of 2.5 mM $CaCl_2$ to the platelet suspension (see below).

Proteolytic degradation of ABP, talin, and myosin heavy chain can be mediated by cytoplasmic calpains which are activated under conditions of increased cytosolic free calcium concentration (Phillips & Jakabova, 1977; White, 1980; Truglia & Stracher, 1981; Fox et al., 1983; Verhallen et al., 1987). Consistent with this, a strict requirement for extracellular calcium was also observed for the degradation of these cytoskeletal proteins upon platelet activation by C5b-9 (Figure 6). This proteolysis was not observed when platelets were exposed to the activated C5b-9 proteins while suspended in calcium-free medium, but it was observed when 2.5 mM $CaCl_2$ was subsequently added.

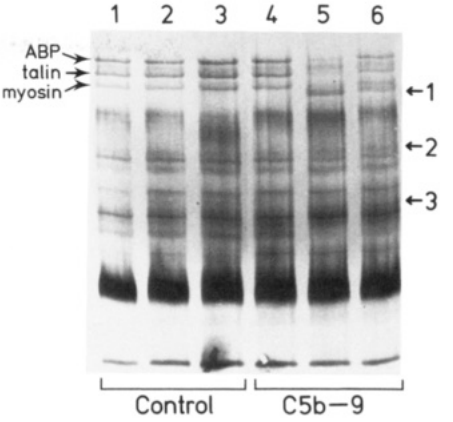

FIGURE 6: Requirement for Ca^{2+} in C5b-9-induced degradation of cytoskeletal proteins. C5b67 platelets were suspended to 10^8/mL in solution II containing either 0.1 mM EGTA (lanes 4 and 5) or 2.5 mM $CaCl_2$ (lane 6), and C5b-9 assembly was then initiated by addition of C8 and C9. After 2 min, 2.5 mM $CaCl_2$ was added to sample 5. Following 10-min incubation at 37 °C, all samples were denatured and analyzed by gel electrophoresis. Also shown are gels of complement-free control platelets incubated in either 0.1 mM EGTA (lane 1), 0.1 mM EGTA followed by 2.5 mM $CaCl_2$ (lane 2), or 2.5 mM $CaCl_2$ (lane 3). Protein bands are identified as detailed in the legend to Figure 3. Data of a single experiment, representative of six similar experiments performed on different days.

In order to demonstrate more directly a role for cytoplasmic calpains in the C5b-9-induced degradation of cytoskeletal proteins, the effect of leupeptin, a calpain inhibitor, was investigated (Umezawa, 1976). In these experiments, the platelet plasma membrane was permeabilized to leupeptin without cell activation by assembling the C5b-9 pore in the presence of 0.1 mM EGTA. Platelet activation was then initiated by adding 2.5 mM $CaCl_2$ (Wiedmer et al., 1987; Experimental Procedures). As shown in Figure 7, degradation of ABP, talin, and myosin heavy chain upon platelet activation was completely blocked when C5b-9-treated platelets had been preequilibrated with >0.1 mM leupeptin before addition of calcium. These results obtained by direct protein staining (Figure 7A) were confirmed when ABP and its degraded fragments were probed with monospecific antibody (Figure 7B). The requirement for calcium (Figures 5 and 6) and inhibition by leupeptin (Figure 7) suggest that the complement-induced proteolysis of these cytoskeletal proteins is mediated by cytoplasmic calpains that are activated by the influx of calcium across the C5b-9 pore.

Relationship of Calpain-Mediated Proteolysis of Cytoskeletal Proteins to Vesiculation of Microparticles from the Platelet Surface. Fox and associates have proposed that the platelet cytoskeleton, which includes actin filaments and ABP, is attached to the plasma membrane through a linkage between the cytoplasmic domain of GP Ib and ABP (Fox, 1985a,b). They further suggest that upon platelet activation, a calpain-mediated cleavage of ABP occurs which liberates GP Ib from its attachment to the cytoskeleton, and thereby facilitates the release of plasma membrane vesicles from the platelet surface (Fox et al., 1988). In order to investigate a possible correlation between calpain-mediated proteolysis of the platelet

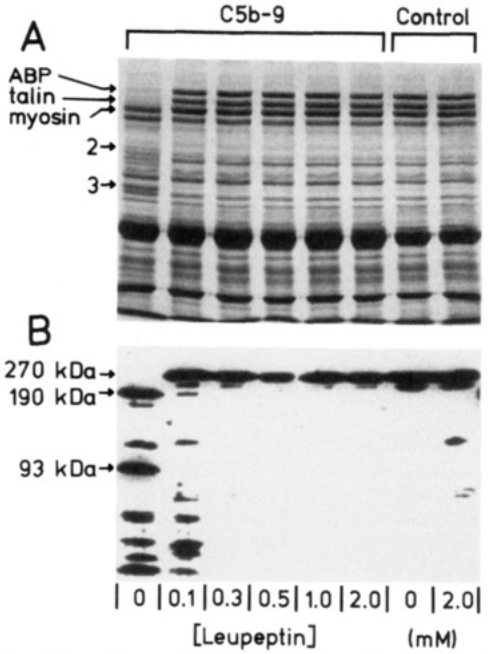

FIGURE 7: Effect of leupeptin on degradation of cytoskeletal proteins. C5b67 platelets were suspended to 5×10^8/mL in solution II containing 0.1 mM EGTA and leupeptin at the concentrations indicated. C8 and C9 were added, and after 2-min incubation at 37 °C, activation of the C5b-9 platelets was initiated by addition of 2.5 mM $CaCl_2$. Ten minutes later, samples were denatured and analyzed by gel electrophoresis. Protein bands are identified as detailed in the legend to Figure 3. (Panel A) Coomassie stain. (Panel B) Immunoblotting of anti-ABP. Data of a single experiment, representative of four similar experiments performed on different days.

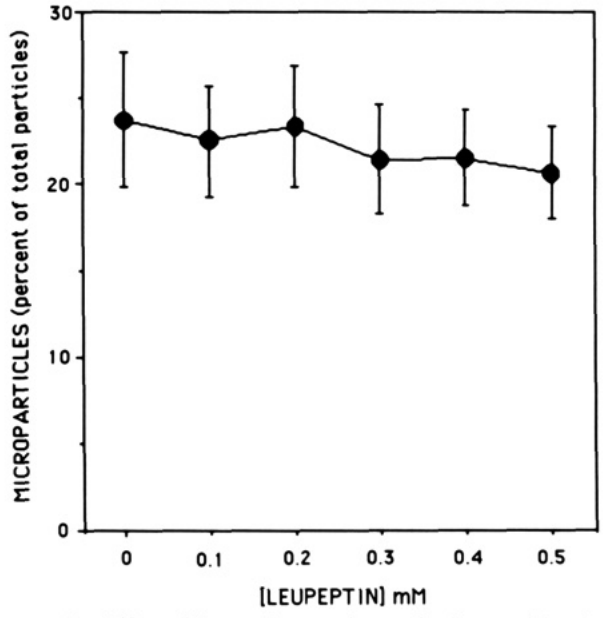

FIGURE 8: Effect of leupeptin on release of microparticles from C5b-9-treated platelets. Microparticle formation measured for C5b-9 platelets preloaded with leupeptin under conditions described for Figure 7. The ordinate denotes the number of microparticles present 10 min after addition of 2.5 mM $CaCl_2$ to the C5b-9 platelets, and is expressed as the percent of total GP Ib positive particles analyzed (see legend of Figure 1). Background of microparticles in complement-free controls was 10.4 ± 0.5%. Data denote mean ± SEM of six determinations.

cytoskeleton and microparticle formation, we examined whether leupeptin incorporated into the platelet cytoplasm would inhibit the release of GP Ib containing microparticles from C5b-9-activated platelets (Figure 8). Under conditions where the proteolysis of ABP and other cytoskeletal proteins

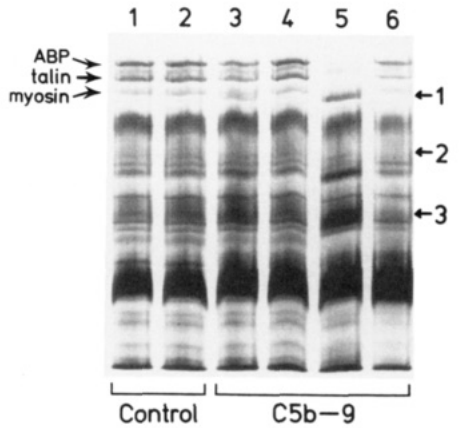

FIGURE 9: Effect of leupeptin on the cytoskeletal proteins associated with microparticles. A total of 5×10^8 C5b67 platelets were suspended in 1 mL of solution II containing 0.1 mM EGTA and either 0 mM (lanes 3 and 5) or 0.5 mM (lanes 4 and 6) leupeptin. C5b-9 assembly, preincubation with leupeptin, and platelet activation by addition of 2.5 mM $CaCl_2$ and incubation at 37 °C were performed as described in Figure 7. All samples were then made 0.5 mM in leupeptin, and the total platelet plus microparticle suspensions (lanes 3 and 4) or the isolated platelet-free microparticles (lanes 5 and 6) were analyzed by gel electrophoresis (see Experimental Procedures). Gels of complement-free control platelets suspended in either 0 mM (lane 1) or 0.5 mM leupeptin (lane 2) are also shown. Protein bands are identified as detailed in the legend to Figure 3. Data of a single experiment representative of two similar experiments performed on different days.

was inhibited by leupeptin, generation of GP Ib containing membrane microparticles by the C5b-9 proteins was unimpaired (cf. Figures 7 and 8). Furthermore, membrane microparticles that were shed from platelets preloaded with leupeptin contained the *undegraded* forms of ABP, talin, and myosin heavy chain (Figure 9). Taken together with the observation that microparticles could be generated from unstirred platelets stimulated with thrombin plus collagen in the absence of cytoskeletal proteolysis (Figures 1 and 3B), these results indicate that the proteolysis of ABP, talin, or myosin heavy chain is not required for the release of membrane vesicles from the platelet surface (see Discussion).

Role of Cytosolic Calpains in C5b-9-Induced Storage Granule Secretion and Expression of Membrane Prothrombinase Sites. There is considerable evidence that the cytoskeleton plays a central role in several processes associated with platelet activation, including cell shape change, translocation of intracellular storage granules to the cell surface during secretion, and loss of transbilayer lipid asymmetry [reviewed by Fox (1986)]. Because platelet activation by C5b-9 is obligatorily linked to the influx of calcium into the cytosol (Wiedmer et al., 1987; and above), we considered the possibility that calpain-mediated proteolysis of cytoskeletal components might play a direct role in the cell responses induced by the assembled complement proteins. However, as illustrated in Figure 10A, we were unable to observe any effect of leupeptin on α- or dense granule secretion induced by C5b-9. In these experiments, preincubation of C5b-9 platelets with concentrations of leupeptin above that required to completely inhibit calpain-mediated proteolysis of ABP, talin, and myosin heavy chain had no effect on the secretory responses observed upon subsequent addition of calcium. As illustrated by the data of Figures 10B and 11, the addition of leupeptin to prevent the proteolysis of cytoskeletal proteins also failed to inhibit the C5b-9-induced increase in membrane binding sites for coagulation factors Va and Xa on either platelets or shed microparticles. Consistent with these data obtained by measurement of factor Va and factor Xa binding, we were also unable to detect any effect of leupeptin on the exposure of

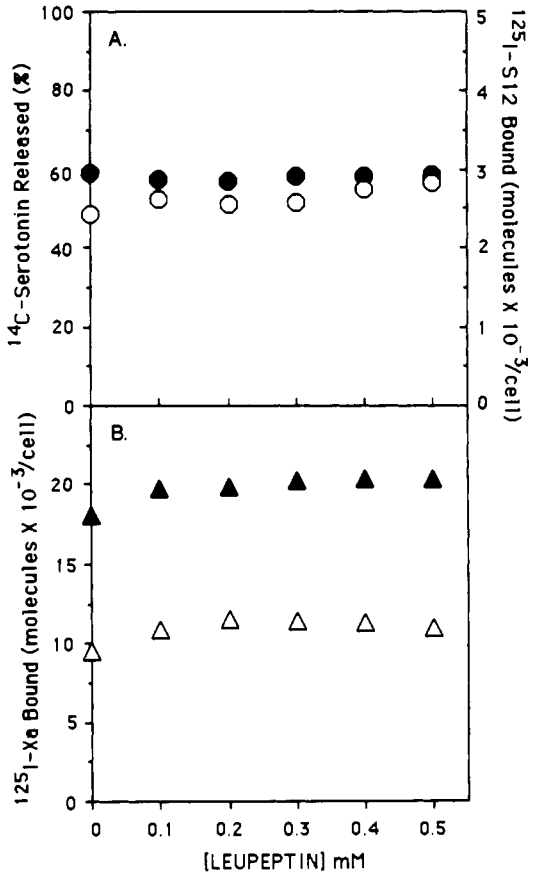

FIGURE 10: Effect of leupeptin on C5b-9-induced platelet secretion and factor Xa binding. C5b-9 platelets were preloaded with leupeptin (0–0.5 mM) before addition of 2.5 mM $CaCl_2$ as described for Figure 7. (Panel A) Dense granule secretion was assessed by measuring release of [^{14}C]serotonin (●). α-Granule secretion was assessed by specific binding of ^{125}I-labeled antibody S12 (○). ^{125}I-S12 specific activity, 522 cpm/ng. (Panel B) ^{125}I factor Xa binding was performed by incubation (30 min, 23 °C) of C5b-9-treated platelets with 0.2 μg/mL ^{125}I factor Xa plus 0 μg/mL (△) or 2 μg/mL (▲) factor Va. ^{125}I-Xa specific activity, 1241 cpm/ng. Nonspecific binding was determined in parallel reaction mixtures containing a 50-fold excess of unlabeled factor Xa. Specific binding (total less nonspecific) is expressed as molecules of ^{125}I-Xa bound per platelet. Data of a single experiment, representative of three similar experiments performed on separate days.

Table I: Effect of Leupeptin on Exposure of Platelet Prothrombinase Sites

stimulus[a]	[leupeptin] (mM)	prothrombinase act. [units of thrombin (10^8 cells)$^{-1}$ min^{-1}]
control	0	8.5
control	0.5	7.1
C5b-9	0	174.9
C5b-9	0.5	174.8

[a] C5b67 platelets were suspended to 10^8 cells/mL in solution II containing 0.1 mM EGTA with 4 μg/mL C8 plus 10 μg/mL C9 in the absence or presence of 0.5 mM leupeptin as indicated. Following incubation for 2 min at 37 °C, 2.5 mM $CaCl_2$ was added to all tubes. After an additional 10 min at 37 °C, platelets were diluted 25-fold; all samples were adjusted to the same final leupeptin concentration (20 μM) and then assayed for prothrombinase activity (see Experimental Procedures). Control refers to complement-free platelets incubated with leupeptin at concentrations indicated. Data of a single experiment are representative of three similar experiments performed on different days.

functional membrane prothrombinase sites induced by C5b-9 (Table I).

Role of Metabolic ATP in Microparticle Formation. The above experiments show that calcium-dependent proteolysis

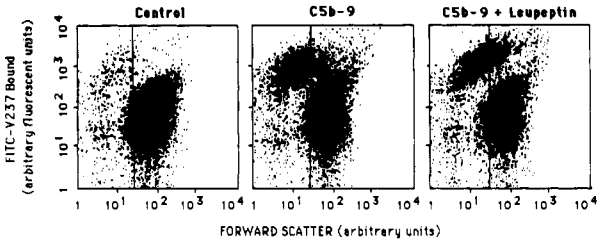

FIGURE 11: Effect of leupeptin on C5b-9-induced expression of binding sites for factor Va. Dot plots illustrate microparticle formation and expression of factor Va binding sites in platelets exposed to C5b-9 proteins. Membrane binding sites for factor Va were probed by incubation (10 min, 23 °C) with 2 μg/mL factor Va light chain, followed by staining with FITC-labeled monoclonal antibody V237 (which recognizes an epitope of factor Va light chain). Each panel denotes the dot plot of forward angle scatter (abscissa) versus FITC-V237 fluorescence (ordinate) plotted on logarithmic scales. C5b-9 assembly was performed in 0.1 mM EGTA and cells preincubated for 2 min with either 0 (middle panel) or 0.5 mM (right panel) leupeptin, before addition of 2.5 mM $CaCl_2$. The left panel represents results for complement-free controls. Vertical lines denote analysis gate of forward angle scatter discriminating platelets and microparticles (see Figure 1). Microparticles represented 9.5% (control), 27.6% (C5b-9), and 25.9% (C5b-9 + leupeptin) of total GP Ib positive particles analyzed. FITC–V237 fluorescence (mean fluorescence intensity per particle) associated with microparticles was 230 (control), 634 (C5b-9), and 617 (C5b-9 + leupeptin). FITC–V237 fluorescence associated with platelets was 100 (control), 268 (C5b-9), and 461 (C5b-9 + leupeptin). Data of a single experiment, representative of six such experiments performed on separate days.

of platelet cytoskeletal proteins is not required for microparticle formation or for expression of membrane prothrombinase activity. Since other platelet responses such as aggregation and secretion require metabolic energy, the effect of metabolic inhibitors on microparticle formation was examined. As shown in Table II, prior metabolic depletion of platelets with a combination of 2-deoxyglucose and antimycin A did not affect either the release of microparticles from C5b-9 platelets or the expression of factor Va binding sites on the microparticles. When exposed to identical concentrations of the C5b-9 proteins, metabolically depleted cells did, however, exhibit *increased* degradation of ABP, talin, and myosin heavy chain (data not shown). In contrast to the results with C5b-9, metabolic depletion inhibited all responses of platelets to thrombin plus collagen, including microparticle formation, platelet α-granule secretion, and the activation of membrane glycoproteins GP IIb–IIIa (as measured by binding of antibody PAC1) (Table II). Although the number of microparticles was greatly reduced, those microparticles vesiculated from ATP-depleted platelets when exposed to thrombin plus collagen showed normal exposure of factor Va binding sites.

Discussion

On the basis of this and previous studies, the proteolysis of cytoskeletal proteins by cytosolic calpain(s) is now known to occur when platelets are activated by the C5b-9 proteins, by thrombin (with or without collagen) under stirred conditions, and by the calcium ionophore A23187 (Phillips & Jakabova, 1977; White, 1980; Truglia & Stracher, 1981; Fox et al., 1983, 1985; Fox, 1985b; Verhallen et al., 1987). This proteolysis can be detected under conditions in which there is activation of platelet fibrinogen receptors, secretion from platelet storage granules, the vesiculation of membrane microparticles from the cell surface, and exposure of membrane binding sites for the prothrombinase enzyme complex. These observations have suggested that there might be a role for activated calpains in these various platelet responses (Baldassare et al., 1985; Fox, 1986; Verhallen et al., 1987, 1988; Fox et al., 1988). On the

Table II: Effect of Metabolic Inhibitors on Platelet Responses to C5b-9 or to Thrombin Plus Collagen[a]

	microparticles				platelets			
	% microparticles		FITC–V237 binding (mean fluorescence intensity)		FITC–S12 binding (mean fluorescence intensity)		FITC–PAC1 binding (mean fluorescence intensity)	
agonists	solution II	+inhibitors	solution II	+inhibitors	solution II	+inhibitors	solution II	+inhibitors
none	11.3 ± 1.9[b]	8.7 ± 1.3	186 ± 64	105 ± 29	93 ± 51	77 ± 37	243 ± 122	63 ± 18
C5b-9	23.2 ± 3.5	26.4 ± 4.3	703 ± 56	781 ± 89	131 ± 64	93 ± 42	362 ± 153	106 ± 55
THR + COLL	47.6 ± 6.2	15.4 ± 2.7[c]	343 ± 49	302 ± 22	361 ± 46	281 ± 56[c]	1285 ± 22	301 ± 120[c]

[a] Platelets (C5b67 or controls) were suspended at 10^8/mL in solution II containing 2.5 mM $CaCl_2$ or in solution II omitting glucose but containing 16.7 mM 2-deoxyglucose, 10 μg/mL antimycin A, and 2.5 mM $CaCl_2$ (+inhibitors). After 10-min incubation at 37 °C, platelet activation was initiated by addition of C8 and C9 to C5b67 platelets (C5b-9) or by addition of thrombin (0.5 unit/mL) plus collagen (10 μg/mL) to an aliquot of control platelets (THR + COLL). After further incubation for 10 min at 37 °C, samples were then prepared for and analyzed by flow cytometry as described under Experimental Procedures. FITC-labeled antibody binding is expressed as the mean fluorescence intensity per particle (microparticle or platelet). [b] Mean ± SEM of three experiments. [c] Indicates the value in the presence of inhibitors is significantly lower than the value without inhibitors at the ≤0.05 level by the paired Student's *t* test.

other hand, the data of the present study indicate that at least for cells stimulated by the C5b-9 proteins or by thrombin plus collagen, each of these platelet responses can be completely uncoupled from the cytoskeletal proteolysis that is mediated by activated intracellular calpains. Therefore, we conclude that the proteolytic degradation of these cytoskeletal proteins (ABP, talin, and myosin heavy chain) that is normally observed when calcium is increased in the platelet cytosol is *not* required for activation of fibrinogen receptors, secretory fusion of the storage granules, release of microparticles from the cell surface, or expression of membrane prothrombinase sites. Nevertheless, we cannot exclude the possibility that these activation-related platelet responses are coupled to a calpain-mediated proteolysis of another platelet protein which might go undetected by our methods.

The expression of a catalytic surface for assembly of the prothrombinase enzyme complex has been related to the release of membrane microparticles from activated platelets and to the exposure of acidic phospholipid through transbilayer migration to the outer leaflet of the plasma membrane (Bevers et al., 1983; Bode et al., 1985; Sandberg et al., 1985; Sims et al., 1988). Zwaal and associates have noted previously that the optimal calcium concentration for calpain-mediated proteolysis of platelet cytoskeletal proteins coincides with the calcium optimum for expression of platelet prothrombinase activity in response to thrombin plus collagen and that these two processes also temporally coincide. From these data, they suggested that a causal relationship exists between generation of a procoagulant platelet surface and the calpain-mediated degradation of ABP, talin, and myosin (Verhallen et al., 1987). Furthermore, they reported that leupeptin inhibits expression of prothrombinase activity in platelets exposed to fluoride plus calcium, further implying that there is a requirement for calpains in the exposure of membrane prothrombinase sites (Verhallen et al., 1988). However, in our experiments, inhibition of cytoplasmic calpains at the time of platelet activation by the C5b-9 proteins had no effect on microparticle formation, the generation of binding sites for factors Va and Xa, or the expression of prothrombinase activity. In addition to differences between the platelet activators employed in these experiments (C5b-9 versus fluoride), one potential explanation for the discrepancy between the results of these studies may derive from a direct inhibitory effect of leupeptin on the prothrombinase assay employed by Zwaal and co-workers. In preparation for the present studies, we noted that when leupeptin was added directly into the platelet prothrombinase assay, it slowed the rate of conversion of prothrombin to thrombin. This inhibitory effect on prothrombin conversion was detected even at micromolar concentrations of leupeptin, and likely reflects the known inhibitory action of leupeptin on thrombin (Ruda & Scrutton, 1987; Rao et al., 1987; Brass & Shattil, 1988), decreasing feedback activation by nascent thrombin generated in the prothrombinase assay. Therefore, in order to avoid any unintended effects of leupeptin in the prothrombinase assays in the present study, leupeptin-treated and control platelets were first activated by incubation with the C5b-9 proteins (15 min, 37 °C), and *then* each was adjusted to the same final leupeptin concentration (20 μM) upon dilution for assay of prothrombinase activity (see Experimental Procedures).

As noted above, we failed to observe any change in C5b-9-induced microparticle formation or factor Va or Xa binding when calpains were inhibited by leupeptin (Figures 8–11). In addition, inhibition of calpains had no effect on the catalytic rate of prothrombin conversion in platelets activated by C5b-9 (Table I). These data imply that exposure of the membrane prothrombinase site is unrelated to the action of calpains upon cytoskeletal proteins. The C5b-9 proteins have been shown to directly induce exchange of phospholipid between inner and outer monolayers in a pure lipid system (Van der Meer et al., 1989). This direct effect on lipids might account for the ability of C5b-9 to increase acidic phospholipid on the platelet surface, thereby providing a catalytic surface for the prothrombinase enzyme complex. Nevertheless, it should be noted that unstirred platelets activated by thrombin plus collagen exhibited a marked increase in the number of microparticles and the expression of membrane prothrombinase sites without detectable proteolysis of the cytoskeletal proteins (Figures 1 and 3B). Thus, it appears that for these agonists as well, calpain-mediated proteolysis is not required for plasma membrane vesiculation and exposure of the factor Va receptor.

Fox and associates have demonstrated that the platelet plasma membrane is associated with a submembrane cytoskeleton. They have suggested that in the unstimulated platelet, the cytoplasmic domain of GP Ib is bound to this cytoskeleton through a direct interaction with ABP (Fox et al., 1983; Fox, 1985a,b). Thus, ABP may in effect serve to tether the plasma membrane to the rest of the cytoskeleton. It has also been reported that upon platelet activation, another cytoskeletal protein, talin, binds to the cytoplasmic domain of GP IIIa (Isenberg et al., 1988). The proteolysis of ABP and talin which is observed when cytosolic calpains are activated could serve to release the plasma membrane from its attachment to the cytoskeleton, thereby facilitating the process of membrane blebbing and vesiculation. The data of our experiments, however, indicate that the proteolysis of ABP or talin is not required for vesiculation. For example, when cytoplasmic calpains were inhibited by leupeptin, the microparticles shed from the C5b-9 platelet surface contained plasma membrane GP Ib plus undegraded components of the platelet cyto-

skeleton, including ABP, talin, and myosin heavy chain (Figure 9). These results suggest that calcium influx across the plasma membrane induces vesiculation in a more direct manner and liberates the plasma membrane from its attachment to the platelet cytoskeleton irrespective of whether the anchoring proteins are also proteolytically degraded. It is conceivable that Ca^{2+} influx induces these membrane changes by causing dissociation of ABP dimers, disruption of the interaction of ABP with actin, or disruption of submembranous actin filaments, for example, through activation of gelsolin. In this context, Stracher and associates have suggested that the phosphorylation of ABP may be required for cross-linking to F-actin, as well as for protection of ABP from calpain-mediated proteolysis (Zhuang et al., 1984). Consistent with this model, we found that metabolically depleted platelets generally exhibited increased proteolysis of ABP and other cytoskeletal proteins after C5b-9 assembly.

In addition to demonstrating that microparticle formation and expression of the prothrombinase site can both be uncoupled from calpain-mediated proteolysis of cytoskeletal proteins, our data also indicate that the metabolic processes required for vesiculation of plasma membrane from the platelet surface differ from those required for other platelet responses such as platelet α-granule secretion and the expression of fibrinogen binding sites on GP IIb-IIIa. For example, concentrations of thrombin plus collagen required for microparticle formation and exposure of factor Va receptors also trigger secretion from storage granules and activate cell surface fibrinogen receptors (Figure 2 and Table II). In contrast, platelet activation by C5b-9 results in storage granule secretion, the vesiculation of microparticles, and the exposure of factor Va receptors, without activating cell surface fibrinogen receptors (Ando et al., 1988, 1989; Sims et al., 1989). ADP and epinephrine can induce fibrinogen receptor exposure but not the formation of microparticles or the exposure of factor Va receptors (Sims et al., 1989). Finally, results obtained with metabolically depleted platelets indicate that normal glucose metabolism is required to initiate microparticle formation with thrombin plus collagen as agonists, but not with C5b-9 (Table II).

The molecular mechanism underlying the formation of platelet microparticles and the expression of the membrane prothrombinase sites remains unresolved. We suggest that microparticle formation and prothrombinase activity are both linked to some common process that is dependent on Ca^{2+} influx across the plasma membrane but is independent of aggregation, secretion, or activation of calpains. This interpretation is supported by recent studies of a patient with an isolated deficiency of platelet procoagulant activity (Weiss et al., 1979; Sims et al., 1989). Her platelets, which exhibited normal secretion and aggregation responses, were defective in both expression of membrane binding sites for factor Va and generation of membrane microparticles in response to either C5b-9, thrombin plus collagen, or A23187. The fact that this patient has a bleeding diathesis underscores the potential physiological significance of the process of microparticle formation when platelets are activated during normal hemostasis.

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Registry No. Ca, 7440-70-2; C5b-9, 82986-89-8; Va, 65522-14-7; Xa, 9002-05-5; calpain, 78990-62-2.

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Identification and Assignment of Base Pairs in Four Helical Segments of *Bacillus megaterium* Ribosomal 5S RNA and Its Ribonuclease T1 Cleavage Fragments by Means of 500-MHz Proton Homonuclear Overhauser Enhancements†

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ABSTRACT: Three different fragments of *Bacillus megaterium* ribosomal 5S RNA have been produced by enzymatic cleavage with ribonuclease T1. Fragment A consists of helices II and III, fragment B contains helix IV, and fragment C contains helix I of the universal 5S rRNA secondary structure. All (eight) imino proton resonances in the downfield region (9–15 ppm) of the 500-MHz proton FT NMR spectrum of fragment B have been identified and assigned as G_{80}·C_{92}-G_{81}·C_{91}-G_{82}·C_{90}-A_{83}·U_{89}-C_{84}·G_{88} and three unpaired U's (U_{85}, U_{86}, and U_{87}) in helix IV by proton homonuclear Overhauser enhancement connectivities. The secondary structure in helix IV of the prokaryotic loop is completely demonstrated spectroscopically for the first time in any native or enzyme-cleaved 5S rRNA. In addition, G_{21}·C_{58}-A_{20}·U_{59}-G_{19}·C_{60}-A_{18}·U_{61} in helix II, U_{32}·A_{46}-G_{31}·C_{47}-C_{30}·G_{48}-C_{29}·G_{49} in helix III, and G_4·C_{112}-G_5·C_{111}-U_6·G_{110} in the terminal stem (helix I) have been assigned by means of NOE experiments on intact 5S rRNA and its fragments A and C. Base pairs in helices I–IV of the universal secondary structure of *B. megaterium* 5S RNA are described.

Ribosomal 5S RNA is known to be an important structural and functional component of the large subunit of all ribosomes. Because of its essential role in protein synthesis in the ribosome (Pieler et al., 1984) and its small size among ribosomal RNA molecules, 5S rRNA has been studied extensively for 2 decades. Since the late 1960s, a large number of 5S rRNAs from many different prokaryotic and eukaryotic ribosomes have been sequenced (Erdmann & Wolters, 1986). Following the general belief that there should be a universal secondary structure for 5S rRNAs, many universal secondary structure models have been proposed on the basis of comparative sequence analysis (Fox & Woese, 1975; Leuhrsen & Fox, 1981; Studnicka et al., 1981; De Wachter et al., 1982; Delihas & Andersen, 1982), enzymatic cleavage and chemical modification (Nishikawa & Takemura, 1974; Pieler & Erdmann, 1982), and physical measurements (Kearns & Wong, 1974; Osterberg et al., 1976; Luoma & Marshall, 1978a,b; Chang et al., 1984).

Of the techniques used for the structural studies of RNA, proton homonuclear Overhauser enhancement (NOE) experiments are the most powerful for the determination of base-paired sequences in nucleic acids (Johnston & Redfield, 1978). Supplemented by the complete structural determination of tRNA by X-ray crystallography (Kim, 1976; Rich, 1977), NOE experiments have successfully identified (as A·U, G·C, or G·U) and assigned (to specific primary sequence bases) virtually all of the secondary and tertiary base pairs in several tRNAs in aqueous solution (Schimmel & Redfield, 1980; Reid, 1981; Johnston & Redfield, 1981; Heerschap et al., 1983a,b; Roy & Redfield, 1983).

Application of NOE techniques to the larger 5S rRNA molecule presents several problems, as noted in a recent review of ^1H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P NMR of 5S rRNAs (Marshall & Wu, 1989). The main obstacles are broader peak widths and more overlap of the proton resonances in the downfield region resulting from more base-paired imino protons than for tRNA. Moreover, the secondary structure and the tertiary folding patterns of 5S rRNA have not yet been established from X-ray diffraction. Although some of the imino proton resonances in the downfield proton NMR spectrum of 5S rRNA have been assigned to specific base pairs in the universal secondary structure models [e.g., Chang and Marshall (1986a)], NOE experiments must be supported by other experiments that shift or simplify the proton NMR spectrum. Among such methods are site-specific spin labeling (Lee & Marshall, 1987) and the use of enzymatic cleavage fragments (Kime & Moore, 1983a,b; Kime et al., 1984; Li & Marshall, 1986; Chen & Marshall, 1986; Li et al., 1987). Proton NMR studies of enzyme-cleaved fragments of 5S rRNA are especially attractive because of greatly reduced peak overlap resulting from fewer, narrower proton resonances.

The secondary structure of prokaryotic 5S rRNA has been studied in *Escherichia coli* (Gram negative) and *Bacillus*

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