Participation of Protein Kinases in Complement C5b-9–Induced Shedding of Platelet Plasma Membrane Vesicles

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The formation of membrane microparticles through vesiculation of the platelet plasma membrane is known to provide catalytic surface for several enzyme complexes of the coagulation system, and to underlie the procoagulant responses elicited with platelet activation. This induced shedding of vesicles from the plasma membrane is most prominent when platelets are activated by the terminal complement proteins, C5b-9, by a Ca²⁺ ionophore, or by the combination of thrombin plus collagen. Although shown to require elevated [Ca²⁺], the cellular events that initiate plasma membrane evagination and fusion to form the shed vesicles remain unresolved. To gain additional insight into the cellular events that regulate membrane microparticle formation, we have examined how this process is influenced by the activity of cellular protein kinases. Cytoplasmic [Ca²⁺] of gel-filtered platelets was increased by membrane assembly of the terminal complement proteins C5b-9 in the presence of selective inhibitors of protein kinase or phosphatase reactions, and resulting microparticle formation was quantitated by fluorescence-gated flow cytometry. Pre-equilibration of the phosphatase inhibitor vanadate into the platelet cytosol increased microparticle formation by as much as 40%, suggesting that vesiculation of the platelet plasma membrane is influenced by the state of phosphorylation of a cellular constituent. By contrast to the stimulatory effects of vanadate, microparticle formation was partially inhibited in platelets treated with the protein kinase inhibitor sphingosine, the myosin light chain kinase inhibitor ML-7, the calmodulin-antagonist W-7, and under conditions of elevated cytosolic concentration of cyclic adenosine monophosphate. These results indicate that complement-induced platelet microparticle formation is influenced by one or more protein kinase(s) as well as by calmodulin, and suggest a role for the platelet myosin light chain kinase or another Ca²⁺-plus-calmodulin-regulated membrane component.

ARTICLE OF platelets by a variety of agonists induces platelet shape change, secretion of α- and dense-granule storage pools, activation of surface receptors for adhesive proteins, and induction of procoagulant activity. Expression of the procoagulant properties of activated platelets has been shown to be related to the exposure of binding sites for assembly of the tenase and prothrombinase enzyme complexes on small membrane vesicles (“microparticles”) that are shed from the platelet surface upon activation.1-4 The mechanism by which this vesiculation of the plasma membrane of activated platelets is initiated and regulated remains unresolved. Cumulative data suggest that the capacity of an agonist to induce formation of these membrane microparticles is directly related to its ability to raise cytosolic [Ca²⁺].5-6 Because proteolytic degradation of membrane-associated cytoskeletal proteins by cytosolic calpains is often observed concomitant with membrane vesiculation, it has been suggested that this action of Ca²⁺-plus-calpain initiates vesiculation by dissociating plasma membrane proteins from the submembrane cytoskeleton.7 The observation that platelet microparticle formation and the expression of platelet prothrombinase activity can proceed unimpeded when intracellular calpains are completely inhibited suggests alternative mechanisms by which Ca²⁺ triggers this response.5,8

We have previously shown that complement proteins C5b-9 differ from other physiologic agonists by their capacity to increase intracellular [Ca²⁺] and to activate cellular protein kinases through direct effects on the plasma membrane, bypassing the normal receptor-coupled pathways that require phospholipase C-mediated hydrolysis of inositol phospholipids to increase cytosolic [Ca²⁺].3 In addition to providing catalytic surface for the prothrombinase and tenase reactions, this vesiculation after C5b-9 assembly serves as a mechanism to remove these pore-forming proteins from the platelet surface, circumventing the cytotytic activity of the C5b-9 complex. Therefore, we have used these complement proteins to examine the role of phosphorylation events in the Ca²⁺-requiring process of plasma membrane vesiculation. Our results suggest that in addition to a dependence on Ca²⁺, the complement-induced shedding of vesicles from the platelet plasma membrane involves participation of one or more protein kinase(s), and is regulated by calmodulin.

MATERIALS AND METHODS

Materials. Apyrase, bovine serum albumin (globulin and fatty-acid free), sodium orthovanadate, adenosine triphosphate (ATP), dibutyryl cyclic adenosine monophosphate (Bt,cAMP), prostaglandin E₁ (PGEl), and D-sphingosine were from Sigma Chemical Co (St Louis, MO); myosin light chain kinase inhibitor ML-7 [1-(5-isopropylpentane-1-sulfonyl)-1-H-hexahydro-1,4-diazepine] and calmodulin-antagonists W-7 [N-(6-aminohexyl)-5-chloro-1-naphtha-

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were added and the samples analyzed by flow cytometry.

Activation of platelets by C5b-9. Gel-filtered human platelets were prepared and collected into buffer 1 at 1 to 2 x 10^9/mL as described previously. Human complement proteins C5b6, C7, C8, and C9 were purified and analyzed for functional activity according to methods previously described. To assemble membrane-bound C5b67 complexes, gel-filtered platelets (10^9/mL) were incubated for 3 minutes at 37°C with C5b6 (10 μg/10^8 platelets) and C7 (2.5 μg/10^6 cells). To assemble membrane-bound C5b-9 complexes, C5b67 platelets were suspended to 10^5 platelets/mL in buffer 2 containing 0.1 mmol/L EGTA. Then C8 (4 μg/10^6 cells) and C9 (10 μg/10^6 cells) were added, and the cells incubated at 37°C without stirring for 2 minutes in the presence or absence of inhibitors of kinase or phosphatase as described in the figure legends. As previously described, assembly of the C5b-9 complex in 0.1 mmol/L EGTA results in the permeabilization of the platelet plasma membrane while maintaining low cytosolic calcium, thereby preventing activation of protein kinases and granule secretion. Platelet activation was then initiated by addition of 1 mmol/L CaCl_2 and incubation continued for another 10 minutes. Activation was stopped by the addition of 2 mmol/L EGTA. In all experiments, comparison was made to identical matched-pair controls (platelets incubated in the absence of the C5b-9 proteins).

Kinase inhibitors and calmodulin antagonists. Sphingosine was dissolved in buffer 2. Staurosporin was delivered to platelets from a stock solution in dimethyl sulfoxide, with the solvent concentration never exceeding 0.6%. ML-7, W-5, and W-7 were maintained as stock solutions in water and diluted into buffer 2 shortly before use.

Protein phosphorylation. Platelets were labeled with 32P, as described elsewhere. C5b-9 assembly was then performed as described above and as detailed in the figure legends. In all cases, platelet activation was stopped 30 seconds after addition of 1 mmol/L CaCl_2 by dilution into hot sodium dodecyl sulfate (SDS) sample buffer as described. Aliquots of 50 μL (1.3 x 10^5 platelets) were loaded onto 12% SDS polyacrylamide gels. The gels were stained, dried, and autoradiographed using Kodak BB-5 Blue Brand x-ray film (Kodak, Rochester, NY).

Labeling of MoAbs. Antibody S12 was labeled with FITC and antibody AP1 was conjugated with N-hydroxysuccinimide biotin ester as described previously.

Preparation of platelets for flow cytometry. C5b-9-treated or control platelets, 5 x 10^6, were incubated in the dark in a total volume of 60 μL for 10 minutes at 23°C in the presence of biotin-AP1 (2 μg/mL) and FITC-S12 (30 μg/mL). Phycoerythrin-streptavidin was added (5 μL of a 1:10 dilution), and the cells were incubated an additional 10 minutes. Then 1-mL aliquots of buffer 2 were added and the samples analyzed by flow cytometry.

Flow cytometry. Samples were analyzed in a Becton Dickinson FACScan flow cytometer (Mountain View, CA) formatted for two-color analysis as described previously. To discriminate between platelets and microparticles, gates for analysis were set as described.

RESULTS

Ca^{2+}-induced vesiculation of the platelet plasma membrane is potentiated by vanadate. Assembly of the terminal complement proteins C5b-9 on the platelet plasma membrane results in an increase in cytosolic [Ca^{2+}], which is accompanied by secretion of the contents of the storage granules and by shedding of plasma membrane-derived vesicles from the platelet surface. In addition to removing the inserted C5b-9 proteins from the platelet surface, these membrane "microparticles" provide catalytic surface for assembly of the prothrombinase and tenase enzyme complexes, accounting for expression of the platelet's procoagulant properties. Although little is known about the mechanism by which C5b-9 assembly induces vesiculation of the platelet plasma membrane, the formation of these microparticles

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Fig 1. Effect of o-vanadate on C5b-9-induced platelet secretion and microparticle formation. C5b67-complexes were assembled on platelets as described in Materials and Methods. These cells were then suspended (10^6 cells/mL) at 37°C in buffer 2 containing 0.1 mmol/L EGTA, 2 mmol/L AtP plus 0 to 1 mmol/L o-vanadate (concentrations shown on abscissa), and C8 and C9 were added to initiate formation of the C5b-9-pore. After 2 minutes, 0 (△) or 1 mmol/L CaCl_2 (A) was added, and incubation continued for another 10 minutes. All samples were stopped by addition of 2 mmol/L EGTA, and analyzed by flow cytometry. (Upper panel) α-Granule secretion was monitored with antibody FITC-S12. (Lower panel) Microparticles are expressed as the percent of all 5,000 GPIIb-positive particles analyzed per sample. Data of single experiment representative of three similar experiments.
has been shown to be regulated by intracellular Ca\textsuperscript{2+} and to be inhibited when the influx of Ca\textsuperscript{2+} is precluded.\textsuperscript{13} To gain additional insight into the obligate role of Ca\textsuperscript{2+} in this process of induced vesiculation of the platelet plasma membrane, we have investigated the contribution of Ca\textsuperscript{2+}-regulated protein kinases to the formation of the platelet membrane vesicles. Gel-filtered platelets were exposed to complement proteins C5b-9 in the presence of 2 mmol/L ATP and increasing concentrations of the phosphatase inhibitor vanadate to elevate the state of phosphorylation of cytosolic proteins.\textsuperscript{14} In these experiments the C5b-9 complex was assembled in the presence of 0.1 mmol/L EGTA to permeabilize the plasma membrane to vanadate and ATP before influx of Ca\textsuperscript{2+}, and platelet activation was subsequently initiated by the addition of 1 mmol/L CaCl\textsubscript{2} (Fig 1, closed symbols). Under these conditions, the addition of vanadate was found to potentiate both C5b-9-induced secretion from \alpha-granules (Fig 1, upper panel) and the formation of plasma membrane-derived microparticles (Fig 1, lower panel) by approximately 40%. These effects of vanadate saturated at [vanadate] > 100 \mu mol/L. In the absence of added Ca\textsuperscript{2+}, both microparticle formation and \alpha-granule secretion were inhibited (open symbols). Furthermore, in contrast to results obtained at elevated [Ca\textsuperscript{2+}], when C5b-9 platelets were maintained in 0.1 mmol/L EGTA, the addition of vanadate caused little increase in microparticle formation, although secretion was near maximally stimulated at 1 mmol/L vanadate. No secretion or microparticle formation was induced when these concentrations of vanadate were added to complement-free controls suspended in either 1 mmol/L CaCl\textsubscript{2} or 0.1 mmol/L EGTA (data not shown). Taken together, these data suggest that the C5b-9-induced vesiculation of the platelet plasma membrane is influenced by both Ca\textsuperscript{2+} and by the extent of phosphorylation of an intracellular component.

\textit{Inhibition by cAMP.} As shown in Fig 2, both \alpha-granule secretion and plasma membrane vesicle formation were inhibited by approximately 25% when C5b-9 platelets were exposed to dibutyryl cAMP before Ca\textsuperscript{2+} addition. Similar results were obtained with PGE\textsubscript{1}. These results suggest that in addition to a dependence on Ca\textsuperscript{2+}, the process of plasma membrane vesiculation is also sensitive to the level of cytoplasmic cAMP.

\textit{Contribution of protein kinases to membrane microparticle formation.} The possible role of a phosphorylated membrane component in the Ca\textsuperscript{2+}-induced shedding of vesicles from the platelet plasma membrane led us to examine the contribution of cellular protein kinases to this process. As shown in Fig 3, sphingosine, a relatively nonspecific protein kinase inhibitor,\textsuperscript{14} had a marked inhibitory effect on the shedding of vesicles from C5b-9-treated platelets, which paralleled its inhibitory effect on \alpha-granule secretion. By contrast, in platelets treated with the relatively selective \textit{C-kinase inhibitor}, staurosporine, only a small inhibition of platelet microparticle formation was observed, despite a marked inhibition of \alpha-granule secretion under these conditions (Fig 4). As illustrated in Fig 5, the concentrations of staurosporine used in these experiments virtually abolished C5b-9-induced phosphorylation of the 40-Kd substrate of protein kinase C, as well as inhibited other phosphorylations, including that of myosin light chain (20 Kd; see below). Taken together with the results in Fig 2, these data suggest that whereas the activity of the platelet C-kinases is required for C5b-9-induced \alpha-granule secretion, it is not required for vesiculation of the plasma membrane, and that another protein kinase(s) sensitive to the effects of both Ca\textsuperscript{2+} and cAMP participates in this event.

\textit{Role of myosin light chain kinase.} Because of the known regulation of the activity of myosin light chain kinase by both Ca\textsuperscript{2+} and cAMP, we next examined the effect of ML-7, a potent inhibitor of this enzyme.\textsuperscript{15} As shown in Fig 6 (lower panel), ML-7 at 50 \mu mol/L reduced microparticle formation by approximately 35%. Under these conditions, \alpha-granule secretion was inhibited by more than 80% (Fig 6, upper panel). As illustrated in Fig 7, at the concentrations of ML-7 required to observe inhibition of secretion and plasma membrane vesiculation (1.6 to 12.5 \mu mol/L), a selective inhibition of the phosphorylation of myosin light chain (20 Kd) was observed, although higher concentrations of this compound partially reduced phosphorylation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Effect of elevated cAMP on C5b-9-induced secretion and microparticle formation. Platelets were incubated in buffer 2 or buffer 2 containing 5 \mu mol/L PGE, or 1 mmol/L dibutyryl-cAMP for 5 minutes at 37°C. C5b-9 assembly was then performed as described in Materials and Methods in buffer 2, or buffer 2 containing PGE, or Bt2cAMP in the above concentration (I). Platelets incubated in the absence of complement proteins are also shown (II). \alpha-Granule secretion (Left panel) and microparticle formation (Right panel) were monitored as described for Fig 1. Error bars denote range of duplicates. Data of single experiment representative of two so performed.}
\end{figure}
DISCUSSION

Our results indicate that the vesiculation of plasma membrane that is observed when \([Ca^{2+}]\) is increased in the platelet cytosol can be modulated by reagents that alter the state of phosphorylation of intracellular phosphoproteins, including inhibitors of protein kinase(s) and a direct calmodulin antagonist. Therefore, these data suggest that the shedding of vesicles from the platelet surface is metabolically regulated by the activity of one or more \(Ca^{2+}\)-dependent protein kinases, in addition to any direct effect that \(Ca^{2+}\) may have on the intrinsic stability of the plasma membrane. This conclusion is consistent with recent observations indicating that \(Ca^{2+}\)-induced vesiculation of the platelet plasma membrane can proceed unimpeded under conditions where proteolytic degradation of the submembrane cytoskeleton by intracellular \(Ca^{2+}\)-activated proteases is prevented, implying a dependence of vesicle formation on other \(Ca^{2+}\)-regulated events that affect cytoskeletal organization and membrane structure.\(^5,6\)

of the 40-Kd C-kinase substrate, pleckstrin. While these results suggest that activation of myosin light chain kinase contributes to membrane microparticle formation, it should also be noted that at concentrations of staurosporine at which significant inhibition of both myosin light chain kinase and C-kinase(s) were observed, vesiculation of the plasma membrane was only slightly inhibited (Figs 4 and 5; see Discussion).

A calmodulin antagonist inhibits vesiculation of the platelet plasma membrane. The effects of these protein kinase inhibitors, plus the obligate requirement for \(Ca^{2+}\), suggested that a calmodulin-regulated kinase, possibly myosin light chain kinase, participates in platelet microparticle formation. Consistent with this interpretation, the calmodulin antagonist \(W-7\) was found to inhibit microparticle formation (Fig 8). Maximum inhibition by \(W-7\) was observed at 50 \(\mu\text{mol}/\text{L}\), a concentration at which the halide-free analogue \(W-5\) (a weak calmodulin antagonist) had virtually no effect.

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Fig 3. Effect of sphingosine on C5b-9-induced secretion and microparticle formation. C5b67-complexes were assembled on platelets as described in Materials and Methods and these cells suspended to \(10^8\) cells/mL in buffer 2 containing 0.1 mmol/L EGTA plus 0 to 25 \(\mu\text{mol}/\text{L}\) D-sphingosine (concentrations shown on abscissa). C5b-9-assembly was then performed as described in Materials and Methods (\(\Delta\)). Complement-free control platelets (\(\Delta\)) are also shown. \(\alpha\)-Granule secretion (Upper panel) and microparticle formation (Lower panel) were monitored as described for Fig 1. Data of single experiment representative of three similar experiments.

Fig 4. Effect of staurosporine on C5b-9-induced secretion and microparticle formation. C5b67-complexes were assembled on platelets as described in Materials and Methods and these cells suspended to \(10^8\) cells/mL in buffer 2 containing 0.1 mmol/L EGTA plus 0 to 10 \(\mu\text{mol}/\text{L}\) staurosporine. After additional incubation for 5 minutes at 37°C, C5b-9 assembly was completed as described in Materials and Methods (\(\Delta\)). Complement-free control platelets (\(\Delta\)) are also shown. \(\alpha\)-Granule secretion (Upper panel) and microparticle formation (Lower panel) were monitored as described for Fig 1. Data of single experiment. Inhibition of microparticle formation at 2 \(\mu\text{mol}/\text{L}\) staurosporine compared with C5b-9-treated platelets in the absence of the inhibitor ranged from 9% to 15% in three separate experiments.
As shown in Fig 1, platelet microparticle formation was potentiated by vanadate, a potent inhibitor of tyrosine phosphatases. Although several protein kinase inhibitors were found to exert an inhibitory effect on the formation of these vesicles, the reported selectivity of the kinase inhibitors used is for enzymes (especially C-kinases and myosin light chain kinase) that mediate serine/threonine phosphorylation. However, it should be noted that in addition to being a potent inhibitor of tyrosine phosphatase reactions, vanadate has also been shown to increase formation of inositol-phosphates and the phosphorylation of serine/threonine phosphoproteins in permeabilized platelets, suggesting that it potentiates the effects of a variety of cellular kinases in addition to preventing dephosphorylation of phosphotyrosines.

Although the increase in microparticle formation observed in vanadate-loaded platelets, and the inhibition of this process by certain protein kinase inhibitors, implicates participation of one or more protein kinases in platelet microparticle formation, these data leave unresolved which kinase(s) are directly involved. Sphingosine (25 μmol/L) virtually abolished C5b-9–induced platelet secretion and inhibited vesicle formation by approximately 50% (Fig 3). This compound, which was originally proposed to be a specific inhibitor of protein kinase C, has more recently been shown to inhibit other kinases as well, including the Ca²⁺-activated myosin light chain kinase and other calmodulin-dependent enzymes. The requirement for Ca²⁺ and the inhibitory effects of both the calmodulin antagonist, W-7, and the myosin light chain kinase inhibitor, ML-7, are each consistent with the participation of myosin light chain kinase in the shape changes leading to evagination and fusion of plasma membrane as required for vesiculation of microparticles from the platelet surface. Furthermore, this enzyme has been implicated in the centralization of platelet storage granules during secretion, a process that also requires reorganization of the platelet cytoskeleton.

In addition to the inhibitory effects of W-7 and ML-7, cAMP was also found to reduce the extent of microparticle formation (Fig 2, right panel). Because a cAMP-dependent
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10⁶ cells/mL in buffer 2 containing 0.1 mmol/L EGTA plus 0 to 50 pmol/L ML-7. After additional incubation for 5 minutes at room temperature C5b-9 assembly was completed as described in Materials and Methods. Thirty seconds after addition of 1 mmol/L CaCl₂, aliquots were diluted into hot SDS sample buffer and subjected to gel electrophoresis. Autoradiograph shows complement-treated (C5b-9) and untreated control (C) platelets. k-

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completely abolish the vesiculation response induced by the C5b-9 proteins, even when added at apparently “saturating” concentrations (eg, see Figs 6 and 7). One possibility is that under the conditions of our experiments, intracellular concentrations of these compounds required for complete enzyme inhibition were not achieved. Alternatively, these data may indicate that a kinase reaction facilitates but is not obligatorily coupled to the Ca²⁺-dependent step that is required for microparticle formation. Although we cannot exclude the possibility that the membrane-inserted C5b-9 proteins directly induce vesiculation of the plasma membrane, irrespective of the metabolic changes that are initiated by influx of Ca²⁺ across the complement pore, it should be noted that: (1) in C5b-9-treated platelets, microparticle formation shows strict dependence on [Ca²⁺], even though membrane insertion of these proteins does not, and (2) in platelets obtained from the patient with Scott Syndrome, vesiculation of the plasma membrane in re-

kinase is known to downregulate the Ca²⁺/calmodulin-dependent activation of myosin light chain kinase, it is possible that the inhibitory effect of cAMP on plasma membrane vesiculation is exerted directly through the phosphorylation of this enzyme. Alternatively, the observed inhibition of vesiculation may be related to decreased cytosolic [Ca²⁺], arising secondarily to elevated cAMP. Despite this cumulative evidence implicating the Ca²⁺-plus-calmodulin-activated myosin light chain kinase in platelet microparticle formation, it should be noted that the selective C-kinase inhibitor staurosporine was also observed to substantially inhibit phosphorylation of myosin light chain under the conditions of our experiments (Fig 4), although it caused only modest inhibition of microparticle formation (Fig 5). Therefore, it is possible that the inhibition of protein phosphorylation that we observe for these various enzyme inhibitors is neither temporally nor functionally related to their effects on microparticle formation.

None of the inhibitors used in this study were found to completely abolish the vesiculation response induced by the C5b-9 proteins, even when added at apparently “saturating” concentrations (eg, see Figs 6 and 7). One possibility is that under the conditions of our experiments, intracellular concentrations of these compounds required for complete enzyme inhibition were not achieved. Alternatively, these data may indicate that a kinase reaction facilitates but is not obligatorily coupled to the Ca²⁺-dependent step that is required for microparticle formation. Although we cannot exclude the possibility that the membrane-inserted C5b-9 proteins directly induce vesiculation of the plasma membrane, irrespective of the metabolic changes that are initiated by influx of Ca²⁺ across the complement pore, it should be noted that: (1) in C5b-9-treated platelets, microparticle formation shows strict dependence on [Ca²⁺], even though membrane insertion of these proteins does not, and (2) in platelets obtained from the patient with Scott Syndrome, vesiculation of the plasma membrane in re-

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response to C5b-9, Ca\(^{2+}\) ionophore, or other agonists that elevate cytosolic [Ca\(^{2+}\)] is markedly impaired. This suggests that membrane insertion of the C5b-9 proteins is not sufficient to induce plasma membrane vesiculation, and that this process normally requires a cellular response to elevated cytosolic [Ca\(^{2+}\)] that is defective in the Scott Syndrome cell. The capacity to modulate platelet microparticle formation by altering the Ca\(^{2+}\)-dependent protein phosphorylations implies that the vesiculation defect observed in Scott Syndrome may be related to an aberrant kinase or phosphoprotein in the platelets of this patient that is normally required to induce vesiculation of the plasma membrane during platelet activation.

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