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Alternations in C3 Activation and Binding Caused by Phosphorylation by a Casein Kinase Released from Activated Human Platelets

Kristina Nilsson Ekdahl and Bo Nilsson

A casein kinase released from activated human platelets phosphorylates a number of plasma proteins extracellularly, and that activation of platelets in systemic lupus erythematosus patients parallels an increase in the phosphate content of plasma proteins, including C3. The present study was undertaken to characterize this platelet protein kinase and to further elucidate the effect(s) on C3 function of phosphorylation by platelet casein kinase. The phosphate content of human plasma C3 was increased from 0.15 to 0.60 mol phosphate/mol of C3 after platelet activation in whole blood or platelet-rich plasma. The platelet casein kinase was distinct from other caseins in terms of its dependence on cations, inhibition by specific protein kinase inhibitors, and immunological reactivity. C3 that had been phosphorylated with platelet casein kinase was tested for its susceptibility to cleavage by trypsin or the classical and alternative pathway convertases and its binding to EAC and IgG. Phosphorylation did not affect the cleavage of C3 into C3a and C3b, but the binding of fragments from phosphorylated C3 to EAC14oxy cells and to IgG in purified systems and in serum was increased by 1.6–4.5 times over that of unphosphorylated C3. A covariation was seen between the enhanced binding of C3 fragments to IgG after phosphorylation and an increased ratio of glycerol/glycine binding, from 2.0 for unphosphorylated C3 to 4.9 for phosphorylated C3. The present study suggests that an overall effect of phosphorylation of C3 by platelet casein kinase is to enhance the opsonization of immune complexes. The Journal of Immunology, 1999, 162: 7426–7433.
The present study was undertaken to characterize this platelet protein kinase and to further elucidate the effect(s) of phosphorylation on C3 functions.

Materials and Methods

Antibodies

Polyclonal rabbit Abs against human C3a and C3c and HRP-conjugated polyclonal Abs against human C3c and mouse IgGs were purchased from Dako (Glostrup, Denmark). Chicken Abs against rat recombinant CK1 were purchased from Immunysystem (Uppsala, Sweden), a mouse monoclonal IgG1 Ab (mAb) clone 1AD9 against the α subunit of human protein kinase CK2 was obtained from Boehringer Mannheim (Mannheim Germany). Polyclonal rabbit Abs against phosphoserine and phosphothreonine were bought from Zymed (San Francisco, CA). HRP-conjugated streptavidin was purchased from Amersham (Slough, U.K.).

Falcon tubes from (Becton Dickinson, Meylan, France) were furnished with Corline heparin surface (Corline, Uppsala, Sweden) according to the manufacturer’s recommendation (15). The surface concentration of heparin was 0.5 μg/cm², corresponding to approximately 0.1 IU/cm², with an anti-thrombin III binding capacity of 2–4 pmol/cm².

Preparation of reagents

C3 and factor B were purified from human plasma according to the procedures described by Hammer et al. (16), and Lambris and Müller-Eberhard (17), respectively. Factor D was purified from peritoneal fluid from patients with renal failure, as described by Catana and Schifferli (18). C3 was digested with trypsin (Sigma, St. Louis, MO) to C3a and C3b (1%/w/v, for 5 min at room temperature), and the fragments were separated on Sephadex G-100 (Pharmacia Upjohn, Stockholm, Sweden) equilibrated in PBS. Nascent C3b, which exposes a free sulfhydryl group, was covalently bound to activated thiol Sepharose (ATS; Pharmacia Upjohn) (19): 80 μg trypsin was added to 10 mg of C3 and 2 mL of ATS and incubated at 37°C for 15 min. The digestion was interrupted by incubation with 0.8 mg of soybean trypsin inhibitor (Sigma) for 15 min, followed by extensive washing of the Sepharose.

Platelet CK was analyzed by the dot-blot technique using Abs against CK1 and CK2. Recombinant rat CK1 served as a positive control when platelet CK was detected by biotinylated polyclonal chicken Abs against CK1, followed by HRP-conjugated streptavidin. Human CK2 on U937-derived microparticles served as a positive control when platelet CK was analyzed using mAb anti-CK2, followed by HRP-conjugated anti-mouse IgGs.

In separate experiments, ATS-C3b was phosphorylated with platelet CK in the presence of 10 mM Mn²⁺ and 0.1 mM ATP (Sigma) and with three different protein kinase inhibitors, present in concentrations of up to 1 mM. The compounds tested were (2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide (CKI-7; Seikaguch, Tokyo, Japan), which is specific for CK1; 24): 5.6-dichloro-1β-ribofuransylbenzimidazole (Calbiochem, La Jolla, CA), which is specific for CK1 (25); and N-(2-aminooethyl)-5-chloro-ronaphthalene-1-sulfonamide HCl (A3; Calbiochem), which is reported to inhibit CK1 and CK2; protein kinases A, C, and G, and myosin light chain kinase (26). After phosphorylation, Sepharose was washed three times with PBS containing 0.1% Tween-20, and the amount of protein-bound radioactivity was measured in a beta counter. The ion dependence of platelet CK was compared with that of rat recombinant CK1 (Calbiochem) using casein (3 μg; Sigma) and C3 (20 μg) as substrates in the presence of 0.1 mM (γ⁻⁳²P)ATP and 5 mM of Mn²⁺, Mg²⁺, or Ca²⁺. To facilitate comparison of the two enzymes, the recombinant CK1 was diluted in albumin-free Tyrode’s medium to yield a sp. act. similar to that of the platelet CK preparation, giving final concentrations of 0.25 mM Mg²⁺ and 0.5 mM Ca²⁺, respectively. After phosphorylation at 37°C for 30 min, the samples were subjected to 10% SDS-PAGE under reducing conditions (27). Exposure of the dried gels and scanning were performed in a PhosphoImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) for calculations.

Phosphorylation of C3 and C3b

Platelets were precipitated from citrated blood and washed as described by Mustard et al. (22), with the modifications described previously (13, 23). Platelet protein kinase was prepared by resuspending the final platelet pellet in 1/3 volume PBS. Nascent C3b, which exposes a free sulfhydryl group, was covalently bound to activated thiol Sepharose (ATS; Pharmacia Upjohn) (19): 80 μg trypsin was added to 10 mg of C3 and 2 mL of ATS and incubated at 37°C for 15 min. The digestion was interrupted by incubation with 0.8 mg of soybean trypsin inhibitor (Sigma) for 15 min, followed by extensive washing of the Sepharose.

Activation of platelets in whole blood or in platelet-rich plasma (PRP)

Twenty milliliters of blood was collected in a 50-ml Falcon tube (Becton Dickinson), which had been furnished with a Corline heparin surface and contained 20 IU of soluble heparin (Bio Iberica, Barcelona, Spain). PRP was produced from blood by centrifugation for 15 min at 150 × g and was used immediately. Platelets in PRP or whole blood were stimulated with 100 μM ADP (Sigma) or HAGG (up to 100 μM/mL) for up to 15 min without agitation at room temperature. After incubation, 10 mM EDTA (κ-fold denaturation) was added, and the cells were centrifuged at 1100 × g for 10 min. Blood collected in Vacutainer tubes containing EDTA (Becton Dickinson, Rutherford, NJ) was used as a control.

C3 was precipitated removed from these samples by incubation with Sepharose-coupled anti-human C3c (13). The samples were then subjected to SDS-PAGE followed by Western blot analysis. A standard curve for the quantification of phosphate content in isolated proteins, ranging from 0.1– to 2.5 μmol of phosphate, was constructed with reduced histone type II-A (Sigma). By using a colorimetric technique (28), this preparation of histone was shown to contain 0.2 mol of phosphate/mol protein. Nitrocellulose membranes with precipitated proteins and histone were incubated in parallel with biotinylated rabbit Abs against phosphoserine and phosphothreonine, followed by HRP-conjugated streptavidin as described previously (14). The membranes were scanned using Studio Scan II (Agfa-Gevaert, Antwerp, Belgium), the intensity of the bands was quantified using NIH Image 1.54 for Macintosh, and the phosphate content of C3 from the samples was calculated from a standard curve on the same gels.

Platelet CK was analyzed by the dot-blot technique using Abs against CK1 and CK2. Recombinant rat CK1 served as a positive control when platelet CK was detected by biotinylated polyclonal chicken Abs against CK1, followed by HRP-conjugated streptavidin. Human CK2 on U937-derived microparticles served as a positive control when platelet CK was analyzed using mAb anti-CK2, followed by HRP-conjugated anti-mouse IgGs.

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Ab for detection, was performed in parallel, with identical Ab dilutions and staining times. This technique had a lower detection limit of 0.5 ng C3/C3 fragments/ml, which corresponds to 0.05 ng of C3/C3 fragments/well. ELISA for detection of C3α. Frozen serum samples were diluted 1/2000 (final dilution) and analyzed as described previously (29). mAb 4SD17.3 was used for capture, and bound C3α was detected with biotinylated anti-C3α followed by HRP-conjugated streptavidin. Zymosan-activated serum, calibrated against a solution of purified C3α, served as the standard.

Cleavage of C3 by trypsin

Phosphorylated or unphosphorylated C3 (10 μg) in PBS was incubated with trypsin in serial dilutions up to 0.5 μg for 30 min at 37°C. The reaction was then stopped by boiling the samples in electrophoresis sample buffer containing 15% 2-ME, and the samples were analyzed by SDS-PAGE, followed by densitometric scanning of the gels.

Activation of C3 by the alternative pathway convertase

Activation by the alternative pathway was assessed by incubating phosphorylated or unphosphorylated C3 (16 μg) with factor B (2.1 μg) and factor D (10 ng) diluted in VBS containing 2.5 mM Mg2+ for up to 5 min at 37°C. In an alternative procedure, phosphorylated or unphosphorylated C3 was added to preformed alternative pathway convertase complex molecules obtained by incubating 2 μg of unphosphorylated C3β in VBS containing 2.5 mM Mg2+ with factors B and D. The resulting cleavage was analyzed by SDS-PAGE, as described above.

Activation of C3 by the classical pathway convertase

Activation by the classical pathway was measured by incubating phosphorylated or unphosphorylated C3 diluted in VBS containing 0.75 mM Ca2+ and 2.5 mM Mg2+ with EAC14 oxy 2 cells bound to microtiter plates as described previously (30); microtiter plates were coated with EAC14 oxy 2 and 2.5 mM Mg2+. The reaction was then stopped by boiling the samples in electrophoresis sample buffer containing 15% 2-ME, and the samples were analyzed by SDS-PAGE, followed by densitometric scanning of the gels.

Binding of trypsin-generated C3 fragments to IgG

Wells of microtiter plates were coated with 10 μg/ml of human IgG ml. Purified phosphorylated or unphosphorylated C3 (0.5 mg/ml) in PBS was incubated with trypsin in serial dilutions from 3.1 μg/ml for 30 min at 37°C. The deposited C3 was detected with HRP-conjugated anti-C3c. Quantitation of the relative amount of bound C3 fragments was made using the sandwich ELISA as described above.

Binding of factor B-generated C3 fragments to IgG

Purified phosphorylated or unphosphorylated C3 (0.5 mg/ml) in VBS containing 2.5 mM Mg2+ was activated in IgG-coated microtiter plates for 30 min at 37°C by incubation with factors B and D in serial dilution from 50 and 0.4 μg/ml, respectively. Detection was performed as described above, and quantitation of the deposited C3 fragments was performed.

Covalent binding of C3 to glycerol and glycine

The binding of glycerol and glycine to C3 was measured by a modification of the technique described by Dodds and Law (31). Phosphorylated or unphosphorylated C3 (10 μg) in VBS containing 1 mM EDTA was activated with trypsin (0.03 μg) in the presence of either 2.5 mM [2-3H]glycerol (41.1 Ci/mmol) or 10 mM [2-3H]glycerol (200 μCi/mmol; both from New England Nuclear, Boston, MA) for 60 min at 37°C. Control samples of phosphorylated and unphosphorylated C3 were incubated in parallel, but without the addition of trypsin. After incubation, the reaction was terminated by boiling the samples in electrophoresis sample buffer, followed by separation by SDS-PAGE. Under these conditions, C3 was quantitatively activated, while no fragments beyond C3b were visible. Thereafter, the gels were cut, and the band in each lane corresponding to the α chain of C3b was incubated with Biolute-B tissue solubilizer (Zissner Analytic, Frankfurt, Germany), and the radioactivity in the samples was determined after the addition of Aquasafe 300 Plus scintillation mixture (Zissner Analytic). The sp. act. of the added glycine and glycyl was determined, and the degree of binding to the C3 samples, expressed as moles per mole of protein, was calculated.

Statistical analyses

The results are expressed as the mean ± SEM. Statistical significance was calculated with Student’s t test for unpaired samples, using StatView 4.01 (Abacus Concepts, Berkeley, CA) for Macintosh.

Results

Partial characterization of platelet CK

We first compared platelet CK to recombinant rat CK1 with regard to its immunological reactivity, dependence on divalent cations, and inhibition by protein kinase inhibitors (Table I). The immu-

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<tr>
<th>Table I. Comparison of platelet CK and recombinant rat CK1</th>
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<tr>
<td><strong>Immunological reactivity</strong></td>
</tr>
<tr>
<td>Anti-CK1</td>
</tr>
<tr>
<td>Anti-CK2</td>
</tr>
<tr>
<td><strong>Inhibitor (specificity)</strong></td>
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<tr>
<td>CK1-7 (CK1)</td>
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<tr>
<td>DRB (CK2)</td>
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<td>A3 (CK1, CK2, MLCK, PKA, PKC, PKG)</td>
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<td><strong>Effect of protein kinase inhibitors</strong></td>
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<tr>
<td><strong>Dependence on divalent cations</strong></td>
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<tr>
<td>Casein</td>
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<td>C3</td>
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* Tested by dot blot using biotinylated polyclonal chicken Abs against rat CK1, HRP-conjugated streptavidin or a mouse mAb against human CK2, and HRP-conjugated anti-mouse Ig.
* Inhibition of platelet CK activity, using AT5-bound C3b as substrate, as described in Materials and Methods.
* Concentration required to achieve 50% inhibition (μM).
* Ki (μM) as reported by the manufacturers.
* Protein kinases were diluted in Tyrode’s buffer to give final concentrations of 0.25 mM Mg2+ and 0.5 mM Ca2+, respectively. Thereafter 5 mM of either Mg2+, Mn2+, or Ca2+ was added together with 0.1 mM [γ-32P]ATP. After phosphorylation, the samples were subjected to SDS-PAGE under reducing conditions, and the dried gels were exposed as described in Materials and Methods.
* Tyrode’s buffer diluted 1/4; 0.25 mM of Mg2+ and 0.5 mM of Ca2+ (final concentrations).
nological identity of platelet CK was checked by means of dot blots using Abs against CK1 and CK2 (Table I). Chicken Abs against CK1 reacted weakly with platelet CK and strongly with rat CK1. In contrast, a rat anti-CK2 mAb reacted with CK2 on U937-derived microparticles (not shown), but failed to react with both platelet CK and CK1.

The activity of platelet CK was then measured in the presence of three protein kinase inhibitors in concentrations up to 1 mM, using ATS-bound C3b as a substrate (Table I). Platelet CK was inhibited by CKI-7 (specific for CK1) and A3 (which inhibits multiple protein kinases, including CK1 and CK2), but not by 5,6-dichloro-1-D-ribofuranosylbenzimidazole (which is specific for CK2). The $K_{i_{50}}$ values obtained were 110 $\mu$M for CKI-7 and 800 $\mu$M for A3, respectively. These values are substantially higher than those cited by the manufacturers for these inhibitors against CK1.

Mn$^{2+}$ and Ca$^{2+}$ were more potent stimulators of platelet CK activity than Mg$^{2+}$ when equimolar amounts of casein and C3 were used as substrates (Table I). In contrast, activation of CK1 was most pronounced in the presence of Mg$^{2+}$, whereas inhibition was seen in the presence of Mn$^{2+}$.

**Phosphorylation of C3 after activation of platelets in whole blood or in PRP**

Western blot analysis (using a mixture of Abs against phosphoserine and phosphothreonine) of C3 that had been isolated from EDTA plasma had a phosphate content of 0.14 $\pm$ 0.02 mol/mol protein. The level was increased in C3 that had been isolated from activated whole blood or PRP. In whole blood the phosphate content in C3 increased after incubation with HAGG (from 0.14 $\pm$ 0.02 to 0.52 $\pm$ 0.08 mol/mol; $n = 7$) or incubation with ADP (to 0.60 $\pm$ 0.14 mol/mol; $n = 7$). A similar increase was seen in PRP from 0.15 $\pm$ 0.02 to 0.56 $\pm$ 0.09 mol/mol ($n = 7$) after incubation with HAGG or to 0.65 $\pm$ 0.14 ($n = 7$) after incubation with ADP (Fig. 1A).

The phosphate content of C3 in whole blood that had been incubated with 100 $\mu$g of HAGG/ml increased continuously during the 15-min incubation, reaching a level more than 4 times the starting level (Fig. 1B).

**Activation of C3 in serum on an IgG-coated surface**

When the activation of C3 and binding of C3 fragments to an IgG-coated surface were assessed by ELISA, significantly higher amounts of split products from phosphorylated C3 than from unphosphorylated C3 were detected in all serum concentrations tested (Fig. 2A). At the highest serum concentration tested (1/3), substitution with unphosphorylated C3 resulted in deposition of approximately 10 ng of C3 fragments/well as assessed by sandwich ELISA, whereas substitution with phosphorylated C3 resulted in deposition of 4 times higher amounts of C3 fragments/well ($n = 3$; $p < 0.0001$).

This higher level of binding of fragments from phosphorylated C3 was accompanied by higher complement activation, as assessed by the generation of C3a (Fig. 2B). At a serum dilution of 1/3 the levels of C3a reached 3000 ng/ml (for phosphorylated C3) and 2200 ng/ml (for unphosphorylated), respectively ($n = 3$; $p = 0.0286$).
Cleavage of C3 by trypsin

There was no significant difference in the rate of trypsin cleavage after phosphorylation of C3 (Fig. 3B). In addition, cleavage generated very similar or identical polypeptide fragments from phosphorylated and unphosphorylated C3 (Fig. 3A).

Activation of C3 by the alternative pathway convertase

Alternative pathway activation of phosphorylated and unphosphorylated C3 was studied by incubation of mixtures of purified factor B, factor D, and C3. The cleavage of phosphorylated C3 occurred at a slightly (but not significantly) lower rate than that of unphosphorylated C3 (Fig. 4, A and B). After 3 min of incubation, 60% of the unphosphorylated C3 remained intact compared with 75% of the phosphorylated C3.

In experiments designed to expose phosphorylated and unphosphorylated C3 to identical amounts of preformed convertase, factor B and factor D were incubated with (unphosphorylated) C3b before the addition of C3. Phosphorylated C3 was marginally more resistant to cleavage than was unphosphorylated C3, with approximately 40% cleavage as compared with 50% for unphosphorylated C3 after 3 min of incubation (Fig. 4, C and D).

Activation of C3 by the classical pathway convertase

Physiological activation of C3 by the classical pathway convertase was measured after incubation of phosphorylated or unphosphorylated C3 with EAC14^Oxy2 cells fixed to microtiter plates. Activation was monitored by measuring the binding of C3 fragments on the EAC14^Oxy2 cell surface. Approximately 2-fold higher binding occurred when fragments were generated from phosphorylated C3 than from unphosphorylated C3 after addition to the cells at concentrations >3 μg/ml (Fig. 5; n = 4; p = 0.0006 for 30 μg/ml). Lower concentrations of added C3 resulted in the same degree of binding.

Binding of trypsin-generated C3 fragments to IgG

The capacity of C3 to bind to IgG was assessed by incubating C3 together with trypsin in wells of microtiter plates coated with IgG. The level of binding of phosphorylated C3 was significantly higher at all concentrations of trypsin tested (Fig. 6A). When trypsin concentrations of 1.5 μg/ml or higher were used, the binding of C3 fragments reached a plateau at approximately 10 ng of C3 fragments/well for unphosphorylated C3 (as estimated by sandwich ELISA) and 1.6 times higher for phosphorylated C3, respectively (n = 4; p < 0.0001).

Binding of factor B-generated C3 fragments to IgG

The capacity of C3 to bind to IgG was further assessed by incubating C3 together with factor B and factor D in wells of microtiter plates coated with IgG. The level of binding of phosphorylated C3 with EAC14^Oxy2 cells fixed to microtiter plates. Activation was monitored by measuring the binding of C3 fragments on the EAC14^Oxy2 cell surface. Approximately 2-fold higher binding occurred when fragments were generated from phosphorylated C3 than from unphosphorylated C3 after addition to the cells at concentrations >3 μg/ml (Fig. 5; n = 4; p = 0.0006 for 30 μg/ml). Lower concentrations of added C3 resulted in the same degree of binding.

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Binding of factor B-generated C3 fragments to IgG

The capacity of C3 to bind to IgG was further assessed by incubating C3 together with factor B and factor D in wells of microtiter plates coated with IgG. The level of binding of phosphorylated C3 was significantly higher at all concentrations of trypsin tested (Fig. 6A). When trypsin concentrations of 1.5 μg/ml or higher were used, the binding of C3 fragments reached a plateau at approximately 10 ng of C3 fragments/well for unphosphorylated C3 (as estimated by sandwich ELISA) and 1.6 times higher for phosphorylated C3, respectively (n = 4; p < 0.0001).
was significantly higher at all tested concentrations of factor B (Fig. 6B). When factor B at concentrations of 6 μg/ml or higher were used, the binding of C3 fragments reached a plateau at an approximately 1.6 times higher level for phosphorylated C3 compared with unphosphorylated C3 (n = 4; p = 0.0001).

**Covalent binding of C3 to glycerol and glycine**

The capacity of C3 to form ester and amide bonds was assessed by examining the binding of radiolabeled glycerol and glycine to unphosphorylated and phosphorylated C3 when trypsin was used to activate the C3. The ratio of trypsin to C3 (indicated by an arrow in Fig. 6A) was the lowest amount that gave maximal binding of C3 fragments to the IgG surface. Under these conditions unphosphorylated C3 was found to bind 0.454 ± 0.047 mol of glycerol/mol of protein, whereas phosphorylated C3 bound 0.726 ± 0.065 mol of glycerol/mol of protein (n = 10; p = 0.0148).

The binding of glycine decreased after phosphorylation, from 0.224 ± 0.025 to 0.149 ± 0.011 mol of glycine/mol of protein, respectively (n = 10; p = 0.0036). Consequently, the glycerol/glycine ratio increased from 2.0 for unphosphorylated C3 to 4.9 for phosphorylated C3 (Table II). The background levels of glycine binding was 0.010 ± 0.002 mol/mol for unphosphorylated C3 and 0.011 ± 0.002 mol/mol for phosphorylated C3 (n = 4). The corresponding values for glycerol binding to control and phosphorylated C3 were 0.020 ± 0.002 and 0.021 ± 0.002 mol/mol (n = 4), respectively.

**Discussion**

We have previously shown that activation of washed platelets leads to the release of a CK that is able to phosphorylate a number of plasma proteins in vitro, including C3, after the addition of ATP and ions. The endogenous supplies of ATP and ions in the platelets are sufficient to support phosphorylation of exogenously added substrates, suggesting that activation of platelets in whole blood might lead to extracellular phosphorylation. Indeed, the phosphate content of plasma proteins increased in systemic lupus erythematosus patients in parallel to platelet activation in vivo, but the direct line of evidence linking platelet activation in vitro to extracellular phosphorylation of proteins in whole blood has been missing until now. In the present study we have demonstrated that the phosphate content of plasma C3 increases by >4-fold after platelet activation, thus confirming that platelet activation in PRP or intact blood indeed leads to extracellular phosphorylation of plasma proteins, without the addition of exogenous ATP or cations. The initial value of 0.14 mol of phosphate/mol of C3 reported here is in good agreement with earlier reports of 0.15 and 0.30 mol of phosphate/mol of C3 detected using other techniques (4, 13).

The protein kinase that is released from platelets and phosphorylates human C3 is a Ser/Thr protein kinase that has CK1-like properties, in that it phosphorylates casein and is not inhibited by heparin; however, in contrast to CK1, its activity is dependent on Mn$^{2+}$ and Ca$^{2+}$ (13, 23). Phosphorylation by platelet kinase made

### Table II. Covalent binding of glycerol and glycine to C3b$^a$

<table>
<thead>
<tr>
<th></th>
<th>Glycerol Binding (mol/mol)</th>
<th>Glycine Binding (mol/mol)</th>
<th>Glycerol/Glycine Ratio</th>
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<tbody>
<tr>
<td>C3$^b$ (activated)</td>
<td>0.454 ± 0.047</td>
<td>0.224 ± 0.025</td>
<td>2.0</td>
</tr>
<tr>
<td>C3 (nonactivated)</td>
<td>0.020 ± 0.002</td>
<td>0.010 ± 0.002</td>
<td>—</td>
</tr>
<tr>
<td>C3-P$^a$ (activated)</td>
<td>0.726 ± 0.065</td>
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<td>4.9</td>
</tr>
<tr>
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$p$ value 0.0148 0.0036

$^a$ Phosphorylated or unphosphorylated C3 (10 μg) was activated by 0.03 μg of trypsin in the presence of 10 mM [2-3H]glycerol or 2.5 mM [2-3H]glycine. Quantitation of the bound molecules was carried out as described in Materials and Methods.

$^b$ C3-P, phosphorylated C3.
bound C3b less susceptible to cleavage by factor I, with factor H as a cofactor (13). To characterize the platelet Ser/Thr protein kinase, we have now compared this protein kinase to commercially available rat recombinant CK1 with regard to its response to specific protein kinase inhibitors, immunological reactivity, and dependence on various divalent cations. Platelet CK was partially inhibited by compounds reported to be specific for CK1, but with a 10-fold higher $K_i$ than that reported for CK1, suggesting similarity, but not identity, between the two enzymes. This interpretation was supported by the observation that Abs raised against CK1 reacted much more weakly with platelet CK than with CK1. CK1 is extremely well conserved between different species, e.g., 94% identity between rat and human CK1$\gamma_2$ (32). There is even a considerable degree of homology between mammalian and yeast CK1 (33). It is therefore most likely that polyclonal Abs raised against rat CK1 should react with human CK1. Both protein kinases phosphorylated C3 and casein, but while Mn$^{2+}$ ions potentiated phosphorylation of both substrates by platelet CK, it inhibited phosphorylation by CK1. Taken together, these results suggest that the platelet protein kinase is a CK that is distinct from CK1. We have earlier excluded the possibility that it is a CK2, since it did not react with a mAb to human CK2, and its activity was not inhibited by heparin or 2,3-DPG (13).

The observation that phosphorylation of C3b by platelet CK decreased the susceptibility for factor I cleavage led us to speculate whether phosphorylation of C3 might increase the amount of C3b that binds to a target surface, e.g., an immune complex. The fact that the half-life of bound C3 fragments in the form of C3b is prolonged should provide greater opportunity for formation of alternative pathway convertase complexes and should thus potentially give rise to increased opsonization. We found that this was indeed the case when serum from a patient deficient in C3 was reconstituted with phosphorylated or unphosphorylated C3 and then incubated with an IgG-coated surface. Phosphorylation increased by 4-fold the amount of generated C3 fragments that bound to IgG. This substantial increase in the binding of C3 fragments was accompanied by a more modest increase in C3a generation, which indicated that other mechanisms were also involved in increased binding.

Phosphorylation of C3 by PKA and PKC has previously been shown to increase resistance to cleavage of C3 to C3a and C3b by the classical and alternative pathway convertases as well as by trypsin (11). We have now conducted similar experiments with purified C3 phosphorylated by platelet CK; exposure of phosphorylated C3 to the classical pathway convertase on EAC14oxy2 cells resulted in approximately 2-fold higher binding of C3 fragments than that for unphosphorylated C3. The binding approached a plateau, indicating binding to a limited number of sites. In this assay the level of binding is dependent upon the efficiency with which the cell-bound convertase molecules cleave C3 and upon the ability of the nascent C3b molecules to form covalent bonds to hydroxyl or amino groups on the cell surface (30). Phosphorylation of C3 by platelet CK did not significantly affect cleavage of fluid phase C3 by a fluid phase C3bBb convertase or by trypsin. It is thus unlikely that the increased binding seen on the EAC14oxy2 cells was due to increased cleavage, since both of the convertases and trypsin cleave C3 at the same peptide bond. On the other hand, it is feasible that the difference in binding was related to an increase in the number of available acceptor sites for phosphorylated C3. This hypothesis was confirmed by our observation of increased binding to surface-bound IgG of fragments generated from phosphorylated C3 by either C3bBb convertase- or tryptic-mediated cleavage. In these experimental systems, as in EAC14 oxy2 cells, the binding of fragments generated from phosphorylated C3 was significantly (~1.6 times) higher than that for fragments from unphosphorylated C3.

The putative phosphorylation site for platelet CK in C3 is located within a tryptic fragment, between Lys$^{979}$ and Lys$^{1014}$, which also comprises the thiol ester as we have reported previously (13). After extensive trypsin digestion of phosphorylated ATS-bound C3b, this fragment containing 90% of the total radioactivity still bound to the ATS. After hydrolysis of the ATS-bound C3 fragment, only Thr-P was detected. Based on these results we concluded that the most likely phosphorylation site for platelet CK is Thr$^{1009}$, 19 aa residues toward the C terminus of the C3 $\alpha$-chain from the thiol ester. The sequence DETEQWE surrounding Thr$^{1009}$ (34) contains several acid amino acid residues, which makes it a potential phosphorylation site for CKs (35). One possible explanation for the difference in binding efficiency reported in the present study is that phosphorylation of C3 in some way alters the binding properties of the thiol ester. To test whether this was the case, we used trypsin to cleave phosphorylated or unphosphorylated C3 in the presence of either [2-$^3$H]glycerol or [2-$^3$H]-glycine. Phosphorylation of C3 increased the glycerol binding capacity (by ~1.6-fold), while it decreased glycine binding, resulting in an increased ratio of glycerol/glycine binding, from 2.0 for unphosphorylated C3 to 4.9 for phosphorylated C3. These changes are sufficient to account for the increased binding of C3 fragments to IgG, since it has been demonstrated that the binding of C3b to IgG mainly occurs via ester bonds that are formed with hydroxyl group-containing residues in the heavy chain of IgG (36–38). It should also be noted that in the present study phosphorylation of C3 brought about an analogous increase in glycero binding and in binding of generated C3 fragments to IgG after activation by trypsin (Fig. 6), emphasizing that the binding to IgG is indeed ester linked. A recently published crystal structure of human C3d (39) confirms that Thr$^{38}$ of C3d (which corresponds to Thr$^{1009}$ of intact C3) is exposed on the exterior of the molecule, in close proximity to a cluster of acidic amino acid residues. In C3d, Thr$^{38}$ and the thiol ester are located at opposite ends of the $\alpha$1 helix, with Thr$^{38}$ at the C terminus. For the formation of ester bonds the thiol ester of C3 requires the sequence Cyx$^{1010}$, Glx$^{1013}$, and His$^{1126}$ (40). In C3d, these amino acid residues correspond to Cyx$^{17}$, Glx$^{29}$, and His$^{133}$, which are located at opposite ends of the T3 segment. The mechanism by which phosphorylation of Thr$^{1009}$ alters the binding properties of C3 remains to be established; one possibility is that the negatively charged phosphate group alters the distance between the thiol ester and His$^{1126}$, thereby contributing to the formation of ester bonds instead of amides.

The overall effect of the phosphorylation of C3 by platelet CK should be to enhance the opsonization of immune complexes in whole blood. Handling of immune complexes involves a sequence of events that includes complement activation and covalent binding of C3b to the immune complex (41). One can propose a pathway in vivo by which platelets that become activated by immune complexes (triggered by Fc receptors, C1q, and/or C5b-9 complexes) mediate the phosphorylation of fluid phase C3 and immune complex-bound C3b. The consequence is an amplification effect by which immune complexes indirectly potentiate their own opsonization.

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References