In Vitro Phosphorylation of Human Complement Factor C3 by Protein Kinase A and Protein Kinase C

Effects on the Classical and Alternative Pathways*

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Complement factor C3, recently found to contain covalently bound phosphate, was phosphorylated in vitro by cyclic AMP-dependent protein kinase (protein kinase A) and Ca"+-activated, phospholipid-dependent protein kinase (protein kinase C). Both protein kinases phosphorylated the same serine residue(s) located in the C3a portion of the α-chain. In addition, protein kinase C phosphorylated the β-chain to a lesser extent. Protein kinase A gave a maximal incorporation of 1 mol of phosphate/mol of C3 while that value with protein kinase C was 1.5 mol of phosphate/mol of C3. The velocity in pmol of [32P]phosphate/(min × unit kinase) was 20 times higher for protein kinase C than for protein kinase A although a 10 times lower ratio of protein kinase to C3 was used in the former case. The apparent K_m for C3 was 2.6 μM when protein kinase C was used.

The phosphorylated C3 was found to be more resistant to partial degradation by trypsin than unphosphorylated C3. It was also found that phosphorylation of C3 in the C3a portion of the α-chain inhibited both the classical and alternative complement activation pathways on an approximately stoichiometric basis.

The complement system plays an important role as a defense system against invading microorganisms, causing cell lysis, induction of inflammatory and immunological responses, opsonization, engulfment, and killing. It can also damage the autologous tissues and cause a variety of inflammatory diseases.

Activation of the complement system occurs by two distinct routes, the classical and the alternative pathways. The former characteristically is triggered by antigen-antibody complexes, while the latter, which does not require immune complexes, can be triggered by polysaccharides, or other less well-defined substances. Both pathways converge on C3, which is activated proteolytically by similar C3 convertases, giving the C3a and C3b fragments. C3a is released into the fluid phase and functions as an anaphylatoxin. As a ligand of various cell receptors, C3b and its degradation products function as powerful mediators of a variety of inflammatory/immunological reactions (for a recent review see Reid (1)).

As presently understood, the complement system, like other proteolytically activated cascade systems in plasma, is regulated by a delicate balance of specific proteases, protease inhibitors, and binding proteins, although recent work suggests that sulfation (2) might also play a part.

Several plasma proteins have been shown to be substrates of protein kinases from blood (3–9). Thus, fibrinogen, a known phosphoprotein (10), is phosphorylated by several different protein kinases (11), and complement S protein, involved in the regulation of the membrane attack complex, is phosphorylated in vivo (12). Recently, complement factor C3 has been found to be phosphorylated in vivo in coagulating and anticoagulated blood (13). This has led us to investigate whether phosphorylation of C3 alters its activity.

Most protein-bound phosphate, at least intracellularly, is of a regulatory type. This implies that regulatory phosphorylation of blood plasma proteins may also occur. This suggestion is supported by the fact that fibrin obtained from fibrinogen phosphorylated by protein kinase C in vitro has a different fiber structure than fibrin from the unphosphorylated or dephosphorylated precursor (14,15).

In this paper experiments are described showing the in vitro incorporation of phosphate into the α-chain of C3 by protein kinase A and protein kinase C, as well as the effect such phosphorylation has on both complement activation systems.

Experimental Procedures

Materials

Histone H1 (Sigma type III-S), mixed histone, bovine serum albumin, 1,2 diolen, L-α phosphatidyl α-serine, and soybean trypsin inhibitor were all products of Sigma.

[γ-32P]ATP was purchased from Du Pont-New England Nuclear. Sephadex, Sepharose, and DEAE-Sepharose were purchased from a Pharmacia (Sweden), and the DEAE-cellulose used was DE52 from Whatman (United Kingdom). The reference proteins used in polyacrylamide gel electrophoresis and the protein dye reagent for the dye binding assay were from Bio-Rad. All other chemicals were of the highest purity commercially available.

Preparation of Protein Kinases

The catalytic subunit of protein kinase A was prepared from pig muscle according to Bechtle et al. (16) with modifications (17) or from rabbit muscle as described by Beavo et al. (18).

Protein kinase C was purified from pig spleen as described for the bovine brain enzyme (19), except for the final chromatography on phenyl-Sepharose which was excluded because of low recovery. However, the results with the less purified kinase were identical to those with the fully purified kinase preparation, as has also been reported for other substrates by Ferrari et al. (20).

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One unit of protein kinase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 pmol of phosphate/min into mixed histone (protein kinase A) or into histone H1 (protein kinase C) under the conditions specified below at a 1 mg/ml concentration of histone.

Preparation of Complement Factor C3 from Human Plasma

C3 was purified according to Hammer et al. (21). After each purification step C3 was detected by fused rocket immunoelectrophoresis (22). The final preparations were immunologically pure. A molecular mass of 185 kDa was used to calculate the molar concentration of C3.

Phosphorylation Experiments

The standard phosphorylation incubation mixture (80 μl) with protein kinase A contained magnesium acetate (8 mM), EDTA (0.5 mM), [32P]ATP (0.5 mM, 100 cpm/pmol), Tris-HCl (50 mM) at pH 8.5, and protein kinase and substrates as indicated. The reaction was carried out at 30 °C.

Incubations with protein kinase C were performed at 30 °C and pH 6.0 in 75 μl of incubation mixtures, containing MES (30 mM), potassium phosphate (14 mM), sodium chloride (43 mM), calcium acetate (0.71 mM), EGTA (0.057 mM), magnesium acetate (5.4 mM), [32P]ATP (0.1 mM, 100 cpm/pmol), phosphatidylserine (64 μg/ml), diolein (1.1 μg/ml), and substrates as indicated.

All phosphorylation reactions were interrupted by the application of aliquots of the mixture to pieces of Whatman 3MM paper, which were washed and dried as described by Humble et al. (23). The radioactivity was then measured as Cerenkov radiation (24).

Polyacrylamide Gel Electrophoresis

Samples were incubated for 1 h at 70 °C in 1% (w/v) sodium dodecyl sulfate, 4 M urea, and 1% (v/v) β-mercaptoethanol, and subjected to electrophoresis in 0.1% sodium dodecyl sulfate (SDS), under reducing conditions (SDS-PAGE). A slab gel system was used (25), as modified by O'Farrel (26). The gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed.

Determination of 32P-Phosphoamino Acids

32P-Labeled or unlabeled C3, 1 mg of each, was precipitated and washed as described for the 32P-phosphoamino acid determination. The samples were hydrolyzed in 2 M HCl at 100 °C for 20 h in sealed Pyrex tubes.

The hydrolysate was dried in vacuo and applied to a Dowex 50 × 8 column (50 ml) which was equilibrated with 10 mM HCl. Phosphoserine and phosphothreonine were eluted with 10 mM HCl. Phosphoserine and phosphothreonine were eluted at 100 °C.

High Voltage Electrophoresis

C3 in 150-μg portions was phosphorylated with each protein kinase, precipitated, and washed as described for the 32P-phosphoamino acid determination. The samples were hydrolyzed in 2 M HCl at 100 °C for 30 min and dried. The hydrolysates were dissolved in water, and material from about 15 μg C3 was analyzed by high voltage electrophoresis. This was performed for 90 min at pH 4.75, in 50 mM citrate buffer or at pH 3.6 in 50 mM pyridine acetate, on Polygram CEL 300 thin layer plates (20 × 20 cm) at 20 V/cm. After drying, the plates were autoradiographed to visualize the labeled phosphopeptides.

Trypsinization of C3

L-1-Tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Worthington Diagnostic Systems Inc.) 0.9% (w/w) was used to partially proteolysize unphosphorylated and phosphorylated C3 in 50 mM phospho-buffered saline, pH 7.5, for short periods of up to 5 min at room temperature as in Ref. 28. The reactions were stopped by adding soybean trypsin inhibitor. The samples were then denatured and subjected to SDS-PAGE.

RESULTS

Phosphorylation of C3 by Protein Kinases A and C

Dependence on pH and Time—It was found that C3 was phosphorylated in vitro by protein kinase A and C. The two protein kinases displayed different pH optima for the phosphorylation of C3. For protein kinase A a high pH was optimal (Fig. 1). The optimum pH for protein kinase C was about 6.0 (Fig. 1). All subsequent phosphorylation experiments were conducted at pH 8.5 and 6.0 for protein kinase A and C, respectively, pH 8.5 was chosen to ensure the stability of C3.

The maximum amount of 32P-phosphate incorporated by protein kinase A was around 1 mol of phosphate/mol of C3 (Fig. 2), and by protein kinase C around 1.5 mol of phosphate/mol of C3 (Fig. 2). The maximal incorporation of phosphate into hemolytically inactive C3, i.e. C3 (H4O) where the thiol-ester bond had been broken by repeated freezing and thawing (33), was approximately 0.2 mol of phosphate/mol of C3 even after 2 h incubation (data not shown).

The higher phosphate incorporation into C3 with protein kinase C indicated that more than one site in C3 was phospho-
Phosphorylation of C3 by Protein Kinases A and C

FIG. 2. Time course of the phosphorylation of C3. The standard phosphorylation incubations were performed as described under "Experimental Procedures." ○—○, 700 units of protein kinase A and 0.33 mg/ml of C3. ●—●, 130 units of protein kinase C and 0.69 mg/ml of C3.

phosphorylated with this kinase. The approximate speed expressed in pmol of phosphate/(min × unit kinase) was 0.0049 for protein kinase A and 0.092 for protein kinase C. The unit kinase/pmol C3 ratio in these experiments was 6 and 0.6, respectively.

Dependence of the Phosphorylation on the Amount of Protein Kinase C and Divalent Cations—When low concentrations of protein kinase C and a saturating concentration of C3 were used, the incorporation of [32P]phosphate into C3 increased proportional to the amount of protein kinase C used. When more than 250 units of protein kinase were used in the test the degree of phosphorylation was independent of the amount of protein kinase (data not shown) and the β-chain of C3 was also phosphorylated, but always to a lesser extent than the α-chain (Fig. 4B).

The rate of C3 phosphorylation varied slightly with the concentration of Mg2+. Maximal incorporation of [32P]phosphate was obtained at 2.5–10 mM Mg2+. The effects of Ca2+ were tested at 0.15, 0.7, and 3.5 mM concentration. The greatest stimulation and incorporation was found at 0.7 mM which was the concentration used in the standard assay. The activity was only slightly less at the other concentrations of Ca2+ (data not shown).

Determination of Apparent $K_m$ for C3 Using Protein Kinase C—A saturating concentration of [32P]ATP was used (i.e. 100 μM) and the concentration of C3 was varied. The data were processed by a computer package (Dare-P, for processing the Michaelis-Menten equation on a Nord 10 minicomputer) determining the apparent $K_m$ to be 2.6 μM C3 (Fig. 3). The apparent $K_m$ value for the other substrate (ATP) was 7 μM when the calculations were made from a Lineweaver-Burk plot (data not shown).

Localization of the 32P-Phosphorylated Site

The chains of C3 phosphorylated with protein kinase A or protein kinase C were separated on SDS-PAGE as described under "Experimental Procedures." After autoradiography of the gels it was found that C3 phosphorylated with protein kinase A showed two bands on the film corresponding to the α-chain of C3 and the N-terminal 47,000 fragment of the α-chain arising from denaturation of C3 in SDS (Fig. 4A).

Because of C3’s unusual thiol-ester bond, a proportion of the C3 molecules undergo hydrolysis of the peptide chain when denatured in SDS (34). C3 phosphorylated with protein kinase C (Fig. 4B), in addition to a heavily phosphorylated α-chain, displayed a phosphorylation site in the β-chain. The latter...
phosphorylation was more stimulated by lipids than the phosphorylation of the α-chain (data not given). This pattern was also obtained with protein kinase C from human platelets (data not shown).

C3 phosphorylated by protein kinase C was partially trypsinized to produce C3a and C3b, subjected to SDS-PAGE, and autoradiographed. Fig. 4B shows that when the C3a portion of the α-chain phosphorylated with protein kinase C was cleaved off by trypsin, the α-chain was converted to an α'-chain lacking any [32P]phosphate. The same result was obtained with C3 phosphorylated by protein kinase A (data not shown).

C3 phosphorylated with each of the protein kinases was partially hydrolyzed in acid, as described under "Experimental Procedures," and the resultant peptides separated by high voltage electrophoresis. A similar pattern of [32P]phosphopeptides was obtained from C3 phosphorylated with either kinase indicating that both kinases phosphorylated the same serine residue(s) (Fig. 5). Some extra phosphopeptides in C3 phosphorylated by protein kinase C can be seen, they probably derived from the site(s) phosphorylated in the β-chain.

When C3 phosphorylated by protein kinase A or protein kinase C was analyzed for the 32P-labeled amino acids it was found that C3 phosphorylated by protein kinase A contained only [32P]phosphoserine, while C3 phosphorylated by protein kinase C gave 65% [32P]phosphoserine and 35% [32P]phosphothreonine (data not shown).

Effect of Phosphorylation on the Rate of the Degradation of C3 with Trypsin

It was obviously of interest to test whether phosphorylation of C3 affected its activity. First, unphosphorylated C3 and in vitro phosphorylated C3 were partially trypsinized for 0–5 min. A marked difference between the two samples in the rates of conversion of C3 to C3a and C3b was noted. This is shown by the gel in Fig. 6 where the α-chain of C3 phosphorylated by protein kinase A apparently was more resistant to proteolysis than the non-phosphorylated α-chain, as judged by comparing the rates of appearance of the α'-chain. Identical results were obtained when C3 was phosphorylated by protein kinase C (data not shown).

Effect of Phosphorylation on the Activity of C3

Because trypsin cleaves the same arginine 77-serine 78 bond in the α-chain as both the classical and alternative pathway C3 convertases, the activity of phosphorylated C3 in classical and alternative pathway activation tests was compared with that of unphosphorylated C3. The results of the classical pathway hemolysis test are shown in Fig. 7, where it is apparent that phosphorylated C3 was less active than unphosphorylated C3. Incubation of C3 with either kinase in the absence of ATP did not reduce the hemolytic activity of C3 (data not given).

When the degree of phosphorylation of the C3 samples was considered, 0.5 mol of [32P]phosphate/mol of C3 for protein kinase A and 0.6 mol of [32P]phosphate/mol of C3 for protein kinase C, it appeared that the inhibition was nearly stoichiometric as there was approximately 45% inhibition of hemolysis with protein kinase C and 65% inhibition with protein kinase A phosphorylated C3.

The same C3 preparations used in the experiments described in Fig. 7 were incubated with the alternative pathway...
**DISCUSSION**

Complement factor C3 was phosphorylated in vitro by protein kinases A and C, the latter being the more active. The two protein kinases had different pH optima for the incorporation of phosphate into C3. The dependence on a high pH for a high rate of the protein A phosphorylation has previously been shown for other proteins located extra- and intracellularly (36, 37). A low pH is also required for the phosphorylation of fibrinogen with protein kinase C where the reported pH optimum is around 6.5 (23).

The possible physiological role of the phosphorylation of C3 is obviously of great interest. Phosphorylation of the protein may occur either before its secretion, as with sulfation (2), or in the blood under normal (13) or pathological conditions. There have been reports of extracellular protein kinase activity in plasma (3-7) and on the surface of cell membranes (8, 9). ATP has been detected in human plasma in micromolar amounts (13, 38). Platelet stimulation has also been reported to release ATP and protein kinase A (39). Thus, the necessary enzymes and substrates for a phosphorylation reaction may be present at sufficient concentrations at least under certain conditions. During tissue injuries the pH decreases locally, making proteins a better substrate for protein kinase C, which is also present in platelets in substantial amounts (40).

The rate and extent of the phosphorylation of C3 supports the view that it might be of biological significance. This is further supported by the fairly low $K_{b}$ value obtained for C3 with protein kinase C (i.e., 2.6 $\mu M$) which was in the same order of magnitude as those given for other protein substrates (41-46). The $K_{b}$ is in the physiological concentration range being approximately a third of the normal plasma concentration of C3.

The phosphorylated part of the $\alpha$-chain was cleaved off by partial trypsinization, under conditions known to liberate the C3a portion of the $\alpha$-chain from C3 phosphorylated with either kinase (Fig. 4B). This fact, as well as the nearly identical phosphopeptides obtained after partial acid hydrolysis, suggest that the same serine residue(s) in the C3a portion were phosphorylated by both protein kinases.

The most convincing results with regard to a biological role of the phosphorylation reactions were obtained when the activity of C3 was tested. Phosphorylated C3 was shown to be less susceptible to partial trypsinization than unphosphorylated C3 regardless of which of two protein kinases were used to phosphorylate it. The same inhibitory effect was found when phosphorylated C3 was used in either classical or alternative pathway activation tests. The degree of inhibition was seen to correlate well with the degree of phosphorylation. Between 30 and 65% inhibition was obtained in the complement activation tests when the in vitro phosphorylated C3 samples contained 0.5 and 0.6 mol of $[^{32}P]$phosphate/mol C3.

Other studies have shown that protein phosphorylation can affect proteolytic susceptibility (47, 48) and can be a regulatory mechanism.

Native C3 contains a thiol-ester bond. If this bond is broken C3 is inactivated (32). Every attempt to phosphorylate C3 in which the thiol-ester bond was already broken proved futile; regardless of which kinase was used the incorporation was always less than 0.2 mol of $[^{32}P]$phosphate/mol C3 (data not shown). The mere fact that the 47 kDa-fragment is phosphorylated (Fig. 4) shows that the thiol-ester bond was intact until the C3 was denatured for SDS-PAGE. Thus, the inhibition seen in Figs. 6-8 was due to phosphorylation of the $\alpha$-chain and the thiol-ester bonds must have remained intact during the phosphorylations.

For the first time an in vitro phosphorylation of C3 resembling the phosphorylation of the $\alpha$-chain of C5 in blood is reported. The low $K_{b}$ value, the inhibition of both the classical and alternative pathways and that only small amounts of $[^{32}P]$phosphate could be incorporated into hemolytically inactive C3 support the physiological relevance of this complement C3 phosphorylation.

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