Phosphorylation of complement factor C3 *in vivo*

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Complement factor C3, the central protein of the complement system, was found to be phosphorylated both in EDTA- and heparin-anticoagulated whole blood and in coagulating blood. Complement S protein (vitronectin) was also found to be phosphorylated under these conditions. Further, purified C3 was found to be a phosphoprotein *in vivo*, containing 0.15 mol of alkali-labile phosphate/mol of protein. The ATP concentration in plasma was measured and found to be about 2 μM .

INTRODUCTION

Fibrinogen was the first plasma phosphoprotein to be identified [1], and the function of its phosphate groups remains unclear 25 years later. Recently complement S protein (vitronectin) has also been found to be a phosphoprotein [2] that can be phosphorylated by a cyclic AMP-dependent protein kinase secreted by thrombin-activated platelets [3]. Furthermore, a number of other reports have appeared on plasma and extracellular protein kinases [4–7], and an ATP concentration of 20 μM has been reported in human serum during coagulation [8].

Intracellularly, protein phosphorylation is a control mechanism that is found to act on regulatory enzymes in metabolic pathways. In blood, the complement cascade can be activated by two independent pathways, both of which converge on C3, making C3 a likely candidate for phosphorylation if regulatory phosphorylation occurs in the blood.

Complement activation by the classical pathway occurs when antibodies react with foreign antigens and involves complement factors C1, C4 and C2, resulting in the proteolytic conversion of C3 into C3a, an anaphylatoxin, and C3b. The alternative pathway is antibody-independent and is activated by bacterial and fungal cell-wall components. Here, Factors D and B convert C3 into C3a and C3b. C3b binds to the convertase, altering its specificity so that it activates the remaining factors C5–C9 of the membrane attack complex, causing lysis of the target bacterium (for a review see [9]). S protein binds to C5b–C7 complexes, keeping them soluble, preventing C9 polymerization and lysis.

Since it was conceivable that protein phosphorylation could occur in plasma or serum, it was decided to investigate whether C3 was phosphorylated *in vivo*.

MATERIALS AND METHODS

Plasma serum protein phosphorylation by endogenous kinases and ATP

Portions (10 μl) of carrier-free [γ - ^{32}P]ATP (New England Nuclear; 37 MBq/100 μl vial, nominally 3.34 μM) were added to 100 μl samples of EDTA-anticoagulated blood. These samples were incubated at 37 °C for 30 min without addition of exogenous kinase, Mg^{2+} or Mn^{2+} and the reaction was stopped by centrifuging the samples in a bench-top microcentrifuge

to prepare plasma for SDS/polyacrylamide-gel electrophoresis. After electrophoresis the 4–20 % gradient gels were immunoblotted in accordance with Burnette [10], with rabbit antisera to human C3a or C3 and horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibodies. Then the immunoblot was dried and autoradiographed.

Immunoprecipitation of C3 from plasma and serum

Heparin-anticoagulated blood and non-anticoagulated blood were incubated with radiolabelled ATP as above. Portions (300 μl) of whole blood were allowed to clot in a polyethylene test tube in the presence of 30 μl of [γ - ^{32}P]ATP. Plasma or serum was prepared after a 60 min incubation. The precipitating antisera were prepared as follows. Portions (10 μl) of anti-(human C3) serum or anti-(human complement S protein) serum, a gift from Dr. D. Tollefsen (Washington University School of Medicine, St. Louis, MO, U.S.A.) were mixed with 250 μl portions of 10 % (v/v) fixed Protein A-containing staphylococci, a gift from Dr. A. Larsson of this institute, and incubated at 4 °C. After 60 min, 110 μl of each Protein A-antibody complex was added to 10 μl of plasma or serum, and incubated at 4 °C for a further 60 min. The samples were pelleted by centrifugation and washed four times with 75 mM-potassium phosphate buffer, pH 7.4, containing 0.9 % NaCl and 0.05 % Tween 20. After the final centrifugation the pellets were re-suspended and denatured with SDS and 2-mercaptoethanol before SDS/polyacrylamide-gel electrophoresis on a 12 % slab gel and autoradiography.

Determination of the alkali-labile phosphate content of C3

C3 was prepared rapidly from 10–15 ml of EDTA-anticoagulated plasma by chromatography on a 20 ml DEAE-Sephacel column equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, containing 32 mM-6-amino-hexanoic acid, 6.4 mM-EDTA and 6.4 mM-benzamidine. A 0–300 mM-NaCl gradient in the same buffer was used to elute C3. The C3 pool, dialysed against four changes of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl, was found to be more than 95 % pure by SDS/polyacrylamide-gel electrophoresis. Rapid purification of C3 (48 h) was necessary for the detection of protein-bound phosphate. Alkali-labile phosphate was

not detectable in C3 from large-scale preparations taking several weeks, and this is believed to be due to phosphatase activity during the isolation procedures.

For the detection of alkali-labile phosphate the procedure of Nimmo *et al.* [11] was used. Portions (0–15 nmol) of dialysed C3 were precipitated by the addition of 10% (w/v) trichloroacetic acid. A 1 mg portion of bovine serum albumin (Sigma Chemical Co.) was used as the zero phosphate control, and 0.3 mg of human fibrinogen (Imco, Stockholm, Sweden) containing about 1 nmol of protein-bound phosphate was used as a positive control. KH_2PO_4 (3 nmol) was added to the final supernatants for phosphate determination to ensure that all were within the 3–15 nmol detection range. Control experiments with albumin and fibrinogen showed that the addition of 3 nmol of phosphate improved the sensitivity of the test. All samples were in triplicate.

Measurement of plasma ATP concentration

Heparinized whole blood was used in this experiment, as heparin inhibits the ATP release from blood cells during haemostasis [8]. The blood was centrifuged at 600 *g* for 10 min and the platelet-rich plasma was removed. The plasma was then centrifuged twice at 1200 *g* to pellet the platelets, and the platelet-poor plasma, checked microscopically, was tested for the presence of ATP by the procedure of Ljungström & Ekman [12], except that 100 000 c.p.m. of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to 27 ml of plasma to allow the calculation of the final recovery of ATP after chromatography on a Dowex 1-X8 column (2 ml). The peak was pooled and the absorbance at 260 nm was measured, with the fractions immediately before and after the peak being used as blank. A molar absorption coefficient of $14\,300\text{ M}^{-1}\cdot\text{cm}^{-1}$ was used to calculate the ATP concentration.

RESULTS

Plasma protein phosphorylation by endogenous kinases and ATP

Experiments to detect endogenous extracellular protein kinase activity in blood were carried out as described above and the results of one such experiment are shown in Fig. 1. Three ^{32}P -labelled protein bands are clearly visible on the autoradiogram, lane 3. The most obvious phosphorylated protein appears to be human serum albumin; the other phosphorylated protein bands are the α -chain of C3, as shown by the co-migration of the α -chain antigens and radioactivity, and the third band, with an M_r of about 75 000, is believed to be the complement S protein previously shown to be phosphorylated *in vivo* by McGuire *et al.* [2]. The amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ added was nominally 330 pmol to 100 μl of blood (final concentration 0.33 μM), and no protein kinase activators or bivalent cations were added, unlike the experiments described in refs. [3] and [2] respectively. The position of C3a is shown to demonstrate that no complement activation had occurred.

Incubation of the blood at 37 °C for periods of up to 30 min did not alter the pH of the blood (result not shown). The protein kinase activity appeared to be associated mainly with the erythrocyte fraction, but was also detected to a lesser extent when plasma alone was incubated with labelled ATP (result not shown).

Fig. 1. Immunoblot of a gel after SDS/4–22%-polyacrylamide-gel electrophoresis and its accompanying autoradiograph

Whole blood, anticoagulated with EDTA, was incubated with carrier-free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Materials and methods section. A 20 μg portion of plasma proteins was added to each lane. The first lane is an anti-C3a serum immunoblot showing that the complement system was not activated during the experiment. The middle lane shows both the α - and β -chains of human C3, and the last lane is the autoradiograph of the middle lane.

In passing one may note the phosphorylation of human albumin in Fig. 1. Albumins from various species have previously been shown to be substrates for some protein kinases *in vitro* [13,14]. This is the first time, however, that an albumin has been found to be phosphorylated under more physiological conditions.

Immunoprecipitation of C3 from plasma and serum

To prove conclusively that C3 can be phosphorylated in blood, it was decided to immunoprecipitate C3 from plasma and serum prepared from blood incubated with labelled ATP as described above and then to autoradiograph the gels obtained after SDS/polyacrylamide-gel electrophoresis. Complement S protein was chosen as a suitable control as it is a phosphoprotein and its peptide chain has a different M_r , 75 000, to those of C3 α -chain, M_r 115 000, and β -chain, M_r 70 000. The results portrayed in Fig. 2 show the ^{32}P -labelled immunoprecipitated peptide chains seen on the autoradiogram. The α -chain of C3 is clearly visible in lanes 1 and 2, and it is apparent that there is another phosphorylated band of M_r 47 000 in both lanes. This fragment arises from the N-terminal of the C3 α -chain upon the denaturation of C3 in SDS. Because of C3's unusual thioester bond, a proportion of the C3 molecules undergo hydrolysis of the peptide chain when denatured

Fig. 2. Autoradiograph of a gel after SDS/12%-polyacrylamide-gel electrophoresis of the products of the immunoprecipitation experiment on heparin-anticoagulated plasma (P) and serum (S) described in the Materials and methods section

Lanes 1 and 2 show the immunoprecipitated C3; lanes 3 and 4 show the immunoprecipitated complement S protein.

Fig. 3. Amount of alkali-labile phosphate detected in three plasma proteins

A negative control (bovine serum albumin, seven determinations), a positive control (human fibrinogen, four determinations) and the protein of interest (human C3, nine determinations) were examined. The vertical bars indicate 1 S.D. Further details of the experiment are given in the Materials and methods section.

in SDS [15]. After autoradiography, the gel was stained for protein and both gel and autoradiogram were scanned by a laser densitometer to calculate the ratio of ^{32}P incorporation per unit of protein. This was set at 100 for S protein in plasma (corresponding to approx. 2 nmol of ^{32}P phosphate/l of plasma); the value for S protein in

serum was 105, while for C3 in plasma it was 55 and in serum 34.

Determination of the alkali-labile phosphate content of C3

Following these experiments, the possibility that C3 already contains protein-bound phosphate *in vivo* was investigated and the results are presented in Fig. 3. This shows that the zero control bovine serum albumin contained 0.002 ± 0.030 mol of phosphate/mol of protein. Human C3 contained 0.156 ± 0.064 mol of phosphate/mol of protein, and human fibrinogen, used here as a positive control, contained 0.833 ± 0.122 mol of phosphate/mol of protein, which is in good agreement with the expected value of about 1 mol of phosphate/mol of protein.

Measurement of plasma ATP concentration

As a result of the ^{32}P incorporation found in Figs. 1 and 2 experiments were undertaken to determine the free ATP concentration in human plasma, since it was believed that the amount of ATP added in those experiments was too small to account for the findings. After correction for losses during the purification the plasma concentration of ATP was found to be $2.25 \pm 0.3 \mu\text{M}$ (mean of the results for three experiments).

DISCUSSION

There is a great deal known about regulatory phosphorylation of intracellular proteins and how it adds another layer of control on to metabolic regulation. Now, attention is shifting to the surface of cells and even extracellularly. The discoveries of soluble protein kinases in plasma [3,5-7], protein phosphorylation in plasma [2,6,7,16] and that the free ATP concentration in serum reaches $20 \mu\text{M}$ during coagulation [8] all suggest that protein phosphorylation plays a regulatory role in the blood. To test this hypothesis, I investigated whether C3 was, or could be, phosphorylated. As the target protein of both the classical and alternative pathways, triggering the amplification mechanism that leads to the production of the cytotoxic membrane attack complexes, it requires close control.

When carrier-free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to EDTA-anticoagulated whole human blood, three main protein bands were phosphorylated, as shown in Fig. 1. The bands were tentatively identified as albumin, complement S protein and the α -chain of C3. Fig. 2 confirmed that the α -chain of C3 was phosphorylated in anticoagulated blood incubated with labelled ATP. Further, that it was also phosphorylated to a lesser extent (62% of the plasma value) in clotting blood. Complement S protein was about twice as heavily labelled as C3 in the plasma tracks and, unlike C3, the incorporation was as high in clotting blood as in anticoagulated blood. This difference between plasma and serum may have some regulatory significance. When acted upon by the C3 convertase, a small peptide (C3a) is cleaved off the N-terminus of the α -chain. This peptide is contained within the 47000- M_r fragment seen to be phosphorylated in Fig. 2, so it is possible that the labelling of the α -chain is on or near enough to the C3a peptide to influence the activity of the C3 convertase. Indeed, phosphorylation *in vitro* of C3 appears to alter its activity (P.-O. Forsberg, S. C. Martin, B. Nilsson, P. Ekman, U. R. Nilsson & L. Engström,

unpublished work). Complement S protein has been shown by McGuire *et al.* [2] to be phosphorylated in plasma, but an incubation time of 3 h and the addition of the non-physiological Mn^{2+} ion at a concentration of 10 mM was required. In the present paper it is shown that, if whole blood was used for the phosphorylation, an almost 5-fold higher incorporation was achieved (by comparison with data given in ref. [2]) and without the addition of bivalent cations such as Mg^{2+} and Mn^{2+} .

The detection of alkali-labile phosphate in C3 shows that C3 is a phosphoprotein *in vivo*. Although the degree of phosphorylation appears modest at 0.15 mol/mol of protein, the values are significantly above those for bovine serum albumin and are most likely underestimates as a consequence of phosphatase activity during preparation. Further, although fibrinogen appears to be stoichiometrically phosphorylated, the 1 mol of phosphate/mol of protein is distributed over at least six different serine residues in the two A α -chains [17].

The finding that plasma contains an ATP concentration of about 2 μ M extends the work of Born & Kratzer [8], who used a luciferin/luciferase technique to measure ATP release during coagulation. They also detected a plasma ATP concentration of 2 μ M, but attributed it to endothelial cell damage during venepuncture. However, since plasma proteins can be phosphorylated in blood and plasma, it is reasonable to believe that micromolar concentrations of ATP exist in the plasma.

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