Phosphorylation of complement component C3 after synthesis in U937 cells by a putative protein kinase, casein kinase 2, which is regulated by CD11b: evidence that membrane-bound proteases preferentially cleave phosphorylated C3

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It was our aim in this study to investigate the possibility that the third component of complement (C3) is phosphorylated during synthesis and secretion in U937 cells. Labelling of U937 cells with [³²P]P_i, followed by immunoprecipitation of C3 from cell lysates and culture supernatants at different time points, showed that C3 was phosphorylated intracellularly immediately before release into the medium, which initiated cleavage of the protein into an iC3b-like fragment. Stimulation of CD11b/CD18 increased phosphorylation 7-fold, from a basal level of 2%. The phosphorylation sites in C3 did not resemble those described previously for casein kinase (CK) 1, cAMP-dependent protein kinase A or calcium- and phospholipid-dependent protein kinase C. Instead, protein kinase CK2 was suggested inasmuch as: (1)

CK2 was detected both on the cell surface and on shed microparticles; (2) phosphorylation of purified C3 by microparticles was abolished by a monoclonal antibody, anti-CK2; (3) the [³²P]P_i tag of both phosphorylated C3 (secreted from U937 cells) and of microparticle-phosphorylated C3, which was cleaved either by membrane proteases or by leucocyte elastase, was found in a 40 and a 70 kDa polypeptide; (4) both secreted C3 and C3 phosphorylated *in vitro* were much more susceptible to cleavage by proteases. Generation of C3 fragments provides a means by which U937 cells can stimulate nearby cells which are expressing complement receptors. The present study demonstrates that the cleavage of C3 is controlled by an intracellular phosphorylation event regulated by CD11b/CD18.

INTRODUCTION

The complement system is a defence against invading micro-organisms and comprises approximately 30 proteins which act in concert, with the ultimate goal being to lyse alien cells and to mediate inflammatory and immunological reactions. The central event of the complement activation cascade is the proteolytic activation and binding of the third complement component (C3) to a target surface, e.g., bacteria or fungi.

Complement components are mostly synthesized in hepatocytes [1–4], but substantial amounts are also secreted by monocytes/macrophages [5–7]; local synthesis of complement proteins by macrophages plays an important role in the inflammatory response [8]. The human monocytic cell line U937 [9] is a suitable model for complement synthesis in monocytes/macrophages, and has been reported to synthesize a number of components, including C3 [10], C4 [11], Factor B [12] and Factor H [13]. The secretion of C3 by U937 is stimulated by, e.g., PMA, [10,14], lipopolysaccharide (LPS), and interferon- γ [11].

Extracellular phosphorylation of several model substrates in the vicinity of three different human leukaemic cell lines, including U937, has been described by Paas and Fishelson [15]. This study describes at least two types of cell-bound protein kinases (ecto-protein kinases), one of which phosphorylates substrate molecules on serine/threonine residues and the other on tyrosine residues. The first mentioned protein kinase also phosphorylates exogenously added C9 on serine residues. Both types of protein kinases are also found on microparticles which are shed spontaneously from these cells [15].

C3 has been reported to be phosphorylated by several protein kinases with different specificities, in different domains of the molecule and with different impacts on C3 function. C3 is phosphorylated *in vitro* by protein kinase (PK) A (cAMP-dependent PK) and PKC (calcium- and phospholipid-dependent PK) [16], as well as by an ecto-PK from the human parasite *Leishmania major* [17]. In all three cases, phosphorylation takes place in the C3a portion of the α -chain and results in a dramatically decreased susceptibility to cleavage by the classical and alternative pathway convertases (PKA and PKC), and against tryptic cleavage (all three PKs). C3 is also phosphorylated in citrated and coagulating blood [18]. We have shown recently that the latter phosphorylation, which occurs in the N-terminal part of the C3d fragment is mediated by a casein kinase (CK), which is secreted from platelets during activation, and that the C3b phosphorylated by this PK is more resistant to Factor-I-mediated cleavage to iC3b [19].

Taken together, earlier studies have shown that C3, which *in vitro* is phosphorylated at different sites in the molecule, is virtually turned off from activation and down regulation. It is thus possible that this is a mechanism by which the nascent C3 molecule is protected as it is produced by hepatocytes or by other cell types. The reversible inactivation of C3 and other complement components could thus be a mechanism to protect the synthesizing cell against attack by the complement components which it secretes.

In the present paper we describe how a subpopulation of newly synthesized C3 is excreted from U937 cells as a phosphoprotein, and how the degree of phosphorylation is altered by ligands to

Abbreviations used: ATS, activated S-Sepharose; C3, third component of complement; C3a, C3b, C3c, C3d, iC3b, proteolytic fragments of C3; CK, casein kinase; 2,3-DPG, 2,3-diphosphoglycerate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; HAGG, heat-aggregated γ -globulin; HRP, horseradish peroxidase; Ig, immunoglobulin; LPS, lipopolysaccharide; mAb, monoclonal antibody; PK, protein kinase; PKA, cAMP-dependent PK; PKC, calcium- and phospholipid-dependent PK; SBTI, soy-bean trypsin inhibitor.

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Fc and C3 receptors. We also suggest that it is mediated by a PK CK2 close to the cell membrane. Phosphorylation occurs both in the α - and the β -chains of C3 and one functional implication is that the molecule is made more susceptible to proteolytic degradation, in contrast to effects on C3 by phosphorylation by other PKs described previously.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium buffered with 25 mM Hepes (lacking methionine and NaHCO_3), glutamine, penicillin and streptomycin were purchased from the Swedish Veterinary Institute, Uppsala, Sweden; fetal-calf serum (FCS) was purchased from Sera Lab, Crawley Down, Sussex, U.K. [^{32}P]P_i, [γ - ^{32}P]ATP, [^{35}S]methionine (> 1000 Ci/mmol) and horseradish peroxidase (HRP)-conjugated streptavidin were purchased from Amersham International, Little Chalfont, Bucks., U.K.; a mouse monoclonal antibody (mAb) IgG1 clone 1AD9, against the α -subunit of human PK CK2, was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. Human IgG (γ -globulin, 165 mg/ml) was purchased from Kabi, Stockholm, Sweden; activated S-Sepharose (ATS), CNBr-Sepharose, pig mucosal heparin (average molecular mass 10000–12000 Da) and Sephadex G-100 were purchased from Pharmacia-LKB, Uppsala, Sweden. An IgG1 mAb against the CD11b subunit of CD11b/CD18, clone 2LPM19c, rabbit antibodies against mouse Igs conjugated with FITC or HRP, unmodified or HRP-conjugated rabbit antibodies against human C3c, and unmodified rabbit antibodies against human C3d were purchased from Dakopatts A/S, Glostrup, Denmark. Chicken antibodies against human C3 and mouse Igs, and biotinylated rabbit anti-chicken antibodies were purchased from Immunsystem, Uppsala, Sweden. Bovine milk casein was from Merck, Darmstadt, Germany. ATP, cAMP, calpain inhibitors I and II, cathepsin G (EC 3.4.21.20), deoxycholate, 2,3-diphosphoglycerate (2,3-DPG), elastatinal, histone-type H1, iodoacetamide, leucocyte elastase (EC 3.4.21.37), LPS (from *Escherichia coli* 0111:B4), 2-mercaptoethanol, methylamine, NaF, Nonidet P40, soy-bean trypsin inhibitor (SBTI), staurosporine, Triton X-100, trypsin, Tween 20 and Zymosan A were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Reagents

Human C3 and Factor I were prepared according to Hammer et al. [20] and Fearon [21] respectively. Factor H was prepared essentially according to Hammer et al. [20] except that the first step consisted of an euglobulin precipitation, as in [22]. C3 was digested with trypsin to C3a and C3b [1 % (w/w) for 5 min at room temperature] or to C3c and C3d [25 % (w/w) trypsin for 10 min at 37 °C] [23]. Subsequently, the fragments were separated by gel filtration on Sephadex G-100 equilibrated in PBS. C3b was converted to iC3b by incubation of 1.65 mg of C3b with 70 μg of Factor I and 330 μg of Factor H for 30 min at 37 °C. Nascent C3b, which exposes a free sulphhydryl group, was covalently bound to ATS, as described in [24]. Briefly, 360 μg of trypsin was added to 45 mg of C3 and 9 ml of ATS and incubated at 37 °C for 15 min. The digestion was terminated by incubation with 3.6 mg of SBTI for 15 min, followed by extensive washing. Methylamine C3 was prepared as in [25].

Zymosan A was boiled as described in [26]. Human IgG (1 mg/ml in PBS) was heat-aggregated to HAGG (heat aggregated γ -globulin) [27] by incubation at 63 °C for 30 min.

Polyclonal antibodies to C3c and C3d were coupled to CNBr-activated Sepharose according to the manufacturer's instructions.

Cell culture

U937 cells were grown in DMEM containing 10 % (v/v) FCS, 2 mM glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin, in a humidified 5 % CO_2 /95 % air atmosphere at 37 °C. On the day before the start of the experiment the cells were routinely washed and the medium was changed using centrifugation at 200 *g* for 10 min.

[^{32}P]P_i-labelling of U937 cells

Before radioactive labelling, U937 cells (5×10^8) were washed twice with sterile PBS. Fresh DMEM (0.5 ml) containing 100 μCi [^{32}P]P_i was then added and the cells were incubated at 37 °C for 60 min. The cells were then washed twice with sterile PBS, divided into ten aliquots and each aliquot was suspended in 5 ml of fresh DMEM containing 2 mM glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (without FCS), and including one of the compounds shown in Table 1. The incubation was continued for 2 h. In experiments where a combination of mouse mAb anti-CD11b and chicken anti-mouse antibodies was used, the antibodies were preincubated at 37 °C for 30 min before the addition of cells. In order to investigate the possible involvement of PKC, cells were pre-treated with staurosporine (up to 20 nM) for 1 h before the addition of P_i. To inhibit cell bound proteases, SBTI (0.1 mg/ml), elastatinal (up to 1 mM), calpain inhibitors I and II (up to 50 μM), purified C3 (0.5 mg/ml) or normal human serum diluted by half, with or without previous heat inactivation, was included in the incubation medium. Following incubation, cells and supernatants were separated by centrifugation at 1000 *g* for 5 min and 125 mM NaF and 1 mM PMSF (final concentrations) were added. The cell pellets and the incubation mediums were immediately frozen in liquid N₂ and stored at -70 °C pending further analysis. The specific activity of intracellular ATP was determined as described previously [28].

Biosynthetic labelling of U937 cells by [^{35}S]methionine

After washing, the cells were rinsed and suspended in Eagle's minimal essential medium, which was buffered with 25 mM Hepes/HCl, pH 7.4, but without methionine and NaHCO_3 . The cells were incubated with 100 μCi [^{35}S]methionine for 30 min, washed twice in sterile PBS and dissolved in DMEM, with or without test ligands, as shown in Table 1. Thereafter, incubation was continued for another 2 h.

Lysis of U937 cells

In experiments intended to liberate intracellular C3, the cells were suspended in 1 ml PBS/0.5 % (v/v) Triton X-100/0.5 % (w/v) deoxycholate/2 mM PMSF/10 mM EDTA/0.1 M KCl/125 mM NaF and lysed by freezing in liquid N₂ and thawing three times. Thereafter, the cell debris was removed by centrifugation at 15000 *g* for 5 min. In order to detect binding of C3 fragments to the cell membranes, the cells were washed twice with ice-cold PBS containing 2 mM Ca^{2+} and 1 mM Mg^{2+} and then incubated with 1 ml of lysis buffer [10 mM phosphate, pH 7.5/0.15 mM CaCl_2 /0.5 mM MgCl_2 /1 % (v/v) Nonidet P40/0.1 mg/ml SBTI/5 mM iodoacetamide] as described by Erdei et al. [29]. After 60 min at 37 °C, the samples were boiled with electrophoresis sample buffer as described below.

Table 1 Modulation of synthesis and phosphorylation of C3 by U937 cells

In each experiment, the cells were labelled with [32 P] P_i or [35 S]methionine as described in the Materials and methods section. Thereafter, incubation was continued for 2 h in the presence of the indicated ligands. The values shown for C3 phosphorylation and C3 production are the percentages obtained when compared with unstimulated control cells (100%). The results are the means $\pm$ S.E.M. of at least four experiments. The values are those obtained with the concentration of ligand giving the maximal effect on C3 phosphorylation. For ligands which did not affect C3 phosphorylation, the results are those of the highest concentration tested. The absolute levels of C3 phosphorylation and C3 synthesis were determined from unstimulated cells. Abbreviations: nd, not determined; secab, secondary antibody, i.e. chicken anti-mouse Igs. Statistical significance: ***, $P < 0.001$; **, $P < 0.01$; unless indicated, results were not significantly different from controls.

Ligand	Concentration range (μ g/ml)	Concentration giving maximal effect (μ g/ml)	C3 phosphorylation (%)	C3 synthesis (%)	Mol P_i /mol C3
No ligand			100	100	0.02
α -CD11b	0.02–2	2	530 $\pm$ 22***	70.9 $\pm$ 1.9**	0.15
α -CD11b + secab	1 + 0.1–10	1 + 10	498 $\pm$ 8***	71.1 $\pm$ 2.3**	0.14
Secab	0.1–10	10	108 $\pm$ 8	nd	—
C3	40–150	150	101 $\pm$ 2	nd	—
Methylamine-C3	40–150	150	99 $\pm$ 4	nd	—
C3b	35–140	140	109 $\pm$ 13	96.7 $\pm$ 8.3	0.02
iC3b	35–140	140	306 $\pm$ 11***	99.7 $\pm$ 6.8	0.06
C3c	5–40	70	296 $\pm$ 37**	92.7 $\pm$ 4.2	0.06
C3d	3–20	20	102 $\pm$ 3	nd	—
LPS	0.001–100	0.1	202 $\pm$ 15***	94.3 $\pm$ 15.6	0.04
HAGG	0.1–100	50	297 $\pm$ 33***	78.8 $\pm$ 10.1	0.07
IgG	0.1–100	50	107 $\pm$ 9	nd	—
Zymosan	1–100	50	47 $\pm$ 11***	96.8 $\pm$ 8.7	0.01

Separation of C3 fragments from samples

Aliquots of cell lysates or culture medium were incubated with 100 μ l of a 25 % suspension of anti-C3c-Sepharose or anti-C3d-Sepharose at room temperature with vigorous shaking for 60 min. The Sepharose was washed 3 times with PBS containing 0.1 % (v/v) Tween 20 and the amount of precipitated 32 P $_i$ or 35 S was measured in a β -counter. The precipitated samples were subjected to SDS/PAGE, under reducing conditions in 10 % (w/v) acrylamide gels [30]. Analysis of the dried gels were made in a PhosphorImagerTM, using ImagequantTM software (Molecular Dynamics, CA, U.S.A.).

Western-blot analysis of C3 extracted from U937 cell supernatants

C3 was extracted from U937 cell supernatants and lysed cells with anti-C3c-Sepharose and subjected to SDS/PAGE, as described above. After transfer to nitrocellulose membranes, the C3 and C3 fragments were detected using chicken polyclonal anti-C3 antibodies, followed by biotinylated rabbit anti-chicken antibodies. The bound antibodies were visualized with HRP-conjugated streptavidin. Purified C3 and iC3b were analysed in parallel. Alternatively, the polypeptide chains of the precipitated C3 were detected using mouse mAbs 7D84.1 against a neoepitope in C3g [31], and 7D169.7 against a neoepitope in the β -chain of C3 [32]. The bound mAbs were detected by HRP-conjugated rabbit anti-(mouse Ig) antibodies.

C3 ELISA

The concentration of human C3 in the cell medium after 24 h in culture was measured by a sandwich ELISA, described previously [33], using polyclonal anti-C3c antibodies for capture and HRP-conjugated polyclonal anti-C3c antibodies for detection. The lower detection limit using this technique was 0.5 ng/ml. In order to avoid interference from bovine C3 in the FCS, which was present in a concentration of 10 % (v/v) in the cell culture

supernatants, the detecting antibody preparation was pre-absorbed with FCS and the resulting antigen-antibody complexes were removed by centrifugation at 4000 g for 15 min.

Preparation of microparticles

Microparticles, which are shed spontaneously from leukaemic cell lines including U937, have been shown to expose identical or very similar PKs to those of the intact cells [15]. U937-derived microparticles were prepared according to the method of Paas and Fishelson [15]. The cells were washed in PBS, diluted to a density of 40×10^6 cells/ml and incubated at 37 °C for 60 min. Thereafter, the cells were removed by centrifugation at 250 g for 5 min, the supernatant was cleared by centrifugation at 6000 g for 30 min and the shed microparticles were precipitated by centrifugation at 100000 g for 30 min. The microparticles were resuspended in PBS and stored at -70 °C until required.

Detection of CK2 on intact U937 cells and on cell-derived microparticles

PK CK2 was detected on the U937 cell membranes using flow cytometry measurements either on unstimulated cells or on cells which had been stimulated with iC3b (140 μ g/ml), C3c (70 μ g/ml) or HAGG (50 μ g/ml) for 60 min at 37 °C. U937 cells (1×10^6) were incubated for 30 min at 37 °C with mAb anti-CK2 or an irrelevant mAb with unknown specificity, followed by FITC-conjugated anti-mouse Igs at room temperature, also for 30 min. In control experiments, cells were incubated with FITC-conjugated anti-mouse Igs only. Fluorescent cells were analysed in a FACStar (Becton Dickinson) equipped with a 5 W argon-ion laser, run at 0.5 W and emitting at 488 nm. Cells which had not been incubated with antibodies were analysed for autofluorescence. Green fluorescence was collected through a 530/30 filter. Data were processed on a Hewlett-Packard 217 computer (Fort Collins, CO, U.S.A.) using Consort 30 software (Becton Dickinson). PK CK2 was detected on the U937-derived microparticles by dot-blot using mAb anti-CK2, followed by HRP-conjugated anti-mouse Igs.

Characterization of U937 PK

The specificity of the U937 PK on intact cells and on microparticles was investigated using various substrates: C3 and C3 fragments (C3b, either in fluid phase or ATS-bound, iC3b, C3c or C3d), casein or histone type H1, together with cations (Mn^{2+} , Mg^{2+} or Ca^{2+}) or EDTA in concentrations up to 5 mM. Experiments were performed to block phosphorylation by preincubating the microparticles with a mAb against the catalytic subunit of PK CK2 for 30 min at 37 °C, before the addition of substrate. An irrelevant mAb of the same subclass but with unknown specificity was used as control. In some cases, heparin (up to 10 $\mu\text{g}/\text{ml}$), 2,3-DPG (up to 25 mM), NaF (up to 50 mM), staurosporine (up to 20 nM), or cAMP (15 μM together with 4 mM Mg^{2+}) was included in the reaction mixture. Higher concentrations of cations or inhibitors were not included in order to avoid aggregation of the substrate.

Phosphorylation of C3 and ATS–C3b with microparticles

The standard phosphorylation mixture consisted of 10 μl of substrate (30 μg of C3 or 28 μg of ATS–C3b), 10 μl of U937-derived microparticles (containing particles shed from approx. 25×10^6 cells under the conditions described above), 5 mM Mn^{2+} and 0.1 mM [γ - ^{32}P]ATP. Phosphorylation was allowed to take place at 37 °C for 15 min. After phosphorylation of fluid phase C3, any insoluble material was removed by centrifugation at 15000 g for 5 min and the supernatants were transferred to new tubes and boiled in 5 μl of electrophoresis sample buffer containing 15% (v/v) β -mercaptoethanol. The phosphorylated ATS–C3b was washed five times by centrifugation at 80 g for 10 min in order to allow quantitative precipitation of the Sepharose and the removal of cell remnants. Non-phosphorylated controls were treated in the same manner as the phosphorylated samples, except that no ATP was added.

Digestion of C3 by cell-bound proteases and binding of generated C3 fragments to membranes

C3 was phosphorylated with U937-derived microparticles as described above. After centrifugation, 3 μg of phosphorylated C3 was mixed with 27 μg of non-phosphorylated C3, intact cells (up to 2×10^8) or microparticles (derived from $\leq 6 \times 10^8$ cells) were added and the mixture was incubated at 37 °C for 2 h. Subsequently, the samples were centrifuged at 1000 g (cells) or 15000 g (particles) for 5 min. The supernatants were then transferred to new tubes and boiled with electrophoresis sample buffer. The cells were washed, either in PBS containing 2 mM Ca^{2+} and 1 mM Mg^{2+} or in PBS with 10 mM EDTA, lysed in 25 μl of buffer as described above [29] and analysed by SDS/PAGE.

The ion dependence of the cell-bound proteases was investigated by incubating phosphorylated C3 in the presence of microparticles and Ca^{2+} in concentrations up to 50 mM. In separate experiments, the elastase inhibitor elastatinal (up to 1 mM) or calpain inhibitor I or II (up to 50 μM) was included during digestion.

Proteolytic digestion of phosphorylated C3

Phosphorylated and non-phosphorylated C3 or ATS–C3b (alone or mixed in a ratio of 1:9, as described above) were digested with purified Factor I, with Factor H as co-factor [up to 6% (w/w) of Factor I and 1% (w/w) of Factor H], trypsin [up to 5% (w/w)], cathepsin G (up to 20 m-units), or leucocyte elastase (up to 44 m-units). All digestions were at 37 °C for 60 min. The reaction was

terminated by boiling in electrophoresis sample buffer, as described above.

RESULTS

Secretion of phosphorylated C3

After labelling of U937 cells with [^{32}P]P_i and withdrawal of C3 from lysed cells and from cell supernatants with Sepharose-coupled polyclonal antibodies against C3c, it was shown that complement component C3 was secreted from U937 cells as a phosphoprotein (Figure 1). SDS/PAGE showed that intact C3, consisting of α - and β -chains, was the predominant form of C3 found in the supernatant, with traces of the 67 kDa fragment of iC3b (Figure 1, upper panel; lane 1). In addition, the heavy chain of the rabbit anti-C3c antibodies used to precipitate C3 is seen in Figure 1 (upper panel, lane 1). Western-blot analysis using chicken polyclonal antibodies against C3 showed several additional bands (Figure 1, upper panel; lane 2). Identical results were obtained using a mixture of HRP-conjugated antibodies to C3c and C3d (results not shown). Western-blot analysis using mouse mAbs to neoepitopes in C3g (lane 3) and in the β -chain (lane 4) confirmed the presence of C3 and iC3b, but these and other mAbs specific for neoepitopes in C3a, C3c 20 kDa, C3d and C3c 40 kDa failed to detect other C3 fragments (results not shown). Western blots of purified C3 (lanes 5 and 6) and iC3b

Figure 1 Analysis of C3 secreted from U937 cells

Upper panel: lanes 1 to 4 represent C3 extracted from U937 cell supernatants with Sepharose-bound anti-C3c antibodies. Lanes 5 and 7 represent Coomassie-Blue-stained SDS-gels and lanes 6 and 8 Western blots obtained using a mixture of HRP-conjugated rabbit polyclonal antibodies to C3c and C3d. Lane 1, Coomassie-Blue-stained SDS-gel; lane 2, Western blot made using chicken polyclonal antibodies to human C3, which were detected by biotinylated rabbit anti-chicken IgG; lane 3, Western blot made using mouse mAbs reacting with a neoepitope in C3g; lane 4, Western blot made using mouse mAbs reacting with a neoepitope in the β -chain; lanes 5 and 6, purified C3; lanes 7 and 8, iC3b. Lower panel: lanes 1 and 2, C3 which has been extracted from U937 cell supernatants; lanes 3 and 4, C3 extracted from lysed cells with Sepharose-bound anti-C3c antibodies. Lanes 1 and 3 show Western blots made using chicken polyclonal antibodies to human C3 which were detected by biotinylated rabbit anti-chicken IgG. Lanes 2 and 4 show phosphorimages of phosphorylated C3. After labelling with [^{32}P]P_i, the cells (5×10^7) were incubated in 5 ml medium for 2 h, precipitated, subjected to SDS/PAGE under reducing conditions and the dried gels were quantified as described in the Materials and methods section.

Figure 2 Time course for secretion of phosphorylated C3

The cells were incubated with [³²P]P_i, washed and incubated in fresh medium. At the time points indicated in the Figure, samples were removed and centrifuged and cells and supernatant were frozen separately in liquid N₂. The frozen cells were solubilized by freezing and thawing in the presence of detergents [0.5 mM Triton X-100 and 0.5% (v/v) deoxycholate], PMSF (2 mM), NaF (125 mM) and EDTA (10 mM). The cell debris was removed by centrifugation and C3 and C3 fragments were extracted quantitatively from cell lysate and culture medium by Sepharose-bound anti-C3c antibodies. After withdrawal, the Sepharose was washed three times with PBS containing 0.1% (v/v) Tween and radiation was measured in a β -counter. (A) Phosphorylated C3 withdrawn from the culture medium. (B) Phosphorylated C3 obtained from the solubilized cells. The data are the means $\pm$ S.E.M. of four experiments.

(lanes 7 and 8) demonstrated that most of the fragments seen in the cell-derived C3 were seen also in both of these preparations. In Figure 1 (lower panel), it is seen that the [³²P]P_i tag of C3 precipitated from the culture medium was found in only two polypeptides with approx. molecular masses of 70 and 40 kDa (lane 2). Identical patterns were seen when the withdrawal was carried out with Sepharose-coupled polyclonal antibodies against C3d instead of C3c (results not shown), suggesting that the phosphorylated protein was either intact C3 or C3b, or had an iC3b-like conformation. C3 extracted from lysed U937 cells consisted of a mixture of intact C3 and a form with a molecular mass greater than 150 kDa (lane 3). Phosphorylation was found in the high molecular-mass protein only (lane 4). Based on the specific activity of the intracellular ATP, it was determined that the released C3 contained approx. 0.02 mol of P_i/mol of protein.

After labelling of U937 cells with [³²P]P_i, intracellular and

extracellular phosphorylated C3 were quantified over a 120 min incubation period. During this time the amount of phosphorylated C3 in the supernatant increased continuously (Figure 2A). After 2 h, more than 98% of the total amount of phosphorylated C3 was found in the supernatant. The amount of intracellularly phosphorylated C3 increased up to 1 h of incubation but decreased thereafter (Figure 2B). The total amount of radioactive [³²P]P_i that was bound to C3 intracellularly was only a minute part (< 5%) of that found in C3 extracellularly.

Modulation of synthesis and phosphorylation of C3 *in vitro*

The total synthesis of C3 and the amount of phosphorylated C3 secreted by U937 cells were studied after incubation with different receptor binding ligands. The tested concentration intervals and optimal conditions are shown in Table 1.

In absolute numbers, as assessed by ELISA, unstimulated cells secreted 2.63 ± 0.29 ng of C3/10⁶ cells in 24 h (mean $\pm$ S.E.M., $n = 4$) into the culture medium. This figure should be compared with earlier reports of 3 ng and 0.5 ng of C3/10⁶ cells in 24 h [11,14]. However, after 24 h of incubation the proportion of intact C3 was lower than after 2 h (results not shown). This is in agreement with the results of Bengio et al. [10], who observed that the haemolytic activity of U937-derived C3 decreased after 6 h in culture due to proteolysis.

All estimates of synthesis during the experimental time of 2 h were made by metabolic labelling of the cells with [³⁵S]methionine, followed by a 2 h incubation in fresh culture medium and quantitative immune precipitation of C3 from the supernatants and solubilized cells. After metabolic labelling of unstimulated cells, half of the total amount of C3 was precipitated from the cell lysates ($49.1 \pm 3.5\%$; mean $\pm$ S.E.M., $n = 4$). Similar distribution between intracellular and extracellular C3 was seen for all ligands tested (results not shown).

The C3 extracted from the supernatants of unstimulated U937 cells contained approx. 0.02 mol of P_i/mol of protein. The effect of CD11b/CD18 was studied using a mAb which increased the degree of phosphorylation 7-fold when present in concentrations of up to 2 μ g/ml (Table 1); 1 μ g of antibody/ml almost doubled the phosphorylation. At the lower dose, the stimulatory effect was strengthened by the addition of a secondary chicken anti-(mouse Ig) antibody preparation, resulting in the same degree of phosphorylation as when the higher dose of antibody was used. The secondary antibody alone did not affect secretion or phosphorylation of C3 when present in concentrations of up to 10 μ g/ml.

The dependence on CD11b/CD18 was further confirmed by using purified C3 and C3 fragments as physiological ligands. The degree of phosphorylation of the newly synthesized C3 was increased approx. 3-fold when the cells were incubated in the presence of C3 fragments with affinity for CD11b/CD18 (iC3b and C3c), but no effect was seen after incubation with native or methylamine treated C3, C3b or C3d.

LPS and HAGG increased phosphorylation of C3 2-fold and 3-fold respectively, without affecting the rate of synthesis significantly. Zymosan caused a significant decrease in the degree of phosphorylation to half the value found for the unstimulated control cells. In all cases, the radioactive label was evenly distributed between the 70 and 40 kDa polypeptide chains (results not shown).

CK2 on microparticles shed by U937 and on the cell surface

Microparticles shed by U937 cells during incubation at 37 °C in PBS have previously been shown to have membrane bound PKs which are identical or very similar to those found on intact cells

Figure 3 Occurrence of PK CK2 on microparticles shed by U937 and on intact cells

Upper panel: flow cytometry analysis of PK CK2 on U937 cells. U937 cells (1×10^6) were incubated with mouse mAbs against PK CK2 (open) or an irrelevant mAb with unknown specificity (filled), followed by FITC-conjugated rabbit anti-mouse IgG. Lower panel: inhibition of PK activity in U937-derived microparticles by anti-CK2 mAb. Microparticles were preincubated with anti-CK2 mAb (□) or an irrelevant control mAb of the same subclass but with unknown specificity (○) for 30 min at 37 °C. Thereafter, 30 μg of C3 was added together with 5 mM Mn^{2+} and 0.1 mM ATP (final concentrations) and incubated for 20 min at 37 °C. The samples were analysed by SDS/PAGE under reducing conditions and the dried gels with radiolabelled proteins were subjected to PhosphorImager analysis, as described in the Materials and methods section. The data are the means $\pm$ S.E.M. of three experiments.

[15]. In the present study, PK CK2 was detected on these microparticles by dot-blot analysis and on the surface of intact U937 cells by FACS analysis, using a monoclonal antibody against PK CK2 (Figure 3, upper panel). Control cells incubated with an irrelevant mAb with unknown specificity, with only FITC-conjugated anti-mouse Ig or without antibodies, gave identical fluorescence (results not shown). Stimulation of the

Figure 4 Digestion of CK2-phosphorylated C3 with elastase

C3 was phosphorylated as described in the Materials and methods section. Subsequently, it was digested with elastase at the concentrations shown in the lower panel for 60 min at 37 °C. Upper panel: lanes 1 and 2, Coomassie-Blue-stained gel; lanes 3 and 4, the corresponding phosphorimage. The concentration of leucocyte elastase used was 10 m-units. Molecular-mass markers are indicated on the left. Lower panel: results of densitometric scanning of gels and phosphorimages showing the disappearance of the α -chain of phosphorylated (○) and non-phosphorylated (□) C3.

cells for 60 min at 37 °C with iC3b (140 $\mu\text{g/ml}$), C3c (70 $\mu\text{g/ml}$) or HAGG (50 $\mu\text{g/ml}$), i.e. the concentrations which gave maximal increase in phosphorylation of the secreted C3 (Table 1), did not affect the expression of PK CK2.

It was demonstrated that both microparticles and intact cells were able to phosphorylate C3 and C3 fragments in the presence of cations and ATP, with similar specificity and ion dependence (see below). Inhibition studies showed that the predominant PK in the microparticle preparation, which phosphorylated C3, was a casein kinase CK2. The activity of this PK was completely abolished by a mAb against the catalytic subunit of this enzyme but an irrelevant control antibody had no effect (Figure 3, lower panel).

Because of the very low concentration of Ca^{2+} in the DMEM (1.8 mM Ca^{2+}) there was no significant phosphorylation of exogenously added C3 to cells in DMEM together with [γ - ^{32}P]ATP, despite the expression of PK CK2 on the cell surface. Extracellular phosphorylation was seen only after further in-

Figure 5 Digestion of CK2-phosphorylated C3 by U937 cells

A mixture of 3 μ g of CK2 phosphorylated C3 and 27 μ g of non-phosphorylated C3 was incubated with U937 cells diluted in 50 μ l of DMEM for 120 min at 37 $^{\circ}$ C. Subsequently, the cells were removed by centrifugation and the soluble proteins were analysed by SDS/PAGE under reducing conditions. The gels containing radioactive proteins were exposed on phosphorimage screens. Upper panel: densitometric scanning of gels and phosphorimages

crease in cation concentration (see below). It was thus ascertained that the phosphorylated C3, which was precipitated from the cell culture supernatants, had not been phosphorylated extracellularly by the cell membrane PK using ATP which had leaked from dead or damaged cells.

Characterization of the PK activity in microparticles

The PKs on the cell-derived microparticles phosphorylated casein and purified C3 and C3 fragments, whereas only negligible phosphorylation of histone H1 was seen. The negligible phosphorylation of casein and C3 obtained in the absence of bivalent cations was considerably enhanced by the addition of 5 mM Mn^{2+} ($1944 \pm 208\%$, $P < 0.001$ versus PBS control) or 5 mM Ca^{2+} ($1071 \pm 198\%$, $P < 0.01$ versus control); it was enhanced to a lesser extent by the addition of 5 mM Mg^{2+} ($390 \pm 15\%$, $P < 0.05$ versus control) and phosphorylation was abrogated by the presence of 5 mM EDTA ($51 \pm 9\%$, not significantly different from the PBS control). Similar results were obtained when the microparticles were replaced by intact U937 cells ($1960 \pm 500\%$ for 5 mM Mn^{2+} , $P < 0.05$ versus control; $1160 \pm 110\%$ for 5 mM Ca^{2+} , $P < 0.01$; 300 ± 70 for 5 mM Mg^{2+} , $P < 0.05$; and 48 ± 8 for 5 mM EDTA, $P < 0.05$).

cAMP (15 μ M) or staurosporine (up to 20 nM) did not affect the phosphorylation of C3 by microparticles ($98 \pm 4\%$, $P = 0.87$, $n = 3$ and $102 \pm 1\%$, $P = 0.49$, $n = 4$ versus uninhibited control cells respectively), which excluded the possible involvement of a PKA or PKC. In addition, no inhibition of phosphorylation of the newly synthesized C3 was seen in cells which had been preincubated with staurosporine in concentrations of up to 20 nM, thus confirming that PKC was not responsible for phosphorylation of C3 in intact cells ($98 \pm 8\%$, $P = 0.92$, $n = 3$ versus uninhibited control cells).

The identification of the cell membrane-bound protein kinase as a CK2 was further strengthened by the use of heparin, 2,3-DPG and NaF, each of which has been reported previously to inhibit PK CK2 activity [34–36]. The concentrations of inhibitors which gave 50% inhibition were 5 μ g/ml of heparin, 3 mM of 2,3-DPG or 0.5 mM NaF respectively.

When native and fragmented C3s were used as substrates with either microparticles or intact cells as a protein kinase source, phosphorylation was seen in the β -chain of C3, C3b, iC3b and C3c (results not shown). The degree of phosphorylation was similar in the α -chain and α' -chain of C3 and C3b and slightly higher in the 40 kDa fragments of iC3b and tryptic C3c. No phosphorylation was seen in the 67 kDa fragment of iC3b or in C3d.

Increased susceptibility to proteolysis of phosphorylated C3

C3 was phosphorylated by cell-derived microparticles containing PK CK2. When a mixture of phosphorylated and unmodified C3 was incubated with leucocyte elastase, the phosphorylated C3 was digested much faster (Figure 4). It was not possible to cleave C3 with cathepsin G (results not shown). In contrast, no differences were found between phosphorylated and non-phosphorylated C3 in the pronounced susceptibility to trypsin or

showing the disappearance of the α -chain of phosphorylated ($\circ$) and non-phosphorylated ($\square$) C3. Middle panel: fluid phase C3 before and after incubation with 50×10^6 U937 cells using the conditions described above. Lanes 1 and 2, Coomassie-Blue-stained gel; lanes 3 and 4, the corresponding phosphorimage. Lower panel: inhibited cleavage of the α -chain of phosphorylated C3 seen in the presence of the protease inhibitors elastatinal ($\diamond$), calpain inhibitor I ($\square$) or calpain inhibitor II ($\triangle$). The uninhibited control is set at 0% of remaining α -chain.

to Factor I with Factor H as co-factor (results not shown). After digestion of ATS-C3b with 3 % (w/w) of Factor I for 60 min at 37 °C, $26.3 \pm 9.4\%$ of the phosphorylated and $24.0 \pm 7.1\%$ of the non-phosphorylated C3b remained ($n = 3$, $P = 0.85$).

Digestion and binding of phosphorylated C3 to U937 cell membranes

The C3 phosphorylated with membrane-bound PK CK2 was also more susceptible to proteolysis by membrane-bound proteases (Figure 5, upper and middle panels). After incubation of a mixture of phosphorylated and non-phosphorylated C3 for 2 h with intact cells or cell-derived microparticles, no proteolysis of the non-phosphorylated C3 could be seen on a gel stained with Coomassie Blue. In contrast, the phosphorylated C3 had been quantitatively converted to a number of fragments with molecular masses of 70 kDa or less. A portion of the phosphorylated C3 fragments of 70 and 40 kDa generated during incubation bound to the cell preparation in an ion-dependent manner (results not shown).

It was confirmed that the cell-associated proteolytic enzyme responsible for digestion of the α -chain of phosphorylated C3 was indeed elastase, as enzymic activity was inhibited by elastatinal and no effect was seen when calpain inhibitors I and II were added (Figure 5, lower panel).

The difference in processing of phosphorylated, exogenously added and endogenously produced C3 was illustrated by the observation that it was impossible to inhibit the proteolytic digestion of newly synthesized phosphorylated C3, which was released from the cells by the addition of elastatinal, SBTI, purified C3 or human serum to the cell medium, suggesting that the endogenous C3 was in closer proximity to cell-bound elastase during transit. In each case, identical amounts of the phosphorylated 70 and 40 kDa fragments, but not phosphorylated C3 fragments with higher or lower molecular masses, were isolated regardless of the additions.

DISCUSSION

We have shown, in this study, that a subpopulation of the C3 produced by the monoblastoid cell line U937 is phosphorylated. The phosphorylated C3 was only approx. 2% of the total amount of synthesized C3 in unstimulated cells. Phosphorylation of C3 took place close to the cell membrane, since incubation of U937 cells with [32 P]P_i showed that the phosphorylated C3 was found in the supernatant shortly after the appearance of phosphorylated C3 intracellularly. In addition, phosphorylated C3 was found almost exclusively in the supernatant, with less than 5% present intracellularly. These results are in contrast with those obtained with HepG2 cells, where the release of phosphorylated C3 was much slower and at least 25% of the total amount of phosphorylated C3 was found intracellularly when similar conditions were used (K. Nilsson Ekdahl and B. Nilsson, unpublished work). It is unlikely that the phosphorylation of newly synthesized C3 extracellularly, utilizing ATP from damaged cells, is significant because there was no detectable phosphorylation of exogenously added C3 unless the ion concentration was increased.

Analysis by PhosphorImager of C3 fragments in the culture supernatants has shown that the major phosphorylated polypeptides were approx. 70 kDa and 40 kDa in size and Western-blot analysis showed low amounts of iC3b in addition to intact C3. In size, the phosphorylated fragments of C3 secreted by the U937 cells resembled those of the polypeptide chains of the iC3b (70, 67 and 40 kDa) or the C3c (70, 40 and 20 kDa) molecules.

These findings, combined with the fact that they were extracted by either anti-C3c or anti-C3d Sepharose, suggest that the phosphorylated molecules were iC3b-like, with phosphorylated 70 and 40 kDa fragments, unless any unusual proteolytic cleavage had taken place, and that the non-phosphorylated majority of C3 molecules consisted of intact α - and β -chains.

The location of the phosphorylation sites and the lack of effect by staurosporine excluded the involvement of PKA and PKC [16] and of the CK1-like kinase released by activated platelets, which we have described recently [19].

Paas and Fishelson [15] have described ecto-PKs on intact cells, e.g. U937, and on microparticles derived from these cells, which, among several other proteins, phosphorylate C9 and to some extent C3. This report and that of Geberhiwot and Skoglund [37] describe several ecto-PKs, in particular the dominating PK which phosphorylates phosvitin, one of the model substrates for CKs. Here we have shown by dot-blot and FACS analysis that both the microparticles and the intact cells have membrane bound PK CK2. This finding agrees with that of Walter et al. [38], who showed that CK1 and CK2 ecto-kinase activity shed from HeLa cells.

We have characterized the C3 phosphorylating PK activity on the U937 microparticles as a CK2, based on its specificity for casein but not histone, the inhibition of the kinase activity by 2,3-DPG and heparin [34] and the total abolition of the phosphorylation of C3 by a mAb specific to CK2. The latter observation ascertained that CK2 was the only PK on these particles which phosphorylated C3. In the study of Paas and Fishelson [15] it appears that the kinase, which phosphorylated C3, had low extracellular activity in the presence of Mg²⁺ under the experimental conditions used. This is compatible with our efforts to phosphorylate purified C3 added to intact cells together with radioactively-labelled ATP, where significant incorporation was observed only after the addition of Mn²⁺ or Ca²⁺. These findings imply that CK2 could be responsible for the phosphorylation of C3 synthesized by U937 cells.

It was shown that the U937-derived PK CK2 phosphorylated at least one site in each of the chains of intact C3. By using C3 fragments, the phosphorylation sites were located to the C-terminal α -chain-derived 40 kDa fragment of iC3b and to the β -chain (70 kDa). The size of the polypeptides phosphorylated with PK CK2 *in vitro* and the phosphorylated C3 fragments secreted by the U937 cells were similar. None of the CK2 phosphorylation sites was identical to the site phosphorylated by the platelet CK1-like PK, which phosphorylated a threonine residue close to the S-ester in the C3d domain [19].

The intracellularly phosphorylated C3 was a high-molecular-mass protein, presumably pro-C3 [10], while the secreted phosphorylated C3 was highly degraded. In contrast, as shown by Western-blot analysis using monoclonal and polyclonal anti-C3 antibodies, most of the total amount of secreted C3 was in the form of undegraded α - and β -chains. Based on previous findings by Kalafatis et al. [39], who reported that phosphorylation of Factor Va by a platelet CK potentiated cleavage mediated by protein C, we hypothesized that the secreted phosphorylated C3 was more susceptible to cleavage by cellular proteases.

U937 cells express the lysosomal proteases elastase and cathepsin G on the membrane surface [40]. Taylor et al. [41] have shown that elastase can cleave C3 to fragments which resemble iC3b, C3c and C3d, and Maison et al. [40,42] have demonstrated that cathepsin G and elastase on U937 membranes are able to cleave C3 into iC3b- and C3d-like fragments. To investigate the hypothesis of increased susceptibility, C3 was phosphorylated by the U937 cell-derived CK2 and thereafter incubated with proteolytic enzymes, intact U937 cells or microparticles. It was

shown that the phosphorylated C3 was indeed much more susceptible to cleavage by leucocyte elastase when compared with non-phosphorylated C3, suggesting that the phosphorylation promoted the cleavage. Elastase generated phosphorylated polypeptide fragments of different sizes, some of which were of a similar size to those of the secreted phosphorylated C3 fragments. Phosphorylation did not affect the cleavage by trypsin or the cleavage by Factor I with Factor H as co-factor. Cathepsin G, at concentrations which cleave C3b, did not cleave C3 at all.

Interestingly, an even more pronounced potentiation of the cleavage of phosphorylated C3 was obtained by the use of intact cells or by cell derived microparticles. In a mixture of pure non-phosphorylated C3 and C3 phosphorylated with U937 CK2, the α -chain of the phosphorylated C3 disappeared completely while the α -chain of the non-phosphorylated C3 was uncleaved. Furthermore, some of the cleaved C3 was shown to bind to the cell membrane. The phosphorylated generated polypeptides were of similar size (40 and 70 kDa) to the secreted phosphorylated C3 fragments from U937 cells. This indicated that membrane proteases were able to cleave phosphorylated C3 in a manner similar to that of the intact cell. Cleavage of phosphorylated C3 on the surface of the cell or the microparticle was inhibited by elastatinal, thus suggesting that it was indeed caused, at least partially, by elastase.

The degree of C3 phosphorylation in U937 cells was regulated by different specific receptor ligands. Stimulation of the U937 cells by ligands to Fc receptors or CD11b/CD18 (iC3b, C3c or mAb anti-CD11b) increased the phosphorylation up to five times. Identical cleavage of the phosphorylated C3 to iC3b-like fragments was seen with all ligands tested.

The existence of a signalling pathway linking stimulation of CD11b/CD18 or Fc receptors to upregulation of PK CK2 remains to be investigated. No second messenger is known for CK2 but its regulation is mediated by the regulatory domain (β -subunit) of PK CK2, which brings about the inhibition of the kinase by heparin and 2,3-DPG, described above [34,43]. Phosphorylation of the β -subunit of starfish and frog CK2 by M-phase-specific cdc2 and PKC respectively, has been reported to increase the activity of the kinase [44,45]. Furthermore, phosphorylation of CK2 in A431 cells as a result of growth-factor stimulation, is known to increase this activity [46].

The function of PK CK2 has been linked to a number of intracellular events associated with cell growth control [47]. The present report suggests that CK2 might also be involved in the regulation of C3 cleavage. It has previously been reported that monocyte/macrophages are able to secrete and cleave C3 into fragments. Unlike the monocyte and U937 cells, the macrophage acquires acceptor sites for C3 and is able to bind C3 fragments to the membrane surface [29,42]. Phosphorylation of a subpopulation of C3, an event which is regulated by CD11b/CD18, provides a mechanism by which monocytes/macrophages can produce intact C3, which is able to participate in the inflammatory process, and C3 fragments simultaneously. The generation of C3 fragments and the fact that complement receptors CD21 (which bind to iC3b and C3dg) and CD11b/CD18 and CD11c/CD18; (which bind to iC3b) are present on a number of cells provides the monocyte/macrophage with a powerful tool to regulate cells involved in the inflammatory process and in the immune system.

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