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Leishmanial protein kinases phosphorylate components of the complement system

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Externally oriented protein kinases are present on the plasma membrane of the human parasite, *Leishmania*. Since activation of complement plays an important role in the survival of these parasites, we examined the ability of protein kinases from *Leishmania major* to phosphorylate components of the human complement system. The leishmanial protein kinase-1 (LPK-1) isolated from promastigotes of *L.major* was able to phosphorylate purified human C3, C5 and C9. Only the α-chain of C3 and C5 was phosphorylated. The β-chain appeared not to be a substrate for this enzyme. C3b which is formed by proteolytic cleavage of C3 was not phosphorylated by LPK-1. Trypsin treatment of phosphorylated C3 (P-C3) resulted in the disappearance of ^{32}P from the α-chain. This was correlated with the conversion of the C3 α-chain to the α'-chain of C3b, and the appearance of a 9 kDa ^{32}P fragment comigrating with the C3a fragment of C3. P-C3 was more resistant to cleavage by trypsin than non-phosphorylated C3. LPK-1 phosphorylated purified C3a and two synthetic peptides, C3a21R and YA-C3a10R, derived from its COOH-terminal end, which contain the C3a binding site to leukocytes and platelets. LPK-1 did not phosphorylate C3a8R. Phosphoamino acid analysis of the synthetic peptides indicated that serine 71 of C3a was phosphorylated by LPK-1. Treatment of C3 with either methylamine or freeze–thaw C3 (H_2O) prevented phosphorylation by the LPK-1 suggesting that substrate conformation may be involved in recognition by the leishmanial enzyme. Viable *L.major* promastigotes could phosphorylate both C3 and C3b implying that more than one protein kinase is probably present on the surface of these parasites. Extracellular protein phosphorylation may play a role in the interaction of the parasite with the host's immune system and in the survival of *Leishmania*.

Key words: Complement proteins/ecto-protein kinases/ *Leishmania major*

Introduction

Leishmania are protozoan parasites of humans with a simple digenetic life-cycle. As flagellated promastigotes, *Leishmania* reside and multiply in the sandfly vector. Upon transmission to an appropriate mammalian host, such as man, the promastigotes are ingested by phagocytes where they transform into obligate intracellular amastigotes. The latter stage of the parasite is responsible for the sundry diseases observed in humans, including the three main forms: cutaneous, mucocutaneous and visceral leishmaniasis (Chang and Bray, 1985; Peters and Killick-Kendrick, 1987).

As the promastigotes develop in the sandfly vector the parasites change from non-infective logarithmic forms to virulent metacyclic forms which pre-adapt to life in the host. These changes can be mimicked *in vitro* using logarithmic and metacyclic stationary phase parasites (Sacks, 1989). In *Leishmania major*, changes in two surface antigens, the promastigote surface protease (also known as gp63) and the lipophosphoglycan, both involved in invasion have been noted during this transformation (Kweider *et al.*, 1987; Sacks *et al.*, 1990).

During axenic transformation to metacyclics, protein kinase activity increases and phosphorylation patterns change in *L.major* (Mukhopadhyay *et al.*, 1988; Hermoso, 1989). Like few other eukaryotic cells, *Leishmania* have been shown to possess an externally oriented surface protein kinase capable of phosphorylating non-endogenous protein substrates (Das *et al.*, 1986; Lester *et al.*, 1990). Post-translational modification of proteins by protein kinases is involved in the regulation of many cellular processes, including cell differentiation, oxidative burst, metabolic pathways and proliferation. Phosphorylation of specific amino acids on proteins can modulate the kinetics of protein proteolytic cleavage in biological processes (Laumas *et al.*, 1989). Nothing is known regarding the biological function of ecto-protein kinase(s), though in *Leishmania* they probably play a role in signal transduction and/or the regulation of host defence mechanisms during invasion.

Prior to macrophage phagocytosis *Leishmania* promastigotes are exposed in the blood to the human complement system. This complex network of proteins is responsible for killing many of the pathogens which invade the human host. However, *Leishmania*, like other virulent pathogens, have developed mechanisms to avoid killing by complement (Joiner, 1988; Fuhrman and Joiner, 1989). The infective metacylic forms of *L.major* only invade human macrophages in the presence of serum, but are not lysed even though 80% of the C3 on their surface is present as C3b (da Silva *et al.*, 1989).

Following activation, by either the alternative or classical pathway, the complement cascade converges at the step of C3b deposition. This involves proteolytic cleavage of C3 by a C3-convertase to give metastable C3b, which can bind covalently to molecules on the activating surface, and C3a, a 9 kDa analphylatoxin (Muller-Eberhard, 1988; Hugli, 1990). Phosphorylation of C3 by protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) has been reported (Forsberg *et al.*, 1990). In this paper we demonstrate that live *L.major* stationary phase promastigotes can phosphorylate both human C3 and C3b. Phosphoryla-

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tion of C3 changes its kinetics of cleavage to C3a and C3b. Using a purified serine protein kinase from *L.major* membranes, LPK-1, we were able to characterize further the phosphorylation of human C3 and identify the C3a portion of C3 as the site of phosphorylation. The potential role of protein phosphorylation in parasite survival is discussed.

Results

Phosphorylation of human complement proteins by pure LPK-1

A membrane-bound leishmanial protein kinase (LPK-1) was purified 250-fold from *L.major*. Additional purification by gel filtration on a Superose-12 column and renaturation following electrophoretic transfer to a polyvinylidene difluoride membrane demonstrated that the 104 000 molecular weight band (Figure 1a) contains the protein kinase activity (Hirschberg *et al.*, manuscript in preparation). This enzyme was used to study the phosphorylation of four purified proteins, C3, C3b, C5 and C9, belonging to the human complement system. Each protein (10 μg) was incubated in the presence of LPK-1 and [γ-^{32}P]ATP. The reactions were analysed by SDS–PAGE and autoradiography (Figure 1b, +). It can be seen that LPK-1 is capable of phosphorylating the 115 kDa α-chains of human C3 and C5, and the 71 kDa C9 protein. Several low molecular weight polypeptides, 10 kDa in C5 and 10 and 26 kDa in C9, were also phosphorylated. These molecules represent either contaminates in complement protein preparations or fragments of the respective proteins, such as C5a and C9a. No phosphorylation of the α'-chain of C3b was observed. In addition, LPK-1 did not phosphorylate the β-chains of C3, C3b or C5. When the leishmanial enzyme was boiled prior to inclusion in the assay mixture, phosphorylation was prevented (Figure 1b, −), demonstrating that phosphorylation was not due to a contaminating protein kinase activity present in the complement protein preparations.

Localization of the site of phosphorylation on C3

The pure leishmanial protein kinase was not able to phosphorylate the α'-chain of C3b (Figure 1). Cleavage of the C3 α-chain between residues 726 and 727 (Arg-Ser) by either the classical or alternative pathway C3-convertase releases the C3a and C3b fragments. The inability of LPK-1 to phosphorylate C3b suggested that the site of LPK-1 phosphorylation is located on the 9 kDa C3a fragment of C3. This was investigated by phosphorylating C3 (P-C3) using the leishmanial protein kinase, LPK-1, and treating P-C3 with trypsin, a serine protease that mimics the action of the C3-convertase. Digestion of either P-C3 or C3 with trypsin for increasing periods of time results in the conversion of both C3s into C3b. This can be readily seen following SDS–PAGE and Coomassie blue staining of the gel (Figure 2, panel C.B.) which shows the disappearance of the C3 α-chain and appearance of the C3b α'-chain following trypsin treatment. As expected, the small 9 kDa C3a fragment produced from the cleavage of C3 to C3b becomes visible following a 1 min incubation with trypsin. Autoradiogram of this gel (Figure 2, panel ^{32}P) demonstrates that the label in the α-chain of P-C3 disappears over increasing times of trypsin treatment. This corresponds with the conversion of P-C3 to C3b, as seen by Coomassie blue, and the appearance of a 9 kDa P-C3a fragment which comigrates with the C3a standard. None of the radioactivity comigrates with the newly formed α'-chain of C3b. Quantitation of the autoradiogram by laser densitometry shows that < 4.2% of the original ^{32}P-labelled C3 α-chain remains intact after 5 min digestion with trypsin.

Effect of phosphorylation on the degradation of C3 by trypsin

The kinetics of cleavage of P-C3 and C3 by trypsin was compared in several experiments to discern whether phosphorylation affected the activity of C3. Results from a representative experiment are shown in Figure 2 C.B. Both samples were treated the same, except that C3 and P-C3 were originally incubated with inactivated and active LPK-1, respectively. The Coomassie blue stained gel was scanned

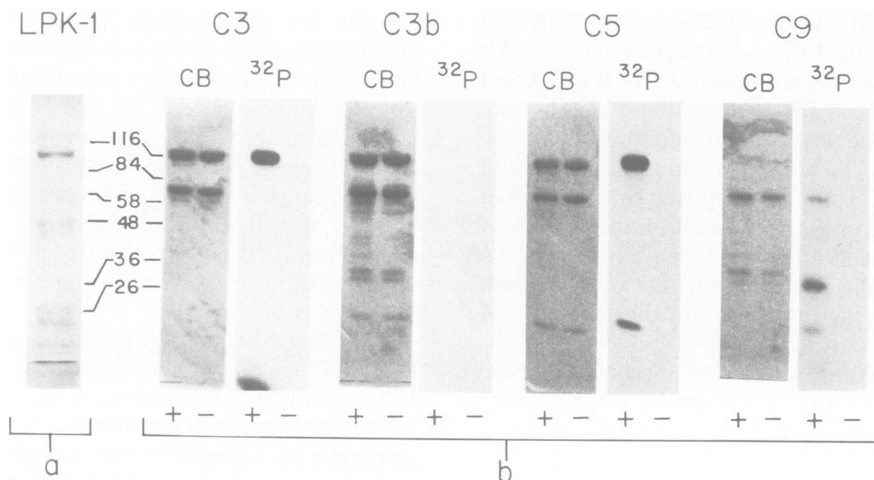

Fig. 1. Phosphorylation of human complement components by purified LPK-1. (**a**) Coomassie blue stained SDS–PAGE of LPK-1 preparation used in these studies. The enzyme eluted at 480 mM sodium acetate from the Mono Q 5/5 column in 20 mM Tris–HCl pH 7.4 buffer containing 1 mM Lubrol-PX (see Materials and methods). (**b**) Phosphorylation of human C3, C3b, C5 and C9 by LPK-1. Coomassie blue stained SDS–PAGE gel (C.B.) and corresponding autoradiogram (^{32}P) are shown. Lanes marked (+) contained active LPK-1. Lanes marked (−) included enzyme boiled prior to use.

by densiometry and the α-, α'- and β-chains of P-C3 and C3 quantified for each time point. Phosphorylation of the α-chain by LPK-1 caused P-C3 to be more slowly cleaved to C3b by trypsin than native C3. After 1 min incubation, 76% of the C3 α-chain was converted to the α'-chain of C3b, compared with 50% of the P-C3 α-chain. By 2 min incubation with trypsin, almost none of the C3 α-chain remains, while 21% of the P-C3 α-chain is still present (Figure 2, C.B.). Similar results were also seen in other experiments.

Serine 71 in C3a is phosphorylated by LPK-1

The polypeptide C3a belongs to a group of biologically active molecules called anaphylatoxins. Human C3a interacts with leukocytes and platelets probably via an active site comprised of the five COOH-terminus amino acids residues 73–77, LGLAR (Table I; Hugli, 1990). Phosphorylation of the C3a fragment by LPK-1 occurs in intact C3. The purified C3a fragment was also a good substrate for LPK-1 and is highly phosphorylated when incubated with the leishmanial enzyme (Table I). This was also seen by SDS–PAGE and autoradiography (data not shown).

In human C3a, serine is present at residue 71 close to the active site of this molecule. We were interested in determining whether this serine could be phosphorylated by LPK-1, since phosphorylation near the active site could be involved in the regulation of C3a activity. Phosphorylation of several peptides, C3a8R, C3a21R and YA-C3a10R, differing in size and all containing the COOH-terminus of C3a was examined. The amino acid sequence of the peptides used is given in Table I. Protamine sulphate was also included as a positive control. The results are shown in Table I. The two peptides, C3a21R and YA-C3a10R, were readily phosphorylated by LPK-1, δc.p.m. – 15600 and 16700, respectively as compared with protamine sulphate, δc.p.m. – 9600. Only the peptide C3a8R was not phosphorylated by the leishmanial enzyme. Phosphoamino acid analysis of each of the phosphorylated peptides showed the presence of phosphoserine (not shown) indicating that Ser71, the only serine present in these peptides, was phosphorylated by LPK-1. Phosphoamino acid analysis of P-C3a also identified phosphoserine. Phosphothreonine was not found in either P-C3a or C3a21R (data not shown).

Protein conformation affects the phosphorylation of C3

Slow spontaneous inactivation of native C3 probably occurs in the fluid phase upon hydrolysis of its thioester bond and formation of C3(H_2O). This process is markedly enhanced by freezing C3 at −20°C and thawing or by treatment of C3 with methylamine and formation of C3(CH_3NH_2) (Pangborn *et al.*, 1981; Isenman *et al.*, 1981). These modified C3s undergo a conformational change and acquire functional properties similar to C3b (Pangburn and Muller-

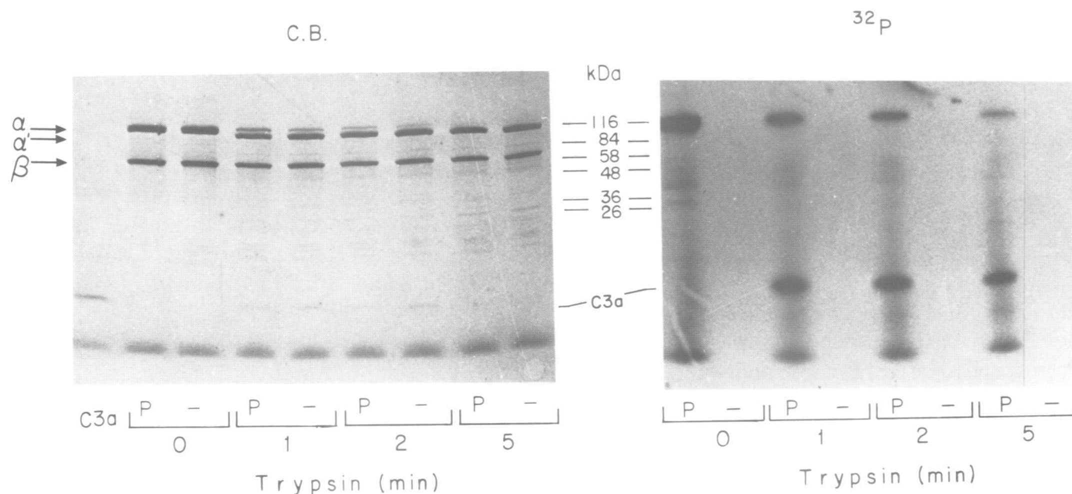

Fig. 2. Cleavage of phosphorylated C3 (P-C3) and native C3 by trypsin. Equal amounts of P-C3 or native C3 were incubated with TPCK-treated trypsin for increasing periods of time up to 5 min. Aliquots containing 5 μg were removed from each reaction and soybean trypsin inhibitor added. The cleavage was analysed by SDS–PAGE and autoradiography. P indicates phosphorylated C3 and (–) indicates native C3. Purified human C3a was included for comparison.

Table I. Phosphorylation of C3a and COOH-terminal peptides of C3a by LPK-1

Substrate	Sequence	Phosphorylation (δ c.p.m.)[a]
Human C3a[b]		74 678
C3a8R	70 … 77 A-S-H-L-G-L-A-R	0
C3a21R	60 C-N-Y-I-T-E-L-R-R-Q-H-A-R-A-S-H-L-Q-L-A-R	15 606
YAC3a10R	Y-A-A-R-A-S-H-L-Q-L-A-R	16 717
Protamine sulphate		9600

[a]Results are presented as mean (c.p.m. substrate + LPK-1) – (c.p.m. substrate + boiled enzyme), n = 3. Average background for LPK-1 in the absence of substrate was 3803 c.p.m.
[b]Results from a separate experiment.

Eberhard, 1984). $C3(H_2O)$ or $C3(CH_3NH_2)$ can form a complex with Factor B in the presence of magnesium ions and upon cleavage by Factor D, form a C3-convertase. While $C_3(H_2O)$ is structurally indistinguishable from native C3 on SDS−PAGE, the convertase formed from $C3(H_2O)$ contains the intact C3 α-chain (Fishelson *et al.*, 1984). This is postulated to be the initial convertase of the alternative complement pathway. We were interested to see if $C3(H_2O)$ or $C3(CH_3NH_2)$, could be phosphorylated by LPK-1. The α-chain of this C3b-like molecule is not susceptible to cleavage by the C3-convertase, but like C3b can be cleaved by Factor I. As already discussed, LPK-1 phosphorylates the α-chain of C3 but cannot phosphorylate the α'-chain of C3b (Figure 1).

Equal amounts of C3 were either frozen and thawed three times or treated with methylamine for 1 h at 37°C to yield $C3(H_2O)$ or $C3(CH_3NH_2)$, respectively. The modified C3s and freshly thawed C3 from the same stock were incubated with LPK-1 and [γ-^{32}P]ATP, and the reactions analysed by SDS−PAGE and autoradiography. Equal amounts of each C3 were loaded on the gel as determined by Coomassie blue staining of the gel (not shown), however, major differences in the phosphorylation of C3 and the modified C3s were seen (Figure 3). When C3 is converted to $C3(H_2O)$ or $C3(CH_3NH_2)$, its ability to be phosphorylated by LPK-1 is significantly reduced. As determined by densitometric analysis of the bands, phosphorylation of $C3(H_2O)$ was only 23% of the control C3 preparation. Likewise, treatment of C3 with 5 mM methylamine resulted in only 59% phosphorylation compared with control C3. Increasing the methylamine concentration to 50 mM decreased phosphorylation of $C3(CH_3NH_2)$ to only 38% of control. These results imply that the conformational change which occurs in these C3b-like molecules renders the C3a portion of their α-chain inaccessible to the leishmanial protein kinase.

Phosphorylation of C3 and C3b by live promastigotes

The ability of stationary phase *L.major* promastigotes to phosphorylate the C3 and C3b components of the complement system was also examined. Viable promastigotes were washed and resuspended in buffer containing either purified human C3, C3b or buffer alone. The reaction buffer contained inhibitors of leishmanial surface proteases and phosphatases. Following incubation with [γ-^{32}P]ATP, the intact parasites were removed by centrifugation and the cell-free supernatant examined by SDS−PAGE. No lysis of the parasites was observed following this treatment. No protein bands, stainable by Coomassie blue, were found in the cell-free supernatant in the absence of the complement proteins (Figure 4A). When complement proteins were included in the reaction mixture, the major bands observed by Coomassie blue staining of the gels were the 115 kDa α- and 75 kDa β-chain of C3 (lane c), and the 106 kDa α'- and 75 kDa β-chain of C3b (lane b). No proteolytic degradation of C3 or C3b by the promastigote surface protease (gp63) occurred under these conditions even though the pure promastigote protease has been reported to cleave C3 (Chaudhuri and Chang, 1988). Examination of the corresponding autoradiogram (Figure 4B) shows that no endogenous parasite phosphoproteins were present in the cell-free supernatant (lane a). When exogenous C3 or C3b is included with the promastigotes, both complement proteins were phosphorylated. However, only the α-chain of C3 (lane c) and the α'-chain of C3b (lane b) were phosphorylated. The β-chain from both C3 and C3b is not phosphorylated by the live parasites. In addition, a 50 kDa phosphoprotein was also seen. This could be a contaminant present in both the C3 and C3b preparations or a leishmanial protein released into the supernatant in response to C3 and C3b.

Discussion

Leishmania major promastigotes possess a cell surface ecto-protein kinase activity (Lester *et al.*, 1990). A similar activity has been described on promastigotes of *Leishmania donovani* (Das *et al.*, 1986). While surface protein kinases are unusual, a few reports on ecto-protein kinases in other eukaryotic cells, such as HeLa, neuronal, rat liver epithelial, 3T3 fibroblasts and human leukaemic cells have also appeared (Kubler *et al.*, 1982; Ehrlich *et al.*, 1986; Kleine and Whit-

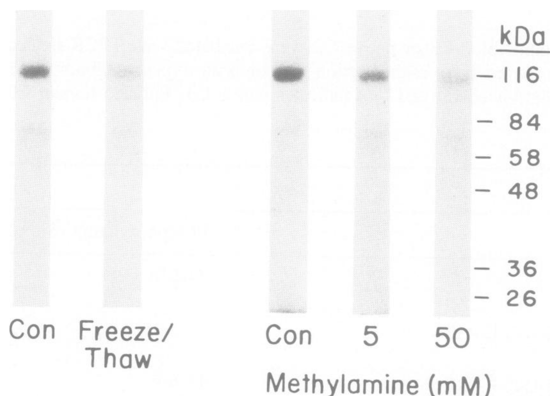

Fig. 3. Effect of conformational changes in C3 on its phosphorylation by LPK-1. C3 frozen−thawed three times, $C3(H_2O)$ and C3 treated with 5 mM or 50 mM methylamine for 1 h at 37°C, $C3(CH_3NH_2)$ were phosphorylated as described in Materials and methods. C3 served as control.

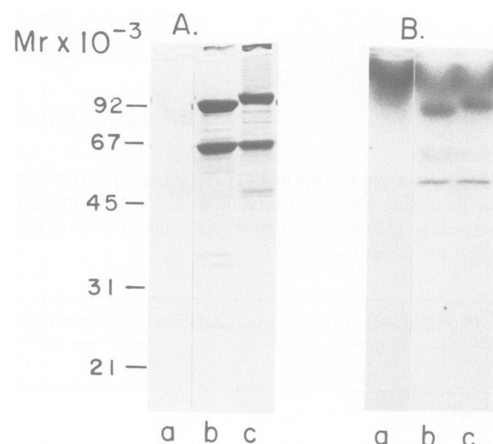

Fig. 4. Phosphorylation of human complement C3 and C3b by live promastigotes of *L.major*. C3 and C3b were mixed with stationary phase promastigotes and incubated in the presence of [^{32}P]ATP for 12 min at 30°C. The cells were removed and the supernatants examined for protein phosphorylation by SDS−PAGE and autoradiography. (**A**) Coomassie blue staining of gel. (**B**) Autoradiogram of same gel. Lane a, promastigotes alone; lane b, promastigotes plus C3b; lane c, promastigotes plus C3.

field, 1987; Fishelson *et al.*, 1989). The function of these extracellular enzymes is unknown, though it has been suggested that such enzymes might be involved in the initiation of DNA synthesis (Kleine *et al.*, 1986) or evasion of complement lysis (Fishelson *et al.*, 1989).

Evasion of the complement cascade by *Leishmania* is an integral part of the parasite life-cycle. Most species of *Leishmania* promastigotes activate the alternative pathway complement (Mosser *et al.*, 1986; Puentes *et al.*, 1989). In the case of *L.major*, C3b is fixed to the parasite surface via lipophosphoglycan, a major parasite glycolipid (Fuentes *et al.*, 1988; Sacks, 1989). However, despite the presence of intact C3b and C5−7 complexes on the metacyclics, assembly of the C5b−9 complexes is blocked (Puentes *et al.*, 1990). It has been suggested that this inhibitory activity may be associated with the developmentally regulated elongation of the lipophosphoglycan (Puentes *et al.*, 1990; Hall and Joiner, 1991). In the presence of serum, the macrophage complement receptor CR1, which interacts with bound C3b, appears to be the major host cell receptor for the virulent *L.major* metacyclics. *L.donovani* enters phagocytes via the macrophage CR3/mannose−fucose receptor (Blackwell *et al.*, 1985). Interestingly, *L.major* metacyclics do not activate the complement cascade via the alternative pathway which is activated by non-virulent logarithmic phase promastigotes (da Silva *et al.*, 1989). The reasons for this difference are still unclear.

Phosphorylation can regulate the susceptibility of proteins to proteolytic cleavage. This has been shown for a 70 kDa PKC substrate from rat fibroblasts which is protected after phosphorylation from proteolytic degradation by cathepsin L or endogenous proteases (Laumas *et al.*, 1989). Our studies show that a purified leishmanial enzyme, LPK-1, is capable of phosphorylating different components of the complement cascade, including C3, C5 and C9. While the role of phosphorylation of complement components in the regulation of the complement system is still unknown, P-C3 has been demonstrated in normal human plasma (Martin, 1989). Proteolytic cleavage of C3 and C5 by serum convertases is an important step in the process of complement activation. Phosphorylation of C3 by LPK-1 reduces the rate at which trypsin cleaves this protein to C3a and C3b. Trypsin mimics the action of the C3-convertase. LPK-1 only phosphorylated the α-chain of C3. This is similar to results obtained *in vitro* using human C3 and PKA prepared from pig muscle (Forsberg *et al.*, 1990). PKA only phosphorylated the α-chain of C3, while PKC phosphorylated both chains under similar conditions. However, phosphorylation with either PKC or PKA also reduced the rate of C3 cleavage by trypsin, LPK-1 has been shown to be distinct from both PKA and PKC (Hermoso and Jaffe, 1991). The activity of LPK-1 is not increased by cyclic-AMP, an activator of PKA, or Ca^{2+}/phospholipid/diolein, a reagent mixture which activates PKC. LPK-1 does not react with antibodies to PKC (unpublished data).

Experiments using synthetic peptides derived from the COOH-terminal of C3a show that LPK-1 recognizes the amino acid sequence, Arg-X-Ser, as a substrate for phosphorylation. The shortest peptide tested, C3a8R, which lacks the Arg69 could not serve as a substrate for phosphorylation by the leishmanial enzyme. On the other hand the presence of Arg69 in the peptides, either YA-C3a10R and C3a21R, converted them into good substrates for LPK-1. Sequences such as Arg-X-Ser are frequently recognized by serine protein kinases, including PKC (Taylor *et al.*, 1990). Thus we have identified Ser71, which is equivalent to Ser720 in C3, as a potential phosphorylation site in C3a and intact C3. This residue is located close to the active site of the anaphylatoxin and to the Arg-Ser site (C3 residues 726−727) cleaved by the C3-convertase.

When the thioester bond is hydrolysed converting C3 to C3(H_2O), the molecule undergoes a conformational change. C3(H_2O) exhibits many biological properties similar to C3b, including the binding of Factors B and H, cleavage by Factor I, binding to C3b cellular receptors and the formation of the C3-convertase (Pangburn and Muller-Eberhard, 1984). C3(H_2O) is less sensitive to cleavage by the C3-convertase or trypsin than native C3, suggesting that the conformational change which occurs upon hydrolysis reduces the accessibility of the Arg-Ser cleavage site (Pangburn and Muller-Eberhard, 1984). Likewise, this same conformational change prevents phosphorylation of the C3(H_2O) by LPK-1 suggesting that the sequence recognized by LPK-1 is no longer available to this parasite protein kinase. Interestingly, phosphorylation of C3 renders the molecule less susceptible to cleavage by trypsin, suggesting that phosphorylation at Ser720 in C3 inhibits the interaction between proteases and the C3-convertase cleavage site or that phosphorylation leads to hydrolysis of the C3 thioester bond. Work is in progress to characterize further the phosphorylation of C3 and to study the effect of Ser720 phosphorylation on the susceptibility of this region to cleavage by trypsin and C3-convertase.

Additionally, the phosphorylation of Ser71 in C3a may also affect the biological activity of human C3a. This factor binds to receptors on cells via the five COOH-terminus amino acids Leu-Gly-Leu-Ala-Arg (C3a−73−77) and is a potent effector of the inflammatory response (Hugli, 1984, 1990; Fishelson, 1985). C3a has been demonstrated to possess immunoregulatory activity (Hugli, 1990). In serum, C3a activity is regulated by carboxypeptidase N which cleaves the COOH-terminal Arg, inactivating the anaphylatoxin (Bokisch and Muller-Eberhard, 1970). Phosphorylation near this residue may affect the half-life of C3a in human serum. Phosphorylated peptides are being used to measure the effect of phosphorylation at serine 71 on the activity of carboxypeptidase N.

The effect of phosphorylation on the biological activities of C5 and C9 has not yet been studied. It may be speculated that phosphorylation of C5 will reduce its reactivity with the C5 convertases and inhibit its cleavage to C5a and C5b. The 10 kDa phosphorylated band in the C5 lane (Figure 1b) may represent some C5a present in the C5 preparation which is phosphorylated by LPK-1. A possible phosphorylation site in C5a is Arg-Ile-Ser42 (Wetsel *et al.*, 1988). The lower phosphorylated bands in C9 (Figure 1b) may also represent fragments of C9. Recent results have demonstrated that an ecto-protein kinase of K562 human erythroleukaemic cells phosphorylates C9 on a serine residue in the C9a portion of C9 (Paas,Y. and Fishelson,Z., manuscript in preparation). Perhaps, LPK-1 phosphorylates amino acids in the C9a region of C9 which are more accessible in the C9a fragment. Potential phosphorylation sites in C9a are Ser47 and Thr80 (Stanley *et al.*, 1985). At present we can only speculate that phosphorylation of C9 will inhibit its polymerization or reduce the stability of the membrane attack complex of complement.

The physiological role of complement phosphorylation *in vivo* as a parasite protection mechanism is of great interest.

Other studies have shown that protein kinase activity is present both in the plasma and on the surface of cell membranes (Lin *et al.*, 1985; Kubler *et al.*, 1987; Kleine and Whitfield, 1987). ATP at micromolar concentrations has been identified in human plasma (Martin, 1989). A recent study on the phosphorylation of human C3 using PKC or PKA *in vitro* showed that phosphorylation may inhibit the activation of both the classical and alternative pathways of the complement cascade (Forsberg *et al.*, 1990). Like LPK-1, the site of phosphorylation for these other protein kinases appears to be located on the C3a polypetide of the C3 α-chain, and is only found in haemolytically active C3.

Materials and methods

Parasites

Leishmania major (MHOM/IL/80/Fredlin) obtained by needle aspiration from infected BALB/c mice was used in this study. The promastigotes were cultured in Schneider's *Drosophila* medium (Gibco Laboratories) containing 10% fetal calf serum and antibiotics. Stationary phase promastigotes were used in all experiments. The parasites were maintained in culture for not more than 10 passages before thawing additional stabilates prepared from the original isolate.

Human complement components

C3, C5 and C9 were purified from human plasma as previously described (Hammer *et al.*, 1981). C3b was generated by trypsin cleavage from purified C3 and isolated over a Sephacryl S-300 (Pharmacia/LKB) column (Reiter and Fishelson, 1989). C3a was isolated from zymosan-activated human serum as described (Chenoweth *et al.*, 1979). The synthetic peptides C3a8R and C3a21R were gifts from Dr Tony Hugli, Research Institute of the Scripps Clinic, La Jolla, CA. The peptide YA-C3a10R was synthesized at the Peptide Synthesis Unit of our institute.

Leishmanial protein kinase-1 (LPK-1)

In brief LPK-I was purified as follows: promastigotes (6×10^{10} total) were lysed by nitrogen cavitation (10 min, 1500 p.s.i.) in 20 mM Tris–HCl buffer, pH 7.4 containing 40 mM NaCl and protease inhibitors (5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin and 2 mM iodoacetamide). Glycerol, 10% final concentration, was added immediately to the lysed parasites, and the homogenate centrifuged for 45 min at 48 000 *g*. The supernatant was collected, concentrated 7-fold by ultrafiltration using an Omega filter, 10 kDa cut-off (Filtron Corporation), and separated on a Sephadex G-75 (Pharmacia) column. The void volume containing the protein kinase activity was further purified on a DEAE–sephacryl column using a 0.0–0.2 M NaCl gradient. The active fraction eluting at 150 mM NaCl was collected and concentrated by HPLC on a Mono Q HR 5/5 column (Pharmacia). The enzyme-containing fractions eluting at 388 mM NaCl were further purified by adjusting the buffer to 1 mM Lubrol-PX and rechromatographing on the Mono Q HR 5/5 column. The purified LPK-1 activity eluted at 480 mM sodium acetate. LPK-1 was analysed both enzymatically and by SDS–PAGE.

Phosphorylation by LPK-1 and live Leishmania

LPK-1 was used to study the phosphorylation of human complement proteins including C3a, C3, C3b, C5 and C9. Each protein, 10 μg, was dissolved in labelling buffer containing 20 mM Tris–HCl pH 7.4, 2 mM EDTA, 10 mM $MgSO_4$ and 0.5 mM DL-dithiothreitol (60 μl). LPK-1 (25–40 μl) was added. In some experiments the LPK-1 was first inactivated by boiling for 1 min. Following a 5 min preincubation at 30°C, the reactions were initiated by the addition of 50 μCi [γ-^{32}P]ATP and 5 μM ATP. After 20 min, the reactions were stopped by the addition of SDS–PAGE sample buffer containing β-mercaptoethanol and analysed by SDS–PAGE and autoradiography.

Phosphorylation of C3a and the COOH-terminal C3a peptides was carried out by incubating each peptide (10 μg) or protamine sulphate (10 μg) dissolved in labelling buffer with LPK-1 (50 μl). Reaction mixtures were preincubated for 5 min at 30°C and initiated by the addition of 1 μCi [^{32}P]ATP plus 5 μM ATP. After 8 min, the reactions were terminated by the addition of 230 mM phosphoric acid. Incorporation of ^{32}P was measured by spotting aliquots onto phosphocellulose paper (Whatman p81). Unbound phosphate was removed by washing four times in 75 mM phosphoric acid. The filter paper was dried and incorporation measured in a 1500 Tri-Carb scintillation counter (Packard Instrument Company). Control reactions were carried out using LPK-1 which was boiled for 1 min.

Live stationary phase promastigotes were washed twice with 20 mM Tris–HCl pH 7.5 containing 150 mM NaCl and 2 mM glucose (buffer A) and resuspended at 10^8 cells in 100 μl buffer A containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, 1 mM ortho-vanadate and proteolytic inhibitors (1 μg/ml leupeptin, 2 mM iodoacetamide and 1 mM phenylmethylsulphonyl fluoride). The cells were mixed with either C3 or C3b and with [γ-^{32}P]ATP (50 μCi) for 20 min at 30°C. The cells were then removed to ice and spun out by centrifugation (5 min, 6000 r.p.m.). The supernatant was recleared for 10 min at 14 000 r.p.m. in an Eppendorf Microfuge. Cold ATP (500 mM, 1 μl) and Na_2HPO_4 (500 mM, 5 μl) was added to the supernatants prior to analysis by SDS–PAGE.

Cleavage of C3 by trypsin

C3 was labelled with [^{32}P]ATP using LPK-1 as described above. P-C3 or C3, 25 μg each, were diluted in 50 mM phosphate buffered saline (5 μl of 25 × PBS) and an aliquot removed at time 0. TPCK-treated trypsin (type XIII, Sigma Chemical Co.), 0.28 μg, was added and aliquots removed from each reaction at 1, 2 and 5 min. The reactions were terminated by adding soybean trypsin inhibitor, SDS–PAGE sample buffer and stored frozen until analysis by gel electrophoresis on 7.5–20% polyacrylamide gels and autoradiography. Coomassie blue stained gels were scanned using a Bio-Rad Model 620 Video Densitomiter and X-ray films were scanned using a Molecular Dynamics Computing Densitomiter (Model 300A, Sunyvale, CA).

Phosphorylation of hydrolysed and methylamine-treated C3

C3(H_2O) was prepared by slowly freezing C3 (10 μg in 60 μl labelling buffer) at −20°C and thawing to room temperature three times. In parallel, an equal amount of C3 was incubated in 20 mM Tris–HCl, pH 7.4 containing 5 or 50 mM methylamine (Sigma Chemical Co.) for 1 h at 37°C. Phosphorylation with LPK-1 of native C3 and the treated samples was carried out as described above. The results were anlaysed by SDS–PAGE and autoradiography.

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