Release of Ecto-protein Kinases by the Protozoan Parasite Leishmania major*

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The protozoan parasite Leishmania is responsible for a wide spectrum of human diseases that cause varying degrees of patient morbidity and mortality and affect more than 12 million people world-wide. Leishmania have a relatively simple life cycle, existing as extracellular flagellated promastigotes in the sandfly vector and following transmission to a mammalian host, as intracellular aflagellated amastigotes in macrophages (1). Throughout its life cycle, Leishmania encounter hostile, changing environments that require rapid responses to ensure survival of the parasites (1, 2). In eukaryotes, protein phosphorylation is a major mechanism for regulating cellular responses to environmental signals, including cell-cell interactions. The role of intracellular protein kinases (PK, EC 2.7.1.37)1 in complex regulatory cascades that control differentiation, metabolism, growth, gene expression, and other cellular processes is well established (3).

Less is known about the functions of externally oriented cell surface PK (ecto-PK), although the potential for their involvement in signal transduction and cell-cell interactions appears great. These ecto-PK utilize extracellular ATP that is present in blood plasma and other body fluids at concentrations from 1 to 30 μM (4). Ecto-enzymes have been demonstrated in a variety of cultured cells, including HeLa cells, fibroblasts, neutrophils, neurons, and others (5–10). Several types of serine/threonine ecto-PK have been identified and include the cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and cyclic nucleotide independent PK (6–8, 10). Recently tyrosine ecto-PK have also been reported (11). Ecto-PK are capable of phosphorylating both endogenous membrane and exogenous foreign substrates. We have identified a cyclic nucleotide-independent ecto-PK activity on viable Leishmania major promastigotes that phosphorylate exogenous substrates, such as mixed histones and protamine sulfate, in addition to 11 endogenous parasite membrane proteins (12). Live parasites can also phosphorylate the C3 and C3β polypeptide components of the human complement system (13). Phosphorylation of C3 was shown to inactivate both the alternative and classical complement pathways (14) and thus may play an important role in parasite survival.

In addition, the inducible release or shedding of ecto-PK in the presence of enzyme substrates has been described for specific PK on the surface of HeLa cells, endothelial cells, fibroblasts, neutrophils, and other cells (6, 7, 9, 15, 16). This activity appears to be similar to casein kinases and was recently purified from HeLa cells and characterized as casein kinase 1 (CK1) and casein kinase 2 (CK2, see Ref. 6).

In this study we show for the first time that parasites are capable of shedding ecto-PK. At least two leishmanial ecto-PK released by promastigotes were identified as follows: first, an ecto-PK that is shed constitutively and phosphorylates phosphatidylinositol; and second, an enzyme released by incubation with PK substrates that phosphorylates protamine sulfate. The constitutively shed enzyme was characterized and shown to be CK1-like. These findings will allow us to further characterize the properties and roles of ecto-PK and the possible ramifications of these enzymes on host-parasite interactions.

EXPERIMENTAL PROCEDURES

Materials—The CK-specific inhibitors, CKI-7 and CKI-8, were purchased from Seikagaku America (St. Petersburg, FL). The CK1- and CK2-specific peptides, RRRDLHDDEEAMSTA and RRRADDSDDDD, respectively, used in the phosphorylation assays were generous gifts from Dr. L. Pinna (University of Padova, Italy). All the other PK inhibitors, protein substrates, and reagents were purchased from Sigma. P-81 phosphocellulose paper was obtained from Whatman Scientific Ltd. (United Kingdom). Rat liver CK2 was purchased from Promega Corp. (Madison, WI).

Cell Cultures—Stationary phase L. major (MHOM/IL/80/Friedlin) promastigotes were used in all studies. Promastigotes were cultured in Schneider’s Drosophila medium containing 10% fetal calf serum and antibiotics (complete medium, see Ref. 17). The B4 clone was isolated by limiting dilution cloning on 1% agar plates made with complete medium
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Parasite Viability—Promastigote viability was assessed by two complementary assays as follows: ethidium bromide (EtBr) incorporation, which measures the percentage of dead or damaged cells; and fluorescein diacetate hydrolysis (18), which measures the percentage of viable cells. The cells were layered over di-N-butyl phthalate (150 μl) and incubated for 20 min at 30 °C. The promastigotes were removed by rapid centrifugation in a Microfuge (Beckman model B, 1 800 × g for 1 min), and the supernatants were used for phosphorylation assays.

Phosphorylation Assays—Released ecto-PK activity was measured in cell-free supernatants, prepared as described above, by adding PK substrates, [γ-32P]ATP (1–10 μCi) and 0.1 mM cold ATP. The reactions were incubated for 10–20 min at 30 °C and stopped by the addition of ice-cold trichloroacetic acid (1 ml, 25%). After 30 min on ice protein phosphatase activity was removed by precipitation of protein or by digestion with 1% dimethyl sulfoxide and 1% bovine serum albumin (BSA). The supernatant was washed with 75 mM phosphoric acid (500 ml, 5 min each wash) to remove the BSA and washed three times with Millipore HAWP filters (0.45 μm), washed four times with ice-cold 5% trichloroacetic acid (2 ml each), and counted in a β-scintillation counter. For gel electrophoresis, the precipitate was centrifuged and washed with 5% trichloroacetic acid (once) and 90% acetone (three times). After the pellet was resuspended in sample buffer, analyzed by 12% SDS-PAGE, and exposed to x-ray film or phosphorimaging. Quantitation of the phosphorylation was carried out by densitometric analysis of the bands. The protein substrates and concentrations used are indicated in the figure legends.

Phosphorylation by live promastigotes. Parasites were washed once by centrifugation with buffer A and resuspended at 5 × 10⁷ cells/ml in buffer A (100 μl). Cells were preincubated at 30 °C with or without the PK substrates for 2–5 min, and the reaction was initiated by addition of [γ-32P]ATP (1–10 μCi) and 0.1 mM cold ATP. After 10–20 min incubation the reactions were stopped by adding trichloroacetic acid and analyzed by filtration and SDS-PAGE as described above.

Peptide Phosphorylation—Phosphorylation of specific peptide substrates for CK1 or CK2 using cell-free supernatants was carried out as described above with the following modifications. The reaction was stopped by the addition of ice-cold 100% trichloroacetic acid (24 μl) and 1% bovine serum albumin (BSA, 40 μl), and co-precipitated on ice (30 min). After centrifugation, samples from the supernatant (20 μl in triplicate) were spotted on P-81 ion exchange chromatography phosphocellulose paper (Whatman Scientific Ltd., UK) and washed three times in 75 mM phosphoric acid (500 ml, 5 min each wash) to remove the unbound phosphate. The paper was dried and 35S incorporation was measured in a β-scintillation counter.

Parasite Viability—Promastigote viability was assessed by two complementary assays as follows: ethidium bromide (EtBr) incorporation, which measures the percentage of dead or damaged cells; and fluorescein diacetate hydrolysis, which measures the percentage of viable cells. Fluorescein diacetate hydrolysis (18) was tested in selected experiments; however, the EtBr assay (19), described below, was used in all experiments. To determine the effect of each treatment on cell viability, promastigotes (5 × 10⁷ cells/200 μl buffer A) were incubated in parallel under identical conditions, except for [γ-32P]ATP, to those described above. At the beginning and end of each incubation 2.3 ml of buffer A containing 50 μM EtBr was added, and the fluorescence was measured 5 min later in a fluorescent spectrophotometer (Perkin-Elmer LS-5B Luminescence Spectrometer, 365 nm excitation, 580 nm emission). Buffer A containing 50 μM EtBr served as the blank. A standard curve using increasing numbers of promastigotes (5–500 × 10³) in buffer A containing digitonin (30 μg/ml) and EtBr (50 μM) was used to calculate the number of dead parasites. In some cases, the percentage of dead or damaged parasites was also determined by counting fluorescent and total parasites using a fluorescent phase microscope (Laborlux K; Leitz, Germany) at 400 × magnification.

Effect of PK Inhibitors on Enzyme Activity—Stock solutions of the PK inhibitors heparin, CKI-7, CKI-8, staurosporine, H-7, and W-7 were prepared in dimethyl sulfoxide or distilled water. The inhibitors or dimethyl sulfoxide alone were diluted with buffer A and added to the reactions just before use. Phosphorylation of the PK substrates and analysis of the reactions by either filtration or SDS-PAGE was carried out as described previously. The final concentrations of inhibitors examined are given in the text.
Figure 2. Shed protein kinase activity of L. major promastigotes. Parasites (5 × 10^7/100 μl) were resuspended in labeling buffer (buffer A, 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM MgCl₂, and 10 mM NaF) and carefully layered onto oil. After 15 min incubation (Inc) at 30 °C in the absence (lanes 2, 5, and 7) or presence (lanes 1, 4, and 6) of hydrolyzed casein (C), the promastigotes were removed by rapid centrifugation, and the cell-free supernatant (Sup) was examined for PK activity. Reactions (Ren) were carried out by adding [γ-32P]ATP and either buffer alone (lanes 2, 5, and 6) or additional substrate, protamine sulfate (PS, lanes 1–3) or casein (lanes 1, 4, and 7), to the supernatant and incubating for 15 min at 30 °C. All reactions were stopped by the addition of trichloroacetic acid and examined by SDS-PAGE and autoradiography.

Release of Ecto-PK Activity from Parasites—Incubation of neutrophils, HeLa cells, and fibroblasts with substrates for PK was shown to induce the release of PK activity from these cells. This activity could be detected in the cell-free supernatants (7, 11, 15, 16). Therefore, we decided to test if phosphorylation of exogenous substrates observed using live parasites was due to enzyme release by the cells. Promastigotes were washed and incubated with or without h-casein for 20 min. After removal of the cells by centrifugation through an oil layer, PK activity of the supernatants was assayed by adding PS where indicated (Fig. 2; lanes 1–5).

Preincubation of promastigotes with h-casein resulted in the release of a PK that phosphorylates both h-casein and PS (lanes 1 and 4). Shedding of the PS phosphorylating activity by the cells required parasite preincubation with h-casein, since no phosphorylation of PS was observed when the parasites were preincubated in buffer alone (lane 2). Preincubation of promastigotes with other PK substrates, including i-casein, phosvitin, or PS, also caused the shedding of a PK activity that could phosphorylate PS. However, no phosphorylation of PS was observed if supernatants from parasites incubated with BSA were used (data not shown). The labeled bands seen in lanes 1 and 4 are not due to phosphorylation of endogenous secreted parasite proteins, since no radiolabeled bands were observed if supernatant alone was used in the phosphorylation reactions (lane 5). Likewise, PS was not phosphorylated when added to the reaction mixture in the absence of supernatants collected from h-casein-treated promastigotes (lane 3).

Shedding of the PK that phosphorylates PS requires promastigote preincubation with h-casein. However, it was not clear whether h-casein phosphorylation (Fig. 2, lane 1) required parasite preincubation with substrate, “induced release,” or if this PK activity was constitutively released by the parasites. Cell-free supernatants were collected from promastigotes incubated with or without h-casein (Fig. 2, lanes 6 and 7). Substrate was added to supernatant obtained from parasites incubated in buffer alone and the phosphorylation reaction carried out. Phosphorylation of h-casein was seen when either procedure was used and did not depend on whether the parasites were incubated with the substrate prior to collection of the supernatant. These results demonstrate that the PK activity that phosphorylates h-casein is constitutively released from the leishmanial parasites. However, phosphorylation of h-casein using supernatants collected from parasites preincubated with substrate was 70% greater than those only incubated with buffer (lanes 6 and 7), suggesting that incubation with casein may induce release of a PK activity capable of phosphorylating h-casein. These findings suggest that at least two different kinase activities are released from intact cells: 1) “substrate-inducible” activity that phosphorylates PS and perhaps h-casein; and 2) a “constitutive” activity that only phosphorylates h-casein and not PS. The “constitutively” shed leishmanial CK-like activity, LCK, was characterized further.

Substrate Specificity of the Constitutive PK Activity—Enzyme activity of cell-free supernatants, collected from promastigotes incubated with buffer alone, was measured using several different PK substrates, including h-casein, i-casein, phosvitin, mixed histones, PS, and BSA. Phosphorylation was examined by SDS-PAGE (Fig. 3 and data not shown). Phosvitin was the best substrate for the constitutively released PK (lane 3), followed by h-casein (lane 1) and i-casein (lane 2). No phosphorylation of PS (Fig. 2, lane 2), mixed histones, or BSA (Fig. 3, lanes 4 and 5, respectively) was observed. Likewise, no endogenous phosphorylation of parasite proteins in the supernatant was observed (lane 6).

Effect of Inhibitors on the Constitutively Released PK—Several different PK inhibitors, including staurosporine, W-7, heparin, CKI-1 and CKI-8, were examined for their ability to block phosphorylation of PS. The antibiotic staurosporine, a competitive inhibitor of ATP binding to PK, inhibits a wide range of enzymes, including PKC, PKA, and Ca²⁺-calmodulin PK, at nanomolar concentrations. Unlike most PK, CK are less sensitive to staurosporine, and the IC₅₀ for CK1 and CK2 is 163 and 19 μM, respectively (20). The IC₅₀ found for the leishmanial CK (LCK), 5 μM, is similar to CK2.

Heparin has been used to distinguish between the two CKs (21). CK2 is strongly inhibited at approximately 1% the concentration that inhibits CK1 (IC₅₀ = CK1 = 24 μg/ml; CK2 = 0.15 μg/ml). Phosphorylation of phosvitin by LCK was measured in the presence of different concentrations of heparin (Fig. 4). This curve shows that relatively high concentrations of heparin (IC₅₀ = 50 μg/ml), more similar to CK1, are needed to inhibit the leishmanial enzyme’s activity. In addition to heparin, the effect of CKI-7, an isooquinoline derivative of W-7, on phosvitin phosphorylation was also examined. CKI-7 is a specific inhibitor of CK1 and CK2 (IC₅₀ = CK1 = 9.5 μM; CK2 = 90
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FIG. 4. Effect of heparin on the constitutively shed leishmanial casein kinase (LCK) activity. Promastigotes were incubated in labeling buffer for 20 min at 30 °C, and the cell-free supernatants were collected by centrifugation through an oil layer. Protein kinase activity was assayed using phosvitin (1 mg/ml), [γ-32P]ATP, and increasing concentrations of heparin. The reaction was stopped after 10 min by the addition of ice-cold trichloroacetic acid and analyzed by 12% SDS-PAGE and autoradiography. Quantitation of the phosvitin phosphorylation was carried out by densitometry.

μM; see Ref. 22). The IC50 for other common PK, such as PKA, PKC, and Ca2+/calmodulin PK, is much higher, 550, >1000, and 195 μM, than for CK. The IC50 found for LCK was 75 μM, closer to that observed for mammalian CK2. Taken together these results using PK inhibitors and substrate specificity strongly suggests that the shed leishmanial enzyme is a CK. LCK was also inhibited by W-7 (IC50 = 10 μM).

Phosphorylation of CK1- and CK2-specific Peptide Substrates—The constitutively released LCK activity and total cellular CK activity were further characterized in four separate experiments using peptides specific for either CK1 (RRKDLH-DDEEDEAMSITA) or CK2 (RRADDSDDDIDDD); Ref. 23). Results typical of these experiments are given in Table I. Phosphorylation of the peptides using parasite lysates, either freeze/thawed or sonicated, showed that both enzymes are present in the parasite (Table I and data not shown). The total activity found in the promastigote lysates for CK1 was approximately 2.6-fold higher than CK2.

When peptide phosphorylation was examined using cell-free supernatants as a source of LCK, the activity was significantly lower than that measured using parasite lysates. In some experiments both CK-specific peptides were phosphorylated (data not shown). However, the CK1 activity was consistently higher, up to 650 times greater, than the CK2 activity (Table I). Phosphorylation of the CK-specific peptides using intact promastigotes gave results similar to that found using the cell-free supernatants. These results strongly suggest that the constitutively released leishmanial enzyme is CK1-like. Furthermore, the finding of high CK1 activity in the cell-free supernatant (9.6% of the parasite lysate) and little or no CK2 activity suggests that the released activity is not due to cell lysis. Promastigote viability in buffer A was >97% over 20 min.

Kinetics of Constitutive PK Release—Release of the CK-like activity over time was followed for 30 min. At each time point, aliquots containing parasites in buffer alone were removed and cell-free supernatants prepared. In parallel, the percentage of dead promastigotes was measured using the EtBr assay. CK-like activity was assayed using phosvitin as substrate and analyzed by SDS-PAGE and densitometry. Results from one typical experiment is shown in Fig. 5. PK activity shed by the parasites into the buffer increased dramatically over the first 10 min of incubation (600%). After peaking, the activity measured in the cell-free supernatants slowly decreased, until it appeared to level off after 25 min at twice the initial activity. The initial time point (t = 0 min) was obtained by adding parasites to buffer and then immediately centrifuging to prepare a cell-free supernatant. All time points were compared with a negative control, phosvitin, in labeling buffer without supernatant. The percentage of dead promastigotes was examined in parallel by EtBr staining and showed no change in parasite viability over the first 15 min in labeling buffer (98% viable; t = 0 and 15 min) and only a small decrease after 30 min (96% viable). The difference in kinetics of CK secretion and change in parasite viability further excludes the possibility that the PK release observed is a result of cell damage.

DISCUSSION

Many eukaryotic cells possess ecto-PK that are capable of phosphorylating foreign and endogenous protein substrates. PK identified include PKC, PKA, CK1, and CK2. In addition, several vertebrate cells were shown to shed ecto-PK from their surface in the presence of PK substrates like phosvitin or casein. Although most cells examined appear to release cyclic nucleotide independent CK, the release of PKA or PKC has been documented in only a few cases.

Parasites have evolved varied strategies to evade host defense mechanisms including the mimicry of host regulatory molecules and enzymes. Leishmania promastigotes also express ecto-PK on their surface that phosphorylate foreign proteins (24). Previous studies showed little or no evidence that the parasite ecto-PK activity was related to PKA or PKC, respectively. Activators and inhibitors of these enzymes had no significant effect on the phosphorylation of exogenous or endogenous substrates (Ref. 12 and data not shown). However, comparative studies between live parasites, which phosphorylate the C3 and C3b polypeptides of the human complement system, and LPK-1, a purified parasite enzyme, which only phosphorylates C3, but not C3b, suggested that promastigotes possess more than one ecto-PK (13). Preliminary experiments show that the constitutively shed leishmanial PK, LCK, phosphorylates C3a (data not shown). Interestingly, a casein kinase shed from human platelets following activation was shown to

TABLE I

| Enzyme source | Peptides phosphorylation rate (Δ cpm) |
|---------------|-------------------------------------|
|               | CK1 | CK2 |
| Shed cLPK     | 653 ± 134 | 1 ± 0 |
| Lysed parasites | 6697 ± 227 | 2446 ± 156 |
| Intact parasites | 368 ± 103 | 11 ± 0 |
| Pure rat liver CK2 | 39 ± 40 | 580 ± 49 |

Δ cpm = (cpm reaction mixture − cpm without peptide).

[...]

PK-2

Phosphorylation of casein kinase (CK) peptide substrates by leishmanial protein kinases

Shed cLPK, promastigotes (5 × 10^7/100 μl) were incubated for 20 min at 30 °C and removed by centrifuging through an oil layer. The cell-free supernatants were used as a source of enzyme. Lysed parasites, promastigotes (5 × 10^7/100 μl) were freeze/thawed three times and used as a source of enzyme. Intact parasites, live promastigotes (5 × 10^7/100 μl) were incubated directly with the CK-specific peptide substrates. CK1 (RRKDLH-DDEEDEAMSITA) or CK2 (RRADDSDDDIDDD) specific peptide, [γ-32P]ATP, and protein kinase were incubated for 10 min, and the reaction was stopped by adding 1% BSA and trichloroacetic acid. After 30 min on ice the precipitate was removed by centrifugation, supernatants in triplicate were spotted on P-81 filters and washed with 75 mM phosphoric acid, and 32P incorporation was measured by liquid scintillation counting. Background cpm. Shed cLPK, 576 ± 74; lysed parasites, 1039 ± 55; intact parasites, 502 ± 85, and CK2, 61 ± 74.

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**Fig. 5. Constitutive secretion of leishmanial casein kinase 1 (LCK1) by promastigotes of L. major.** Effect of incubation time on released protein kinase activity and cell viability was measured. Promastigotes in labeling buffer were layered on oil and incubated at 30 °C. At three time points samples were taken to check parasite viability by staining with ethidium bromide and counting in a fluorescent microscope. In parallel, the promastigotes were removed by rapid centrifugation, and the cell-free supernatants were assayed for LCK1 activity by the addition of phosphovitin and [γ-32P]ATP. Reactions were analyzed by 12% SDS-PAGE, autoradiography, and densitometric scanning of the film. ●, LCK1 activity; ■, parasite viability.

phosphorylate both C3 and C3b (25). To obtain a better understanding of the role of ecto-PK in host-parasite interactions, it will be necessary to characterize parasite ecto-enzymes and the physiological substrates involved in these processes.

In this study we demonstrate that *Leishmania* promastigotes have at least two types of ecto-PK, both of which can be released by the parasites. Unlike most cells examined so far, one type of activity appears to be shed continuously in the absence of substrate, whereas the second activity, similar to other eukaryotic cells, is shed only when incubated with substrate. These activities, constitutive and inducible, are easily distinguished by their ability to phosphorylate PS, since the former enzyme(s) shows no activity when assayed with this substrate, and the latter readily phosphorylates PS.

These shed ecto-PK are not due to cytoplasmic leakage from damaged or dead cells. Although both histone and PS are cytotoxic for eukaryotic cells (5, 16, 26) and parasites, little or no cytotoxicity was found when cells (5, 7, 16, 26) or parasites were incubated with phosphovitin or casein. Initial parasite viability in the studies described herein always exceeded 97%, as measured by two fluorescent assays, and changed by <2% following incubation either in the presence or absence of casein or phosphovitin. This percentage was identical to that found following incubation with buffer alone. The small amount of dead parasites was not responsible for the induced PK activity observed, since no phosphorylation of PS was noted using cell-free supernatants from promastigotes incubated either with BSA or buffer alone, whereas good phosphorylation of PS was found with as little as 2.5 × 10^6 freeze/thawed parasites (data not shown). Furthermore, no correlation was found between the release of constitutive ecto-PK activity in the cell-free supernatants and decreasing cell viability. Ecto-PK activity peaked rapidly at 10 min and then gradually decreased with time, whereas cell viability remained essentially constant over the first 15 min and then decreased only slightly, by 2%, after 30 min.

We decided to focus on the characterization of the constitutively shed leishmanial ecto-PK activity that appears to be related to casein kinases. Substrate specificity was typical of these enzymes. Phosvitin, followed by h-casein and i-casein, was the best substrate for the enzyme(s), and neither mixed histones nor PS were phosphorylated by the ecto-PK.

The parasite activity is different from a spontaneously shed human leukemic cell line serine/threonine ecto-PK that was recently reported (11). The latter activity phosphorylates PS and histone H2B, as well as casein, phosphovitin, and the human complement polypeptide C9, and may contain more than one PK. PKI, a specific PKA inhibitor, was found to inhibit the phosphorylation of histone H2B but not C9 by the leucemic cell ecto-PK. The latter activity was postulated to be CK-like. Little evidence was found in our study or previous studies for either an intracellular or externally oriented leishmanial PKA (12). However, we have recently cloned and characterized the genes for two PKA catalytic subunits from *L. major* (27).

The effect of several PK inhibitors on the leishmanial ecto-PK was examined. Unlike most PK that are inhibited by nanomolar concentrations of staurosporine (20), the IC_{50} values for casein kinases are in the μM range (CK1 and CK2, 163 and 19 μM, respectively). The IC_{50} value for the leishmanial ecto-PK (5 μM) was similar to CK2. The high concentration of drug need to inhibit the leishmanial activity is not due to the intrinsic resistance of parasite enzymes to staurosporine. The phosphorylation of PS by *Leishmania aethiopica* promastigotes, either particulate or soluble fractions or live parasites, is strongly inhibited by staurosporine. Low concentrations of drug (50 nM) inhibited the phosphorylation of PS by >80% when parasite fractions were used and by approximately 45% using live parasites (28). Furthermore, staurosporine concentrations similar to those that inhibit the constitutively shed ecto-PK are cytostatic and/or cytotoxic to the promastigotes and induce pronounced morphological changes (29).

Heparin and CKI-7, both specific CK inhibitors, also blocked the leishmanial ecto-PK activity at concentrations similar to those reported for mammalian and yeast CK and confirmed that the constitutively shed parasite enzyme is CK-like. However, no conclusion regarding the type of CK in the cell-free supernatants could be made based on IC_{50} values for these inhibitors, since the constants found using heparin or CKI-7 each implicated the presence of a different CK, either CK1 or CK2, respectively. Interestingly, heparin concentrations (10 μM) similar to those that inhibit LCK were also shown to significantly reduce endogenous protein phosphorylation by live parasites (30).

Finally, we were able to identify the constitutively shed PK by examining the phosphorylation of specific peptide substrates for CK1 and CK2. Only the CK1-specific peptide substrate was phosphorylated confirming that the constitutively shed LCK activity is CK1-like. This conclusion was further supported by the finding that the LCK, similar to other CK1, only utilizes ATP, whereas CK2 utilizes both ATP and GTP for phosphorylation (data not shown).

Although spontaneously shed ecto-PK activity has not been observed with cell-free supernatants from HeLa cells or neutrophils (7, 16), ecto-CK released from HeLa cells by incubation with phosphovitin were recently purified and characterized (6). Differences in the sensitivity of the leishmanial and mammalian ecto-PK to different inhibitors suggest that it may be possible to design drugs that specifically inhibit the parasite but not the host enzymes. However, this will require the purification and characterization of the parasite enzyme(s).

The CK1 family has been found in all eukaryotic cells exam-
ined so far and is believed to be involved in the regulation of nuclear and cytoplasmic processes. These PK consist of monomeric proteins that vary considerably in size from 25 to 55 kDa and have been found in the nucleus, cytoplasm, membrane, and cytoskeleton. Several different isoforms of CK1 have been identified in mammalian cells and yeast using molecular techniques. In *Saccharomyces cerevisiae* two essential genes have been sequenced and found to encode a carboxyl-terminal prenylation motif, believed to target them to the plasma membrane. Using a nested polymerase chain reaction with degenerate oligonucleotides to conserved regions of CK1, we have amplified a 342-base pair fragment from *L. major* that shows 74.4% identity over 336 base pairs to human CK1-e.2 We expect that molecular analysis of the leishmanial-CK1 gene (*lck1*), the recent availability of molecular techniques for the production of null *Leishmania* mutants, and further biochemical characterization of parasite ecto-PK will prove invaluable in understanding the role of these enzymes in parasite-host interactions.

REFERENCES
1. Peters, W., and Killick-Kendrick, R. (eds) (1987) *The Leishmaniasis in Biology and Medicine*, p. 941, Academic Press, London
2. Zilberstein, D., and Shapira, M. (1994) *Annu. Rev. Microbiol.* 48, 449–470
3. Hunter, T. (1995) *Cell* 80, 225–236
4. Gordon, J. L. (1986) *Biochem. J.* 233, 309–319
5. Vilgrain, I., and Baird, A. (1991) *Mol. Endocrinol.* 5, 1003–1012
6. Walter, J., Schnolzer, M., Pyerin, W., Kinzel, V., and Kubler, D. (1996) *J. Biol. Chem.* 271, 111–119
7. Skubitz, K. M., Ehresmann, D. D., and Ducker, T. P. (1991) *J. Immunol.* 147, 638–650
8. Kubler, D., Pyerin, W., Bill, O., Hotz, A., Sonka, J., and Kinzel, V. (1989) *J. Biol. Chem.* 264, 14549–14555
9. Friedberg, I., Beiber, I., Oged-Plesz, O., and Kuebler, D. (1995) *J. Biol. Chem.* 270, 20560–20567
10. Hogan, M. V., Pawlowska, Z., Yang, H.-A., Kornecki, E., and Ehrlich, Y. H. (1995) *J. Neurochem.* 65, 2022–2030
11. Paas, Y., and Fishelson, Z. (1995) *Arch. Biochem. Biophys.* 316, 780–788
12. Lester, D. S., Hermoso, T., and Jaffe, C. L. (1990) *Biochem. Biophys. Acts* 1052, 293–298
13. Hermoso, T., Fishelson, Z., Becker, S. L., Hirschberg, R., and Jaffe, C. L. (1991) *EMBO J.* 10, 4061–4067
14. Forsberg, P.-O., Martin, S. C., Nilsson, B., Ekman, P., Nilsson, U. R., and Engstrom, L. (1990) *J. Biol. Chem.* 265, 2941–2946
15. Hartmann, M., and Schrader, J. (1992) *Biochem. Biophys. Acts* 1136, 189–195
16. Kubler, D., Pyerin, W., Burrow, E., and Kinzel, V. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 80, 4021–4025
17. Jaffe, C. L., Grimaldi, G., and McMahon-Pratt, D. (1984) in *Genes and Antigens of Parasites: A Laboratory Manual* (Morel, C. M., ed) pp. 47–91, UNDP/WHO/World Bank, Rio de Janeiro
18. Jackson, P. R., Pappas, M. G., and Hansen, B. D. (1985) *Science* 227, 435–438
19. Dey, C. S., and Majumder, G. C. (1988) *Biochem. Int.* 17, 367–374
20. Meggio, F., Deana, A. D., Ruzzene, M., Brunati, A. M., Cesaro, L., Guerra, B., Meyer, T., Metti, H., Fabbro, D., Furet, P., Dohrowlska, G., and Pinna, L. A. (1995) *Eur. J. Biochem.* 234, 317–322
21. Corbin, J. D., and Hardman, J. G. (1983) *Methods Enzymol.* 99, 317–331
22. Kubler, D., Pyerin, W., Bill, O., Hotz, A., Sonka, J., and Kinzel, V. (1989) *J. Biol. Chem.* 264, 4924–4927
23. Marin, O., Meggio, F., and Pinna, L. A. (1994) *Biochem. Biophys. Res. Commun.* 198, 898–905
24. Hermoso, T., and Jaffe, C. L. (1996) in *Molecular and Immune Mechanisms in the Pathogenesis of Cutaneous Leishmaniasis* (Tapia, F. J., Caceres-Dittmar, G., and Sachez, M. A., eds) pp. 71–87, R. G. Landes Co., Austin, Texas
25. Ekdahl, K. N., and Nilsson, B. (1995) *J. Immunol.* 154, 6592–6510
26. Kubler, D., Pyerin, W., and Kinzel, V. (1982) *Eur. J. Cell Biol.* 26, 306–309
27. Siman-Tov, M. M., Aly, R., Shapira, M., and Jaffe, C. L. (1996) *Mol. Biochem. Parasitol.* 77, 201–215
28. Ausefa, D., Worku, Y., and Skoglund, G. (1995) *Biochem. Biophys. Acts* 1270, 157–162
29. Becker, S., and Jaffe, C. L. (1997) *Parasitol. Res.* 83, 275–289
30. Mukhopadhyay, N. K., Shome, K., Saha, A. K., Hassell, J. R., and Glew, R. H. (1989) *Biochem. J.* 264, 519–525

* N. Sacerdoti-Sierra and C. L. Jaffe, unpublished data.