THE UNIVERSITY OF CALGARY

MOLECULAR CLONING, CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF CYSTEINE PROTEASE cDNAs FROM *LEISHMANIA DONOVANI CHAGASI* AND *TRYPANOSOMA CRUZI*

by

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THIS THESIS IS DEDICATED TO

MY LATE FATHER ALON OPYENE AND

MY MOTHER ERUVONIA AUMA FOR

ALL THE SACRIFICES WHICH THEY

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
CIAP	calf intestinal alkaline phosphate
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetra-acetate
EtBR	ethidium bromide
GST	glutathione-S-transferase
HEPES	N-[2-hydroxyethyl]piperizine-N'-[2-ethanesulphonic acid]
hr	hour
LB	Lauria-Bertani (broth)
LMP	low melting point (agarose)
LSL	*Leishmania*-spliced leader
µCi	microCurie (10^{-6} Curie)
mg	milligram (10^{-3} gram)
min	minute
ml	milliter (10^{-3} liter)
µl	microliter (10^{-6} liter)

mM	millimolar (10^{-3} moles/liter)
µM	micrmolar (10^{-6} moles/liter)
µmol	millimole (10^{-3} moles)
MOPS	3-(N-morpholino)-propanesulphonic acid
MTPBS	mouse tonicity phosphate saline
ng	nanogram (10^{-9} gram)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCI	phenol-chloroform-isoamyl alcohol
PCR	polymerase chain reaction
PFUs	plaque forming units
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase PCR
TEMED	tetramethylethylethylenediamine
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
v/v	ratio of volume to volume
vol	volume
w/w	ratio of weight to weight
Ldccys1	*Leishmania donovani chagasi* cysteine protease 1
Ldccys2	*Leishmania donovani chagasi* cysteine protease 2

Lddcys1	*Leishmania donovani donovani* cysteine protease 1
Lddcys2	*Leishmania donovani donovani* cysteine protease 2

ABSTRACT

Two distinct cysteine protease cDNAs were isolated from *Leishmania donovani chagasi* (*L. chagasi*) cDNA libraries using PCR with primers from conserved regions of cysteine proteases from closely related organisms. One of the *L. chagasi* cysteine protease cDNAs was isolated from a cDNA library prepared from total promastigote RNA and has been designated Ldccys2 cDNA while the other cDNA was isolated from a cDNA library prepared from total amastigote RNA and has been designated Ldccys1 cDNA. Ldccys2 has an open reading frame of 471 amino acids and Ldccys1 has an open reading frame of 447 amino acids. Each of the two cDNAs is distinct in genomic arrangement and chromosome location. While Ldccys1 belongs to a family of cysteine proteases encoded by tandemly organized genes, Ldccys2 appears to be a single gene. Ldccys2 is expressed as a 2.6 kb transcript in all stages of development. Ldccys1 is expressed abundantly as 2.4, 4.0 and 5.0 kb transcripts in the amastigotes recovered from infected hamsters, but is expressed at low level in promastigote stage of development.

A new member of *T. cruzi* cysteine protease genes (*T. cruzi* CP) was isolated from a *T. cruzi* cDNA library prepared from the epimastigote stage of *T. cruzi* and was found to have an open reading frame coding for a polypeptide of 471 amino acids. The *T. cruzi* CP gene is arranged in tandem in the genome of *T. cruzi* and is expressed as a 2.2 kb RNA transcript. The *T. cruzi* CP was expressed in a bacterial expression system but cysteine protease is produced in this system as inactive insoluble inclusion bodies.

All the three cDNAs of cysteine proteases were overexpressed in *L. chagasi* and *Leishmania major* cells and the over-produced cysteine proteases are biologically active and

are inhibited by cysteine protease inhibitors. The *L. major* overexpressing these cysteine proteases were used to examine the impact of overexpression on *Leishmania* virulence. It was found that a virulent strain of *L. major* overexpressing cysteine proteases from *L. chagasi* become avirulent to susceptible BALB/c mice and could not be recovered when the tissues from the sites of inoculation were cultured. Importantly, the *L. major* overexpressing Ldccys1 cysteine proteases induce protective immunity against *L. major*. Thus, we have shown that *L. major* overexpressing Ldccys1 cysteine protease is safe and induce protective immunity against *L. major*.

INTRODUCTION

INTRODUCTION

A. General

*Leishmania*spp and *Trypanosoma cruzi* are members of the family Trypanosomatidae and are the causative agents of important human diseases termed leishmaniasis and Chagas' disease respectively. The two diseases are very important in tropical and subtropical regions of the World.

B. The Discovery of Leishmaniasis

The historical information on leishmaniasis has been reviewed by Katz *et al.*, (1989) and the following description is based on their account. The causative agent of visceral leishmaniasis was discovered simultaneously in India, in 1903, by two physicians, Leishman and Donovan. At the time of the discovery both physicians were working in India, Leishman in the British army and Donovan in the Indian Medical Service. Sir Ronald Ross named the genus and species after these physicians in recognition of their landmark discovery. The morphology of newly named species *Leishmania donovani* was similar to the organism that was described earlier in 1885 by Cunningham and later named *Leishmania tropica*. In 1908, Nicolle discovered that other mammals such as the dogs are reservoir hosts of the parasite. In 1942, Swaminath and colleagues proved in human volunteers that leishmaniasis was transmitted by the phelobotomus flies.

C. The Discovery of Chagas' Disease.

The historical information on Chagas' disease has been reviewed by Katz *et al.*, (1989) and the following description is based on their account. The infective stage of *T. cruzi* was discovered in Brazil, in 1909, by Chagas, after whom the disease was named. Chagas described the major clinical features of the disease, the morphology of the trypomastigote stage of the causative agent and named the organism after his mentor, Oswaldo Cruz. In 1912, Brumpt described the complete life cycle of *T. cruzi*. In 1916, Vianna described the pathological changes caused by the organism.

D. Leishmaniasis.

Leishmaniasis is a complex spectrum of diseases caused by different species of *Leishmania* and presents a large variety of disease manifestations differing markedly in their severity and health impact. The clinical manifestations of each disease are determined by the species of *Leishmania* that infects the host and the response of the host to the parasite. The clinical signs of leishmaniasis have been described by Katz *et al.*, (1989) and the following description is based on their account. The three major clinical forms of the disease are: (i) Visceral leishmaniasis or Kala-azar. This is the most severe form of the diseases and is caused by *L. donovani* in the Old World and *L. chagasi* in the New World. Kala-azar causes fever, enlarged spleen, anaemia, kidney damage, and internal bleeding. Without treatment it has a high mortality rate especially in children; (ii) Mucocutaneous leishmaniasis is the name applied only to the New World leishmaniasis known as "espundia", which is caused by *L. braziliensis* or *L. panamensis*. This form of the disease is very disfiguring and the complications

associated with it can be fatal; (iii) Cutaneous leishmaniasis exists in both the Old and New Worlds. Cutaneous leishmaniasis of the Old World which is normally caused by *L. tropica* and *L. major* starts as a nodule and develops into an ulcer which heals gradually. New World cutaneous leishmaniasis is caused by *L. peruviana* and *L. guyanensis*; (iv) Diffuse cutaneous leishmaniasis also exists in both the Old and New Worlds. It is caused by *L. aethiopica* in the Old World and *L. mexicana* in the New World.

Cutaneous leishmaniasis due to *L. major* is becoming a public health problem of considerable magnitude. For example, in Algeria, Morocco, the Sudan (Khartoum) and Tunisia, epidemics involving thousands of cases have occurred (WHO, Technical Report Series, 1990). Visceral leishmaniasis or Kala-azar due to *L. donovani* is also becoming a public health problem of considerable magnitude especially in the event of epidemics. For example in 1991, 70 million people in north eastern India suffered from the worst kala-azar epidemic since the late 1970s when over 20,000 people died of the disease (Alexander and Russel, 1992). In addition, the Southern Sudan also suffered the worst kala-azar epidemic in 1995. Thus, leishmaniasis presents a public health problem and an economic drain in most of the developing countries. The World Health Organization has estimated that some 350 million people are at risk of acquiring the disease and approximately 12 million are currently infected (WHO, Technical Report Series, 1990). The magnitude of the disease also seems to be increasing as new risk population groups for HIV-Leishmania co-infection emerge. For example, in Spain it has recently been estimated that 50% of visceral leishmaniasis in adults are HIV-Leishmania co-infections (Manington *et al.*, 1992). In addition, there is now a mounting concern over the development of resistance to drug therapy and the vector

becoming insensitive to insecticide. Because of the serious health problems it presents, leishmaniasis has become one of the six major diseases targeted by the WHO (WHO, Technical Report Series, 1990).

Definitive diagnosis of the leishmaniasis is based on the demonstration of the parasites in the infected tissue. For visceral leishmaniasis this is achieved by aspiration of bone marrow, spleen, a lymph node, or liver (Katz *et al.*, 1989; Evans, 1993). Immunodiagnosis is feasible because antibodies to *Leishmania* can be demonstrated by immunofluorescence in about 90 percent of infected people (Katz *et al.*, 1989). DNA-based methods such as PCR and Southern blot using probes which are specific for *Leishmania* have been proposed (Howard *et al.*, 1992; Lopes and Wirth, 1986; Baker, 1987). However, the high cost and high technical skills required make these techniques prohibitive in countries where the disease is endemic.

E. Current Management of Leishmaniasis

Although leishmaniasis constitutes an enormous health problem in the developing countries, current treatment of this disease leaves much to be desired. Most drugs still in use, such as antimonials, pentamidine, amphotericin B, were developed in the first half of this century. Most of these drugs are highly toxic to man and in fact some of them would not pass today's stringent standards for drug safety. In addition, there is mounting problem of drug resistance. Strains of parasites which do not respond to treatment with these drugs have emerged. The search for new drugs has been hampered by high costs of developing new

drugs with little hope of recovering the money invested in research and development of such drugs (Fairlamb, 1990; Olliaro and Bryceson, 1993).

F. Vaccine Against Leishmaniasis.

The only vaccine against leishmaniasis which has been shown to yield effective immunization in humans is the inoculation of virulent strains of *Leishmania major* to form a small lesion, as a prevention of subsequent *L. major* infection (Modabber, 1989; Greenblatt, 1988). This type of vaccination was practised in Iran (Naim and Javadian, 1988), Israel (Koufman *et al.*, 1978; Katznellenbogan, 1944), and the former Soviet Union (Sergiev *et al.*, 1970; Shuikna *et al.*, 1968). In these countries, people were infected with virulent *L. major* on regions of the body where resultant scarring is hidden. This practice is known as "leishmanization" (Modabber, 1989; Greenblatt, 1988). Life-long immunity is known to develop following spontaneous resolution of cutaneous leishmaniasis (Liew, 1992). However, this practice has been discontinued because of the problems associated with virulence of live *L. major*. More recently, it has been shown that BALB/c mice infected with very low dose of *L. major* can contain the infection and develop long-lasting immunity (Bretscher *et al.* 1992). The development of an attenuated *L. major* lacking the dihydrofolate reductase/thymidilate synthetase gene, but which could invade macrophages and persist for several days, has raised hope for the development of a safe attenuated vaccine (Titus *et al.* 1995). The partial immunization of mice against malaria using recombinant *Leishmania* (Wang *et al.* 1995), suggest that *Leishmania* is a potential vector for the delivery of vaccines

against intracellular pathogens. Therefore, immunization with attenuated recombinant *Leishmania* may be an ideal method for inducing protective immunity against leishmaniasis.

G. Chagas' Disease

Chagas' disease is endemic in Latin America where some 18 million people are currently estimated to be infected with its aetiologic agent, *Trypanosoma cruzi* (TDR NEWS, 1996). However, a few cases of *T. cruzi* infection have been diagnosed in Texas and California (Woody and Woody, 1955; Schiffler *et al.*, 1984). Two forms of the disease are recognized: (i) the acute form is characterized by fever, tachycardia, lymphadenopathy, splenomegaly and subcutaneous edema; (ii) the chronic form of the disease which is seen in about 10 to 30 percent of the patients is characterized by a slowly progressive cardiomyopathy which results in congestive heart failure (Hagar and Rahimtoola, 1991). Other abnormalities seen in the chronic form of the disease such as dilation of the oesophagus (megaesophagus) and colon (megacolon) may result in regurgitation, dysphagia, malnutrition and severe constipation (Reed, 1988; Schumis, 1991).

Definitive diagnosis of the acute form of Chagas' disease is based on the demonstration of the parasite in the peripheral blood. However, diagnosis of chronic form of the disease requires highly sensitive immunological assays such as the indirect immunofluorescence (IIF), complement fixation (CF), and indirect hemagglutination test to demonstrate the anti-parasite IgG antibodies (Camargo, 1988; Katz *et al.*, 1989). DNA-based methods such as PCR have also been explored (Moser *et al.*, 1989; Sturm *et al.*, 1989).

H. Current Management of Chagas' Disease

The current management of Chagas' disease has recently been reviewed by Urbina *et al.*, (1996) and the following description is based on their account. Currently, there is no treatment available for the long-term form of Chagas' disease, which is prevalent and leads in 30 to 40 percent of the cases to irreversible cardiac and gastrointestinal lesions. Nitrofurans such as nifurtimox (Bayer, recently discontinued) and nitroimidazoles such as benznidazole (Roche) have been used in short-term cases, but their efficacy varies between geographical areas, probably as a consequence of variation in parasite strains. In addition, both drugs require long term treatment and yet they have very severe side effects including anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy, which are believed to be due to oxidative or reductive damage in the host tissues.

Recently, a new drug that is capable of treating both the short- and long-term Chagas' disease was reported. The drug termed D0870, a bis-triazole derivative, was found to cure 70 to 90 percent of the animals (Urbina *et al.*, 1996). Therefore, D0870 may become available for the treatment of human long-term Chagas' disease, a condition which is currently incurable.

I. Life Cycles of the Causative Agents

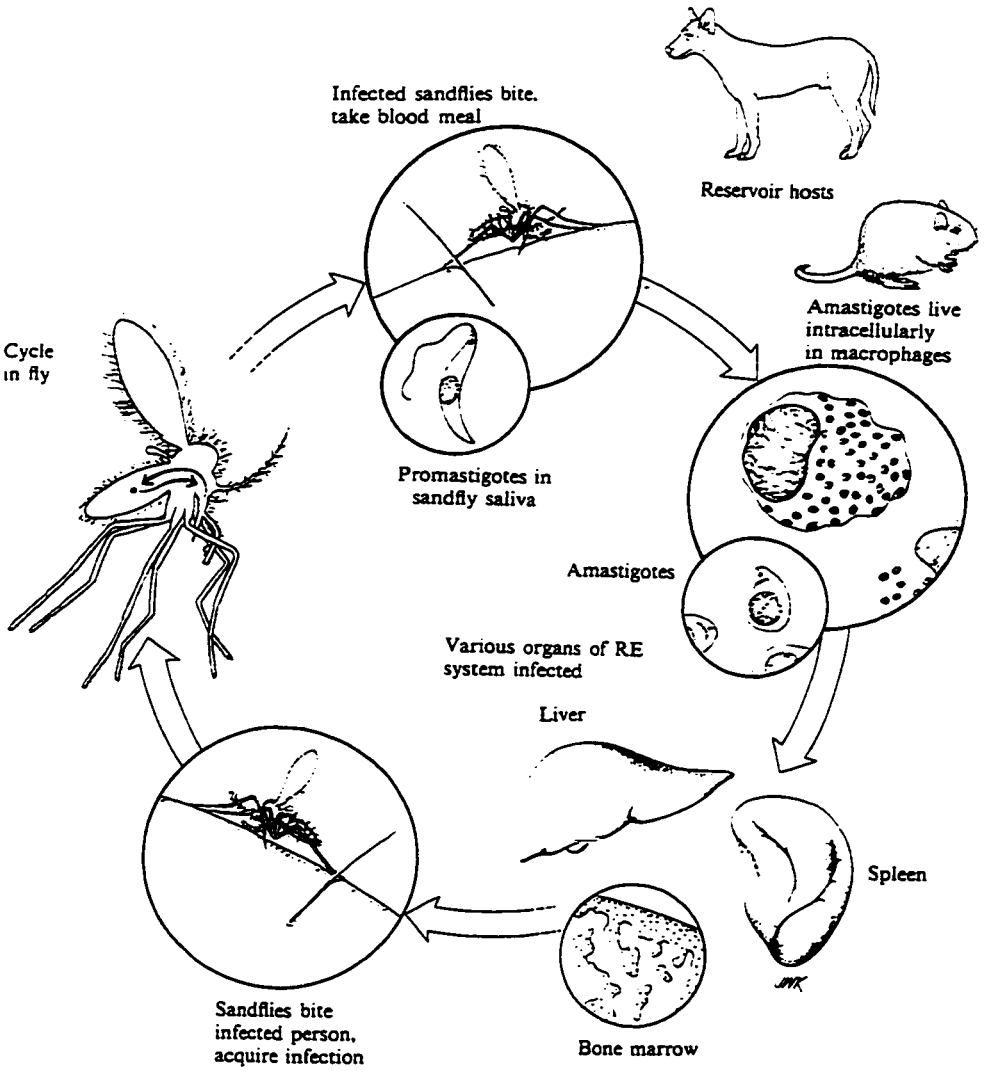
(i). Life Cycle of *Leishmania chagasi* (*L. chagasi*)

L. chagasi and *L. donovani* are the causative agents of visceral leishmaniasis in the New world and Old World respectively. *L. chagasi* and *L. donovani* are transmitted by sandflies of the genera *Lutzomyia* in the New World and *Phlebotomus* in the Old World

respectively. *L. chagasi*, *L. donovani* and *L. infantum* belong to the *L. donovani* complex (Peters and Killicck-Kendrick, 1987). The life cycles of *L. chagasi* and *L. donovani* have been reviewed by Peters and Killick-Kendrick (1987) and Katz *et al.* (1989) and the following description is based on their account.

Human infection begins with a bite of an infected sandfly which inoculates metacyclic promastigotes into subcutaneous tissue where they are taken up by tissue macrophages, without directly entering the blood stream (Fig. 1). In the macrophages, promastigotes establish themselves within the phagolysosomes where they transform into a non-flagellated form called amastigotes. Although lysosomes fuse with the vacuole containing the parasite and release acid hydrolases, *Leishmania* resist destruction by these enzymes. How the *Leishmania* amastigotes escape hydrolysis by lysosomal enzymes is not well understood. The amastigotes multiply within the macrophage eventually filling the cytoplasm. The infected cell dies and releases the amastigotes which go on to re-infect other surrounding macrophages. The life cycle is completed when the sandfly feeds on an infected individual, ingesting amastigotes which go through a number of intermediate forms (haptomonad, paramastigote, nectomonad) before converting to the metacyclic promastigotes found in the proboscis. The process of this transformation is termed metacyclogenesis (Sacks, 1989).

Figure 1. Life Cycle of *Leishmania chagasi* (Adapted from Katz *et al.*, 1989; Permission granted by Dr. Dickson Despommier [one of the authors of Parasitic Disaeses, 2nd edition by M. Katz, D.D. Despommier and R. Gwadz] and John W. Karapelou from Biomedical Illustrations, 3932 Blueberry Hollow Road, Columbus OH 34230. USA)


Infected sandflies bite,
take blood meal
Reservoir hosts
Amastigotes live
intracellularly
in macrophages
Cycle
in fly
Promastigotes in
sandfly saliva
Amastigotes
Various organs of RE
system infected
Liver
Spleen
Sandflies bite
infected person,
acquire infection
Bone marrow

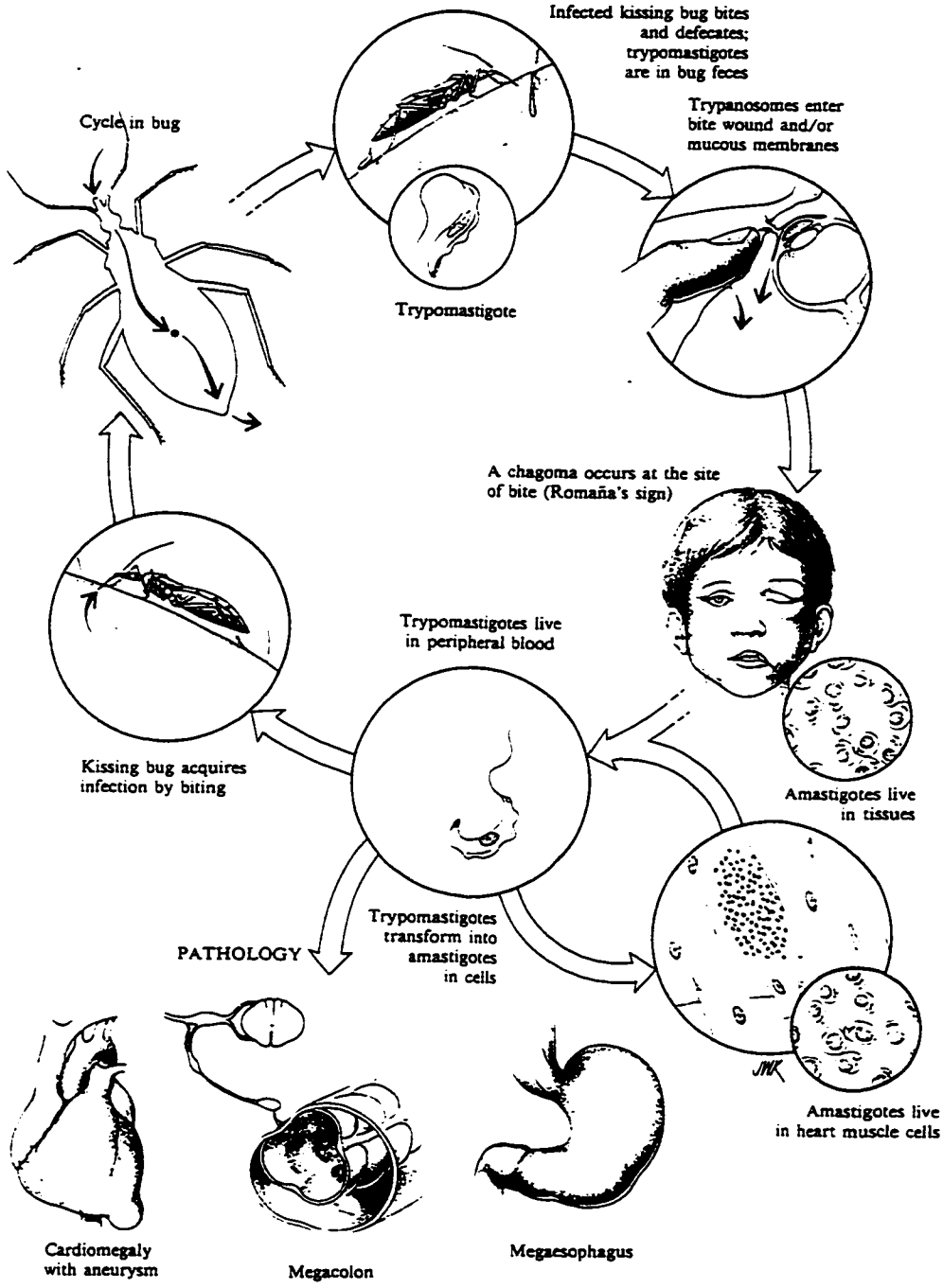
(ii). Life Cycle of *Trypanosoma cruzi* (*T. cruzi*)

The life cycle of *T. cruzi* has been reviewed by Katz *et al.*, (1989) and the following description is based on their account. *T. cruzi* is transmitted by vectors of the order Hemiptera, family Redvidae, subfamily Triatominae which consists of nine genera, the most common of which are *Panastrongylus* and *Rhodnius*. These vectors are commonly referred to as triatomid or kissing bugs and characteristically feed at night on sleeping people. The bug bites, around the mouth and eyes, ingests blood, and defecates immediately near the site of the bite. The bite itself is painless, but subsequently the affected area begins to itch, forcing the victim to rub the bug faeces and thus the trypomastigotes (which are in the bug faeces) into the wound or mucous membranes (Fig. 2).

Once introduced into the bite site or the mucous membrane, the trypomastigotes invade a wide variety of cells and transform themselves within these cells into amastigotes (Fig. 2). The amastigotes divide several times and then transform into trypomastigotes. The infected cells die and release the trypomastigotes, which find their way to the bloodstream and become distributed throughout the body. The trypomastigotes infect many different tissues including glial cells in the central nervous system, the heart muscle, the myenteric plexus in the gut, the urogenital tract, and the reticuloendothelial system. In contrast to the African trypanosomes, *T. cruzi* is essentially an intracellular parasite.

The early lesion of the infection which is usually on the face is known as primary chogma and contains only intracellular amastigotes (Fig. 2). Triatomids acquire infection by feeding on an infected individual and the ingested trypomastigotes migrate to the midgut of the bug where they transform into epimastigotes which divide many times resulting in

Figure 2. Life Cycle of *Trypanosoma cruzi*. (Adapted from Katz *et al.*, 1989; Permission granted by Dr. Dickson Despommier [one of the authors of Parasitic Disaeses, 2nd edition by M. Katz, D.D. Despommier and R. Gwadz] and John W. Karapelou from Biomedical Illustrations, 3932 Blueberry Hollow Road, Columbus OH 34230. USA)


Infected kissing bug bites and defecates; trypomastigotes are in bug feces
Trypanosomes enter bite wound and/or mucous membranes
Cycle in bug
Trypomastigote
A chagoma occurs at the site of bite (Romaña's sign)
Trypomastigotes live in peripheral blood
Kissing bug acquires infection by biting
Amastigotes live in tissues
Trypomastigotes transform into amastigotes in cells
PATHOLOGY
Amastigotes live in heart muscle cells
Cardiomegaly with aneurysm
Megacolon
Megaesophagus

thousands of the organisms. The epimastigotes in the bug transform into metacyclic trypomastigotes and migrate to the hind gut, whence they are excreted with faeces. In addition, the infection can also be transmitted by transfusion or when the faeces of the bug fall onto sleeping people who can become infected by rubbing the trypomastigotes int to the mucous membranes. Transmission can also occur between partners during sexual intercourse and to the foetus across the placenta.

J. *Leishmania* entry into macrophages

The first step in the interaction between promastigotes and macrophages is a receptor-mediated event followed by phagocytosis. Experiments conducted *in vitro* have identified two highly abundant parasite surface molecules capable of mediating attachment to the macrophage: the glycoprotein gp63 and a lipophosphoglycan (LPG) molecule (Russell and Talamus-Rohana, 1989). gp63 binds to the macrophage via the complement receptor CR3 at a site responsible for binding the Arg-Gly-Asp (RGD) sequence (Russell and Wright, 1988; Russell and Talamus-Rohana, 1989). The LPG molecule may also bind to the CR3 receptor at a site distinct from the RGD binding site (Talamas *et al.*, 1990). In addition, LPG has been reported to bind the p150,95 receptor (Russell and Talamus-Rohana, 1989; Talamas *et al.*, 1990). Both CR3 and p150,95 are members of the CD18 family of inter grins and have considerable homology in their α-chains which is thought to be the binding site of LPG (Russell and Talamus-Rohana, 1989). There is also evidence that the gp63 molecule from *Leishmania* is capable of interacting with the macrophage mannose-fucose receptor (Blackwell *et al.*, 1986).

K. Reservoir hosts for *Leishmania*

There are two types of leishmaniasis: (i) zoonotic leishmaniasis, in which the reservoir hosts are wild animals, commensals or domestic animals and (ii) anthropotic leishmaniasis, in which the reservoir is man (WHO, Technical Report Series, 1990). However, there are notable exceptions to such generalizations. For example, cutaneous leishmaniasis caused by *L. tropica* is derived from rodents or dogs and not from human reservoir hosts. On the other hand, although leishmaniasis caused by *L. major* is typically zoonotic, man with chronic lesions may constitute a secondary reservoir host in the absence of rodent reservoir hosts (WHO, Technical Report Series, 1990).

L. Molecular biology of Trypanosomatids

Lack of condensed chromosomes in any phase of mitotic cycle in trypanosomatids has made it difficult to establish a *Leishmania* karyotype by conventional means such as microscopy. However, attempts have been made using pulse field electrophoresis (Van der Ploeg *et al.*, 1984; Scholler *et al.*, 1986) which permits the separation of chromosome sized DNA into distinct bands (Schwartz and Cantor, 1984). Using this technique, it has been shown that the *Leishmania* genome comprises 36 chromosomes which are conserved across widely divergent human pathogenic species (Wincker *et al.*, 1996). The genome size of *Leishmania* has been estimated to vary from 10^7 to 5×10^7 bp in different species (Landfear *et al.* 1983).

Protozoan parasites of the family Trypanosomatidae exhibit a number of novel molecular phenomena (Borst, 1986; Benne, 1985). Among these are the unusual structure

of the cellular mRNAs, which exhibit a bipartite structure in which a common 35 nucleotide extension is found on the 5' end (Walder *et al.*, 1986; Cornelson *et al.*, 1986). This sequence, referred to either as the "mini-exon" (De Lange *et al.*, 1984) or the "spliced leader sequence" (Parsons *et al.*, 1984), is encoded by a separate genetic locus from the body of mRNAs (Nelson *et al.*, 1983; Campell *et al.*, 1984), and is added by a mechanism(s) which is unknown. This is an example of trans-splicing mechanism. The gene encoding the *Leishmania* spliced leader sequence has been cloned and characterized (Miller *et al.*, 1986). There are approximately 150 copies of this gene comprising 0.1% of the parasite genome (Miller *et al.*, 1986). This gene encodes a 85 nucleotide transcript that contains the 35 SL sequence at its 5' end (Miller *et al.*, 1986). This 35 bp sequence and the regions immediately 5' and 3' to it are highly conserved across Trypanosomatids (De Lange *et al.*, 1984; Miller *et al.*, 1986).

Although the promoter has not been identified in trypanosomatids a number of recent reports have shown the significance of the 3' untranslated region (UTR) and intergenic regions in regulating gene expression on trypanosomatids (Jefferies *et al.*, 1991; Ramamoorthy *et al.*, 1992; Teixeira *et al.*, 1995; Charest *et al.*, 1996). Since signals for trans-splicing and polyadenylation are contained in these regions, the mRNA abundance could potentially be regulated at one or both of these steps. Alternatively, the 3' UTR could contain binding sites for proteins that influence the stability of mRNA.

M. The state of knowledge of cysteine protease

Cysteine protease is a group of proteolytic enzymes whose cysteine or thiol groups are involved in the cleavage of substrate. These enzymes have been described in many pathogenic organisms, such as bacteria (Morihara, 1974), viruses (Bazan and Fletterick, 1988), fungi (Apodaca and McKerrow, 1989), and parasites (Robertson and Coombs, 1994; North *et al.*, 1990; Eakin *et al.*, 1990; North and Coombs, 1981; Coombs, 1982; Purpkis and Coombs, 1984; Pupkis *et al.*, 1984; Mottram *et al.*, 1992; Souza, *et al.*, 1992; Traubo-Cseko *et al.*, 1993; Pamer *et al.*, 1989; Campetella *et al.*, 1990; Lockwood *et al.*, 1987). While there are structural variations among the cysteine proteases of different phyla, all are endopeptidases that range in molecular weight from 15,000 - 80,000 daltons and have a critical cysteine residue at the active site involved in the hydrolysis of the substrate (Apodaca and McKerrow, 1989). The overall structure of cysteine protease consists of Pre, Pro, protease core and c-terminal (Fig. 3).

Although the exact roles of cysteine proteases in *Leishmania* pathogenesis have yet to be defined (McKerrow, 1984), these enzymes appear to be crucially important for the survival of both the promastigote and amastigote forms of these parasites. Evidence in support of this view first came from studies which show that inhibitors of cysteine proteases arrest the growth of the amastigote within macrophages (Coombs *et al.*, 1982; Coombs and Baxter, 1984) but these reports were controversial because these inhibitors are also toxic to macrophages. The controversies have been resolved by evidence which show that two fluoromethyl ketone-derivatized peptide inhibitors of cysteine proteases are effective in killing both the promastigote and amastigote forms of the parasites but not the host cells

Figure 3. The overall structure of cysteine protease


PRE
PRO
PROTEASE CORE
C-TERM

(McKerrow, 1993). The effectiveness of these inhibitors in killing the parasites have led some investigators to propose that cysteine proteases are potential chemotherapeutic targets (McKerrow, 1993; Robertson and Coombs, 1994; North *et al.*, 1990). Recently, evidence from the disruption of *Leishmania mexicana* cysteine protease gene array (lmcpb) suggest these enzymes are virulence factors (Mottram *et al.*, 1996).

All the cysteine protease genes which have been cloned from both *Leishmania* and *T. cruzi* appear to be developmentally regulated (Mottram *et al.*, 1992; Souza *et al.*, 1992; Traubo-Cseko *et al.*, 1993; Pamer *et al.*, 1989; Campetella *et al.*, 1990). However, prior to our work, efforts to detect cysteine proteases from the *L. donovani* and *L. major* using gelatin SDS-PAGE have been unsuccessful (Coombs, 1982; Pupkis *et al.*, 1986).

N. Possible Roles of Cysteine Protease in the Pathogenesis of *Leishmania*

The pathogenesis of leishmaniasis is influenced by the $CD4^+$ T_H cell subset that dominates shortly after infection. A dominant $CD4^+T_H2$ response favours parasite multiplication resulting in uncontrolled lesion development, metastasis of the parasite and eventual death of the patient, whereas a dominant $CD4^+$ T_H1 response controls parasite multiplication resulting in healing. Recent *in vitro* and *in vivo* studies strongly suggest that the presence of IL-12 during the primary stimulation of $CD4^+$ T cells is required for T_H1 cell development (Hsieh *et al.*, 1993; Manetti *et al.*, 1993; Heinzel *et al.*, 1993; Sypek *et al.*, 1993). There is also compelling evidence from both *in vivo* and *in vitro* studies that the presence of IL-4 during the primary stimulation of $CD4^+$T cells is required for T_H2 cell development (Swain *et al.*, 1990; Heish *et al.*, 1992; Seder *et al.*, 1992; Chatelain *et al.*, 1992;

Heinzel *et al.*, 1989). The recent report that $CD4^+$T cells from naive IL-$4^{-/-}$ mice failed to produce T_H2-derived cytokines after *in vitro* stimulation (Kopf *et al.*, 1993), confirms that IL-4 is required for the generation of T_H2-derived cytokines. However, how the initial IL-4 is generated is not very well understood (Paul and Seder, 1994). A useful clue comes from the report of Killar *et al.* (1989) that injection of recombinant IL-1 into mice induces the expression of mRNA for IL-4, IL-2 and IFN-γ. This report suggests that the expression of IL-4 is stimulated by IL-1. This is supported by the observation that macrophages from genetically susceptible mice secrete large amounts of IL-1 in response to infection with *L. major*, while those from resistant mice produce significantly lower levels of IL-1 (Cillari *et al.*, 1989). The large amounts of IL-1 secreted by macrophages infected with *L. major* may have been generated by cysteine protease from *Leishmania* since it has been demonstrated that cysteine protease from several sources cleaves IL-1β precursor to mature IL-1β (Thornberry *et al.* 1992; Kapur *et al.*, 1993). Another useful clue comes from the reports of Urban *et al.*, (1992) and Finkelman and Urban (1992) that injection of mice in the footpad with cysteine protease stimulates an increase in IL-4 gene expression in the drainage lymph node 1 hour later. These reports suggest that cysteine protease up-regulates IL-4 gene expression. There are also reports that macrophages infected with *L. amazonensis* and *L. brazilliensis* secrete large amounts of biologically active TGF-β which promotes the replications of amastigotes (Barral-Netto *et al.* 1992; Barral *et al.* 1993). As described by Barral-Netto *et al.* 1992, TGF-β is a 24 kDa protein produced by many cells including B and T lymphocytes and activated macrophages. TGF-β is secreted as a latent precursor that requires enzymatic cleavage of the carbohydrate groups or transient acidification to release

the active cytokine. Although the enzyme which cleaves the precursor TGF-β is yet to be identified, there is a growing suspicion that *Leishmania* utilizes cysteine protease to cleave preTGF-β into active TGF-β which then enables *Leishmania* to survive within macrophages. This suspicion stemmed from the recent observation which revealed the involvement of cysteine protease in the cleavage of IL-1β precursor to mature IL-1β (Thornberry *et al.* 1992). In addition, it has recently been demonstrated that CHO cells transfected with pre-TGF-β is processed into active TGF-β by *T. cruzi* cysteine protease (Reed, 1995). As suggested by Barral-Netto *et al.* (1992), it is possible that the increased production of active TGF-β during *Leishmania* infection constitutes the principal mechanism of survival of the parasite within macrophages. This is understandable because TGF-β is known to play a role in the survival of *Leishmania* in the macrophages (discussed below and summerized in Fig. 4).

The recent observation that *Leishmania* promastigotes evade IL-12 induction by macrophages but stimulate the induction of mRNAs for IL-2, IL-4, IL-10 and IL-13 in $CD4^+$ T cells (Reiner *et al.*, 1994), suggest that it is the *Leishmania* parasite itself which drives the $CD4^+$ precursors towards the $CD4^+T_H2$ phenotype. Indeed, it is known that IL-3, IL-4, IL-10 and TGF-β inhibit the expression of nitric oxide synthase (NOS) which macrophages use to kill *Leishmania* while migration inhibition factor (MIF), IFN-γ, TNF-α induce the expression of NOS (Liew and O'Donnell, 1993). In addition, there is a report which suggests that *Leishmania* promastigotes utilize lipophosphoglycan surface antigen (LPG) to inhibit the induction of IL-12 at the onset of the infection (Reiner *et al.*, 1994). This stage-specific evasion of IL-12 induction delays the development of T_H1 cells to allow transformation of

promastigotes to amastigotes which express high-activity cysteine protease abundantly. Since cysteine protease has been reported to up-regulate the expression of IL-4 gene expression (Urban *et al.*, 1992), it is possible that the striking burst in IL-4 transcription which occurs in the $CD4^+$ T cells at the onset of *Leishmania* infection (Reiner *et al.*, 1994) is brought about by cysteine protease from *Leishmania*.

In addition, studies carried out in trypanotolerant and trypanosusceptible cattle reveal that trypanotolerant cattle mount a strong immune response to cysteine protease from *Trypanosoma congolense* while trypanosusceptible cattle do not (Authie, 1994). It appears that the primary function of anti-cysteine protease antibodies produced by trypanotolerant cattle is to inhibit the enzyme activity of cysteine protease. This report strongly suggests that trypanotolerant cattle control trypanosomiasis by neutralizing the cysteine protease from the trypanosomes. If this analysis is true, then this report implies that cysteine protease is a virulent factor.

O. Possible Role of Cysteine Protease in the Pathogenesis of *T. cruzi*

Recent evidence suggest that cysteine protease may be important in the replication of *T. cruzi*, since two fluoromethyl ketone-derivatized cysteine protease inhibitors arrest *T. cruzi* replication (Hart *et al.*, 1993; McKerrow, 1993). The efficacy of these inhibitors have led some investigators to propose that cysteine protease may be a target for chemotherapy (McKerrow, 1993).

P. Macrophage Activation and Parasite Killing

One of the most effective immune responses involves macrophage killing of either intracellular or extracellular pathogens. The microbicidal activity of macrophage is mediated by several distinct mechanisms. (i) Innate microbicidal activity occurring when phagosomes containing ingested organisms fuse with lysosomes containing proteases. (ii) Respiratory burst leading to production of toxic oxygen metabolites such as superoxide anion and hydrogen peroxide. These mechanisms kill many non pathogenic microorganisms and was assumed for a long time to be involved in the killing of intracellular parasites (Hugies, 1988). This changed when it was shown that macrophage cell line (Ic-21) deficient in respiratory burst was as efficient at killing *Leishmania* amastigotes and promastigotes as normal peritoneal macrophages when activated by lymphokines (Scott *et al.*, 1985). Before this report, it was already known that in order to eliminate protozoans like *Toxoplasma*, *Leishmania* and *T. cruzi*, macrophages must be activated by exposure to cytokines (Borges and Johnson, 1975; Noguiera and Cohn, 1978; Nacy *et al.*, 1981). The key observation was that incubation of macrophages with supernatants derived from T cells led to marked inhibition of replication and/or killing of each of these intracellular parasites (Borges and Johnson, 1975; Noguiera and Cohn, 1978; Nacy *et al.*, 1981). Similar observations were later reported in malaria (Ockenhouse *et al.*, 1984) and schistosomula of *Schistosoma mansoni* (Mahmoud *et al.*, 1979; Pearce and James, 1986). Finally, in *Leishmania*, activated macrophages do not only kill these parasites intracellularly, but also inhibit infection (Davis *et al.*, 1988). The mechanisms by which activated macrophages resist infection by *Leishmania* is unknown (Nacy and Meltzer, 1991).

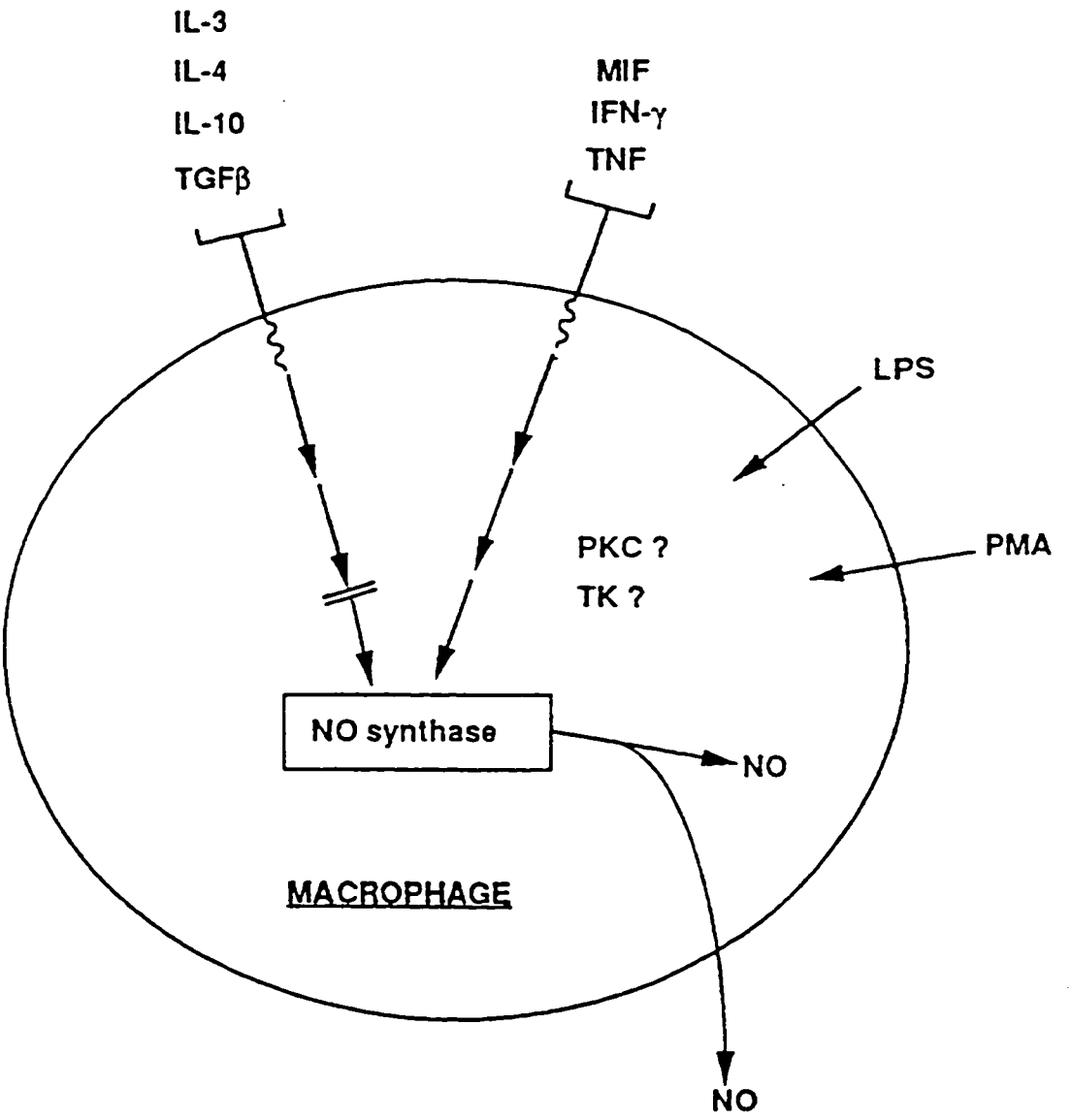
The activation of macrophages requires two signals (Nacy and Meltzer, 1991). The first signal primes the macrophage, while the second signal triggers activation. IFN-γ is the primary priming signal while TNF-α is the second signal that triggers activation. Thus, the current model of macrophage mediated killing of Leishmania postulates that IFN-γ primes macrophages, and the infection by the parasite itself induces the macrophage to produce the TNF-α required to trigger the activation (Green, Crawford *et al.*, 1990).

Macrophage mediated killing of intracellular parasites is now believed to be due to reactive nitrogen intermediates (Nacy and Meltzer, 1991; Liew and Cox, 1991; James, 1991; Green *et al.*, 1990). Nitric oxide is derived from a reaction with the terminal guanidino nitrogen of the amino acid L-arginine and molecular oxygen, resulting in the formation of L-citrulline and NO. This reaction is catalysed by the enzyme nitric oxide synthase (NOS). The resulting NO is very unstable, having a half-life of 3 - 5 seconds. However, NO reacts with itself, water and oxygen to generate nitrogen dioxide (NO_2) and finally a stable nitrite (NO_2^-) and nitrate (NO_3^-). The products generated are collectively known as reactive nitrogen intermediates (RNI). This hypothesis is supported by several experimental results which show that ablation of nitric oxide production by addition of arginine analogues inhibits activated macrophage killing of *Leishmania*, *Toxoplasma*, *T. cruzi* and *S. mansoni* (Green, Crawford *et al.*, 1990; Green, Meltzer *et al.*, 1990; Liew *et al.*, 1990; Liew and Millott, 1990; Adams *et al.*, 1990, James and Glaven, 1989; Gazzinelli *et al.*, 1992). The most dramatic result was presented by Liew *et al.*, (1990). When these investigators treated CBA mice infected with *L. major* with arginine analog for 3 weeks, these mice exhibited a dramatic increase in the numbers of *Leishmania* parasites compared with controls. These results have been confirmed

recently using mice lacking inducible nitric oxide synthase (iNOS) (Wei *et al.*, 1995). The result of this study showed that in contrast to the wild-type and heterozygous mice which are highly resistant to the protozoa parasite *Leishmania major* infection, iNOS mutant mice are uniformly susceptible. In one malaria experiment, the ability of IFN-γ to inhibit intrahepatocyte *P. berghei* development was also abrogated when an arginine analog was included in the medium (Mellouk, *et al.*, 1991). Taken together, these results demonstrate that nitric oxide is involved in the killing of the parasites. However, the mechanism by which nitric oxide kills either intracellular or extracellular parasite is unknown.

The mechanism by which macrophages are regulated by cytokines in the production of NO is summarized in Fig. 4. IFN-γ, TNF-α and migration inhibition factor (MIF) occupying their respective receptors on macrophage, send a series of signals which together with those of co-simulators (eg LPS and other bacterial or parasite components), lead to the induction of NOS (Liew and O'Donnel, 1993). Other cytokines, such as IL-3, IL-4, TGF-β and IL-10 also send a series of signals which act in the opposite direction, inhibiting the expression of NOS (Liew and O'Donnel, 1993).

Figure 4. The regulation of nitric oxide (NO) synthesis in macrophages by two opposing sets of cytokines; IFN-γ, interferon γ; Il, interleukin; LPS, lipopolysaccharide; MIF, migration inhibitory factor; PMA, phorbol myristate acetate; PKC, protein kinase C; TGFβ, transforming growth factor β. (Adapted from Liew and O'Donnel, 1993; Permission granted by Academic Press Limited, London, U.K.).

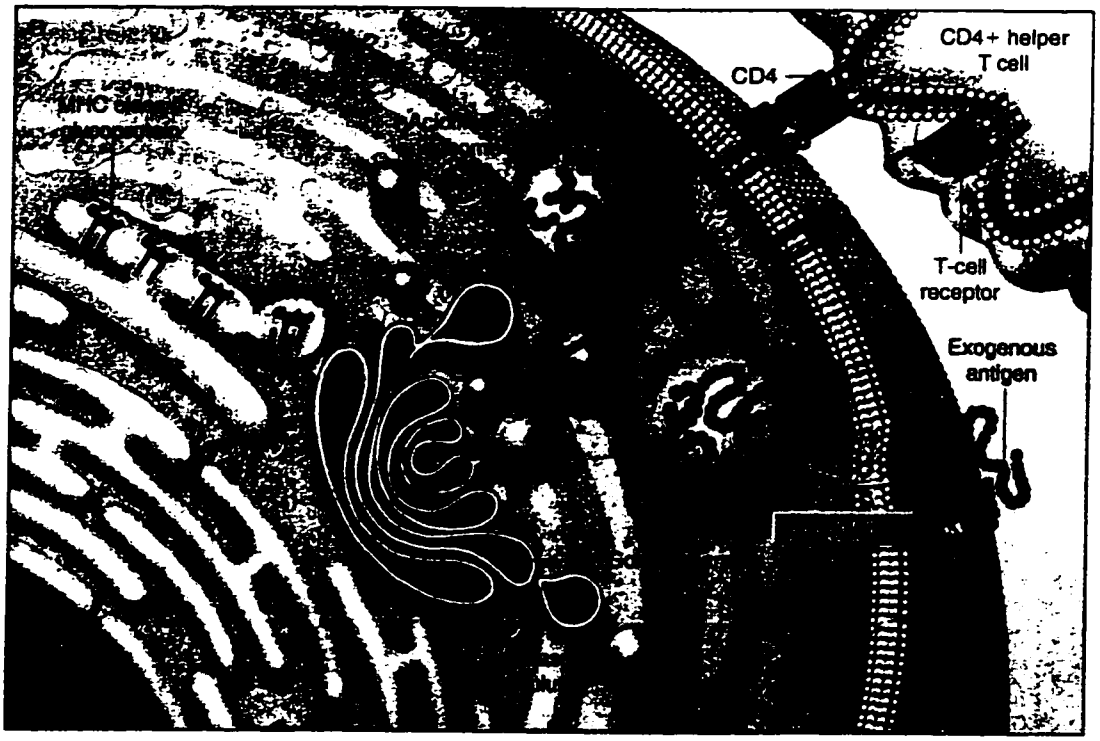

IL-3
IL-4
IL-10
TGFβ
MIF
IFN-γ
TNF
LPS
PKC ?
TK ?
PMA
NO synthase
NO
MACROPHAGE
NO

Q. Antigen Processing and Presentation

Numerous studies have identified two main pathways of antigen processing and presentation involving the products of the MHCI or MHCII (reviewed by Germain, 1994; Germain and Margulies, 1994). Antigens produced in the cytosol of the antigen presenting cell (APC) are cleaved into short peptides (composing 8 to 10 amino acids) and enter the endoplasmic reticulum via transport-associated proteins (TAP1 and TAP2) and bind to MHC class I molecules. After binding, the complex is transported through the Golgi apparatus to the cell surface, where it can be recognized by cytotoxic T cells ($CD8^+$) (Fig. 5; Monacco, 1995; Elliott *et al.*, 1995; Heemeles and Ploegh, 1994; van Endert *et al.*, 1994; Hill and Ploegh, 1992; McDonnell and Askari, 1996). The same antigen as an extracellular soluble protein will enter APC either by endocytosis or phagocytosis. MHC class II molecules in the endoplasmic reticulum pass through the Golgi apparatus and enter acidified endosomes in which the foreign protein has been fragmented into peptides. The class II MHC-peptide complex is then brought to the cell surface, where it can be recognized by helper T cells ($CD4^+$) (Fig. 5; McDonnell and Askari, 1996; Barnes and Mitchel, 1995; Qui, *et al.*, 1995; Cresswell, 1994; Harding *et al.*, 1990).

Figure 5. Presentation of Antigen to T cells through the MHC Class I and Class II. Panel A shows the endogenous pathway. Panel B shows the exogenous pathway. (Adapted from McDonnell and Askari, 1996; Permission granted by the Massachusetts Medical Society).


CD8+ cytotoxic
T cell
CD8
T-cell receptor
DNA


CD4+ helper
T cell
CD4
T-cell
receptor
Exogenous
antigen

R. DNA Immunization

Direct DNA injection technology was first reported by Ulmer *et al.*, (1993). A plasmid DNA construct, encoding the nucleoprotein of influenza A virus was injected directly into the quadriceps muscles of mice. This vaccine protected 90% of the mice. The nucleic acids are known to be taken up and expressed by host cells and the corresponding protein is made inside the cell (Waine and McManus, 1995; McDonnell and Askari, 1996). An important advantage of this system is that the protein enters the cell's major histocompatibility complex (MHC) class I pathway (Fig. 6; McDonnell and Askari, 1996). As already described above under antigen presentation, only proteins that originate inside a cell are processed by this pathway. MHC class I molecules carry peptide fragments of the protein to the cell surface, where, by stimulating $CD8^+$ cytotoxic T cells, they trigger cell-mediated immunity (Fig. 6; McDonnell and Askari, 1996; Ulmer *et al.*, 1996). In fact, immune responses include not only the production of cytotoxic T lymphocytes (CTL), but also antibody induction and T-cell activation with cytokine secretion (Ulmer *et al.*, 1996).

Figure 6. Molecular Route of DNA Vaccine.

Naked DNA vaccines are plasmids that contain a gene from the target pathogen, in our case it is the gene encoding cysteine protease from *Leishmania chagasi*. Gene transcriptions are regulated by a cytomegalovirus promoter element in the plasmid. The DNA plasmid enters the cell and the nucleus, where the gene is transcribed into messenger RNA (mRNA). Cysteine protease mRNA is translated into protein by ribosomes in the rough endoplasmic reticulum (not shown). In the cytosol the protein is cleaved by proteasomes, and the short peptides (composing 8 to 10 amino acids) are transported into the endoplasmic reticulum, where they bind to MHC molecules. The complex is transported to the cell surface, where binding to cytotoxic T cells and stimulation of cell-mediated immunity occur. (Adapted from McDonnell and Askari, 1996. Permission granted by the Massachusetts Medical Society).


Plasmid DNA Vector
Viral promoter
Viral nucleoprotein gene
Translation
Transcription
CD8
CD8+ cytotoxic T cell
T-cell receptor

HYPOTHESES AND OBJECTIVES

HYPOTHESES

The hypotheses of this study were:

(1) Cysteine proteases are virulent factors in the pathogenesis of leishmaniasis.

(2) Cysteine proteases are immunogens which induce protective immunity against leishmaniasis.

OBJECTIVES

The objectives of this study were:

(1) To isolate and characterize cysteine protease cDNAs from *L. chagasi* and *T. cruzi*.

(2) To compare the virulence of wild type *L. major* and those transfected with constructs having cysteine protease cDNAs.

(3) To determine the potential use of transfected *L. major* as a vaccine.

MATERIALS AND METHODS

MATERIALS AND METHODS

A. Composition of Commonly Used Buffers

1. Lysis buffer for the isolation of genomic DNA

10 mM Tris-HCl [pH 8.3], 50 mM EDTA [pH 8.0], and 1% SDS.

2. TELT lysis buffer for the isolation of genomic DNA

50 mM Tris-HCl (pH 8.0), 62.5 mM EDTA (pH 8.0), 2.5 M LiCl, 4% Triton X-100.

3. PCI

Phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v).

4. TE

10 mM Tris-HCl (pH 8.0), 1 mm EDTA (pH 8.0).

5. DNA loading dye

0.25% bromophenol blue, 0.25% xylene cyanol (optional), 15% Ficoll (type 400; Pharmacia).

6. 10 X PCR buffer

100 mM Tris-HCl (pH8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin.

7. 10 X Ligase buffer

660 mM Tris- HCl (pH 7.6), 66 mM $MgCl_2$, 10 mM DTT, 660 mM ATP, 500 mg/ml

8. Denaturing solution

1.5 M NaCl, O.5 M NaOH.

9. Neutralization buffer

0.5 M Tris-HCl pH 7.2, 1.5 M NaCl, 0.001 M EDTA.

10. 1 X SSC

150 mM NaCl, 15 mM sodium citrate adjusted to pH 7.0 with NaOH.

11. Denhardt's solution

0.02% BSA, 0.02% polyvinyl pyrrolidine, 0.02% ficoll.

12. Solution D for RNA isolation

4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 100 mM β-mercaptoethanol

13. Formaldehyde (RNA) loading dye

50% glycerol, o.25% bromophenol blue, o.25% xylene cyanol, 1 mM EDTA.

14. Acrylamide Solution for SDS-PAGE

Acrylamide/Bis (30% T, 2.6% C).

15. Running buffer (for separating gel) for SDS-PAGE

1.5 M Tris-HCl, pH 8.8. Adjust to pH 8.8 with 10 N HCl.

16. Stacking buffer for SDS-PAGE

0.5 M Tris-HCl, pH 6.8. Adjust to pH 6.8 with 10 N HCl.

17. 5 X Sample buffer (SDS reducing buffer SDS-PAGE; BIORAD)

62.5 mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS, 5% β-mercaptoethanol.

18. 1 X MTPBS

150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 (pH 7.3)

19. 1 X PBS

10 mM KH_2PO_4, 2 mM NaH_2PO_4, 140 mM NaCl, 40 mM KCl (pH 7.4)

20. LB broth

1% tryptone, 0.5% yeast extract, 1% NaCl. ph 7.5 with NaOH

21. NZY broth (per liter)

5 g NaCl, 2 g $MgSO_4$,.H_2O, 5 g yeast extract, 10 g casein hydrolysate; pH adjusted to 7.5 with NaOH.

22. SM buffer

100 mM NaCl, 5 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5), 0.01% gelatin.

23. *Leishmania* electroporation buffer

137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, 21 mM HEPES (pH 7.5).

24. Colony lysis buffer

1% Triton X-100, 20 mM Tris-Hcl (pH 8.5), 2 mM EDTA.

25. 1 X TAE

40 mM Tris-acetate, 1 mM EDTA.

26. 1 X TBE

89 mM Tris-borate, 89 mM boric acid, 1 mM EDTA.

27. 20% Acrylamide mix (per 500 ml) for sequencing

96.5 g acrylamide, 3.35 g N,N'-methylene bisacrylamide, 233.5 g urea in TBE.

28. Urea mix (per 500 ml)

233.5 g urea in 1 X TBE

B. Growth and isolation of parasites

A Brazilian isolate of *Leishmania donovani chagasi* was kindly provided by R. Pearson, University of Virginia, Charlottesville, VA. Promastigotes from this isolate were cultured at 26°C in a modified minimal essential medium (HOMEM) (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal calf serum and hemin (GIBCO Laboratories). The parasites were harvested in the logarithmic (log) or stationary phase of growth. A Sudanese strain of *L. donovani donovani* (LD3) was kindly provided by T. Pearson, University of Victoria, Victoria, B.C., Canada. Promastigotes from this species of *Leishmania* were cultured at 26°C in Schneider's Drosophila medium (GIBCO Laboratories) supplemented with 20% heat-inactivated fetal calf serum (GIBCO Laboratories). To maintain infectivity, hamsters were infected with the parasites by intracardiac inoculation at a rate of 1×10^7 promastigotes per animal. The animals were sacrificed 4 weeks post infection and the amastigotes recovered from the spleens as described by others previously (Pearson and Steigbigel, 1980) Amastigotes convert to promastigotes when cultured at 26°C in HOMEM medium with hemin and heat-inactivated foetal calf serum (Pearson and Steigbigel, 1980).

C. Isolation of Nucleic Acids

(i). Isolation of parasite genomic DNA

Genomic DNA was prepared from early stationary phase promastigotes (6 day culture). A pellet of about 10^{10} promastigotes was lysed in 10 ml of lysis buffer (10 mM Tris-HCl [pH 8.3], 50 mM EDTA [pH 8.0], and 1% SDS). The lysate was incubated for 1 hour at 37°C with 1 mg of RNase A then overnight at 42°C with 100 µg/ml of proteinase K (both

RNase A and Proteinase K were from Boehringer Mannheim). The DNA was then extracted four times: first with phenol equilibrated with Tris-HCl [pH 8.0], second with phenol/chloroform/isoamyl and lastly twice with chloroform/isoamyl (24:1). DNA was then precipitated out using cold 95% ethanol and immediately spooled out, washed with 70% ethanol, dried and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). Genomic DNA from *T. cuzi* was a gift from Dr. Steven G. Reed (Infectious Diseases Research Institute, Seattle, WA 98109).

(ii). Total RNA Isolation

Total RNA was isolated from promastigotes in logarithmic and stationary growth phases using the guanidium-phenol-chloroform method as described by Chomczynski and Sacchi (1987) with some modifications. Briefly, cells from a 50 ml culture were harvested by centrifugation at 800 x g. The cells were washed twice with sterile PBS and lysed in 600 µl of solution D. After that 1 vol of phenol (water saturated), 0.1 vol of 2 M sodium acetate (pH 4.0), and 0.2 vol of chloroform-isoamyl alcohol (49:1) were sequentially added with thorough mixing after addition of each reagent. The mixture was kept on ice for 10 min and centrifuged at full speed in a microcentrifuge for 15 min at room temperature. The upper phase was transferred to a new tube and mixed with an equal vol of isopropanol and kept at -20°C for 1 hr or overnight. After centrifugation at 4 C for 10 min the pellet was resuspended in 500 µl of solution D and RNA was precipitated with 1 vol of isopropanol in -20°C for 1 hr or overnight. RNA was again pelleted by centrifugation followed by washing with 70% ethanol, dried and resuspended in appropriate vol of DEPC-treated water. Total

RNA from infected and non-infected spleens of hamsters was a gift from Dr. Mary E. Wilson (Departments of Medicine and Microbiology, University of Iowa). Total RNA from epimastigote stage of *T. cuzi* was a gift from Dr. Steven G. Reed (Infectious Diseases Research Institute, Seattle, WA 98109).

(iii). Plasmid Miniprep From *E. coli*

Plasmid DNA was isolated from *E. coli* using the alkaline lysis method described by Sambrook *et al.* (1989). Briefly, five ml of LB broth with appropriate antibiotics was inoculated with a single colony of *E. coli* and grown for 16-18 hours at 37°C with shaking at 300 rpm. Overnight cultures were centrifuged at 3000 rpm for 10 min and the pellet resuspended in 200 µl of SET buffer. After adding 5 µl (10 mg/ml) of Rnase solution, the cells were vortexed to homogeneity. Following complete resuspension of the cells, 350 µl of lytic buffer (1% SDS, 0.2 M NaOH) was added and the tube was inverted several times before placing on ice for 5 min. After that 250 µl of 1.5 M potassium acetate (pH 4.8) was added and the tube inverted several times before placing on ice for 20 min. The sample was centrifuged at full speed for 10 min and the supernatant transferred to clean tube. The supernatant was extracted once with 800 µl of phenol:chloroform (1:1) and plasmid was precipitated with 800 µl of isopropanol and centrifugation at full speed for 5 min. The pellet was washed with 70% ethanol, dried and resuspended in 20 µl of TE buffer.

D. Cloning of DNA Fragments

(i). PCR amplification of cysteine protease DNA Fragment from genomic DNA

Two oligonucleotide primers used to amplify the cysteine protease gene fragments were based on conserved regions from closely related organisms as reported by Eakin *et al.*, (1990). The sequences of the primers are:

Sense Primer (TCCP-1):

5' ACAGAATTCCAGGGCCAGTGCGGCTCGTGCTGG 3'.

The underline indicates *Eco*RI restriction site.

Antisense Primer (TCCP-2):

5' CGCAAGCTTCCACGAGTTCTTCACGATCCAGTA 3'.

The underline indicates *Hin*dIII restriction site. The conditions for the PCR were: 100 pmoles of each the two primers together with 100 ng of genomic DNA as template were used in 100 µl reaction mixture containing 10 mM Tris-HCl, [pH 8.3], 50 mm KCl, 1.5 mM MgC_2, 200 mM dNTPS, 2.5 units of recombinant Taq DNA polymerase (Pharmacia, Sweden). Reactions were performed using the Techne PHC-2 Dri-Block Cycler (Cambridge, UK). After 5 minutes of template denaturation at 94°C, the reactions mixture was subjected to 30 cycles of 94°C for 1 min, 42°C for 2 min and 72°C for 2 min followed by an extension at 72°C for 10 min.

(ii). LMP Purification of DNA Fragments

DNA fragments generated by either PCR amplification or restriction enzyme digestion were purified in LMP agarose (Dr. J. Wisniewski, personal communication). Briefly, after

electrophoresis, LMP part containing DNA fragments were excised using a clean surgical blade. One tenth of vol of 3 M sodium acetate (pH 5.2) was added and the agarose containing DNA was melted at 68°C for 10-15 minutes. The melted agarose was mixed with an equal vol of phenol (Tris-HCl pH 8.0 saturated) and vortexed briefly. The mixture was kept on ice for 15 min and centrifuged for 15 min at full speed and at room temperature. The upper phase containing DNA was transferred into new tubes, mixed 2 vol of absolute ethanol and put at -20°C Freezer for one hour or overnight. Samples were centrifuged for 30 min at 4°C washed with 70% ethanol and resuspended in appropriate vol of sterile water.

(iii). Ligation of DNA Fragment into Plasmid Vectors

Prior to the ligation, PCR products of expected length were digested with appropriate restriction enzymes to completion. The digested plasmid vectors were dephosphorylated using CIAP and linearized plasmids were purified through LMP agarose.

A typical ligation reaction was carried out by mixing 150 ng dephosphorylated vector DNA, 5 fold molar excess of insert and 8 units of T_4 DNA ligase and 1 x ligation buffer. The ligation mixture was incubated at 12°C overnight.

To determine the amount of insert needed for a ligation reaction using 5:1 molar ratio of insert:vector and 150 ng of vector DNA the following formula was used:

$$\text{Insert in ng} = \frac{5 \times 150 \times \text{insert}}{\text{Vector size}}$$

(iv). Transformation

In most experiments, *E. coli* K12 strain DH5α was used for transformation. When pCR II vector (TA cloning kit, Invitrogen, CA) was used, competent *E. coli* strain INVα supplied by the manufacturer was used. Both chemical method and electroporation were used to transform *E. coli*. When chemical method was used, fresh competent cells were prepared by $CaCl_2$ method described by Hanahan (1983) with some modifications. Briefly, a single colony of DH5α was inoculated into 5 ml broth without antibiotic and grown overnight in shaking incubator at 37°C. Two hundred microliter of saturated overnight culture was used to inoculate 30 ml of LB broth without antibiotic and grown for 2-3 hours. Cells were harvested by centrifugation at 4°C, the pellet resuspended in 10 ml of ice-cold 50 mM $CaCl_2$ and kept on ice for 30 min. After that, the cells were centrifuged again and the pellet resuspended in 1 ml of ice-cold 50 mM $CaCl_2$ and kept on ice for 1 hr. Competent cells were used immediately in aliquots of 100 µl. When electroporation was used, the competent DH5α cells were prepared as described (Miller and Nickoloff, 1995) with some modifications. Briefly, 500 ml flask of LB broth without antibiotics was inoculated with 25 ml of a fresh overnight culture of DH5α cells and grown at 37°C with vigorous shaking to an OD_{600} of 0.4 (mid log phase). To harvest, the flask was chilled on ice for 20 min and centrifuged at 4°C, 3000g for 10 min. After discarding the medium, each pellet was resuspended in 250 ml ice-cold sterile water and centrifuged at 4°C, 3000g for 10 min. The washing with ice-cold sterile water was performed twice. The cells were then resuspended in 20 ml of 10% glycerol and centrifuged at 4°C, 3000g for 5 min. After that pellet was resuspended to a final volume of 1.5 ml in 10% glycerol. The competent cells were dispensed in 40 µl aliquots and either

used immediately or flash-frozen in an ethanol/dry ice and stored at -80°C. The cells are good for at least 6 months under these conditions.

To use competent cells prepared for chemical transformation, the ligation reaction of 20 µl was diluted to 100 µl using distilled water. Fifty microliter of the diluted ligation mixture was added to 100 µl of competent cells and kept on ice for 10 min. This was incubated at 45°C for exactly 2 min followed by another incubation of 10 min at room temperature. After that, 1 ml of LB broth was added to the competent cells having the ligation reaction mixture and then incubated for 1 hr in a 37°C shaking incubator. The cells were pelleted by a quick spin and 800 µl of the supernatant discarded. The cells were resuspended in the remaining LB media and spread over LB plates containing 100 µg/ml of ampicillin or in the case of PCR vector of 50 µg/ml of kanamycin, and incubated at 37°C. Resistant colonies which grew on the plates were patched into a new LB plate and incubated at 37°C. Plasmid DNA was isolated using the alkaline lysis method described by Sambrook *et al.*, (1989) and subjected to restriction analysis.

To use electroporation, the 40 µl aliquot of competent cells was mixed with 2 µl of a 20 µl ligation reaction mixture and placed in cold 0.2 cm electroporation cuvette. Electroporation was performed once using Gene Pulser (BIO-RAD) delivering a pulse of 2.5 kV and capacitance at 250 microfarads and 200 ω resistance. The cuvette was removed from the chamber and immediately 1 ml of SOC medium (at room temperature) was added to the cuvette and the entire mixture was transferred to a sterile 1.5 ml microfuge tube. This was incubated for 1 hr at 37°C with gentle shaking. The plating of the cells and their analysis were carried out as described for chemical transformation above.

(E). Preparation of Radiolabelled (^{32}P) Probes

(i). PCR Method of Labelling Probes

Probes were mainly labelled using PCR by mixing 1 µl (2 ng) of target DNA, 1 µl of primer 1, 1 µl of primer 2, (both primers were 20 µM stock), 5 µl (50 µCi) of [α-^{32}P]dCTP, 1 µl of 250 µM reaction mix (dATP, dGTP, dTTP), 1 µl of 10 x PCR buffer and 0.5 µl of Taq DNA Polymerase (Pharmacia, Sweden) in a total vol of 10.5 µl. This was overlaid with 20 µl of mineral oil. PCR was performed as described earlier.

(ii). Random Priming Method of Labelling Probes

On some occasions, DNA fragments were labelled using random priming method to a specific activity of $>10^9$ cpm/ug with [α-^{32}P]dCTP using T_7 QuickPrime Kit (Pharmacia Biotech, Sweden). The labelling was done according to the instructions of the manufacturer as described by Feinberg and Volgelstein (1984). Briefly, 50-200 ng of denatured DNA fragment was labelled in a 50 µl reaction volume containing 10 µl of reaction mix (buffered solution containing dATP, dGTP, dTTP and random 9-mers supplied by the manufacturer), 5 µl (50 µCi) of [α-^{32}P]dCTP, 4-8 units of T_7 DNA polymerase and water. Tubes containing the mixture were incubated at 37°C for 30 min and the reaction stopped by adding 2 µl of 0.5 M EDTA.

(iii). Removal of Unincorporated (α-^{32}P)dCTP from the Probes

In order to remove the unincorporated [α-^{32}P]dCTP from the probes generated by PCR and random priming, the vol of the probe made by PCR reaction was increased to 50

µl using TE. The probes were then mixed with equal vol of the DNA dye (2% Blue dextran, 0.5% Orange G) and the mixture was passed through an equilibrated G-50 Sephadex column (Pharmacia Biotech, Sweden). The column was eluted with sterile TE buffer. The blue dextran dye co-eluted with [α-^{32}P]dCTP-labelled probe and therefore the fraction containing the dye was collected in a tube. One 5 µl aliquot of 1:50 dilution of the probe was transferred GF/C Glass Microfibre Filter (Whatman No. 1822 024) and was used to determine total count. Another 5 µl aliquot of same dilution of the probe was added to 25 µg of carrier DNA from T_7 QuickPrime Kit (Pharmacia Biotech, Sweden). To the aliquot in 25 µg of carrier DNA, 1 ml of 10% TCA was added and kept on ice for 10 min. The mixture was passed through a GF/C filter, washed sequentially with 10 ml of 10% cold TCA and 10 ml of cold absolute ethanol and used to determine precipitate count which is an indication of the actual count of probe. The dried filters were submerged in a scintillation cocktail fluid (Ecolume No. 882470, ICN, Costa Mesa, CA) and counted in a scintillation counter (LKB, Sweden).

(F). Screening, isolation and sequence determination of cysteine protease cDNAs from *L. donovani chagasi* and *Trypansoma cruzi*

Two *L. donovani chagasi* cDNA libraries in λ-ZAP II were kindly provided by Dr. Mary E. Wilson, Departments of Medicine and Microbiology, University of Iowa. The first cDNA library screened was prepared from total promastigote RNA. This library was prepared using Stratagene's λ-ZAP II Unidirectional Cloning System. The cDNA inserts were cloned into *Eco*RI site at the 5' end and *Xho*I site at the 3' end. The second cDNA library screened was prepared from total amastigote RNA as described (Wilson *et al.*, 1995).

The amastigotes used were purified from the spleens of hamsters infected with *L. d. chagasi* and are believed to represent the true amastigotes found in clinical cases in terms of gene expression. This library was prepared using Stratagene's λ-ZAP II Bidirectional Cloning System. The cDNA inserts were cloned into *Eco*RI site.

The *T. cruzi* cDNA library in λ-ZAP II was a gift from Dr. Steven G. Reed (Infectious Diseases Research Institute, Seattle, WA 98109). This cDNA library was prepared from total epimastigote RNA of *T. cruzi* and was constructed using Stratagene's λ-ZAP II Unidirectional Cloning System. The cDNA inserts were cloned into *Eco*RI site at the 5' end and *Xho*I site at the 3' end.

(i). PCR Amplification of Cysteine Protease Genes from the cDNA libraries

Before embarking on the screening of cDNA libraries, the three cDNA libraries were checked for the presence of cysteine protease cDNA(s). The two primers used earlier to detect cysteine protease from the genomic DNA was again used. A 10 µl aliquot of the phage lysate from each library was heated at 70°C for 5 min to denature the phage. The two primers together with 10 µl of a cDNA library as template were used in 100 µl reaction volume. PCR conditions were as described before. PCR products of expected length (500 bp) were digested sequentially with *Hin*dIII and *Eco*RI, gel purified and subcloned in pGEM^{R}-2 (Promega). Plasmid DNA was isolated using the alkaline lysis method described by Sambrook *et al.* (1989), sequenced directly using universal and SP6 primers as described below.

(ii). Amplification and Titering of cDNA Libraries

All the cDNA libraries were amplified as follows. Aliquots of phage containing approximately 10^5 were added to 600 µl of host XL1-blue strain of *E. coli* which had been adjusted to $O.D._{600}$ of 0.5 with 10 mM $MgSO_4$. The mixture was incubated at 37°C for 15 min, mixed with 10 ml of NZY top agarose which was kept at 48°C and plated onto 150 mm NZY plates. After the top agarose had solidified, the plates were inverted and incubated at 37°C overnight. The following morning, the plates were flooded with 10 ml of SM buffer and kept at 4°C with gentle shaking overnight. The eluted phages were aspirated into 50 ml polypropylene tubes and 5% chloroform was added followed by mixing to lyse *E. coli*. The phage suspension was cleared of debris by centrifugation at 300 rpm in a Sorval HH60 for 10 min, transferred into new tubes and stored at 4°C.

Prior to screening procedures the titres of the cDNAs were determined as follows: Serial dilution of phage suspension (10^{-3} to 10^{-6}) were made in SM buffer and 200 µl of XL1-Blue cells prepared as described above were mixed with 100 µl of each of the phage dilution. The mixture was incubated 37°C for 15 min, then mixed with 4 ml of top agarose before plating onto 80 mm NZY plates. After the top agarose had solidified, the plates were inverted and incubated at 37°C overnight. The following morning, the number of the plaques were counted and the titer of each cDNA library determined.

(iii). Screening of cDNA Libraries

After confirming the presence of cysteine protease in the cDNA libraries, a total of 80,000 PFUs (20,000 PFUs/150 mm plate) were plated from each cDNA library. Duplicate

nylon membranes (Amersham, UK) were lifted, the first lift after 1 minute and the second lift after 2 minutes on the plates. Before lifting, each filter was marked for orientation by placing three asymmetrical holes through the filter onto the plates using 16 gauge needle. Duplicate filters were lifted from each plate. The filters were air dried (the plaque side facing up), then subjected to denaturation (in 1.5 M NaCl + 0.5 M NaOH) for 7 minutes and two sequential neutralizations (in 1.5 M NaCl + 0.5 M Tris-HCl [pH 7.2] + 0.001 M EDTA [pH 8.0]) for 3 minutes. The denaturation and neutralization was carried out on 3MM Whatman papers pre-soaked with the appropriate buffers. The filters were then washed briefly in 2X SSC, air dried and baked at 80^{o}C for 2 hours before hybridization. For *L. donovani chagasi*, the probe was the 500 bp fragment obtained by PCR from genomic DNA and was ^{32}P labelled using PCR (the probe was a mixture of 500 bp fragment from promastigote and amastigote cysteine proteases). For *T. cruzi*, the probe was from the cDNA library from this organism. Areas on the plates containing strong duplicated signals were picked and the phages submitted to secondary screening. Each positive clone was screened separately. Only plaques with strong and duplicated signals were picked. For each cDNA clone picked, a Bluescript plasmid derivative was excised from the λ-ZAP II recombinant phages *in vivo* with the helper phage R-408 (Stratagene protocol). Sequence analyses were performed using dideoxy chain termination method first developed by Sanger *et al.*, (1977). Briefly, sequencing reactions were performed on double-stranded DNA plasmids with either T7 sequencing mixes or the Deaza T7 sequencing mixes (Pharmacia) and labelled nucleotides (^{35}Sα-dATP or ^{35}Sα-dCTP; 1000 mCi/mmol; Dupont). Reactions were analyzed on 8% denaturing polyacrylamide gels and all dried gels were autoradiographed on Kodak X-Omat

films with an intensifying screen for 15 to 72 hours. The sequencing strategies and primers used in sequencing have been described in Appendices 1, 2 and 3.

G. Southern blot analysis.

Genomic DNA (10 µg/lane) was digested with appropriate restriction enzyme and separated electrophorectically in 1% agarose gels containing 1.5 µg of ethidium bromide per ml. The gels containing separated genomic DNA were first depurinated in 0.25 M HCl for 30 minutes, then submerged in a denaturing solution (1.5 M NaCl and 0.5 M NaOH) for another 30 minutes and finally submerged in a neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2] and 0.001 M EDTA [pH 8.0]) also for 30 minutes. Between each of these procedures the gels were rinsed in distilled water several times. The separated genomic DNA was then transferred to nylon membrane (Hybond-N hybridization transfer membrane [Amersham]) using 20X SSC (1X SSC consists of 0.15 M NaCl plus 0.015 M sodium citrate pH 7.0) and capillary blotting described by Sambrook *et al.*, (1989). The blots were washed briefly in 2X SSC, air dried and baked at 80°C for 2 hours.

H. Pulsed Field Gel Electrophoresis (PFGE)

Agarose blocks containing *L. donovani chagasi* or *L. donovani donovani* chromosomes were prepared from early stationary phase promastigotes. A New England BioLabs Imbed Kit, including low-melting-point agarose, was employed for embedding the genomic DNA as described by Liu and Sanderson (1992) with some modifications. Briefly, a pellet of 3.4 X 10^9 promastigotes was resuspended in 1 ml of cell suspension solution (10

mM Tris-HCl [pH 7.2], 20 mM NaCl, 100 mM EDTA) and mixed quickly with 1 ml of 1.8% low-melting-point agarose. The mixture was drawn in a GelSyringe Mold and cooled to room temperature. The hardened agarose rod was then sliced into pieces (1 mm thick) and treated with lysozyme at 37°C for 2 hours and with proteinase K at 42°C for 18 hours. After incubation in 1 mM phenylmethylsulfonyl fluoride (PMSF) to destroy proteinase K, the agarose blocks were washed twice in wash solution (20 mM Tris-HCl [pH 8.0], 50 mM EDTA) and twice in storage solution (10-fold-diluted wash solution) and then stored in storage solution at 4°C. The samples remain intact in this buffer for at least 20 months.

The chromosomes were separated electrophorectically by using a Bio-Rad DRII contour-clamped homogeneous electric field apparatus or a Bio-Rad Mapper. The pulse times of 60 - 150 seconds were used. The total running time was 21 hours. The gels contained 0.5X TBE buffer (1X buffer contains 90 mM Tris, 90 mM boric acid, 2 mM EDTA [pH 8.0]) with 1.5 μg of ethidium bromide per ml and 0.7% agarose. Electrophoresis was carried out in the same buffer without ethidium bromide. The gels were photographed, and the positions of the various chromosomes marked using an 18 gauge needle attached to syringe filled with India ink. The gels containing separated chromosomes were then processed exactly as the gels for Southern blot described above.

I. Northern blot analysis

RNA samples (15 μg/lane) were treated with 1.25 μl of 20x MOPS, 4.5 μl of deionized formamide and 12.5 μl of formaldehyde then heated at 65°C for 10 min and fractionated in 1.0% agarose gels containing 1.2% formaldehyde and 1.5 μg of ethidium

bromide per ml. After electrophoresis, the ethidium bromide stained gels were photographed to show equal loading. The gels were then rinsed in distilled water for 15 minutes and in 20X SSC for 30 minutes then transferred to nylon membrane (Hybond-N hybridization transfer membrane [Amersham]) using 20X SSC and capillary blotting described by Sambrook *et al.*, (1989). The blots were washed briefly in 2X SSC, air dried and baked at 80°C for 2 hours.

J. Hybridization of Southern and Northern blots

Hybridizations of both Southern and Northern blots were carried out using Rapid-hyb buffer (Amersham). The buffer was used at a rate of 100 µl per cm^2 of the hybridization membrane. The blots were prehybridized in this buffer at 60°C for 1 hour then hybridized in the same buffer and at the same temperature with denatured probe added at a rate of 1×10^6 cpm per ml of the buffer used. The probe was the 500 bp fragment obtained by PCR from *L. donovani chagasi* genomic DNA or *T. cruzi* cDNA library and was ^{32}P labelled using PCR. As an internal control in Northern blot analysis, *L. d. chagasi* ribosomal phosphoprotein (LcPo) which is produced constitutively and the message is at 1.4 kb was used (cDNA of LcPo was kindly provided by Dr. Steven G. Reed, Infectious Diseases Research Institute, Seattle, WA 98109). Two washes were performed in 2 x SSC + 0.1% sodium dodecyl sulphate (SDS), followed by two washes in 0.5x SSC + 0.1% SDS and lastly one or two washes in 0.1 x SSC + 0.1% SDS. All washes were performed for 15 minutes and at 60°C. All blots were autoradiographed on Kodak X-Omat films with an intensifying screen for 15 to 72 hours.

K. Expression of *T. cruzi* cysteine protease in *E. coli*

Two oligonucleotide primers containing *Bam*HI restriction sites were used in PCR to amplify the cysteine protease coding region from the long cDNA. The sense primer (TCCP-GST1) was based on direct sequence of the *T. cruzi* CP cDNA around the ATG with modifications to create *Bam*HI restriction site outside the coding region. The antisense primer (TCCP-GST2) was based on the direct sequence of the long cDNA around the TGA with modifications to create *Bam*HI restriction site outside the coding region. The sequences of the two primers are:

Sense Primer (TCCP-GST1):

5' CAAGGAAGGATCCACACCCACGCAAGCAGT 3'.

Antisense Primer (TCCP-GST2):

5' GAGAAAAGGATCCATCAGAAACAAAGTGTGCC 3'.

The underline indicates the position of *Bam*HI restriction sites in both primers. Other details about the primers are provided in Appendix 4.

The PCR reactions were performed as described above except the annealing temperature was raised to 50^{o}C as calculated from the Td (Td= 4[G+C] + [A+T]) of the two primers. The PCR product which represented the coding region of the cDNA was digested with *Bam*HI, gel purified and ligated into the *Bam*HI site of the pGEX-2T vector in frame with GST (Smith and Johnson, 1988). The bacterial clones carrying the pGEX-2T + cysteine protease was selected and induced to express cysteine protease using a procedure similar to the one described by others (Smith and Johnson, 1988). Briefly 50 ml overnight cultures of *E. coli* transformed with pGEX-2T + cysteine protease were spun down, resuspended in 250

ml of fresh LB broth (ampicillin 100 μg/ml), induced with 0.5 mM isopropyl-β-D-thiogalactopyranosde (IPTG, Sigma) and was grown for 5 hours at 37°C with vigorous shaking. The cells were pelleted, washed once in MTPBS (150 mM NaCl, 16 mM Na_2HPO_4 4 mM NaH_2PO_4 [pH 7.3]), resuspended in 5 ml of MTPBS and incubated for 20 min with 1 mg/ml lysozyme (Boehringer Mannheim) followed by treatment with 1% Triton X-100 for 10 min on ice. After lysis by mild sonication (10 x 15 seconds bursts on ice), the bacterial lysate was subjected to centrifugation at 10,000 x g for 5 min at 4°C. The bacterial proteins in the supernatant and pellets were analyzed by sodium dodecyl polyacrlyamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer of Laemmli (1970).

L. Overexpression of cysteine proteases in *L. d. chagasi* cells

Two oligonucleotide primers containing *Bam*HI restriction sites were used in PCR to amplify the cysteine protease coding region from the each of the two distinct cDNAs from *L. d. chagasi*. The sense primer (4-1-1-GST1) was based on direct sequence of the Ldccys1 cDNA around the ATG with modifications to create *Bam*HI restriction site outside the coding region. The antisense primer (4-1-1-GST2) was based on the direct sequence of Ldccys1 cDNA around the TGA with modifications to create *Bam*HI restriction site outside the coding region. Similarly, the sense primer (34-1-2-GST1) was based on direct sequence of Ldccys2 cDNA around the ATG with modifications to create *Bam*HI restriction site outside the coding region. The antisense primer (34-1-2-GST2) was based on the direct sequence of Ldccys2 cDNA around the TGA with modifications to create *Bam*HI restriction site outside the coding region. The sequences of the four primers are:

Sense Primer (4-1-1-GST1):

5' TTCTCTTCCCCTCGCTGGATCCTCCGCGATGG 3'.

Antisense Primer (4-1-1-GST2):

5' AGACGCAGGATCCACCCCTACGTG 3'.

Sense Primer (34-1-2-GST1):

5' GCACGTTGCACCAACAAGGATCCGCAAAG 3'.

Antisense Primer (34-1-2-GST2):

5' GCACAGTGGATCCTGCCAGTCCTA 3'.

The underline indicates the position of *Bam*HI restriction sites in all the four primers. The number 4-1-1 stands for Ldccys1 cDNA and the number 34-1-2 stands for Ldccys2 cDNA. Other details about the primers are provided in Appendices 5 and 6.

To express cysteine protease cDNA from *Trypanosoma cruzi* in *L. d. chagasi* cells, the coding region of cysteine protease cDNA from *Trypanosoma cruzi* was amplified by PCR using the primers described above under "Expression of *T. cruzi* cysteine protease in *E. coli*".

The PCR reactions were performed as described earlier. The PCR products which represented the coding region of each of the three cysteine protease cDNAs was digested with *Bam*HI, gel purified and ligated into the *Bam*HI site of pX (kindly provided by Dr. Steven Beverley from Havard Medical School, Boston, MA). The ligation reaction was used to transform DH5α strain of *Escherichia coli* by electroporation as described earlier and in the literature (Miller and Nickoloff, 1995). Plasmid clones containing the cDNAs were selected by restriction analysis and sequencing. For cysteine protease from *T. cruzi*, two types of clones were selected: (i) 7pX+TCCP having the insert in the correct orientation and (ii)

8pX+TCCP having the insert in the opposite direction. For both amastigote and promastigote specific cysteine proteases only one clone, pX4-12 and p34-1 respectively were selected. Transfections of *L. d. chagasi* promastigotes were performed according to protocols described by others (Laban *et al.*, 1990; Beverley and Clayton, 1993). Briefly, log phase *L. d. chagasi* promastigotes were washed twice in PBS and resuspended at a rate of 1 x 10^8/ml in electroporation buffer (21 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 6 mM glucose) and 50 μg of plasmid pX+4-1-1 or pX+34-1-2 or pX alone. The promastigotes resuspended in electroporation buffer with 50 μg of plasmid DNA (total volume 400 μl) were placed in 0.2 cm cuvette and electroporation was performed using Gene Pulser (BIO-RAD) delivering a pulse of 1.2 kV and capacitance at 250 microfarads and 200 ω resistance. Electroporation was performed three times at 10 min intervals with promastigotes in cuvette sitting on ice in between. Transfected promastigotes were transferred to drug-free HOMEM media for overnight. After selection in HOMEM media containing 50 μg/ml of G418 (GIBCO BRL) for about 2 weeks, resistant promastigotes appeared. The concentration of G418 was then increased at a weekly interval by 50 μg/ml until it reached 400 μg/ml.

M. Cloning of cysteine protease cDNAs from *Leishmania donovani chagasi* in pALT (Neo) vector.

The coding regions of Ldccys1 and Ldccys2 cysteine protease cDNAs from *Leishmania donovani chagasi* were amplified by PCR using primers described above. The PCR products which represented the coding region of each of the two cysteine protease

cDNAs were digested with *Bam*HI, gel purified and subcloned into the *Bam*HI sites of the *Leishmania* expression vector pALT-Neo (kindly provided by Dr. Dyann Wirth respectively; Havard Medical School, Boston, MA). The ligation reaction was used to transform DH5α strain of *E. coli* by electroporation as described (Miller and Nickoloff, 1995). Plasmid clones containing the cDNAs were selected by restriction analysis and sequencing. The constructs were used to transfect avirulent and virulent strains of *Leishmania major* (kindly provided by Dr. Steven Beverley). Transfections of *L. major* promastigotes were performed as described above for *L. d. chagasi* promastigotes.

N. Isolation of Genomic and Plasmid DNA from transfected *Leishmania* cells

Total genomic and plasmid DNA was prepared from early stationary phase promastigotes (6 day culture). A pellet of about 1 x 10^8 promastigotes was lysed in 200 µl of TELT lysis buffer (50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA [pH 8.0], 2.5 M LiCl, and 4% Triton X-100). The parasite lysate was extracted with an equal volume of phenol:chloroform (1:1) by gentle inversion for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The aqueous phase was transferred into a new tube and DNA was precipitated by mixing with two volumes of absolute ethanol followed by spinning at 14,000 rpm for 5 minutes. The pellet was washed with 70% ethanol, dried and resuspended in 50 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) containing RNase at a concentration of 1 µg/µl (RNase was purchased from Pharmacia).

O. Southern blot analysis of total genomic and plasmid DNA.

To confirm that transfected *Leishmania* cells contained the cysteine protease constructs, genomic and plasmid DNA (10 µg/lane) was digested with *Bam*HI and separated electrophorectically in 1% agarose gels containing 1.5 µg of ethidium bromide per ml. The gels containing separated genomic and plasmid DNA were prepared for blotting and blotted as described before. Hybridizations of the Southern blots were carried out as described before.

P. Assays for proteolytic activities of cysteine protease

Biological activity of recombinant cysteine protease was detected in gelatin SDS-PAGE (non-denaturing) prepared according to recipe of Hotez *et al.*, (1992) with some modifications. Briefly, the separating gel contained 2.8 ml of 1.5 M Tris-HCl [pH8.8] and 0.4% SDS, 3.0 ml of 28.4% acrylamide and 1.6% bisacrylamide (final concentration was equal to 12.5%), 0.8 ml of 1% gelatin (while still hot), 0.8 ml of water, 10 µl of TEMED (N,N,N',N'-tetrmethylethylenediamine), and 60 µl of 10% ammonium persulfate. The stacking gel contained, 1.25 ml of 0.5 M Tris-HCl [pH 6.8], and 0.4% SDS, 0.5 ml of 28.4% acrylamide and 1.6% bisacrylamide, 0.5 ml of 1% gelatin (while still hot), 2.75 ml of water, 10 µl of TEMED, and 50 µl of 10% ammonium persulfate. The protein samples were applied to the gels in an SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol and bromophenol blue) without heating. The procedure was modified to include SDS in both the gel and the running buffer so as to allow separation of cysteine protease as a function of molecular weight. The gel was run at low voltage (approx. 50 V) to prevent heating during

the run. Upon completion of the run, the gel was washed once in 2.5% Triton X-100 (Sigma) for 1 hour then washed in 0.1 M sodium acetate [pH 5.5] and 1 mM DDT (Sigma) for 1 hour. The gel was then incubated in fresh 0.1 M sodium acetate [pH 5.5]+ 1 mM DDT + 20 μM Z-Phe-Arg-MCA (Peninsula Laboratories Inc. California) overnight at 37°C. The gels were stained the next day by placing in 50% methanol, 10% acetic acid, containing 0.2% coomassie blue for 30 minutes. The gel was then destained for at least 2 hours in a solution containing 7% methanol, 5% acetic acid in water. Once bands of proteolysis became visible, gels were photographed. To confirm that the enzymatic activity was due cysteine protease, inhibitors of cysteine protease (2 mM leupeptin and 2 mM iodoacetamide both from Sigma) were included in all the buffers used to incubate control gels.

Q. Generation of antibody against cysteine protease by DNA immunization of mice.

The direct DNA injection technology (Ulmer, *et al.* 1993; Waine and McManus, 1995; McDonnell and Askari, 1996; Ulmer *et al.*, 1996; Donnelly, *et al.*, 1996; Manickan *et al.*, 1997) was employed to generate antibody against the Ldccys1, Ldccys2 and *T. cruzi* cysteine proteases. Briefly, the coding regions of the three cDNAs of cysteine proteases were amplified by PCR using the same primers described under overexpression and subcloned into the *Bam*HI site of a plasmid VR1012 containing a eukaryotic promoter (plasmid VR1012; kindly provided by Dr. Jon Norman, Vical Inc. San Diego). The plasmid clones having inserts in the correct orientation were selected by restriction analysis and confirmed by sequencing. These constructs were delivered to experimental mice by direct injection into the hind limb quadriceps muscles (Ulmer, *et al.* 1993). The BALB/c mice used were bred and housed in

the animal facility at the Department of Biological Sciences, University of Calgary. A total of 25 BALB/c mice weighing 18-20 grams in groups of five were immunized and boosted with DNA as described above. One group of 5 mice received DNA plasmid encoding Ldccys1, another group of 5 mice received DNA plasmid encoding Ldccys2, another group of 5 mice received DNA plasmid encoding *T. cruzi*, yet another group received the plasmid VR1012 without any insert and the last group of 5 mice were injected saline (the last two groups served as controls). Each mouse in all 4 groups which received DNA was injected with 50 μg of appropriate DNA in saline and in a total volume of 100 μl and mice in the last group of controls also received 100 μl of saline. All mice were boosted after 21 days and tested for antibody response seven days after the boost. To monitor for antibody response, the mice were anaesthetized and bled through orbital plexus by a licensed veterinarian and 100 μl of blood was drawn. The blood was allowed to clot for 24 hours at 4^{o}C and serum obtained was tested for antibody using Western blot. Some mice were boosted for a second time after another 21 days from the first boost. Mice were anaesthetized, bled out through cardiac puncture and sacrificed when a titre of 1:500 as detected by Western blot was achieved.

R. SDS-PAGE and Western blot analysis of *Leishmania* cells overexpressing cysteine protease

SDS-PAGE was performed according to the method of Laemmli (1970) using 12% polyacrylamide gels. Transfected and untransfected promastigotes were washed and resuspended in lysis buffer (20 mM Tris pH 7.3; 40 mM NaCl; 10 mM EDTA and 20 mM

PMSF) (Duboise *et al.* 1994) and sonicated for 5 x 15 seconds on ice with Virsonic cell disrupter at a setting of 60 using small probe. Cell lysate was treated with an equal volume of 2% SDS and one part of 5 x sample buffer (formulated as described by BIO-RAD) was added to four parts of the solubilized protein. The proteins were then denatured for 3 minutes in 100°C boiling water bath and loaded into wells of a 4% stacking. After electrophoresis the protein was transferred to PVDF-Plus transfer membrane (Micron Separations Inc.) and probed with antibody raised against amastigote specific cysteine protease through direct DNA injection technology. Reactivity was detected by using the ECL system (Amersham).

Molecular Cloning, Characterization and Overexpression of Two Distinct Cysteine Protease cDNAs from *Leishmania donovani chagasi*

RESULTS

A. Summary

We have cloned and characterized two distinct cysteine protease cDNAs from *Leishmania donovani chagasi*. One of the cDNAs was isolated from a cDNA library prepared from total promastigote RNA and has been designated Ldccys2 while the other cDNA was isolated from a cDNA library prepared from total amastigote RNA and has been designated Ldccys1. Ldccys2 has an open reading frame of 471 amino acids and Ldccys1 has an open reading frame of 447 amino acids. Comparison of the predicted protein sequences of the two distinct cysteine proteases with those of cysteine proteases from *Leishmania pifanoi*, a member of *L. mexicana* complex, showed that the cysteine proteases from the two species of *Leishmania* have a high degree of homology. Each of the two cDNAs is distinct in genomic arrangement and chromosome location. Ldccys1 belongs to a family of cysteine proteases encoded by tandemly organized genes located on chromosome 7 while Ldccys2 appears to be a single cysteine protease gene located on chromosome 10. The organization of the two families of cysteine proteases in *L. donovani donovani* was also found to be similar. In this species, Lddcys1 (which correspond to Ldccys1) are located on chromosome 5 while Lddcys2 (which corresponds to Ldccys2) is located on chromosome 8. Ldccys1 genes are expressed abundantly in the amastigotes recovered from infected hamsters, but at very low level in the promastigote stage of development. On the other hand, Ldccys2 gene is expressed in both the promastigote and amastigote stages. We have overexpressed the two cDNAs of cysteine proteases in *Leishmania* cells and the over-produced cysteine proteases

are biologically active and are inhibited by cysteine protease inhibitors. Furthermore, the over-produced and indigenous amastigote specific cysteine protease reacted with polyclonal antibodies raised against this protein.

B. PCR amplification of cysteine protease genes

In order to determine the population of cysteine protease genes in the genome of *L.d. chagasi* we carried out the PCR amplification of cysteine protease DNA fragment from genomic DNA using two primers (LCP1 and LCP2) designed as described in Fig. 7. A 500 bp PCR product was obtained (Fig. 7). The PCR product was digested sequentially with *Hin*dIII and *Eco*RI, gel purified and subcloned in pGEM-2 (Promega). Plasmid DNA from a large number of clones were isolated and sequenced. Our results showed the presence two distinct types of cysteine protease DNA sequence which we designated as Ldccys1 and Ldccys2. Ldccys1 was encountered more frequently than Ldccys2 suggesting Ldccys1 is more abundant in the *L. d. chagasi* genome. We also found the presence of a related variant of Ldccys1 which only differs with the deletion of 13 amino acids close to the LCP2 primer and this variant was under-represented in the genome (data not shown).

C. Screening, isolation and sequence determination of cysteine protease cDNAs from *Leishmania donovani chagasi*

In order to isolate full length cDNAs representing the Ldccys1 and Ldccys2 cysteine protease genes, we screened promastigote and amastigote specific *L. donovani chagasi* cDNA libraries in λ-ZAP II. We used the 500 bp PCR product amplified from the genomic DNA containing all the cysteine protease genes as a probe. From each cDNA library we first sequenced the 500 bp of all positive clones, then sequenced several selected positive clones to completion (Appendices 1 and 2).

Figure 7. Designing of LCP-1 and LCP-2 Primers for cloning.

Two highly conserved regions in the sequences described by Eakin *et al.*, (1992) were chosen for the design of two primers. The two regions chosen were Q G Q C G S C W and Y W I V K N S W . These amino acid residues were translated to nucleotides using the *Leishmania* codon bias. Although these primers were built to amplify cysteine protease from *Leishmania* genomic DNA, they turned out to be general primers which can amplify cysteine protease from virtually all trypanostomatids. The lower panel shows the expected PCR product of 500 bp.

T. cruzi	Q	G	Q	C	G	S	C	W	.	.	.	.	.	Y	W	I	V	K	N	S	W
T. brucei	Q	G	Q	C	G	S	C	W	.	.	.	.	.	Y	W	I	V	K	N	S	W
E. hist.	Q	G	Q	C	G	S	C	W	.	.	.	.	.	Y	W	I	V	K	N	S	W
CHICK CATH L	Q	G	Q	C	G	S	C	W	.	.	.	.	.	Y	W	I	V	K	N	S	W
D. discoideum	Q	G	Q	C	G	S	C	W	.	.	.	.	.	Y	W	I	V	K	N	S	W
S. mansoni Sm31	Q	S	R	C	G	S	C	W	.	.	.	.	.	Y	W	L	I	A	N	S	W
PAPAIN	Q	G	S	C	G	S	C	W	.	.	.	.	.	Y	I	L	I	K	N	S	W

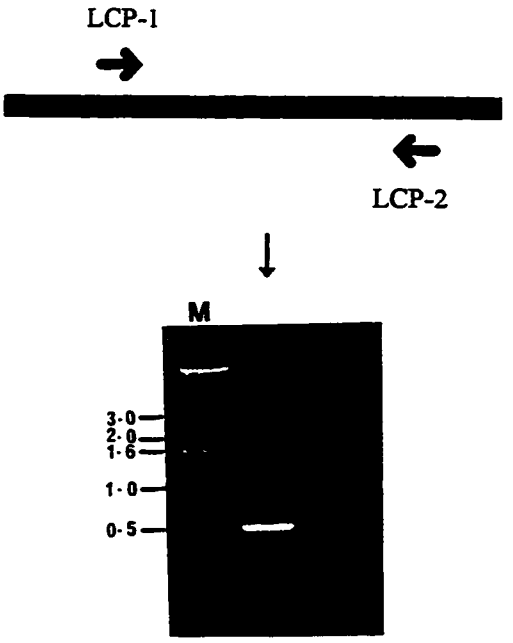
LCP-1 **LCP-2**

LCP-1: 33 bp. 5' ACAGAATTCCAGGGCCAGTGCGGCTCGTGCTGG 3'

Underline indicates *Eco*RI site.

LCP-2: 33 bp. 5' CGCAAGCTTCCACGAGTTCTTCACGATCCAGTA 3'

Underline indicates *Hin*dIII site.



All the 16 clones from the promastigote specific cDNA library corresponds to Ldccys2 as determined by sequencing within the 500 bp. Similarly, all the 20 clones from the amastigote specific cDNA library corresponds to Ldccys1 (as determined by sequencing within the 500 bp) except one clone which contains the variant form. In addition to the 13 amino acid deletion, this variant form also differ extensively 3' to LCP2 towards the carboxyl-terminal end (data not shown). Furthermore, when amastigote cDNA library blots were stripped and rehybridized with the Ldccys2 probe, 4 clones were picked from positions which differed from amastigote clones. However, we did not further analyze these clones. These findings suggest that Ldccys1 is amastigote specific, while Ldccys2 appears not to be stage-specific.

The sequences of Ldccys1 and Ldccys2 cDNAs are shown in Figures 8 and 9 respectively. These cDNAs may not be full length since the 5' ends of both Ldccys1 and Ldccys2 do not coincide with the published sequence for *L. donovani* splice leader (Wilson *et al.*, 1991). The cDNAs were sequenced completely. Ldccys1 has an open reading frame of 447 amino acids and Ldccys2 has an open reading frame of 471 amino acids (Figs. 8 and 9 respectively). The difference in length between the two proteins is mainly due to a 21 amino acid deletion from the carboxyl terminal region of Ldccys1 (Fig. 10). It is interesting to note that there is an open reading frame of 23 amino acids in the 5'-untranslated region of the Ldccys1 cDNA. At the amino acid level, comparative analysis of the cDNA sequences for Ldccys1 and Ldccy2 show a similarity of 40%, suggesting the two cDNAs are distinct and have an ancient divergence.

Figure 8. Sequence of Ldccys1 cDNA from *Leishmania donovani chagasi*. The cDNA was isolated from a cDNA library constructed from total RNA of the amastigotes purified from hamsters infected with *L. d. chagasi*; all the clones of cysteine proteases isolated from this library belonged to this family. The cDNA has open reading frame of 447 amino acids. In the 5' untranslated region of Ldccys1, an open reading frame of 23 amino acids is shown in bold. The LCP-1 and LCP-2 primers used to amplify the 500 bp fragment are underlined. *Leishmania donovani chagasi* Ldccys1 cDNA has been assigned EMBL/GenBank nucleotide accession number AF004592.

```
                                                                                CGGCCGCGGATTATATTCCAAAAAGAAATTGCATGTGGGAGT

ATATCTCCGTTAGATCTTTGCTACGCCAACCAAGATCTGCACTAGAGGCCGGTCCATCTTAGGCTC

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CGCCCTTACCAAAGGAATAATGGGCAATCGCAGAATTCGCGGCCGCTATACACCCGCCACGGTCTTAGCGTGCGAG

CTGTGGCCTCTGCGTTGCACGTGCTCTCCCCTCGCTGCCCTCGTTGCCTCCTTCTCTTCCCCTCGCTGGCGCCTCC

    M   A   T   S   R   A   A   L   C   A   V   A   V   V   C   V   V   L      18
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A   A   A   V   A   A   R   A   I   Y   V   G   T   P   A   A   A   L   F      37
GCG GCT GCC GTC GCT GCG CGC GCG ATA TAC GTG GGC ACG CCG GCT GCT GCG CTG TTC

E   E   F   K   R   T   Y   R   G   E   Y   R   T   L   A   E   E   Q   Q      56
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R   L   A   N   S   E   R   N   L   E   L   M   R   E   D   Q   A   E   N      75
CGG CTC GCG AAC TCC GAG CGC AAC CTG GAG CTG ATG CGC GAG GAT CAG GCG GAG AAC

P   H   A   R   F   G   I   T   K   F   F   D   L   S   E   A   E   F   A      94
CCA CAC GCG AGG TTC GGG ATC ACG AAG TTC TTC GAC CTG TCC GAG GCG GAG TTC GCC

A   R   Y   L   N   G   A   A   Y   F   A   A   A   K   Q   H   A   G   Q     113
GCC CGC TAC CTG AAC GGC GCC GCG TAC TTC GCA GCG GCG AAG CAG CAC GCC GGC CAG

H   Y   R   K   A   R   A   D   L   S   A   V   P   D   A   V   D   L   R     132
CAC TAC CGC AAG GCG CGC GCG GAC CTG TCG GCG GTG CCT GAT GCG GTG GAC TTG CGC

E   K   G   A   V   T   P   V   K   N   Q   G   A   C   G   S   C   W   A     151
GAG AAG GGC GCC GTG ACG CCG GTG AAG AAT CAA GGC GCG TGC GGG TCG TGC TGG GCG
                                        ------------------------------>

F   S   A   V   G   N   I   E   S   Q   W   A   R   A   G   H   G   L   V     170
TTC TCG GCG GTC GGC AAC ATC GAG TCG CAG TGG GCC CGT GCC GGC CAC GGC TTG GTG

S   L   S   E   Q   L   V   S   C   D   D   K   D   N   G   C   N   G   G     189
AGC CTG TCG GAG CAG CTG GTG AGC TGC GAT GAC AAA GAC AAT GGC TGC AAC GGC GGG

L   M   L   Q   A   F   E   W   L   L   R   H   M   Y   G   I   V   F   T     208
CTG ATG CTG CAG GCG TTC GAG TGG CTG CTG CGA CAC ATG TAC GGG ATC GTG TTC ACG

E   K   S   Y   P   Y   T   S   G   N   G   D   V   A   E   C   L   N   S     227
GAG AAG AGC TAC CCC TAC ACG TCC GGC AAC GGT GAT GTG GCC GAG TGC TTG AAC AGC

S   K   L   V   P   G   A   Q   I   D   G   Y   V   M   I   P   S   N   E     246
AGT AAA CTC GTT CCC GGC GCG CAA ATC GAC GGC TAC GTG ATG ATC CCG AGC AAC GAA

T   V   M   A   A   W   L   A   E   N   G   P   I   A   I   A   V   D   A     265
ACG GTT ATG GCT GCG TGG CTT GCG GAG AAT GGC CCC ATC GCG ATT GCG GTC GAC GCC

S   S   F   M   S   Y   Q   S   G   V   L   T   S   C   A   G   D   A   L     284
AGC TCC TTC ATG TCT TAC CAG AGC GGC GTG CTG ACC AGC TGC GCT GGC GAT GCA CTG

N   H   G   L   L   L   V   G   Y   N   K   T   G   G   V   P   Y   W   V     303
AAC CAC GGC TTG CTG CTC GTC GGG TAC AAC AAG ACC GGT GGG GTT CCG TAC TGG GTG
                                                            <------------

I   K   N   S   W   G   E   D   W   G   E   K   G   Y   V   R   V   V   M     322
ATC AAG AAC TCG TGG GGT GAG GAC TGG GGC GAG AAG GGC TAC GTG CGC GTG GTC ATG
---------------

G   L   N   A   C   L   L   S   E   Y   P   V   S   A   H   V   P   R   V     341
GGG CTG AAC GCG TGC CTG CTC AGT GAA TAC CCC GTG TCC GCG CAT GTG CCG CGA GTC

S   P   L   A   R   A   R   E   R   G   A   P   P   K   R   V   T   V   E     360
TCA CCC CTG GCC CGG GCA CGA GAG CGA GGA GCC CCC CCT AAA CGG GTG ACG GTG GAG

Q   M   M   C   T   D   M   Y   C   R   E   G   C   K   K   S   L   L   T     379
CAG ATG ATG TGC ACC GAT ATG TAC TGC AGG GAG GGG TGC AAG AAG AGT CTT CTC ACC
```

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A   K   V   C   Y   K   N   G   G   G   G   S   S   M   T   K   C   G   P     398
GCG AAG GTG TGC TAC AAG AAC GGG GGA GGC GGC TCC TCT ATG ACG AAG TGC GGT CCG

Q   K   V   L   M   C   S   Y   S   N   P   H   C   S   Y   S   N   P   H     417
CAG AAG GTG CTG ATG TGC TCG TAC TCG AAC CCT CAT TGC TCG TAC TCG AAC CCT CAT

C   F   G   P   G   L   C   F   E   T   P   D   G   K   C   A   P   Y   F     436
TGC TTT GGT CCT GGG CTG TGC TTC GAG ACT CCT GAT GGC AAG TGC GCG CCG TAC TTC

L   G   S   I   M   N   T   C   Q   Y   T   .                                 447
TTG GGC TCG ATC ATG AAC ACC TGC CAG TAC ACG TAG GGGTGGAGCGTGCGTCTAGGTGTGGGT

GGCGTTGTGTGTGTGTGTGTGGTGGCAGCGCCGTCGCGCGCGTGAGGGGGCCTCCTTGGGTGCGCGCGCGCCAT

GGCCGCTCCGCGTGGACGCGATATGTGAGGCCGAGGAAGAAGAAAGCGACGCTGCCGAAGGCGCCGCGAGGTGTTG

CGGAGCGTAGGGGGAAAATGGACGAACAGGCGCGCTGTGAATAGGAGCTGCGGCACCACGCAACTCGTGGTGTACC

GCCAATGGCTATTCCGCCCGCCCTCGCTTCACGCCTCGTCCCCCCCCCCCTCGCGCGCCCTCCCTTGTTATCCCTCT

CTTTCACGCACACACGTACACACACGCACGCCCACACACACGCGCACACACACGCGCGCCCGTTATTCTGCCTTCG

TCTGCCTCTTTATGTGTTTTGTGTGTTTCTTTGTTTTGCTTGCTTCTTCTGCACACTTCTGTTTTCTGCTTCAGCG

ATATCTCCCACTCCTTGTTTTCTCTTCCCCCTCGGTGAGCGCTTCGTTTGTGGTTTGACTGTGGTGCGTCTGTGCC

GGATCATCTACCGGCGTGCGCACCTCCCCCATCTCCCTGCATCGTGTGCGTGCACTGCCTTCACACCGCGCTCGCG

TGCATGTACATGTGCGCACTGCTTGTCGGACGCCTTCCGCCCGCGTTGGCCCCCCGTTTCCGCATCCACGGTTTGT

TTATCGGTTGTGCTTGCTTCACCGGCTCTTCTCTGCGCGCGTGCGTGTGTGTGCGTGCGTGTGCGTGAGGCGCAAC

GGTCCCCAGAGCAAAAAAAAAAAAAAAA.
```

Figure 9. Sequence of Ldccys2 cDNA from *Leishmania donovani chagasi*. The cDNA was isolated from a cDNA library constructed from total RNA of the promastigotes; all the clones of cysteine proteases isolated from this library belonged to this family. The cDNA has open reading frame of 471 amino acids. The LCP-1 and LCP-2 primers used to amplify the 500 bp fragment are underlined. *Leishmania donovani chagasi* Ldccys2 cDNA has been assigned EMBL/GenBank nucleotide accession number AF004593.

```
                CACGAGCTTTATTGCTTTATTGTCGCCGCTTTCGCACCACACACTACCTGTTTGCTTGATTCTCTTGT

TTTTTTTTGTTTGTACTCCTCAGAACGCAGAAACTACTAGCGCGCGCACACGCACACACCTCGCCCAACCAGTGCTT

TTAAACCCCTCTTGCCTCCACTATACCCGCTCACTTCCACAGCAGCACCTGCTACACACTGTACCTTTTCCCGCCTT

GTTTTTCGTATTCGCCCTTTGTTTTTTTTTTTGCTTTTTTGATTCCAACGCTCTCTTCTCCGCTCACGCATCCGCCG

                                                                  M   A   R     3
CCCCCCCCCCTGCACCCCGTCACGTCTTCTCGTGCGCACGTTGCACCAACAAGTATCGGCAAAG ATG GCG CGC

N   P   F   F   F   A   I   V   V   T   I   L   F   V   V   C   Y   G   S    22
AAC CCC TTT TTT TTT GCG ATA GTG GTA ACT ATC CTG TTC GTG GTG TGC TAC GGT TCC

A   L   I   A   Q   T   P   L   G   V   D   D   F   I   A   S   A   H   Y    41
GCT CTT ATC GCC CAG ACA CCT CTC GGC GTC GAC GAC TTC ATT GCC TCG GCG CAT TAC

G   R   F   K   K   R   H   G   K   P   L   G   E   D   A   E   E   G   R    60
GGA CGC TTT AAG AAG CGC CAC GGC AAG CCC TTA GGA GAG GAC GCC GAG GAG GGT CGA

R   F   N   A   F   K   Q   N   M   Q   T   A   S   F   L   N   A   H   N    79
CGC TTC AAC GCC TTC AAG CAG AAC ATG CAG ACA GCC TCG TTC CTC AAT GCG CAC AAC

P   H   A   H   Y   D   V   S   G   K   F   A   D   L   T   R   Q   E   F    98
CCG CAC GCG CAC TAC GAC GTG TCC GGT AAG TTC GCG GAC CTC ACC CGG CAG GAG TTC

A   K   L   Y   L   N   P   N   Y   Y   A   R   H   G   K   V   T   R   S   117
GCC AAG TTG TAC CTG AAC CCC AAC TAC TAC GCG CGC CAC GGT AAG GTT ACA AGG AGC

N   V   H   V   D   D   S   V   R   S   G   V   M   S   V   D   W   R   E   136
AAC GTG CAC GTC GAC GAC AGC GTC CGC AGT GGT GTG ATG TCT GTG GAC TGG CGT GAG

K   G   V   V   T   P   V   K   N   Q   G   M   C   G   S   C   W   A   F   155
AAG GGT GTC GTG ACG CCG GTG AAG AAC CAG GGA ATG TGC GGC TCG TGC TGG GCC TTC
                                    ------------------------------------>

A   T   T   G   N   I   E   G   Q   W   D   L   K   N   H   S   L   V   S   174
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L   S   E   Q   V   L   V   S   C   D   N   I   D   D   G   C   N   G   G   193
CTG TCG GAG CAA GTC CTC GTG TCG TGT GAC AAC ATC GAT GAT GGG TGC AAC GGC GGG

L   M   Q   Q   A   M   Q   W   I   I   N   D   H   N   G   T   V   P   T   212
CTG ATG CAA CAG GCA ATG CAA TGG ATC ATC AAC GAT CAC AAC GGC ACT GTG CCC ACG

E   D   S   Y   P   Y   T   S   A   G   G   T   R   P   P   C   H   D   N   231
GAG GAC AGC TAC CCC TAC ACC TCT GCC GGC GGC ACG AGG CCT CCG TGC CAT GAC AAC

G   H   V   G   A   K   I   K   G   Y   M   S   L   P   H   D   E   E   E   250
GGA CAC GTC GGC GCC AAA ATA AAA GGT TAC ATG TCC CTG CCG CAT GAC GAG GAG GAG

I   A   A   Y   V   G   K   N   G   P   V   A   V   A   V   D   A   T   T   269
ATC GCG GCT TAT GTA GGG AAG AAC GGC CCA GTC GCC GTC GCC GTC GAC GCG ACA ACC

W   Q   L   Y   F   G   G   V   V   T   L   C   F   G   L   S   L   N   H   288
TGG CAG CTG TAC TTT GGC GGT GTG GTC ACC CTC TGC TTC GGG TTG TCG CTC AAC CAC

G   V   L   V   V   G   F   N   R   Q   A   K   P   P   Y   W   I   V   K   307
GGT GTG CTC GTT GTC GGC TTC AAC AGA CAA GCG AAA CCG CCG TAC TGG ATC GTG AAG
                                                        <------------------

N   S   W   G   S   S   W   G   E   K   G   Y   I   R   L   A   M   G   S   326
AAC TCG TGG GGC TCC TCG TGG GGT GAG AAG GGG TAC ATC CGC CTT GCC ATG GGC AGC
---------

N   Q   C   L   L   K   N   Y   V   V   T   A   T   I   D   D   S   N   T   345
AAC CAG TGC TTG CTG AAG AAT TAC GTC GTG ACG GCC ACG ATT GAC GAC TCC AAC ACC

S   Q   V   P   T   T   A   A   M   P   E   P   S   P   T   A   L   V   Q   364
TCA CAG GTG CCG ACG ACA GCG GCC ATG CCG GAG CCC AGC CCC ACG GCG CTT GTG CAG

M   N   C   L   S   G   C   A   R   L   C   T   N   T   T   Y   P   T   G   383
ATG AAC TGT TTG AGT GGG TGC GCG CGG CTG TGC ACA AAC ACG ACC TAT CCG ACC GGT
```

```
V   C   L   K   R   K   N   G   G   S   A   Q   V   A   C   G   K   S   E   402
GTG TGC CTG AAG CGT AAG AAT GGC GGC TCT GCC CAG GTA GCC TGT GGC AAA TCC GAG

T   V   E   L   L   Y   D   S   P   G   C   S   G   P   A   N   K   A   R   421
ACG GTG GAG CTT CTC TAC GAC TCG CCG GGC TGC TCC GGG CCT GCC AAC AAG GCC CGC

F   P   P   S   L   C   M   C   S   Y   A   G   Y   L   Q   N   V   R   S   440
TTC CCG CCG AGC CTA TGT ATG TGC AGC TAC GCC GGC TAC CTC CAG AAC GTC CGC AGT

E   S   V   A   A   V   R   T   N   D   A   V   S   N   L   A   K   P   L   459
GAG TCT GTC GCT GCG GTG AGA ACC AAC GAC GCC GTC TCT AAC TTG GCC AAG CCG CTC

I   L   G   Q   P   H   A   R   H   A   D   I   .                           471
ATC TTA GGC CAA CCA CAC GCG AGG CAC GCA GAC ATC TAG GACTGGCAGGATGCACTGTGCGCT

GTGCACCTCAGCCGCACCGATGCGATCCCTCACTCAACCTCTCTTTGGATGCCTCTCTGCCTTCCCCCGAGTGCCA

TGAAGTCATGACCCTACCGTATACCTCTGCGGTAGTCCCTTTCGAAGTGCACCTACTCGCGACGAGTCCGCGCGGC

TTGTCACAGTCCATTGCGACTCGCAGCTCGTCAGCTTGCACAACGCGATCTGGCGCACAGCTGATAAGACAGTGCA

AGAGCACCGTGCCGGCCACCGCCACCCCCTGCCTCCACCACCATCGCGAGCATCGTACTCTCTTGCTTTACCTGAA

GAGCGACGACGGTCTCTGCCCCGTCTCTCTGTCCGTTTGCTGTTCCTTTCATATTTGTTGTTTCGCCTTCACCACC

CCGTTCTTTTCGTGCTTTTTGCTTTTCCTTCCGACTGACATGCACGCATATATATATATATATATATTTGCATATA

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CCTCCTCCCCTTCCCAAGCCAGCAAGCGAGTGCGTCGGTCCGTGCGAATATATGTAATGCTTCTTTGGTTTCCTTT

ACTTCTTCCTCTTATTTATTTGTGCACGGTTTGCTCCGAGCACAACGGTGCGGCGAGATCGGAGGACAACGGCGTT

TTCTTGTCGTCGTCTAGGGGCAGCGAGACCTTTGTTTTACCCGACAAAGTCACTGCAGGCCTCCGCCCTTTTTGAG

AGAGGGCCCCAGTGACCTTACACGGACCATTTTTTTTTGTTGTTTCCACGGTCCTTGCCTTCAGCGCAAGTTTCTT

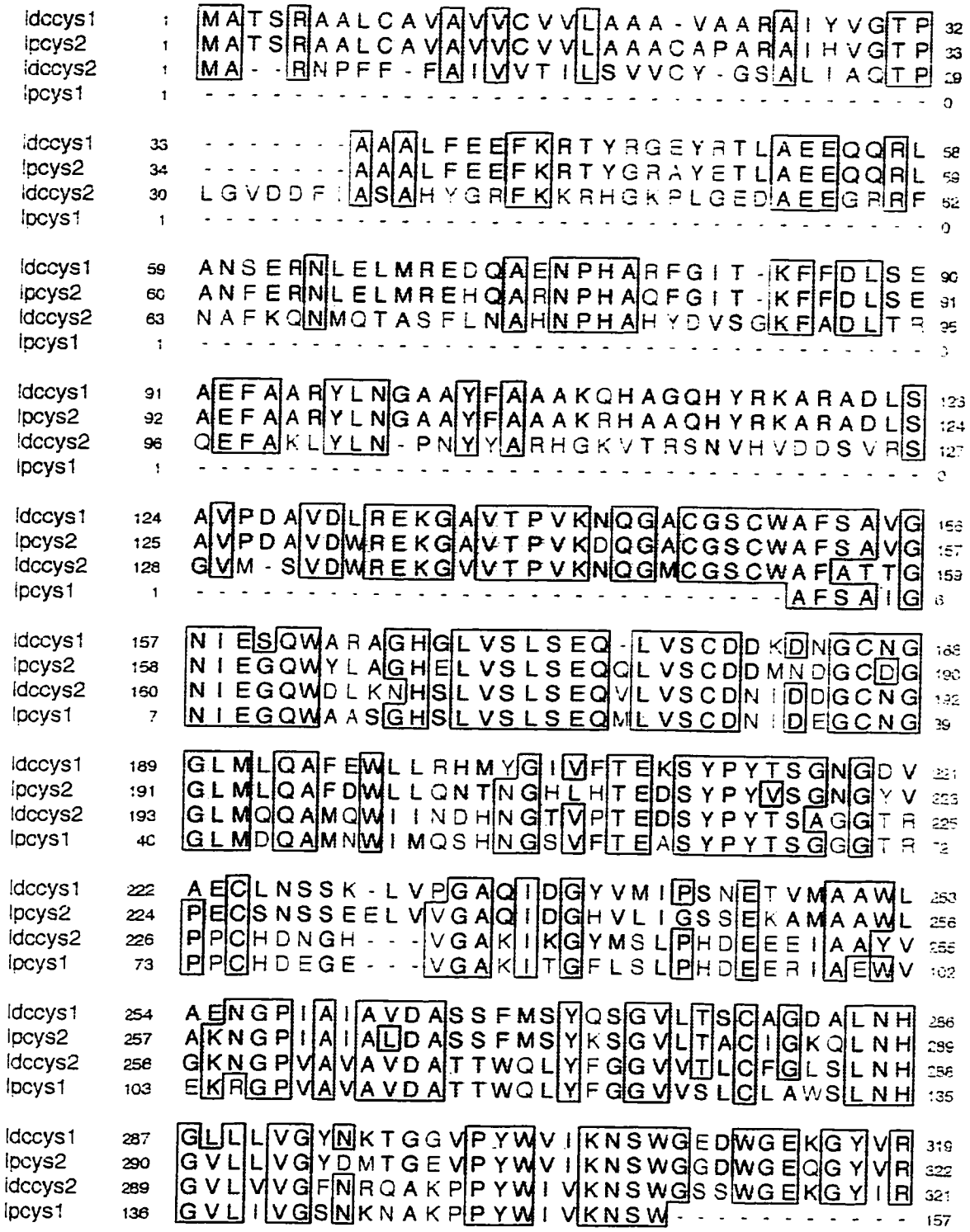
CGCCCATCCGACCTTGTTTATGTCCGCACCCGGTGAAACGGGTGGCCCCGC (A67) .
```

In order to determine the degree of homology, we aligned the deduced amino acid sequences of the two distinct cysteine proteases with those of cysteine proteases from *Leishmania pifanoi*, a member of *L. mexicana* complex (Fig. 10). Amino acid sequence of Ldccys1 when compared with that of Lpcys2 which is the corresponding gene from *L. pifanoi* show 75% homology while Ldccys2 is 78% similar to the available amino acid sequence of Lpcys1 which is the corresponding gene from *L. pifanoi* (Traub-Cseko *et al.*, 1993).

The deduced amino acid sequence from Ldccys1 and Ldccys2 cDNAs contain all the features characteristic of the different members of the cysteine protease family of proteins (Fig. 10). Thus, it contains the active site cysteine at positions 149 and 150 in Ldccys1 and Ldccys2 respectively. Glycine which is involved in substrate binding in papain is also present at positions 147 (in Ldccys1) and 148 (in Ldccys2). In addition, other amino acid residues important in catalysis including histidine and asparagine are present. Histidine is located at positions 286 (in Ldccys1) and 289 (in Ldccys2) while asparagine is present at positions 306 and 309 in Ldccys1 and Ldccys2 respectively. All these key residues are bound by other well conserved amino acids as in other cysteine proteases (Souza *et al.*, 1992; Traub-Cseko *et al.*, 1993).

From this amino acid sequence alignment (Fig. 10) and sequence information on other cysteine proteases (Souza *et al.*, 1992), Ldccys1 and Ldccys2 have pre- and pro-regions from residues 1 to 123 and 1 to 127 respectively. The N-terminal regions (pre-) of both cysteine protease sequence are hydrophobic and appears to be represented by residues 1-37 and 1-39 for Ldccys1 and Ldccys2 (Fig. 10).

Figure 10. Comparison of the deduced amino acid sequences from Ldccys1 and Ldccy2 from *Leishmania donovani chagasi* to the deduced amino acid sequences of the Lpcys1 and Lpcy2 from *Leishmania pifanoi*. Ldccys1 is homologous to Lpcys2; Ldccys2 is homologous to Lpcys1. For alignment of amino acid sequences the Clustal V program was used. Amino acid residues common to the cysteine proteases are boxed. Shaded residues imply similarity/identity of amino acids among the cysteine proteases. The "-" symbol indicates gap introduction into the sequences to maximize alignment.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ldccys1 33 -------AAAALFEEFKRTYRGEYRTLAEEQQRL 58
lpcys2 34 -------AAAALFEEFKRTYGRAYETLAEEQQRL 59
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lpcys2 60 ANFERNLELMREHQARNPHAQFGITKFFDLSE 91
ldccys2 63 NAFKQNMQTASFLNAHNPHAHYDVSGKFADLTR 95
lpcys1 1 ---------- 3
ldccys1 91 AEFAARYLNGAAYFAAAKQHAGQHYRKARADLS 123
lpcys2 92 AEFAARYLNGAAYFAAAKRHAAQHYRKARADLS 124
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lpcys1 1 ---------- 0
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lpcys2 125 AVPDAVDWREKGAVTPVKDQGACGSCWAFSAVG 157
ldccys2 128 GVM-SVDWREKGVVTPVKNQGMCGSCWAFATTG 159
lpcys1 1 ----------AFSAIG 6
ldccys1 157 NIESQWARAGHGLVSLSEQ-LVSCDDKDNGCNG 188
lpcys2 158 NIEGQWYLAGHELVSLSEQQLVSCDDMNDGCDG 190
ldccys2 160 NIEGQWDLKNHSLVSLSEQVLVSCDNIDDGCNG 192
lpcys1 7 NIEGQWAASGHSLVSLSEQMLVSCDNIDEGCNG 39
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lpcys2 191 GLMLQAFDWLLQNTNGHLHTEDSYPYVSGNGYV 223
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lpcys1 40 GLMDQAMNWIMQSHNGSVFTEASYPYTSGGGTR 72
ldccys1 222 AECLNSSK-LVPGAQIDGYVMIPSNETVMAAWL 253
lpcys2 224 PECSNSSEELVVGAQIDGHVLIGSSEKAMAAWL 256
ldccys2 226 PPCHDNGH---VGAKIKGYMSLPHDEEEIAAYV 255
lpcys1 73 PPCHDEGE---VGAKITGFLSLPHDEERIAEWV 102
ldccys1 254 AENGPIAIAVDASSFMSYQSGVLTSCAGDALNH 286
lpcys2 257 AKNGPIAIALDASSFMSYKSGVLTACIGKQLNH 289
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ldccys1 287 GLLLVGYNKTGGVPYWVIKNSWGEDWGEKGYVR 319
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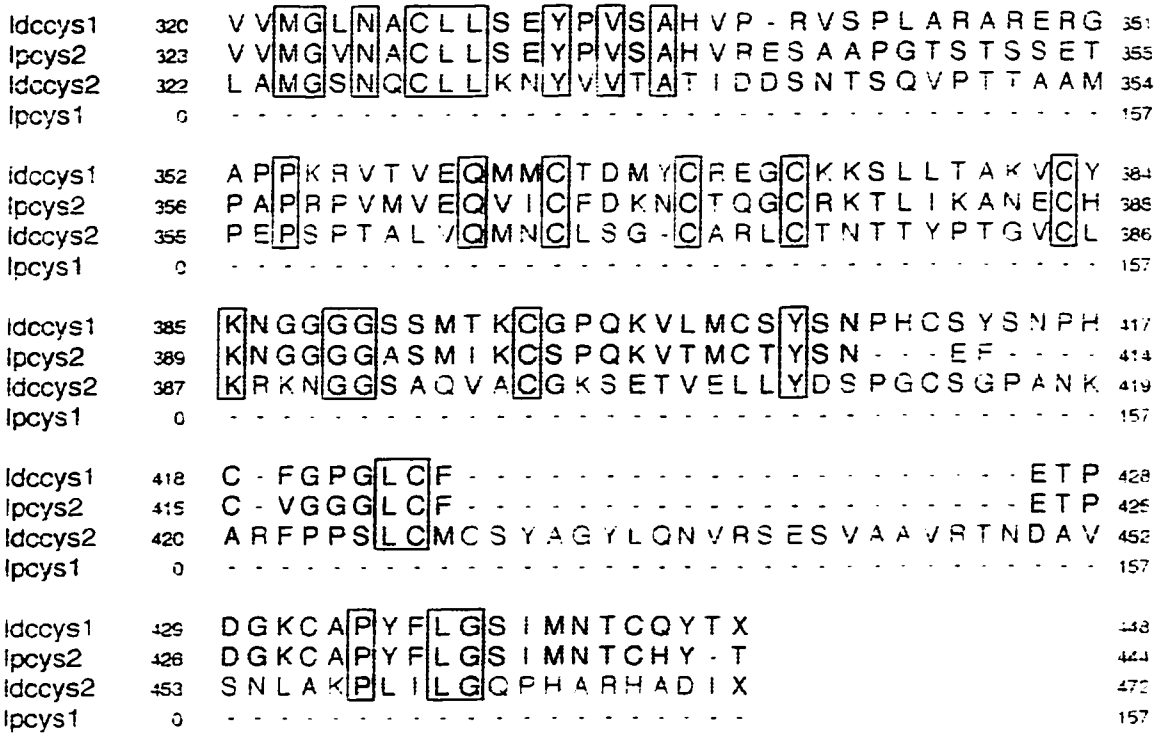

ldccys1 320 V V M G L N A C L L S E Y P V S A H V P - R V S P L A R A R E R G 351
lpcys2 323 V V M G V N A C L L S E Y P V S A H V R E S A A P G T S T S S E T 355
ldccys2 322 L A M G S N Q C L L K N Y V V T A T I D D S N T S Q V P T T A A M 354
lpcys1 0 - 157
ldccys1 352 A P P K R V T V E Q M M C T D M Y C R E G C K K S L L T A K V C Y 384
lpcys2 356 P A P R P V M V E Q V I C F D K N C T Q G C R K T L I K A N E C H 388
ldccys2 355 P E P S P T A L V Q M N C L S G - C A R L C T N T T Y P T G V C L 386
lpcys1 0 - 157
ldccys1 385 K N G G G G S S M T K C G P Q K V L M C S Y S N P H C S Y S N P H 417
lpcys2 389 K N G G G G A S M I K C S P Q K V T M C T Y S N - - - E F - - - - 414
ldccys2 387 K R K N G G S A Q V A C G K S E T V E L L Y D S P G C S G P A N K 419
lpcys1 0 - 157
ldccys1 418 C - F G P G L C F - E T P 428
lpcys2 415 C - V G G G L C F - E T P 425
ldccys2 420 A R F P P S L C M C S Y A G Y L Q N V R S E S V A A V R T N D A V 452
lpcys1 0 - 157
ldccys1 429 D G K C A P Y F L G S I M N T C Q Y T X 448
lpcys2 426 D G K C A P Y F L G S I M N T C H Y - T 444
ldccys2 453 S N L A K P L I L G Q P H A R H A D I X 472
lpcys1 0 - - - - - - - - - - - - - - - - - - 157

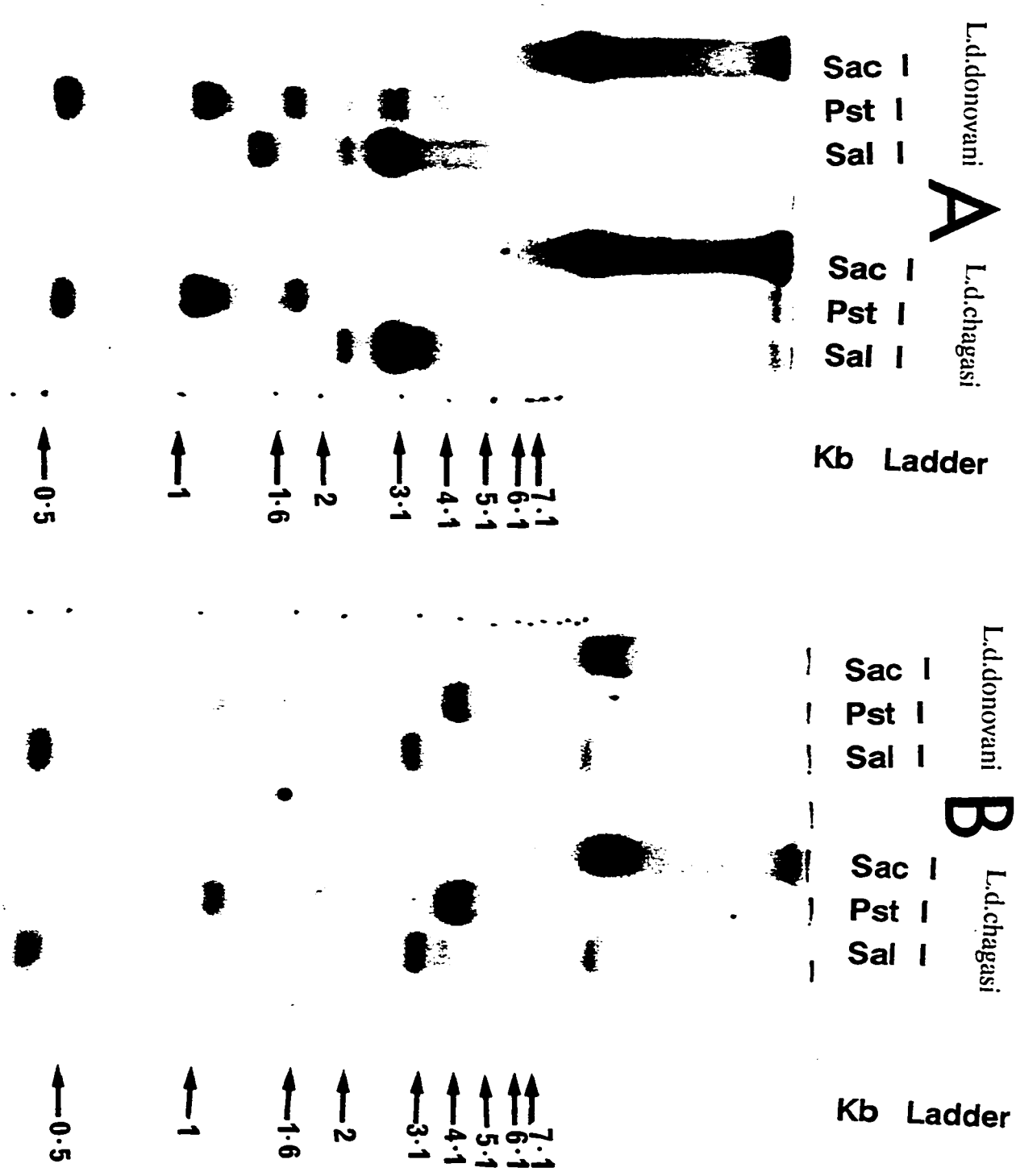
The pro-region for Ldccys1 appears to be cleaved between amino acid residues 123 and 124 generating an alanine as in the case of Lpcys2 and other cysteine proteases. Similarly, the pro-region of Ldccys2 also appears to have a cleavage site between amino acids 127 and 128 resulting in a glycine and this position has been shown to be different in other cysteine proteases (Fig. 10; Souza *et al.*, 1992). Like other cysteine proteases, the pro-region of both Ldccys1 and Ldccys2 is followed by a mature protease core and a C-terminal extension region. The cleavage site between the mature protease core and the C-terminal extension region found in cruzain (Eakin *et al.*, 1992), which is Val-Gly/-Gly-Pro was not found in both *L. d. chagasi* cysteine proteases and thus, the cleavage site at this point is not clear. In addition, a stretch of amino acid sequences rich in Ala, Gly, Pro, Ser, and Thr which have been described downstream of the cleavage site of the mature protease core and C-terminal region for *L. pifanoi* and *L. mexicana* cysteine proteases were not quite evident in both *L. d. chagasi* cysteine proteases. In the C-terminal region, Ldccys1 and Ldccys2 contain 10 and 7 cysteine residues respectively which align with the cysteine residues in other cysteine proteases (Fig. 10; Souza *et al.*, 1992). In addition, Ldccys2 has two major insertions between amino acids 30-36 and 429-449 (Fig. 10). Taken together, these structural comparisons strongly suggest that the cDNAs presented in chapter 1 of this thesis are cysteine proteases.

D. Southern blot analysis

In order to understand the organization of cysteine protease genes in the *Leishmania* genome, two identical Southern blots of *L. chagasi* genomic DNA digested with *Sac*I, *Pst*I

and *Sal*I restriction enzymes were prepared as described under materials and methods. One blot was hybridized with ^{32}P labelled 500 bp fragment of Ldcys1 and the other blot was hybridized with ^{32}P labelled 500 bp fragment from Ldccys2. The results from these experiments are shown in Fig. 11. In the blot hybridized with the probe from Ldccys1 (panel A); *Sac*I digest gave a single hybridizable band of about 15 kb (this was clear when the blot was underexposed); *pst*I digest gave a strong band at about 1.1 kb and two fainter bands of 0.6 kb and 1.7 kb. *Sal*I gave a strong band at about 3.1 kb with two fainter bands at 3.3 kb and 2.2 Kb. On the other hand, in the blot hybridized with probe Ldccys2 probe (panel B); *Sac*I gave two closely related bands at about 14 kb and 15 kb, *pst*I digest gave a strong band of 4.1 kb and a faint one of 1.2 kb. *Sal*I gave a strong band at about 3.1 Kb and two faint bands at 0.45 kb and 14 kb. Similar bands were detected in the blots of *L. d. donovani* hybridized with probes from amastigote and promastigotes specific cysteine protease genes. This analysis shows that the amastigote and Ldccys1/Lddcys1 cysteine protease genes display different patterns in *L. d. chagasi* and *L. d. donovani*. However, the pattern of cysteine protease hybridization of Ldccys1/Ldccys1 as well as Ldccys2/Ldccys2 cysteine protease genes between *L. d. chagasi* and *L. d. donovani* is similar. Taken together, this data indicates that the two *L. d. chagasi* cysteine protease genes show different patterns of hybridization and therefore represent two distinct genes and the corresponding genes exist in *L. d. donovani* and are arranged in a similar manner.

Figure 11. Southern blot analysis of cysteine protease of *L. donovani donovani* and *L. donovani chagasi*. Panel A was hybridized with a probe from Ldccys1 and exposed for 12 hours. Panel B was hybridized with a probe from Ldccys2 and exposed for 48 hours.


A
L.d.donovani
Sac I
Pst I
Sal I
L.d.chagasi
Sac I
Pst I
Sal I
Kb Ladder
0·5
1
1·6
2
3·1
4·1
5·1
6·1
7·1
B
L.d.donovani
Sac I
Pst I
Sal I
L.d.chagasi
Sac I
Pst I
Sal I
Kb Ladder
0·5
1
1·6
2
3·1
4·1
5·1
6·1
7·1

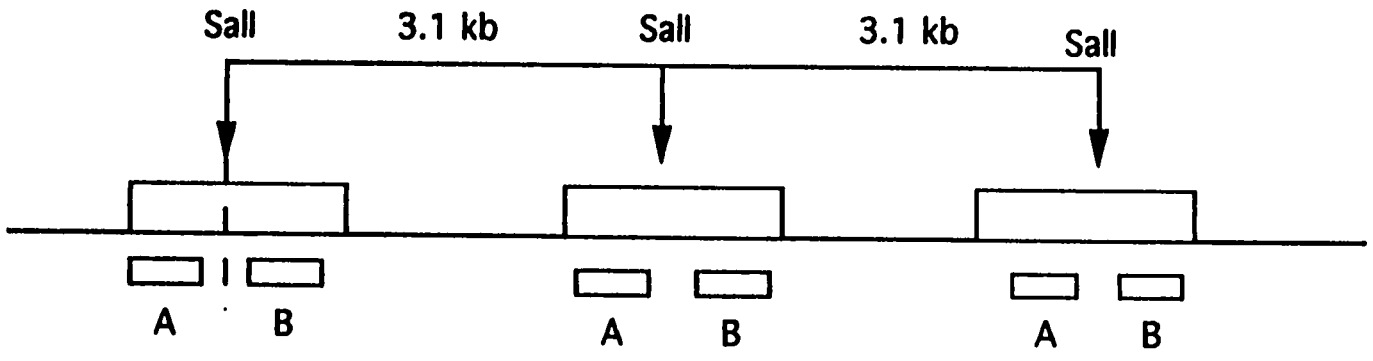
E. Test for genes which are arranged in tandem

The theory of the test for genes which are arranged in tandem states that a restriction enzyme which cuts the gene cloned once such as *SalI* in Fig. 12 must first be identified. Then two identical Southern blots of genomic DNA digested with that restriction enzyme are prepared (Fig. 12). One blot is hybridized with a probe representing sequences 5' end to the *SalI* site (probe A) while the other is hybridized with a probe representing sequences 3' end to the *SalI* site (probe B) (Fig. 12). A band of the same size in both blots such as 3.1 kb in Fig 12 indicates that the gene being tested belongs to a family of genes which are arranged in tandem.

F. Arrangement of cysteine protease in the *L. d. chagasi* genome

In order to determine the arrangement of cysteine protease genes in the genome of *L. d. chagasi*, we tested whether the cysteine protease genes we have isolated are arranged in tandem. To do this, a restriction enzyme which cuts the cDNA cloned once was first identified. *SalI* and *StuI* were found to cut amastigote specific cDNA (Ldccys1) and promastigote specific cDNA (Ldccys2) respectively once. Thus, genomic DNA from *L. d. chagasi* was digested with these enzymes separately to completion and the two identical blots were prepared for each digest. A duplicate blot from *SalI* digest was hybridized with a probe representing sequences 5' end to the *SalI* site. The other duplicate blot was hybridized with a probe representing sequences 3' end to the *SalI* site.

Figure 12. Illustration of the theory of test for genes which are arranged in tandem. The states that a restriction enzyme which cuts the gene cloned once must first be identified. For example, we found that SalI cuts Ldccys1 once. One blot is hybridized with a probe representing sequences 5' end to the *SalI* site (probe A) while the other is hybridized with a probe representing sequences 3' end to the *SalI* site (probe B). Detection of bands of equal size (eg. 3.1 kb) in both blots is suggestive of a tandem arrangement of genes.



SalI Southern

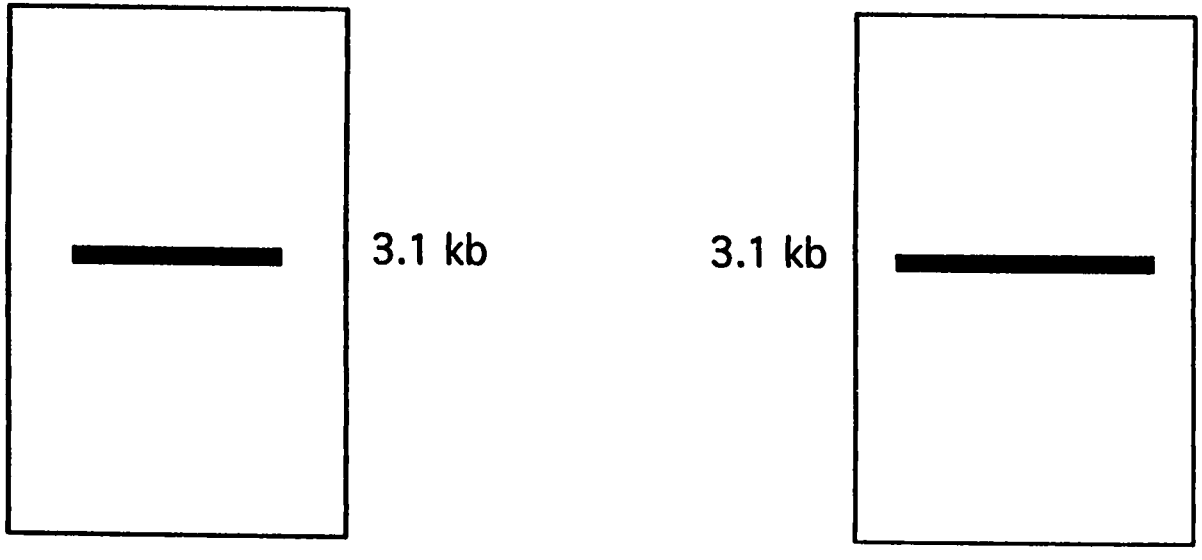
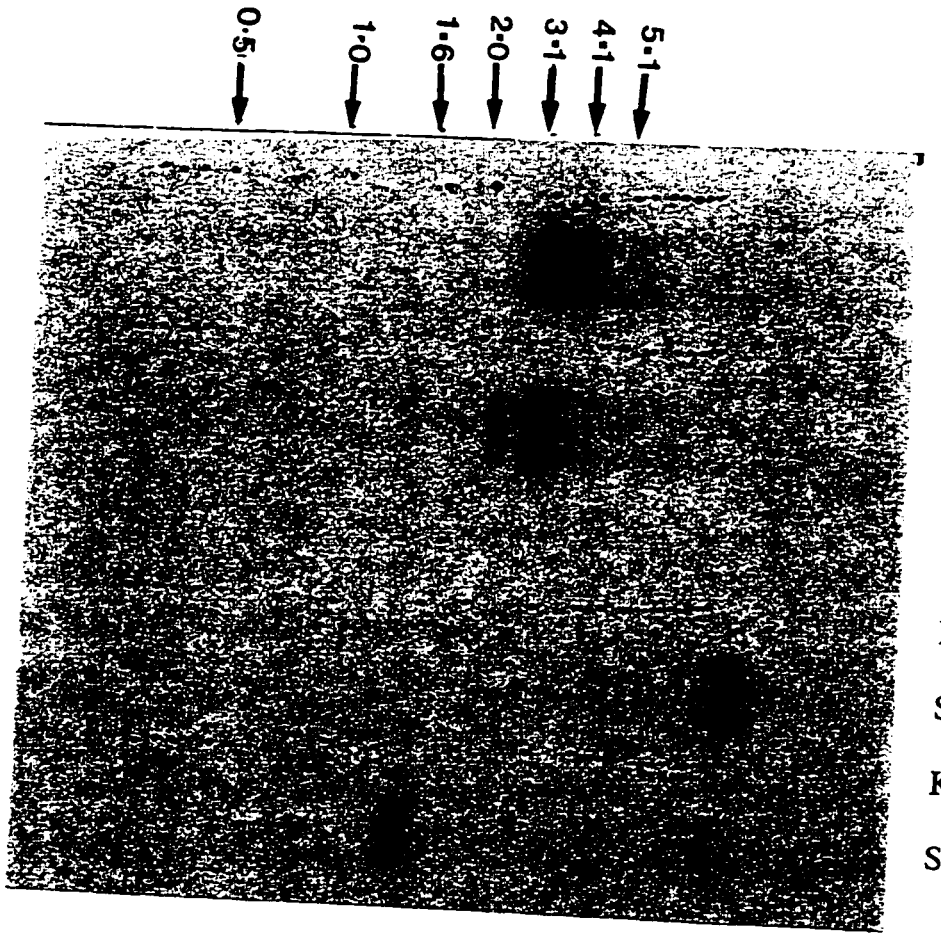


Figure 13. Arrangement of Ldccys1 and Ldccys2 genes. Autoradiographic results from the Southern blot analysis. Panel A: The lane labelled *Sal*I 5' was hybridized with probe representing sequences 5' to *Sal*I site in Ldccys1. The lane labelled *Sal*I 3' was hybridized with probe representing sequences 3' to *Sal*I site in Ldccys1. Panel B: The lane labelled *Stu*I 5' was hybridized with probe representing sequences 5' to *Stu*I site in Ldccys2. The lane labelled *Stu*I 3' was hybridized with probe representing sequences 3' to *Stu*I site in Ldccys2. Ldccys1 = amastigote specific cysteine protease from *L. d. chagasi*; Ldccys2 = promastigote specific cysteine protease from *L. d. chagasi*.


0·5
1·0
1·6
2·0
3·1
4·1
5·1
Kb
Sal I: 5'
Kb
Sal I: 3'
A
Kb
Stu I: 5'
Kb
Stu I: 3'
B

Similarly, hybridization was carried out using probes representing sequences 5' end and 3' end of *Stu*I site of promastigote specific cDNA. The results of these tests are shown in Fig. 13. As shown in panel A, a 3.1 kb fragment hybridized when the blot was probed with either probes. However, the 5' end probe hybridized to a 3.6 kb fragment. On the other hand, a 2.4 kb fragment hybridized faintly to the 3' end probe. The 3.6 kb and 2.4 kb bands are probably the 5' and the 3' ends of the amastigote specific cysteine protease gene cluster. Similar analysis was performed on promastigote specific cysteine protease gene(s). As shown in panel B, 15 kb and 2.8 Kb hybridizable bands were obtained using 5' end and 3' end probes respectively. From this data, it appears that there is at least one Ldccys2 cysteine protease gene in the *L. d. chagasi* genome while the Ldccys1 cysteine protease genes are represented by multi-copy genes which are arranged in tandem.

G. Chromosomal localization of cysteine protease genes

In an attempt to find out the chromosomes where each of the cysteine protease genes is located in the *Leishmania* genome, pulsed field gel electrophoresis was used to resolve chromosome size DNA. The resolved DNA was blotted and hybridized separately with ^{32}P labelled 500 bp fragment as probe containing (i) both Ldccys1 and Ldccys2, (ii) Ldccys1 and (iii) Ldccys2. The results of these analysis are shown in Figures 14, 15 and 16. Ethidium bromide stained gels showing the separation of *L. d. chagasi* (Ldc) and *L. d. donovani* (Ldd) are presented in panels A of Figures 14, 15 and 16. When probes containing a mixture of Ldccys1 and Ldccys2 sequences were used, two hybridizable bands, one very strong and one very faint, were detected in both *L. d. chagasi* and *L. d. donovani* (Fig. 14; panel B). In *L.*

d. chagasi, the strong band is located on chromosome 7 and a weak one on chromosome 10. On the other hand, in *L. d. donovani*, the strong and the faint bands are located on chromosomes 5 and 8 respectively. The chromosomal numbering was on the basis of counting from the smallest visible chromosome size DNA.

In order to determine the chromosomal localization of each of the cysteine protease gene, we hybridized the blot with specific probes. When the blot was hybridized with the probe containing Ldccys1 sequences, only the strong band was observed in both *L. d. chagasi* and *L. d. donovani* genomes (Fig. 15; panel B). This data suggested that in *L. d. chagasi*, the Ldccys1 cysteine protease genes are located on chromosome 7 while the corresponding cysteine protease genes in *L. d. donovani* (Lddcys1) are located on chromosome 5. On the other hand, when the blot was hybridized with the probe containing Ldccys2 sequences, two hybridizable bands were obtained in both *L. d. chagasi* and *L. d. donovani* genomes (Fig. 16; panel B). The stronger bands were on chromosome 10 for *L. d. chagasi* and 8 of *L. d. donovani* genomes. The fainter bands were on chromosome 7 of *L. d. chagasi* and 5 of *L. d. donovani* genomes. Interestingly, the position of the faint hybridizable bands is in the same chromosomes for Ldccys1 cysteine protease genes. It is not clear at present whether a Ldccys2 cysteine protease gene is located on that same chromosome.

Figure 14. Chromosomal location of cysteine protease genes from *L. d. chagasi* (Ldccys1 and Ldccys2) and and the corresponding genes from *L. d. donovani* (Lddcys1 and Lddcy2). Panel A ethidium bromide stained gel showing separation of chromosome from *L. d. chagasi* (L.d.c) and *L. d. donovani* (L.d.d.) using pulsed field gel electrophoresis (PFGE). Panel B is Southern blot of PFGE hybridized with the 500 bp DNA fragment from both Ldccys1 and Ldccys2. The numbers in panel A indicate the chromosomal location.

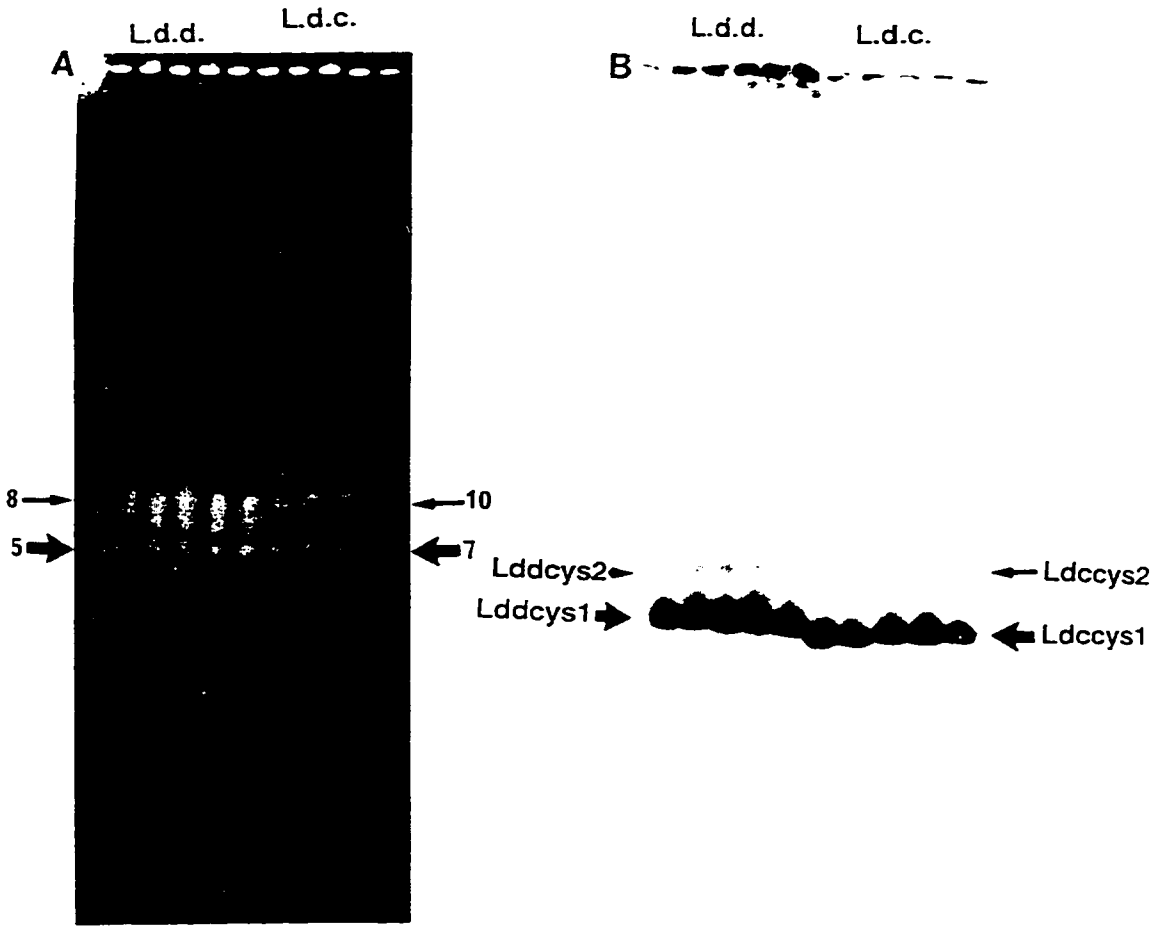

L.d.d.
L.d.c.
A
8
5
10
7
L.d.d.
L.d.c.
B
Lddcys2
Lddcys1
Ldccys2
Ldccys1

Figure 15. Chromosomal location of Ldccys1 cysteine protease genes from *L. d. chagasi* and the corresponding Lddcys1 cysteine protease genes from *L. d. donovani*. Panel A ethidium bromide stained gel showing separation of chromosome from *L. d. chagasi* (L.d.c) and *L. d. donovani* (L.d.d.) using pulsed field gel electrophoresis (PFGE). Panel B is Southern blot of PFGE hybridized with the 500 bp DNA fragment from Ldccys1. The numbers in panel A indicate the chromosomal location.

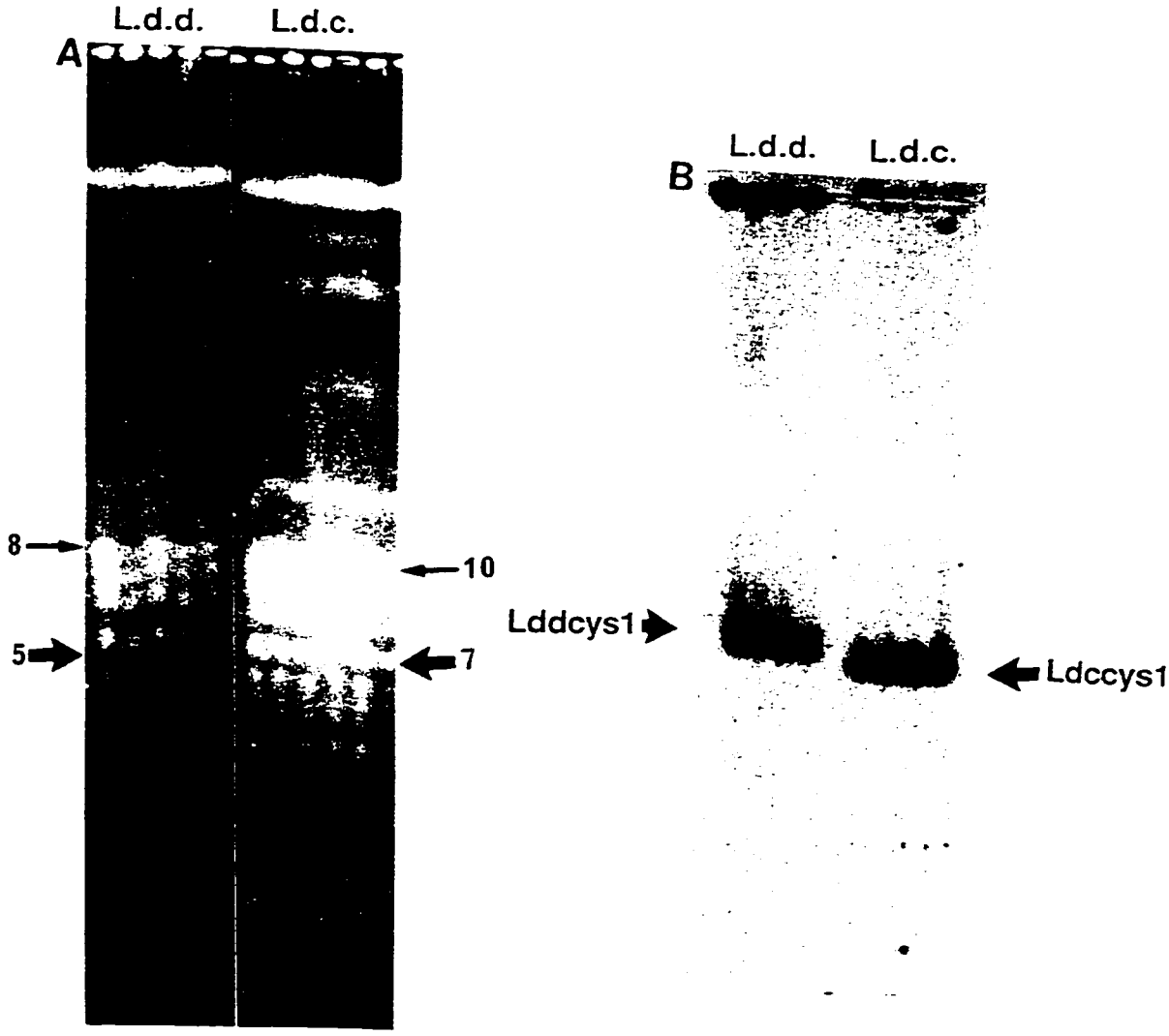
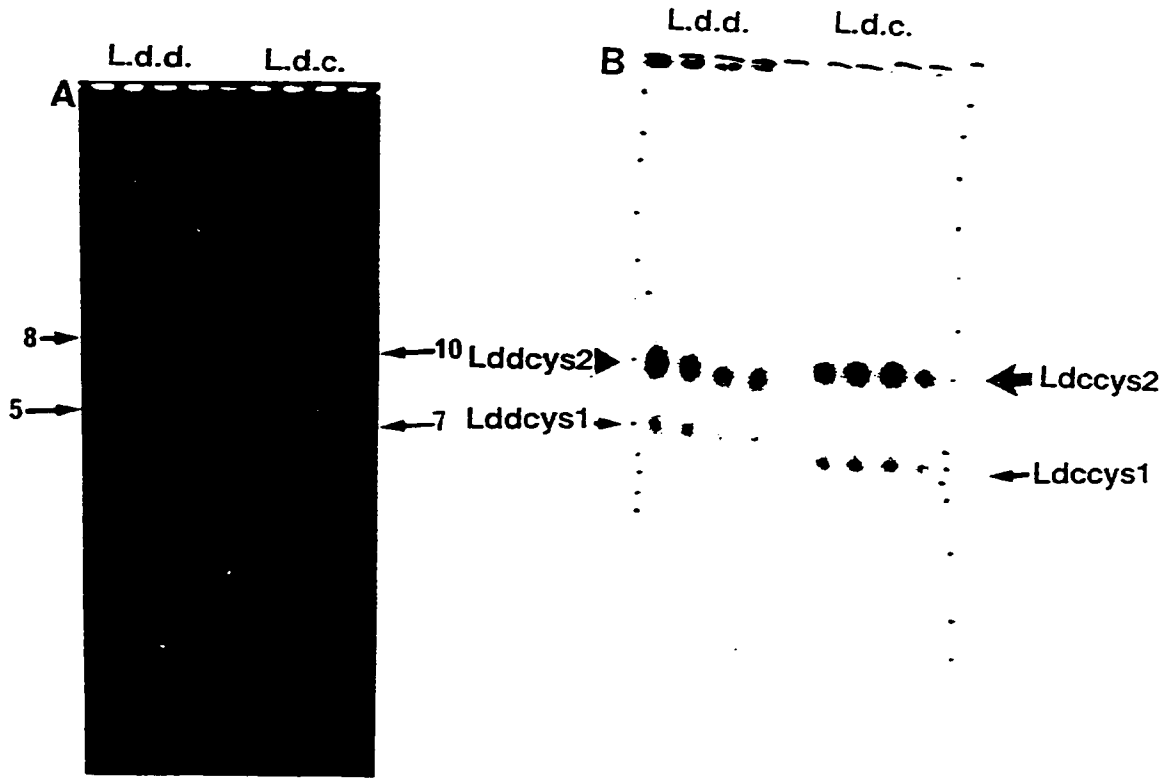

L.d.d.
L.d.c.
A
8
10
5
7
B
L.d.d.
L.d.c.
Lddcys1
Ldccys1

Figure 16. Chromosomal location of Ldccy2 cysteine protease gene from *L. d. chagasi* and the coresponding Lddcy2 cysteine protease gene *L. d. donovani*. Panel A ethidium bromide stained gel showing separation of chromosome from *L. d. chagasi* (L.d.c) and *L. d. donovani* (L.d.d.) using pulsed field gel electrophoresis (PFGE). Panel B is Southern blot of PFGE hybridized with the 500 bp DNA fragment from Ldccys2. The numbers in panel A indicate the chromosomal location.

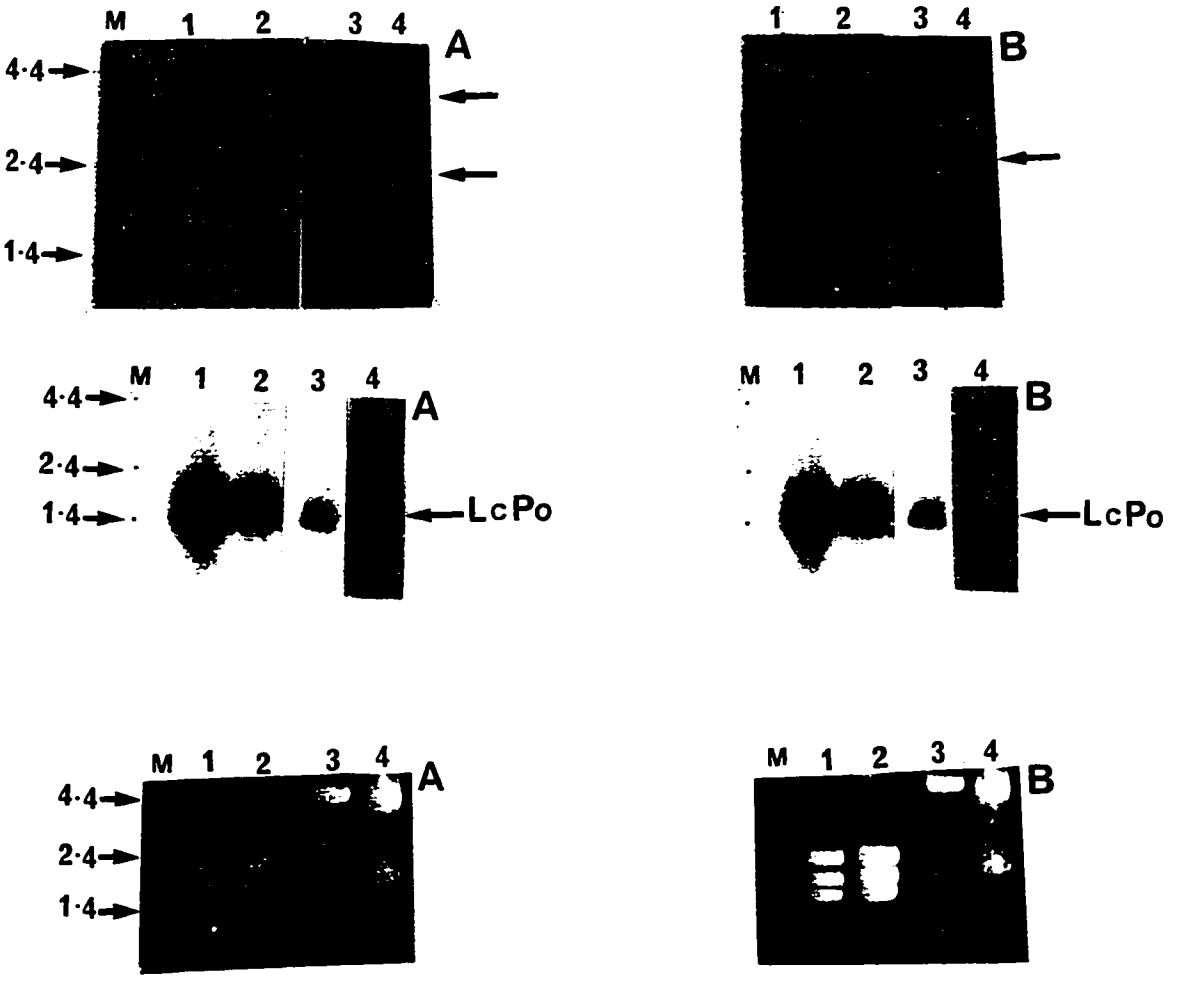

L.d.d.
L.d.c.
A
8
5
10 Lddcys2
7 Lddcys1
L.d.d.
L.d.c.
B
Ldccys2
Ldccys1

Alternatively, the probe may have cross-reacted with related sequences in the Ldccys1 cysteine protease gene cluster. In any event, this data suggest that the Ldccys2 cysteine protease gene is located on chromosome 10 of *L. d. chagasi* and 8 of *L. d. donovani* genomes. Taken together, this data shows that the two distinct cysteine protease genes are located on two separate chromosomes in *L. d. chagasi* genome and the organization of the corresponding genes are similar in *L. d. donovani* genome.

H. mRNA expression of Ldccys1 and Ldccys2

In order to determine the expression pattern of the two distinct cysteine protease genes, we examined the level of mRNAs using Northern blot analysis. For this purpose, we utilized the 500 bp fragment probe specific for each cysteine protease gene. We isolated total RNA from logarithmic and stationary stages of promastigote as well as from amastigotes as described in materials and methods. As shown in Fig. 17 B (top panel), a 2.6 kb message (this mRNA is the expected size from Ldccys2 cysteine protease cDNA sequence) hybridized to the Ldccys2 probe in the logarithmic (lane 1), stationary (lane 2) and amastigote (lane 3) and this message is not detectable in the spleens from uninfected hamsters (lane 4). On the other hand, when the same blot was hybridized with Ldccys1 cysteine protease probe, a predominant 2.4 kb message (this mRNA is the expected size from the Ldccys1 cysteine protease cDNA sequence) with moderately abundant 4.0 kb transcript and very low level of transcript at approximately 5.0 kb were detected in the RNA from amastigote stage (Fig. 17

Figure 17. Northern blot analysis of Ldccys1 and Ldccys2 transcripts. Total RNA isolated from: lane 1 = log phase promastigotes; lane 2 = stationary phase promastigotes; lane 3 = spleens of hamsters infected with *L. d. chagasi*, and Lane 4 = spleens of uninfected hamsters. Top panels A and B are blots hybridized with Ldccys1 and Ldccys2 respectively. The blot shown in panel A was first hybridized with a Ldccys1 probe, then it was stripped and rehybridized with a Ldccys2 probe (pane B). The middle panels are blots hybridized with LcPO probe which is constitutively expressed in *L. d. chagasi* and used as internal control. All blots were exposed for 16 hours except lanes 3 and 4 (top panel A) which were exposed for 48 hours. The bottom panels A and B are ethidium bromide stains of RNA used as loading controls. *Leishmania* RNA has three bands. RNA from spleens of infected hamsters shows 28S and 18S (strong bands). The two faint bands are due to *Leishmania* RNA. However, the third band co-migrate with the 18S RNA from macrophages of infected hamsters. The arrows indicate transcript sizes and the size of the RNA marker (M) is also shown. *L. chagasi* ribosomal phosphoprotein (LcPO) which is produced constitutively and the message is at 1.4 kb (cDNA of LcPo was kindly provided by Dr. Steven G. Reed, Infectious Disease Research Institute, Seattle, WA 98109).


M 1 2 3 4
A
4·4
2·4
1·4
1 2 3 4
B
M 1 2 3 4
A
4·4
2·4
1·4
LcPo
M 1 2 3 4
B
LcPo
M 1 2 3 4
A
4·4
2·4
1·4
M 1 2 3 4
B

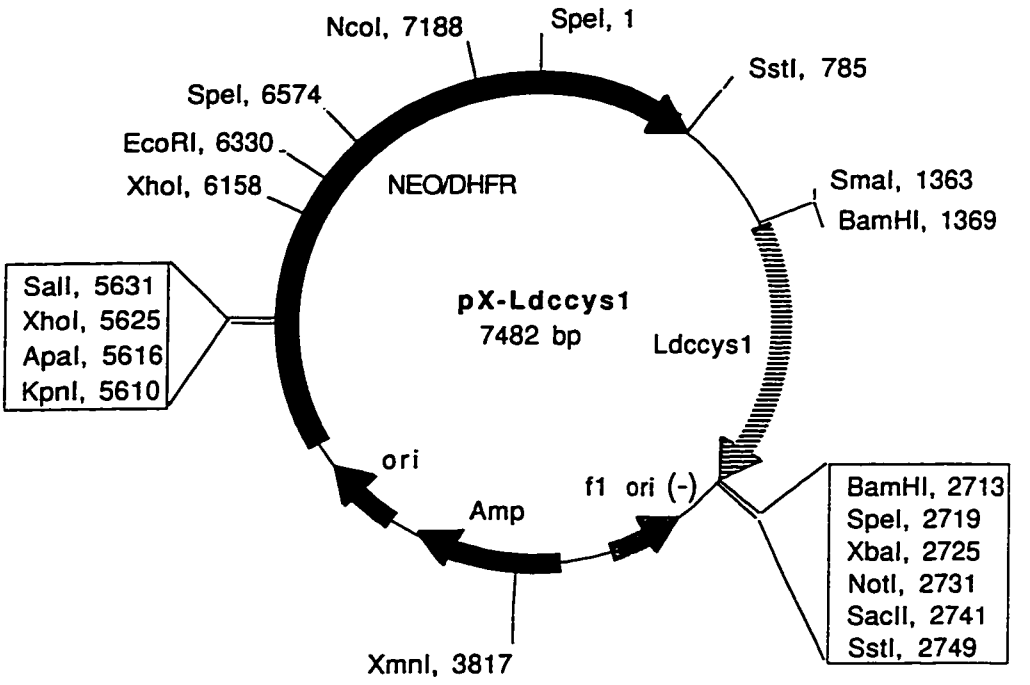
A; lane 3), but not in the logarithmic and stationary stages of the promastigote (Fig 17 A; lanes 1 and 2) and in the spleens of uninfected hamsters (Fig. 17 A; lane 4). It is interesting to note that the level of Ldccys1 expression is significantly higher than that of Ldccys2 in the amastigotes considering that the same blot was used for both hybridizations (Fig. 17 top panels A and B) after stripping the first probe used from the membrane. The levels of loading were assessed by stripping the blots and rehybridizing with LcPO which is expressed constitutively (Fig 17 A and B; middle panels) as well as from ethidium bromide stained gels (Fig. 17 A and B; lower panels). The faint hybridizable LcPO bands in the amastigote is due to low amount of the RNA from the amastigotes. Taken together, our data suggest that Ldccys1 genes are expressed abundantly in the amastigote stage of development but at very low level in the promastigote stage of development. Conversely, the Ldccys2 gene is expressed in both the amastigote and promastigote stages of development.

I. Overexpression of cysteine protease in *L. d. chagasi* cells

In order to show that the two cDNAs code for cysteine proteases, each cDNA was cloned in the pX vector (Fig. 18; panels A and B). *L. d. chagasi* promastigotes were transfected with the recombinant plasmids and selected with G418. The presence of the plasmids containing the cDNAs were confirmed by Southern blot analysis of *Bam*HI digested total DNA from transfected cells. As shown in Fig. 19, the cells transfected with recombinant plasmids show hybridizable bands at positions

Figure 18. Top panel: Map of pX vector showing how the coding region of Ldccy1 cysteine protease was ligated at the *BamH*I site giving recombinant plasmid pX-Ldccy1. The coding region of Ldccys1 is 1344 bp.

Bottom panel: Map of pX vector showing how the coding region of Ldccys2 cysteine protease was ligated at the *Bam*H I site giving recombinant plasmid pX-Ldccy2. The coding region of Ldccys2 is 1416 bp.


NcoI, 7188
SpeI, 1
SstI, 785
SpeI, 6574
EcoRI, 6330
XhoI, 6158
NEO/DHFR
SmaI, 1363
BamHI, 1369
SalI, 5631
XhoI, 5625
ApaI, 5616
KpnI, 5610
pX-Ldccys1
7482 bp
Ldccys1
ori
f1 ori (-)
Amp
BamHI, 2713
SpeI, 2719
XbaI, 2725
NotI, 2731
SacII, 2741
SstI, 2749
XmnI, 3817

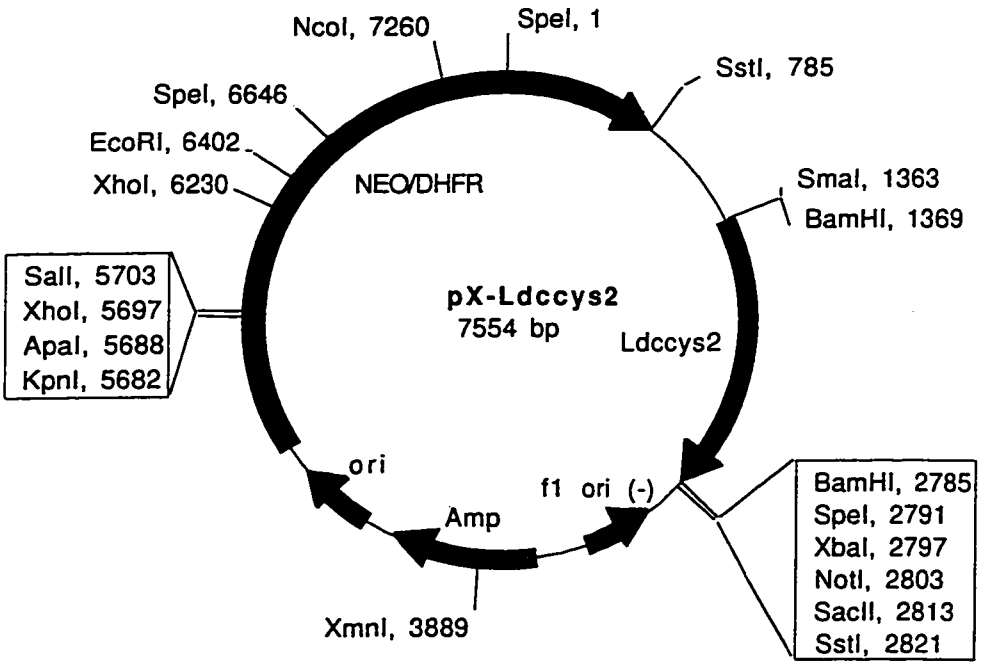
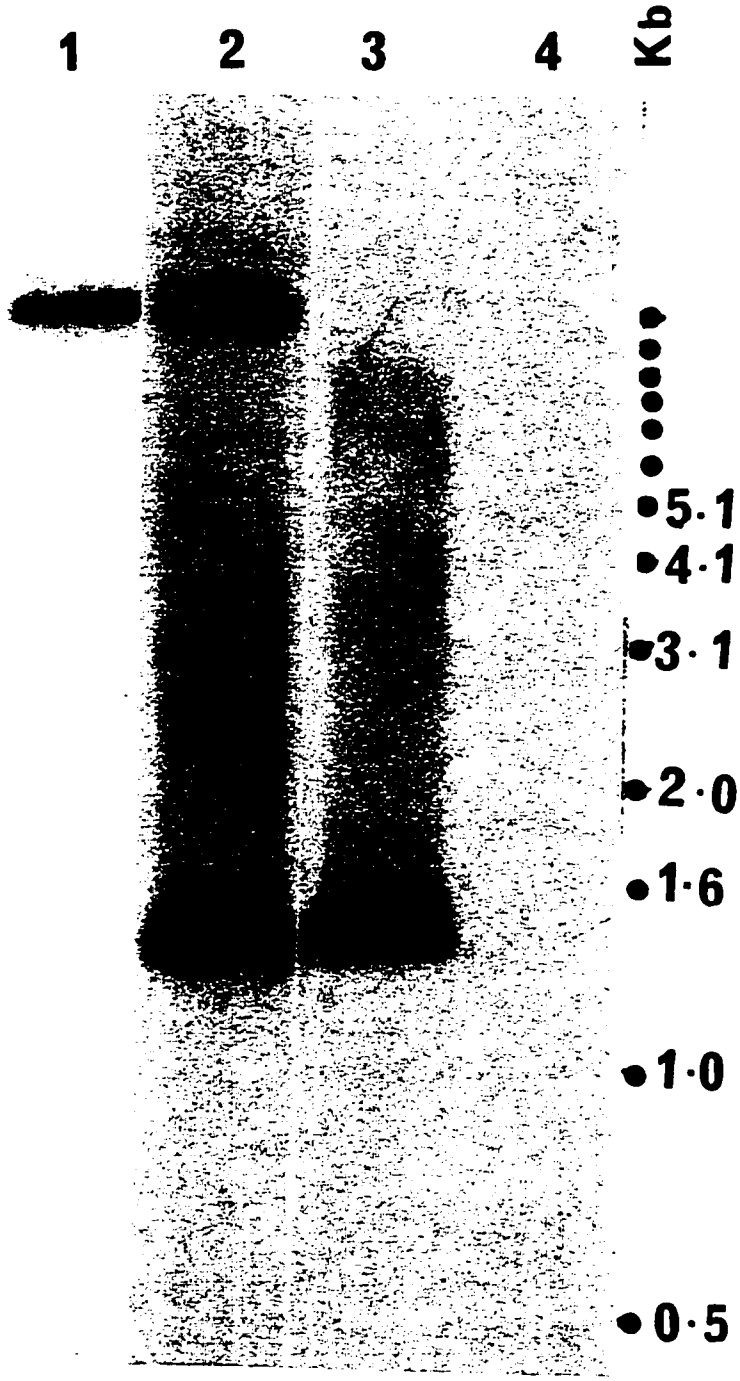

NcoI, 7260
SpeI, 1
SstI, 785
SpeI, 6646
EcoRI, 6402
XhoI, 6230
NEO/DHFR
SmaI, 1363
BamHI, 1369
SalI, 5703
XhoI, 5697
ApaI, 5688
KpnI, 5682
pX-Ldccys2
7554 bp
Ldccys2
ori
f1 ori (-)
Amp
BamHI, 2785
SpeI, 2791
XbaI, 2797
NotI, 2803
SacII, 2813
SstI, 2821
XmnI, 3889

Figure 19. Southern blot analysis of total DNA from transfected *L. d. chagasi* cells. The total DNA was digested with *Bam*H I. Lanes 1 and 4 = pX transfected cells. Lanes 2 and 3 = pX containing Ldccys1 and Ldccys2 cysteine protease cDNAs respectively. Lanes 1 and 2 were hybridized with Ldccys1 cDNA probe while Lanes 3 and 4 were hybridized with Ldccys2 cDNA probe. The blots were exposed for 1 hour. The strong bands at 1.3 Kb and 1.4 Kb in lanes 2 and 3 indicate the presence of the coding regions of Ldccys1 and Ldccys2 respectively. The upper bands in lanes 1 and 2 are due to indigenous Ldccys1 in *L. d. chagasi* genome. Indigenous Ldccys2 could not be detected in lanes 3 and 4 during the 1 hour exposure of the blots because it is less abundant in *L. d. chagasi* genome.


1
2
3
4
Kb
5·1
4·1
3·1
2·0
1·6
1·0
0·5

1.3 kb (lane 2) and 1.4 kb (lane 3) for Ldccys1 and Ldccys2 cysteine protease genes respectively. However, such bands were absent from cells transfected with the pX vector alone (lanes 1 and 4). In addition, a band representing each cysteine protease gene in the genome was also detected. These results demonstrate the presence of the constructs in the transfected cells.

In order to show that the transfected cells are over-producing biologically active cysteine proteases, enzymatic activities of cell lysates were analyzed in gelatin SDS-PAGE (non-denaturing). Only cells transfected with recombinant constructs were found to over-produce biologically active cysteine proteases (Fig. 20, panel A; lanes 1 and 3). Enzymatic activities were seen at about 43 kDa and 30 kDa (lanes 1 and 3), the latter appears to be the processed form of cysteine proteases (Duboise *et al.*, 1994). When a similar gel is incubated in a buffer which included specific inhibitors to cysteine proteases, the enzymatic activities seen at about 43 kDa and 30 kDa disappeared (Fig. 20; panel B), confirming that the enzymatic activities are indeed due to the over-expressed cysteine proteases. This data shows that biologically active cysteine proteases are over-produced in *L. d. chagasi* promastigotes transfected with the recombinant plasmids.

In order to further demonstrate the overexpression, protein extracts from cells transfected with the recombinant plasmid containing Ldccys1 cysteine protease cDNA were analyzed by Western blot analysis using a specific polyclonal antibody generated by direct DNA injection technology as described in the literature (Ulmer *et al.*, 1993; Waine and McManus, 1995; McDonnell and Askari, 1996; Ulmer *et al.*, 1996). We used the recombinant plasmid outlined in Figure 21 (top panel) to generate the antibody. As shown

in Fig. 22, a 43 kDa band corresponding to the unprocessed form of cysteine protease was detected. This band was present at higher level in the transfectants (lanes 1 and 2) compared to the wild type transfected with the pX vector alone (lane 3). In addition, there are minor bands at about 26 kDa which are probably due to the processed forms of this enzyme. This data further confirms the overexpression of Ldccys1 cysteine proteases in *Leishmania* cells. However, the difficulty of raising polyclonal antibody to Ldccys2 cysteine protease using recombinant plasmid outlined in Figure 21 (bottom panel) prevented us from carrying out similar analysis on protein extracts from cells transfected with the recombinant plasmid containing Ldccys2 cysteine protease cDNA.

Figure 20. Panel A: Gelatin SDS-PAGE (non-denaturing) showing the enzymatic activity of biologically active cysteine proteases over-expressed in *Leishmania* cells. Panel B: Shows a similar gel incubated with inhibitors of cysteine proteases (2 mM leupeptin and 2 mM iodoacetamide). The enzymatic activity of wild type gp63 at 63 KDa and above was not affected by inhibitors of cysteine proteases and confirms equal loading. Labels M = marker; 1 = Ldc+pX+4-12 = clone of *Leishmania* cells transfected with pX having Ldccys1 cysteine protease cDNA in the correct orientation; 2 and 4 = Ldc+pX = clone of *Leishmania* cells transfected with plasmid pX only and 3 = Ldc+pX+34-1 = clone of *Leishmania* cells transfected with plasmid pX having Ldccys2 cysteine protease cDNA in the correct orientation. The enzymatic activity seen at 63 kDa and above are wild type gp63 which confirms equal loading. The size of the proteins is represented by the marker lane (M).

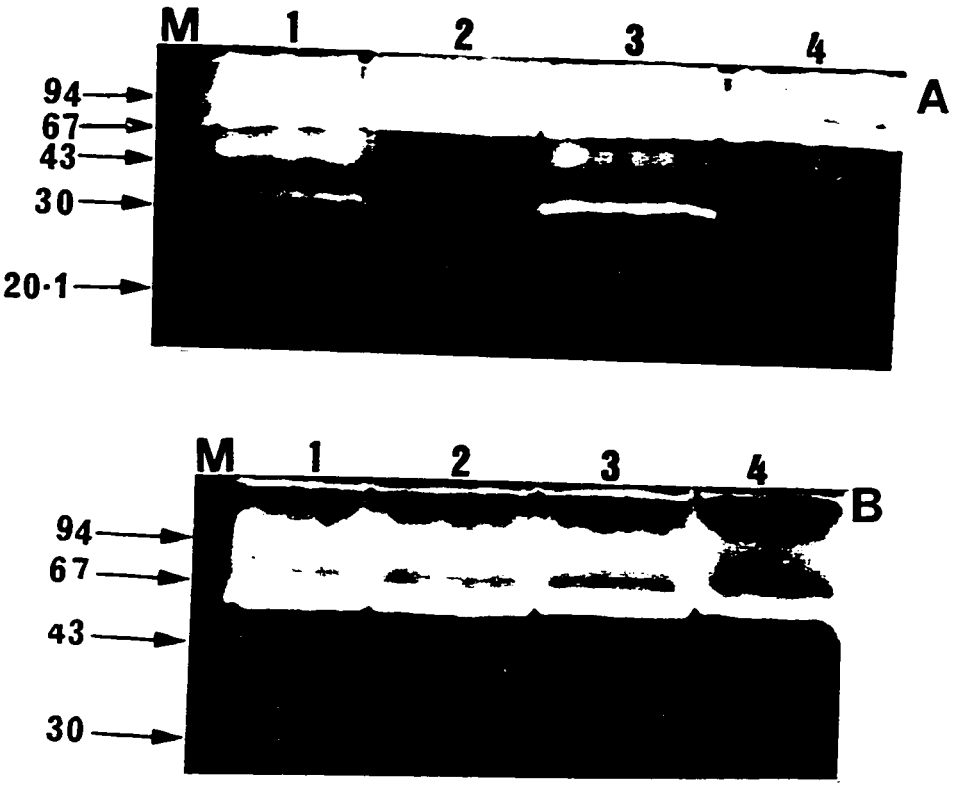
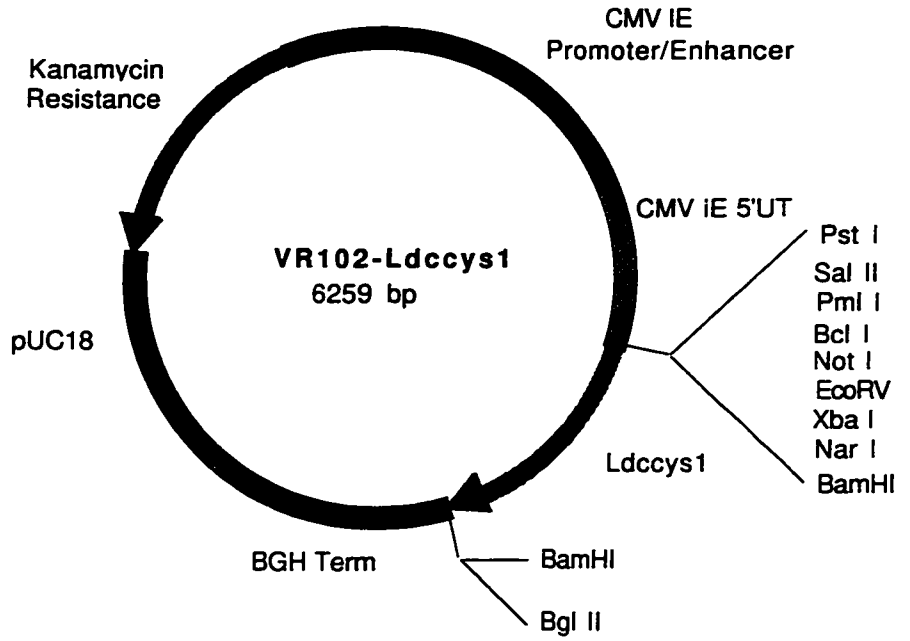

M
1
2
3
4
A
94
67
43
30
20·1
M
1
2
3
4
B
94
67
43
30

Figure 21. Top panel: Map of VR1012 vector showing how the coding region of Ldccy1 cysteine protease cDNA was ligated at the *Bam*HI site giving recombinant plasmid V1012-Ldccy1 which is 6177 bp. The coding region of Ldccys1 is 1344 bp. Bottom panel: Map of VR1012 vector showing how the coding region of Ldccys2 cysteine protease cDNA was ligated at the *Bam*HI site giving recombinant plasmid VR1012-Ldccy2 6259 bp. The coding region of Ldccys2 is 1416 bp. We were not successful in generating antibody using this construct.


CMV IE
Promoter/Enhancer
Kanamycin
Resistance
CMV IE 5'UT
VR102-Ldccys1
6259 bp
Pst I
Sal II
Pml I
Bcl I
Not I
EcoRV
Xba I
Nar I
BamHI
pUC18
Ldccys1
BGH Term
BamHI
Bgl II

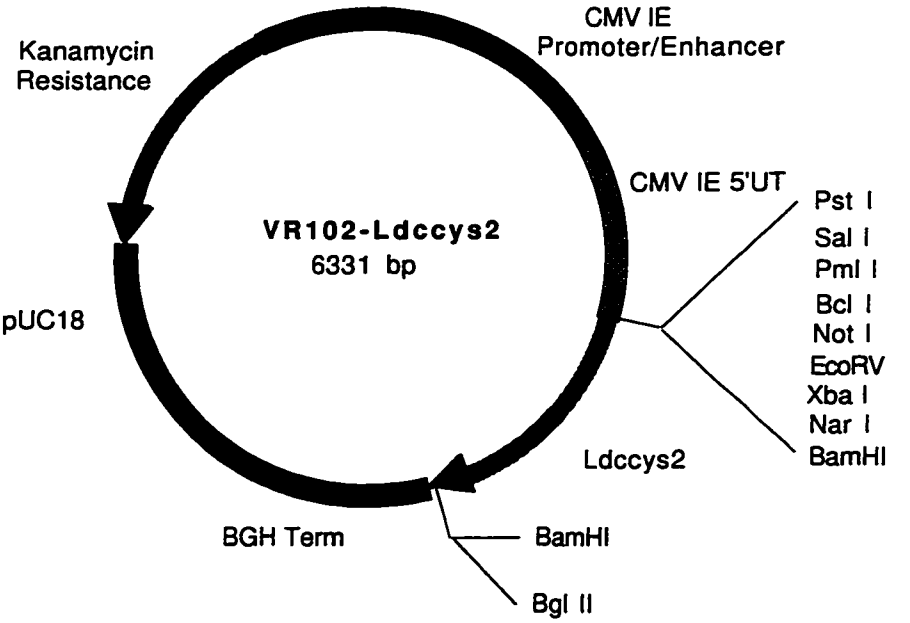
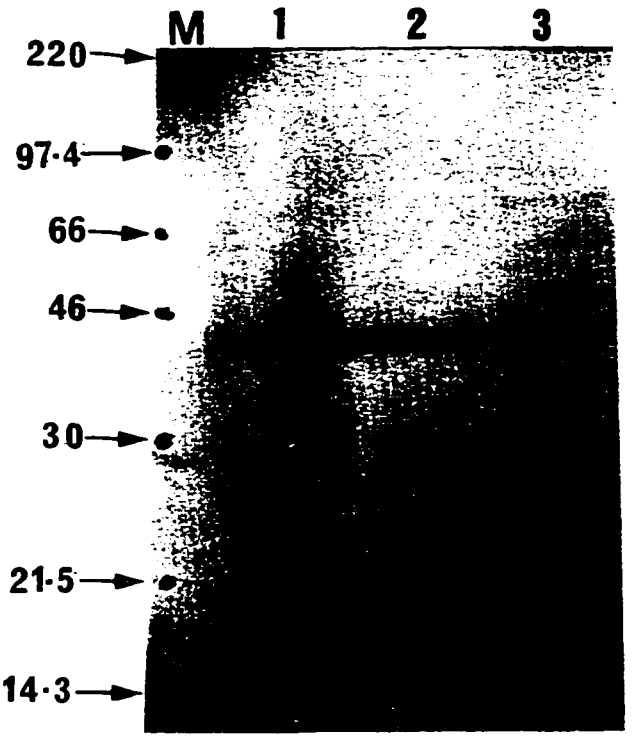

CMV IE
Promoter/Enhancer
Kanamycin
Resistance
CMV IE 5'UT
VR102-Ldccys2
6331 bp
Pst I
Sal I
Pml I
Bcl I
Not I
EcoRV
Xba I
Nar I
BamHI
pUC18
Ldccys2
BGH Term
BamHI
Bgl II

Figure 22. Western blot analysis which demonstrates that Ldccys1 cysteine protease has been overexpressed in *Leishmania* cells. After M = marker in kDa, the two lanes labelled 1 and 2 = Ldc+pX+4-12 = the two cultures of *Leishmania chagasi* cells transfected at two separate occasions with the same plasmid clone (pX+4-12) containing Ldccys1 cysteine protease cDNA. The lane labelled 3 = Ldc = *Leishmania* cells (wild type) which has not been transfected. Antibody was raised in BALB/c mice using direct DNA injection technology described in the thesis.


M
1
2
3
220
97·4
66
46
30
21·5
14·3

DISCUSSIONS

Discussion

For the first time we show that there are two distinct cysteine protease genes in the genome of *Leishmania donvani chagasi*, a member of the *Leishmania donovani* complex, which causes visceral leishmaniasis. The two genes isolated were characterized and found to have structural characteristics of cysteine proteases which have previously been isolated from other Trypanosomatids. However, the two genes differ at the levels of amino acid sequence, genomic organization and gene expression. At the level of the amino acid sequence, there is a 40% homology between Ldccys1 and Ldccys2 which is comparable to the similarity found between Lpcys2 and Lpcys1 of *L. pifanoi* (Traub-Cseko *et al.*, 1993) as well as between lmcpb and lmcpa of *L. mexicana* (Mottram *et al.*, 1992; Souza *et al.*, 1992). This fact indicates that the two genes are distinct and have ancient divergence. However, there is a 75% homology in amino acid sequence between Ldccys1 and Lpcys2 from *L. pifanoi* while Ldccys2 is 78% homologous to Lpcys1 of *L. pifanoi*. This data show that the corresponding cysteine proteases from the two species of *Leishmania* have a high degree of homology.

At the level of genomic organization, we show that Ldccys1 belongs to a family of cysteine proteases encoded by tandemly organized genes while Ldccys2 appears to be a single cysteine protease gene. The organization of the two families of cysteine proteases in *L. d. donovani* was also found to be similar. In this regard, Ldccys1 is similar to other multi-copy cysteine protease genes such as lmcpb from *L. mexicana* (Souza *et al.*, 1992), Lpcys2 from *L. pifanoi* (Traub-Cseko *et al.*, 1993), cysteine protease from *T. brucei* (Mottram *et al.*, 1989) and cruzain from *T. cruzi* (Campetella *et al.*, 1992). On the other hand, Ldccys2

resembles other single copy cysteine protease genes such as lmcpa from *L. mexicana* (Souza *et al.*, 1992) and Lpcys1 from *L. pifanoi* (Traub-Cseko *et al.*, 1993).

At the level of gene expression, we show that the Ldccys1 genes are expressed predominantly in the amastigote stage of development but at very low level at the promastigote stage of development. Thus, this gene is expressed in a stage-specific manner. On the other hand, the Ldccys2 gene is expressed throughout the parasite development, albeit, at a comparable level. Furthermore, the Ldccys1 gene is expressed at a significantly higher level than the Ldccys2 gene at the amastigote stage. Although further functional analysis is required, these results suggest that the Ldccys1 genes are important and may play a significant role at the amastigote stage of development. The transcript size of Ldccys2 is 2.6 Kb. On the other hand, Ldccys1 has a 2.4 Kb abundant transcript with a relatively high level transcript of 4.0 Kb and a weak 5.0 Kb transcript. Traub-Cseko *et al* (1993) found Lpcys1 which is homologous to Ldccys2 and Lpcys2 which is homologous to Ldccys1 are expressed in both promastigotes and amastigotes of *L. pifanoi*, although the levels of expression is higher for both in the amastigotes. The predominant mRNAs for Lpcys1 are 2.6 kb and 4.6 kb with weaker transcripts of approximately 7.5 and 8.1 kb. However, for Lpcys2 the predominant mRNA is 2.3 kb, with a moderately abundant transcript of 4.4 kb and very low levels of transcripts of approximately 7 kb (Robertson and Coombs, 1994). Similar observation was also made for amastigotes derived from tissue infected with *L. amazonensis* and hybridized with probes for the two genes (Traub-Cseko *et al.* 1993). Similarly, Souza *et al* (1992) have cloned a multi-copy cysteine protease gene (lmcpb) from *L. mexicana* which is expressed predominantly in the amastigote stage of development as an abundant 2.3 Kb

mRNA with less abundant 5.0 Kb and 8.0 Kb transcripts. In addition, Mottram *et al* (1992) have cloned a single copy cysteine protease gene (lmcpa) from *L. mexicana* which is developmentally regulated with a transcript size of 3.5 Kb. Therefore, the expression of Ldccys1 resembles the expression of other cysteine protease genes such as lmcpb from *L. mexicana* (Souza *et al.* 1992) but differ from the expression of Lpcys2 of *L. pifanoi*. However, the expression pattern of the Ldccys2 gene of *L. d. chagasi*, is similar to Lpcys1 of *L. pifanoi* and lmcpa of *L. mexicana*. In the present study, the mechanisms responsible for the stage specific expression of Ldccys1 is not yet clear. It is possible that regulation may be effected at the level of transcription. Alternatively, regulation of RNA abundance may be due to post-transcriptional events. A number of recent reports have shown the significance of the 3' untranslated region (UTR) and intergenic regions in regulating gene expression of trypanosomatids at the post-transcriptional level (Jefferies *et al.* 1991, Ramamoorthy *et al.* 1992, Teixeira *et al.* 1995, Charest *et al.* 1996). Since signals for trans-splicing and polyadenylation are contained in these regions, the mRNA abundance could potentially be regulated at one or both of these steps. Alteratively, the 3' UTR could contain binding sites for proteins that influence the stability of mRNA. Future experiments are required to address the issue of stage specific expression of these genes.

Here we also show the overexpression the two distinct cysteine proteases cDNAs in *Leishmania chagasi* cells. The over-produced cysteine proteases are biologically active and are inhibited by cysteine protease inhibitors. Furthermore, the over-produced and indigenous Ldccys1 cysteine protease reacted with polyclonal antibodies raised against this protein. Taken together, this confirms that the cDNAs we have cloned are cysteine proteases. The

difficulty in demonstrating cysteine proteases in *Leishmania donovani* complex using gelatin SDS-PAGE led earlier investigators to suggest that cysteine proteases were not present in the amastigotes of *L. donovani* and *L. major* (Coombs, 1982; Pupkis *et al.*, 1986). Our data refutes this suggestion.

The functions of cysteine proteases are currently not well defined. Recently, evidence from disruption of cysteine protease gene array of *L. mexicana* (lmcpb), suggest that these enzymes are virulence factors (Mottram *et al.*, 1996). However, Ilga *et al*, (1994) have reported that immunization with recombinant cysteine protease from *L. mexicana* and T cell stimulation experiments using this recombinant enzyme show that the enzyme is a T cell immunogen allowing the development of a protective T_H1 helper cells. These reports suggest two potential functions for cysteine proteases. In order to understand the functions of cysteine proteases from *L. d. chagasi*, we examined the impact of *Leishmania* overexpressing cysteine proteases on *Leishmania* pathogenesis and virulence.

Molecular Cloning, Characterization and Overexpression of Biologically Active Cysteine Protease from *Trypanosoma cruzi* in *Leishmania* cells

RESULTS AND DISCUSSIONS

A. Summary

We have cloned and characterized a cDNA encoding a new member of the cysteine protease family of proteins (*T. cruzi* CP) from a *Trypanosoma cruzi* cDNA library in λ-ZAP II. The isolated cDNA contains an open reading frame coding for a polypeptide of 471 amino acids. The predicted amino acid sequence displays extensive similarity to the cysteine protease (cruzain) previously isolated from the genomic library of *T. cruzi* by Eakin *et al.* (1992), as well as the *Trypanosoma brucei* Cysteine protease (*T. brucei* CP) and *Trypanosoma congolenese* cysteine protease (congopain). However, the predicted amino acid sequence in the C-terminal region differs between *T. cruzi* CP and cruzain in that *T. cruzi* CP has an extra 6 amino acids and the last 24 amino acids differ from the last 18 amino acids of cruzain. In addition, there are 11 amino acid differences between these two proteins. The *T. cruzi* gene is arranged in tandem in the genome of *T. cruzi* and is expressed predominantly as 2.2 kb RNA transcript. We have expressed the cDNA in a bacterial expression system but cysteine protease is expressed in this system as inactive insoluble inclusion bodies. To evade these problems, we have examined the possibility of expressing cysteine protease cDNA from *T. cruzi* in the *Leishmania* cells. The coding region of the *T. cruzi* cysteine protease cDNA was cloned into the *Leishmania* overexpressing vector, pX, in two different orientations and these constructs were transfected into *Leishmania donovani chagasi* promastigotes. The presence and expression of these constructs in the transfected *L. d. chagasi* promastigotes were confirmed by Southern and Northern blot analysis respectively. Our results show that

both clones of the transfected *Leishmania* cells produced biologically active cysteine proteases and the activity is inhibited by specific inhibitors of cysteine protease leupeptin and iodoacetimide.

B. REASON FOR CLONING CYSTEINE PROTEASE cDNA FROM *T. CRUZI*

Cysteine proteases have been implicated in numerous processes that are crucial for both infectivity and pathogenesis of *Trypanosoma cruzi* (McKerrow, 1991). Some of these processes are intracellular metabolism, parasite-macrophage interaction, host cell invasion and adhesion to target cells (Calderon *et al.*, 1989; Piras *et al.*, 1985; Souto-Padron *et al.*, 1990). Recent evidence that two fluoromethyl ketone-derivatized cysteine protease inhibitors arrest *T. cruzi* replication (Harth *et al.*, 1993; Mckerrow, 1993), suggest that cysteine protease is important in the replication of *T. cruzi*. In addition, it is possible that this enzyme has other functions which have not been identified. We cloned cysteine protease from *T. cruzi* in order to study it as a vaccine against trypanosomatids.

C. PCR amplification of cysteine protease gene

In order to determine the population of cysteine proteases in the cDNA library of *T. cruzi*, we carried out the PCR amplification of cysteine protease DNA fragment from the cDNA library using two primers (TCCP1 and TCCP2). A 500 bp PCR product was obtained (similar to that shown in Figure 7). The PCR was digested sequentially with *Hin*d III and *Eco*RI, gel purified and ligated in pGEM-2 (Promega). Plasmid DNA from a large number of clones were isolated by alkaline lysis and sequenced. Our result showed the presence of one type of cysteine protease DNA sequence.

D. Screening, isolation and sequence determination of cysteine protease cDNA from *Trypanosoma cruzi*

In order to isolate full length cDNA representing the cysteine protease DNA fragment amplified from the cDNA library by PCR, we screened *T. cruzi* cDNA library in λ-ZAP II. We used the 500 bp PCR product amplified from the cDNA library as a probe. We isolated 26 clones of cysteine proteases from the screening exercise but further analysis revealed that only one of the cDNAs had the entire coding region. We sequenced the cDNA having the entire coding region (*T. cruzi* CP) as well as 3 partial cDNAs in order to determine the differences which might exist between them. *T. cruzi* CP cDNA described in this thesis may not be full length because the published sequence for splice leader which is present at the 5' ends of all *Trypanosoma* mRNAs (Lang, *et al.*, 1984) was not found. The *T. cruzi* CP cDNA has an open reading frame encoding 471 amino acids (Fig. 23). The deduced amino acid sequence contain all the features characteristic of different members of cysteine protease. Thus, it contains the active site cysteine at positions 147. Glycine which is involved in substrate binding is also present at position 145. In addition, other amino acid residues important in catalysis including histidine and asparagine are present. Histidine is located at position 285 while asparagine is present at position at 305. All these key residues are bound by other well conserved amino acids as in other cysteine proteases (Fig. 24).

In order to determine the degree of homology, we aligned the deduced amino acid sequence of *T. cruzi* CP cDNA to the deduced amino acid sequence from the

Figure 23. Sequence of the cysteine protease cDNA which we isolated from *Trypanosoma cruzi* cDNA library. The isolated cDNA contains an open reading frame coding for a polypeptide of 471 amino acids. The LCP-1 and LCP-2 primers used to amplify the 500 bp fragment are underlined. The *T. cruzi* cysteine protease cDNA has been assigned EMBL/GenBank nucleotide accession number AF004594.

```
                                                                  M  S  G  W
[EcoRI]GGCACGAGGCTGTTGAAGGCACACAAGGAAGGAAACACACCCACGCAAGCAGTA ATG TCT GGC TGG      4

 A  R  F  V  L  L  A  A  V  L  V  V  M  A  C  L  V  P  A  A
GCC CGG TTC GTG TTG CTC GCG GCC GTC CTG GTC GTC ATG GCG TGC CTC GTC CCC GCG GCG   24

 T  A  S  L  H  A  E  E  T  L  T  S  Q  F  A  E  F  K  Q  K
ACG GCG AGC CTG CAT GCG GAG GAG ACG CTG ACG TCG CAA TTC GCA GAA TTC AAG CAG AAG   44

 H  G  R  V  Y  E  S  A  A  R  R  L  P  L  S  V  F  A  E  N
CAT GGC AGG GTG TAC GAG AGC GCC GCG AGG AGG CTT CCG CTG AGC GTG TTC AGG GAG AAC   64

 L  F  L  A  R  L  H  A  A  A  N  P  H  A  T  F  G  V  T  P
CTG TTT CTT GCG AGG CTG CAC GCC GCG GCA AAC CCA CAC GCG ACC TTC GGC GTC ACG CCC   84

 F  S  D  L  T  R  E  E  F  R  S  R  Y  H  N  G  A  A  H  F
TTC TCG GAC CTC ACG CGC GAG GAA TTC CGG TCC CGC TAC CAC AAC GGC GCG GCG CAC TTT  104

 A  A  A  Q  E  R  A  R  V  P  V  K  V  E  V  V  G  A  P  A
GCG GCG GCG CAG GAG CGC GCG AGA GTG CCG GTG AAG GTG GAG GTA GTT GGC GCG CCC GCG  124

 A  V  D  W  R  A  R  G  A  V  T  A  V  K  D  Q  G  Q  C  G
GCA GTG GAT TGG CGT GCG AGA GGC GCC GTG ACA GCC GTC AAG GAC CAG GGC CAA TGC GGT  144

 S  C  W  A  F  S  A  I  G  N  V  E  C  Q  W  F  L  A  G  H
TCG TGC TGG GCC TTC TCC GCC ATT GGC AAC GTT GAG TGC CAG TGG TTT CTT GCC GGT CAC  164

 P  L  T  N  L  S  E  Q  M  L  V  S  C  D  K  T  D  F  G  C
CCG CTG ACG AAC CTG TCG GAG CAG ATG CTC GTG TCG TGC GAC AAA ACG GAC TTT GGC TGC  184

 S  G  G  L  M  N  N  A  F  E  W  I  V  Q  E  N  N  G  A  V
AGT GGT GGC CTG ATG AAC AAC GCC TTT GAG TGG ATT GTG CAG GAG AAT AAC GGC GCC GTG  204

 Y  T  E  D  S  Y  P  Y  A  S  G  E  G  I  S  P  P  C  T  T
TAC ACG GAG GAC AGC TAC CCT TAT GCG TCG GGC GAG GGG ATA TCG CCG CCG TGC ACG ACG  224

 S  G  H  T  V  G  A  T  I  T  G  H  V  E  L  P  Q  D  E  A
TCA GGC CAC ACG GTT GGT GCC ACG ATT ACC GGT CAC GTT GAA TTA CCT CAG GAC GAG GCC  244

 Q  I  A  A  C  V  A  V  N  G  P  V  A  V  A  V  D  A  S  S
CAA ATA GCC GCA TGC GTT GCA GTC AAT  GGC CCG GTT GCC GTT GCC GTC GAC GCC AGC AGC 264

 W  M  T  Y  T  G  G  V  M  T  S  C  V  S  E  Q  L  D  H  G
TGG ATG ACC TAC ACG GGC GGC GTT ATG ACG AGC TGC GTC TCC GAG CAG CTG GAT CAC GGC  284

 V  L  L  V  G  Y  N  D  S  A  A  V  P  Y  W  I  I  K  N  S
GTT CTT CTC GTC GGC TAC AAT GAC AGC GCC GCA GTG CCG TAC TGG ATC ATC AAG AAC TCG  304

 W  T  T  Q  G  E  E  G  Y  I  R  I  A  K  G  S  N  Q  C  L
TGG ACC ACG CAG GGT GAG GAA GGC TAC ATC CGC ATT GCA AAG GGC TCG AAC CAG TGC CIT  324
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 V  K  E  E  A  S  S  A  V  V  G  G  P  G  P  T  P  E  P  T
GTC AAG GAG GAG GCG AGC TCC GCG GTG GTC GGT GGT CCC GGA CCC ACT CCC GAG CCA ACC  344

 T  T  T  T  T  S  A  P  G  P  S  P  S  Y  F  V  Q  M  S  C
ACC ACG ACA ACC ACA AGT GCC CCA GGA CCG TCC CCA TCG TAC TTT GTG CAG ATG TCC TGC  364

 T  D  A  A  C  I  V  G  C  E  N  V  T  L  P  T  G  Q  C  L
ACT GAC GCT GCG TGC ATT GTC GGG TGC GAG AAC GTG ACG TTA CCG ACC GGT CAG TGT CTC  384

 L  T  T  S  G  V  S  A  I  V  T  C  G  A  E  T  L  T  E  E
CTG ACC ACC AGC GGC GTC TCT GCC ATT GTC ACG TGC GGT GCT GAG ACT CTC ACA GAA GAA  404

 V  F  L  T  S  T  H  C  S  G  P  S  V  R  S  S  V  P  L  N
GTC TTC CTT ACG AGT ACG CAC TGC AGC GGC CCA TCG GTG AGG TCC TCT GTT CCT CTC AAC  424

 K  C  N  R  L  L  R  G  S  V  E  F  F  C  G  S  S  S  S  G
AAA TGC AAC CGG CTT TTA AGA GGC TCC GTT GAG TTC TTC TGC GGC TCC AGC TCC AGT GGC 444

 R  L  A  T  W  T  G  S  V  A  I  S  H  T  T  A  V  I  A  A
CGA CTG GCG ACG TGG ACA GGC AGC GTC GCC ATC AGC CAT ACC ACA GCC GTC ATC GCC GCC 464

 S  E  G  T  L  C  F STOP
TCT GAG GGC ACA CTT TGT TTC TGA TGCTGCCTTTTCTCTCATTTTACTTTTTTATTATTATTGAGCGAGCGT

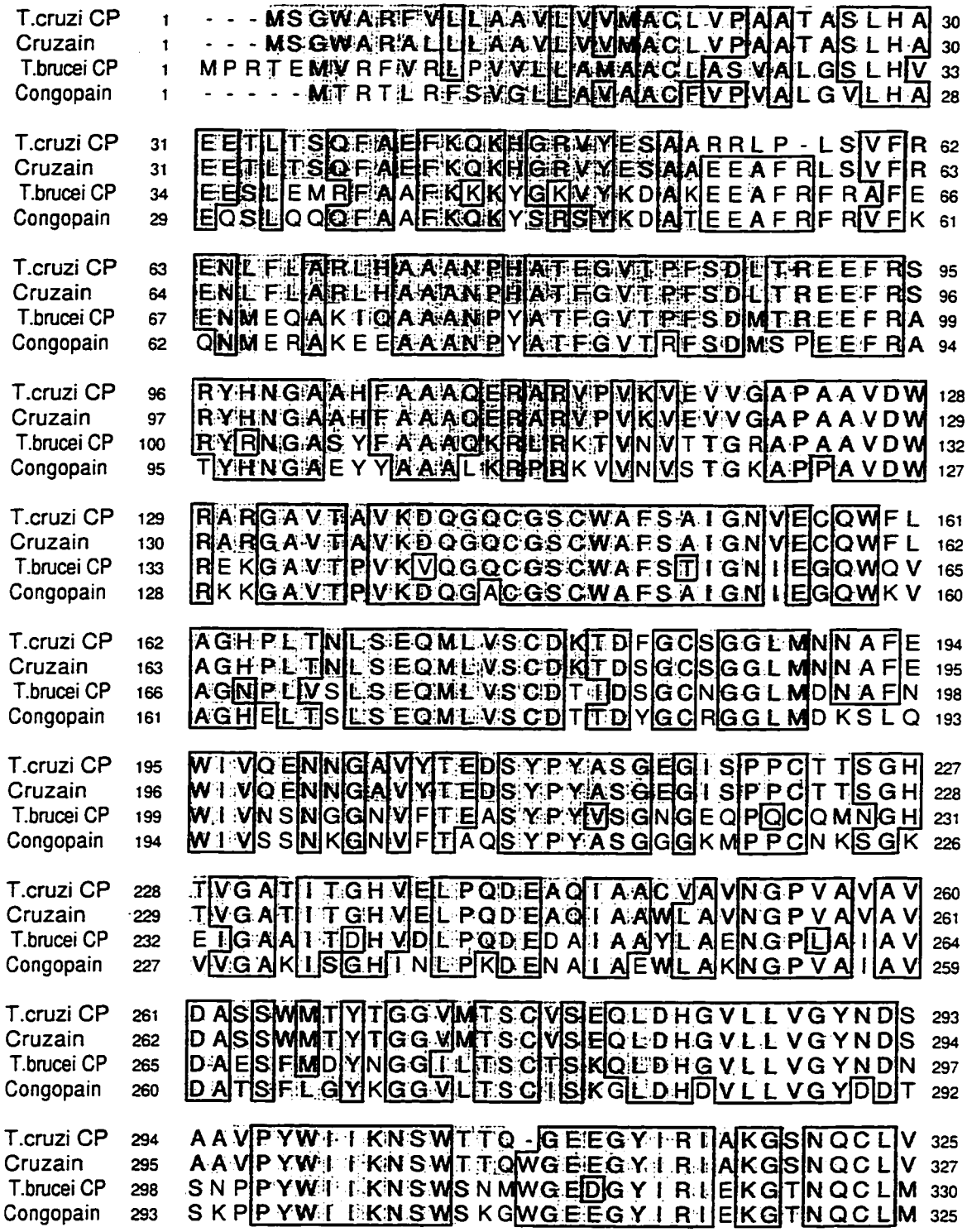
CGGGCTGCGGCACACTGGGCAAATAAGTCCCAGTGGAGTGGATTTGTTTATTGATTTGCTCCATGTATGTGTTTTCTTTTT

TGTGCACAACTGCAAATATGTGTCCGCGATCGACGTCTCAGCCACGCGTGTGTTTTTTCTATTATATGATTATGTTG

(A)43[XhoI].
```

T. cruzi cysteine protease (cruzain) (Eakin *et al.*, 1992); *T. brucei* cysteine protease (Mottram *et al.* 1989; Pamer *et al.*, 1989) and *T. congolense* cysteine protease (congopain) (Fish *et al.*, 1995). The predicted amino acid sequence of *T. cruzi* CP displays extensive similarity to the cysteine protease (cruzain) previously isolated from the genomic library of *T. cruzi* by Eakin *et al.*, (1992) within the preregion, propeptide region and mature protease core. The pre- and pro-region are from residues 1 to 122. The N-terminal regions (pre) of all the cysteine proteases from trypanosomes which are shown in Fig. 24 are hydrophobic. The pro-region has a putative cleavage site between 121 and 122 generating an alanine as in the case of cruzain (Eakin *et al.*, 1992) and other cysteine proteases (Fig. 24). Like other cysteine proteases, pro-region of *T. cruzi* CP is followed by a mature protease core and a C-terminal extension region. The cleavage site between the mature protease core and the C-terminal extension found in cruzain (Eakin *et al.*, 1992), Val-Gly/-Gly-Pro was found between 335 and 336 (Fig. 24). However, the predicted amino acid sequence in the C-terminal region differs in the two genes in that *T. cruzi* CP has an extra 6 amino acids and the last 24 amino acids differ from the last 18 amino acids of cruzain (Fig. 24). The DNA sequence representing this region of the cDNA is shown in Figure 25 and clearly demonstrates that this gene is different from that of cruzain (Eakin *et al.*, 1992). In addition, we have noticed other differences. These differences include changes in amino acid 7 and 8 where *T. cruzi* CP shows Phe and Val, in cruzain they are Ala and Leu respectively. There is a difference of 5 amino acids

Figure 24. Panel A shows the alignment of *T. cruzi* CP with cysteine proteases from other species of trypanosomes. The deduced amino acid sequence *T. cruzi* CP is aligned to the *T. cruzi* cysteine protease (cruzain) (Eakin *et al.*, 1992); *T. brucei* cysteine protease (Mottram *et al.*, 1989; Pamer *et al.*, 1990) and *T. congolense* cysteine protease (congopain) (Fish *et al.*, 1995). The amino acid alignment was performed using the Clustal W program and Seqvu 1.1 software (The Garavan Institute of Medical Research, Sydney, Australia).


T.cruzi CP 1 - - - MSGWARFVLLAAVLVVMACLVPAATASLHA 30
Cruzain 1 - - - MSGWARALLLAAVLVVMACLVPAATASLHA 30
T.brucei CP 1 MPRTEMVRFVRLPVVLLAMAACLASVALGSLHV 33
Congopain 1 - - - - - MTRTLRFSVGLLAVAACFVPVALGVLHA 28
T.cruzi CP 31 EETLTSQFAEFKQKHGRVYESAAARRLP - LSVFR 62
Cruzain 31 EETLTSQFAEFKQKHGRVYESAAEEAFRLSVFR 63
T.brucei CP 34 EESLEMRFAAFKKKYGKVYKDAKEEAFRFRAFE 66
Congopain 29 EQSLQQQFAAFKQKYSRSYKDATEEAFRFRVFK 61
T.cruzi CP 63 ENLFLARLHAAANPHATFGVTPFSDLTREEFRS 95
Cruzain 64 ENLFLARLHAAANPHATFGVTPFSDLTREEFRS 96
T.brucei CP 67 ENMEQAKTQAAANPYATFGVTPFSDMTREEFRA 99
Congopain 62 QNMERAKEEAAANPYATFGVTRFSDMSPEEFRA 94
T.cruzi CP 96 RYHNGAAHFAAAQERARVPVKVEVVGAPAAVDW 128
Cruzain 97 RYHNGAAHFAAAQERARVPVKVEVVGAPAAVDW 129
T.brucei CP 100 RYRNGASYFAAAQKRLRKTVNVTTGRAPAAVDW 132
Congopain 95 TYHNGAEYYAAALKRPRKVVNVSTGKAPPAVDW 127
T.cruzi CP 129 RARGAVTAVKDQGQCGSCWAFSAIGNVECQWFL 161
Cruzain 130 RARGAVTAVKDQGQCGSCWAFSAIGNVECQWFL 162
T.brucei CP 133 REKGAVTPVKVQGQCGSCWAFSTIGNIEGQWQV 165
Congopain 128 RKKGAVTPVKDQGACGSCWAFSAIGNIEGQWKV 160
T.cruzi CP 162 AGHPLTNLSEQMLVSCDKTDFGCSGGLMNNAFE 194
Cruzain 163 AGHPLTNLSEQMLVSCDKTDSGCSGGLMNNAFE 195
T.brucei CP 166 AGNPLVSLSEQMLVSCDTIDSGCNGGLMDNAFN 198
Congopain 161 AGHELTSLSEQMLVSCDTTDYGCRGGLMDKSLQ 193
T.cruzi CP 195 WIVQENNGAVYTEDSYPYASGEGISPPCTTSGH 227
Cruzain 196 WIVQENNGAVYTEDSYPYASGEGISPPCTTSGH 228
T.brucei CP 199 WIVNSNGGNVFTEASYPYVSGNGEQPQCQMNGH 231
Congopain 194 WIVSSNKGNVFTAQSYPYASGGGKMPPCNKSGK 226
T.cruzi CP 228 TVGATITGHVELPQDEAQIAACVAVNGPVAVAV 260
Cruzain 229 TVGATITGHVELPQDEAQIAAWLAVNGPVAVAV 261
T.brucei CP 232 EIGAAITDHVDLPQDEDAIAAYLAENGPLAIAV 264
Congopain 227 VVGAKISGHINLPKDENAIAEWLAKNGPVAIAV 259
T.cruzi CP 261 DASSWMTYTGGVMTSCVSEQLDHGVLLVGYNDS 293
Cruzain 262 DASSWMTYTGGVMTSCVSEQLDHGVLLVGYNDS 294
T.brucei CP 265 DAESFMDYNGGILTSCTSKQLDHGVLLVGYNDN 297
Congopain 260 DATSFLGYKGGVLTSCISKGLDHDVLLVGYDDT 292
T.cruzi CP 294 AAVPYWIIKNSWTTQ-GEEGYIRIAKGSNQCLV 325
Cruzain 295 AAVPYWIIKNSWTTQWGEEGYIRIAKGSNQCLV 327
T.brucei CP 298 SNPPYWIIKNSWSNMWGEDGYIRLEKGTNQCLM 330
Congopain 293 SKPPYWIIKNSWSKGWGEEGYIRIEKGTNQCLM 325

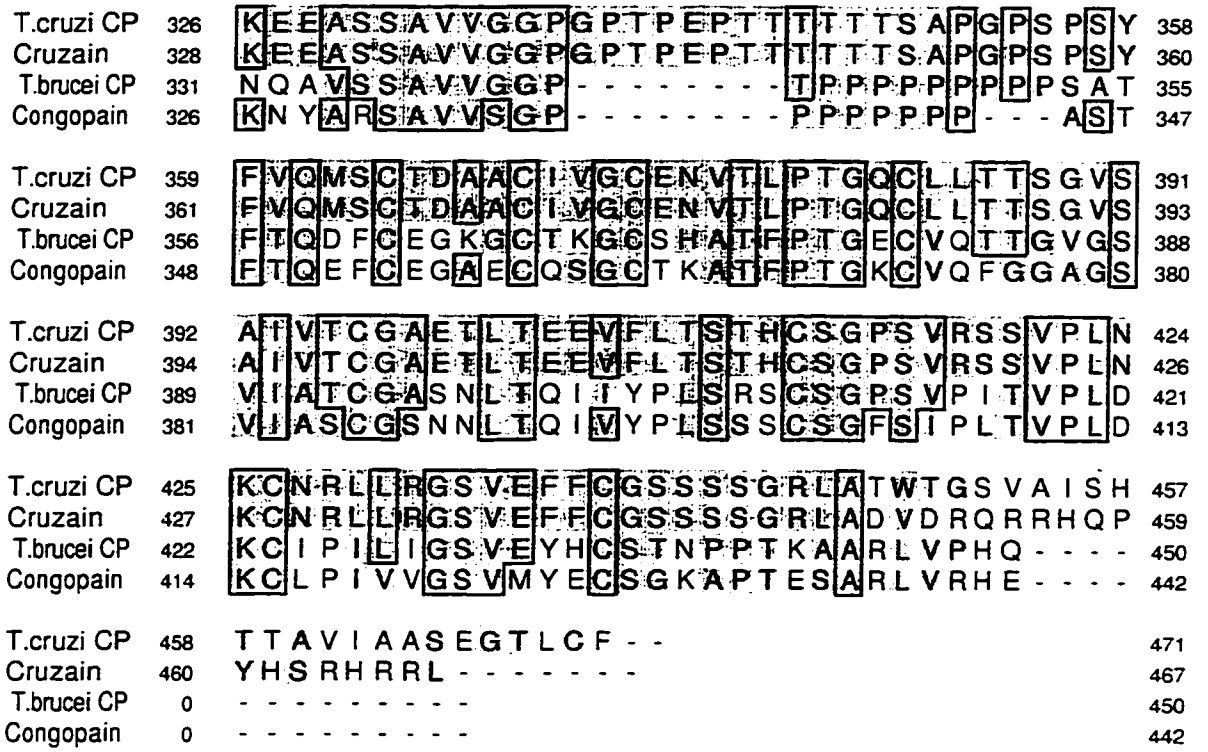
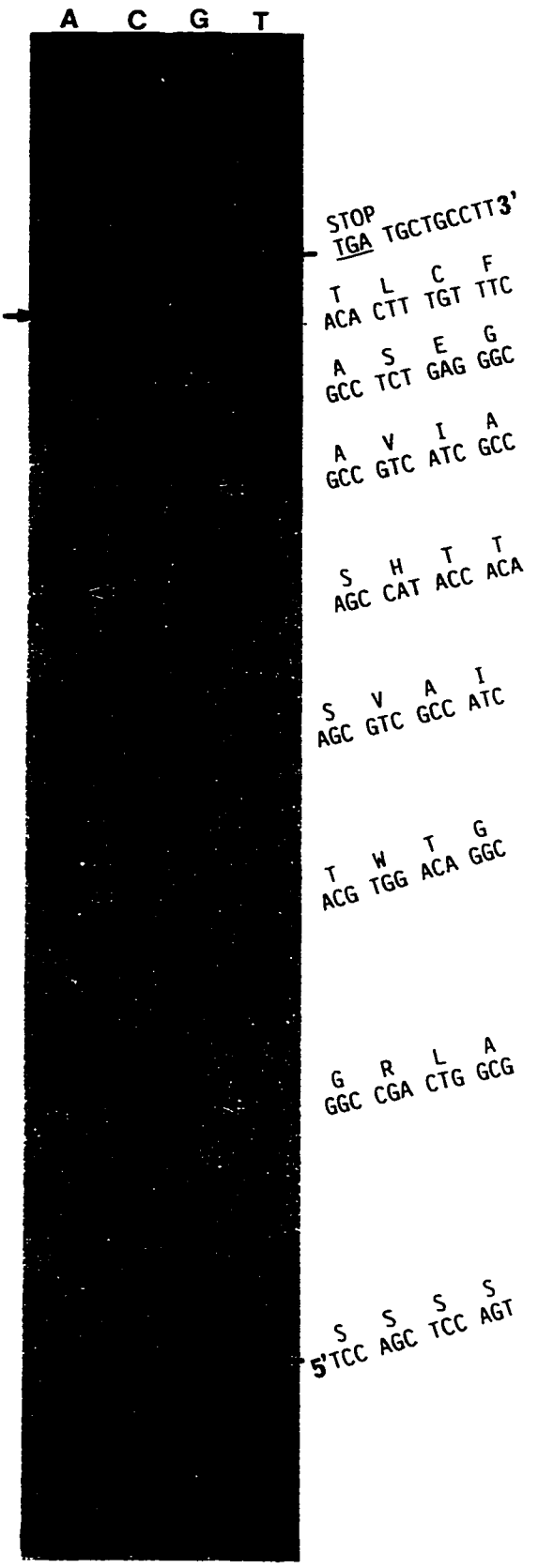

T.cruzi CP 326 KEEASSAVVGGPGPTPEPTTTTTTSAPGPSPSY 358
Cruzain 328 KEEASSAVVGGPGPTPEPTTTTTTSAPGPSPSY 360
T.brucei CP 331 NQAVSSAVVGGP--------TPPPPPPPPPSAT 355
Congopain 326 KNYARSAVVSGP--------PPPPPPP--- AST 347
T.cruzi CP 359 FVQMSCTDAACIVGCENVTLPTGQCLLTTSGVS 391
Cruzain 361 FVQMSCTDAACIVGCENVTLPTGQCLLTTSGVS 393
T.brucei CP 356 FTQDFCEGKGCTKGCSHATFPTGECVQTTGVGS 388
Congopain 348 FTQEFCEGAECQSGCTKATFPTGKCVQFGGAGS 380
T.cruzi CP 392 AIVTCGAETLTEEVFLTSTHCSGPSVRSSVPLN 424
Cruzain 394 AIVTCGAETLTEEVFLTSTHCSGPSVRSSVPLN 426
T.brucei CP 389 VIATCGASNLTQITYPLSRSCSGPSVPITVPLD 421
Congopain 381 VIASCGSNNLTQIVYPLSSSCSGFSIPLTVPLD 413
T.cruzi CP 425 KCNRLLRGSVEFFCGSSSSGRLATWTGSVAISH 457
Cruzain 427 KCNRLLRGSVEFFCGSSSSGRLADVDRQRRHQP 459
T.brucei CP 422 KCIPILIGSVEYHCSTNPPTKAARLVPHQ---- 450
Congopain 414 KCLPIVVGSVMYECSGKAPTESARLVRHE---- 442
T.cruzi CP 458 TTAVIAASEGTLCF-- 471
Cruzain 460 YHSRHRRL------- 467
T.brucei CP 0 ---------- 450
Congopain 0 ---------- 442

Fig. 25 Panel B shows the actual sequences of the region where *T. cruzi CP* differs from the sequences of cruzain reported by Eakin *et al.*, (1992). We have shown the position from the four S but the major differences between *T. cruzi CP* and cruzain start from amino acid T indicated by star:

```
                                    *
  S   S   S   S   G   R   L   A   T   W   T
5'TCC AGC TCC AGT GGC CGA CTG GCG ACG TGG ACA

  G   S   V   A   I   S   S   T   T   A   V
 GGC AGC GTC GCC ATC AGC CAT ACC ACA GCC GTC

  I   A   A   S   E   G   T   L   C   F  STOP
 ATC GCC GCC TCT GAG GGC ACA CTT TGT TTC TGA TGCTGC3'
```

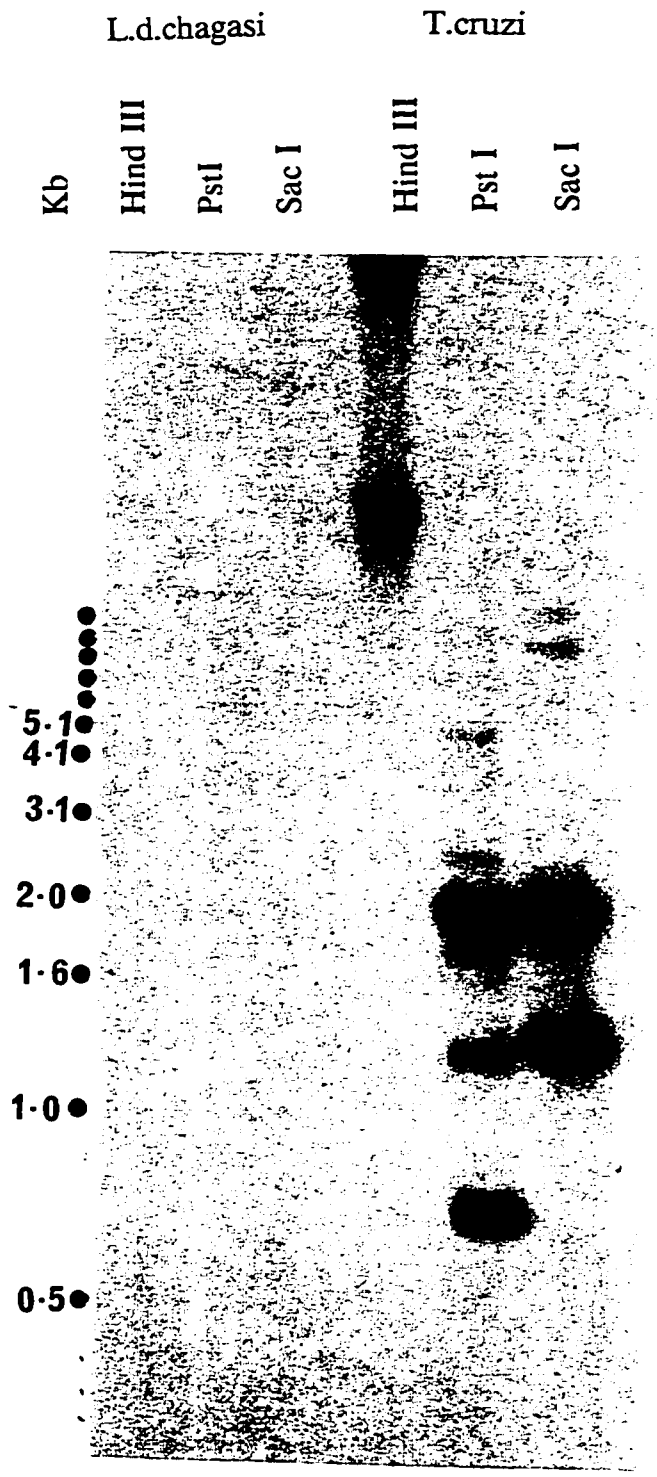

A C G T
STOP
TGA TGCTGCCTT 3'
T L C F
ACA CTT TGT TTC
A S E G
GCC TCT GAG GGC
A V I A
GCC GTC ATC GCC
S H T T
AGC CAT ACC ACA
S V A I
AGC GTC GCC ATC
T W T G
ACG TGG ACA GGC
G R L A
GGC CGA CTG GCG
S S S S
5'TCC AGC TCC AGT

between 53 and 57 of *T. cruzi* and cruzain. In these positions, cruzain has amino acids as in *T. brucei* CP and congopain. Furthermore, in amino acid positions 182, 249 and 250 amino acids Phe, Cys and Val in *T. cruzi* CP cDNA are substituted by Ser, Trp and Leu in cruzain respectively. Finally, in amino acid position 309 one amino acid is deleted in *T. cruzi* CP cDNA while cruzain as well as *T. brucei* CP and congopain all have Trp (Fig. 24). Taken together, these differences suggest that the cDNA we have isolated is a new member of the cysteine protease family of proteins from *T. cruzi*.

E. Southern blot analysis.

In order to understand the genomic organization of cysteine protease, genomic DNA from *T. cruzi* was digested separately with *Hin*dIII, *Pst*I and *Sac*I restriction enzymes and the digested DNA was used to prepare Southern blots as described under materials and methods. *L. d. chagasi* genomic DNA was similarly digested and run along side in the gel to see if there is cross reaction between the cysteine proteases of the two organisms. The blot was hybridized with ^{32}P labelled 500 bp fragment of cysteine protease from *T cruzi*. The results of hybridization is shown in Fig. 26. In lane where *T. cruzi* genomic DNA was digested *Hin*dIII, there was a single hybridizable band at above 15 kb. In the lane where *T. cruzi* genomic DNA was digested with *Pst*I there were two strong hybridizable bands at about O.7 kb and 2.0 kb with weaker hybridizable bands at about 1.2 kb, 1.7 kb, 2.5 kb and 5.1 kb. In lane where *T. cruzi* genomic DNA was digested with *Sac*I there were two strong hybridizable

Figure 26. Southern blot analysis of cysteine protease of *T. cruzi*. Genomic DNA (5 µg/lane) was digested with the restriction enzymes shown, run in 1% agarose gels and transferred to nylon membrane. The blot was hybridized with a probe from *T. cruzi* CP cDNA.


L.d.chagasi
T.cruzi
Kb
Hind III
PstI
Sac I
Hind III
Pst I
Sac I
5·1
4·1
3·1
2·0
1·6
1·0
0·5

bands at about 1.2 kb and 2.0 kb with two faintly hybridizable bands at about 9.5 kb and 10 kb. The probe did not cross react with genomic DNA from *L. d. chagasi*.

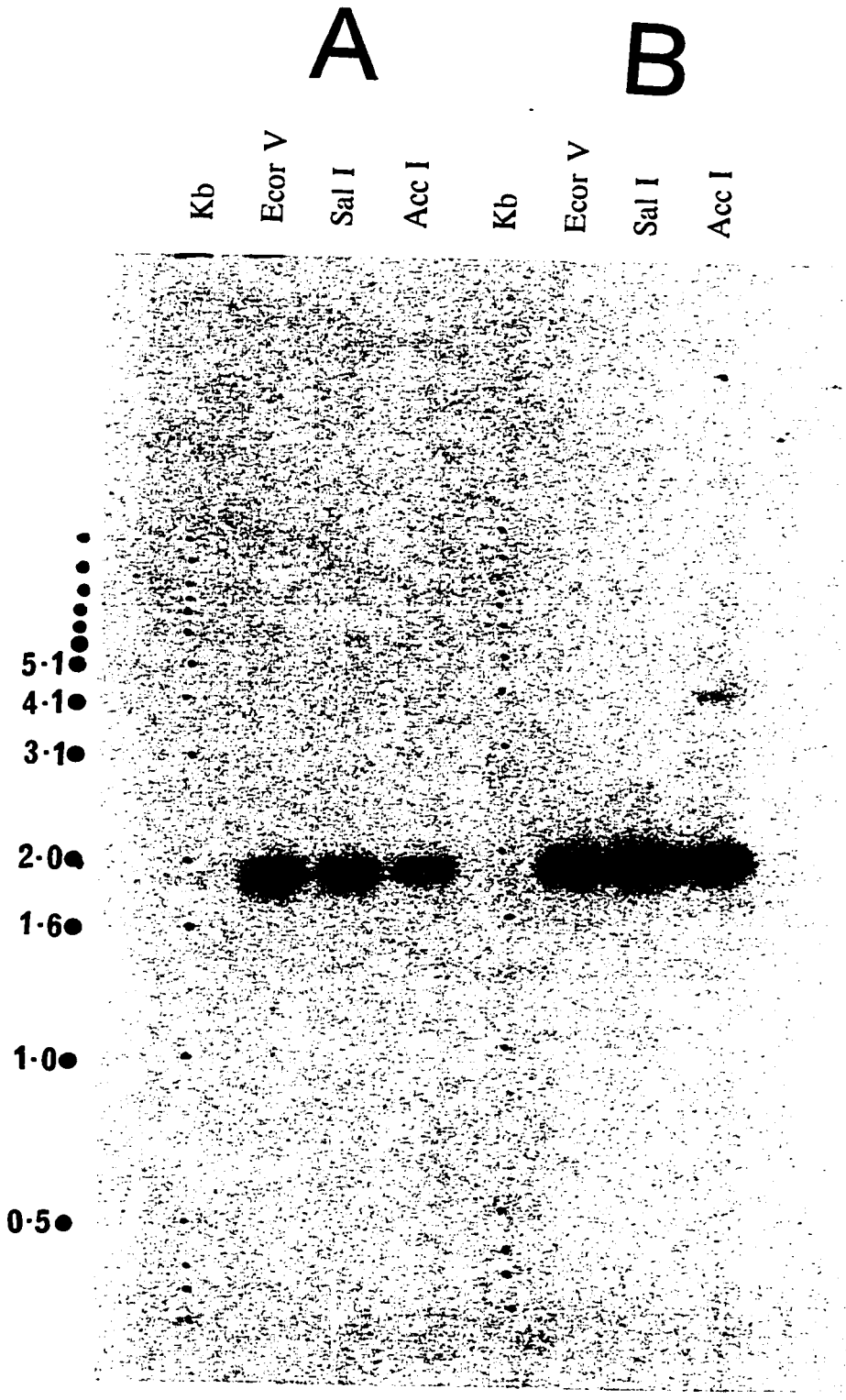
F. Arrangement of cysteine protease in the *T. cruzi* genome

In order to determine the arrangement of cysteine protease in the genome of *T. cruzi*, we tested whether the cysteine protease cDNA we have isolated is a member of cysteine protease genes which are arranged in tandem. To do this, we employed the theory of the test for genes which are arranged in tandem as described before on page 87 and Fig. 12. Thus, a restriction enzyme which cuts the gene cloned once was first identified. *Eco*RV, *Sal*I and *Acc*I were found to cut the cDNA once. Thus, genomic DNA from *T. cruzi* was digested separately with each of these restriction enzymes and two identical blots containing *Eco*RV, *Sal*I and *Acc*I digests were prepared. A duplicate blot was hybridized with a probe representing sequences 5' end to these restriction enzymes. The other duplicate blot was hybridized with a probe representing sequences 3' end to these restriction enzymes. The results of these tests are shown in Fig. 27. As shown in panels A and B of Fig. 27, a 2.0 kb fragment hybridized with either probes. This data indicates that the cysteine protease cDNA which we have isolated is a member of the cysteine protease genes which are arranged in tandem in the genome of *T. cruzi*.

G. mRNA expression of *T. cruzi* cysteine protease.

In order to show the expression of the cysteine protease gene in *T. cruzi*, we utilized the 500 bp fragment probe specific for *T. cruzi* cysteine protease. We used total RNA

Figure 27. Southern blot analysis demonstrating tandem arrangement of *T. cruzi* CP gene. Panel A was hybridized with probe A while panel B was hybridized with probe B from the *T. cruzi* CP cDNA.


A
B
Kb
Ecor V
Sal I
Acc I
Kb
Ecor V
Sal I
Acc I
5·1
4·1
3·1
2·0
1·6
1·0
0·5

isolated from the epimastigote stage of development of this organism. For control purposes, we also included total RNA isolated from *L. d. donovani* and *L. d. chagasi* promastigotes. As shown in Fig. 28, an abundant transcript of about 2.2 kb size (this mRNA is the expected size from the *T. cruzi* cysteine protease cDNA sequence) and a minor transcript at about 4.4 kb hybridized to the *T. cruzi* cysteine protease probe in lane of *T. cruzi* RNA. The 4.4 kb message which hybridized to the probe in lane of *T. cruzi* is probably a precursor message to the 2.2 Kb message. A 2.6 kb message also hybridized very faintly to this probe in the lane of *L. d. donovani* and is probably due to cross-reaction with the cysteine protease mRNA in this organism.

H. Expression of cysteine protease from *T. cruzi* in *E. coli*

In an attempt to produce cysteine protease protein from the cDNA, we first used the bacterial expression system. The coding region of *T. cruzi* CP cDNA was amplified by PCR and ligated into the *Bam*HI site of the pGEX-2T vector in frame with GST (Fig. 29). The bacterial clones carrying pGEX-2T + *T. cruzi* cysteine protease were induced as described in materials and methods. Our results show that the abundant cysteine protease produced in this system are in the form of inactive insoluble inclusion bodies (Fig. 30). Cysteine protein was produced as a fusion protein with GST giving a product of about 80 kDa. The product of clone in lane 2 is shorter than the other clones and this was found to be due to a deletion of 13 amino acids around the ATG which left the rest of the coding regiom in frame.

Figure 28. Northern blot analysis of cysteine protease from *T. cruzi*. The blot was hybridized with the probe from the *T. cruzi* CP cDNA.

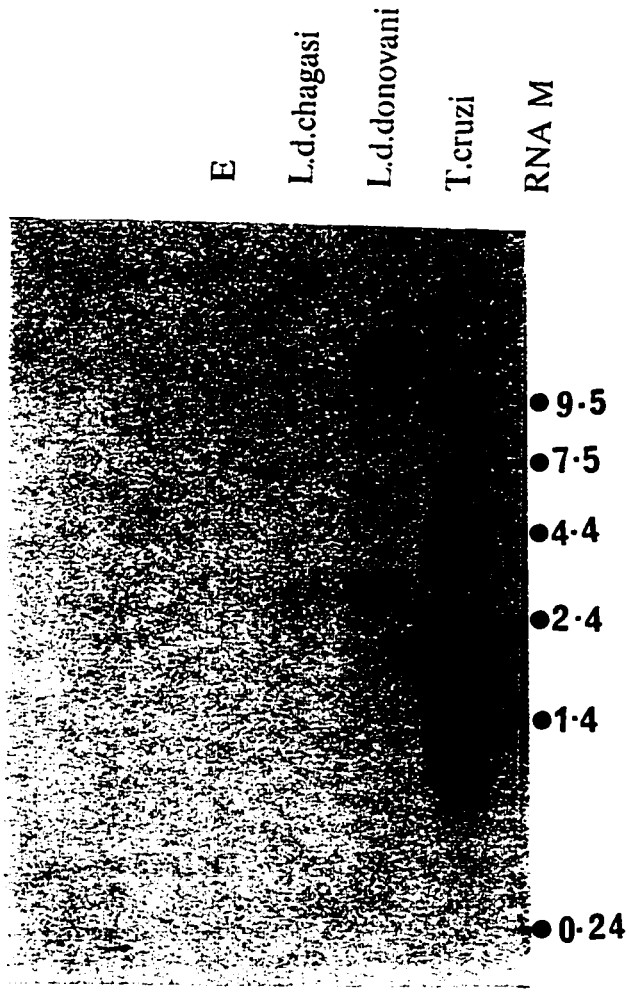

E
L.d.chagasi
L.d.donovani
T.cruzi
RNA M
9·5
7·5
4·4
2·4
1·4
0·24

Figure 29. Map of pGEX-2T vector showing how the coding region of *T. cruzi* CP cDNA was ligated into the *Bam*HI site of the vector in frame with GST.

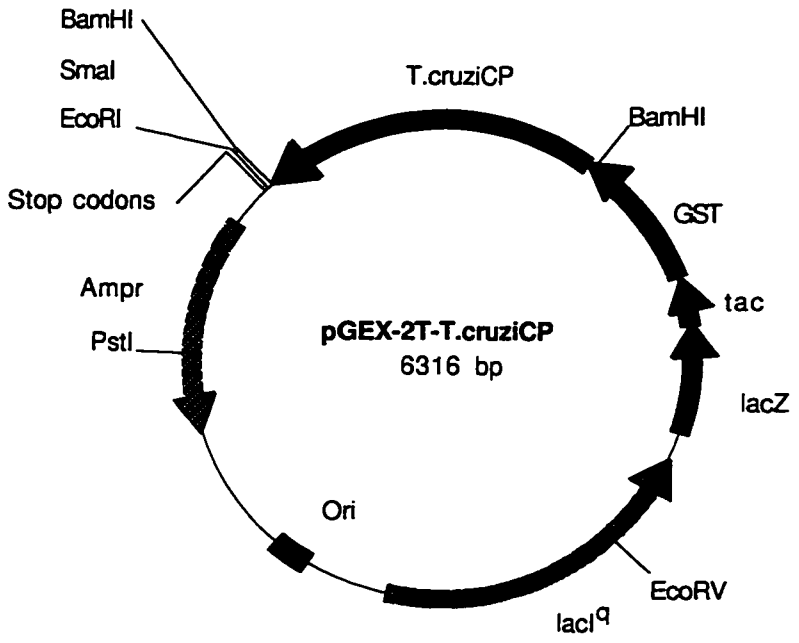
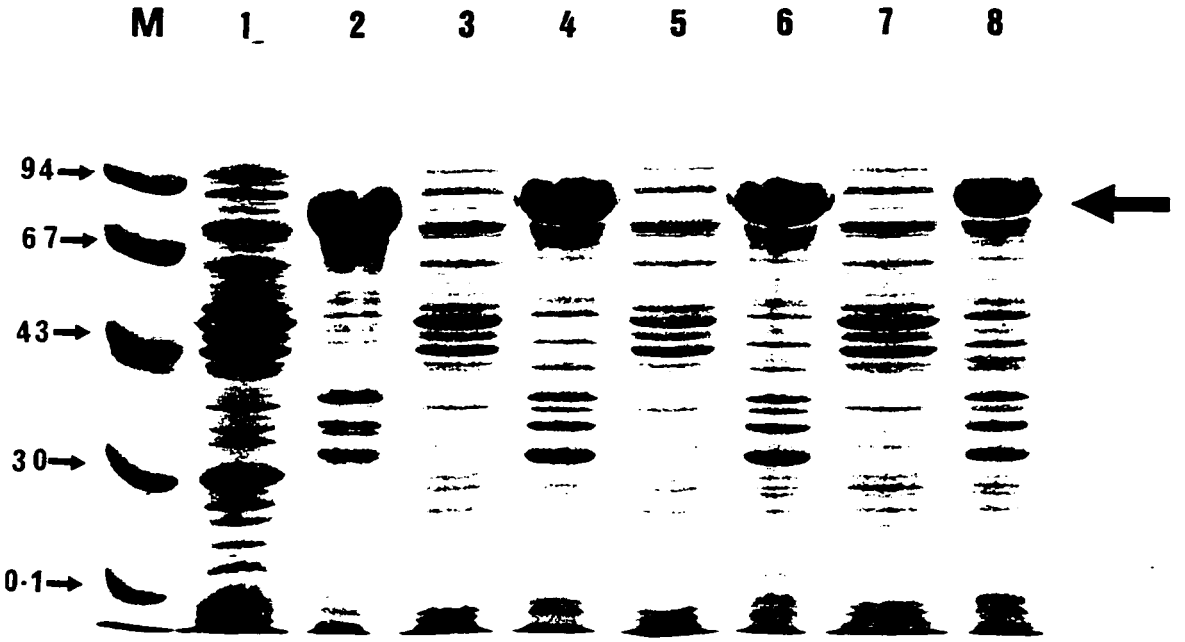

BamHI
SmaI
EcoRI
Stop codons
T.cruziCP
BamHI
GST
tac
lacZ
EcoRV
lacI^q
Ori
PstI
Ampr
pGEX-2T-T.cruziCP
6316 bp

Figure 30. SDS-PAGE analysis of proteins from *E. coli* expressing GST+cysteine protease fusion protein. Lane M = marker in kDa; lanes 1, 3, 5, and 7 are supernatants of bacterial lysates; lanes 2, 4, 6 and 8 are GST+cysteine protease fusion protein as insoluble inclusion bodies. The gel was stained with coomassie blue.

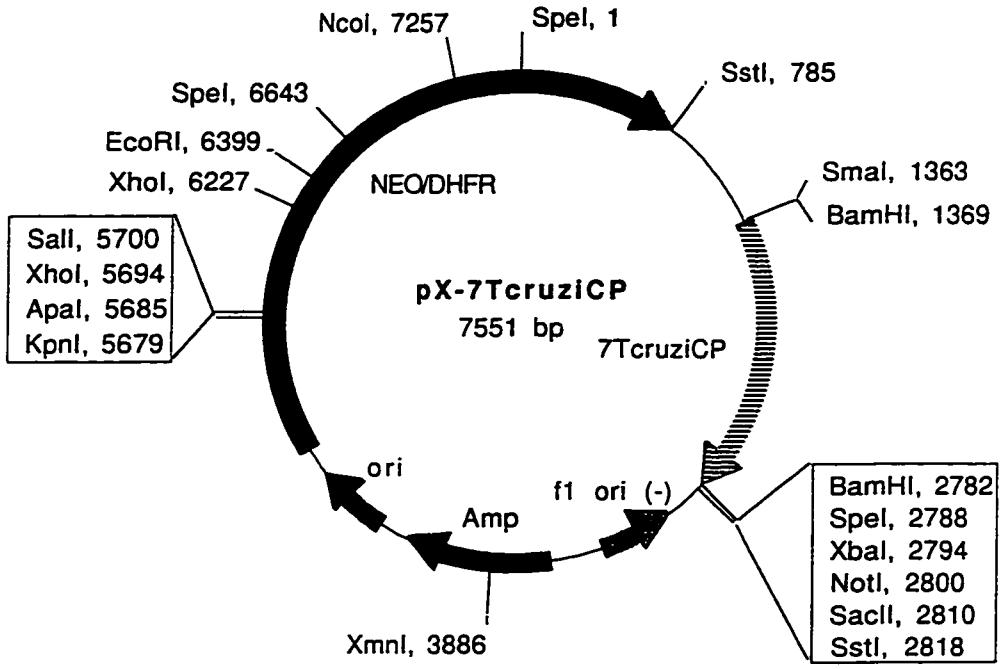

M
1
2
3
4
5
6
7
8
94
67
43
30
0·1

I. Expression of cysteine protease from *T. cruzi* in *Leishmania* cells

To evade the problem encountered in the expression of cysteine protease in bacteria, we examined the possibility of expressing this enzyme in *Leishmania* cells. The coding region of the *T. cruzi* cysteine protease cDNA was cloned into the *Leishmania* overexpressing vector, pX, in two different orientations (Fig. 31) and these constructs were transfected into *Leishmania donovani chagasi* promastigotes. The presence and expression of these constructs in the transfected *L. d. chagasi* promastigotes were confirmed by Southern (Fig. 32) and Northern (Fig. 33) blots analysis respectively. Our results show that both clones of the transfected *Leishmania* cells produced biologically active cysteine proteases (Fig. 34 panel A). The unique enzymatic activity seen at about 43 kDa in lanes labelled 1 and 2 are due to unprocessed forms of over-expressed cysteine protease. A small amount processed form of cysteine protease can be seen at about 30 kDa ins lane labelled 3 and 4. When a similar gel is incubated in a buffer which included specific inhibitors of cysteine proteases such as leupeptin and iodoacetimide, the enzymatic activities seen at about 43 kDa and 30 kDA disappeared (Fig. 34 panel B), confirming that the enzymatic activities are indeed due to the overexpressed cysteine protease. The enzymatic activity seen at 63 kDa and above in both panels A and B of Fig. 34 are wild type gp63 which confirms equal loading. This data demonstrates that it is possible to express *T. cruzi* cysteine protease in *Leishmania* cells using the pX vector. Furthermore, the *T. cruzi* cysteine protease cDNA was expressed in both orientations. However, the level was higher when the correct orientation was employed. Similar observations have been made by other investigators. The mechanism is not yet understood.

Figure 31. Top panel: Map of pX vector showing how the coding region of cysteine protease from *T. cruzi* was ligated at the *Bam*HI site giving recombinant plasmid 7pX+TCCP. The coding region of *T. cruzi* cysteine protease is 1416.

Bottom panel: Map of pX vector showing how the coding region of cysteine protease from *T. cruzi* was ligated at the *Bam*HI site in the opposite direction giving recombinant plasmid 8pX+TCCP.


NcoI, 7257
SpeI, 1
SstI, 785
SpeI, 6643
EcoRI, 6399
XhoI, 6227
NEO/DHFR
SmaI, 1363
BamHI, 1369
SalI, 5700
XhoI, 5694
ApaI, 5685
KpnI, 5679
pX-7TcruziCP
7551 bp
7TcruziCP
ori
f1 ori (-)
Amp
BamHI, 2782
SpeI, 2788
XbaI, 2794
NotI, 2800
SacII, 2810
SstI, 2818
XmnI, 3886

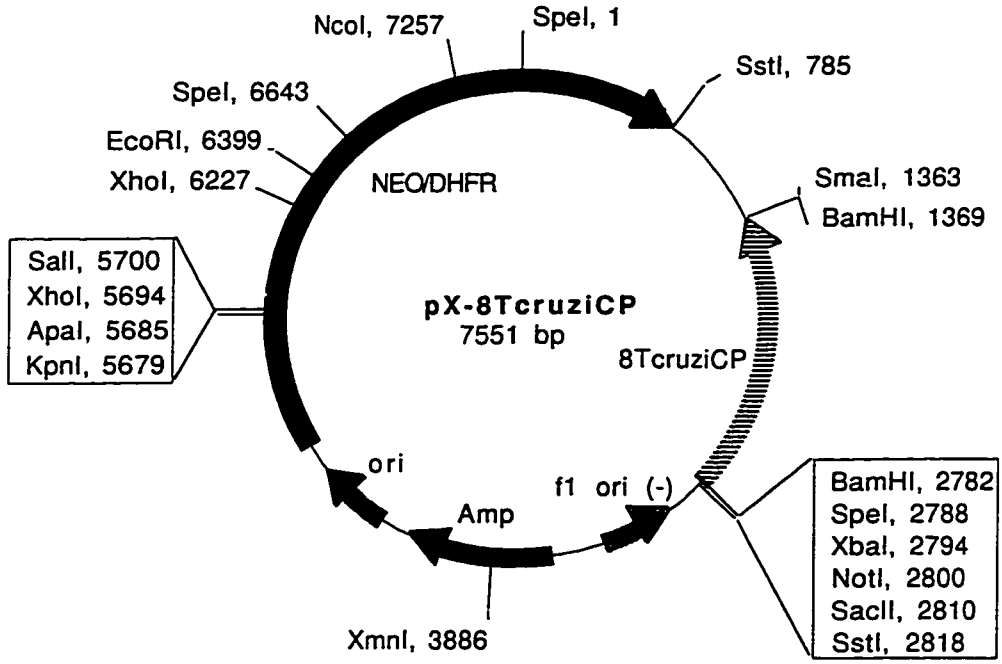

NcoI, 7257
SpeI, 1
SstI, 785
SpeI, 6643
EcoRI, 6399
XhoI, 6227
NEO/DHFR
SmaI, 1363
BamHI, 1369
SalI, 5700
XhoI, 5694
ApaI, 5685
KpnI, 5679
pX-8TcruziCP
7551 bp
8TcruziCP
ori
f1 ori (-)
Amp
BamHI, 2782
SpeI, 2788
XbaI, 2794
NotI, 2800
SacII, 2810
SstI, 2818
XmnI, 3886

Figure 32. Southern blot analysis demonstrating the presence of the coding region of *T. cruzi* CP gene in *Leishmania donovani chagasi* cells. Left: ethidium bromide picture of the genomic DNA from transfected *L. d. chagasi* cells. Right: autoradiographic results from the Southern blot analysis confirming the presence of the coding region of *T. cruzi* CP gene in *L. d. chagasi* cells. Labels kb = marker in kb; 1 = Empty well; 2 = Ldc+pX = clone of *L. d. chagasi* cells transfected with pX only. 3 = Ldc+7pX+TCCP = clone of *L. d. chagasi* cells transfected with pX having cysteine protease in the correct orientation; 4 = Ldc+8pX+TCCP = clone of *L. d. chagasi* cells transfected with pX having cysteine protease in the opposite orientation.

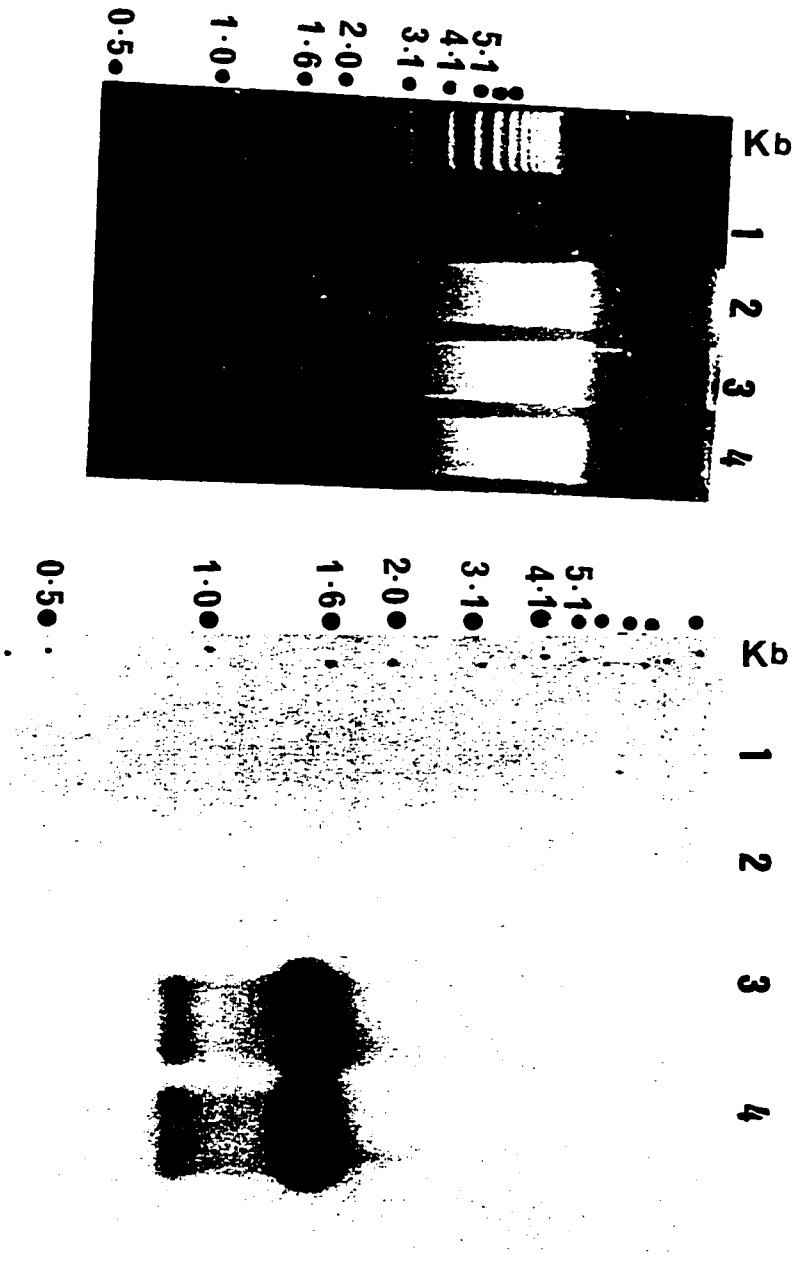

0·5
1·0
1·6
2·0
3·1
4·1
5·1
Kb
1
2
3
4
0·5
1·0
1·6
2·0
3·1
4·1
5·1
Kb
1
2
3
4

Figure 33. Northern blot analysis showing that cysteine protease mRNA from *T. cruzi* is produced in *L. d. chagasi* cells. Left: ethidium bromide picture of the total RNA transfected *L. d. chagasi* cells. Right: Northern blot analysis confirming that cysteine protease mRNA from *T. cruzi* is being produced in *L. d. chagasi* cells. Labels kb = marker; 1 = Ldc+pX = clone of *L. d. chagasi* cells transfected with pX only; 2 = Ldc+7pX+TCCP = clone of *L. d. chagasi* cells transfected with pX having cysteine protease in the correct orientation; 3 = Ldc+8pX+TCCP = clone of *L. d. chagasi* cells transfected with pX having cysteine protease in the opposite orientation.

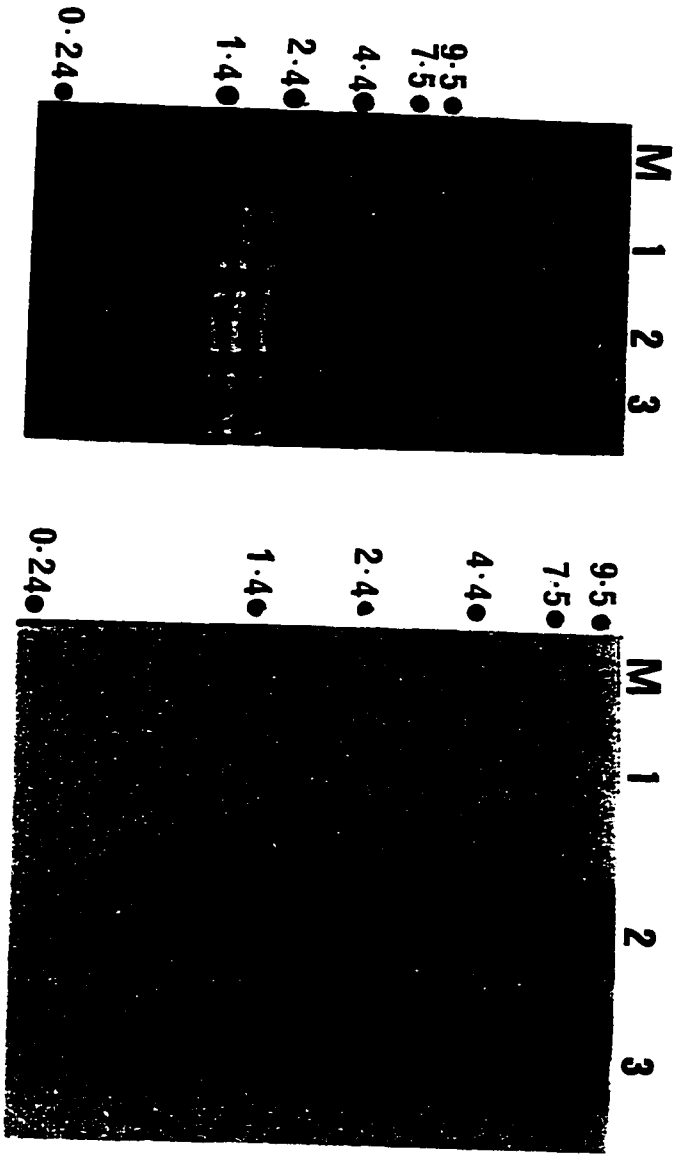
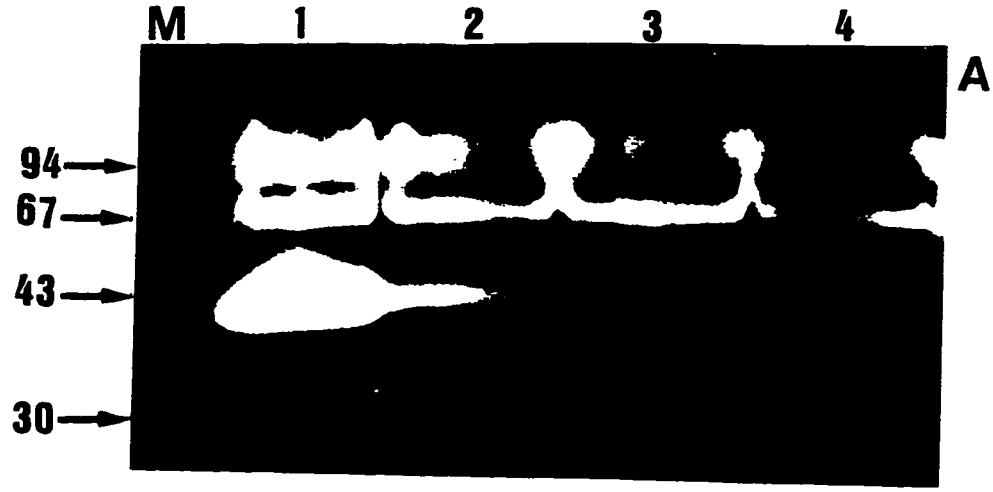
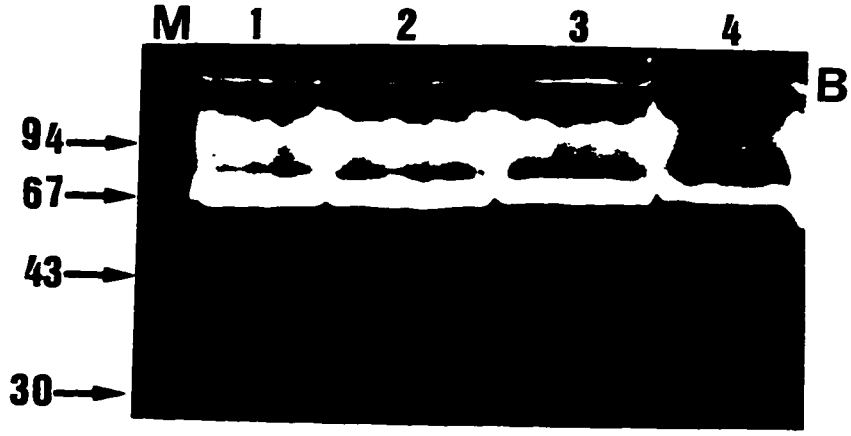

M
1
2
3
9·5
7·5
4·4
2·4
1·4
0·24
M
1
2
3
9·5
7·5
4·4
2·4
1·4
0·24

Figure 34. Panel A: Gelatin SDS-PAGE (non-denaturing) showing the enzymatic activity of the cysteine proteases expressed in *L. d. chagasi* cells. Panel B: a similar gel incubated with inhibitors of cysteine proteases. Labels M = marker in kDa; 1 = Ldc+7pX+TCCP = clone of *L. d. chagasi* cells transfected with pX having cysteine protease in the correct orientation; 2 = Ldc+8pX+TCCP = clone of *L. d. chagasi* cells transfected with pX having cysteine protease in the opposite orientation; 3 = Ldc+pX = clone of *L. d. chagasi* cells transfected with pX only and 4 = Ldc = wild type *L. d. chagasi*.


M
1
2
3
4
A
94
67
43
30


M
1
2
3
4
B
94
67
43
30

Development of a Safe Live Recombinant *Leishmania* Vaccine Line by Overexpression of Cysteine Proteases in *Leishmania major*

MATERIALS AND METHODS

A. Animals and Parsites

BALB/c mice weighing 18-20 grams were obtained from Charles River Canada, 188 Lasalle St-Constant, Quebec, Canada. C.B-17 scid.Biege (SCID) mice weighing 18-20 grams were kindly provided by Dr. Lung-Ji Chang, Department of Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada. The mice were housed in the animal facility at the Department of Biological Sciences, University of Calgary. Before injecting mice with parasites, they were anaesthetized with metofane (methoxyflurane; inhalation anaesthetic for veterinary use) supplied by Janssen Pharmaceutica, 19 Green Belt Drive, North York, Ontario, Canada.

Virulent and avirulent *Leishmania major* was kindly provided by Dr. Steven Beverley (strain of the parasites is described in Titus *et al.* 1995). *Leishmania major* promastigotes were cultured at 26°C in a modified minimal essential medium (HOMEM) (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal calf serum and hemin (GIBCO Laboratories). The parasites were harvested in the stationary phase of growth.

B. Overexpression of cysteine proteases in *Leishmania major* cells

Leishmania major cells were transfected with constructs shown in Figures 18, 31 (top panel) and 35 using procedures described in the thesis under the overexpression of cysteine proteases in *L. d. chagasi*. The constructs were used to transfect avirulent and virulent strains of *Leishmania major* (kindly provided by Dr. Steven Beverley).

C. Virulence of *L. major* promastigotes overexpressing cysteine proteases

The avirulent and virulent strains of *L. major* overexpressing recombinant cysteine proteases were used to examine the impact of cysteine protease on *Leishmania* virulence. Fifty BALB/c mice in groups of 5 were infected with avirulent and virulent strains of *L. major* overexpressing recombinant cysteine proteases as well as controls which were *L. major* transfected with pX and pALT(Neo) and wild type *L. major*. All infections were performed by injection of mice with appropriate dose of the parasites (Table 1) in the right hind footpad by Dr. Douglas Morck (licensed veterinarian). Infected mice were monitored for lesions visually and daily. The BALB/c mice which were injected with *L. major* overexpressing *L. d. chagasi* cysteine protease did not develop lesions by 10 weeks (Table 1) and were challenged with a virulent strain of *Leishmania major*. In addition, 32 SCID.Berge mice in groups of 4 were similarly infected with avirulent and virulent strains of *L. major* overexpressing recombinant cysteine proteases as well as controls which consisted of wild type virulent *L. major* and virulent strains of *L. major* transfected with vectors pX and pALT(neo) (Table 2). Again SCID.Berge mice which were injected with *L. major* overexpressing *L. d. chagasi* cysteine protease did not develop lesions by 10 weeks (Table 2) and were challenged with a virulent strain of *Leishmania major*. As shown in table 1, in the first two experiments injections with *L. major* overexpressing *L. d. chagasi* cysteine protease were performed with 5×10^6 parasites per mouse. When the results from the first two experiments showed that 5×10^6 virulent *L. major* cells overexpressing cysteine proteases from *L. chagasi* were not causing any lesions in BALB/c mice after 10 weeks, the third and fourth experiments were performed with 10^7 and 10^8 transfected parasites respectively to test

whether large numbers of the transfected parasites would induce cutaneous lesions. Large numbers of these parasites (10^7 and 10^8) were used in the last two experiments because other investigators who used these strains of *L. major* have tested their avirulent strain with similar large numbers before (Titus *et al.* 1995). SCID mice were injected with 5×10^6 transfected parasites and the experiment was performed once because of lack of facilities for SCID mice in the Department of Biological Sciences. Both BALB/c and SCID mice which did not develop lesions were challenged with 10^6 virulent *L. major* (Titus *et al.*, 1995). All challenge injections were performed on the right hind footpad by Dr. Douglas Morck. Challenged mice were monitored for lesions visually and daily. All challenged mice were sacrificed as soon as lesions developed in groups that were not protected (at 2 weeks post challenge) to comply with the requirement of the Animal Committee of the University of Calgary. However, in one experiment the protected BALB/c mice in groups 1 and 2 (Table 1) were observed for 10 weeks post challenge.

RESULTS

A. Summary

We have overexpressed cysteine proteases in the *Leishmania major* cells and examined the impact of overexpression on *Leishmania* virulence. We found that a virulent strain of *Leishmania major* overexpressing cysteine proteases from *Leishmania donovani chagasi* failed to cause lesions in susceptible BALB/c mice and could not be recovered when the tissues from the sites of inoculation were cultured. However, the same strain of *L. major* overexpressing cysteine protease from *Trypanosoma cruzi* as well as controls transfected with vectors pX and pALT(neo) caused lesions in susceptible BALB/c mice. When the same cysteine proteases were overexpressed in an avirulent strain of *Leishmania major*, the parasites remained avirulent for susceptible BALB/c mice. In contrast to the wild type *L. major* as well as controls transfected with vectors pX and pALT(neo) which caused lesions in SCID mice, the virulent strain of *L. major* overexpressing cysteine proteases from *L. d. chagasi* failed to cause lesions in the SCID mice. However, when these BALB/c and SCID mice were challenged with a virulent strain of *Leishmania major* 10 weeks after injection with *Leishmania* overexpressing cysteine proteases, only BALB/c mice injected with *Leishmania major* overexpressing Ldccys1 cysteine protease were protected. Thus, we have shown that *Leishmania major* overexpressing Ldccys1 cysteine protease is safe and induce protective immunity against *L. major*.

B. Reasons for overexpressing cysteine proteases in *Leishmania major* cells

The functions of cysteine proteases are currently not well defined. Recently, evidence from disruption of cysteine protease gene array of *L. mexicana* (lmcpb), suggest that these enzymes are virulence factors (Mottram *et al.* 1996). However, Ilga *et al.* (1994) have reported that immunization with recombinant cysteine protease from *L. mexicana* and T cell stimulation experiments using this recombinant enzyme show that the enzyme is a T cell immunogen allowing the development of a protective T_H1 helper cells. In addition, cysteine protease purified from amastigotes of *L. pifanoi* have been shown to protect mice against cutaneous leishmaniasis (Soong *et al.*, 1995). These reports suggest two potential functions for cysteine proteases. In order to confirm or refute these potential functions for cysteine proteases from *L. d. chagasi*, we overexpressed cysteine proteases in *L. major* cells and examined the impact of *L. major* overexpressing cysteine proteases on *Leishmania* pathogenesis. *L. major* was chosen for this study because it causes cutaneous leishmaniasis which is easy to monitor visually.

C. Overexpression of cysteine proteases in *Leishmania major* cells

In order to confirm that the transfected *Leishmania major* cells contained the constructs shown in Figures 18, 31 (top panel) and 35, total genomic and plasmid DNA (10 µg/lane) was digested with *Bam*HI and used for Southern blot analysis. The blot was hybridized with ^{32}P labelled 500 bp fragment from the appropriate cysteine protease cDNA. The results of Southern blot analysis are shown in Fig. 36. The blot (lanes 1 and 2) confirms the presence of the coding region of Ldccys1 cysteine protease gene which is 1344 bp. The

blot (lanes 3 and 4) confirms the presence of the coding region of Ldccys2 cysteine protease gene which is 1416 bp. The blot (lanes 5 and 6) confirms the presence of the coding region of *T. cruzi* cysteine protease gene which is 1416 bp. Lastly, the control blots (lane 9 and 10) confirm that virulent *Leishmania major* were transfected with pALT(Neo) and pX without inserts. These data confirm that all transfected *Leishmania major* cells contain the appropriate constructs.

In order to confirm that the constructs containing the coding regions of Ldccys1 cysteine protease cDNA were expressed in *Leishmania* cells, we used Western blot analysis (we had antibody to Ldccys1 cysteine protease only). One clone was found which expressed high level of cysteine protease (Fig. 37). This was a clone generated by transfecting *L. major* with pALT+4-1 (pALT-Neo containing Ldccys1 cysteine protease gene). However, the expression of the gene could not be detected in the clone generated by transfecting pX+4-12 (pX containing Ldccys1 cysteine protease gene) (Fig. 37).

Figure 35. Top: Map of the plasmid pALT(Neo) showing how the coding region of Ldccys1 cysteine protease cDNA was ligated at the *Bam*HI site giving recombinant plasmid pALT-Ldccy1 (the construct used was pALT4-1). The coding region of Ldccys1 is 1344 bp. The *Leishmania* tubulin intergenic regions are shaded black. The Neo^r gene is shown by broken lines. The Ldccys1 cysteine protease gene is shown as spotted. Bottom: Map of the plasmid pALT(Neo) showing how the coding region of Ldccys2 cysteine protease cDNA was ligated at the *Bam*HI site giving recombinant plasmid pALT-Ldccy2 (the construct used was pALT34-3). The coding region of Ldccys2 is 1416 bp. The *Leishmania* tubulin intergenic regions are shaded black. The Neo^r gene is shown by broken lines. The Ldccys2 cysteine protease gene is shown as spotted.

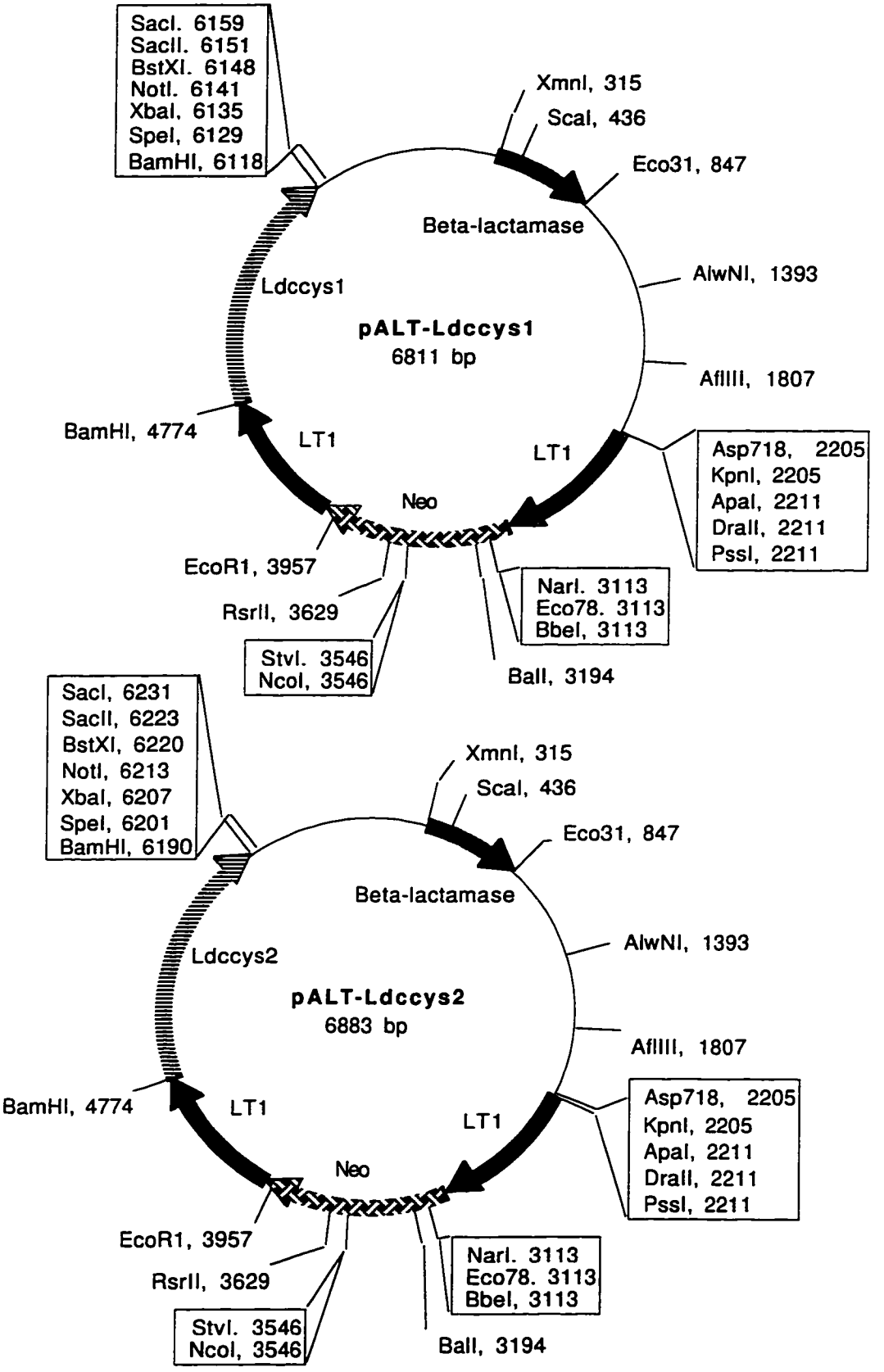

SacI. 6159
SacII. 6151
BstXI. 6148
NotI. 6141
XbaI. 6135
SpeI. 6129
BamHI. 6118
XmnI, 315
ScaI, 436
Eco31, 847
Beta-lactamase
AlwNI, 1393
AflIII, 1807
Asp718, 2205
KpnI, 2205
ApaI, 2211
DraII, 2211
PssI, 2211
NarI. 3113
Eco78. 3113
BbeI, 3113
BalI, 3194
StvI. 3546
NcoI, 3546
RsrII, 3629
EcoR1, 3957
Neo
LT1
LT1
BamHI, 4774
Ldccys1
pALT-Ldccys1
6811 bp
SacI, 6231
SacII, 6223
BstXI, 6220
NotI, 6213
XbaI, 6207
SpeI, 6201
BamHI, 6190
XmnI, 315
ScaI, 436
Eco31, 847
Beta-lactamase
AlwNI, 1393
AflIII, 1807
Asp718, 2205
KpnI, 2205
ApaI, 2211
DraII, 2211
PssI, 2211
NarI. 3113
Eco78. 3113
BbeI, 3113
BalI, 3194
StvI. 3546
NcoI, 3546
RsrII, 3629
EcoR1, 3957
Neo
LT1
LT1
BamHI, 4774
Ldccys2
pALT-Ldccys2
6883 bp

Figure 36. Southern blot analysis demonstrates the presence of the Ldccys1 and Ldccys2 cysteine protease genes as well as *T.cruzi* cysteine protease gene in the transfected *Leishmania major*. Kb = marker (DNA ladder), 9 = virulent *Leishmania major* transfected pALT (neo) alone; 1 = virulent *Leishmania major* transfected with (pALT+4-1) containing Ldccys1 cysteine protease (the coding region of Ldccys1 is 1344 bp.); 2 = avirulent *Leishmania major* transfected with (pX+4-12) containing Ldccys1 cysteine protease; 3 = avirulent *Leishmania major* cells transfected with (pX+34-1) containing Ldccys2 cysteine protease; 4 = virulent *Leishmania major* cells transfected with (pALT+34-3) containing Ldccys2 cysteine protease (the coding region of Ldccys2 is 1416 bp.); 5 and 6 = avirulent and virulent *Leishmania major* cells respectively transfected with (7pX+TCCP) containing *T. cruzi* cysteine protease (the coding region of *T. cruzi* CP is 1416 bp.) and 10 = virulent *Leishmania major* transfected with pX alone. Top band in Panel A in each lane represents Ldccys1 cysteine protease genes of the parasite. NB. The Ldccys2 cysteine protease gene in Panels B was not picked up during hybridization as it is a single gene. However, longer exposure shows its presence. *T. cruzi* cysteine protease did not hybridize with the *Leishmania* cysteine protease genes. Lower band (panels A, B and C) are cysteine proteases from recombinant vectors.

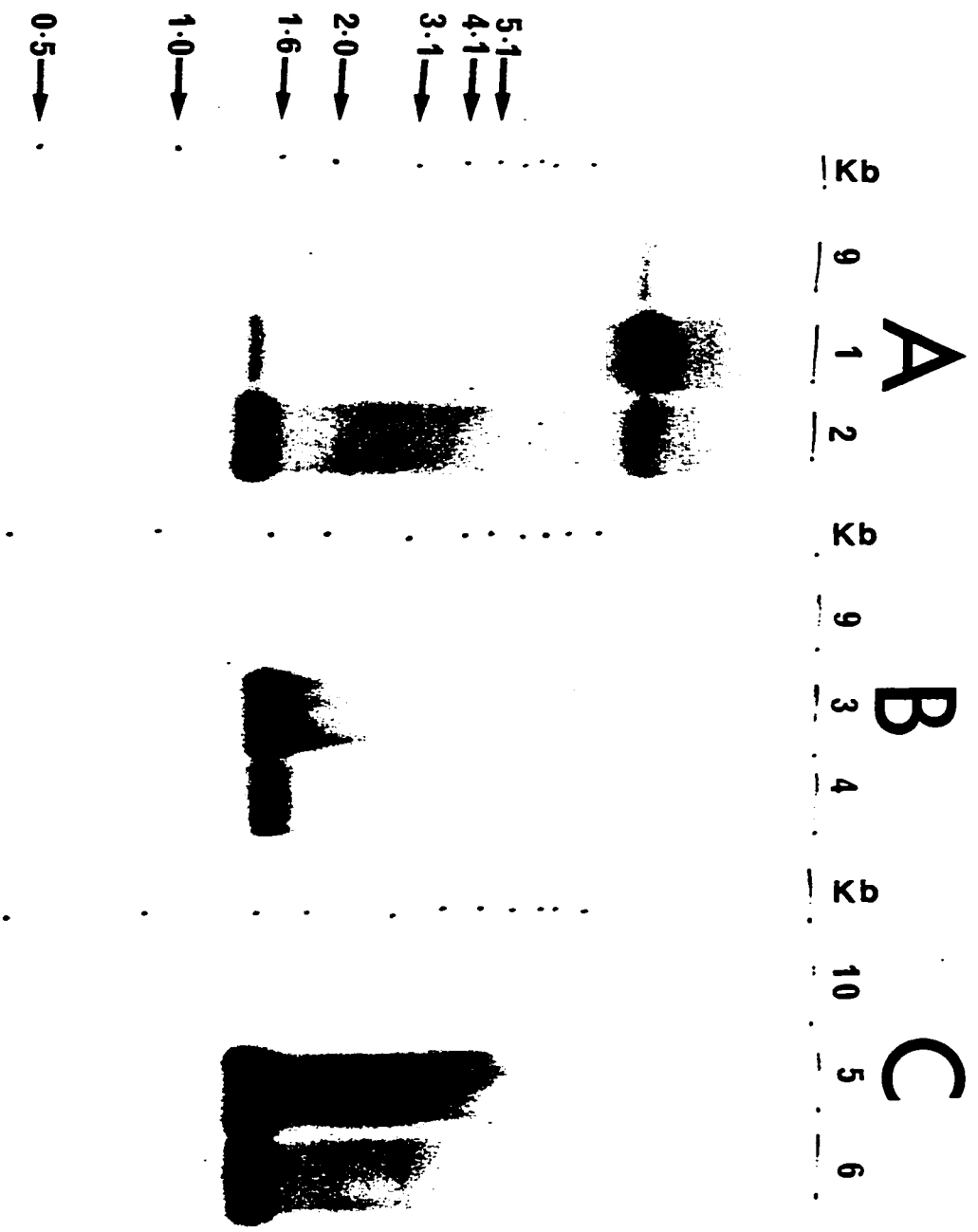
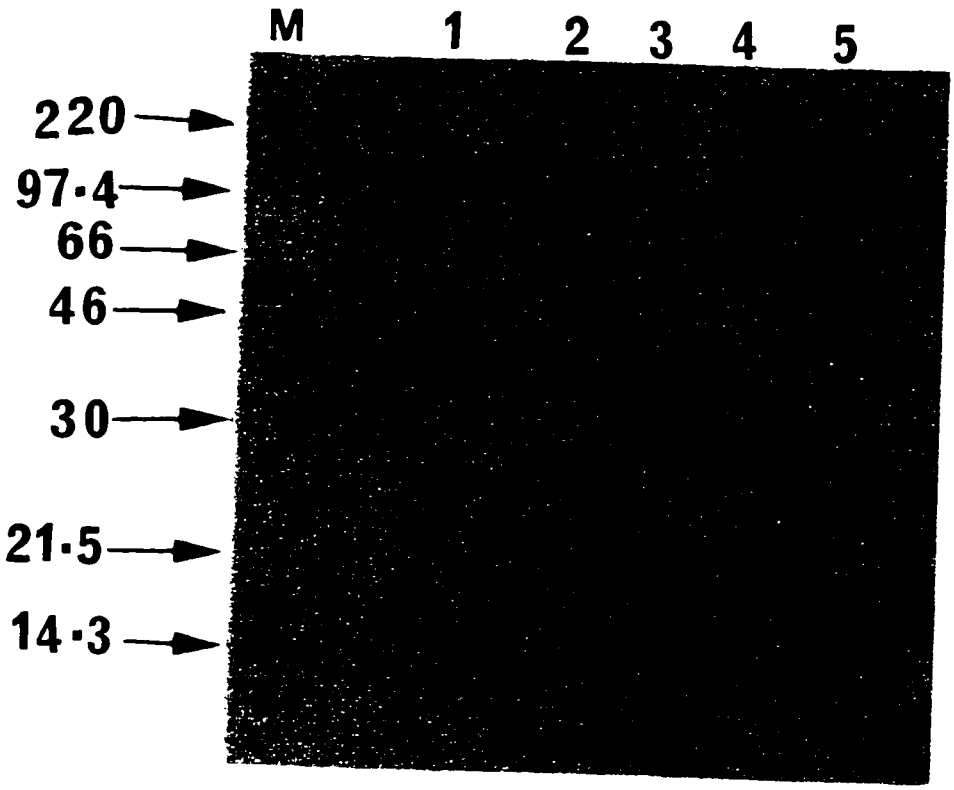

0·5
1·0
1·6
2·0
3·1
4·1
5·1
Kb
9
1
2
A
Kb
9
3
4
B
Kb
10
5
6
C

Figure 37. Western blot analysis which demonstrates that Ldccys1 cysteine protease has been expressed at high levels in *Leishmania major* cells. M = marker in kDa, 1 = virulent *Leishmania major* transfected with (pALT+4-1); 2 = avirulent *Leishmania major* transfected with pX+4-12; 3 = virulent *Leishmania major* transfected with pALT (neo) alone; 4 = virulent *Leishmania major* transfected with pX alone and 5 = untransfected virulent *Leishmania major*. The expressed cysteine protease is seen at about 43 kDa in lane 1. The antibody was raised by direct DNA injection technology described in the text. The marker was the Rainbow coloured protein molecular weight markers (Amersham RPN 756).


M
1
2
3
4
5
220
97·4
66
46
30
21·5
14·3

D. Virulence of transfected promastigotes

We overexpressed cysteine proteases in *Leishmania major* cells and examined the impact of overexpression on *Leishmania* virulence. As shown in Table 1, all BALB/c mice were infected on day 0 by subcutaneous injections of stationary-phase organisms at the shallow site in the right hind footpad. Stationary-phase organisms were used because they provide the infective metacyclic stage (Sacks and Perkins, 1984) of *L. major*. At 2 weeks, only BALB/c mice infected with wild type virulent *L. major* as well as virulent *L. major* strains transfected with pX and pALT(Neo) vectors developed lesions (lesions became noticeable at 10 days post infection and increased to a size shown in Fig. 38 right panel by 14 days). At 6 weeks, BALB/c mice infected with virulent *Leishmania major* cells expressing *T. cruzi* cysteine protease developed lesions (lesions became noticeable at 5 weeks post infection and increased to a size shown in Fig. 38 right panel by 6 weeks). At 10 weeks post infection, none of the BALB/c mice infected with virulent and avirulent *Leishmania major* transfected with pALT+4-1 and pX+4-12, respectively (both of which expressed the Ldccys1 cysteine protease) developed lesions (Table 1, Fig. 38 left panel). Similarly, avirulent and virulent *Leishmania major* cells transfected with pX+34-1 and pALT+34-3, respectively, both of which expressed Ldccys2 cysteine protease, did not develop any lesions (Table 1, Fig. 38 left panel). Lastly, avirulent *Leishmania major* transfected with 7pX+TCCP (expressing the *T. cruzi* cysteine protease) also did not develop any lesion. These data showed that when cysteine proteases from *Leishmania donovani chagasi* were overexpressed in virulent strain of *Leishmania major*, the parasites failed to cause lesions in susceptible BALB/c mice. Virulent strain of *Leishmania major* expressing *T. cruzi* cysteine protease delayed in causing

lesions (Table 1). When the same cysteine proteases were overexpressed in an avirulent strain of *Leishmania major*, the parasites remained avirulent for susceptible BALB/c (Table 1). Since *Leishmania* is known to persist in tissues without causing disease (Titus, Marchand, *et al.*, 1985; Muller, 1992; de Rossell *et al.*, 1993; Aebischer *et al.*, 1993; Peter *et al.*, 1990), tissues from the inoculation sites of *L. major* cells overexpressing *L. d. chagasi* cysteine proteases were cultured but no parasites were recovered. However, when these BALB/c mice were challenged with virulent *L. major* 10 weeks after injection with *L. major* overexpressing cysteine proteases, only BALB/c mice injected with *L. major* cells overexpressing amastigote specific cysteine protease were protected for two weeks post challenge. In one experiment, the protected BALB/c mice in groups 1 and 2 were observed for 10 weeks post challenged. All 5 BALB/c mice in group 1 never developed any lesions within this period suggesting 100% protection. On the other, 2 out of 5 BALB/c mice in group 2 developed lesions after 6 and 8 weeks respectively suggesting 60% protection. This partial protection may be due low level of Ldccys1 cysteine protease expressed by transfected parasites of group 2 (expression could not be detected by Western blot). Taken together, these findings suggest that cysteine proteases from *L. d. chagasi* may have biochemical/enzymatic effects and their overexpression in *L. major* promastigotes prevent *L. major* promastigotes from surviving *in vivo*. The findings further suggest that Ldccys1 cysteine protease is an immunogen which if expressed at high level provides protection against *L. major*. The latter suggestion is plausible because protection was 100% in group 1 where expression of Ldccys1 cysteine protease gene could be detected by Western blot and only 60% in group 2 where expression of the gene could not be detected by Western blot.

We also examined the impact of the overexpression in SCID mice. In contrast to the wild type *L. major* as well as controls transfected with vectors pX and pALT(neo) which caused lesions in SCID mice, the virulent strain of *L. major* cells overexpressing cysteine proteases from *L. d. chagasi* also failed to cause lesions in the SCID mice (Table 2). However, when these SCID mice were challenged with a virulent strain of *Leishmania major* 10 weeks after injection with *L. major* cells overexpressing cysteine proteases, none of the SCID mice injected with *Leishmania major* cells overexpressing cysteine protease were protected. These findings again suggest that overexpression of cysteine proteases cause loss of virulence in *L. major* but do not induce protection in SCID mice. Since SCID mice are deficient in lymphocytes, these findings also suggest that protection seen in BALB/c may be due lymphocytes. The findings further suggest that *Leishmania major* cells overexpressing cysteine protease are not able to survive *in vivo*.

Table 1. Shows that *Leishmania major* promastigotes overexpressing cysteine proteases from *L. d. chagasi* become avirulent and the ones overexpressing Ldccys1 cysteine protease induce protective immunity against *Leishmania major* in BALB/c mice. All BALB/c mice were infected on day 0. At 2 weeks only BALB/c mice infected with a virulent *L. major* as well as virulent *L. major* strains transfected with pX and pALT(Neo) vectors developed lesions. At 6 weeks BALB/c mice infected with virulent *Leishmania major* cells transfected with (7pX+TCCP) containing *T. cruzi* cysteine protease developed lesions. At 10 weeks post infection all BALB/c mice infected with virulent and avirulent *Leishmania major* transfected with (pALT+4-1) and (pX+4-12) respectively (both constructs contained Ldccys1 cysteine protease) did not develop any lesions. Similarly, avirulent and virulent *Leishmania major* cells transfected with (pX+34-1) and (pALT+34-3) respectively (both constructs contained Ldccys2 cysteine protease) did not develop any lesions. Lastly, avirulent *Leishmania major* transfected with (7pX+TCCP) containing *T. cruzi* cysteine protease also did not develop any lesion. When these BALB/c mice which did not develop any lesions were challenged with full virulent strain of *Leishmania major* at 10 weeks after infection, only BALB/c mice injected with *Leishmania major* cells overexpressing Ldccys1 cysteine protease were protected.

Table 1: Summary of infection and challenge of BALB/c mice

CONSTRUCTS	DAY 0	2 Weeks	6 Weeks	10 Weeks	2 Weeks PC
1. *L.. major* FVI + amastigote specific cysteine protease in pALT(Neo)	Infected[1,2]	No Lesion	No Lesion	Challenged[4]	Protected
2. *L.. major* FAI + amastigote specific cysteine protease in pX	Infected	No Lesion	No Lesion	Challenged	Protected
3. *L.. major* FAI + promastigote specific cysteine protease in pX	Infected	No Lesion	No Lesion	Challenged	Lesions
4. *L.. major* FVI + promastigote specific cysteine protease in pALT(Neo)	Infected	No Lesion	No Lesion	Challenged	Lesions
5. *L.. major* FAI + *T. cruzi* cysteine protease	Infected	No Lesion	No Lesion	Challenged	Lesions
6. *L.. major* FVI + *T. cruzi* cysteine protease	Infected	No Lesion	Lesions		
7. *L.. major* FAI (avirulent)	Infected	No Lesion	No Lesion	Challenged	Lesions
8. *L.. major* FVI (virulent)	Infected	Lesions[3]			
9. *L.. major* FVI +pALT (Neo)	Infected	Lesions			
10.*L.. major* FVI + PX	Infected	Lesions			

1 = 5x 10^6 promastigotes in first two experiments; 10^7 and 10^8 promastigotes in the last two experiments.

2 = 5 BALB/c mice per group.

3 = All lesions were similar in size to the one shown in Fig 38 right.

4 = 10^6 virulent *L. major* promastigotes.

Table 2. Shows that *Leishmania major* promastigotes overexpressing cysteine proteases from *L. d. chagasi* failed to cause lesions in C.B17 SCID.Biege mice but none of the SCID mice was protected when challenged with virulent *L. major*. All C.B17 SCID.Biege mice infected on day 0. At 2 weeks only C.B17 SCID.Biege mice infected with wild type virulent *L. major* developed lesions. At 10 weeks post infection all C.B17 SCID.Biege mice infected with virulent and avirulent *Leishmania major* transfected with (pALT+4-1) and (pX+4-12) respectively (both constructs contained Ldccys1 cysteine protease) did not develop any lesions. Similarly, all C.B17 SCID.Biege mice infected with avirulent and virulent *Leishmania major* cells transfected with (pX+34-1) and (pALT+34-3) respectively (both constructs contained Ldccys2 cysteine protease) did not develop any lesions. When all the C.B17 SCID.Biege mice which did not develop any lesions were challenged with a virulent strain of *Leishmania major* at 10 weeks after infection, all of them developed lesions.

Table 2: Summary of infection and challenge of C.B17 SCID.Bierge mice

CONSTRUCTS	DAY 0	2 Weeks	6 Weeks	10 Weeks	2 Weeks PC
1. *L.. major* FVI + amastigote specific cysteine protease in pALT(Neo)	Infected[1,2]	No Lesion	No Lesion	Challenged[4]	Lesions
2. *L.. major* FAI + amastigote specific cysteine protease in pX	Infected	No Lesion	No Lesion	Challenged	Lesions
3. *L.. major* FAI + promastigote specific cysteine protease in pX	Infected	No Lesion	No Lesion	Challenged	Lesions
4. *L.. major* FVI + promastigote specific cysteine protease in pALT(Ne	Infected	No Lesion	No Lesion	Challenged	Lesions
7. *L.. major* FVA (avirulent)	Infected	No Lesion	No Lesion	Challenged	Lesions
8. *L.. major* FVI (virulent)	Infected	Lesions[3]			
9. *L.. major* FVI pALT (Neo)	Infected	Lesions			
10. *L.. major* FVI pX	Infected	Lesions			

1 = 5×10^6 promastigotes.
2 = 4 SCID mice per group.
3 = All lesions were similar in size to the one shown in Fig. 38 right.
4 = 10^6 virulent *L. major* promastigotes.

Figure 38. Shows that *Leishmania major* promastigotes overexpressing cysteine proteases from *L. d. chagasi* become avirulent. Left = BALB/c mouse from a group of mice infected with large numbers (1×10^8) of virulent strains of *Leishmania major* in which amastigote specific cysteine protease was over-expressed and developed no lesion within 10 weeks. None of the BALB/c mice which received 5×10^6, 1×10^7 and 1×10^8 virulent strain of *Leishmania major* in which Ldccys1 and Ldccys2 cysteine proteases were overexpressed developed lesions within 10 weeks. In addition, when the tissues from the sites where the parasites were inoculated were cultured at 10 weeks post challenge, no parasites were recovered. However, all the BALB/c mice which received 5×10^6 virulent *Leishmania major* in which *T. cruzi* cysteine protease was overexpressed developed lesions at 6 weeks. All the BALB/c mice which received 5×10^6, 1×10^7, 1×10^8 avirulent *Leishmania major* in which cysteine proteases were overexpressed did not develop any lesion as the BALB/c infected with avirulent *Leishmania major* in which no cysteine protease was over-expressed. Right = BALB/c mouse from a group of mice infected with 5×10^6 virulent *Leishmania major* transfected with pALT (neo) alone; All of them developed lesions of a similar size at 2 weeks and when the tissues from the lesions were cultured, the parasites were recovered.

DISCUSSIONS

DISCUSSION

We show here that *Leishmania major* promastigotes overexpressing cysteine proteases from *L. d. chagasi* become avirulent and the ones overexpressing Ldccys1 cysteine protease induce protective immunity against *Leishmania major* in BALB/c mice. This conclusion is based on several observations. The virulent strain of *Leishmania major* overexpressing cysteine proteases from *Leishmania donovani chagasi* failed to cause lesions in susceptible BALB/c mice and could not be recovered from tissues at sites of inoculation, whereas the same strain of *L. major* overexpressing cysteine protease from *Trypanosoma cruzi* as well as controls transfected with vectors pX and pALT(neo) caused lesions in susceptible BALB/c mice. When the same cysteine proteases were overexpressed in an avirulent strain of *Leishmania major*, the parasites remained avirulent for susceptible BALB/c mice. The same virulent strain of *Leishmania major* overexpressing cysteine proteases from *L. d. chagasi* also failed to cause lesions in the SCID mice which are deficient in T cells but wild type virulent *Leishmania major* caused lesions in the scid mice. However, when these BALB/c mice were challenged with a virulent strain of wild type *Leishmania major* 10 weeks after injection with *Leishmania* overexpressing cysteine proteases, only BALB/c mice injected with *Leishmania major* overexpressing Ldccys1 cysteine protease were protected. Taken together, these findings suggest that (i) cysteine proteases from *L. d. chagasi* decrease *L. major* virulence and (ii) Ldccys1 cysteine protease is an immunogen which can provide protection against *Leishmania major*.

We expected to show that virulent *L. major* overexpressing cysteine proteases cause more severe lesions in BALB/c mice than the virulent wild type *L. major*. We also expected

show that avirulent *L. major* overexpressing cysteine proteases would become virulent and cause lesions in BALB/c. These results would have confirmed our hypothesis that cysteine proteases are virulent factors in *Leishmania* pathogenesis. Instead, we found that virulent *L. major* overexpressing cysteine proteases from *L. d. chagasi* became avirulent and *L. major* overexpressing Ldccys1 induced protection against *L. major*. In addition, avirulent *L. major* overexpressing cysteine proteases remained avirulent. These findings supported our hypothesis that cysteine proteases are immunogens which induce protective immunity against leishmaniasis. However, the mechanism through which protection is achieved is yet to be defined. Interestingly, other investigators have reported that cysteine proteases are T cell immuogens which allow the development of a protective T_H1 helper cells (Ilga *et al.*, 1994; Soong *et al.*, 1995). Furthermore, it is possible that cysteine proteases from *L. d. chagasi* have biochemical/enzymatic effects which prevent the *L. major* overexpressing them from surviving *in vivo*. However, evidence from disruption of cysteine protease gene array of *L. mexicana* (lmcpb) suggest that these enzymes are virulent factors (Mottram *et al.* 1996). Therefore, more experiments are required to confirm the functions of cysteine proteases.

The decrease in *Leishmania* virulence has been described in the literature before. Many investigators have shown that immunologic interventions made at the time of infection, such as acute $CD4^+$ depletion using anti-CD4 monoclonal antibody (Titus, Ceredig *et al.*, 1985; Sadick *et al.*, 1987), sublethal irradiation (Howard *et al.*, 1981), and administration of anti-IL-4 antibody (Sadick *et al.*, 1990; Chatelin *et al.*, 1992) each enable BALB/c mice to control the parasite and develop effective immunity. In all these cases parasite control were associated with the appearance of T_H1 cells. The type of immune response induced in

successful vaccination with molecularly defined antigens has only been characterized for gp63, gp46/M2 and promastigote surface antigen 2 (Champsi and McMahon-Pratt, 1992; Jardim *et al.*, 1990; McMahon-Pratt *et al.*, 1993; Sadick *et al.*, 1990; Handman *et al.*, 1994) and in all these cases it was T_H1 type of response. We have yet to show that protection induced by our recombinant *Leishmania* is due to a T_H1 type of response.

Although other investigators have previously reported the production of biologically active human p53 (Zhang *et al.*, 1993), murine IFN-γ (Tobin *et al.*, 1993), *Leishmania* gp63 (Liu and Chang, 1992; Roberts *et al.*, 1996) and *Plasmodium yoelii* circumsporozoite protein (Wang *et al.*, 1995) in *Leishmania* cells, only one group of these investigators (Wang *et al.*, 1995) has previously reported the use of *Leishmania* expressing a foreign protein in immunization trials. Thus, this approach represents a new method for using recombinant organisms overexpressing proteins as vaccines and may prove to be very useful in the future.

CONCLUSION

AND

FUTURE RESEARCH

CONCLUSIONS AND FUTURE EXPERIMENTS

Cysteine proteases are a group of proteolytic enzymes whose cysteine or thiol groups are involved in the cleavage of substrate. These enzymes have been described in many pathogenic organisms but their role in the pathogenesis or virulence of those organisms are yet to be defined. In this study, two distinct cysteine protease cDNAs from *L. d. chagasi* and a cDNA encoding a new member of the cysteine protease family of proteins from *Trypanosoma cruzi* were cloned and characterized. However, attempt to express the cysteine protease cDNAs from *L. d. chagasi* in bacterial expression system using several vectors such as pGEX-2T, pET29b, pTrxFus (Appendices 5 and 6) were unsuccessful. We were able to express the cysteine protease cDNA from *T. cruzi* in a bacterial expression system using pGEX-2T (Fig. 30) but cysteine protease is expressed in this system as an inactive insoluble protein. Other investigators working on *T. cruzi* cysteine protease have also reported a similar finding (Eakin *et al.* 1992). Therefore, future experiments should focus on the expression of all the cDNAs in other expression systems such as the baculovirus expression system which has been successful for gp63 (Button *et al.*, 1993). Pure biologically active cysteine proteases are needed for immunization trials to confirm or refute the data presented in this thesis which suggest that cysteine proteases may be immunogens which induce protective immune response against leishmaniasis.

In an attempt to evade the problems encountered in the bacterial expression system, we overexpressed all the three cDNAs in *L. d. chagasi* (Figures 20 and 34). Although we have demonstrated the production of biologically active cysteine proteases in *Leishmania*

cells, we have yet to purify cysteine protease from this system. Since we have shown in this thesis that it possible to raise antibody to Ldccys1 cysteine protease in BALB/c by DNA immunization, this technology will be used to generate monoclonal antibody. BALB/c mice will be immunized with the same construct described in this thesis (Fig 21 top panel) and the spleen cells of BALB/c mice which have responded to DNA immunization will be fused to myeloma cells. Clones secreting antibody which is specific for Ldccys1 cysteine protease will be selected and used in the production of monoclonal antibody to Ldccys1 cysteine protease. The monoclonal antibody will be used in immunoaffinity chromatography (Soong *et al.*, 1995) to purify the biologically active Ldccys1 cysteine protease produced in the *Leishmania* cells. Such purified Ldccys1 cysteine protease is needed for immunization trials to confirm or refute the data presented in this thesis which suggest that Ldccys1 cysteine protease may be an immunogen which induces protective immune response against leishmaniasis.

In an attempt to understand the functions of cysteine proteases, we overexpressed all the three cDNAs in *L. major* cells (Fig. 36) and used the *L. major* cells overexpressing the cysteine proteases to examine the impact of overexpression on *Leishmania* virulence. We found that *L. major* promastigotes overexpressing cysteine proteases from *L. chagasi* became avirulent and the ones overexpressing Ldccys1 cysteine protease induced protective immunity against *L. major* in BALB/c mice. However, SCID mice which are deficient in T cells were not protected and this finding suggests that the protection seen in BALB/c mice may be due to T cell mediated response. In addition, the fact that *L. major* promastigotes overexpressing cysteine proteases did not cause disease in SCID mice suggest that these parasites are not able

to survive *in vivo*. Taken together, these data suggest that *L. major* overexpressing Ldccys1 cysteine protease is safe and induce protective immunity against *L. major*. Future experiments are required to determine the mechanism of this protection.

To understand the molecular and cellular basis of the protection described above, future experiments should focus on dissecting the type of immune response(s) responsible for the above protection. There will be need to purify $CD4^+$, $CD8^+$ and $NK1.1^+$ cells from the popliteal lymph nodes draining the infected footpads and spleens by magnetic beads (Reiner *et al.* 1994) at various times after infections and challenge. Such purified cells need to be subjected to two types of analysis. First, these cells need to be cultured separately with and without antigens to determine the subset of cells which are secreting INF-γ or IL-4 in the popliteal lymph nodes draining the infected footpads and spleens after infection and challenge. Secondly, there is need to employ a system of competitive quantitative reverse transcriptase-PCR (RT-PCR) (Reiner *et al.* 1993; Reiner *et al.* 1994) to measure the various cytokines in the popliteal lymph nodes draining the infected footpads and spleens of the protected mice and the mice that develop lesions to see whether they correlate with the established T_H1 or T_H2 cytokine pattern. Thus, there is need to extract total RNA from the $CD4^+$, $CD8^+$ and $NK1.1^+$ cells purified from the popliteal lymph nodes draining the infected footpads and spleens after infection and challenge. The total RNA extracted will then be subjected to RT-PCR using protocols described (Reiner *et al.*, 1993) to determine mRNAs for INF-γ and IL-4 in these cells at various times after infections and challenge. There will be need to examine the infected and challenge mice for at least 12 weeks to ensure immune response has reached maximum level (Menon and Bretscher, 1996). Data from these analysis will enable us to

determine the type of immune response(s) involved in protection.

The requirement by the Animal Committee of the University of Calgary that mice be sacrificed as soon as they developed lesions prevented us from measuring lesion sizes. There is need to keep all infected and challenged mice for at least 12 weeks and to measure lesion sizes every week so that the data presented in tables 1 and 2 can be translated into graphs as reported (Bretscher *et al.* 1992; Titus *et al.* 1995). Apparently, the results of FACs analysis presented (Appendix 9, Table 3) is not conclusive because the mice were sacrificed too early for the response of the protective cells to show up (Menon and Bretscher, 1996). There is therefore need to repeat FACS analysis on the cells from the popliteal lymph nodes draining the infected footpads and spleens at 12 weeks after challenge. Such analysis may reveal the expansion of the protective subset of lymphocytes in the popliteal lymph nodes and spleens.

In collaboration with Dr. Mary Wilson from Iowa University, we have started examining the impact of overexpressing of cysteine protease in *L. chagasi* virulence. Preliminary data indicates that overexpression of cysteine proteases from *L. chagasi* in *L. chagasi* promastigotes cause loss of virulence (data not shown). The same strain of *L. chagasi* transfected with pX vector were also avirulent (*L. chagasi* used has been maintained in culture for 5 years without passage in animal and might have lost virulence). However, when these BALB/c mice were challenged with a virulent strain of *L. chagasi* 4 weeks after injection with *L. chagasi* overexpressing cysteine proteases, only BALB/c mice injected with *L. chagasi* overexpressing Ldccys2 cysteine protease were protected. This data suggests that *L. chagasi* overexpressing Ldccys2 cysteine protease induce protective immunity against *L. chagasi* in BALB/c mice. This experiment need to be repeated to verify whether it is the

Ldccys1 or Ldccys2 cysteine protease which is protective and to initiate studies to determine the cellular and molecular basis of this protection.

A number of recent reports have shown the significance of the 3' untranslated region (UTR) and intergenic regions in regulating gene expression on trypanosomatids (Jefferies, *et al.*, 1991; Ramamoorthy *et al.*, 1992; Teixeira *et al.*, 1995; Charest *et al.*, 1996). Since signals for trans-splicing and polyadenylation are contained in these regions, the mRNA abundance could potentially be regulated at one or both of these steps. Alternatively, the 3' UTR could contain binding sites for proteins that influence the stability of mRNA. Future experiments are required to address the issue of stage specific expression of Ldccys1 cysteine protease genes.

We have screened *L. donovani chagasi* genomic library in λ ZAP II and have isolated seven clones (clones # 1, 2, 4, 5, 7, 8 and 10). We have partially characterized these clones and confirmed that they contain Ldccys1 cysteine protease genes which are arranged as several copies in tandem (data not shown in this thesis). The sequencing of these clones will be carried out using an automated DNA sequencer which is available at the Regional Laboratory at the University of Calgary. Since we have already shown that Ldccys1 cysteine protease genes are in tandem arrangement (Fig. 13), several overlapping phage clones will be used to construct a contiguous map of the tandem array of Ldccys1 cysteine protease genes. This experiment will enable us to perform gene knock out experiments.

Gene replacement by homologous recombination is a powerful technique that has been used in testing gene function in different organisms (Cruz and Beverley, 1990; Curotto de Lafaille and Wirth 1992). We therefore propose to use homologous gene recombination to

delete the cysteine protease gene locus on one allele by replacement with the NEO gene and selection in G418 followed by sequential replacement of the other locus by HYG gene and selection in hygromycin B. Plasmids containing selectable markers (Neo and Hygro) were kindly provide by Drs. S. Beverley and D.F. Wirth, Harvard School of Public Health, Boston, MA). *Leishmania* that are resistant for both G418 and hygromycin B will then be selected and used in functional analysis of cysteine protease as described by others recently (Mottram *et al.*, 1996).

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APPENDIX

APPENDIX 1

PRIMERS FOR SEQUENCING Ldccys1 CYSTEINE PROTEASE

The following primers were built for sequencing Ldccys1 cysteine protease cDNA from *Leishmania donovani chagasi*:

PLdc1-1: 18 bp. 5' TGCTCCGACAGGCTCACC 3'.

PLdc1-2: 18 bp. 5' GGTGGGGTTCCGTACTGG 3'.

PLdc1-3: 21 bp. 5' ACGGAGAAGAGCTACCCCTAC 3'.

PLdc1-4: 19 bp. 5' GCTGTTCAAGCACTCGGCC 3'.

PLdc1-5: 18 bp. 5' ACCGCGAACGTGTGCTAC 3'.

PLdc1-6: 17 bp. 5' TGCTGCTTCGCCGCTGC 3'.

PLdc1-7: 18 bp. 5' CCGTACTTCTTGGGCTCG 3'.

PLdc1-8: 18 bp. 5' TACGTCCGCTTGAACTCC 3'.

PLdc1-10: 18 bp. 5' ACGTGCAACGCAGAGGCC 3'.

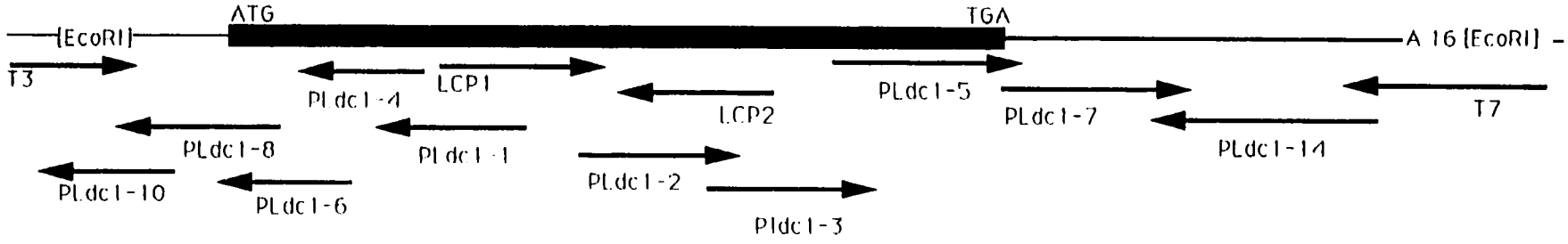
PLdc1-14: 17 bp. 5' TCCGGCACAGACGCACC 3'.

The following primers were built to amplify cysteine protease from *Leishmania* genomic DNA and it turned out to be general primers which can amplify cysteine protease from virtually all the Trypanosomatids:

LCP-1: 33 bp. 5' ACAGAATTCCAGGGCCAGTGCGGCTCGTGCTGG 3' Underline indicates *Eco*RI site.

LCP-2: 33 bp. 5' CGCAAGCTTCCACGAGTTCTTCACGATCCAGTA 3' Underline indicates *Hin*dIII site.

The positions of these primers are shown by arrow on the diagram on the left.


ATG
TGA
(EcoRI)
A 16 (EcoRI) -
T3
PLdc1-4
LCP1
LCP2
PLdc1-5
PLdc1-7
T7
PLdc1-8
PLdc1-1
PLdc1-14
PLdc1-10
PLdc1-6
PLdc1-2
PLdc1-3

APPENDIX 2

PRIMERS FOR SEQUENCING Ldccys2 CYSTEINE PROTEASE

The following primers were built for sequencing Ldccys2 cysteine protease cDNA from *Leishmania donovani chagasi*:

PLdc3-3: 19 bp. 5' GTAGCTGTCCTCCGTGGGC 3'.

PLdc3-4: 19 bp. 5' GTCGACGCGACAACCTGGC 3'.

PLdc3-5: 19 bp. 5' CGATGTTGCCAGTGGTGGC 3'.

PLdc3-6: 18 bp. 5' CCCACGGCGCTTGTGCAG 3'.

PLdc3-7: 18 bp. 5' GCTGTCTGCATGTTCTGC 3'.

PLdc3-8: 18 bp. 5' CTATGTATGTGCAGCTAC 3'.

PLdc3-9: 18 bp. 5' GCGGCGGATGCGTGAGCG 3'.

PLdc3-12: 17 bp. 5' CCGACTGACATGCACGC 3'

The following primers were built to amplify cysteine protease from *Leishmania* genomic DNA and it turned out to be general primers which can amplify cysteine protease from virtually all the Trypanosomatids:

LCP-1: 33 bp. 5' ACAGAATTCCAGGGCCAGTGCGGCTCGTGCTGG 3' Underline indicates *Eco*RI site.

LCP-2: 33 bp. 5' CGCAAGCTTCCACGAGTTCTTCACGATCCAGTA 3' Underline indicates *Hin*dIII site.

The positions of these primers are shown by arrow on the diagram on the left.

T3
EcoRI
AIG
pLdc3-9
pLdc3-7
LCP1
pLdc3-5
pLdc3-4
LCP2
pLdc3-6
pLdc3-8
pLdc3-10
pLdc3-12
TGA
A 67 [XhoI]
T7

APPENDIX 3

PRIMERS FOR SEQUENCING CYSTEINE PROTEASE FROM *TRYPANOSOMA CRUZI*

The following primers were built for sequencing cysteine protease cDNA from *Trypanosoma cruzi*:

LCP-3: 25 bp. 5' GGTCACGTTGAATTACCTCAGGACG 3'

LCP-4: 18 bp. 5' CGACAGGTTCGTCAGCGG 3'

LCP-5: 18 bp. 5' CCGCTGAGCGTGTTCAGG 3'

LCP-6: 21 bp. 5' CTGGATCATCAAGAACTCGTG 3'

LCP-7: 18 bp. 5' ACGTGACGTTACCGACCG 3'

LCP-8: 18 bp. 5' AGGGCGTGACGCGAAGTC 3'

LCP-9: 18 bp. 5' TTAAGAGGCTCCGTTGAG 3'

LCP-10: 17 bp. 5' GTCACGGCGCCTCTCGC 3'

LCP-11: 18 bp. 5' CATTACTGCTTGCGTGGG 3'

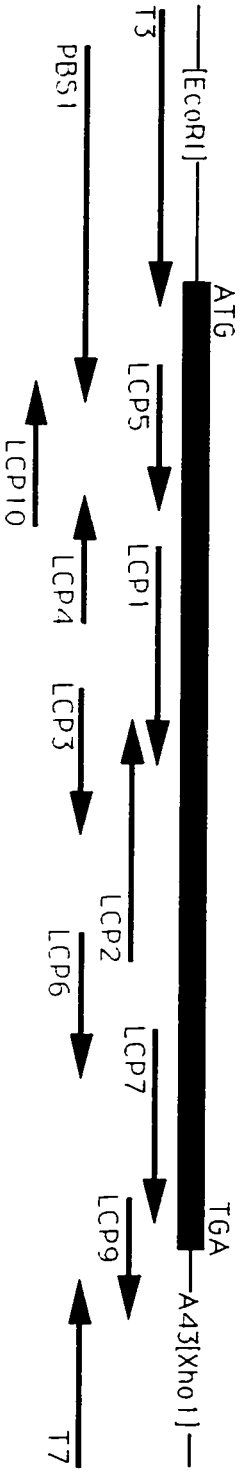
LCP-12: 21 bp. 5' CGTGACGCCGAAGGTCGCGTG 3'

The following primers were built to amplify cysteine protease from *Leishmania* genomic DNA and it turned out to be general primers which can amplify cysteine protease from virtually all the Trypanosomatids:

LCP-1: 33 bp. 5' ACAGAATTCCAGGGCCAGTGCGGCTCGTGCTGG 3' Underline indicates *Eco*RI site.

LCP-2: 33 bp. 5' CGCAAGCTTCCACGAGTTCTTCACGATCCAGTA 3' Underline indicates *Hin*dIII site.

The positions of these primers are shown by arrow on the diagram on the left.


[EcoRI]
T3
PBS1
ATG
LCP5
LCP10
LCP4
LCP1
LCP3
LCP2
LCP6
LCP7
LCP9
TGA
A43[Xho1]
T7

APPENDIX 4

PRIMERS FOR SUBCLONING

Overexpression of cysteine protease from *Trypansoma cruzi* in *Escherichia coli* and *Leishmania* cells.

The sequences of the two primers are used to amplify the coding region of cysteine protease cDNA from *Trypanosoma cruzi* for subcloning into pGEX-2T, pX, pALT(Neo) are:

Sense Primer LCP-GST1: 5'CAAGGAAGGATCCACACCCACGCAAGCAGT 3'.

Antisense Primer LCP-GST2:

5'GAGAAAAGGATCCATCAGAAACAAAGTGTGCC 3'.

The underline indicates the position of *Bam*HI restriction sites in both primers.

The sequences of the two primers are used to amplify the coding region of cysteine protease cDNA from *Trypanosoma cruzi* for subcloning into *Bacillus* vector are:

Sense Primer LCP-B1F:

5' GCAACTCAAGCTTTTGCCAGCCTGCATGCGGAGGAGAC 3'

Antisense Primer LCP-B1R:

5' GAGAGAAAAAAGCTTTTATCAGAAACAAAGTGTGCCCTC 3'

The underline indicates the position of *Hin*dIII restriction sites in both primers.

APPENDIX 5

PRIMERS FOR SUBCLONING

Overexpression of Ldccys1 cysteine protease in *Escherichia coli* and *Leishmania* cells.

4-1-1-GST1: 32 bp. 5' TTCTCTTCCCCTCGCTGGATCCTCC 3'.

4-1-1-GST2: 24 bp. 5' AGACGCAGGATCCACCCCTACGTG 3'.

4-1-1-pET3': 38 bp. 5'

GGGGGGATCCTTCGTGTACTGGCAGGTGTTCATGATCG 3'.

4-1-1-pTrix: 37 bp. 5'

GGGGGATCCTATGGCGACGTCGAGGGCCGCTCTCTGC 3'.

The underline indicates the position of *Bam*HI restriction sites in all the four primers.

4-1-1-GST1 + 4-1-1-GST2 were used to amplify the coding region of Ldccys1 cysteine protease cDNA for subcloning into pGEX-2T, pX, and pALT(Neo).

4-1-1-GST1 + 4-1-1-pET3' were used to amplify the coding region of Ldccys1 cysteine protease cDNA for subcloning into pET29b$^+$ vector.

4-1-2-pTrix1 + 4-1-1-GST2 were used to amplify the coding region of Ldccys1 cysteine protease cDNA for subcloning into pTrxFus (Invitrogen).

APPENDIX 6

PRIMERS FOR SUBCLONING

Overexpression of Ldccys2 cysteine protease in *Escherichia coli* and *Leishmania* cells.

34-1-2-GST1: 29 bp. 5' GCACGTTGCACCAACAAGGATCCGCAAAG 3'

34-1-2-GST2: 24 bp. 5' GCACAGTGGATCCTGCCAGTCCTA 3'

34-1-2-pET3': 36 bp. 5'

GGGGGGATCCTTGATGTCTGCGTGCCTCGCGTGTGG 3'

34-1-2-pTrx1: 31 bp.

5' GGGGGATCCTGCAAAGATGGCGCGCAACCCC 3'

The underline indicates the position of *Bam*HI restriction sites in all the four primers.

34-1-2-GST1 + 34-1-2-GST2 were used to amplify the coding region of Ldccys2 cysteine protease cDNA for subcloning into pGEX-2T, pX and pALT(Neo).

34-1-2-GST1 + 34-1-2-pET3' were used to amplify the coding region of Ldccys2 cysteine protease cDNA for subcloning into pET29b^{-} vector.

34-1-2-pTrx1 + 34-1-2-GST2 were used to amplify the coding region of Ldccys2 cysteine protease cDNA for subcloning into pTrxFus (Invitrogen).

APPENDIX 7

PRIMERS FOR SUBCLONING

Overexpression of cysteine proteases with HisTag in *Leishmania* cells.

The following primers were built to subclone all genes which were first subcloned in pET vector into pX and pALT (Neo). The protein was expected to be produced as a fusion with HisTag at the end.

pX-HisI 32 bp. 5' CCCTGACTAGACTAGTCGCGGTTCCTTGGCGATATCG 3'. This primer was not used because it has *Spe*I site which is also in pX. Underline is the *Spe*I site.

pX-HisIII 32 bp. 5' CCCTGTCTAGACGCGGTTCCTTGGCGATATCG 3'. This primer was used instead of pX-HisI. The underline is *Xba*I site.

pX-HisII 32 bp. 5' CGGTGGTCTAGACCAACTCAGCTTCCTTTCGGGC 3'. Underline is the *Xba*I site.

APPENDIX 8

PRIMERS FOR SUBCLONING

Overexpression of cysteine proteases with GST in *Leishmania* cells.

The following primers were built to amplify all genes which were first subcloned in pGEX-2T vector for subsequent subcloning into pX and pALT (Neo). The protein was expected to be produced as a fusion with GST.

5'*Xba*I-GST 36 bp.

5' ACGTCATCTAGAATGTCCCCTATACTAGGTTATTGG 3'. Underline is the *Xba*I site.

3'*Xba*I-GST 36 bp.

5' AGCTCGATCTAGACCCGGGCGTCAGTCAGTCACGAT 3'. Underline is the *Xba*I site.

5'GST 40 bp.

5' ACGTCAGAAGCTTCATATGTCCCCTATACTAGGTTATTGG 3'. Underline is the *Hin*dIII site.

APPENDIX 9

MATERIALS AND METHODS

A. Preparation of crude antigens for the stimulation of lymphocytes

The parasites were cultured for seven days before use in the preparation of crude antigens. Washed transfected and untransfected promastigotes were resuspended in PBS at a rate of 2×10^8 per ml and sonicated under sterile conditions for 5 x 15 seconds on ice with Virsonic cell disrupter at a setting of 60 using small probe. Protein concentration of total cell lysates was estimated using the BCA technique (Pierce Chemical Co.).

B. Harvest and culture of lymphocytes

The animals were sacrificed after two weeks post infection or challenge (i.e. as soon as the lesions developed) to comply with the requirement of the animal committee of the University of Calgary. Popliteal lymph nodes draining the infected foot pads were excised from infected and challenged BALB/c mice, dispersed into single cell suspensions using the rough edge of slide. Single cell suspensions were filtered through sterile nylon mesh (pore size: 40 microns; supplied by Small Parts Inc. Miami Lakes Fl 33014-0650). Lymphocytes were counted and two aliquots of 1×10^6 lymphocytes were used for FACS analysis. The rest of the lymphocytes were cultured in RPMI 1640 supplemented with 3 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, 20 mM HEPES (pH 7.4), 10% heat-inactivated foetal calf serum (GIBCO BRL), 200 U/ml penicillin, and 100 μg/ml streptomycin (complete media). Lymphocytes were seeded at a rate of 5×10^6/ml in complete media and incubated for 96 hours at 37°C under 5% CO_2 in 12-well Costar plates. The cells in culture

were stimulated with approximately 20 μg/ml of crude *L. major* antigen derived from recombinant *L. major* promastigotes used for the initial immunization (Soong *et al.*, 1995).

C. Two-colour fluorescence staining and FACS analysis

Lymphocytes (total cells) were obtained from the popliteal lymph nodes draining the infected foot pads. Cells at a concentration of 1 x 10^6 were stained with a mixture of FITC labelled anti-mouse CD4 (PharMingen; Cat #06064D) and PE labelled anti-mouse CD8 (PharMingen; Cat #01045B). Cells were gated using forward and side scatters utilizing a Becton Dickinson FACScan flow cytometer and Cell Quest Software (Becton Dickinson, San Jose, CA) and were analyzed for marker expression. The gate for viable cells was determined using propidium iodide exclusion.

D. Statistical Analysis

The data from FACS Analysis was analyzed using one way analysis of variance using ANOVA procedure from the MINITAB, available in the Computer laboratory at the Department of Mathematics and Statistics, University of Calgary (MINITAB is a registered trademark of Minitab Inc., 3081 Enterprise, State College PA 16801 USA).

E. Cytokine assays

Concentrations of INF-γ, as well as IL-4 in the supernatant of lymphocytes (total cells) from the popliteal lymph nodes draining the infected foot pads, were measured with two-sandwich ELISAs as described by others (Wilson *et al.*, 1995; Wilson *et al.*, 1996). To

measure INF-γ, plates were coated with mAb HB170 to IFN-γ (American Type Culture Collection, Rockville, MD.) and incubated with supernatant. INF-γ, was detected with polyclonal rabbit anti-INF-γ followed by biotinylated goat anti-rabbit IgG (Acurate), Streptavidin-horseradish peroxidase, and ABTS substrate (Zymed, San Francisco, CA.) (Wilson *et al.*, 1996). IL-4 was captured with 11B11 (HB191, DNAX Research Institute, Palo Alto, CA.) and detected with biotinylated BVD6 (kindly provided by Drs. Kevin Moore and John Abrams, DNAX) (Wilson *et al.*, 1996).

RESULTS AND DISCUSSIONS

A. FACS analysis

In an attempt to understand the cellular basis of protection observed in BALB/c mice, lymphocytes (total cells) were obtained from the popliteal lymph nodes draining the infected foot pads. Using FACS analysis, we examined T cell subsets from the popliteal lymph node draining the infected foot pad after infection and challenge (Fig. 39). Further examination of the data obtained from FACS analysis revealed that there was increase in the population of double negative cells (i.e. cells expressing neither CD4 nor CD8 which may be NK or B cells) in the popliteal lymph nodes of protected BALB/c mice (groups 1 and 2) and in most unprotected mice (groups 2, 3, 4, 5, 6, 8 and 9). In one unprotected group of mice (group 9) the increase in the population of double negative cells in the popliteal lymph nodes was not significantly higher from mice injected with saline (control group). When the data from FACS Analysis was analyzed using one way analysis of variance using ANOVA procedure from the MINITAB there was significant difference between the population of double negative cells in the popliteal lymph nodes of mice injected with saline and those from the unprotected mice in groups 2, 3, 4, 5, 6, 8 and 9 (the P value was 0.000). However, there was no significant difference between the population of double negative cells in the popliteal lymph nodes of mice injected with saline and those from group 10 which was also unprotected. The fact that double negative cells in the popliteal lymph nodes of both the protected BALB/c mice and most unprotected mice increased, suggest that this increase may not be important. If the increase is due to B cells, these cells will produce antibody which is not important in the

control of leishmaniasis. However, if these increase is due to NK cells, they may be important because these cells have been shown to be the source of INF-γ that drives differentiation of $CD4^+$ T cell subsets and induces early resistance to *Leishmania major* in mice (Scharton and Scott, 1993).

The ratio between $CD4^+$ and $CD8^+$ subsets of T cell population decreased substantially when compared with that found in normal life (Table 3). The decrease in the ratio between $CD4^+$ and $CD8^+$ T cells was mainly due to a decrease in the $CD4^+$ T cell population (Table 3). This was surprising, because there is compelling evidence that the T_H1 type of $CD4^+$ T cells and the cytokines which they secrete play a major role in the resolution of primary infection in *L. major* (Lie, 1989; Locksley and Scott, 1991; Mitchell, 1984; Scott *et al.*, 1989). It is possible that FACS analysis may not be a good technique to look at mechanism of protection in leishmaniasis.

B. Cytokine Analysis

In an attempt to understand the molecular basis of protection seen in the protected BALB/c mice (Table 1), we measured the concentrations of INF-γ and IL-4 in the supernatant of lymphocytes (total cells) from the popliteal lymph nodes draining the infected foot pads using ELISA. Our results showed an increase in the levels of INF-γ in the supernatants derived from the lymphocytes of the protected BALB/c mice and low levels of this cytokine in similar supernatants derived from the lymphocytes of the BALB/c mice which developed lesions (Table 4). The levels of IL-4 was very low in both groups of mice (data not shown). The low levels INF-γ and IL-4 may be due to the fact that the mice were

sacrificed too early to comply with the requirement of the Animal Committee of the University of Calgary. This is possible because Menon and Bretscher (1996) showed that during the first few weeks after infection with *L. major* the levels of these cytokines are very low. The levels of these cytokines become high at 12 weeks post infection (Menon and Bretscher, 1996).

Figure 39. FACS Analysis show the abundance of $CD4^+$ and $CD8^+$ subsets of T cell population. 1 = Lymphocytes from BALB/c mice injected with saline; 2 and 3 = Lymphocytes from BALB/c mice injected with virulent *Leishmania major* transfected with (pALT+4-1) containing Ldccys1 cysteine protease and challenged with virulent *Leishmania major*; 4 = Lymphocytes from BALB/c mice injected with avirulent *Leishmania major* transfected with (pX+4-12) containing Ldccys1 cysteine protease and challenged with virulent *Leishmania major*; 5 = Lymphocytes from BALB/c mice injected with avirulent *Leishmania major* cells transfected with (pX+34-1) containing Ldccys2 cysteine protease and challenged with virulent *Leishmania major*; 6 = Lymphocytes from BALB/c mice injected with virulent *Leishmania major* cells transfected with (pALT+34-3) containing Ldccys2 cysteine protease and challenged with virulent *Leishmania major*; 7 = Lymphocytes from BALB/c mice injected with virulent *Leishmania major* cells transfected with (pALT+34-3) containing Ldccys2 cysteine protease and challenged with virulent *Leishmania major*. 8 = Lymphocytes from BALB/c mice injected with avirulent *Leishmania major* cells transfected with (7pX+TCCP) containing *T. cruzi* cysteine protease and challenged with virulent *Leishmania major*. 9 = Lymphocytes from BALB/c mice injected with virulent *Leishmania major* cells transfected with (7pX+TCCP) containing *T. cruzi* cysteine protease and not challenged. 10 = Lymphocytes from BALB/c mice injected with wild type virulent *Leishmania major* (post infection). 11 = Lymphocytes from BALB/c mice injected with virulent *Leishmania major* transfected with pALT(Neo) alone (post infection) and 12 = Lymphocytes from BALB/c mice injected with virulent *Leishmania major* transfected with pX alone (post infection). In all cases lower left quadrant = double negative (i.e. cells expressing neither CD4 nor CD8 which may be NK or B cells); Upper left quadrant = FL2-H = PE labelled $CD8^+$ T cells; Lower right quadrant = FL1-H = FTC labelled $CD4^+$ T cells; Upper right quadrant = double positives suggesting immature lymphocytes expressing both $CD4^+$ and $CD8^+$.

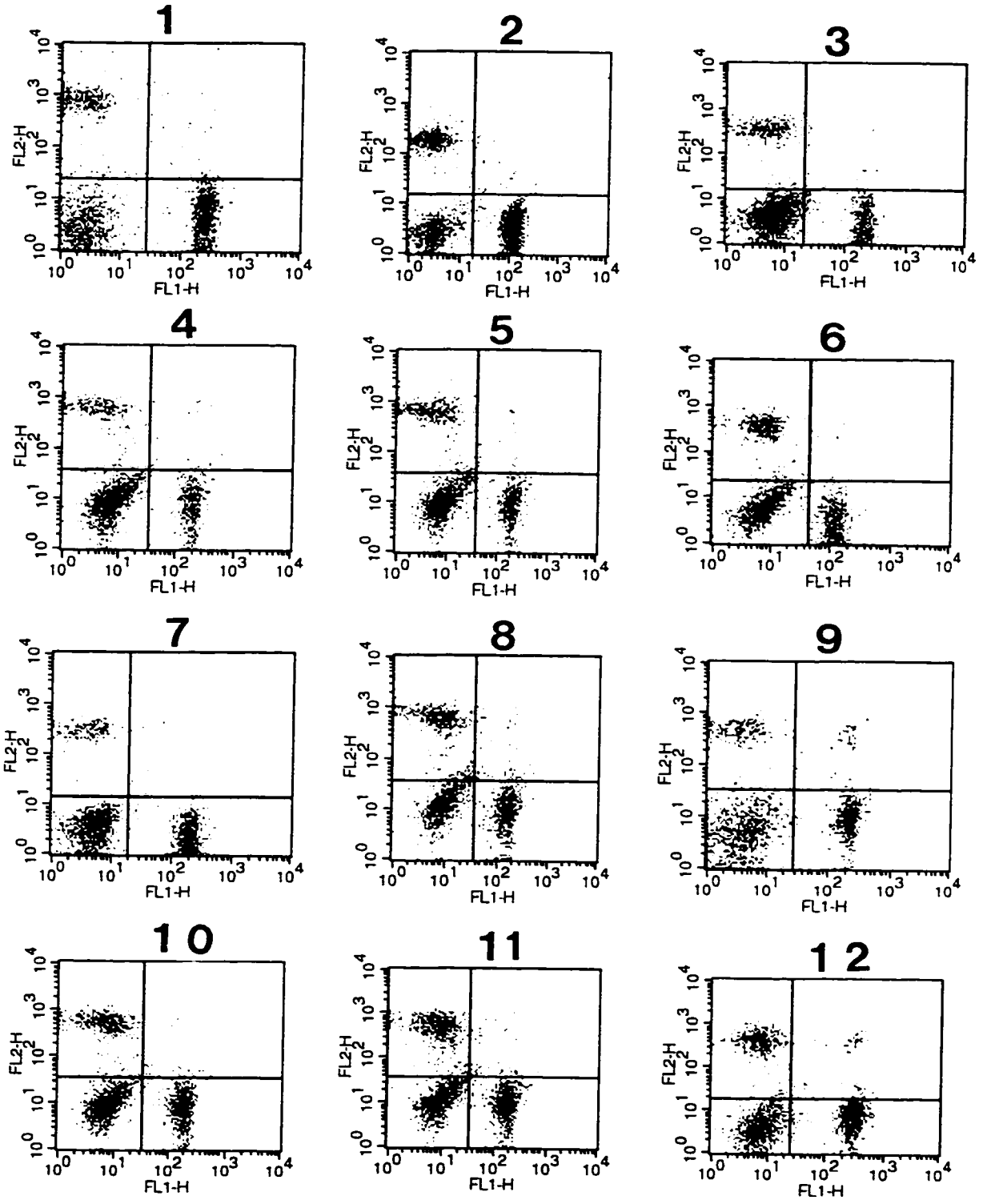

1
2
3
4
5
6
7
8
9
10
11
12
FL2-H
FL1-H
10^0 10^1 10^2 10^3 10^4

Table 3. Further examination of FACS Analysis show increase in the population of double negative cells in the popliteal lymph nodes of protected BALB/c mice as well as unprotected mice. There was also a substantial decrease in the ratio between $CD4^+$ and $CD8^+$ subsets of T cell population when compared with that found in normal life. DN = double negative (i.e. cells expressing neither CD4 nor CD8 which may be NK or B cells); CD8 = $CD8^+$ T cells; CD4 = $CD4^+$ T cells; DP = double positives suggesting immature lymphocytes expressing both CD4 and CD8 and CD4:CD8 = the ratio between $CD4^+$ and $CD8^+$ subsets of T cell population.

Table 3: Further Examination of FACS Analysis

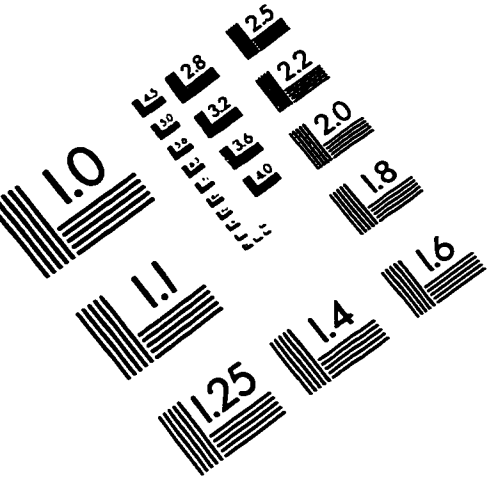
GROUPS	%DN	%DP	%CD4	%CD8	CD4:CD8
Control: Saline	31.12	2.6	49.96	17.8	2.8:1
Group1: pALT4-1	56.77	2.31	26.72	14.15	1.9:1
Group 2: pX4-12	54.12	1.1	31.27	13.27	2.3:1
Group 3: pX34-1	47.37	1.13	31.95	12.47	2.6:1
Group 4: pALT34-3	47.36	1.03	36.22	15.41	2.4:1
Group 5: pX-7TcruziCP	45.42	3.34	29.3	21.89	1.3:1
Group 6: pX-7TcruziCP	55.18	2.87	26.9	15.05	1.8:1
Group 8: virulent L. major	58.75	2.47	23.9	14.85	1.6:1
Group 9: pALT (Neo)	52.21	1.15	29.39	16.62	1.7:1
Group 10: pX	39.03	7.05	34.08	19.93	1.7:1

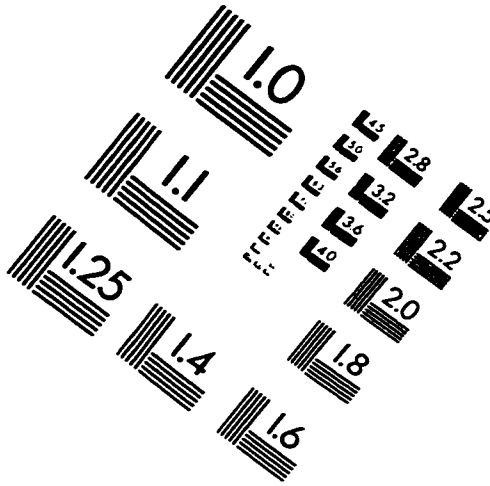
Table 4. The release of IFN-γ by cells from the popliteal lymph node draining the infected foot pad exposed to the antigens of *L. major* which was used to infect the mice.

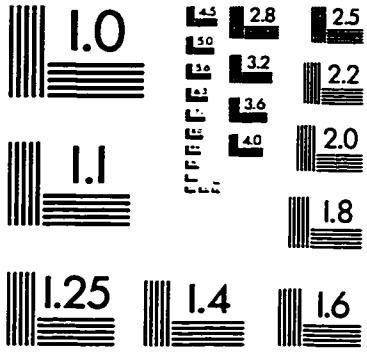
Table 4: Release of IFN-g by cells from the popliteal lymph node draining the infected foot pad.

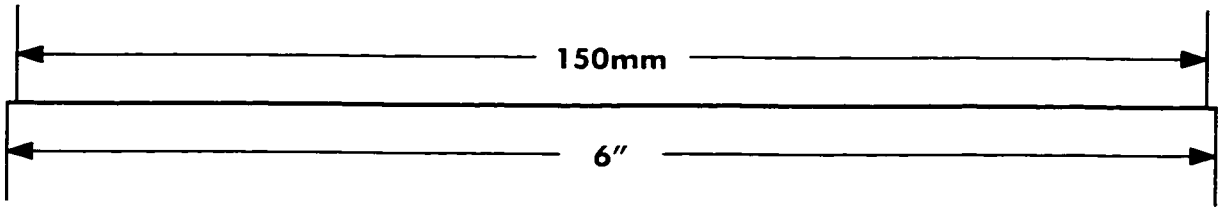
Conditions	IFN-g (ng/ml) Mean
L. major + Amastigote specific cysteine protease	
C1-1	9.05
C1-2	11.37
C1-3	9.05
C1-4	11.37
C1-5	9.25
C1-6	8.39
C1-7	10.37
C1-8	9.45
C1-9	10.88
C1-10	8.64
Mean	9.78
L. major + Promastigote specific cysteine protease	
C4-1	0.52
C4-2	5.31
C4-3	0.21
C4-4	0.02
Mean	1.51
L. major + pALT(neo)	
L-1S	1.93
L-2S	3.60
L-3S	4.78
L-4S	1.30
L-5S	2.70
Mean	2.87
Virulent L. major	
FVI -1	7.00
FVI- 2	3.77
FVI -3	8.80
Mean	6.52

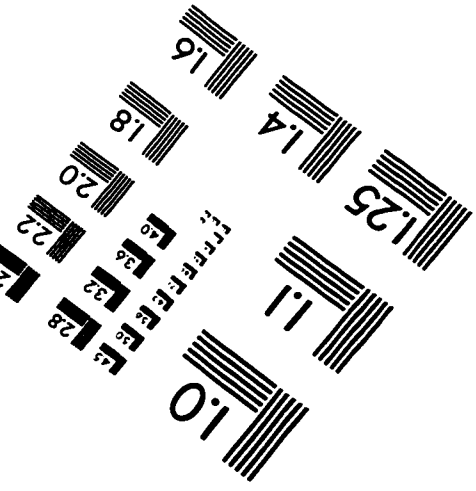
IMAGE EVALUATION
TEST TARGET (QA-3)

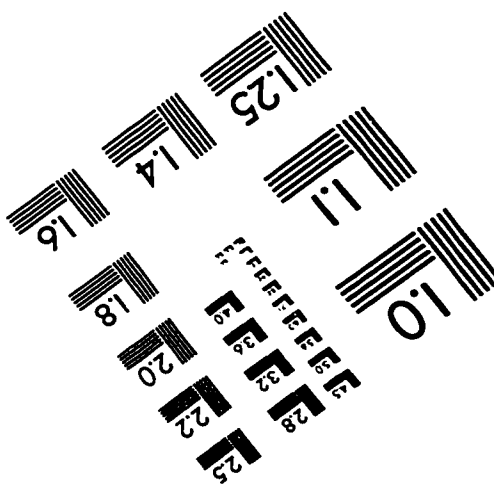












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