Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moiety from host serum

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African trypanosomes contain cysteine proteases (trypanopains) the activity of which can be measured by *in vitro* digestion of fibrinogen, after electrophoresis in fibrinogen-containing SDS/polyacrylamide gels. When assessed by this procedure, trypanopain from *Trypanosoma brucei* (trypanopain-Tb) is estimated to have a molecular mass of 28 kDa. However, two additional bands of trypanopain activity (87 kDa and 105 kDa) are observed if serum is added to the trypanopain before electrophoresis. Formation of the 87 and 105 kDa bands is frequently accompanied by a reduction in the intensity of the 28 kDa activity which suggests that the extra bands are complexes of the 28 kDa trypanopain-Tb and a molecule from rat serum called rat trypanopain modulator (rTM). The rTM-induced activation of cysteine proteases is not restricted to *T. brucei* as it is also observed with proteases from other protozoan parasites such as bloodstream forms of *Trypanosoma congolense* and the mammalian-infective *in vitro*-derived promastigote forms of *Leishmania donovani* and *Leishmania major*. The physical properties of rTM resemble those of the kininogen family of cysteine

protease inhibitors. rTM is an acidic (pI 4.7) heat-stable 68 kDa glycoprotein with 15 kDa protease-susceptible domains. This resemblance between rTM and kininogens was confirmed by the positive, albeit weak, immunoreactivity between anti-(human low-molecular-mass kininogen) antibody and rTM as well as anti-rTM antibody and human low-molecular-mass kininogen. Furthermore, commercial preparations of human-low-molecular-mass kininogen and chicken egg white cystatin mimicked rTM by forming extra bands of proteolytic activity in the presence of trypanopain-Tb. In some instances, low-molecular-mass kininogen was also observed to increase the rate of hydrolysis of 7-(benzyloxycarbonyl-phenylalanyl-arginyl-amido)-4-methylcoumarin by live *T. brucei*. Although this effect was rather erratic, in no instance was significant inhibition observed when this putative cysteine protease inhibitor was used under these conditions. The activation of parasite cysteine proteases by commonly accepted cysteine protease inhibitors is unexpected and may have important pathological repercussions.

INTRODUCTION

Bloodstream and metacyclic forms of African trypanosomes such as *Trypanosoma brucei* and *Trypanosoma congolense* endocytose exogenous proteins through their flagellar pockets. These molecules are then delivered, via a complex endosomal network, into the lysosomes of the parasites (Langreth and Balber, 1975; Webster and Grab, 1988; Webster, 1989) where they are assumed to be hydrolysed by proteases (Lonsdale-Eccles and Grab, 1987a; Mbawa et al., 1992). Although the function of the proteases has not been clearly defined, they may play a role in parasite evasion of the host immune system. For example, bloodstream forms of *T. brucei* that were incubated with antibodies specific for the variant surface glycoprotein were shown to endocytose and degrade the immunoglobulins (Lonsdale-Eccles et al., 1993; Russo et al., 1993). However, when the parasites were incubated in the presence of protease inhibitors, there was a massive accumulation of IgG within the endosomal/lysosomal system of the parasites. Procyclic forms of *T. brucei*, which do not possess a variant surface glycoprotein, have minimal or no lysosomal cysteine protease activity (Lonsdale-Eccles and Mpimbaza, 1986; Pamer et al., 1989; Mbawa et al., 1991b).

Lysosomal cysteine proteases have been found in a wide variety of protozoans (e.g. North et al., 1983; Bontempi et al., 1984, 1989; Grab et al., 1987; Lonsdale-Eccles and Grab, 1987a; Cazzulo et al., 1989; Mbawa et al., 1991a) and the importance of these proteases to protozoan viability has been established in a number of systems. For example, specific inhibitors of lysosomal cysteine proteases kill American and African trypanosomes (Ashall et al., 1990; Mbawa et al., 1992) and block the development of *Plasmodium falciparum* (Rosenthal et al., 1989; Rockett et al., 1990). The molecular-mass values of the cysteine protease from *T. brucei* (trypanopain-Tb) and *T. congolense* (trypanopain-Tc) are about 28 and 32 kDa respectively. However, the molecular-mass values of crude trypanopain preparations have proved to be variable when analysed by either contact print zymography (Rautenberg et al., 1982) or electrophoresis in substrate-containing (e.g. fibrinogen) SDS/polyacrylamide gels [Fbg/SDS/PAGE (Lonsdale-Eccles and Mpimbaza, 1986)]. Some of this variability may be caused by the presence of molecules derived from host plasma or serum in the parasite preparations. Indeed, the pattern obtained varies according to the animal species from which the serum is obtained (Lonsdale-Eccles and Grab, 1987a). As well as changing the zymographic

Abbreviations used: Trypanopain-Tb, lysosomal cysteine protease from *Trypanosoma brucei*; trypanopain-Tc, lysosomal cysteine protease from *Trypanosoma congolense*; Fbg/SDS/PAGE, SDS/PAGE on fibrinogen-containing gels; rTM, rat trypanopain modulator; Z-Phe-Arg-NH-Mec, 7-(benzyloxycarbonyl-phenylalanyl-arginyl-amido)-4-methylcoumarin; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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molecular-mass patterns, the addition of serum to the trypanosome lysates causes an apparent increase in the total amount of proteolysis when the mixture is analysed by Fbg/SDS/PAGE. Here we describe the isolation and partial characterization of a serum-derived molecule that actuates this modulation of trypanopains.

MATERIALS AND METHODS

Materials

Dithiothreitol, fibrinogen, Mes, Hepes, BSA, cystatin, trypsin, and phospholipase C were obtained from Sigma Chemical Co. Glycosidases were from Seikagaku Fine Biochemicals and kininogens from Protogen AG. Methanol, acetic acid and glycine were purchased from BDH Chemical Co. Tris (hydroxymethyl)-aminomethane and Coomassie Brilliant Blue were from E. Merck. SDS and poly(ethylene glycol) were obtained from Serva. Ampholytes were from LKB-Produktor AB and Sephacryl S-200 and the molecular-mass markers (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soya-bean trypsin inhibitor, 21.1 kDa; α -lactalbumin, 14.4 kDa) were from Pharmacia Fine Chemicals Co. Matrix Gel Blue A (lot JD-1462) was purchased from Amicon Corp. Linoleic acid and DE-53 (DEAE-cellulose) were obtained from Aldrich Chemical Co. and Whatman respectively. [7-¹⁴C]Phenylglyoxal (855 MBq/mmol) was from Amersham. Human high- and low-molecular-mass kininogens and anti-(human low-molecular-mass kininogen) antibodies were purchased from Protogen AG or Calbiochem-Novabiochem AG. 7-(Benzyloxycarbonyl-phenylalanyl-arginyl-amido)-4-methylcoumarin (Z-Phe-Arg-NH-Mec), leupeptin and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) were from Cambridge Research Biochemicals.

Sample preparation and analysis

Bloodstream forms of the African trypanosomes *T. brucei*, MITat 1.2, and *T. congolense*, clone IL 3000 (Fish et al., 1989), were grown in lethally irradiated randomly bred Sprague-Dawley rats (600–900 rad). The parasites were isolated on the day 3 after infection and purified by centrifugation through Percoll (Grab and Bwayo, 1982) and by DEAE-cellulose chromatography in PBS/glucose buffer, pH 8.0 [prepared as described by Lanham and Godfrey (1970)] containing 100 μ M hypoxanthine (Lonsdale-Eccles and Grab, 1987b). After elution from the DEAE-cellulose, the parasites were washed three times with the hypoxanthine-containing PBS/glucose, pH 8.0, suspended in the same buffer and stored in 1 ml portions of 2×10^8 parasites/ml at -70°C .

Leishmania donovani (NLB-065) and *Leishmania major* (NLB-144) promastigotes were grown by *in vitro* culture in Schneider's *Drosophila* medium (Gibco Laboratories) (Childs et al., 1978) and medium 199 (Flow Laboratories) (Hendricks et al., 1978) respectively. The parasites were harvested at the stationary phase of growth, washed three times in PBS/glucose and resuspended at a final concentration between 5×10^8 and 1×10^9 parasites/ml and either used directly or stored at -70°C until required.

Proteolytic activity in the parasite lysates was detected by Fbg/SDS/PAGE (Lonsdale-Eccles and Mpimbaza, 1986). Analysis of rat trypanopain modulator (rTM) during its purification was performed by mixing the rTM-containing samples (20–100 μ l) with a trypanosome lysate (4×10^6 trypanosomes in 20 μ l) and subjecting the mixture to Fbg/SDS/PAGE. The molecular masses of the active bands were determined from a calibration curve of relative mobility against log (molecular

mass) prepared using Bio-Rad molecular-mass markers as standards. To test serum albumin, kininogens and cystatin for their ability to modulate trypanopain activity, 20 μ l containing 4×10^6 trypanosomes mixed with 20 μ l of the protein (1.05 mg/ml) was analysed by Fbg/SDS/PAGE. In the case of the *Leishmania* samples, 20 μ l of 5.2×10^6 promastigotes/ml was used. After electrophoresis (40 mA per gel at 12°C) of the mixtures, the Fbg/SDS/polyacrylamide gels ($1.5 \times 135 \text{ mm} \times 145 \text{ mm}$) were rinsed in distilled water for 15 min followed by overnight incubation in a shaking water bath (37°C) in a buffer mixture containing 1 mM dithiothreitol, 50 mM each of Mes, Hepes and citric acid (pH 6.0). The gels were stained in 0.1% (w/v) Coomassie Brilliant Blue R250 in methanol/acetic acid/water (3:1:6, by vol.) and destained in the same solution without Coomassie Brilliant Blue. Quantitative analysis of the gels was performed using a Molecular Dynamics personal densitometer.

Purification of rTM

Rat serum (1 ml) was added to a 5 ml packed volume of Matrex Gel Blue A or Matrex Gel Green A that had been prewashed in 50 mM Tris/HCl, pH 8.0, and then resuspended in 5 ml of the same buffer. The serum and beads (total volume 11 ml) were mixed on a rotary mixer at 4°C for 2 h to remove serum albumin. Samples were then centrifuged in a bench Minifuge set at 4°C for 20 min at 3000 *g*. The supernatant was used immediately or stored at -20°C until required.

A total of 15 ml of Matrex Gel Blue A-treated serum supernatant was applied to a column of DE-53 DEAE-cellulose (65 ml) equilibrated in 50 mM Tris/HCl buffer, pH 8.0. The column was washed with 250 ml of the same buffer. A 200 ml linear gradient (0–500 mM NaCl in 50 mM Tris/HCl, pH 8.0) was then applied to the column to fractionate proteins bound to the column. The chromatographic fractions were analysed for protein by SDS/PAGE (Laemmli, 1970), and rTM was analysed by Fbg/SDS/PAGE (Lonsdale-Eccles and Mpimbaza, 1986). Fractions containing rTM activity were pooled and dialysed against water (2×2 litres for 4 h each).

The pooled and dialysed fractions from the DE-53 columns were subjected to isoelectric focusing in 1% (v/v) ampholytes (pH range 3.5–10) in a 5–50% (w/v) sucrose gradient for 18 h at 4°C and 20 W constant power (final voltage 2000 V). The fractions containing rTM were pooled, dialysed against 4×2 litres of 10 mM Tris/HCl buffer, pH 8.0, over 8 h, lyophilized and reconstituted in double-distilled deionized water (to give a final concentration of Tris/HCl of 50 mM). The pooled material was loaded on to a Sephacryl S-200 gel-filtration column ($100 \text{ cm} \times 1.6 \text{ cm}$, equilibrated in 100 mM Tris/HCl buffer, pH 8.0) and the sample was eluted with the same buffer. Fractions containing rTM were concentrated by lyophilization, reconstituted in 10 ml of water, dialysed against water (4 litres for 4 h) and stored at -70°C . The purified rTM was used to prepare anti-rTM in rabbits (Harlow and Lane, 1988).

Characterization of rTM

Reduced and unreduced samples of rTM were analysed by SDS/PAGE using the discontinuous buffer system described by Laemmli (1970). The gels were stained with 0.1% Coomassie Brilliant Blue R250 in methanol/acetic acid/water (3:1:6, by vol.), and then destained in the same solution without Coomassie Brilliant Blue.

Enzymic digestion of rTM was performed by incubating 7.5 μ g of rTM with trypsin, glycosidase or phospholipase C (0.1 μ g each) for 1 h at 37°C in a total volume of 10 μ l of 25 mM Tris/HCl, pH 8.0, before boiling for 5 min to inactivate the

enzymes. The samples were cooled to room temperature, added to a lysate of 4×10^6 trypanosomes/ml and subjected to Fbg/SDS/PAGE. Samples of purified rTM ($7.5 \mu\text{g}$ in $5 \mu\text{l}$) were also added to various concentrations of linoleic acid (0 – $750 \mu\text{M}$). The mixtures were incubated for approx. 1 min and then lysates of trypanosomes, in sample buffer, were added before the samples were analysed by Fbg/SDS/PAGE. The areas corresponding to the high-molecular-mass bands were scanned using the LKB laser densitometer. For Western-blot analysis, samples of purified rTM, human low molecular-mass kininogen and human high-molecular-mass kininogen were subjected to SDS/PAGE before immunoblotting (Harlow and Lane, 1988).

Arginine residues on the purified rTM were radiolabelled by treatment with $[7\text{-}^{14}\text{C}]$ phenylglyoxal by the procedure of Takahashi (1968). To $100 \mu\text{g}$ of rTM in $200 \mu\text{l}$ of 50 mM Tris/HCl, pH 8.0, was added $2.16 \mu\text{mol}$ ($50 \mu\text{Ci}$) of $[7\text{-}^{14}\text{C}]$ phenylglyoxal for 90 min at room temperature. The reaction by-products from the above were separated from radiolabelled rTM by chromatography through Sephadex G-25 (medium grade) equilibrated in PBS. The radiolabelled rTM was stored at -70°C until required.

Z-Phe-Arg-NH-Mec hydrolysis by live *T. brucei*

T. brucei (1.8×10^6 parasites/ml; stored on ice until required) were incubated at 25°C and 37°C with the fluorogenic trypanopain substrate Z-Phe-Arg-NH-Mec ($5 \mu\text{M}$) in PBS/glucose, pH 7.4 (Lanham and Godfrey, 1970) containing $100 \mu\text{M}$ hypoxanthine. Z-Phe-Arg-NH-Mec, which is an excellent substrate for trypanopains (Mbawa et al., 1991b, 1992), permits the *in situ* assessment of the effect of various inhibitors on trypanosomal proteases without the need to purify reaction products from the parasites. Hydrolysis of Z-Phe-Arg-NH-Mec was monitored continuously for up to 5 min on an Hitachi F-200 spectrofluorimeter (excitation wavelength 370 nm; emission wavelength 460 nm) in the presence and absence of E64 (0.1 mg/ml), leupeptin (0.1 mg/ml) or low-molecular-mass kininogen (0.1 mg/ml).

RESULTS

Lysates of *T. brucei* contain lysosomal cysteine protease activity, called trypanopain-Tb, which has a molecular mass of 28 kDa when analysed by Fbg/SDS/PAGE. The addition of a trace (0.01%) of heated (boiled for 5 min at 93°C ; altitude 1800 m) or unheated rat plasma or serum to such a lysate of *T. brucei* results in the generation of two (87 kDa and 105 kDa) or more extra bands of proteolytic activity when analysed by Fbg/SDS/PAGE (not shown). Similar results are obtained with serum from different animal species, although the apparent molecular masses of the additional band(s) of activity vary according to the particular animal species studied (Lonsdale-Eccles and Grab, 1987a). When the trypanosome lysates are boiled, no activity is observed in either the presence or absence of added serum. Thus rat serum contains a heat-stable molecule (rTM) that seems to alter the apparent molecular mass and activity of *T. brucei* proteolytic activity.

The initial steps of the purification of rTM involved the removal of serum albumin by chromatography on Amicon Matrex Gel Blue A; rTM does not bind to Amicon Matrex Gel Blue A whereas serum albumin does (Fulton, 1980). Further purification of rTM was then accomplished by a combination of gel-filtration and ion-exchange column chromatography and isoelectric focusing.

Fbg/SDS/PAGE of the fractions obtained from Sephacryl S-200 chromatography of rTM showed that rTM was eluted slightly before a 68 kDa molecule that might be serum albumin

Figure 1 Chromatography of rTM on Sephacryl S-200

A column of Sephacryl S-200 was equilibrated and run in 100 mM Tris/HCl (pH 8.0; 4°C). Fractions of volume 5 ml were collected and $50 \mu\text{l}$ samples assayed with $20 \mu\text{l}$ of lysates of 2×10^6 trypanosomes/ml by Fbg/SDS/PAGE. The fraction number is indicated at the bottom of the Figure. The peak of rTM activity, fraction 48, corresponded to a molecular mass of 120 kDa.

Figure 2 Chromatography of rTM on DE-53 cellulose

After equilibration in 50 mM Tris/HCl (pH 8.0), a $16 \text{ cm} \times 2.5 \text{ cm}$ column of DE-53 cellulose was loaded with rTM. The column was washed with 4 bed vol. of 50 mM Tris/HCl, and then a 200 ml gradient of 0 – 500 mM NaCl in 50 mM Tris/HCl was applied. Fractions (2.5 ml) were collected and assayed by Fbg/SDS/PAGE as described in the Materials and methods section. The destined areas of the gel, corresponding to the peaks of proteolytic activity, were measured by densitometric scanning. Areas lacking rTM activity were not scanned. ○, 87 kDa activity band; ◇, 105 kDa activity band; ●, 87 kDa + 105 kDa activity. rTM was eluted between 110 and 160 mM NaCl.

(Figure 1). The peak of rTM activity from the Sephacryl column corresponded to a molecular mass of 120 kDa and may be a dimer of an approx. 70 kDa molecule. Figure 1 also illustrates the dramatic enhancement of proteolytic activity that can occur in the presence of rTM.

A fairly broad peak of rTM was eluted from DE-53 cellulose at approx. 140 mM NaCl (Figure 2). A trace amount of an approx. 70 kDa protein, which may be serum albumin, was also eluted from the same column slightly after the peak of rTM activity at about 150 mM NaCl (not shown). Fbg/SDS/PAGE of rTM that had been purified by isoelectric focusing indicated that it has a pI of approx. 4.7 (not shown). SDS/PAGE shows

Figure 3 Coomassie-stained SDS/polyacrylamide gel of purified rTM

Lane 1, reduced rTM (7.5 µg of rTM/lane; the sample was boiled in sample buffer for approx. 3 min); lane 2, non-reduced unboiled rTM; lane 3, molecular-mass standards; lane 4, purified rat serum albumin.

Figure 4 Dose-response of rTM with trypanosome lysate

Increasing volumes of rTM (2.6 µg/ml) were added to 20 µl containing 4×10^6 trypanosomes. The mixtures were then subjected to Fbg/SDS/PAGE. The gel was stained and destained with Coomassie Brilliant Blue and photographed to obtain a negative image. The negative was then scanned and the relative amounts of enzyme activity in each band determined: Δ , 87 kDa band of activity; ∇ , 105 kDa band of activity; $\diamond$, 37 + 105 kDa band of activity; $\blacksquare$, 28 kDa band of activity.

that the molecular mass of rTM is very similar to that of bovine and rat serum albumin (Figure 3). Reduction and boiling increases the apparent molecular mass of purified rTM when analysed by SDS/PAGE but provides no evidence for the presence of different subunits that may be held together by disulphide bridges.

The addition of increasing amounts (volumes) of rTM to trypanopain-Tb results in saturable increases in higher-molecular-mass proteolytic activity accompanied by a decrease in the 28 kDa band. In the dose-response curve (Figure 4), the 87 kDa band is more prominent than the 105 kDa band at high doses of

Figure 5 Effect of enzymes on rTM

Purified rTM (5 µl; 7.5 µg) was incubated for 1 h at 37 °C before Fbg/SDS/PAGE with the enzymes indicated. Lane 1, trypanosome lysate alone; lane 2, trypanosome lysate + purified rTM; lane 3, trypanosome lysate + rTM treated with trypsin (5 µl of 0.01%, w/v); lane 4, trypanosome lysate + rTM treated with glycosidase-S (5 µl of 0.01%, w/v; 1 h; 37 °C); lane 5, trypanosome lysate + rTM treated with phospholipase C (5 µl of 0.01%, w/v).

rTM, whereas at low doses the reverse applies. Although the observation of activation is reproducible, precise quantification of the enhancement of the trypanopain proteolytic activity in the presence of rTM has not yet been achieved despite the knowledge that the densitometric readings are proportional to the amount of activity applied to the gel (Lonsdale-Eccles and Mpimaza, 1986). This is because of the variation that occurs between different preparations of rTM and parasite extracts. Nevertheless, extrapolation of an inverse plot of a similar experiment, in which less than 10% diminution of the 28 kDa band was observed, suggested that the activation may approach 20-fold. This is five-fold greater than that reported for the cystatin activation of cathepsin L (Dennison et al., 1992) but is similar to the 24-fold activation of papain by a protein in blood (Senthilmohan and Topping, 1994) and the 28-fold increase in proteolytic activity of an *Entamoeba histolytica* cysteine protease when released into serum-containing medium (Avila and Calderon, 1993).

The ability of trypsin or a mixture of glycosidases to cause partial breakdown of the active protein with the high molecular mass (Figure 5) suggests that the activator is a glycoprotein. When the high-molecular-mass bands were excised and subjected again to Fbg/SDS/PAGE, several additional discrete bands of activity were found between about 105 kDa and 30 kDa (Table 1). Thus the rTM-enzyme complex appears to undergo self-catalysed limited proteolysis. If, as suggested above, the high-molecular-mass bands of activity are composed of a complex of the trypanopain and rTM, then the apparent molecular-mass values of the fragmentation products appear to correspond to cleavages at about 14–15 kDa intervals in the complex.

In order to assess the nature of the rTM-trypanopain complex, rTM was treated with radiolabelled phenylglyoxal which modifies arginine residues. The specific radioactivity of the [7- 14 C]phenylglyoxal-labelled protein was greater than 7400 d.p.m./µg of protein. However, the treatment of rTM with this reagent had no obvious inhibitory or enhancing effect on the interaction of rTM with trypanopain-Tb (not shown). In contrast, hydrophobic interactions would appear to be an important component of the complex-formation. Linoleic acid, when added to a final concentration of 500 µM, completely prevented the formation of the high-molecular-mass bands of activity. A 50%

Table 1 Molecular masses of trypanopain-rTM complexes and auto-digestion products

The value α is obtained by subtracting the molecular mass of the native cysteine protease (28 kDa for trypanopain-Tb and 32 kDa for trypanopain-Tc) from the observed molecular mass of the proteolytically active fragments. The integer (β) corresponds to a hypothetical repetitive domain structure that could accommodate the observed molecular mass. Numbers in parentheses refer to bands of weak proteolytic activity.

	Molecular mass (kDa)	α	β	α/β
<i>T. congolense</i>	106	74	5	14.8
	88	56	4	14.0
	(74)	42	3	14.0
	(61)	29	2	14.5
	46	14	1	14.0
	32	0	0	—
			$\bar{x} =$	14.3
<i>T. brucei</i>	105	77	5	15.4
	87	59	4	14.8
	60	32	2	16.0
	43	15	1	15.0
	28	0	0	—
			$\bar{x} =$	15.3

Figure 6 Cystatin and kininogen interaction with trypanopain-Tb

Chicken cystatin and human kininogen were subjected to Fbg/SDS/PAGE in the presence and absence of trypanopain-Tb. Lane 1, cystatin alone; lane 2, cystatin + trypanopain-Tb; lane 3, low-molecular-mass kininogen alone; lane 4, low-molecular-mass kininogen + trypanopain-Tb; lane 5, rat serum alone; lane 6, rat serum + trypanopain-Tb; lane 7, human serum alone; lane 8, human serum + trypanopain-Tb.

inhibition of the production of the high-molecular-mass bands, as assessed by a 50% reduction in the areas of the scanned bands, occurred between 100 and 250 μ M linoleic acid. SDS also completely inhibited the formation of the 87 and 105 kDa bands of activity when used at 0.25% (8.7 mM). This is just above its critical micelle concentration of 8.2 mM (Helenius and Simons, 1975). However, when used at the concentrations employed in SDS/PAGE (350 μ M), SDS does not prevent the interaction.

The ability of rTM to interact with cysteine proteases, coupled with its acidic isoelectric point and approx. 70 kDa molecular mass, suggested to us that it might be a kininogen-like molecule.

Figure 7 Western-blot analysis of rTM and kininogens

Purified rTM (lane 1), human-low-molecular-mass kininogen (lane 2) and human high-molecular-mass kininogen (lane 3) were subjected to SDS/PAGE and Western blotting with anti-rTM (a) and anti-(human low-molecular-mass kininogen) antibodies (b). (c) Negative control serum blot. Lane M, molecular-mass markers. The arrowhead indicates the position of rTM. Strong reactivity is observed in this position using anti-rTM and a weak but positive reaction with anti-(human low-molecular-mass kininogen).

We therefore investigated whether kininogen and/or cystatin cause similar activation. Both did so (Figure 6). The molecular mass of the additional bands from the kininogen were in the same range as that of rTM whereas that of cystatin, as expected, was considerably smaller. Serum albumins also have similar molecular masses (Figure 3) and isoelectric points to low-molecular-mass kininogens, but neither rat serum albumin (see Figure 8) nor BSA (not shown) caused formation of the additional bands of Fbg/SDS/PAGE-detectable activity. [Interestingly, a commercial preparation of high-molecular-mass kininogen was observed to contain endogenous leupeptin-inhibitable Fbg/SDS/PAGE-detectable activity (results not shown) which precluded its use for the analysis of rTM-like activity.] Further indication that rTM may be a kininogen-like molecule was provided by Western-blot analysis using anti-(human low-molecular-mass) and anti-rTM antibodies (Figure 7). As might be expected, the cross-reactivity between the different species was rather weak. Nevertheless, anti-rTM antibody reacted with a commercial human kininogen preparation to give two weak bands, and anti-(human low-molecular-mass kininogen) antibody produced a single 68 kDa cross-reactive band against the purified rTM.

Very variable results were obtained with live parasites. Suspensions of live *T. brucei* hydrolyse the fluorogenic peptide Z-Phe Arg-NH-Mec (see also Lonsdale-Eccles et al., 1989). In the presence of leupeptin the hydrolysis of Z-Phe Arg-NH-Mec by live *T. brucei* was inhibited by almost 100%. In contrast, in the presence of E64 only about 50% of the activity was inhibited. This difference probably reflects the presence of two predominant but distinct trypanosomal enzymes that hydrolyse Z-Phe-Arg-NH-Mec; leupeptin inhibits both enzymes but E64 only the cysteine protease (Lonsdale-Eccles, 1991). In our preliminary

Figure 8 Effect of rTM on purified trypanopain-Tc

Trypanopain-Tc was purified rapidly on a column of cystatin–Sephacryl as described by Mbawa et al. (1991a), and subjected to Fbg/SDS/PAGE in the presence of rTM. Lane 1, molecular-mass markers; lane 2, trypanopain-Tc alone; lane 3, trypanopain-Tc + commercial rat serum albumin; lane 4, trypanopain-Tc + rTM; lane 5, commercial rat serum albumin; lane 6, rTM alone.

experiments we observed an approx. 200% increase in the rate of hydrolysis of Z-Phe-Arg-NH-Mec by the parasites in the presence of low-molecular-mass kininogen. Unfortunately, subsequent attempts to reproduce this effect showed minimal activation (approx. 10–20%) or no significant activation. It should be noted, however, that we saw no significant inhibition of the hydrolysis of Z-Phe-Arg-NH-Mec by live parasites at 37 °C when using this putative cysteine protease inhibitor. The variability in activation is similar to the variability observed using the Fbg/SDS/PAGE procedure with differing purified rTM and/or trypanopain sources.

In order to determine whether the protease-activating effect of rTM is restricted to bloodstream forms of *T. brucei*, bloodstream forms of *T. congolense* were also assayed for interaction with rTM (Figure 8). A trypanopain-Tc preparation was prepared from *T. congolense* by rapid affinity chromatography on cystatin–Sephacryl (Mbawa et al., 1991a). This result shows that activation can occur with purified as well as crude preparations of trypanosomal enzymes. The result also shows that rTM can interact with a cysteine protease of a different species of African trypanosome.

The formation of similar additional bands of high-molecular-mass activity was observed when rTM was added to lysates of stationary-phase (metacyclic) promastigote forms of either *L. donovani* or *L. major* (Figure 9). Because *Leishmania* are human infective parasites, we investigated whether human serum could mimic rTM by generating additional bands of proteolytic activity. Human serum alone exhibited no proteolytic activity (Figure 9, lane 7), but formed two extra bands of higher-molecular-mass proteolytic activity when added to *Leishmania* extracts (Figure 9, lane 8). Thus human serum must contain a similar molecule to rTM that can modulate the proteolytic activity of the *L. major* parasites.

The protease profiles of *L. major* promastigotes, like those of *Leishmania mexicana* (Robertson and Coombs, 1990; Coombs et al., 1991), were considerably more complex than those of *L. donovani* or the African trypanosomes. We have not specifically

Figure 9 Effect of rTM and human serum on *Leishmania* sp

Lysates of *L. donovani* and *L. major* were subjected to Fbg/SDS/PAGE in the absence of rTM (lanes 1, 3 and 5) and in its presence (lanes 2, 4 and 6). Lane 7 shows human serum alone and lane 8 human serum in the presence of *L. major*. The *Leishmania* samples in lanes 1–4 and 8 were freshly prepared whereas those in lanes 5 and 6 were stored frozen at –70 °C for several days. Lanes 1 and 2, *L. donovani* (7.6×10^6 parasites/ml); lane 3 and 4, *L. major* (1.7×10^6 parasites/ml); lanes 5, 6 and 8, *L. major* (10.3×10^6 parasites/ml). The arrowheads indicate the positions of the additional bands of activity in the presence of rTM or human serum and the arrow indicates the induction of an approx. 60 kDa band of activity observed in *L. major* after storage. The dashes on the left of each panel indicate the positions of the 94, 67, 43 and 30 kDa molecular-mass markers from top to bottom respectively.

characterized each of the bands of proteolytic activity from the *Leishmania* and so one or more of these bands of activity may be related to the surface metalloprotease of these parasites. However, over 90% of the protease activity in *Leishmania* is attributable to cysteine proteases (Coombs et al., 1991) and studies on the metalloproteases from *L. major*, *Leishmania amazonensis* and *L. mexicana* show that these proteases are optimally active at neutral to basic pH and that the metalloprotease from *L. amazonensis* is not able to digest fibrinogen at acid pH after SDS/PAGE (Etges et al., 1986; Chaudhuri et al., 1989; Ip et al., 1990). Thus, although we cannot unequivocally eliminate the possibility that some of the bands of activity may be metalloprotease, this seems unlikely, particularly in view of the acidic pH in which our assays are performed.

After storage of *L. major* at –70 °C for 24 h or more, enhanced proteolytic activity was observed at an approx. molecular mass of 60 kDa that was only moderately apparent in the freshly prepared sample (compare lanes 3 and 4 with lanes 5 and 6 respectively of Figure 9). Indeed, the stored sample had to be diluted about 6-fold in order to obtain similar levels of proteolytic activity to those observed before storage. The extra activity may result from activation of the zymogen-like precursors, predicted from cDNA sequence analysis of the structure of several protozoan cysteine proteases [reviewed in Coombs et al. (1991)], but this remains to be shown.

DISCUSSION

African trypanosomal cysteine proteases have molecular-mass values of approx. 30 kDa when assayed by Fbg/SDS/PAGE in the absence of serum. However, if a trace amount of serum is added to the trypanosome lysates, there is an increase in total proteolytic activity that can be correlated with the formation of additional high-molecular-mass bands of activity (Lonsdale-Eccles and Grab, 1987a). The appearance of the additional high-

molecular-mass bands of activity (87 and 105 kDa) frequently occurs with a concomitant decrease in the amount of the 30 kDa band which suggests that the additional enzyme activity is formed by the binding of the rTM to the 30 kDa trypanopain, presumably by causing the trypanopain to change its conformation into a more active and/or stable form. The mechanism by which this proteolytic activity is increased is not clear as it may be an allosteric process that resembles the activation of intracellular proteases by ATP, fatty acid or a Ca^{2+} -dependent protease regulator (De Martino and Goldberg, 1979; De Martino and Blumenthal, 1982; Dahlmann et al. 1985). Nor have we eliminated the possibility that rTM may enhance the minimal endogenous activity found in some zymogens (Lonsdale-Eccles et al., 1978) but, in such a case, the rTM preparation must also cause the inhibition of the 30 kDa enzyme. This seems unlikely, particularly in view of the recent report of the activation of papain by a molecule in blood (Senthilmohan and Topping, 1994).

Interaction between rTM and trypanopains appears to reduce the pH optimum of the enzyme activity from about pH 5.3 to about pH 4.8 (Lonsdale-Eccles and Mpimbaza, 1986). This lower value is close to the intralysosomal pH (4.2) reported for an African trypanosome (Mbawa et al., 1992) and may result in enhanced lysosomal proteolytic activity *in vivo*.

The ability of rTM to form an rTM-trypanopain complex is dependent on the handling of the various preparations. High-molecular-mass bands of activity were not seen with highly purified enzyme that had been isolated from lysates of *T. brucei* by slow conventional chromatography on DE-52 cellulose and Sephacryl S-200. Similarly, repeated freezing and thawing of the trypanosome lysates also results in a diminished capacity to generate the high-molecular-mass bands of activity. However, when the cysteine protease from *T. congolense* was purified rapidly by affinity chromatography, complex-formation was observed with the pure enzyme, although the activities of the high-molecular-mass bands were weak. Similar erratic activation was observed with live parasites. The reason for such variability, which has made it difficult to quantify the extent of activation, is not known and will be the subject of future study. Possibly the domain to which the rTM binds is very susceptible to (self) proteolysis. Limited proteolysis may also explain why the 105 kDa band is frequently less intense than the 87 kDa band.

Early work on the cysteine proteases of African trypanosomes led to the suggestion that trypanopains may be composed of several 14–15 kDa structural domains (Lonsdale-Eccles, 1985) but subsequent sequence studies provided no support for such a repetitive structure within the proteases themselves (Mottram et al., 1989; Pamer et al., 1990). It now seems likely that the early studies were performed on enzyme-rTM complexes and so the repetitive structural motifs may be present in the rTM itself rather than in the enzyme. These domains could be analogous to the similar-sized cystatin-like domains of kininogens (Ohkubo et al., 1984; Müller-Esterl et al., 1985) with the cleavages occurring in the 'hyperfrangible regions I and II' (Vogel et al., 1988) between domains 1 and 2 and domains 2 and 3 (Salvesen et al., 1986). The association of the enzyme with fragments obtained by cleavage at each of these sites would be sufficient to explain the autodigestion products seen with rTM and trypanopain-Tb (Table 1).

Although the idea that a cysteine protease inhibitor may interact with its target enzyme to form an active complex seems counterintuitive, it should be born in mind that not all cysteine proteases (e.g. bromelain) are inhibited by kininogens (Barrett et al., 1986). Furthermore, purified chicken cystatin and human kininogen react in a similar manner with trypanopain to form

active complexes like those seen with trypanopain and rTM. The cross-reactivity between anti-(human kininogen) and rTM, and between anti-rTM and human kininogen, lends credence to the idea that rTM contains amino acid sequence domains that are common to those in human kininogen. Also the active inhibitor-protease complexes are similar to those observed with cystatin (or stefin B) and cathepsin L (Pike et al., 1992; Dennison et al., 1992). The similarity between rTM and kininogens is also supported by the observations that α_1 -cysteine protease inhibitor, T-kininogen and thioastatin (Esnard and Gauthier, 1983; Enjyoji et al., 1988; Rusiniak et al., 1991) are single-chain glycoproteins with similar molecular masses (68 kDa) and similar pIs (pI 4.75) to rTM, and the carbohydrate moiety is as important for the interaction between thioastatin and papain as it is for the interaction between trypanopain-Tb and rTM.

The results in this study are based primarily on SDS/PAGE analyses and so the phenomena may be artifacts, a consequence of the proteins interacting under partially denaturing conditions. Nevertheless, we have seen that, in the presence of live *T. brucei*, hydrolysis of the small substrate Z-Phe-Arg-NH-Mec may also be enhanced by kininogen, albeit erratically. Furthermore, recent studies with cystatin or kininogen showed that proteolytically active complex-formation is possible between those inhibitors and cathepsin L in the absence of SDS (Dennison et al., 1992; Pike et al., 1992; Dennehy et al., 1994).

An activator of trypanopains has been observed in the serum of several mammalian species (Lonsdale-Eccles and Grab, 1987a), and rat TM enhances the *in vitro* activity of cysteine proteases in *T. brucei*, *T. congolense*, *L. major* and *L. donovani*. However, we have been unable to demonstrate any reactivity between rTM and the cysteine protease activities in rat skin or liver, the proteolytic activity of which is 100–200-fold lower than that present in the trypanosomes (J. D. Lonsdale-Eccles and G. W. N. Mpimbaza, unpublished work). The activation or stabilization of the trypanosomatid cysteine proteases by mammalian kininogen-like cysteine protease inhibitors may have profound implications with respect to disease pathology as a consequence of the inappropriate control of these proteases when they are released into the hot bloodstream and tissues (Nwagwu et al., 1988; Authié et al., 1992). It has been proposed that these cysteine protease inhibitors contribute to the body's defence against foreign organisms that invade by using cysteine proteases (Green et al., 1984), and yet our observations suggest that this may not always be the case. Recently, papain was reported to be activated 24-fold by a molecule in blood (Senthilmohan and Topping, 1994), and Avila and Calderon (1993) have reported that the proteolytic activity of a cysteine protease of *E. histolytica* was increased 28-fold when released into medium containing 15% bovine serum. Despite the strong inhibition of some cysteine proteases by the cystatin/kininogen family of proteins [e.g. the inhibition of papain by the acute-phase reactant α_1 -cysteine proteinase inhibitor; $K_i = 5 \times 10^{-11}$ M (Esnard and Gauthier, 1993)], these reports and our observations strengthen the argument that a reassessment of the regulation of cysteine protease activity in blood is probably in order.

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