Journal of Cellular Biochemistry 40:1–13 (1989)
Cellular Proteases and Control Mechanisms 87–99

An Inhibitor Specific for the Mouse T-Cell Associated Serine Proteinase 1 (TSP-1) Inhibits the Cytolytic Potential of Cytoplasmic Granules but Not of Intact Cytolytic T Cells

Markus M. Simon, Marlot Prester, Michael D. Kramer, and Uli Fruth

Max-Planck-Institut für Immunbiologie, Freiburg (M.M.S., U.F., M.P.), and Universitätshautklinik, Heidelberg (M.D.K.), Germany

We have investigated a proteinase inhibitor, designed according to the preferred amino acid sequence that is cleaved by the murine T-cell specific serine proteinase 1 (TSP-1) for its effect on the cytolytic potential of cloned cytotoxic T-cell lines (CTLL) and of cytoplasmic granules, derived from these cells. Pretreatment of effector cells with H-D-Pro-Phe-Arg-chloromethyl-ketone (PFR-CK) prior to the cytotoxicity assay did not result in inhibition of cytolytic activity of three independent CTLL and did not effect their granule-associated TSP-1 activity after extraction with Triton X-100. Furthermore, PFR-CK did not interfere with cytolysis of target cells by CTLL when present for the entire incubation period. In contrast, PFR-CK inhibited in a dose-dependent manner both TSP-1 activity and the hemolytic/cytolytic potential of isolated cytoplasmic granules after their pretreatment with high-salt concentration. We interpret these results to mean that cytolysis of target cells by CTLL involves the granule-associated proteinase TSP-1, which probably becomes active upon exocytosis following effector-target cell interactions.

Key words: cytolytic T-lymphocytes, cytolytic activity, granule secretion, proteolytic activity, inhibition

Cytolytic T lymphocytes (CTL) constitute a major effector cell population of the immune system with the ability to specifically lyse target cells [1]. Although it is well recognized that CTL play an important role in the host control of virus replication [2] and of tumor development [3], our knowledge of the molecular basis of cytolysis is still fragmentary.

According to the exocytosis model, CTL-target cell interaction leads to secretion of a cytolytic molecule, termed synonymously cytolysin [4], perforin [5,6], or pore-forming protein (PFP) [7], which is associated with cytoplasmic granules of CTL. Assembly of cytolysin/perforin/PFP molecules into polymers and generation

Received March 30, 1988; accepted August 31, 1988.

of channel structures in the membrane of target cells have been taken as evidence that cell death is caused by colloid osmotic lysis [8–11]. Recently, another TNF-like, soluble cytotoxic mediator (cytotoxin), which is distinct from cytolysin/perforin/PFP in both structure and function, has been found in CTL and is also suggested to play an important role in the lytic process [12].

Besides cytolysin/perforin/PFP and cytotoxin, another group of functional molecules, that is, proteinases, have been implied in cell-mediated lympholysis (CML). Evidence in support for a role of these enzymes in cytolysis is based on the finding that inhibitors of proteinases interfere with CML [13–15]. Moreover, these studies indicate that more than one proteinase is involved in different stages of CML [14].

We [16] and others [17–19] have recently isolated and purified to homogeneity a serine proteinase, termed synonymously T-cell specific proteinase 1 (TSP-1) [16], BLT esterase [17], granzyme A [18], or SE 1 [19] from a long-term culture CTL (CTLL). TSP-1, which is a disulphide-linked homodimer with a molecular mass of 60 kDa, is associated with cytoplasmic granules from CTLL [16,18] and specifically released together with other granule components during CTLL-target cell interaction [17,20,21]. One of the prominent features of TSP-1 is its high activity on the model peptide substrate with the amino acid (AA) sequence H-D-Pro-Phe-Arg (PFR) [16]. In order to explore the role of TSP-1 in cytolysis, we have used this information to design a noncompetitive inhibitor, PFR-chloromethylketone (PFR-CK), which specifically interacts with TSP-1, but not with other serine-type molecules contained within cytoplasmic granules, by binding covalently to its active site [22, and unpublished results].

We now show that pretreatment of intact CTLL with the inhibitor PFR-CK abolishes neither their TSP-1 activity nor their cytolytic potential; however, the same reagent inhibits both, TSP-1 and cytolytic activities of cytoplasmic granules derived from the same CTLL after their preexposure to high-salt concentration. The data suggest the involvement of TSP-1 in the terminal stages of cytolysis, possibly steps that are responsible for the processing of structures located within the cytoplasmic granules or on the target cell membrane. These results have been presented elsewhere in preliminary form [23].

MATERIALS AND METHODS

Materials

The enzyme substrates H-D-Pro-Phe-Arg-p-nitroanilide (NA; S-2302) and the proteinase inhibitor H-D-Pro-Phe-Arg-chloromethylketone (PFR-CK; A-17896) were purchased from Bachem AG, Bubendorf, Switzerland. The enzyme inhibitors, phenylmethylsulfonyl fluoride (PMSF; P-7626) and Nα-p-tosyl-L-lysine chloromethylketone (TLCK; T-7254), were from Sigma, Munich, FRG.

T-Cell Lines

CTLL 1.D9, an allospecific CTLL with specificity for H-2D^d was derived from a lymphocyte culture containing female C57B2/6(B6) responder T cells and irradiated female DBA/2 stimulator cells by limiting dilution procedures as described in detail previously [24]. This T-cell line was maintained on antigen and supernatant from ConA-activated rat spleen cells (ConASN) and subcultured at weekly intervals.

CTLL HY3-Ag3 (provided by Dr. H. Hengartner, Zürich, Switzerland) was

derived from a female B6 CTLL with specificity for the minor histocompatibility antigen HY. In culture, these cells lost their specific cytolytic activity on HY-bearing target cells and acquired natural killer (NK)-like activity as defined by their ability to lyse YAC tumor target cells. Furthermore, this cell line was adjusted to grow on interleukin 2 (IL-2) in the absence of antigen [25].

CTLL 1.3E6 was also derived from a female B6 CTLL specific for the minor histocompatibility antigen HY. After 3 months in culture, one line, 1.3E6, was adjusted to grow in IL-2 conditioned medium in the absence of antigen and subsequently was recloned by limiting dilution procedures in the presence of ConASN. Lymphocytes of this line have lost their specific cytolytic activity on male B6 target cells, but have acquired the ability to specifically lyse P815 mastocytoma target cells. These cells have been termed aged killer cells (AK) [24].

Subcellular Fractionation

CTLL were taken from exponentially growing cultures and washed twice in phosphate-buffered saline (PBS). Cell pellets were then resuspended in ice-cold relaxation buffer (100 mM KCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1.25 mM piperazine-*N*-*N*'-bis-2-ethanesulfonic acid [Pipes], pH 6.8) containing 0.25 M sucrose (isotonic) at a cell density of 2.5×10^7/ml [9,26]. Cellular plasma membranes were disrupted by nitrogen cavitation after equilibration in N_2 for 10 min at 10 atm at 4°C in an Artisan (Waltham, MA) pressure chamber. The lysate was collected and freed from nuclei by 10-min centrifugation at 400 *g*, resulting in material termed the "postnuclear supernatant" (PNSN). Six-milliliter aliquots of the PNSN were layered on 20 ml of 48% (v/v) Percoll at 4°C in polycarbonate ultracentrifugation tubes, and subcellular fractionation was performed according to Millard et al. [27]. The Percoll stock solution was prepared by sterile addition of 1 part 2.5 M sucrose, 100 mM HEPES to 9 parts of Percoll, adjusted to pH 6.8. This stock (9.6 ml) was mixed with 10.4 ml relaxation buffer (see above). The tubes were centrifuged at 29,000 rpm (60,000 *g*) in a 60 Ti rotor (Beckman, Munich) for 30 min at 0°C. The profile of buoyant density was determined by the use of density marker beads. Fractions of 1 ml were collected from the bottom of the tube by carefully inserting a glass capillary (1 mm diameter) through the gradients. Fractions were freed from Percoll by centrifugation at 40,000 rpm (105,000*g*) in a 50 Ti rotor for 2 h at 0°C. All fractions were tested for TSP-1 and cytolytic activities, and those with peak activities for both TSP-1 and cytolysin in cytoplasmic granules [22] were used in this study.

Cell Lysates

Detergent lysis by Triton X-100 was achieved by incubation of cells in 0.01 M Tris (Roth, Karlsruhe, FRG), pH 8.5, containing 0.1% Triton X-100 at 4°C for 1 h. Afterwards the lysate was freed from the nuclei and particulate material by centrifugation at 10,000 *g*. Alternatively, lysates were prepared by freezing and thawing the cells five times in 300 μl TBS buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) [24].

Assay for Cell-mediated Cytotoxicity

Specific ^{51}Cr release was determined by incubating serial dilutions of effector cells from bulk culture with a fixed number (2×10^3/well) of ^{51}Cr-labeled day-2 ConA-activated blasts from male or female B6 mice, or with YAC tumor cells ($H\text{-}2^a$),

P815 mastocytoma cells (H-2^d), or EL-4.F15 thymoma cells (H-2^b) in the presence or absence of phytohemaglutinin (PHA; Gibco 670/0576; 4% of stock solution) in a final volume of 0.2 ml for 4 h. Afterwards, plates were centrifuged and 100 μl of the supernatant (SN) was removed for counting. The percentage specific lysis was calculated using the following equation: % ^{51}Cr release = $(x - y/(z - y) \times 100$, in which (x) is cpm in the SN of the target cells mixed with the effector cells, (y) is cpm of the SN of target cells incubated alone, and (z) is cpm after disintegration of target cells in 1 N HCl.

Induction of Proteinase Release from CTLL

CTLL were washed and incubated at 4×10^5 cells with or without the indicated tumor target cells (4×10^5 cells) in the presence or absence of PHA (Gibco, 670/76; 4% of stock solution) in round-bottom microtiter plates (0.2 ml) in RPMI medium (without phenol red; Gibco, Wiesbaden, FRG) supplemented with 0.01 M HEPES and bovine serum albumin (Boehringer, Mannheim, FRG) at 1 mg/ml. The plates were incubated at 37°C for 3 h, and afterwards SN was removed. In order to determine the total amount of proteinase expressed, effector cells were lysed in Triton X-100 buffer as described above. All supernatants were tested for amidolytic activity on H-D-Pro-Phe-Arg-NA.

Assay for Amidolytic Activity

Cell lysates of CTLL, the SN from CTLL incubated with target cells, and preparations of cytoplasmic granules or of purified enzyme (100 μl) were titrated in serial dilutions in 0.1 M Tris/HCl, pH 8.5, and were mixed with 100 μl (0.3 mM) of the chromogenic peptide substrate H-D-Pro-Phe-Arg-NA. Enzyme-catalyzed hydrolysis was measured spectrophotometrically at 405 nm and 37°C as release of p-nitroanilin. One unit is defined as the absorbance (A) of 0.01 per h and 0.2 ml. The molar absorption for the resulting p-nitroanilin at 405 nm = $10.8\ l \times \mu ol^{-1} \times cm^{-1}$ [28].

Assay for Hemolytic and Cytolytic Activity

Cytolysin activity was determined by a modification of the method described by Masson et al. [29]. Dilutions from granule preparations or from whole TBS cell lysates in PBS (100 μl) were incubated at 37°C for 20 min with 100 μl of 6% sheep red blood cells (v/v) in Veronal buffer (5 mM Veronal, 150 mM NaCl, 5 mM $CaCl_2$, 0.3 M $MgCl_2$, 0.1% gelatin, pH 7.3). After pelleting unlysed cells by centrifugation, hemolysis was determined by measuring the release of hemoglobin at 540 nm (absorbance, A). Results are expressed either in Z units by using the Poisson analysis $Z = -\ln (1 - y)$ where y is the fraction of cells lysed [9] or as A at 540 nm. Alternatively, cytolysin activity was determined on [^{51}Cr]-labeled EL-4.F15 thymoma cells (2×10^3/well) at 37°C for 20 min in the same buffer. The percentage specific lysis was calculated as described above for cell-mediated cytotoxicity.

Treatment With Inhibitors

Aliquots either of intact CTLL or the SN from CTLL previously incubated with target cells or of purified enzyme were preincubated with various concentrations of inhibitors for 1 h at 37°C. Preparations of isolated cytoplasmic granules were made 2 M in NaCl, in order to disrupt the granule membrane [26], and were diluted 1:10 in

TABLE I. Cytolytic,* Cytolysin,† and Amidolytic,† Activities Expressed by B6 TLC

CTLL	Original specificity	Culture conditions	Cytolytic activity‡ (% ^{51}Cr release) on B6 ♂/♀	P815 (H-2^d)	P815 + PHA	YAC (H-2^a)	EL-4 (H-2^b)	Cytolysin activity (Z/10^6 cells)	Proteinase activity (U/10^6 cells) on H-D-Pro-Phe-Arg-NA
1.D.9	H-$2D^d$	Antigen/ConASN	0/0	92	100	53	20	5.3	187
HY3-Ag3	HY	ConASN	0/0	33	85	44	n.d.	8.2	396
1.3E6	HY	ConASN	0/0	36	43	0		5.3	460

*Cytolytic activity of intact cells was tested by incubating serial dilutions of CTLL on the indicated target cells.
†Cytolysin and proteinase activities were determined in cell lysates from CTLL as described in Materials and Methods.
‡Effector to target cell ratio = 25:1.

relaxation buffer prior to incubation with inhibitors. Afterwards, samples were assayed for amidolytic and cytotoxic/hemolytic activities.

RESULTS

For studies on the role of TSP-1, [16] in CML, three cytolytic Ly-2$^+$ T lymphocyte clones (CTLL) have been used (Table I). CTLL 1.D9 is a cloned B6 CTLL specific for H-2D^d. CTLL HY3-Ag3 [25] and CTLL 1.3E6 [24] are long-term cultured T-cell lines that have lost their original specificity for H-Y target cells and show high cytolytic activity on YAC and/or P815 target cells [24,25].

Cell lysates of all three CTLL were shown to express high, though variable, amidolytic activities (Table I) on the model peptide substrate H-D-Pro-Phe-Arg-NA, which is a good substrate for TSP-1 [16]. All three lysates contained comparable amounts of hemolytic activity (cytolysin/perforin, PFP [4–7]) as determined on SRBC (Table I). Moreover, it has been demonstrated before that in all three CTLL studied, TSP-1 activity is associated with cytoplasmic granules [22] that are exocytosed during CML [20,21]. It is seen from Table II that TSP-1 secretion from the individual CTLL is only induced by those target cells which are lysed by the respective effector cell population.

These data suggest that besides other granule components, TSP-1 may also play a role in the process of killing. Previous studies, however, have shown that TSP-1 is not cytotoxic on its own [16]. We have now employed a synthetic proteinase inhibitor molecule, PFR-CK, which specifically and irreversibly inhibits TSP-1 [22], to elucidate further the role of this serine proteinase in CML. As shown in Figure 1, PFR-CK was able to completely inhibit at μM (1—10 μM) concentrations the proteolytic activity present in the cell lysate of CTLL (1.D.9, Fig. 1a), in the SN of CTLL 1.D.9 incubated with P815 tumor target cells (Fig. 1b), in the enriched granule fraction from CTLL 1.D9 (Fig. 1c), or in a purified TSP-1 preparation (Fig. 1d). The same results were obtained using the equivalent preparations from CTLL HY3-Ag3 and 1.3E6 (data not shown).

Preincubation of CTLL with PFR-CK (0.1 μM–1 mM) did not result in inhibition of cytolysis of any of the three intact CTLL on relevant target cells, even at concentrations of 1 mM (Figs. 2–4, upper panels, hatched bars). This treatment also did not interfere with TSP-1 activity detected in CTLL cell lysates (Figs. 2–4, upper panels, open bars), suggesting that PFR-CK does not reach TSP-1 in granules of intact CTLL under these conditions. Similarly, PFR-CK had also no effect on both activities when present during the entire CML assay (data not shown). In contrast, the

TABLE II. TSP-1 Secretion by CTLL in Response to Various Target Cells

	Enzyme activity (U/10^6 cell equivalents[a]) released in response to				
CTLL	None	P815	P815/PHA	EL4	YAC
1.D9	12	188	179	16	n.d.
HY3-Ag3	16	n.d.	70	14	42
1.3E6	10	150	155	14	10

[a]TSP-1 activity was determined in supernatants of CTLL after 3-h incubation with the indicated target cells as described in Materials and Methods.

cytotoxic potential of all three CTLL was strongly inhibited after pretreatment with 0.1 mM to 1 mM TLCK. As shown before [16], this inhibitor has no effect on the enzyme activity of a purified TSP-1 preparation and also did not interfere with the granule associated TSP-1 in intact CTLL (Figs. 2–4, upper panels). Preincubation of CTLL with another proteinase inhibitor, PMSF, at concentrations of 1–2 mM, resulted in the reduction of both TSP-1 and cytotoxic activity of CTLL 1.3E6 (Fig. 4, upper panel). On the other hand, the same inhibitor only partially reduced both activities in CTLL 1.D9 (Fig. 2, upper panel) or CTLL HY3-Ag3 (Fig. 3, upper panel).

The effect of preincubation of cytoplasmic granules isolated from the three CTLL with various concentrations of PFR-CK, PMSF, or TLCK on their TSP-1 activity (open bars) and their cytolytic potential on SRBC (hatched bars) is seen in Figures 2–4 (lower panels). The data clearly show a dose-dependent inhibition by PFR-CK of both TSP-1 and hemolytic activities of the granules at μM concentrations. The inhibitory activity of PFR-CK on both activities was evident only after previous treatment of granules with high-salt concentrations. One should note in this context that granules of all three CTLL contain more than one species of proteinase-like molecules as revealed by affinity binding of [^{3}H]diisopropyl-fluorophosphate (DFP), a specific inhibitor of serine proteinases [30]. However, in our hands, TSP-1 is the only species in cytoplasmic granules that expresses high proteolytic activity and that interacts with PFR-CK [22]. It is also obvious from Figures 2–4 (lower panels) that PMSF similarly inhibited TSP-1 and cytotoxic activities in granules from all three CTLL, although only at much higher concentrations (1–2 mM). In contrast and as expected from earlier studies [16], pretreatment of granules with TLCK (0.1 mM–1 mM) did not interfere with the TSP-1 activity in any of the three preparations. However, the same agent strongly inhibited the cytolytic potential of granules derived from CTLL HY3-Ag3 (Fig. 3, lower panel), but only marginally reduced cytotoxic activities in similar preparations from either CTLL 1.D9 (Fig. 1, lower panel) or CTLL 1.3E6 (Fig. 4, lower panel). Essentially the same results were obtained when cytoplasmic granules from HY3-Ag3 were pretreated with inhibitors and were tested for cytolytic activity on ^{51}Cr-labeled EL-4.F15 tumor target cells rather than on SRBC (Fig. 5).

DISCUSSION

Here we show that pretreatment of three independent CTLL with PFR-CK interfers neither with their cytolytic potential nor with their granule-associated TSP-1 activity. On the other hand, incubation of disrupted cytoplasmic granules isolated from the same CTLL with PFR-CK at μM concentrations resulted in a dose-dependent reduction of both TSP-1 and hemolysin/cytolysin activities.

The results obtained with PFR-CK contrasts the effects seen with TLCK, another inhibitor for proteolytic enzymes, which has also been shown before to interfere with the cytolytic potential of intact cells [13,14]. However, the fact that TLCK does not inhibit TSP-1 ([16,31] and data shown) suggests that the molecules involved in TLCK-mediated inhibition of CML-mediated cytolysis are distinct from those for PFR-CK. This is also supported by the notion that the molecules of intact CTLL that react with TLCK (not necessarily proteases) are primarily associated with the cell surface membrane of the effector cell [32].

The finding that PMSF but not PFR-CK was able to inhibit, though to a variable extent, the cytolytic potential of three independent CTLL and of their cytoplas-

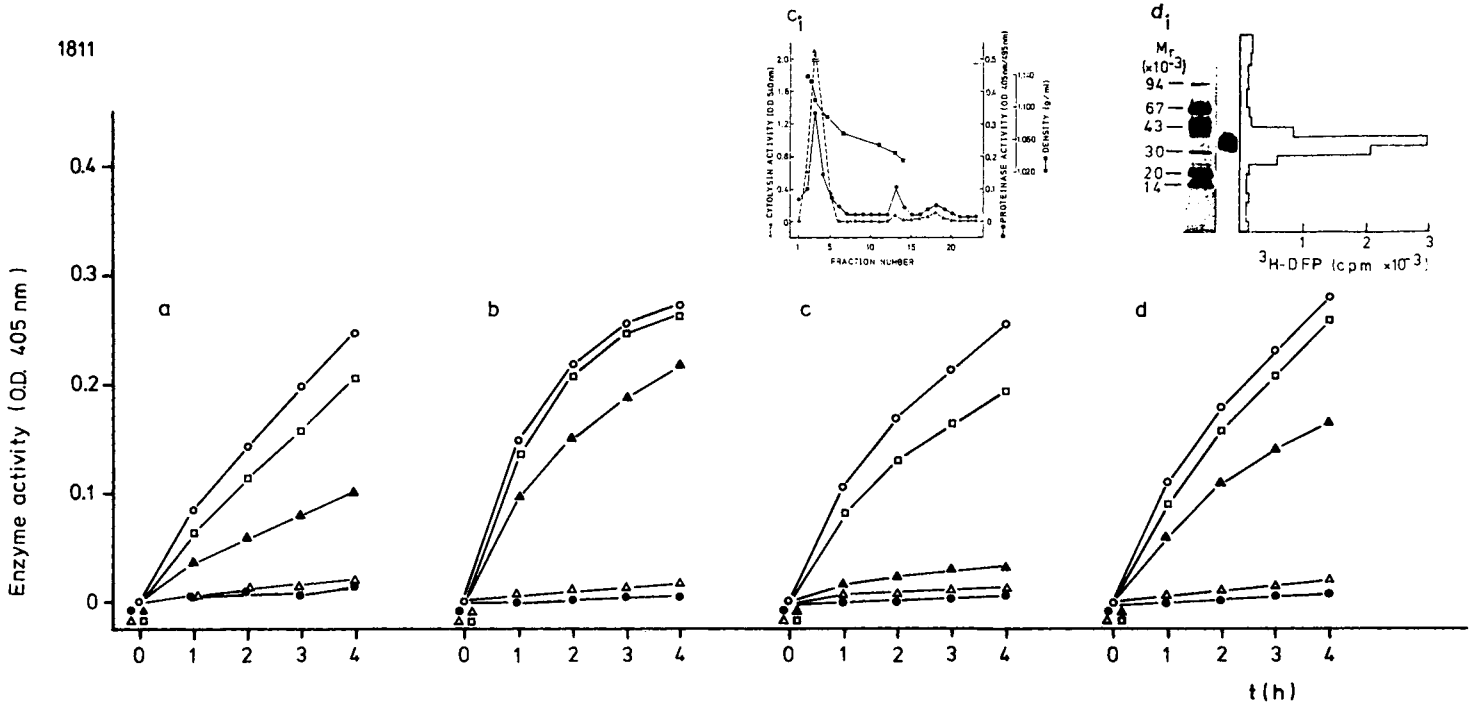
1811
Enzyme activity (O.D. 405 nm)
0.4
0.3
0.2
0.1
0
a
b
c
d
0 1 2 3 4
t (h)
c_1
FRACTION NUMBER
d_1
M_r (×10⁻³)
94
67
43
30
20
14
^{3}H-DFP (cpm ×10⁻³)

Figure 1.

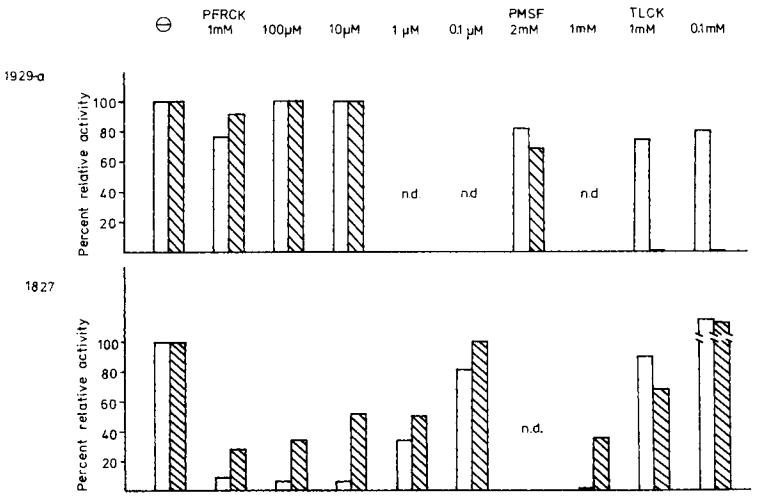

Fig. 2. Effect of proteinase inhibitors on TSP-1 and cytolytic activity expressed by CTLL 1.D9 lymphocytes and their cytoplasmic granules. Upper panel, intact cells: 2×10^6 CTLL 1.D9 were preincubated with either none or the indicated concentrations of PFR-CK, PMSF, or TLCK for 1 h at 37°C. Afterwards, lymphocytes were tested (at E:T = 20:1) for cytotoxicity on ^{51}Cr labeled P815 tumor target cells (hatched bars; 100% relative activity = 81% specific cytolysis). Triton X-100 cell lysates (5×10^4 cell equivalents) of the same groups of pretreated cells were tested for TSP-1 activity on H-D-Pro-Phe-Arg-NA (open bars; 100% relative activity = 0.107 A at 405 mm, 1 h). Lower panel, cytoplasmic granules: aliquots of the enriched cytoplasmic granule fraction (5×10^5 cell equivalents) from CTLL 1.D9 were disrupted in the presence of 2 M NaCl and subsequently treated with the indicated inhibitors. TSP-1 activity (Triton X-100 lysate, 1×10^5 cell equivalents) was tested in each group on H-D-Pro-Phe-Arg-NA (open bars; 100% relative activity = 0.131 A at 405 nm, 1 h); hemolytic activity (2×10^5 cell equivalents) was tested after freezing and thawing samples (5x) on SRBC. Release of hemoglobin was tested after 20 min at 540 nm. (hatched bars; 100% relative activity = 0.479 A).

mic granules may be due to its distinct physicochemical properties. PMSF seems to be able to penetrate cellular membranes under the experimental conditions, whereas PFR-CK clearly does not: pretreatment of CTLL with PMSF but not with PFR-CK followed by washing did interfere with granule-associated TSP-1 activity.

In similar studies on the role of TSP-1 in the CML, Henkart et al. [33] found that although PMSF inhibited proteolytic activity in intact CTL by more than 98% they still retained almost 50% of their lytic activity. At first sight, these results are not

---

Fig. 1. Effect of PFR-CK on TSP-1 activity in cell lysates, cell culture SN, and enriched enzyme preparations. Aliquots of Triton X-100 cell lysates of (**a**) CTLL 1.D9 (10^4 cell equivalents, of **b**), SN from a cell culture of CTLL 1.D9 (10^5 cell equivalents), and P815 tumor target cells; of (**c**) the fraction enriched for cytoplasmic granules from CTLL HY3-Ag3 (10^6 cell equivalents; and of **d**) purified TSP-1 (10 U) were preincubated for 30 min at 37°C with PFR-CK: none (○), 0.1 μM (□), 1 μM(▲), 10 μM (△), 100 μM (●), and tested on H-D-Pro-Phe-Arg-NA. A was tested over the time period of 4 h at 405 nm as described in Materials and Methods. Inset $\mathbf{c}_i$: Percoll gradient separation of cell lysate from CTLL HY3-Ag3; aliquots of each fraction were tested for both TSP-1 and cytolysin-activity. Fraction 3 contained optimal activities for TSP-1 (●—●) cytolysin (-) and the cytoplasmic granules as revealed by examination in the electron microscope [22]. Inset $\mathbf{d}_i$: radiochromatogram and SDS-PAGE (reducing conditions) of purified TSP-1 previously affinity labeled with ^3H-DFP [22].

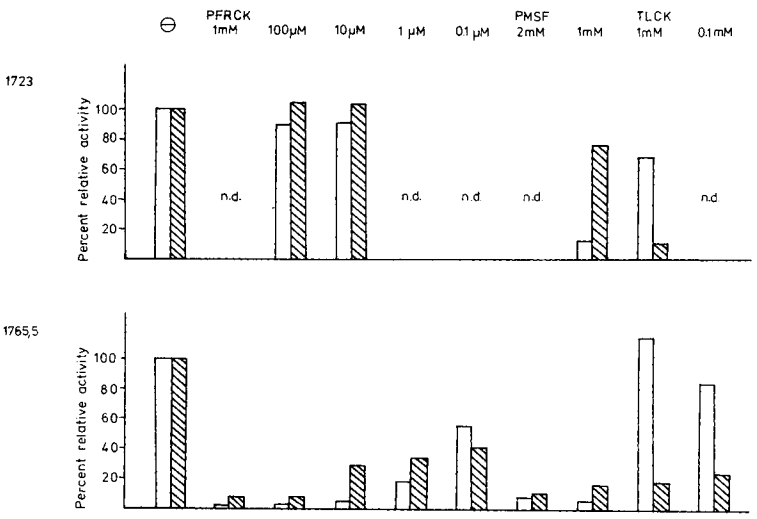

Fig. 3. Effect of proteinase inhibitors on TSP-1 and cytolytic activity expressed by CTLL HY3-Ag3 lymphocytes and their cytoplasmic granules. For details, see legend to Figure 2. Upper panel, intact cells: TSP-1 activity (open bars; 100% relative activity = 0.153 A, at 405 nm, 1 h, 5×10^4 cell equivalents); cytotoxicity was determined on ^{51}Cr labeled P815 tumor targets in the presence of PHA (hatched bars; 100% relative activity = 66% specific cytolysis, E:T = 30:1). Lower panel, cytoplasmic granules: derived from CTLL HY3-Ag3; TSP-1 activity (open bars; 100% relative activity = 0.176 A at 405 nm, 1 h, 1×10^5 cell equivalents); hemolytic activity (hatched bars; 100% relative activity = 0.512 A at 540 nm, 20 min; 3×10^5 cell equivalents).

in favor of a role of TSP-1 in cytolysis. However, it is not clear from their data whether PMSF is able to inactivate TSP-1 in situ, that is, whether PMSF can reach the enzyme contained within the cytoplasmic granules or whether TSP-1 is inhibited during the preparation of cell lysates by PMSF previously entrapped in the cytoplasm. In the latter case, TSP-1 could still exert its enzyme activity after fusion of cytoplasmic granules with the cellular membrane and exocytosis into the extracellular space.

It is shown here that PFR-CK was able to inhibit both TSP-1 activity and the cytolytic potential of isolated cytoplasmic granules derived from three independent CTLL in a dose-dependent manner. Most importantly, this effect was observed only after previous treatment of cytoplasmic granules with high-salt concentration or after freezing and thawing of the granule preparation several times (data not shown). These results indicate that the inhibitor is able to reach and to interact with the granule-associated target molecule TSP-1 only after its disintegration from supramolecular structures. In fact, it has been shown before that cytoplasmic granules contain highly negatively charged proteoglycans of the chondroitin sulphate A type [34,35]. These structures seem to form tight complexes with TSP-1 in situ, which, however, dissociate at high-salt concentrations ([31] and J. Tschopp, personal communication).

Since PFR-CK interacts only with TSP-1 but not with other granule-associated DFP-binding molecules, that is, serine proteinases [20], the data strongly suggest that the abolishment of the granule-associated cytolytic potential by this inhibitor is due mainly to the inactivation of TSP-1. The possibility that the abrogation of granule-associated cytolysis by PFR-CK is due to a nonspecific alkylation, that is, by virtue of

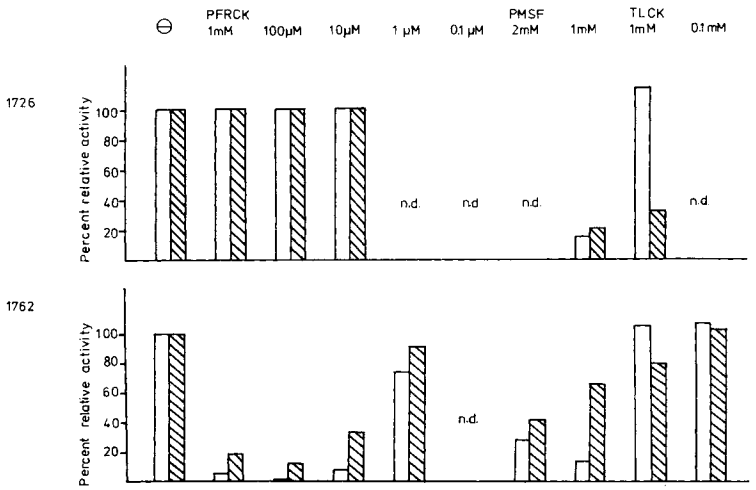

Fig. 4. Effect of proteinase inhibitors on TSP-1 and cytolytic activity expressed by CTLL 1.3E6 lymphocytes and their cytoplasmic granules. For details see legend to Figure 2. Upper panel, intact cells: TSP-1 activity (open bars; 100% relative activity = 0.207 A at 405 nm, 1 h; 5×10^4 cells equivalents); cytotoxicity was determined on ^{51}Cr labeled P815 tumor target cells, in the presence of PHA (hatched bars; 100% relative activity = 33% specific cytolysis, E:T = 25:1). Lower panel, cytoplasmic granules: derived from CTLL 1.3E6; TSP-1 activity (open bars; 100% relative activity = 0.218 A at 405 nm, 1 h, 1×10^5 cell equivalents); hemolytic activity (hatched bars; 100% relative activity = 0.377 A at 540 nm, 20 min; 3×10^5 cell equivalents).

its highly reactive chloromethyl ketone group, of other molecules relevant for the killing of target cells is rather unlikely. Chloromethyl ketones distinct from PFR-CK such as TLCK and Tosyl-phenylalanine-CH_2Cl (TPCK) had no or only marginal effects on the cytolytic potential of cytoplasmic granules at μmolar concentrations ([36] and data not shown). Our data are in line with a similar study recently reported by Hudig et al. [36]. These authors showed that the inhibition of granule-associated proteases by

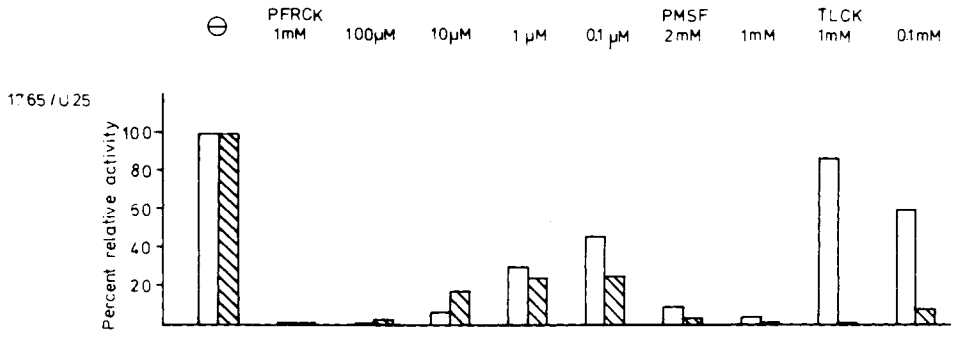

Fig. 5. Effect of proteinase inhibitors on TSP-1-activity and cytolytic activity in *cytoplasmic granules* derived from CTLL HY3-Ag3. For details see legends to Figures 2, 3. TSP-1 activity (open bars; 100% relative activity = 0.165 A at 405 nm, 5×10^4 cell equivalents), cytolytic activity was determined on ^{51}Cr EL4 tumor target cells (hatched bars; 100% relative activity = 59% cytolysis, 1×10^5 cell equivalents).

mechanism-based isocoumarin inhibitors abrogates or delays granule-mediated cytolysis, suggesting the requirement for two independent enzymes with either trypsin- or chymotrypsin-like activity, respectively, in granule cytolysis.

One possible mechanism by which TSP-1 could control the cytolytic activity of CTL is by cleaving appropriate substrates within cytoplasmic granules under physiological conditions. This is, however, unlikely because the enzyme has optimal activity at neutral pH and is inactive at pH 5–6, a pH range, normally taken as that of lysosomal and endosomal compartments [19]. It is therefore reasonable to assume that TSP-1 is operative only during exocytosis of the granule contents into the intercellular space. There are at least two possibilities to explain the dose-dependent inhibition of cytotoxic activity in isolated by cytoplasmic granules PFR-CK: 1) TSP-1 may either act directly on cytolysin/perforin/PFP or a precursor thereof, thereby activating or amplifying its cytolytic potential. In preliminary experiments in which untreated or PFR-CK treated cytoplasmic granules were incubated in the absence or presence of exogenous TSP-1 and analyzed by SDS-PAGE, no significant proteolytic digestion of granule-associated proteins, including cytolysin/perforin/PFP, was seen (data not shown). Together with the finding by Masson and Tschopp that functionally active cytolysin/perforin/PFP can be isolated in the presence of PMSF [6], the data disfavour but do not rule out the possibility that TSP-1 acts by processing either cytolysin/perforin/PFP or its precursor. Alternatively, it is conceivable that TSP-1 acts on relevant structures associated with the surface of target cells. The fact that cytolysin/perforin/PFP may cause significant target cell lysis on its own [5,6,37,38] does not exclude the possibility that under physiological conditions TSP-1 may contribute to cytolysis by reducing the locally required dose of cytolysin/perforin/PFP for an effective lysis to occur.

The experiments presented lead us to conclude that CTL utilize TSP-1 in the lytic process conveyed by granule exocytosis. The determination of the physiological substrate of TSP-1 should provide more information on the complex process of T-cell-mediated cytolysis.

ACKNOWLEDGMENTS

We thank Dr. H. Hengartner for providing us with CTLL HY3-Ag3 and Mss. G. Prosch and R. Schneider for expert technical assistance. This study was supported in part by grant Si 214/5-4/5 from the Deutsche Forschungsgesellschaft. M.D.K. is supported by a Postdoktoranden-Programm of the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Nabholz M, MacDonald HR: Ann Rev Immunol 1:273, 1983.
2. Zinkernagel RM, Doherty PC: Nature 251:547, 1974.
3. Haskell JS, Häyry P, Radio LA: Contemp Top Immunobiol 8:107, 1978.
4. Henkart PA: Ann Rev Immunol 3:31, 1985.
5. Podack ER: Immunol Today 6:21, 1985.
6. Masson D, Tschopp J: J Biol Chem 260:9096, 1985.
7. Young JD-E, Hengartner H, Podack ER, Cohn ZA: Cell 44:849, 1986.
8. Kupfer A, Dennert G: J Immunol 133:2762, 1984.

9. Podack ER, Königsberg PJ: J Exp Med 160:695, 1984.
10. Dennert G, Podack ER: J Exp Med 157:157, 1983.
11. Berke G: Ann Inst Pasteur Immunol 138:304, 1987.
12. Liu C-C, Steffen M, King F, Young JD-E: Cell 51:393, 1987.
13. Chang TW, Eisen HN: J Immunol 124:1028, 1980.
14. Redelmann D, Hudig D: J Immunol 124:870, 1980.
15. Redelmann D, Hudig D: Cell Immunol 81:1, 1983.
16. Simon MM, Hoschützky H, Fruth U, Simon H-G, Kramer MK: EMBO J 5:3267, 1986.
17. Pasternak MS, Verret CR, Lin CC, Eisen HN: Nature 322:740, 1986.
18. Masson D, Nabholz M, Estrade C, Tschopp J: EMBO J 5:1595, 1986.
19. Young JD-E, Leong LG, Lin CC, Wall A, Cohn ZA: Cell 47:183, 1986.
20. Takayama H, Sitkovsky MV: J Exp Med 166:725, 1987.
21. Simon H-G, Fruth U, Geiger R, Kramer MD, Simon MM: Adv Exp Biol Med 1989 (in press).
22. Fruth U, Prester M, Golecki JR, Hengartner H, Simon H-G, Kramer MD, Simon MM: Eur J Immunol 17:613, 1987.
23. Simon MM, Fruth U, Simon H-G, Kramer MD: Ann Inst Pasteur Immunol 138:309, 1987.
24. Simon MM, Weltzien HU, Bühring HJ, Eichmann K: Nature 308:367, 1984.
25. Acha-Orbea H, Groscurth P, Lange R, Sitz L, Hengartner H: J Immunol 130:2052, 1983.
26. Henkart PA, Millard PS, Reynolds C, Henkart MP: J Exp Med 160:75, 1984.
27. Millard PJ, Henkart MP, Reynolds CW, Henkart PA: J Immunol 132:3197, 1984.
28. Erlanger BF, Kokowsky N, Cohen W: Arch Biochem Biophys 95:271, 1961.
29. Masson D, Corthesy P, Nabholz M, Tschopp J: EMBO J 4:2533, 1985.
30. Barret AJ, Salvesen G (eds): "Proteinase Inhibitors," Amsterdam: Elsevier, 1986.
31. Jenne EJ, Tschopp J: Immunol Rev 103:53, 1988.
32. Pasternack MS, Sitkovsky MV, Eisen HN: J Immunol 131:2477, 1983.
33. Henkart PA, Berrebi GA, Takayama H, Munger WE, Sitkovsky MV: J Immunol 139:2398, 1987.
34. MacDermott RP, Schmidt RE, Caulfield JP: J Exp Med 162:1771, 1985.
35. Tschopp J, Conzelmann A: Immunol Today 7:135, 1986.
36. Hudig D, Gregg NJ, Kam C-M, Powers JC: Biochem Biophys Res Commun 149:882, 1987.
37. Young JD-E, Cohn ZA: Cell 46:641, 1986.
38. Henkart PA, Yue CC, Yang J, Rosenberg SA: J Immunol 137:2611, 1986.