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Human T cell specific proteinase (HuTSP) is encoded by the T cell and natural killer cell specific human Hanukah factor (HuHF) gene

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HuTSP, a serine proteinase which is specifically associated with activated T lymphocytes, was purified to homogeneity and characterized. N-terminal amino acid sequencing revealed identity with a sequence predicted from the human Hanukah factor (HF) gene, which was isolated from a human T cell cDNA library. The dimeric structure of HuTSP, together with its extensive sequence homologies with the murine T cell specific proteinase, MTSP-1, suggests phylogenetic conservation of this serine proteinase family.

Serine proteinase; (Human T cell, Murine T cell)

1. INTRODUCTION

The molecular mechanisms by which T cells elicit their multiple effector functions is still a matter of debate. However, with the aid of classical biochemistry as well as recombinant DNA technology, a great number of T cell derived soluble mediators such as lymphokines [1,2], leukolysins [3] and serine proteinases [4–7] have been defined which are believed to play important roles in various T cell mediated processes such as cytolysis [8] and regulation of cellular and humoral immune responses [1,2,9]. We [4] and others [5–7] have previously isolated and characterized a serine proteinase termed synonymously mouse T cell associated proteinase 1 (MTSP-1) [4], BLT-esterase [6], Granzyme A [5] and SE-1 [7] from cloned mouse $CD8^+$ T lymphocytes (TLC). It was shown that this enzyme is encoded by the Hanukah factor (HF) gene isolated from $CD8^+$ TLC by subtraction cDNA methodology [10–12]. Functional studies suggest that MTSP-1 which is specific for T cells and represents the major proteolytic activity in T effector cells is involved in processes such as T cell mediated killing [13], induction of B cells [4] and extravasation [14,15].

Recently, we have succeeded in isolating a similar serine proteinase from a human $CD8^+$ T cell clone [16]. Here, we report that this enzyme, termed human T cell associated proteinase (HuTSP), has an amino-terminal sequence identical with that predicted from a human $CD8^+$ TLC derived cDNA clone termed human HF [17].

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2. MATERIALS AND METHODS

2.1. *T cells and culture medium*

The $CD8^+$ T lymphocyte clone (TLC) UA B34.C7 was derived from the synovial fluid of patient UA with reactive arthritis after rubella infection as described [18,19]. Culture medium was RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), 5% pooled AB serum and recombinant interleukin 2 (50 U/ml; rIL-2 kindly provided by Dr E. Liehl, Sandoz Forschungsinstitut, Vienna). At bi-weekly intervals cells were restimulated with irradiated (3000 rad) peripheral blood mononuclear cells in the presence of phytohemagglutinin (PHA, 2.5 μg/ml, Gibco).

2.2. *Assays for amidolytic and esterolytic activity*

Amidolytic activity was determined using Tos-Gly-Pro-Arg-*p*-nitroanilide (NA; Chromozym TR4, Boehringer Mannheim,

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FRG). 100 μl of a dilution of test samples were mixed with 100 μl of 0.1 M Tris-HCl (pH 8.5), containing 3×10^{-4} M substrate. Incubation was performed at 37°C, the absorbance of the resulting *p*-nitroaniline was measured after different time intervals at 405 nm and amidolytic activity was standardized defining one arbitrary unit (U) as an absorbance value of 0.01/h. Esterolytic activity was determined by mixing 20 μl of sample dilution with 180 μl substrate reaction mixture [0.1 M Tris-HCl (pH 8.0), 10^{-4} M *N*-α-benzyloxycarbonyl lysyl thiobenzyl ester (BLT) and 10^{-4} M dithiobis(nitrobenzoic acid)] and reading the absorbance at 405 nm after 30 min at room temperature. One BLT unit (U) was arbitrarily defined as an absorbance value of 1.0/30 min.

2.3. *Purification of huTSP and SDS gel electrophoresis*

TLC UA B34.C7 cells were lysed by suspending cells from exponentially growing cultures in 10 mM Tris-HCl (pH 7.5), containing 0.1% (v/v) Triton X-100 at a cell density of 5×10^7/ml. After 1 h on ice, lysates were centrifuged at 3000 × *g* to remove particulate material and stored at −70°C. HuTSP was purified from lysates previously adjusted to 1 mg/ml heparin on a *p*-aminobenzamidine-Sepharose column (Pharmacia, Freiburg; 8 ml gel bed, flow rate 0.1 ml/min) equilibrated in 0.1 M Tris-HCl (pH 8.0), 1 mg/ml heparin (buffer A). The bulk of irrelevant protein was removed from the column with a linear salt gradient ranging from 0 to 1 M NaCl in buffer A. Thereafter, elution was performed with 1 M arginine-HCl in buffer A with or without heparin. Fractions containing HuTSP activity were pooled and analysed under reducing and non-reducing conditions in 10% SDS-polyacrylamide gels (SDS-PAGE) according to Laemmli. Proteins were stained with silver using the method of Ansorge [20].

2.4. *Affinity labeling of huTSP and analysis of labeled protein*

Purified HuTSP (without heparin) was dialysed against 20 mM NH_4HCO_3 (pH 8.5), lyophilized and redissolved in 100 μl of 10 mM Tris-HCl (pH 8.0) containing 30 μCi [^{3}H]diisopropyl fluorophosphate (DFP; Amersham, Braunschweig). Aliquots were run on 10% Laemmli gels under reducing and non-reducing conditions. Gels were subsequently soaked in a fluorographic enhancer (EN^{3}HANCE; NEN, Dreieich), dried and autoradiographed on Kodak X-Omat AR films at −70°C for 7 days.

2.5. *Amino acid sequence analysis*

HuTSP protein (10 μg ≈ 200 pM) was run on SDS-PAGE under non-reducing conditions, electroblotted onto a siliconized glass-fiber sheet, detected by Coomassie blue staining, excised and applied to a gas-phase sequencer (type 470A, Applied Biosystems) according to Eckerskorn et al. [21]. Analysis of phenylthiohydantoin derivatives was performed with an isocratic HPLC system [22].

3. RESULTS AND DISCUSSION

HuTSP was isolated from detergent lysates of CD8$^+$ TLC UA B34.C7. The enzyme was purified to homogeneity by virtue of affinity chromatography on *p*-aminobenzamidine-Sepharose using a two-step gradient. Proteinase activity in individual fractions was monitored with the unspecific esterase substrate BLT. Enzyme activity of pooled fractions was determined using the model peptide Tos-Gly-Pro-Arg-pNA which was shown before to be an optimal substrate for HuTSP [16]. Table 1 lists the results of a typical purification of HuTSP from 7×10^8 cells. The proteinase was purified about 150-fold, yielding 57% residual activity. Notably, the amount of active enzyme recovered by this method by far exceeds that obtained with another purification protocol described before [16]. When analysed in SDS-PAGE (fig.1A), HuTSP migrated at a molecular mass of ~50 kDa under non-reducing conditions and as one band of ~25 kDa under reducing conditions, suggesting that it is a disulfide-linked homodimer, a structure also found recently for MTSP-1 [4]. That HuTSP is a serine proteinase is based on its sensitivity to inhibitors such as PMSF and antithrombin III [16] as well as its reactivity with the catalytic site specific ligand [^{3}H]DFP (fig.1B). The substrate specificity of HuTSP as determined on a panel of chromogenic model peptides revealed that the enzyme has an exquisite specificity for the tripeptide Gly-Pro-Arg [15], an amino acid sequence also recognized by human thrombin [23].

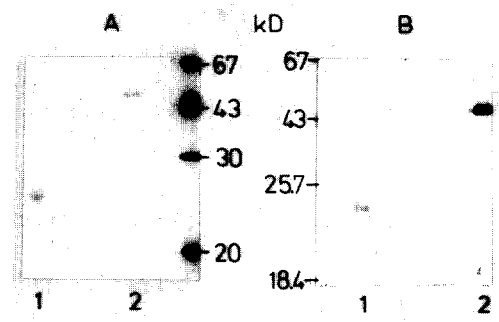

Fig.1. SDS-PAGE analysis of unlabeled or [^{3}H]DFP-labeled huTSP under reducing (1) and non-reducing (2) conditions. (A) 100 ng samples of affinity-purified HuTSP were run on 10% SDS-polyacrylamide gels after pretreatment in the presence or absence of 10 mM DTT. Protein was detected by silver staining. (B) 100 ng huTSP were incubated in buffer containing 30 μCi [^{3}H]DFP for 12 h at 37°C. [^{3}H]DFP-binding protein was revealed as described in section 2.

Alignment of the amino-terminal amino acid sequence, i.e. 18 amino acids, of HuTSP to that predicted from HuHF cDNA recently isolated by

Table 1

Summary of huTSP affinity purification from TLC UA B34.C7

Step	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg protein)	Purification (-fold)	Recovery (%)
(1) Triton X-100 (7×10^8 cells)	28	34320	1125	(1)	(100)
(2) Affinity chromatography	0.04	19680	160333	150	57

Gershenfeld et al. from a cDNA library by hybridization with a mouse HF probe [17] revealed identity in all 18 residues. In parallel studies, another amino-terminal sequence identical with HuHF in 20 out of 22 positions and with HuTSP in 17 out of 18 amino acids has been described for a $CD8^+$ T cell-derived serine proteinase termed Q31 [24]. As with HuTSP, Q31 was found to be a disulfide-linked homodimer with a molecular mass of 45 kDa under non-reducing conditions. In spite of similarities between HuTSP and Q31, the latter enzyme differs markedly from HuTSP in its substrate specificity as well as in its biochemical characteristics on a benzamidine Sepharose column. Thus it is possible that HuTSP and Q31 belong to a family of related serine proteinases expressed by human T effector cells. This assumption would be in line with the recent demonstration by Masson and Tschopp [25] of a family of related serine proteinases associated with cytoplasmic granules from mouse $CD8^+$ T lymphocytes. Amino-terminal sequence analysis revealed that these enzymes exhibit variations in residues 5–8 and identity in residues 1–4 and 9–16 [24]. In addition to HuTSP and Q31, a further serine proteinase that is very similar to the murine

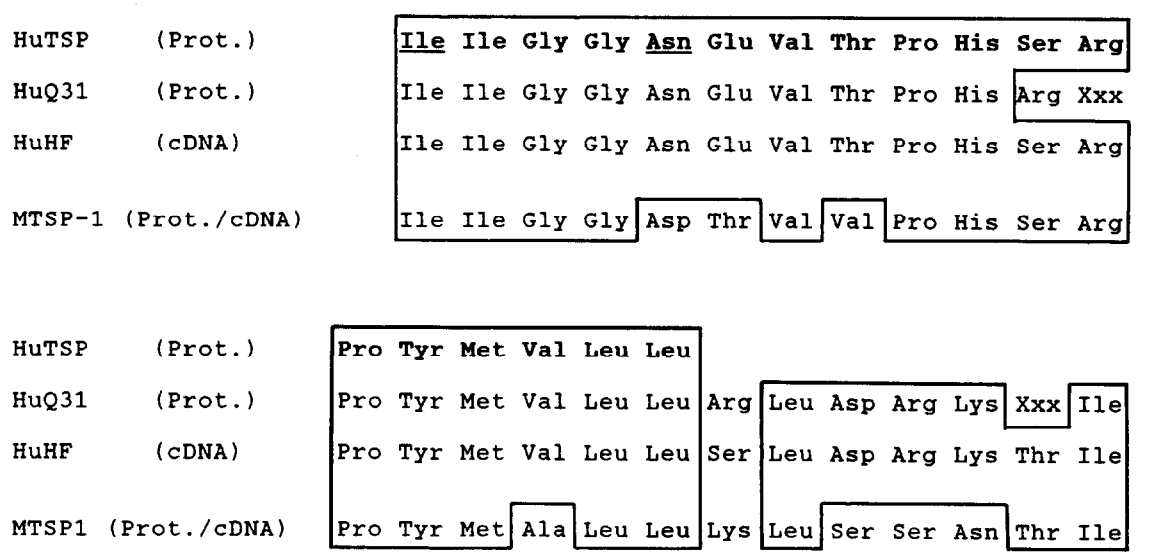

Fig.2. N-terminal amino acid sequence analysis of HuTSP. A sequence of 18 amino acids of HuTSP was determined and compared to the primary structure of T cell tryptase Q31, HuHF and MTSP-1. HPLC chromatograms for underlined amino acids in the HuTSP sequence showed contaminants/degradation products, but still allowed identification of the respective residues. Regions of identical residues are boxed.

counterpart MTSP-1 [4], BLT-esterase [6], granzyme A [5] and SE-1 [7] in substrate and inhibitor specificities and molecular mass has been isolated from cloned human cytolytic T lymphocytes [26]. However, since the amino-terminal sequence for this enzyme has not been determined the exact relationship of this serine proteinase with HuTSP and Q31 is unclear at present.

The biological function of HuTSP and related enzymes remains unknown thus far, but is a matter of intensive investigation. However, functional studies in the mouse system indicate that MTSP-1 which, as HuTSP is secreted by T effector cells upon ligand binding to the T cell receptor-CD3 complex [16,27,28], is involved in T cell mediated processes such as cytolysis [13], regulation of B cell responses [4] and extravasation of activated T cells [14,15].

The simple technique to purify biochemically HuTSP from $CD8^+$ human TLC described here and the perspective of generating large amounts of the enzyme by DNA technology will allow similar functional studies to be initiated with HuTSP in the human system.

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