# Murine T cells expressing high activity of prolyl endopeptidase are susceptible to activation-induced cell death

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**Abstract Prolyl endopeptidase (PEP) is widely distributed and thought to play an important role in the degradation of peptide hormones and neuropeptides, but its biological role is totally unknown. In this study, we examined PEP activity in subpopulations of murine T cells and found that PEP activity was significantly higher in immature thymocytes than in mature thymocytes or in peripheral T cells. Stimulation of murine peripheral T cells time-dependently increased PEP activity. Although murine T cell hybridomas exhibited high PEP activity, the PEP activity was fully inhibited by treatment with PEP inhibitor. The pretreated T cells were found to be resistant to activation-induced cell death (AICD). Similar results were obtained in murine thymocytes as well as in activated peripheral T cells. PEP activity in T cell hybridomas remained unchanged during AICD. These results suggest that T cells expressing high PEP activity are susceptible to ACID.** 

*Key words:* Prolyl endopeptidase; T cell; Activation-induced cell death; Prolyl endopeptidase inhibitor

## 1. Introduction

Prolyl endopeptidase (PEP) was found as an oxytocin-inactivating enzyme in human uterus by Walter et al. [1] in 1971. Since then, it has been found to be ubiquitously distributed among mammalian tissues and various organisms including plants and bacteria [2]. Such a wide distribution suggests the general importance of this enzyme. PEP is one of several proteases that are specific for proline [3]. However, unlike other proline-specific aminopeptidases, carboxypeptidases, or dipeptidases, PEP generally hydrolyzes the peptide bond within polypeptides of $< 30$ amino acids at the C-terminal side of prolyl residues [4–6]. PEP is shown to be capable of rapidly degrading proline-containing neuropeptide and hormones, such as substance P, bradykinin, angiotensin II, and thyrotropin-releasing hormone (TRH) in vitro [7,8], and the enzyme is presumed to participate in their maturation and degradation. PEP has also been reported to be involved in DNA synthesis [9], depression [10], amnesia [11], and the generation of amyloid β-protein [12–14]. However, the contribution of this enzyme in Alzheimer's pathology has been controversial, because a recent study indicated that PEP is not involved in the formation of amyloid β-protein [15].

PEP gene has been cloned from several sources including human lymphocytes or a human T cell line MOLT4 and characterized [16,17]. Northern blot analysis revealed that human thymus and spleen also express PEP [17]. However, the physiological functions of PEP in thymus and spleen are currently unknown. To define the role of this enzyme in T cells, we examined PEP activity in subpopulations of murine T cells by its ability to hydrolyze a fluorescent substrate and found that PEP activity of immature thymocytes exhibited significantly higher than that of mature thymocytes or of peripheral naive T cells. In addition, when murine peripheral T cells were stimulated, PEP activity was increased in a time-dependent manner. Upon TCR stimulation with antigen (Ag), resting T cells are activated to proliferate and produce cytokines. When the activated T cells are restimulated with Ag through TCR, those T cells undergo apoptosis, which is termed activation-induced cell death (AICD). AICD is thought to play an important role in the deletion of autoreactive T cells in thymus or in periphery, and the maintenance of immune homeostasis [18,19]. Murine T cell hybridomas have been widely used for the study of the mechanisms of AICD [20–22], and we observed that murine T cell hybridomas also showed high PEP activity. Accordingly, we considered it plausible that T cells with high PEP activity might be sensitive to AICD. In this study, we tested this hypothesis in murine T cell hybridomas, thymocytes, and activated peripheral T cells using specific PEP inhibitors.

## 2. Materials and methods

### 2.1. Mice

BALB/c female mice were maintained in our animal facility and used at new-born, 8 weeks of age.

### 2.2. Antibodies and reagents

Anti-mouse CD3ε mAb (145-2C11) was purified from ascites fluid

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*Abbreviations:* AICD, activation-induced cell death; DICS, death-inducing signaling complex; DN, double negative; DP, double positive; DMSO, Dimethyl sulfoxide; FCS, fetal calf serum; PEP, prolyl endopeptidase; PI, propidium iodide; SP, single positive; Suc-Gly-Pro-MCA, succinyl-Gly-Pro-4-methyl-coumaryl-7-amide; ZPP, *N*-benzyloxycarbonyl-prolyl-prolinal

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by protein A affinity chromatography. Anti-CD28 (37.51) was purchased from PharMingen (San Diego, CA, USA). 24-well culture plates were coated with purified anti-CD3 antibody and/or anti-CD28 antibody as described previously [22]. All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted.

*2.3. Preparation of T cell subpopulations*

Thymocytes were prepared from thymus glands of BALB/c mice, and a part of them was used as unfractionated thymocytes. Double negative (DN) or double positive (DP) thymocytes were prepared from thymocytes stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5; PharMingen) and phycoerythrin (PE)-conjugated anti-CD8 (53-6.72; PharMingen) by cell sorting on FACS Vantage (Becton Dickinson, Mountain View, CA, USA). For preparation of single positive (SP) thymocytes, thymuses were removed from mice 48 h after administration with 125 mg/kg hydrocortisone sodium phosphate, and SP thymocytes were obtained by cell sorting from thymocytes stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8. Unfractionated thymocytes were washed twice and then resuspended at $1\times10^7$/ml in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA)/5% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan, NY, USA) containing 10% culture supernatant of antibody J11d. The cells were incubated for 45 min on ice, pelleted by centrifugation, and then resuspended in 5% guinea pig serum as a source of complement. After an additional incubation at 37°C for 45 min, the cells were filtered through nylon mesh to remove aggregated debris, washed twice, and used as $J11d^-$ thymocytes. After spleen cell suspensions were prepared, contaminating RBC were removed by treatment with erythrocyte-lysing buffer (0.15 M $NH_4Cl$), and splenic T cells were enriched using nylon wool columns. T cells of lymph node cells were also enriched using nylon wool columns.

*2.4. Assay for PEP activity*

PEP activity was determined as described previously [17]. $2\times10^6$ cells were suspended in extraction buffer (50 mM Tris–HCl pH 7.2, 1 mM EDTA containing the protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, antipain, and pepstatin A) and repeated freezing and thawing. The homogenate was centrifuged at 15,000 rpm for 30 min, and thereafter the supernatant was collected and kept at −70°C before use. PEP activity was assayed in 20 mM sodium phosphate buffer (pH 6.0) containing 0.5 mM EDTA, 0.5 mM succinyl-Gly-Pro-4-methyl-coumaryl-7-amide (Suc-Gly-Pro-MCA) (Peptide Institute, Osaka, Japan) and 100 μg of cytosolic extracts in a total volume of 100 μl. After incubation for 30 min at 37°C, the reactions were terminated by addition of 2 ml of 1 M sodium acetate buffer (pH 4.2), and fluorescence intensity was measured at excitation and emission wavelengths of 380 and 460 nm, respectively. Increase in fluorescence was standardized using free 7-amino-4-methyl-coumarin (AMC) (Peptide Institute). Values are given as release of AMC in nmol/min/mg protein.

*2.5. Cells and culture*

Ovalbumin-specific T cell hybridoma N3-6-71 [22] was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, $5\times10^{-5}$ M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. To induce AICD, N3-6-71 cells were seeded into anti-CD3 (100 μg/well)-coated 24-well culture plates at a density of $5\times10^5$ cells/well. As control of PEP inhibitor, dimethyl sulfoxide (DMSO) was used at a final concentration of 0.02% (v/v). In the experiments for evaluation of the influence of PEP inhibitors on T cells, the concentration of FCS was reduced to 1%. Thymocytes were prepared from thymus glands of 8-week-old BALB/c mice and suspended in RPMI 1640 medium supplemented with 1% FCS and other supplements. To induce apoptosis in thymocytes, the cells were cultured on 24-well culture plates coated with anti-CD3 (20 μg/well) and anti-CD28 (20 μg/well) at a density of $5\times10^6$ cells/well. Splenic T cells and lymph node T cells of 8-week-old mice were resuspended in RPMI 1640 containing 10% FCS and other supplements. They were stimulated with Con A (2.5 μg/ml) for 48 h, washed three times and subsequently cultured with 50 U/ml IL-2 for additional 48 h. For restimulation, the cells were resuspended in the medium as for thymocytes and cultured on anti-CD3 (20 μg/well)-coated 24-well culture plates at a density of $5\times10^6$ cells/well.

*2.6. Determination of cell viability*

*2.6.1. Trypan blue dye exclusion assay.* N3-6-71 cells were seeded into anti-CD3-coated plates or into control plates. At 24 h of culture, the cells were harvested by vigorous pipetting and the cell viability was determined by the trypan blue dye exclusion test.

*2.6.2. Propidium iodide (PI) uptake.* Thymocytes or peripheral T cells were harvested, stained with PI (2 μg/ml), and then the cell viability was analyzed by a FACScan. The percentage of hypodiploid cells represented the percentage of apoptotic cells.

*2.7. Assay for caspase-3 or caspase-8 activity*

Caspase-3 or caspase-8 activity in cell lysates was measured using caspase-3 or caspase-8 colorimetric protease assay kit (Medical and Biological Laboratories, Nagoya, Japan). Briefly, $2\times10^6$ cells were lysed in 100 μl extract buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.2, 1% NP-40 containing the protease inhibitors), centrifuged at 15000 rpm for 15 min, and the supernatant was kept at −70°C. Cytosolic extracts (100 μg) were diluted in an equal volume of 2× reaction buffer containing 10 mM DTT and incubated at 37°C for 1 h with 200 μM colorimetric substrate for caspase-3 and caspase-8 being Ac-DEVD-pNA (Ac-Asp-Glu-Val-Asp-*p*-nitroanilide) and Ac-IETD-pNA (Ac-Ile-Glu-Thr-Asp-*p*-nitroanilide), respectively. Production of cleaved *p*-nitroanilide was monitored on a microplate reader at an absorbance of 410 nm.

## 3. Results

*3.1. Murine immature thymocytes exhibit high PEP activity*

We prepared the extracts of subpopulations of T cells from BALB/c mice and measured their PEP activity using a fluorescent substrate, Suc-Gly-Pro-MCA [23]. DN or DP thymocytes of 8-week-old mice were obtained by cell sorting. Thymocytes from neonatal mice were also used, and FACS analysis confirmed that >97% T cells were $CD4^+CD8^+$ (data not shown). To obtain DP-depleted thymocytes, thymocytes were killed with J11d antibody and complement. As previously described [24], the residual populations were SP (85%) and DN (12%) as determined by FACS analysis (data not shown). SP thymocytes were obtained by cell sorting from thymocytes of 8-week-old mice 48 h after administration with glucocorticoid. Unfractionated thymocytes or peripheral T cells were obtained from 8-week-old mice. As shown in Fig. 1, DN thymocytes, DP thymocytes, and neonatal thymocytes

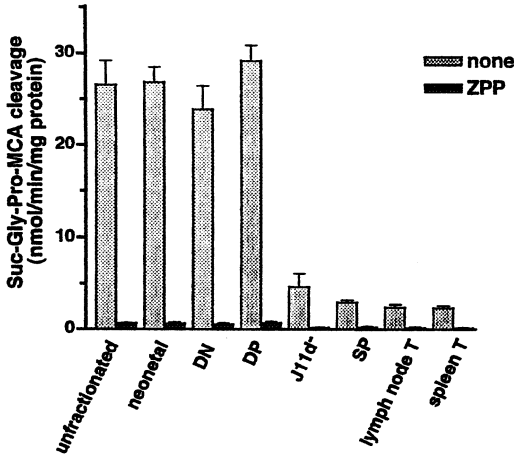


Fig. 1. PEP activity in subpopulations of T cells from BALB/c mice. Whole thymocytes of neonatal mice, unfractionated and fractionated thymocytes of 8-week-old mice, or peripheral T cells of 8-week-old mice were prepared, and PEP activity in each T cell subset was determined using a fluorescent substrate, Suc-Gly-Pro-MCA. As indicated, ZPP was added to the reaction mixture at a concentration of 1 μM.

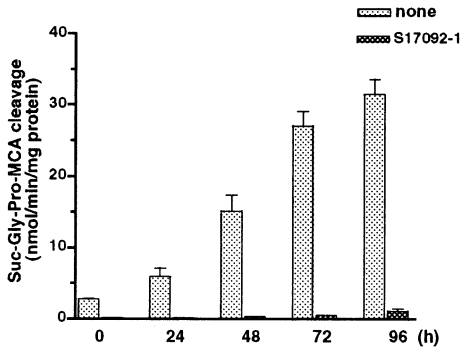


Fig. 2. Increased PEP activity during activation of murine peripheral T cells. Peripheral T cells of 8-week-old BALB/c mice were stimulated with Con A for 48 h, and subsequently cultured with IL-2 for an additional 48 h. At indicated period, PEP activity was determined using Suc-Gly-Pro-MCA. As indicated, S17092-1 was added to the reaction mixture at a concentration of 1 μM.

showed high PEP activities, which were completely inhibited by a specific inhibitor of PEP, *N*-benzyloxycarbonyl-prolyl-prolinal (Z-Pro-prolinal, ZPP) [25]. Conversely, PEP activities in the extracts of mature thymocytes or peripheral T cells, i.e. splenic T cells or lymph node T cells were rather low, compared with those of immature thymocytes.

### *3.2. Increased PEP activity during activation of murine peripheral T cells*

To examine PEP activity of peripheral naive T cells during activation, spleen T cells and lymph node T cells were prepared from 8-week-old BALB/c mice, stimulated with Con A for 48 h, and subsequently cultured with IL-2 for 48 h. At 24-h intervals, the cells were harvested and PEP activity in the cells was assessed by their Suc-Gly-Pro-MCA-hydrolyzing activity. As shown in Fig. 2, PEP activity in stimulated T cells was increased in a time-dependent manner. The activity was completely inhibited by S17092-1, a highly potent PEP inhibitor [26]. We confirmed that freshly isolated peripheral T cells were resistant to AICD, but continuous culture in the presence of Con A followed by IL-2 rendered them sensitive to AICD (data not shown).

### *3.3. Pretreatment of murine T cell hybridomas with PEP inhibitor results in the resistance to AICD*

Murine immature thymocytes are susceptible to AICD whereas mature thymocytes are resistant to AICD [27–30]. Naive mature T cells at early stages of activation are resistant to AICD but become sensitive at late stages of activation [18,19]. These observations led us to assume that murine T cells with high PEP activity might be susceptible to AICD. Murine T cell hybridoma N3-6-71 was also found to express high PEP activity (Fig. 3A). When N3-6-71 cells were treated with either PEP inhibitor, ZPP or S17092-1, PEP activity was decreased in a dose-dependent manner (data not shown). A 100 μM concentration of S17092-1 caused a nearly complete inhibition of PEP activity by 4 h. Similarly, ZPP (150 μM)-treated T cell hybridomas displayed reduced PEP activity. Because optimal inhibition of PEP activity was achieved at 150 μM of ZPP or 100 μM of S17092-1, these concentrations were used in subsequent analyses. Pretreatment of N3-6-71 cells with ZPP or S17092-1 did not modulate the cell surface expression levels of TCR/CD3 (data not shown). Next, we examined whether murine T cell hybridomas that were pretreated with PEP inhibitor could escape from AICD. After pretreatment with either inhibitor for 4 h, the cells were stimu-

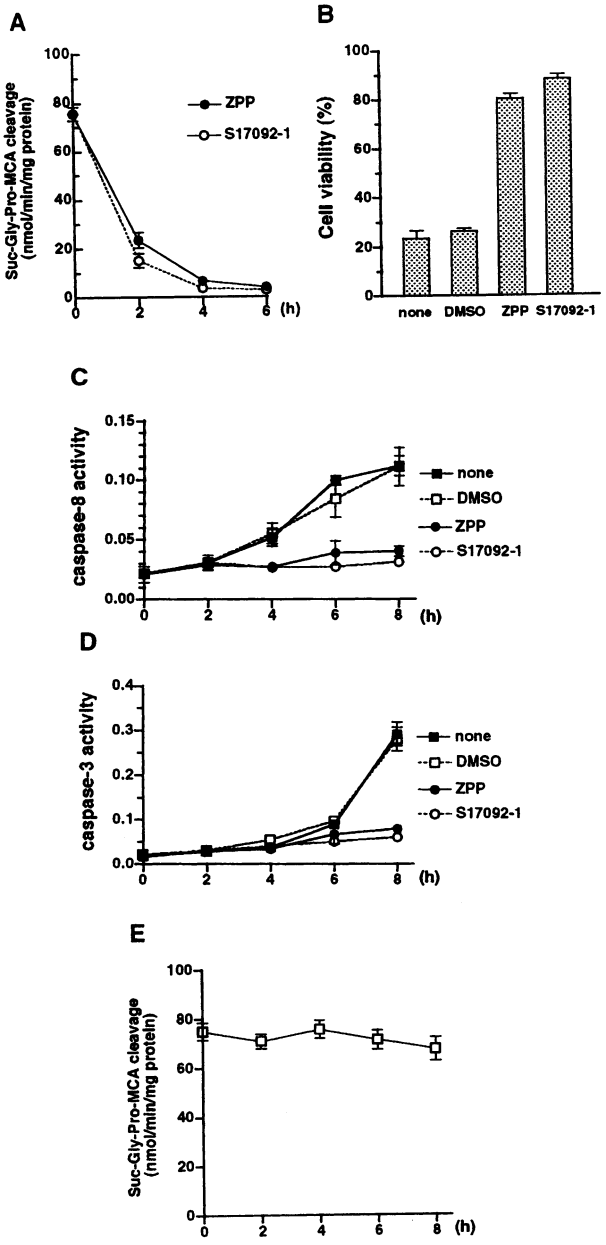


Fig. 3. Pretreatment of murine T cell hybridomas with PEP inhibitor results in the resistance to AICD. A: PEP activity in T cell hybridomas exposed to PEP inhibitor. After N3-6-71 cells were treated with 150 μM of ZPP or 100 μM of S17092-1 for indicated periods, the cells were harvested and PEP activity was determined using Suc-Gly-Pro-MCA. B: Pretreatment of T cell hybridomas with PEP inhibitor blocks AICD. After pretreatment with either PEP inhibitor or DMSO for 4 h, N3-6-71 cells were seeded into control plates or into anti-CD3 antibody-coated plates. At 24 h of culture, the cell viability was determined. C, D: PEP inhibitors suppress activation-induced caspase-8 activity (C) and caspase-3 activity (D) in murine T cell hybridomas. After pretreatment of N3-6-71 cells with ZPP, S17092-1, or DMSO for 4 h, the cells were cultured in anti-CD3 antibody-coated plates. At indicated times, caspase-8 or caspase-3 activity was determined using a colorimetric protease assay kit. E: PEP activity during AICD in T cell hybridomas. After N3-6-71 cells were cultured in anti-CD3-coated plates for indicated periods, PEP activity was determined using Suc-Gly-Pro-MCA.

lated with a higher density of immobilized anti-CD3 antibody for 24 h. As shown in Fig. 3B, ~75% of control T cells were dead as assessed by trypan blue dye exclusion. By contrast, AICD was apparently inhibited in N3-6-71 cells pretreated with ZPP or S17092-1.

Induction of apoptosis via Fas is characterized by cross-linking of Fas with trimeric Fas ligand (FasL) and recruitment of components of death-inducing signaling complex (DICS) [31]. Activation of the receptor-proximal caspase-8 in the DICS has been shown to initiate a cascade of active caspases including caspase-3 [32,33]. To investigate whether the inhibitory action of ZPP or S17092-1 in AICD might affect the initiator caspase or downstream effector caspases, we prepared cytosolic extracts from N3-6-71 cells upon stimulation with anti-CD3 antibody in the presence or absence of pretreatment with ZPP or S17092-1, and measured the caspase-8 activity and the central executioner caspase-3 activity in the cytosolic extracts using colorimetric substrates Ac-IETD-pNA and Ac-DEVD-pNA, respectively. Activation-induced apoptosis in N3-6-71 cells is detected after a period of 6–8 h, as evaluated by DNA cleavage [22]. As shown in Fig. 3C, caspase-8 activity was detected 6 h after activation with anti-CD3 antibody and increased up to 8 h. Similarly, an enhanced caspase-3 activity was also detectable at 6 h after activation (Fig. 3D). The pretreatment with ZPP or S17029-1 that was able to entirely block AICD in N3-6-71 cells inhibited the inducible activities of both caspase-8 and caspase-3. In addition, we examined whether PEP activity in N3-6-71 cells might be increased during AICD and observed that PEP activity was unchanged up to 10 h after stimulation with anti-CD3 antibody (Fig. 3E). The same results were obtained in the experiments using another T cell hybridoma, 2-45-12 [22] (data not shown).

### 3.4. *Inhibition of AICD in murine thymocytes or in activated peripheral T cells upon pretreatment with PEP inhibitor*

We examined whether PEP inhibitors also have inhibitory effects on AICD in immature thymocytes or in activated mature T cells. When thymocytes of BALB/c mice were treated with 150 μM ZPP or 100 μM S17092-1 for 2 h, reduced PEP activity was observed as assessed by Suc-Gly-Pro-MCA cleavage (data not shown). These pretreated thymocytes or untreated thymocytes were further incubated in immobilized anti-CD3 and anti-CD28 antibodies for 24 h, and then the cell viability was evaluated by PI staining (Fig. 4A). Either of PEP inhibitors showed little or no toxicity when added alone at the concentrations tested. After stimulation of untreated thymocytes, ~38% dead cells were detectable. In contrast, thymocytes pretreated with ZPP or S17092-1 decreased cell death comparable to spontaneous cell death in culture. Peripheral T cells of BALB/c mice were activated with Con A for 2 days, followed by culture in the presence of IL-2 for another 2 days. The cells were treated with ZPP or S17092-1 for 4 h. At this period, PEP activity was reduced to back ground levels (data not shown). When activated T cells were further restimulated with immobilized anti-CD3 antibody for 24 h, ~60% of T cells was dead (Fig. 4B). In contrast, the T cells pretreated with ZPP or S17092-1 showed a reduced cell death. Thus, pretreatment with PEP inhibitor prevented TCR-mediated apoptosis in thymocytes or in activated peripheral T cells.

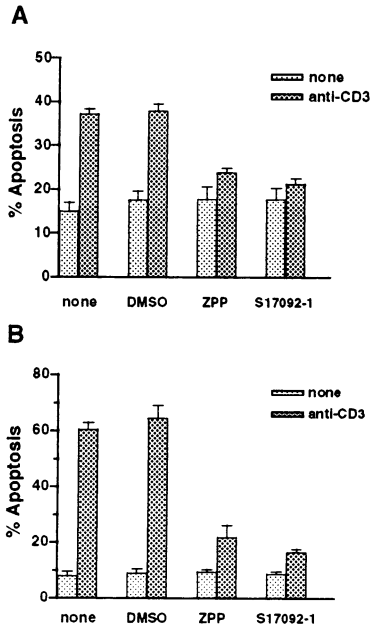


Fig. 4. Inhibition of AICD in murine thymocytes or activated peripheral T cells upon pretreatment with PEP inhibitor. A: Inhibition of AICD in PEP inhibitor-pretreated thymocytes. Thymocytes of 8-week-old BALB/c mice were treated with or without 150 μM ZPP, 100 μM S17092-1, or DMSO for 2 h. These thymocytes were then further incubated for 24 h in the absence or presence of immobilized anti-CD3 and anti-CD28. B: Inhibition of AICD in PEP inhibitor-pretreated peripheral T cells. Peripheral T cells of BALB/c mice were stimulated for 4 days as described in Fig. 2. The cells were then treated with or without 150 μM ZPP, 100 μM S17092-1, or DMSO for 4 h. These cells were then further incubated for 24 h in the absence or presence of immobilized anti-CD3 antibody. A, B: Percentage of apoptosis was determined by PI staining.

## 4. Discussion

We investigated PEP activity in subpopulations of murine T cells and found that PEP activity of immature thymocytes was ~10 times higher than that of mature thymocytes or of peripheral naive T cells. Dipeptidyl peptidase IV (CD26) is a serine protease that selectively hydrolyzes a peptide bond on the carboxyl side of proline when proline is the penultimate residue from the free amino terminus. This enzyme occurs preferentially on the surface of $CD4^+$ T cells and the amount of enzyme activity increases with T cell activation [34,35]. S17092-1 is shown to be unable to inhibit dipeptidyl peptidase IV, aminopeptidase B, and M [26], and we can exclude out the possibility that the activities of these peptidases might contribute to the PEP activity we assessed.

Murine T cell hybridomas undergo apoptosis upon activation with a high density of immobilized anti-CD3 antibody, and T cell hybridomas also showed high PEP activity. The PEP activity in T cell hybridoma N3-6-71 was unchanged during AICD. When N3-6-71 cells, whose PEP activity was reduced by treatment with specific PEP inhibitor, were exposed to anti-CD3 antibody, the cells were protected from AICD. In addition, when murine peripheral T cells were activated with Con A followed by IL-2, PEP activity was increased up to 96 h. Even in murine thymocytes as well as in activated peripheral T cells, pretreatment with PEP inhibitor prior to stimulation with anti-CD3 antibody prevented from AICD. These results suggest that T cells expressing high PEP

activity are sensitive to ACID and that PEP activity in T cells may determine their susceptibility to AICD. In addition, pretreatment with PEP inhibitor interfered with inducible activation of caspase-8, an upstream component of the caspase cascade during AICD, and caspase-3, preventing subsequent apoptotic cell death. The precise mechanism(s) by which inhibition of PEP activity prevents from AICD in T cells is at present unclear. It has been shown that T cells produce substance P, one of the well-characterized PEP substances, upon TCR/CD3 triggering and then substance P contributes to T cell proliferation in an autocrine fashion [36,37]. Therefore, further experiments will be done to determine whether substance P might be synthesized in T cells upon TCR/CD3 triggering and whether PEP might be responsible for the regulation of metabolism of substance P during AICD.

In summary, PEP activity in immature thymocytes was higher than in mature thymocytes or in peripheral naive T cells. These results suggest that PEP activity may be used as a marker to define the maturation of T cells. In addition, we indicated that T cells expressing high PEP activity are sensitive to ACID, suggesting that PEP may contribute to the apoptotic response observed during T cell activation.

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