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Dipeptidyl Peptidase I and Granzyme A Are Coordinately Expressed During CD8+ T Cell Development and Differentiation

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Dipeptidyl peptidase I (DPPI), formerly known as cathepsin C, is a cysteine protease that is capable of removing dipeptides from the amino-terminus of various peptides and protein substrates (1–6). This enzymatic activity is expressed at higher levels in cytotopic lymphocytes and myeloid cells than in lymphocytes without cytotopic potential or cells of non-bone marrow origin (7–9). Within cytotopic lymphocytes and myeloid cells, DPPI colocalizes within specialized granules with proteases from the families of structurally related granule serine proteases expressed in these cells (10). The results of a variety of studies indicate that DPPI plays a requisite role in the posttranslational processing and activation of these granule serine proteases by the removal of an activation dipeptide (10–13). Moreover, inhibiting DPPI activity during CD8+ T cell responses to alloantigens impairs both the activation of granzyme serine protease activity and the generation of cytotoxic effector function (14–17).

During thymocyte development and differentiation, a number of genes encoding putative CTL effector molecules are expressed (18, 19). Granzyme A mRNA expression has been noted in both immature thymocytes devoid of CD4 and CD8 epitopes and in more differentiated CD8+ CD4– thymocytes (18, 19). A multiphasic induction of granzyme genes during T cell ontogeny has been previously proposed (20, 21). While the expression of granzyme A and granzyme B mRNA has been noted during thymocyte differentiation, high levels of these enzymatic activities are expressed late in the course of CD8+ T cell responses to immune stimuli, and granzyme expression has been used as a marker of differentiated CTL effector cells (22). In contrast, the expression of high levels of DPPI has been noted in both CTL precursor and effector cells (9, 10). In a recent study assessing DPPI mRNA levels during the in vitro activation of lymphokine-activated killer cells, increased DPPI gene expression was noted following IL-2 stimulation of human PBLs, suggesting that, similar to granzyme A and granzyme B expression, DPPI may be up-regulated in response to stimuli that elicit cytotoxic lymphocyte responses (23). However, other investigators have noted significant differences in the levels of DPPI mRNA expressed in freshly isolated mouse splenocytes vs in vitro-activated murine lymphokine-activated killer cells or alloAg-activated splenocytes (24). Neither of these previous studies examined DPPI gene expression in purified populations of lymphocytes. Thus, the degree to which DPPI gene expression is regulated in CD8+ CTLs following various immune stimuli remains unclear. In addition, the stage at which DPPI expression is up-regulated in CTLs during T cell ontogeny is unknown. Our studies examine the expression of DPPI mRNA and enzymatic activity during the maturation and activation of CD8+ T cells. These data indicate that DPPI gene expression varies significantly
during the course of lymphocyte ontogeny and CD8⁺ T cell differ-
entiation. Moreover, the patterns of DPPI expression observed in
the course of these studies suggest that DPPI expression is reg-
ulated in a manner that results in the availability of high levels of
DPPI in T cells synthesizing the preprogranzymes that are report-
edly the physiologic substrates of this granule protease.

Materials and Methods

Cell lines

CTLL-2 cells were obtained from the American Type Culture Collection,
(Manasas, VA) and were cultured in RPMI 1640 medium (BioWhittaker,
Walkersville, MD) supplemented with 10% FBS (Life Technologies,
Gaithersburg, MD). 1 mM sodium pyruvate, 5 × 10⁻³ M 2-ME, 5 mM
HEPES (Sigma, St. Louis, MO), 0.3 mg/ml l-glutamine, 200 U/ml peni-
cillin G, and 10 μg/ml gentamicin. These cultures were supplemented
with 25 U/ml of rIL-2 twice per week.

Mice

We obtained 5- to 8-wk-old C57BL/6J and DBA/2J from The Jackson
Laboratory (Bar Harbor, ME). All animal research protocols used in this
project were reviewed and approved by the University of Texas South-
western Medical Center at Dallas Institutional Animal Care and Use Com-
mittee (Dallas, TX). Animals were 10 to 12 wk old at the time of use.

Monoclonal Ab

Anti-CD4 (GK 1.5) (25), anti-CD8 (YTS.169.4) (26), anti-NK (3A4) (27),
anti-1A²b-6 (MS114.15-2) (28), and anti-I-A² (25–5–165) (29) were pre-
pared as culture supernatants of hybridoma cells or were staphylococcal
protein A-purified from hybridoma culture supernatants as previously de-
scribed (30). FITC-conjugated anti-CD3 (145-2CII) was purchased from
PharMingen (San Diego, CA).

Cell purification and isolation

Thymocytes were performed on C57BL/6J mice by careful dissection,
and organs were thoroughly rinsed twice with HBSS (Life Technologies)
before dispersion into single-cell suspensions. Spleens were minced and
filtered through nylon mesh. Viable thymocyte and splenocyte populations
were separated by Hypaque 1083 (Sigma) density gradient centrifugation
for 30 min at 1500 revolutions per min after suspension in RPMI 1640
medium supplemented with 0.3% FBS, 25 mM HEPES, 100 μM 2-ME,
200 U/ml penicillin G, and 10 μg/ml gentamicin. Thymocyte or T cell
subsets were purified and isolated using magnetic cell sorting with a Vari-
omacs cell sorter (Miltenyi Biotec, Auburn, CA) (31). Briefly, cells were
suspected in PBS buffer supplemented with 5 mM EDTA and 1% FBS and
incubated for 15 min at 4°C with Ab-coated microbeads. Positive and
negative selection columns were used where appropriate, and cell popula-
tions were washed with PBS following separation and isolation as directed
by the manufacturer. Upon a subsequent flow cytometric analysis of
splenocytes and single-positive thymocytes following enrichment with the
magnetic-activated cell sorter magnetic bead isolation system, >95% pu-
rity of selected cell populations was routinely observed.

CD3⁺ CD4⁻ CD8⁻ thymocytes

Thymocytes were incubated with FITC anti-CD3, anti-NK (3A4), and anti-
I-A² (MS 114.15-2 and 25-5-165) Abs for 30 min at 4°C. Following
washing, the cells were incubated with goat anti-mouse Ig, anti-FITC, anti-
CD4 (L3T4), and anti-CD8 (Ly-2) microbeads as previously described, and the
CD3⁺ CD4⁻ CD8⁻ thymocytes were collected as the unbound fraction
using a negative selection column.

CD4⁺ CD8⁻ thymocytes

Thymocytes were incubated with fluorescein-conjugated anti-CD8 (Life Technolo-
gies) and biotinylated anti-CD4 (Life Technologies) for 30 min at 4°C.
Cells were washed and incubated with avidin-phycocerythrin (Life Techno-
lologies) for 30 min on ice before washing, and the selection of cells
positively stained with both anti-CD8 and anti-CD4 by FACS using a
FACStar (Becton Dickinson, Mountain View, CA) as previously described
(16). Upon subsequent flow cytometric analysis, >95% purity of positively
selected cell populations was routinely observed.

CD4⁻ CD8⁺ and CD8⁻ CD4⁻ thymocytes

Thymocytes were incubated with anti-CD4 (L3T4) or anti-CD8 (Ly-2)
microbeads. The unbound fraction following selection with anti-CD4 mi-
crobes was then incubated with anti-CD8 (Ly-2) microbeads and posi-
tively selected as CD4⁺ CD8⁻ cells. The unbound thymocytes following
initial selection with anti-CD8 microbeads were then incubated with anti-
CD4 (L3T4) microbeads and positively selected as CD4⁺ CD8⁻ cells.

Isolation of CD8⁺ C57BL/6J splenocytes

Splenocytes were incubated with anti-CD8 (Ly-2) microbeads, and posi-
tively selected CD8⁺ T cells were isolated.

Generation of in vitro-activated CD8⁺ B6 anti-H-2b-specific
CTLs

CD8⁺ CTLs were activated in MLCs containing both 40 × 10⁶ C57BL/6J
(H-2b) responder spleen cells and 10 × 10⁶ irradiated (1500 cGy) T cell-
depleted DBA/2J (H-2b) stimulator spleen cells in 20 ml of culture me-
dium. Before irradiation, the DBA/2J cells were separated from other cel-
ular elements by Hypaque 1083 (Sigma) density centrifugation. The cells
were also depleted of T cells by incubation with HO 13.4 anti-Thy-1 Ab
(32) (1:30 dilution) for 30 min on ice and subsequent treatment with rabbit
complement (1:6 dilution) for 50 min at 37°C. The cells were passed
through 30-μM nylon filters and irradiated with 1500 cGy. Each culture
was suspended in 20 ml of complete media. AlloAg-activated cells were
harvested on days 1 through 5 and positively enriched for CD8⁺ CTLs
using anti-CD8 (Ly-2) microbeads (Miltenyi Biotec).

Protein, DPPI, and N-α-benzoylxy-carbonyl-l-lysine thiobenz-
ester (BLT) esterase (granzyme A) enzymatic activity assays

The protein concentration was assessed by the bicinchoninic acid method
using BSA as the standard as previously described (10). Assay reagents
were purchased from Pierce (Rockford, IL). DPPI activity was assayed by
hydrolysis of glycylphenylalanine-β-naphthylamide (Sigma) as previously
described. BLT esterase activity was assayed by hydrolysis of BLT (Cal-
biochem-Behring, La Jolla, CA) as previously described (10, 33). The re-
sults are given as the mean ± SE of the mean for triplicate determinations.

Generation of DPPI, granzyme A, and glyceraldehyde-3-
phosphate dehydrogenase (G3PDH) probes for semiquantitative
PCR

Those primers (M4I) (5’-CACAACATTGTGAAGGCCATCAAT-3’) and
(B34) (5’-TCACTAGTCTCTTCTGGG-3’) that had been used previously
to amplify a 830-base pair (bp) product of mouse DPPI cDNA (33) were
used to establish an RT-PCR assay for DPPI mRNA expression. These
primers are complementary to sequences in the 4th and 7th exon of the
murine DPPI gene (24); consequently, the primers yield distinctive m.w.
products when used to amplify genomic DNA. Oligonucleotides were ob-
tained from Integrated DNA Technologies (Coralville, IA). A 177-nucleo-
tide bp internal probe was obtained from the previously cloned mouse
DPPI cDNA (34) by digestion with BamHI and EcoRI (Life Technologies)
and gel purification. The sequence of granzyme A mRNA was analyzed, and a
5’ primer from the N terminus (5’-CCCGTGTGTCCTCACTAAA-GACC) and a
3’ primer from the C terminus (5’-TGGCGATCTCCA CACTTCTCCACC) were
synthesized (Integrated DNA Technologies). cDNA encoding a portion of mouse granzyme A was obtained by RT-PCR
using CTL-2 RNA as a template and cloned in the PCR 3.1 vector. The
cloned cDNA was verified as mouse granzyme A by sequence analysis. A
372-nucleotide bp internal probe was obtained by digestion with AvrII and
BglII (Life Technologies) and gel purification. The cloned murine G3PDH
sequence was analyzed, and a 5’ primer from the N terminus (5’-ACCGTGTTGTGCCCATCAAT-GACC) and a 3’ primer from the C terminus (5’-
CCACACCCCTGGTGTCGTGACC) were synthesized (Integrated DNA Technologies). cDNA encoding a 450-nucleotide bp segment of mouse
G3PDH was obtained by RT-PCR using CTL-2 RNA as a template and cloned in the PCR 3.1 vector as described above. The cloned cDNA was
verified by sequencing and used as a probe following gel purification as
previously described (34).

Semiquantitative RT-PCR

Cell populations (1.0 × 10⁶) were individually pelleted and treated for 15
min at 15 to 30°C with TRIZol reagent (Life Technologies). Total RNA
was isolated per the manufacturer’s recommendations. The isolated RNA
was solubilized in 30 μl of RNase-free sterile water at 70°C for 10 min.
First strand cDNA was synthesized by RT reaction with 1 μg of starting
RNA in a 22 μl reaction volume. Following denaturing and annealing with
1 μl of oligo-dT (Life Technologies) (0.5 μg/μl), the reaction was equili-
brated and incubated at 45°C for 1 h with 1 μl of Superscript (Life Tech-
nologies) RT (200 U/μl). Specimens were immediately removed from the
primers produced a 450-bp product (lane 1), was amplified with DPPI- and G3PDH-specific primers by PCR for 28 cycles. DPPI primers produced a 830-bp product (lanes 5 and 6). Lane 4 displays the electrophoretic patterns of a 100-bp ladder (Life Technologies) marker DNA including prominent 600-bp, 1500-bp, and 2000-bp bands. B, the 0.8% agarose gel from A was transferred to a Nytran membrane by capillary action, the DNA was fixed to the membrane in a vacuum oven, and DPPI- and G3PDH-specific [α-32P]deoxyCTP radiolabeled probes were used to quantitatively assess mRNA expression using the Ambis computer-controlled β emission counter. C shows quantitatively the number of cpm/mm² detected by the Ambis counter during a 12-h scan.

In vitro alloAg-activated CD8+ cells

In vitro alloAg-activated CD8+ CTLs were generated in a 5-day MLC. Cells were harvested daily for 5 days, and highly purified CD8+ CTLs were selected as described in Materials and Methods.
and assayed for DPPI and granzyme A mRNA expression (Fig. 4) and enzymatic activity (Fig. 5). In contrast to the readily detectable levels of DPPI and granzyme A mRNA noted in freshly isolated CD8\(^{+}\) spleen cells before allostimulation, the levels of both types of mRNA were greatly reduced or undetectable after 1 to 2 days in MLCs. In contrast to granzyme A mRNA expression, which reappeared on day 3 of MLC and continued to increase through day 5 of activation, DPPI mRNA levels were up-regulated earlier and peaked by day 3 or day 4 of culture as noted in the two representative experiments outlined in Figure 4.

As described in Figure 5, in vitro-activated CD8\(^{+}\) CTLs displayed a reduction in DPPI enzymatic activity on day 2 of the MLC, with a subsequent up-regulation of DPPI enzymatic activity by day 3 that continued throughout the remainder of the 5-day MLC. In contrast, BLT esterase activity, a measure of trypsin-like protease activity that is mediated largely, although not exclusively, by granzyme A in CD8\(^{+}\) T cells (35), decreased following initial stimulation, returned to preactivation levels at day 3, and then continued to increase to much higher levels by day 5 of the MLC.

Discussion

DPPI is a lysosomal cysteine protease previously noted to play a role in the posttranslational processing and activation of members of the family of granule serine proteases or granzymes expressed in activated CTLs. The present findings indicate that the expression of high levels of DPPI antecedes or is concomitant with that

![Figure 2](image-url)  
**Figure 2.** Expression of mRNA in thymocytes and spleen cells. Highly purified CD3\(^{+}\)CD4\(^{+}\)CD8\(^{+}\) (DN Thy), CD4\(^{+}\)CD8\(^{+}\) (DP Thy), CD4\(^{+}\)CD8\(^{-}\) (CD4\(^{+}\) Thy), and CD8\(^{+}\)CD4\(^{-}\) (CD8\(^{+}\) Thy) thymocytes as well as CD4\(^{+}\) or CD8\(^{+}\) spleen cells were isolated and assessed by RT-PCR for DPPI, granzyme A, and G3PDH mRNA. The results represent the means ± SEM of values obtained from three independent experiments.

![Figure 3](image-url)  
**Figure 3.** Expression of DPPI enzymatic activity in thymocytes and spleen cells. Highly purified thymocyte and spleen cell populations as described in Figure 2 were lysed and assessed for hydrolysis of the DPPI substrate glycylphenylalanyl-\(\beta\)-naphthylamide. DPPI-specific activity is expressed as units of activity per mg protein (U/mg) with 1 U = 1 nmol \(\beta\)NA released per minute. The results represent the means ± SEM of values obtained from three independent experiments.

![Figure 4](image-url)  
**Figure 4.** DPPI and granzyme A mRNA expression following alloAg stimulation. B6(H-2\(^{b}\)) spleen cells were cultured with irradiated, T cell-depleted DBA/2(H-2\(^{d}\)) stimulator cells in a 5-day MLC. At the indicated time points, CD8\(^{+}\) T cells were isolated and assayed for DPPI, granzyme A, and G3PDH mRNA expression; two representative experiments are presented.
of granzyme A not only during the activation of CTLs but also during the earlier stages of T lymphocyte ontogeny.

When thymocyte subsets were examined for DPPI mRNA expression and enzymatic activity, DPPI mRNA expression was found to be significantly higher in immature CD3\(^+\)CD4\(^-\)CD8\(^-\) thymocytes and more differentiated CD8\(^-\)CD4\(^-\) thymocytes than in intermediate stage CD4\(^+\)CD8\(^-\) thymocytes or in the corresponding CD4\(^-\)CD8\(^-\) population of more differentiated, single-positive thymocytes. Of interest, even though CD3\(^-\)CD4\(^-\)CD8\(^-\) thymocytes expressed very high levels of DPPI mRNA, these cells were found to express similar or only slightly greater amounts of DPPI enzymatic activity than other thymocyte subsets expressing little or no DPPI mRNA. In addition, while DPPI mRNA levels appear to fall to very low or undetectable levels in CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^-\) thymocytes, low levels of DPPI enzymatic activity remain detectable in these cells. The disparate expression of DPPI mRNA and enzymatic activity in these thymocyte populations likely relates to differences in mRNA and protein half-life or to other posttranscriptional regulatory mechanisms.

The present findings suggest that DPPI gene expression is activated before or immediately after the migration of T cell precursors to the thymus. The concomitant expression of both DPPI and granzyme A (our observations and Refs. 18 and 19) as well as granzyme B (18, 19) at this early stage of T cell development suggests that enzymatically active forms of granzyme A and B could theoretically be produced in these cells. However, we have not found detectable levels of BLT esterase activity in lysates of double-negative thymocytes in additional studies (data not shown). These findings are in agreement with the observation by Ebnet et al. (19) that BLT esterase activity was not able to be detected by histochemical techniques in CD4\(^+\)CD8\(^-\) thymocytes (19) but not in splenic CD8\(^+\) T cells. In intermediate stages of thymocyte differentiation, BLT esterase activity was not found detectable levels of BLT esterase activity in lysates of double-negative thymocytes. These findings are in agreement with the observation by Ebnet et al. (19) that BLT esterase activity was not able to be detected by histochemical techniques in CD4\(^+\)CD8\(^-\) thymocytes (19) but not in splenic CD8\(^+\) T cells. In contrast, a subset(s) of CD8\(^+\) T cells, such as cells that have recently emigrated from the thymus or T cells recently stimulated by Ag. However, the results of previously published studies using dipeptide ester substrates of DPPI to identify cells expressing high levels of this enzyme suggest that the majority of human peripheral blood T cells continue to express high levels of DPPI enzymatic activity (15, 16).

In contrast to the similarities between the patterns of DPPI and granzyme A expression in thymocytes and spleen T cells, granzyme B mRNA is reportedly expressed equally in CD4\(^+\)CD8\(^-\) and CD8\(^-\)CD4\(^-\) thymocytes (19) but not in splenic CD8\(^+\) T cells. In additional studies (data not shown) using granzyme B-specific PCR primers and RT-PCR mRNA assay conditions similar to those that detect granzyme A and DPPI mRNA in CD8\(^-\) spleen T cells, we were unable to detect granzyme B mRNA in CD8\(^-\) spleen T cells.

When spleen T cells were stimulated with alloAg, it was observed that the levels of both granzyme A and DPPI mRNA in CD8\(^+\) cells declined to very low or undetectable levels during the first 1 to 2 days of the 5-day MLC. Following an initial reduction in expression during the first 2 days of culture, DPPI mRNA expression in CD8\(^+\) T cells was induced over the next 24 to 48 h; peak levels of DPPI mRNA expression in CD8\(^+\) T cells were observed during day 3 or day 4 of activation. In contrast, granzyme A mRNA expression was highest at the end of the 5-day MLC.

The present findings suggest that DPPI mRNA is constitutively expressed in both resting and in vitro-activated mouse spleen cells (24). The discrepancies may be secondary to the use of highly purified CD8\(^+\) T cells in the present studies, while previous attempts to follow DPPI mRNA levels during the activation of mouse spleen T cells assessed mRNA levels in whole spleen cell populations and thus

**FIGURE 5.** Expression of DPPI and BLT esterase activities following in vitro alloAg stimulation. CD8\(^+\) B6 spleen T cells were activated in MLCs as described in Figure 4 and then assessed for DPPI and BLT activity. These data correspond to experiment 1 only.
could not distinguish the expression by CD8+ T cells from DPPI mRNA expressed by macrophages, NK cells, or other spleen cell populations. The present findings are in agreement with prior reports that human lymphocyte DPPI mRNA expression is significantly up-regulated following in vitro activation (23).

The observation that DPPI mRNA expression and levels of enzymatic activity peak earlier in the course of CTL activation than does granzyme A expression is consistent with the proposed requisite role that DPPI appears to play in posttranslational processing and the activation of granzymes. Thus, DPPI is expressed immediately before and during periods of peak granzyme A expression during both lymphoid ontogeny and again during the Ag-specific activation of CTLs. These data are consistent with other observations suggesting that a critical level of DPPI enzymatic activity is required for the production of active granzyme B in transfected COS cells (11). Therefore, the observed temporal sequence of DPPI and granzyme A expression during alloAg stimulation is part of a multigene activation sequence leading to the expression of high levels of granule proteins. Thus, DPPI is expressed immediately before and during periods of peak granzyme A expression in highly purified dipeptidyl amineopeptidase I (cathpesin C) and the elimination of their activities from preparations used to sequence peptides. Biochem. Biophys. Res. Commun. 46:52.

References