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

Christopher L. Mabee, Michael J. McGuire, and Dwain L. Thiele

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. This enzymatic activity is expressed at higher levels in cytotoxic lymphocytes and myeloid cells than in lymphocytes without cytotoxic potential or cells of non-bone marrow origin. Within cytotoxic lymphocytes and myeloid cells, DPPI colocalizes within specialized granules with proteases from the families of structurally related granule serine proteases expressed in these cells. 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. Moreover, inhibiting DPPI activity during CD8+ T cell responses to alloantigens (alloAgs) impairs both the activation of granzyme serine protease activity and the generation of cytotoxic effector function.

During thymocyte development and differentiation, a number of genes encoding putative CTL effector molecules are expressed. Granzyme A mRNA expression has been noted in both immature thymocytes devoid of CD4 and CD8 epitopes and in more differentiated CD8+CD4- thymocytes. A multiphasic induction of granzyme genes during T cell ontogeny has been previously reported. 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. In contrast, the expression of high levels of DPPI has been noted in both CTL precursor and effector cells.

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. The degree to which DPPI gene expression is regulated in CD8+ CTLs following various immune stimuli remains uncertain. 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.

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4 Abbreviations used in this paper: DPPI, dipeptidyl peptidase I; BLT, N-benzylloxy-carbonyl-l-lysine thiobenzyl ester; alloAg, alloantigen; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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during the course of lymphocyte ontogeny and CD8+ T cell differentiation. Moreover, the patterns of DPPI expression observed in the course of these studies suggest that DPPI expression is regulated in a manner that results in the availability of high levels of DPPI in T cells synthesizing the preprogranzymes that are reportedly the physiologic substrates of this granule protease.

Materials and Methods

Cell lines

CTLL-2 cells were obtained from the American Type Culture Collection, (Manassas, 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 penicillin 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 southwestern Medical Center at Dallas Institutional Animal Care and Use Committee (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-I-Ab-h (MS/114.15-2) (28), and anti-I-Aa (25–5–165) (29) were prepared as culture supernatants from hybridoma cells or were staphylococcal protein A-purified from hybridoma culture supernatants as previously described (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 VariMACS cell sorter (Miltenyi Biotec, Auburn, CA) (31). Briefly, cells were suspended 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 populations 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% purity of selected cell populations was routinely observed.

CD3+ CD4+ CD8+ thymocytes

Thymocytes were incubated with FITC anti-CD3, anti-NK (3A4), and anti-I-Aa (MS 114.15.2 and 25–5–165) (28) 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 cDNA encoding a portion of mouse granzyme A was obtained by RT-PCR using CTLL-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 for gel purification. The sequence of granzyme A mRNA was analyzed, and a 5’ primer from the N terminus (5’-CAAGTTGTGTCCTCACTCAA GACC) and a 3’ primer from the C terminus (5’-TGCCGATTCTCACACTTACCAC) were synthesized (Integrated DNA Technologies). cDNA encoding a portion of mouse granzyme A was obtained by RT-PCR using CTLL-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 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’-CACGTTGTGTCCTCACTCAA GC) and a 3’ primer from the C terminus (5’-TGGCGATTCTCACA CTTACCAC) were synthesized. cDNA encoding a portion of mouse granzyme A was obtained by RT-PCR using CTLL-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 for gel purification as previously described (34).

Semiquantitative RT-PCR

Cell populations (1.0 × 10^6) 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 equilibrated and incubated at 45°C for 1 h with 1 μl of Superscript (Life Technologies) RT (200 U/μl). Specimens were immediately removed from the
lanes 5

was amplified with DPPI- and G3PDH-specific primers by PCR for 28 cycles. DPPI primers produced a 830-bp product (lanes 1 and 2), and G3PDH primers produced a 450-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.

FIGURE 1. Semiquantitative RT-PCR assays of DPPI mRNA. A, 0.25 μg of RNA from CTLL-2 and mouse brain were reverse transcribed, and cDNA was amplified with DPPI- and G3PDH-specific primers by PCR for 28 cycles. DPPI primers produced a 830-bp product (lanes 1 and 2), and G3PDH primers produced a 450-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.

Results

**DPPI and granzyme A mRNA expression in thymocytes and splenocytes**

The expression of DPPI and granzyme A mRNA was assessed in various subsets of thymocytes and splenocytes as described in Figure 2. Both DPPI mRNA and granzyme A mRNA expression were detected in CD3+ CD4+ CD8+ thymocytes, while the expression of neither DPPI mRNA nor granzyme A mRNA was detected in CD4+ CD8+ thymocytes. CD8+ thymocytes were found to express higher levels of both DPPI mRNA and granzyme A mRNA than CD4+ thymocytes. Although the expression of granzyme A mRNA was modestly reduced in CD8+ spleen cells, this cell population continued to express detectable levels of granzyme A mRNA as well as high levels of DPPI mRNA.

**DPPI enzymatic activity in thymocytes**

Whole cell lysates from each thymocyte and spleen cell population were assayed for DPPI activity. As described in Figure 3, CD3+ CD4+ CD8+ thymocytes were found to express DPPI enzymatic activity at a level that was about threefold less than that detected in peripheral CD8+ splenocytes. Progressively lower levels of DPPI activity were detected in CD4+ CD8+ thymocytes and CD4+ CD8+ thymocytes. CD8+ CD4+ thymocytes were found to express higher levels of DPPI than CD4+ CD8+ thymocytes, with even higher levels of DPPI expressed in CD8+ splenocytes.

**DPPI and granzyme A expression in 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. 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 assayed 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. 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. 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 appeared 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 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 appeared 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.

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Following intrathymic selection and differentiation, single-positive lymphocytes emerge from the thymus and migrate to peripheral lymphoid organs, in which mature CD8^- T cells continue to express both DPPI mRNA and enzymatic activity. In agreement with previously published studies (10) in which we detected immunoreactive and enzymatically active granzyme A in CD8^- spleen T cells, modest but readily detectable levels of granzyme A expression were detected in the freshly isolated spleen CD8^- T cell populations examined in the present studies. It remains unclear whether this low level of granzyme A expression is present only in 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 dipeptidyl ester substrates of DPPI to identify cells expressing high levels of this enzyme suggest that the majority of human peripheral blood and mouse spleen CD8^- 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.

These findings regarding the expression of DPPI during alloAg-induced activation of CD8^- T cells are in contrast to recent reports which 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
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 teleologically appropriate. These findings suggest that the expression of high levels of DPPI is part of a multigene activation sequence leading to the expression of high levels of granule proteases in CTLs at selected stages of T cell ontogeny and T cell immune responses.

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