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Carboxypeptidase E, an essential element of the regulated secretory pathway, is expressed and partially co-localized with chromogranin A in chicken thymus

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Abstract

Although the functions of hormones and neuropeptides in the thymus have been extensively studied, we still do not know whether these intrathymic humoral elements are released in a stimulated manner via the regulated secretory pathway or in a constitutive manner. Carboxypeptidase E (CpE) and chromogranin A (CgA) are functional and structural hallmarks of the regulated secretory pathway in (neuro) endocrine cells. Whereas we have previously shown a CgA-positive neuroendocrine population in the chicken thymus, the current study assesses the expression of CpE in the thymus, both at the mRNA and the protein level. Our immunohistochemical studies provide evidence for the co-existence of CgA and CpE in identical neuroendocrine cells in the thymus. CpE and CgA dual-positive cells have primarily been found in the transition zone between the cortex and medulla of the thymus, an area known to contain numerous arterioles and to be innervated by the autonomic nervous system. Our findings suggest that the diffuse neuroendocrine system serves as a relay for nervous stimuli delivered by the sympathetic and/or parasympathetic nervous system. Thus, these newly defined neuroendocrine cells might play an important role in the immuno-neuro-endocrine cross-talk in the thymus, potentially enabling thymopoiesis to be fine-tuned via the regulated secretory pathway by a variety of physical and environmental factors.

Keywords

Carboxypeptidase E; Thymus; Chromogranin A; Neuroendocrine; Chicken

Introduction

The thymus is the primary lymphoid organ for T-cell development and thus plays a pivotal role in the vertebrate cellular immune system. Considerable evidence has been presented that the thymus is also the source of an evergrowing list of hormones and neuropeptides (for reviews, see Lamberts 1999; Bodey 2002). The immunological significance of these neuroendocrine elements has been intensively investigated. Hormones take an essential part in establishing central T-cell tolerance (Kyewski and Klein 2000). For example, thymic epithelial cells express insulin causing the clonal deletion of insulin-reactive T-cells (Palmer 2003). However, mounting evidence shows that thymic neuropeptides also function as humoral signaling factors affecting T-cell development and maturation. In this context, neuropeptides act as immunomodulators (Lamberts 1999) that participate in homeostatic regulation, providing essential growth regulatory signals (ranging from proliferative to pro-apoptotic) to thymocytes. In addition, they affect cytokine secretion (Mukamoto et al. 1999; Bodey et al. 2000; Mukamoto and Kodama 2000; Bodey 2002). For instance, somatostatin, a neuropeptide mainly produced in the brain, is highly expressed in murine thymus and has been shown to be able to regulate thymocyte development and migration (Solomou et al. 2002).

Neuropeptide expression in the thymus is evolutionarily well conserved in vertebrates (Silva et al. 2006). In birds, various thymic neuropeptide-positive cells, containing peptides such as neurotensin, met-enkephalin, neuropeptide Y, substance P, and vasoactive intestinal polypeptide have been demonstrated immunohistochemically (Atoji et al. 1997). Other studies have demonstrated the presence of immunoreactive pro-opiomelanocortin-derived molecules in the thymus of 4-day-old chickens, with cell numbers increasing with ageing (Ottaviani et al. 1997; Franchini and Ottaviani 1999). In addition, nitrergic and peptidergic innervation of the chick thymus has been described by Gulati et al. (1997, 1998).

Although the immune effects of these humoral elements on T-cell physiology are now being elucidated, their pathway of secretion is largely unknown. Neuroendocrine cells secrete proteins via one of two distinctively different pathways, i.e., either the constitutive secretory pathway or the regulated secretory pathway. In the regulated secretory pathway, proteins such as pro-hormones and pro-neuropeptides mature in the secretory granules of the neuroendocrine cell through a number of post-translational modifications, and a specific extracellular (depolarizing) stimulus is required for delivery (Ca^{2+} -dependent exocytosis) of the secretory products to the outside of the cell. Chromogranin A (CgA), a cargo protein in large dense-core vesicles (LDCV), is essential for regulated secretory vesicle biogenesis in (neuro) endocrine cells. Secretory granule formation has been shown to be largely lost when CgA expression is inhibited, whereas the knock-in of bovine CgA into CgA-deficient PC12 cells restores the regulated secretory pathway (Kim et al. 2001, 2002). Some controversy exists regarding these *ex vivo* findings, since a CgA knock-out mouse has been found to have no phenotype with respect to granule biogenesis (Hendy et al. 2006). However, other knock-out mice generated by Mahata's group (Mahapatra et al. 2005) and transgenic mice harboring short-interfering RNA for CgA (Kim et al. 2005) have provided further evidence in support of an essential role for CgA in regulating dense-core secretory granule biogenesis. These CgA-lacking mice exhibit a decreased number, size, and electron density of granules formed in adrenal chromaffin cells (Kim et al. 2005; Mahapatra et al. 2005). CgA has also been shown to up-regulate LDCV biogenesis by activating the expression of a protease inhibitor, viz., protease nexin-1, to stabilize LDCV cargo proteins against degradation in the Golgi apparatus in endocrine pituitary cells (Kim and Loh 2006).

Another crucial element in the process of sorting and processing of prohormones appears to be carboxypeptidase E (CpE, previously also referred to as CpH; Hook et al. 2004)). CpE

per se is an exoproteolytic processing enzyme present in the Golgi apparatus and secretory granules of neural/neuroendocrine cells. Its function is to remove basic amino acid residues exposed upon endoproteolytic cleavage of the hormone precursor by a specific prohormone convertase at the site of a dibasic pair (e.g., Lys-Lys). The membrane-bound form of the enzyme is anchored in the wall of the secretory granules through its COOH-terminal, which also serves as a sorting receptor for several prohormones and proneuropeptides in neuroendocrine cells (Zhang et al. 1999; Dhanvantari et al. 2002).

In our previous study, we have shown the existence of a complex neuroendocrine cell population within the chicken thymus, including a CgA-immunoreactive cell population (Oubre et al. 2004). Here, we further investigate the expression of CpE in the chicken thymus and, more specifically, in the previously described CgA-positive cells. The co-existence of two LDCV markers, CpE and CgA, in the same cells would suggest that a neuroendocrine subtype cell population exists in the chicken thymus, and that the release of the neuropeptides in the thymus occurs, at least partly, through the regulated secretory pathway.

In this study, the cDNA sequence of chicken CpE was first assembled *in silico*, and a reverse transcription polymerase chain reaction (RT-PCR) was performed to examine the production of CpE mRNA within chicken thymus. In addition, an immunocytochemical approach was used to confirm the presence of CpE in broilers and laying hens at various ages and to assess its cellular co-localization with CgA, and with a neuropeptide, somatostatin.

Materials and methods

Chicken CpE cDNA sequence

A chicken CpE cDNA contig was assembled *in silico* based on the public chicken expressed sequence tag (EST) and chicken genome databases. The human CpE cDNA sequence (GI: 31565486) was compared with the available sequences in the chicken EST database (http://www.chickest.udel.edu/Cogburn_CAP3_DB/blast.html). The resulting EST sequences with high homology were assembled into one consensus sequence by using the CAP3 program at <http://deepc2.zool.iastate.edu/aat/cap/cap.html>. The assembled chicken CpE cDNA sequence was then verified by comparison with the chicken whole genome database (<http://genome.ucsc.edu/cgi-bin/hgBlat>). The predicted amino acid sequence of chicken CpE was deduced by use of the NCBI ORF (Open Reading Frame) Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

CpE RT-PCR and sequencing

Total RNA was extracted from normal broiler chicken thymus by using Trizol (Invitrogen, Carlsbad, Calif., USA) according to standard protocols. Total RNA was reverse-transcribed to cDNA with Omniscript reverse transcriptase (Qiagen, Valencia, Calif., USA) by using random hexameroligos as the primer (1 μ M). The cDNA (5 μ l) was then amplified by PCR for the detection of CpE. The PCR mixture contained 2 mM MgCl₂, 50 mM KCl, dNTP mix (200 μ M each dNTP), and 1 μ l BD Advantage 2 Polymerase Mix, including BD TITANIUM *Taq* DNA polymerase, a small amount of proofreading polymerase, 1.1 μ g/ μ l BD *Taq*Start Antibody (Clontech, Mountain View, Calif., USA), and 0.4 μ M primers. The primer pair was designed by using Primer Express 4.0 (ABI, Foster City, Calif., USA). The sequence of the forward primer was: *TTGGTCGAAGTAATGCCCAGG* (Tm 61°C) and that of the reverse primer was: *TCCTCCCAATAGCCCTTCAGA* (Tm 60°C). The expected size of the PCR product was 550 bp. PCRs were carried out under the following conditions: 95°C for 1.5 min, and 30 cycles of 95°C for 25 s and 68°C for 3 min. The PCR product was

then sequenced in the Gene Technologies Laboratory, Texas A&M University, by using an ABI Prism 377XL DNA Sequencer.

Immunocytochemistry

White Leghorn chicks (8 days, 6 weeks, and 17 weeks old) were obtained from a local hatchery (Hy-line). Thymic, pituitary, and adrenal tissues were harvested and fixed for 24 h at room temperature in Bouin-Hollande sublimate. The tissue blocks were then processed for paraffin embedding by using routine laboratory protocols. Tissue sections (9-10 μ m thick) were cut on a rotary microtome MT 980 (Boeckeler Instruments, Tucson, Ariz., USA)

Immunohistochemical reagents and primary antibodies

TBST is a TRIS-buffered saline containing 0.1% Triton X-100, pH 7.4. Trizma base, Triton X-100, and diaminobenzidine (DAB) were obtained from Sigma (St. Louis, Mo., USA). Peroxidase-conjugated secondary antibodies, biotinylated goat anti-rabbit Ig, rhodamine red-conjugated goat anti-mouse Ig, and fluorescein isothiocyanate (FITC)-and rhodamine red-conjugated streptavidin were obtained from Jackson Laboratories (West Grove, Pa., USA). Cytoseal (a xylene-based mounting medium) was obtained from Stephens Scientific (Kalamazoo, Mich., USA). Vectashield (a water-soluble mounting medium) was purchased from Vector Laboratories (Burlingame, Calif., USA). Monoclonal anti-CgA antibodies were as previously characterized (Proudman et al. 2003; Oubre et al. 2004). Rabbit anti-CpE C-terminal antibodies were as described in Dhanvantari et al. (2002). In the negative control, 1 ml anti-CpE antibody (1:2000) was pre-adsorbed with 10 μ g CpE C-terminal peptide. Rat anti-somatostatin antibody (Mab 354) was purchased from Chemicon/Millipore (Billerica, Mass., USA).

CgA/CpE and CpE/somatostatin immunofluorescent double-staining

A direct comparison between the CgA-immunoreactive (ir) and CpE-ir cell populations was achieved by immunofluorescent double-staining on the same tissue section. The detailed procedure was as described elsewhere (Oubre et al. 2004). Briefly, upon dewaxing and rehydration, the sections were incubated overnight simultaneously with both primary antibodies, i.e., monoclonal mouse anti-CgA (ascites fluid diluted 1:3000) and rabbit anti-CpE (1:1000). The next day, the sections were rinsed with TBST and incubated simultaneously with rhodamine-red-conjugated goat anti-mouse Ig (1:600) and biotinylated goat anti-rabbit Ig (1:500) for 90 min. Upon rinsing, the sections were finally incubated with FITC-conjugated streptavidin (3 μ g/ml TBST) for 30 min, rinsed, and coverslipped with Vectashield. A similar protocol was used for CpE and somatostatin immunofluorescent dual-staining, with primary antibodies were described above. Immunoreactive cells were observed with a Zeiss Axioplan Microscope and a Zeiss Axiophot Camera Module. Photomicrographs were taken with a Spot camera.

SDS-polyacrylamide gel electrophoresis and Western blot

Chicken thymic lobes, adrenal gland, and pituitary from a 6-week-old broiler were homogenized in 0.32 M sucrose buffered with 10 mM HEPES (pH 7.4) with a glass Dounce homogenizer. Ten percent (v/v) of a protease inhibitor cocktail (Sigma P2714) was added to the homogenate. All the reagents and samples were cooled on ice. Protein extracts were mixed with identical volumes of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer (Laemmli 1970). Equal amounts of proteins (12 μ g) were loaded onto a 8 $\times$ 8 cm 10% TRIS-HCl precast polyacrylamide gel (Bio-Rad, Hercules, Calif., USA). Odyssey protein weight markers were obtained from Li-Cor (Lincoln, Nebraska). The proteins were separated for 1 h at a constant voltage of 100 V. Immediately after separation, the proteins were electro-transferred onto a nitrocellulose membrane by using a NOVEX

blotting system at 0.8 mA per cm² for 1 h. The membrane was blocked with blocking buffer (Li-COR) and incubated with primary antibody (1:4000) for 5 h. Goat anti-rabbit IgG conjugated to Alexa Fluor 680 (1:5000, Invitrogen, Carlsbad, Calif., USA) was applied for signal detection. The membrane was scanned with an Odyssey Infrared Imaging System (Li-COR).

Results

Chicken CpE structure and synthesis

A chicken CpE cDNA contig of 2240 bp was assembled (data not shown). The positions of exons and introns were examined by aligning the cDNA contig with the chicken genomic sequence at <http://genome.ucsc.edu/cgi-bin/hgGateway>. The chicken CpE gene is located on chromosome 4 and consists of nine exons. Synthesis of CpE mRNA in the chicken thymus was established by RT-PCR (Fig. 1) and confirmed by sequencing of the resulting PCR product. The predicted chicken CpE protein (Fig. 2) consisted of 469 amino acids, where mouse and human have been reported to consist of 476 amino acids. The calculated theoretical pI and molecular weight of chicken CpE is 5.1 and 52.4 kDa, respectively. The cDNA and protein sequence of chicken CpE, and the secondary structure, are highly conserved. The C-terminal of CpE is believed to anchor the molecule to the membrane of the secretory granules, and its sequence is identical between species. The chicken CpE contains a Zn₂-pept domain starting at position 168 and ending at position 458. The Zn₂-pept domain (a Zn-binding catalytic domain of zinc metallopeptidases) is conserved among many other members of the carboxypeptidase protein family (SMART, <http://smart.embl.de/>).

CpE protein expression in chicken thymus

Western blotting—Chicken CpE was shown to be expressed in chicken thymus at the protein level by SDS-PAGE and Western blot (Fig. 3). A clear band with a molecular weight of approximately 53 kDa was observed in the total protein extract from chicken thymus in Western blots. Chicken pituitary and adrenal protein extracts were used as positive controls (see Fig. 3).

Immunocytochemical localization—To confirm further the expression of CpE in the chicken thymus, immunocytochemistry was carried out with the same antibody as that used for Western blot. Paraffin sections of chicken thymus and pituitary were stained with an anti-CpE C-terminal antibody (Fig. 4). In the thymus, cells displaying CpE-like immunoreactivity were located primarily on the cortico-medullary border, a region heavily innervated by the autonomous nervous system and numerous blood capillaries. Whereas the morphology of the CpE-ir cells was heterogeneous, most of the cells were round, although some had a star-like appearance with conspicuous extensions (Fig. 4c, arrows). Some cells were double-stained, expressing both CpE and CgA (Fig. 5c, arrows). No signal was detected on negative control slides (omission of primary antibody).

As a positive control, the pituitary showed specific typical cytoplasmic staining in groups of adjacent cells sharing an almost identical morphology reminiscent of pro-opiomelanocortin cells, although the phenotype of these cells in the pituitary was not determined in this experiment. However, both lobes contained CpE-positive cells (results not shown). We also found that, in the chicken thymus, some CpE-positive cells were dual-stained for somatostatin, a classic thymic neuroendocrine peptide (Fig. 6). In addition, CpE was expressed in chicken thymus of both young (8-day-old) and older (17-week-old) chickens and in broilers and laying hens (Fig. 7).

Discussion

In the regulated secretory pathway of neuroendocrine cells, neuropeptide precursors are transported from the endoplasmic reticulum, the site of synthesis, to the trans-Golgi network where they are sorted into the secretory granules (LDCVs) for processing and maturation (Brakch et al. 2002; Loh et al. 2004). The present study has aimed at assessing the coexistence of two classical markers of the regulated secretory pathway, CgA and CpE, in the diffuse neuroendocrine system in the thymus of the chicken. The immunohistochemical evidence presented in this investigation, combined with the identification of the chicken CpE mRNA by RT-PCR, has allowed us to demonstrate the co-existence of CgA and CpE in identical neuroendocrine cells in chicken thymus. To our knowledge, this has not been shown previously in any lymphoid tissue of any vertebrate species, including human thymus.

Recently, a predicted CpE mRNA of 2034 bp was added to the GenBank (XM_420392.1 GI:50746181) based on the chicken (jungle fowl) genome sequence. Compared with the nucleotide sequence that we assembled *in silico*, based on the publicly available chicken EST database, the ORFs of both sequences were almost identical but the EST-based sequence was about 200 bp longer in the 3' non-coding region. The chicken CpE gene consists of nine exons, as is also the case in humans. The predicted chicken CpE protein sequence consists of 469 amino acids and shows 87%, 87%, 88%, and 90% homology with rat, mouse, human, and bovine CpE, respectively. The C-terminal sequence of CpE, which serves as an anchor to the membrane of secretory granules, is perfectly conserved between birds and mammals. The major portion of the chicken CpE protein structure consists of a Zn₂pept domain (residues 168–458). This domain is conserved in many other members of the carboxypeptidase protein family in mammals (Rawlings and Barrett 1995). The highly conserved CpE sequence between species suggests a conserved function and distribution of this molecule. Preliminary RT-PCR experiments have demonstrated the expression of CpE mRNA in human thymus (data not shown).

Immunofluorescent dual-staining has clearly demonstrated the co-localization of CpE and CgA in chicken thymus. Importantly, this evidence suggests that, in the chicken thymus, some of the locally produced neuroendocrine molecules are processed and released through the regulated secretory pathway. This, in turn, implies that an extra-cellular specific stimulus is responsible for the release of neuropeptides under the proper physiological conditions. In addition, we have shown that some CpE-positive cells co-localize with somatostatin, a classic neuroendocrine peptide, in chicken thymus, indicating that CpE is involved in the maturation of pro-somatostatin (Fig. 6). Furthermore, we have also revealed that CpE is expressed in chicken thymus at both an early and an older age and in broilers and laying hens (Fig. 7), suggesting that CpE-positive neuroendocrine cells play a role in many different stages of the thymic microenvironment. The detailed functions of CpE, such as differential hormone sorting or regulated secretion in these cells of chicken thymus, need further investigation.

As previously described, the CpE-CgA double-positive cells are primarily found in a transition zone between the cortex and the medulla of the thymic lobules. This area is known to contain numerous arterioles and is heavily innervated by the autonomous nervous system (al-Shawaf et al. 1991). Therefore, we are tempted to speculate that the diffuse neuroendocrine system serves as a relay for nervous stimuli delivered by the sympathetic and/or parasympathetic nervous system and/or humoral factors delivered by the circulation, thereby allowing thymopoiesis to be fine-tuned by a variety of physical and environmental factors (Millington and Buckingham 1992).

References

- al-Shawaf AA, Kendall MD, Cowen T. Identification of neural profiles containing vasoactive intestinal polypeptide, acetylcholinesterase and catecholamines in the rat thymus. *J Anat.* 1991; 174:131–143. [PubMed: 1827784]
- Atoji Y, Yamamoto Y, Komatsu T, Suzuki Y. Localization of neuropeptides in endocrine cells of the chicken thymus. *J Vet Med Sci.* 1997; 59:601–603. [PubMed: 9271458]
- Bodey B. Neuroendocrine influence on thymic haematopoiesis via the reticulo-epithelial cellular network. *Expert Opin Ther Targets.* 2002; 6:57–72. [PubMed: 11901481]
- Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. The role of the reticulo-epithelial (RE) cell network in the immuno-neuroendocrine regulation of intrathymic lymphopoiesis. *Anticancer Res.* 2000; 20:1871–1888. [PubMed: 10928121]
- Brakch N, Allemandou F, Cavadas C, Grouzmann E, Brunner HR. Dibasic cleavage site is required for sorting to the regulated secretory pathway for both pro- and neuropeptide Y. *J Neurochem.* 2002; 81:1166–1175. [PubMed: 12068065]
- Dhanvantari S, Arnautova I, Snell CR, Steinbach PJ, Hammond K, Caputo GA, London E, Loh YP. Carboxypeptidase E, a prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion. *Biochemistry.* 2002; 41:52–60. [PubMed: 11772002]
- Franchini A, Ottaviani E. Immunoreactive POMC-derived peptides and cytokines in the chicken thymus and bursa of Fabricius microenvironments: age-related changes. *J Neuroendocrinol.* 1999; 11:685–692. [PubMed: 10447807]
- Gulati P, Tay SS, Leong SK. Nitroergic, peptidergic and substance P innervation of the chick thymus. *J Hirnforsch.* 1997; 38:553–564. [PubMed: 9476219]
- Gulati P, Leong S, Chan AS. Ontogeny of NADPH-d expression in the thymic microenvironment of the chick embryo. *Cell Tissue Res.* 1998; 294:335–343. [PubMed: 9799449]
- Hendy GN, Li T, Girard M, Feldstein RC, Mulay S, Desjardins R, Day R, Karaplis AC, Tremblay ML, Canaff L. Targeted ablation of the chromogranin a (Chga) gene: normal neuroendocrine dense-core secretory granules and increased expression of other granins. *Mol Endocrinol.* 2006; 20:1935–1947. [PubMed: 16556729]
- Hook V, Yasothornsrikul S, Greenbaum D, Medzihradszky KF, Troutner K, Toneff T, Bunday R, Logrinova A, Reinheckel T, Peters C, Bogoy M. Cathepsin L and Arg/Lys aminopeptidase: a distinct prohormone processing pathway for the biosynthesis of peptide neurotransmitters and hormones. *Biol Chem.* 2004; 385:473–480. [PubMed: 15255178]
- Kim T, Loh YP. Protease nexin-1 promotes secretory granule biogenesis by preventing granule protein degradation. *Mol Biol Cell.* 2006; 17:789–798. [PubMed: 16319172]
- Kyewski RB, Klein L. Making central T-cell tolerance efficient: thymic stromal cells sample distinct self-antigen pools. *Curr Top Microbiol Immunol.* 2000; 251:139–145. [PubMed: 11036769]
- Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Chromogranin A, an “on/off” switch controlling dense-core secretory granule biogenesis. *Cell.* 2001; 106:499–509. [PubMed: 11525735]
- Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Large dense-core secretory granule biogenesis is under the control of chromogranin A in neuroendocrine cells. *Ann N Y Acad Sci.* 2002; 971:323–331. [PubMed: 12438143]
- Kim T, Zhang CF, Sun Z, Wu H, Loh YP. Chromogranin A deficiency in transgenic mice leads to aberrant chromaffin granule biogenesis. *J Neurosci.* 2005; 25:6958–6961. [PubMed: 16049171]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
- Lamberts S. The thymus: at the interface between immunology and neuroendocrinology. *Ann Med.* 1999; 31(Suppl 2):3–4. [PubMed: 10574148]
- Loh YP, Kim T, Rodriguez YM, Cawley NX. Secretory granule biogenesis and neuropeptide sorting to the regulated secretory pathway in neuroendocrine cells. *J Mol Neurosci.* 2004; 22:63–71. [PubMed: 14742911]
- Mahapatra NR, O'Connor DT, Vaingankar SM, Hikim AP, Mahata M, Ray S, Staite E, Wu H, Gu Y, Dalton N, Kennedy BP, Ziegler MG, Ross J, Mahata SK. Hypertension from targeted ablation of

- chromogranin A can be rescued by the human ortholog. *J Clin Invest*. 2005; 115:1942–1952. [PubMed: 16007257]
- Millington G, Buckingham JC. Thymic peptides and neuroendocrine-immune communication. *J Endocrinol*. 1992; 133:163–168. [PubMed: 1613418]
- Mukamoto M, Kodama H. Regulation of early chicken thymocyte proliferation by transforming growth factor-beta from thymic stromal cells and thymocytes. *Vet Immunol Immunopathol*. 2000; 77:121–132. [PubMed: 11068070]
- Mukamoto M, Kodama H, Baba T. Effects of cytokines from thymocytes and thymic stromal cells on chicken intrathymic T cell development. *Vet Immunol Immunopathol*. 1999; 67:223–233. [PubMed: 10195461]
- Ottaviani E, Franchini A, Franceschi C. Evolution of neuroendocrine thymus: studies on POMC-derived peptides, cytokines and apoptosis in lower and higher vertebrates. *J Neuroimmunol*. 1997; 72:67–74. [PubMed: 9003246]
- Oubre CM, Zhang X, Clements KE, Porter TE, Berghman LR. Immunohistochemical assessment of the neurosecretory cells of the chicken thymus using a novel monoclonal antibody against avian chromogranin A. *Dev Comp Immunol*. 2004; 28:337–345. [PubMed: 14698219]
- Palmer E. Negative selection? Clearing out the bad apples from the T-cell repertoire? *Nat Rev Immunol*. 2003; 3:383–391. [PubMed: 12766760]
- Proudman JA, Clerens S, van den Bergh G, Garrett WM, Verhaert PD, Vandesande F, Berghman LR. Immunohistochemical localization of chromogranin A in gonadotrophs and somatotrophs of the turkey and chicken pituitary. *Gen Comp Endocrinol*. 2003; 132:293–303. [PubMed: 12812778]
- Rawlings ND, Barrett AJ. Evolutionary families of metallopeptidases. *Methods Enzymol*. 1995; 248:183–228. [PubMed: 7674922]
- Silva AB, Aw D, Palmer DB. Evolutionary conservation of neuropeptide expression in the thymus of different species. *Immunology*. 2006; 118:131–140. [PubMed: 16630030]
- Solomou K, Ritter MA, Palmer DB. Somatostatin is expressed in the murine thymus and enhances thymocyte development. *Eur J Immunol*. 2002; 32:1550–1559. [PubMed: 12115637]
- Zhang CF, Snell CR, Loh YP. Identification of a novel prohormone sorting signal-binding site on carboxypeptidase E, a regulated secretory pathway-sorting receptor. *Mol Endocrinol*. 1999; 13:527–536. [PubMed: 10194759]

Fig. 1. Agarose gel electrophoresis of CpE RT-PCR product. The specific amplicon with the expected size of 550 bp was sequenced and was shown to be 98% identical with the predicted cDNA sequence

[illegible]

Fig. 2. Predicted chicken CpE protein sequence aligned with the human (gi:6429043) and mouse (gi:22203763) CpE sequence. The amino acid sequence of CpE appears to be highly conserved among chicken, mouse, and human (*asterisks* residues in this column are identical in all sequences in the alignment, *filled circle* semi-conserved substitutions)

Fig. 3. Western blot identification of CpE (*lane 1* protein standards, *lane 2* pituitary, *lane 3* thymus, *lane 4* adrenal). Equal amounts of total protein extracts (12 μ g per lane) from chicken adrenal, thymus, and pituitary were separated by SDS-PAGE on a 10% TRIS-HCl precast polyacrylamide gel, blotted onto a nitrocellulose membrane, and analyzed with a rabbit polyclonal antiserum (1:4000) against a synthetic C-terminal peptide of CpE. Goat anti-rabbit IgG antibody conjugated to Alexa Fluor 680 was used as the detection reagent. CpE was expressed in chicken thymus and in pituitary and adrenal (positive controls)

Fig. 4.

Six-week-old chicken pituitary (**a**) and thymus (**b**, **c**) was stained with rabbit anti-CpE antiserum. In the pituitary, typical cytoplasmic staining was readily detectable in groups of adjacent cells sharing an almost identical morphology reminiscent of pro-opiomelanocortin cells (**a**). In thymus, CpE-immunoreactive (CpE-ir) cells (**b**, **c**) were located primarily on the cortico-medullary border (*arrows* in **c** cells with a star-like appearance with conspicuous extensions). Bars 25 μm (**a**, **c**), 50 μm (**b**)

Fig. 5. Immunofluorescent CgA and CpE double-staining. Thymic cells were stained with a monoclonal antibody against CgA (*red*, rhodamine-labeled) and a rabbit polyclonal antiserum against a synthetic C-terminal peptide of CpE (*green*, FITC-labeled). **a** Single red channel (*arrow* single-stained red CgA-positive cell). **b** Single green channel. **c** Composite image. Double-stained cells were readily observed (*arrows* dual-stained cells). Bars 10 μm

Fig. 6. Thymic cells stained by immunofluorescence with a rat monoclonal antibody against somatostatin and a rabbit polyclonal antibody against CpE (C-terminal). Cells labeled *red* are somatostatin-ir cells (**b, e**), and the cells labeled *green* are CpE-positive cells (**a, d**). Double-stained cells in the overlay images (**c, f**) show that some CpE-positive cells in chicken thymus produce somatostatin, a classic neuropeptide. Bars 50 μm (**a-c**), 15 μm (**d-f**)

Fig. 7. Immunocytochemical detection of CpE in the thymus of broiler and laying hens. **a** Broiler (6 weeks). **b** Laying hens (17 weeks). **c** Eight-day-old chicken. **d** Negative control (primary antibody was preadsorbed with excess antigen). Bars 25 μ m