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Pancreatic Hormone Expression in the Murine Thymus: Localization in Dendritic Cells and Macrophages

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ABSTRACT

The expression of preproinsulin (ppIns), proglucagon, prosomatostatin, and propancreatic polypeptide was investigated in thymic extracts, thymic cells, and thymic cell lines from C57BL/6 mice by RT-PCR. The expression of pancreatic hormones was similar in thymic extracts taken from neonatal and 2-, 4-, and 8-week-old animals, but was decreased in 20-week-old animals. Pancreatic hormone expression was not observed in mouse liver, salivary gland, or spleen. Analysis of thymic cell populations revealed a 10- to 20-fold enrichment in expression of all hormones in low buoyant density cells. No expression was detected in high buoyant density cells (predominantly thymocytes) or in thymic epithelial cell lines, primary cultures of epithelial cells, or peripheral macrophages. In addition, immunoreactive insulin, measured by specific RIA, was detectable in the low buoyant density population, but not in high buoyant density cells. The enriched cell population was depleted of contaminating lymphocytes and sorted based on reactivity to the cell surface markers F4/80 (macrophage) or N418 (dendritic cells). Cells gated for N418 demonstrated expression for ppIns, but not the other pancreatic hormones. Conversely, expression for proglucagon, prosomatostatin, and propancreatic polypeptide, but not ppIns, was detected in F4/80-gated cells. Our data indicate that pancreatic endocrine hormones are differentially expressed by dendritic cells and macrophages in a normal mice. (*Endocrinology* **139:** 2399–2406, 1998)

THE THYMUS, as a primary lymphoid organ, is responsible for promoting the proliferation and differentiation of a specific T cell repertoire through which the immune system is able to distinguish self antigen from invading pathogens and mutagens (1). To facilitate this activity, the thymus depends on both cell-cell contact between surface ligands such as the major histocompatibility complex and the T cell receptor, and humoral interaction (2, 3). In addition to the many classical cytokines that exert their influence in an autocrine or paracrine manner, the thymus produces a number of unique humoral factors and many neuroendocrine and peripheral hormones (4). Some of these, such as PRL and GH, have well characterized effects on thymocyte differentiation and proliferation (5). Others, such as atrial natriuretic peptide and β-endorphin, have been identified but have not been assigned a defined function within the thymus (6, 7).

The endocrine pancreatic hormones are a group of four polypeptides produced by different cell types that together constitute the islets of Langerhans (8). Insulin, which is synthesized by β-cells, maintains metabolic homeostasis by regulating cellular uptake of glucose. Glucagon, somatostatin, and pancreatic polypeptide, each synthesized by a distinct cell type, modulate insulin secretion, among other functions (8). A striking feature of insulin expression is its almost complete restriction to β-cells of the islet in normal mammals (9). Apart from the β-cell, insulin has only been detected in the fetal yolk sac and transiently in the fetal brain (10, 11). It is for this reason that the rat insulin promoter is used in many transgenic models to direct site-specific synthesis of transgenes (12–17). Surprisingly, in several transgenic models specifically designed to investigate mechanisms of peripheral tolerance, a very low expression of the constructs was detected in the thymus, suggesting that the insulin promoter is active outside the pancreas (12, 17). Further analysis with RT-PCR for several pancreatic endocrine and exocrine hormones revealed low, but detectable, expression for all but carboxypeptidase A and amylase (12). More recently, in humans, low preproinsulin (ppIns) expression has been reported in both fetal and infant thymuses (18, 19). Here we confirm the murine thymus as a site of pancreatic hormone synthesis and identify both dendritic cells and macrophages as capable of their expression.

Materials and Methods

Animals

Male and female C57BL/6 mice were bred under specific pathogen-free conditions in our own facility. They were fed standard food pellets and water *ad libitum* and kept on a 12-h light, 12-h dark cycle. The animal facilities and care followed the norms stipulated by the European Community for the care and use of laboratory animals.

Monoclonal antibodies (mAbs)

The mAbs used for depletion were anti-CD4 (clone GK1.5), anti-CD8 (clone 53.6.7), anti-B220 (clone RA3-6B2), anti-Mac-1 (clone M1/70), and anti-Gr1 (clone RB6-8C5). For immunofluorescent staining and sorting the mAbs were anti-Thy-1.2 biotinylated (clone 30H-12; pan-thymocyte marker) (20), anti-CD11c (clone N418; dendritic cell marker) (21), and anti-F4/80 (pan-macrophage marker) (22); all were fluorescein isothiocyanate conjugated. Biotinylated Abs were labeled with streptavidin-

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conjugated phycoerythrin (Caltag Laboratories, South San Francisco, CA).

Isolation of low buoyant density cells

To enrich the stromal elements of the thymus, a previously described density cut separation procedure was used with few modifications (23, 24). Media were adjusted to be isoosmotic with mouse serum. Metrizamide (grade II; Sigma Chemical Co., Saint Quentin Fallavier, France) was prepared at a concentration of 0.308 M and diluted to a density of 1.071 g/cm^3 with PBS-EDTA as previously described (23).

Thymuses were removed from 2-week-old C57BL/6 mice and washed three times in PBS. Tissues were cut into very small pieces with sharp scissors and digested in 7.5 ml isoosmotic RPMI 1640 supplemented with 25 mM HEPES, 2% FCS, 1 mg/ml collagenase, and 20 ng/ml deoxyribonuclease (DNase) for 25 min at room with agitation. EDTA (0.099 M) was added to the digest, and the incubation was continued for 5 min. Undigested fragments were removed by passage through gauze. Cold FCS, supplemented with 0.099 M EDTA, was layered underneath the digest, and cells were recovered by centrifugation at 500 × *g* for 7 min. Supernatant was removed, and the cell pellet was dispersed in 7 ml metrizamide by vortexing. Metrizamide (5 ml) was layered underneath, and PBS supplemented with 5 mM EDTA was layered on top; cells were centrifuged at 1700 × *g* for 10 min. Buoyant density cells were taken from the upper interface, diluted 5-fold with PBS supplemented with 5% FCS-EDTA and 5 mM EDTA (PBS-FCS-EDTA), and centrifuged. Pelleted cells were diluted and recovered for comparison. Both cell suspensions were resuspended in PBS-FCS-EDTA on ice for cell counting and determination of viability. Occasionally, mechanically dispersed cells were used in place of enzyme-digested preparations for metrizamide separation.

Immunomagnetic bead depletion

To remove contaminating thymocytes, B cells, and granulocytes, low buoyant density cells were subjected to depletion by incubation with rat mAbs then antirat mAb-conjugated magnetic beads. Cell suspensions were incubated with a mixture of pretitrated rat mAbs for 20 min at 4 C; the mixture typically contained antibodies against CD4 (T cells), B220 (B cells), and Gr1 (granulocytes). In some experiments, a carefully titrated amount of Mac-1 antiserum was added to cells destined for sorting by N418 (dendritic), or alternatively, anti-CD8 antiserum was added to cells sorted by F4/80 (macrophages). After washing, the cells were incubated as a concentrated slurry with antirat Ig-coated magnetic beads at a ratio of 3:1 beads/cell, gently rotating the mixture for 15 min at 4 C. The slurry was diluted, and coated cells were removed by applying a Dynal magnet twice (Dynal Corp., Chantilly, VA). The depleted population was either extracted directly or stained for sorting.

Immunofluorescent staining and flow cytometry

Cell preparations were preincubated with an Fc receptor antiserum at 4 C for 20 min to diminish nonspecific binding. Then they were incubated with a fluorescein-conjugated mAb against F4/80, a hamster mAb against CD11c, or a biotinylated mAb against Thy-1.2 using PBS-FCS-EDTA as a diluent for 30 min at 4 C. Biotinylated Abs were labeled with streptavidin-conjugated phycoerythrin. Sorting was performed with a FACS Vantage cell sorter, and single parameter analysis was performed with a FACScan (Becton Dickinson Co., Mountain View, CA). Forward light scatter gates were set to exclude dead cells and debris.

Messenger RNA (mRNA) isolation

Total RNA was isolated from mouse tissues and cell preparations using a proprietary modification of the single step extraction method described by Chomczynski and Sacchi (25). Briefly, animals were rapidly killed by cervical dislocation, and tissues were removed as quickly as possible and placed in 1 ml Trizol (Life Technologies, Cergy Pontoise, France)/100 mg tissue. Tissues were homogenized for 0.5–1.0 min with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Cells in suspension were spun down, resuspended in 1 ml Trizol/1 × 10^6 cells, and lysed by passage through a 21-gauge needle. Adherent cells were lysed directly on the plate. After separation in the presence of chloroform, the aqueous phase was precipitated with an equal volume of isopropyl alcohol. Samples were resuspended, treated with proteinase K (100 μg/ml) for 15 min at 50 C in the presence of 1% SDS and 10 mM EDTA, phenol/chloroform extracted, and further subjected to DNase treatment to remove contaminating genomic DNA. Digestion was carried out with ribonuclease-free RQ1 DNase I (50 U/ml; Promega, Charbonnieres, France) for 30 min at 37 C in Tris-EDTA, pH 8.0, containing 10 mM $MgCl_2$, 5 mM dithiothreitol, and 500 U/ml RNasin (Promega). After a final phenol/chloroform extraction, samples were resuspended in sterile ribonuclease-free water at a concentration of 1 μg/μl after the addition of RNasin (500 U/ml). The integrity of RNA was assessed by inspection of 28S and 18S band intensities after agarose gel electrophoresis. Special care was taken not to cross-contaminate dissection and homogenization equipment; before handling each tissue, both were washed with 0.2 M NaOH, sterile water (three times), and alcohol.

RT-PCR analysis

RT was performed on 3 μg total RNA in the presence of 1 mM deoxy (d)-NTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$, 500 U/ml RNasin, 500 U/ml avian reverse transcriptase (AMV; Promega), and 10 mM random hexamer primers. Tubes were incubated for 10 min at 25 C and for 90 min at 42 C, and the reaction was terminated with a 5-min incubation at 95 C followed by cooling on ice. Each reaction was diluted 5-fold and stored at −20 C before PCR amplification.

For semiquantitative PCR, a series of 4-fold dilutions of complementary DNA (cDNA) was prepared in distilled H_2O and amplified in 50-μl reactions using 10 μl of the diluted cDNA as template mixed with 40 μl PCR mix [20 mM Tris-HCl (pH 8.4); 50 mM KCl; 1.0 mM $MgCl_2$; 200 μM dATP, dCTP, dGTP, and dTTP (Pharmacia, Saint-Quentin-Yvelines, France); 1 μM of each primer; and 1.5 U *Taq* DNA polymerase (Life Technologies); all given as the final concentration]. Mineral oil (50 μl) was added to each tube, and amplification was carried out under standard thermal cycling conditions; a single denaturing step at 94 C/2 min was followed by the chosen number of cycles of the profile 94 C for 45 sec, 60 C for 45 sec, 72 C for 1 min, and a final extension step at 72 C for 7 min. The primers for the pancreatic genes (26), actin (27), and β_2-microglobulin (β_2m) (28) have been described previously. Cycle numbers used for each primer pair were adjusted to ensure linear amplification. Reaction products were separated on 0.5 × TBE (0.13 M Tris base, 80 mM boric acid, and 0.25 mM EDTA)-1.8% agarose gels containing 100 ng/ml ethidium bromide. PCR products were visualized under short wave UV light, captured with a CCD camera (Ikegami Tsushinki, Tokyo, Japan, and digitally printed with a video copy processor (Mitsubishi, Tokyo, Japan). For analysis, photographs were scanned at high resolution, and the integrated density of the bands was calculated using Scan Analysis (Biosoft, Cambridge, UK).

For autoradiography, gels were denatured and transferred to positively charged nylon membranes under vacuum for 2 h. The nucleic acid was stabilized on the membrane by UV cross-linking and immersed in prehybridization buffer [6 × SSC, 10 mM Na_2PO_4 (pH 6.8), 1 mM EDTA (pH 8), 0.5% SDS, 100 μg/ml single stranded DNA, and 0.1% nonfat dried milk] for 1 h at 68 C. A 30-bp oligonucleotide (CAGCAAGCAGGTTATTGTTTCAACATGGCC), specific for the insulin PCR product and spanning the first intron of the insulin gene (to exclude genomic DNA contamination), was labeled at the 5′-end with [γ-^{32}P]ATP by T4 polynuclease kinase. Unincorporated nucleotide was removed by alcohol precipitation. Thirty nanograms per ml probe were added to the prehybridization buffer, and the hybridization was continued overnight. The gel was washed in 5 × SSC at room temperature for 10 min, at 42 C for 10 min, at 47 C for 10 min, and at 52 C for 10 min. The nylon membrane was then exposed to autoradiographic film at −70 C.

RIA

Immunoreactive (ir-) insulin was assayed by a Bi-Insulin RIA kit (ERIA Diagnostics Pasteur, Paris, France) using rat insulin (Novo Nordisk, France) as standard. Thymic cells separated by density gradient were washed twice in PBS and resuspended in 1 M acetic acid and 100 μg/ml PMSF (Sigma). Cells were sonicated immediately and then centrifuged for 30 min at 16,000 × *g*. The supernatant was recovered, frozen, and lyophilized. Samples and standards were resuspended for assay in insulin-depleted human serum by activated charcoal filtration. The

detection limit of the assay was 15 pmol/liter, and the molar cross-reactivity with proinsulin was approximately 60%.

Statistical analysis

The ratio of pancreatic hormone expression to actin expression (as determined from OD measurements of ethidium bromide-stained gels) is expressed as the mean ± SEM of three separate experiments. Data were analyzed by ANOVA, followed by Duncan's multiple range test for individual differences with a $P < 0.05$ or $P < 0.01$ level of significance.

Results

Tissue-specific gene expression in the thymus

A RT-PCR assay was developed to compare the mRNA expression of ppIns, proglucagon (pGlu), prosomatostatin (pSom), and propancreatic polypeptide (pPP) in the thymus to levels expressed in the pancreas (Fig. 1). In 2-week-old animals, thymic expression of ppIns mRNA was approximately 10,000 times less than that measured in the pancreas (Fig. 2a). The magnitude of difference between thymic and pancreatic pGlu and pSom was 1000-fold (Fig. 1, b and c, respectively) and was between 50- and 100-fold for pPP mRNA (Fig. 1d). No specific band for ppIns mRNA or the other hormones was detected in the brain, salivary gland, liver, or spleen, except for pSom expression in the brain. No band was observed for any sample when reverse transcriptase was omitted (data not shown).

The expression of pancreatic hormones in the thymus was investigated over the developmental period of the animal and in relation to gender. Total RNA extracts from three individual animals were analyzed for all four hormones at 1–2 days (NN) and 2, 4, 8, and 20 weeks of age (Table 1). In thymic samples, amplification was linear for all hormones at 32 cycles and for actin, after a 100-fold dilution, at 22 cycles (see Fig. 1f). Hormone expression was compared as a ratio of actin expression in the same sample to correct for variations in RNA concentration. The expression of ppIns, pGlu, pSom, and pPP was not significantly different in females from 2–8 weeks. However, the expression of all hormones, except pPP, was significantly decreased at 20 weeks of age; ppIns expression was the most notably diminished. No difference in the expression of pancreatic hormones was observed between male and female mice at 2 weeks of age (Table 1).

Expression of pancreatic hormones in thymic cell populations

We investigated hormone expression in different thymic cell populations using several methods. Initially, cells were separated based on adherence. Using this method, the signal was only detectable when more than 5×10^7 nonadherent cells were extracted. To separate different populations of cells from the overwhelming majority of thymocytes, we used density gradient separation. In this method, cells from collagenase-digested tissue were passed through a medium of defined density to separate the more buoyant dendritic cells and macrophages from the denser lymphocytes (23, 24). After separation, macrophages and dendritic cells were enriched 10- to 20-fold, as assessed by flow cytometry, but few macrophages or dendritic cells were detected in cells that passed through the medium (data not shown). Total RNA was extracted from 1×10^7 cells of each population and analyzed for pancreatic hormone expression by RT-PCR as described above. Expression of ppIns mRNA was not detected in cells that were of sufficient density to pass through the medium (Fig. 2b); however, considerable enrichment in ppIns mRNA expression was observed in buoyant density cells (Fig. 2a). The expression of β_2m mRNA, included as a control, was also slightly enriched in the buoyant density fraction (Fig. 2, a and b). The same phenomenon was observed with actin mRNA expression, although the same number of cells was extracted in each group, as determined

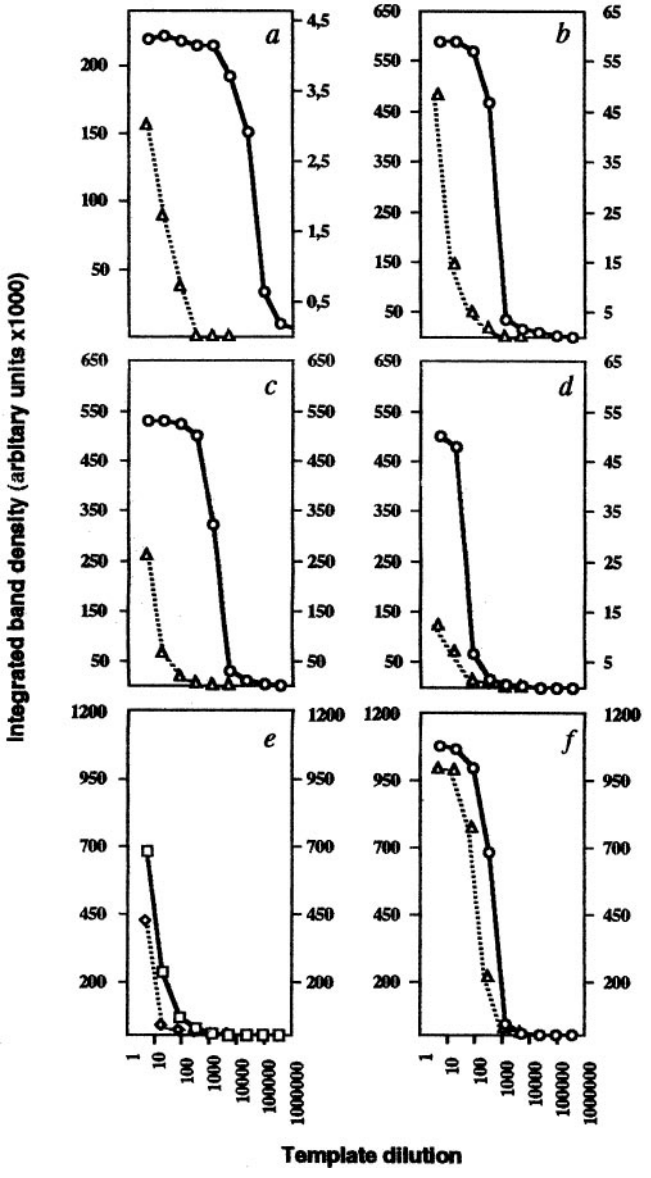

FIG. 1. Comparison of pancreatic and thymic expression of pancreatic endocrine hormones at 2 weeks of age in C57BL/6 mice. Total RNA (3 μg) was subjected to RT-PCR (32 cycles) for ppIns (a), pGlu (b), pSom (c), and pPP (d). After electrophoresis of reaction products in the presence of ethidium bromide, band density was measured under UV light for each hormone and the ubiquitously expressed controls, β_2m (e; 25 cycles of PCR) and actin (f; 22 cycles of PCR). The *left y*-axis of each graph represents the integrated density of the PCR product in the pancreas (○); the *right y*-axis represents the thymus (△), both in arbitrary units.

TABLE 1. Thymic expression of pancreatic hormones measured over the developmental period of the mouse

Age (weeks)	Sex	Preproinsulin	Proglucagon	Prosomatostatin	Propancreatic polypeptide
Neonatal	Female	1.28 ± 0.44	1.16 ± 0.47	1.37 ± 0.30	1.11 ± 0.41
2	Female	1.19 ± 0.44	1.23 ± 0.30	2.04 ± 0.40	0.64 ± 0.28
4	Female	0.93 ± 0.41	1.17 ± 0.24	1.54 ± 0.46	0.61 ± 0.23
8	Female	1.25 ± 0.29	1.46 ± 0.19	1.10 ± 0.35	0.94 ± 0.04
20	Female	0.37 ± 0.19[a]	0.89 ± 0.22[a]	0.64 ± 0.15[a]	0.75 ± 0.14
2	Male	1.33 ± 0.32	1.11 ± 0.24	1.68 ± 0.43	1.02 ± 0.19

Results are expressed as the ratio of hormone expression over actin expression from the same cDNA sample. Each value represents the mean of three determinations ± SEM.

[a] Significant difference between 2 and 20 week levels ($P < 0.05$). Significant difference between 8 and 20 week levels ($P < 0.05$).

by optical density measurements and visual inspection of ethidium bromide-stained agarose gels (data not shown).

The identity of the amplified product from the enriched population of buoyant density cells was confirmed by restriction digest with the endonuclease *Ava*I that produced the expected band at 272 bp (Fig. 2c, lane 1). Note that in the control lane two bands were observed (lane 2, *white arrows*); the primers for insulin were degenerate for the two insulin genes found in the mouse, generating products of 348 and 355 bp for the insulin I gene and the insulin II gene, respectively. The presence of the two bands, visible here due to the greater gel resolution of PAGE, suggests that both genes are transcribed in the thymus.

The same total RNA extracts were amplified for pGlu, pSom, pPP, and ppIns with a different degenerate primer pair (Fig. 3). Expression was detectable for each hormone even at the highest dilution of cDNA. No band was detected for any hormone in RT-PCR samples that had been processed with the omission of RT (data not shown).

In addition to the expression of the ppIns mRNA, ir-insulin was detected in the low buoyant density fraction of cells at a concentration of 35–25 fmol/1 $\times$ 10^7 cells in three separate experiments. However, no ir-insulin was detected in the high buoyant density fraction or dispersed thymic cells before separation, consistent with the findings for mRNA expression,

Pancreatic hormone expression in cells purified by sorting

To further define the identity of hormone-expressing cells, the enriched cell population was depleted of residual lymphocytes using antibodies against cell surface markers, CD4 (T cells), B220 (B cells), and Gr1 (granulocytes), indirectly conjugated with magnetic beads. Two populations were prepared, one that also included an antibody for CD8, which is expressed on mouse thymic dendritic cells and a subpopulation of T cells, but not on macrophages, and one with an antibody against Mac-1, which is strongly expressed on macrophages. Cells from the first group (depleted of dendritic cells) were separated based on reactivity to the cell surface marker F4/80 (pan macrophage marker), and cells from the second group (depleted of macrophages) were separated with CD11c (dendritic cell marker). After cell sorting, serial dilutions of the cells from each gate were extracted immediately for total RNA as described in *Materials and Methods*. The experiment was repeated three times; a representative experiment is presented in Fig. 4.

RT-PCR amplification of total RNA extracts for the four pancreatic hormones revealed expression for pGlu, pSom, and pPP (Fig. 4) in as few as 1.5 $\times$ 10^4 cells gated for the macrophage marker F4/80 (lanes 6–9). It was not possible to quantify directly the level of total RNA from cell extracts; however, the level of β_2m mRNA expression was consistent with cell number and dilution. Note also that although the relative levels of expression were different between hormones, staining in the F4/80 gate was comparable in all cases with staining of the hormone PCR product generated from 0.1 μg total RNA from age-matched thymic extracts processed at the same time (lanes 1–4).

Expression for ppIns was not detected in the F4/80 gates after ethidium bromide staining; however, a faint band was present in cells gated for N418. The gel was transferred to a membrane and hybridized with a 30-bp oligonucleotide specific for an internal sequence in the PCR product. The resulting autoradiography clearly shows ppIns expression in 3 $\times$ 10^4 cells sorted for the dendritic cell marker N418 (lane 11). The relative expression of ppIns compared with thymic extracts appears to be 10-fold higher than that of the other hormones; however, autoradiography is more sensitive and is probably more quantitative than ethidium bromide staining; thus, the relative expression of the other hormones may be underestimated. Faint bands were also observed in lanes representing 1.5 $\times$ 10^5 cells before and after sorting for macrophages (lanes 5 and 6, respectively); however, in contrast to cells depleted of macrophages and sorted for N418 (lanes 10 and 11, respectively), there was no enrichment in expression after sorting for F4/80. Likewise, a faint band for pSom and pPP was observed in N418-gated cells. The significance of this expression is unclear; however, no expression for any pancreatic endocrine hormone was observed in 2 $\times$ 10^5 cells gated for cells expressing high levels of Thy-1.2 (lane 17), an F4/80 negative gate (lane 18), or a serial dilution of viable N418-negative cells (lanes 14–16). As a further control, the expression of insulin (Fig. 5) and the other pancreatic hormones was tested in five well characterized cortical (1.4C18 and 1.2C1) and medullary (2.3, 3.10, and 1C6) thymic epithelial cell lines (29), in cells from deoxyguanosine-treated thymic explants, which consist primarily of epithelial cells and some fibroblasts, and in thioglycolate-elicited peritoneal macrophages, and splenic macrophages, no expression for any of the pancreatic hormones was detected by RT-PCR.

Discussion

In addition to specific thymic hormones, such as thymulin, that are synthesized exclusively by epithelial cells, the expression of several hormones has been reported in the normal thymus, such as the pituitary hormones, PRL, GH, oxy-

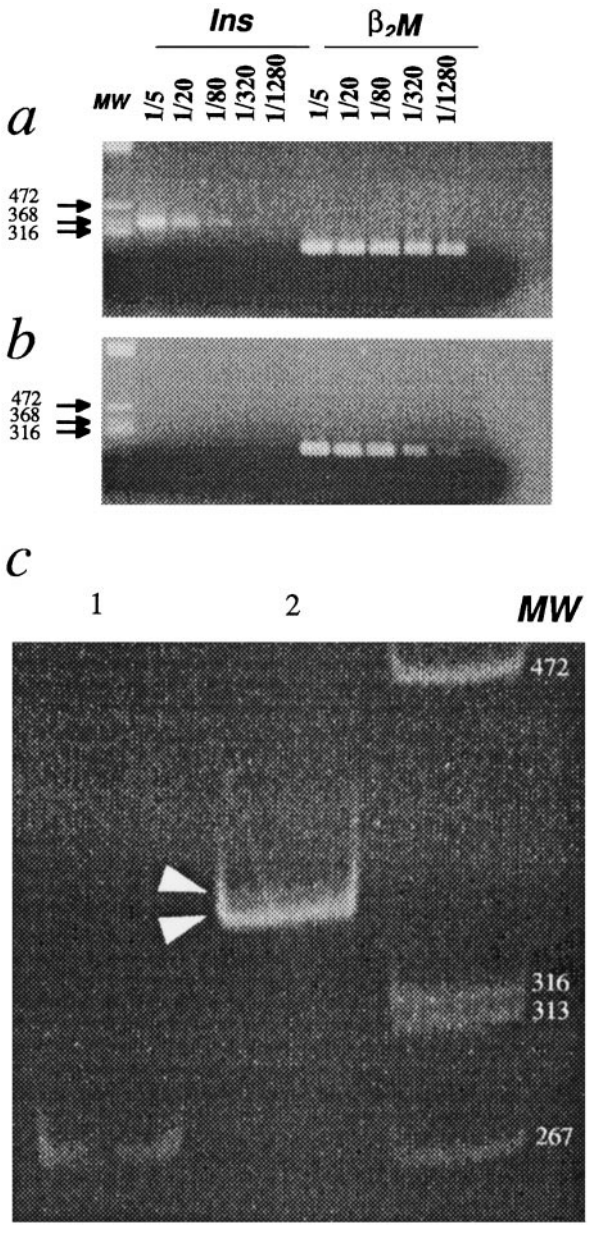

FIG. 2. Thymic expression of ppIns mRNA in fractions of thymic cells separated by density gradient centrifugation. Total RNA from 1×10^7 cells of the low buoyant density cell population (a) and the high density cell population (b) were reverse transcribed, serially diluted, and subjected to 32 cycles of PCR for ppIns (product size, 348 bp for InsI and 355 bp InsII) and β_2m (25 cycles; product size, 219 bp). After electrophoresis, reaction products were visualized with ethidium bromide under UV light; the product size of the molecular weight marker (MW) is indicated on the *left*. c, The amplified product was subjected to restriction analysis with *Ava*I for 60 min, then was separated on an 8% polyacrylamide gel. Lane 1, Amplified product digested with *Ava*I; a band is present with the predicted size of 272 bp. Lane 2, Amplified product without digestion; note the presence of two distinct bands (*white arrowheads*). The mol wt with DNA size, represented in base pairs, are indicated on the *right*.

tocin, and arginine vasopressin (4). In most cases, these hormones have been demonstrated to modulate immunological function. Here, we present evidence that another group of hormones, the pancreatic endocrine hormones, are differentially expressed by thymic cell populations.

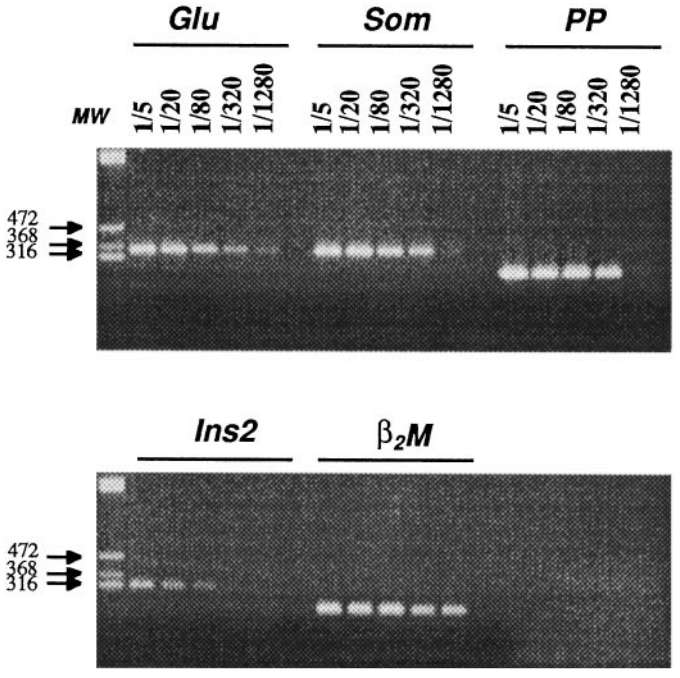

FIG. 3. Thymic expression of pGlu (Glu), pSom (Som), pPP (PP), ppIns (alternative set of primers; Ins2), and β_2m (β_2M) in fractions of thymic cells separated by density gradient centrifugation. Total RNA from 1×10^7 cells of the buoyant density cell population was reverse transcribed in the presence of RT, serially diluted, and subjected to PCR for 32 cycles (25 cycles for β_2m). After electrophoresis, reaction products were visualized with ethidium bromide under UV light. The product size of the mol wt marker (MW) is indicated on the *left*.

The level of ppIns expression measured in the thymus is between 4–5 orders of magnitude lower than that in the pancreas. This makes it highly unlikely that insulin of thymic origin would influence the circulating level of the hormone. Like ppIns, there are several orders of magnitude difference in the expression of pGlu and pSom, but the level of pPP is far closer to that of pancreatic expression. This probably reflects the small number of cells positive for the pancreatic polypeptide in the pancreas (<1%) (8).

In contrast to previous reports (12) we did not observe in the murine thymus a difference in pancreatic endocrine hormone expression with age in young animals. However, at 20 weeks of age, expression of all hormones was diminished. This was particularly true of insulin, and may result from thymic involution that occurs with aging and involves a reduction in the number of bone marrow-derived cells in the tissue.

Expression of all transcripts was enriched in a low buoyant density fraction of thymic cells, in which macrophages and dendritic cells are concentrated from the overwhelming majority of thymocytes and in which epithelial cells were not included. However, from this population of cells, only those selected by FACS sorting for reactivity to the cell surface marker N418 demonstrated expression of ppIns. The expression was observed in 3×10^4 cells, which represents less than 0.05% of the total thymic population, demonstrating a significant enrichment of transcript expression, but still below what might be expected if all

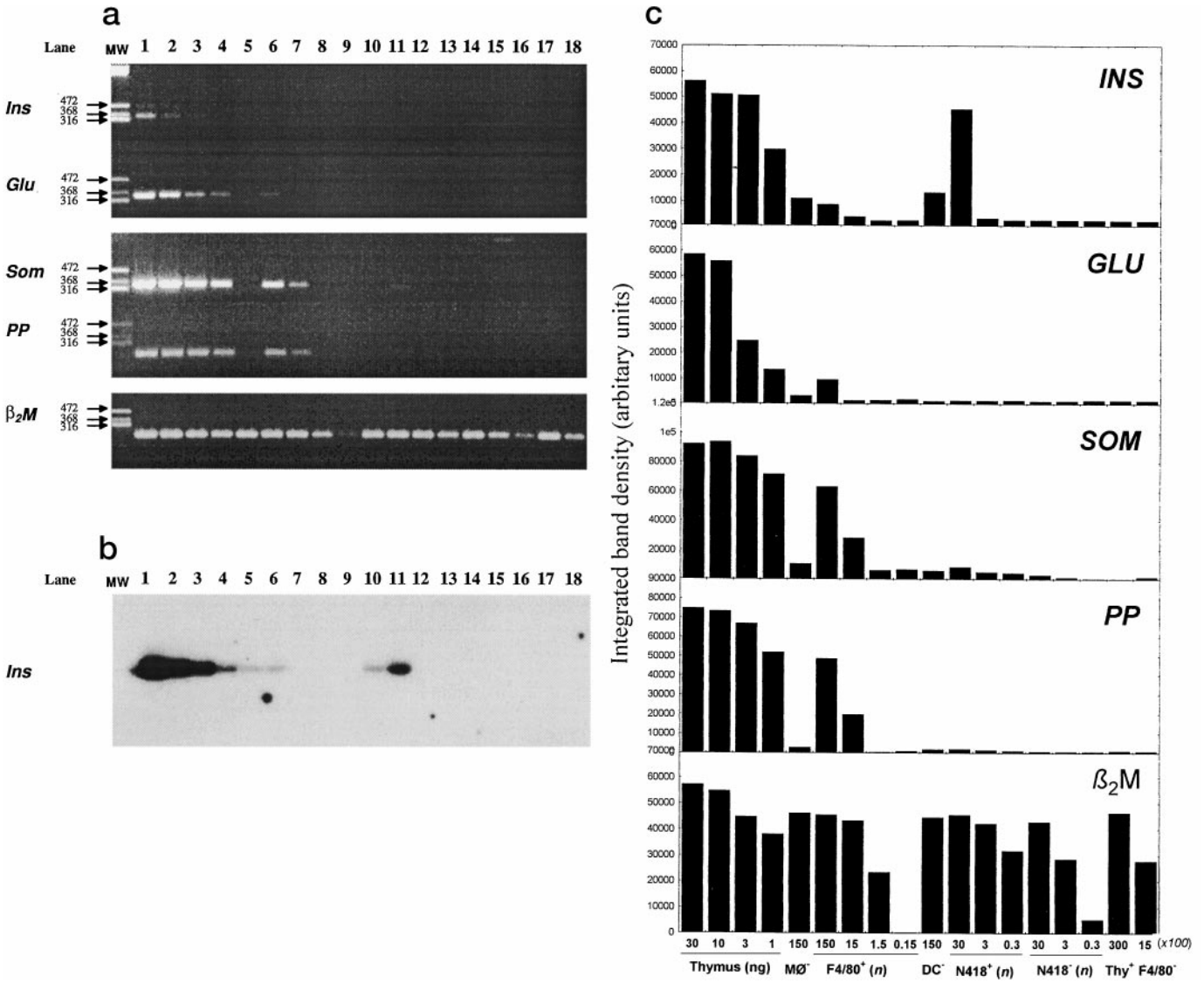

FIG. 4. a, Expression of ppIns (Ins), pGlu (Glu), pSom (Som), pPP (PP), and β_2m (β_2M) in thymic cells sorted by flow-activated cell sorting on the basis of their reactivity to the surface markers F4/80 (pan-macrophage), N418 (dendritic cells), and Thy-1.2 (pan-T cell). Lanes 1–4, Three, 1, 0.3, and 0.1 μg total RNA from 2-week-old C57Bl/6 thymuses, respectively; lane 5, 1.5×10^5 cells depleted of thymocytes, B cells, granulocytes, and CD8-positive dendritic cells; lanes 6–9, 1.5×10^5, 1.5×10^4, 1.5×10^3, and 1.5×10^2 cells, respectively, sorted for F4/80 reactivity from the same cell population as that in lane 5; lane 10, 1.5×10^5 cells depleted of thymocytes, B cells, granulocytes, and Mac 1-positive macrophages; lanes 11–13, 3×10^4, 3×10^3, and 3×10^2 cells, respectively, sorted for N418 reactivity from the same cell population as that in lane 10; lanes 14–16, 3×10^4, 3×10^3, and 3×10^2 cells, respectively, sorted from an N418-negative gate from the same cell population as that in lane 10; lane 17, 3×10^5 cells sorted for reactivity to Thy-1.2; lane 18, 1.5×10^4 cells sorted from an F4/80-negative gate from the same cell population as that in lane 5. Cells were extracted and subjected to RT-PCR. After electrophoresis, reaction products were visualized with ethidium bromide under UV light. The product size of the mol wt marker (MW) is indicated on the *left*. b, Autoradiograph of the first panel (Ins) hybridized with a radiolabeled 30-bp oligonucleotide specific for an internal sequence in the insulin PCR product. c, Densitometric measurements of ethidium bromide-stained cDNA in a and b.

dendritic cells constitutively synthesized the protein. This might be an artifact of the cell isolation and sorting procedure, which, although carried out at 4 C, still requires many steps and around 6–7 h to complete, and/or might represent a very low basal rate of transcriptional activity.

Importantly, we were able to demonstrate, by RIA, the presence of ir-insulin in the same population of cells that specifically expressed ppIns mRNA in the thymus. The amount detected, although above the level of sensitivity of the assay, was very low. However, as the antiserum used in the assay recognizes epitopes on both insulin and proinsulin, it is not possible to determine the concentration or identity of the hormone exactly. Unfortunately, immunohistochemical studies on cryostat or paraffin-embedded sections to locate the site of ir-proinsulin/insulin production *in situ* were unsuccessful (data not shown). In the human thymus, ir-insulin levels are reported to be 10^4- to 10^5-fold lower than those in the pancreas (18, 19), consistent with our findings in the mouse. In these studies, the level of ir-proinsulin was 4-fold greater than that of ir-insulin, and the researchers

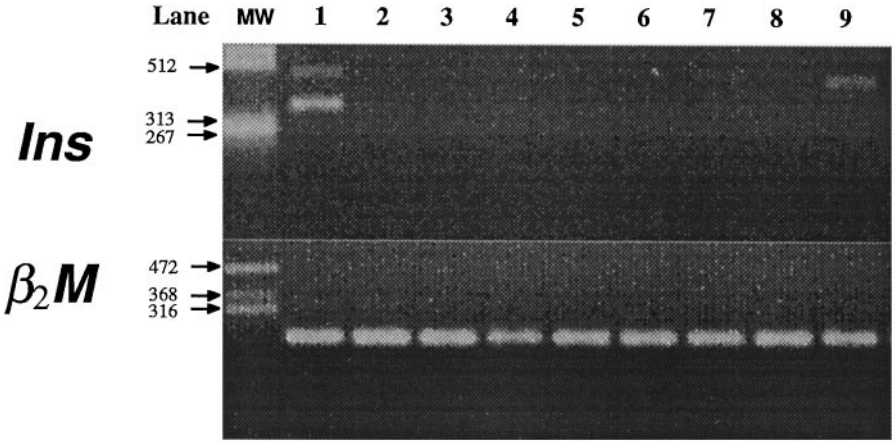

FIG. 5. Analysis of ppIns mRNA expression in thymic epithelial cells and peripheral macrophages. Lane 1, One microgram of total RNA from 2-week-old C57Bl/6 thymuses. Lanes 2–4, Total RNA from 1×10^7 splenic macrophages/dendritic cells isolated by adherence after 4 h in culture (lane 2), 1×10^7 thioglycolate-elicited peritoneal macrophages (lane 3), and 5×10^4 cells purified from deoxyguanosine-treated neonatal thymic lobes consisting of more than 98% epithelial and fibroblastic cells (lane 4). Lanes 5–9, Total RNA (1 μg) from epithelial cell line 1.4C18 (lane 5), 1.2C1 (lane 6), 2.3 (lane 7), 3.10 (lane 8), and 1C6 (lane 9). Total RNA was reverse transcribed and subjected to 32 cycles of PCR for a) preproinsulin (product size, 348 bp for Ins I and 355 bp for Ins II) and b) β_2m (25 cycles; product size, 219 bp). After electrophoresis, reaction products were visualized with ethidium bromide under UV light; the product size of the mol wt marker (MW) is indicated on the *left*.

concluded that proinsulin was probably the major translational product, as the levels of ir-insulin detected were in the range of control tissues and thus likely to be circulating, rather than endogenously produced, hormone.

Surprisingly, when tested for the expression of the three other hormones, F4/80-sorted cells demonstrated a specific band for each one. The level of expression relative to that in the serially diluted, age-matched thymus tissue included as a positive control was roughly similar for all of these hormones, in contrast to observations in the pancreas. This finding probably indicates that a similar number of cells is responsible for hormone synthesis. Whether the same cell synthesizes all hormones cannot be determined in the present study. The faint bands observed for pSom and pPP in the N418-gated extracts and the presence of autoradiographic signal for ppIns in F4/80-gated cells are unlikely to be the result of inadvertent contamination, as the cell populations included as controls and processed in parallel were all negative. Thus, the possibility that both cell types might produce multiple hormones cannot be excluded.

The macrophage belongs to the heterogeneous myeloid lineage of cells whose members are present in most tissues of the body and in the circulation (22, 30). The anatomical, morphological, and functional characteristics of these cells vary greatly and depend on the environment in which they seed. In the thymus, macrophages have two major functional roles: phagocytosis of apoptotic lymphocytes (immature T cells that are unable to develop further) and, to a lesser extent, antigen presentation to developing lymphocytes (31, 32). Dendritic cells are also present throughout the body, where they are critical in displaying antigen to initiate immune responses (24, 33). In the thymus, dendritic cells are derived from early lymphocyte progenitors (34). Thus, it is interesting to note that hormone expression was not present in any other tissue investigated, whether lymphoid or nonlymphoid. This suggests either that the specific thymic microenvironment modulates pancreatic hormone expression or that tissue-specific subpopulations of cells are responsible for their expression.

It is unexpected that insulin expression would be found in a different population of cells from that of the other pancreatic endocrine hormones. Of particular interest are the functional differences between these two cell types. The dendritic cell has a far greater potential to present antigen in the thymus for selection purposes (32, 34). Insulin has been identified as a potential autoantigen in type I diabetes. Thus, any alteration in the expression of insulin in these cells could influence the ability of the thymus to select against the generation of autoreactive T cells for insulin-derived peptides. The implication of this finding in the spontaneous model of type I diabetes, the NOD (nonobese diabetic) mouse, is now being explored.

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