The expression of regulated endocrine-specific protein of 18 kDa in peptidergic cells of rat peripheral endocrine tissues and in blood

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Abstract

We examined the cellular localization of regulated endocrine-specific protein of 18 kDa (RESP18) and mRNA in peripheral endocrine tissues. *In situ* hybridization and immunocytochemistry identified RESP18 mRNA in most cells of the anterior and intermediate pituitary, with RESP18 protein apparent in many anterior pituitary cells but very few intermediate pituitary cells. In the adrenal medulla and superior cervical ganglion, RESP18 mRNA co-localized with dopamine β-mono-oxygenase and neuropeptide Y. In the thyroid, RESP18 mRNA was localized to C-cells. RESP18 mRNA was expressed in most of the cells of the pancreatic islets, co-localizing with insulin, glucagon, and somatostatin. No RESP18 mRNA or protein was detected in the adrenal cortex, ovary, neural lobe of the pituitary, parathyroid, exocrine pancreas, thyroid follicular cells, placenta, mammary tissue, liver, lung, or atria. As in the intermediate lobe of the pituitary, high levels of RESP18 mRNA in the pancreatic islets and adrenal medulla did not always correlate with immunodetectable RESP protein, suggesting that post-transcriptional mechanisms are important in controlling RESP18 expression. Western blot analyses identified 18 kDa RESP and higher molecular weight isoforms of RESP in most tissues and in plasma. Subcellular fractionation of the anterior pituitary identified 18 kDa RESP18 in fractions enriched in endoplasmic reticulum and secretory granules, with the higher molecular weight isoforms of RESP18 concentrated in fractions enriched in secretory granules. The broad neuroendocrine distribution of RESP18 suggests that it subserves an important function in the secretory pathway that is common to the production of many secreted peptides.

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Introduction

Regulated endocrine-specific protein of 18 kDa (RESP18) was isolated from a rat neurointermediate pituitary cDNA library in a search for transcripts whose levels are regulated by dopaminergic drugs in parallel with pro-opiomelanocortin (POMC) (Bloomquist *et al.* 1994*a*). The maturation of POMC involves several enzymes (e.g. prohormone convertases (PC) 1 and 2, carboxypeptidase H, and peptidylglycine α-amidating mono-oxygenase) that are regulated in parallel with POMC (Bloomquist *et al.* 1994*a*, Oyarce *et al.* 1996). RESP18 is a novel protein with a short region exhibiting homology to the lumenal domain of phogrin and several other receptor-type protein tyrosine phosphatases (Wasmeier & Hutton 1996). RESP18 has a functional NH_2-terminal signal sequence and newly synthesized RESP18 enters the lumen of the endoplasmic reticulum (ER) (Bloomquist *et al.* 1994*a*,*b*, Schiller *et al.* 1995). In AtT-20 corticotrope tumor cells, endogenous RESP18 protein has a half-life of less than 20 min due to proteolysis in a post-ER/pre-Golgi compartment (Bloomquist *et al.* 1994*b*, Schiller *et al.* 1995). However, RESP18 protein has been shown to be present in compartments distal to the ER and is released into the medium in stably transfected AtT-20 cells overexpressing RESP18, or in AtT-20 cells treated with a calpain protease inhibitor that blocks RESP18 degradation (Schiller *et al.* 1995). In the rat anterior pituitary, higher molecular weight isoforms of RESP18 were detected by Western blot analysis and are due, at least in part, to O-glycosylation, probably at the level of the Golgi (Schiller *et al.* 1995).

The expression of RESP18 mRNA is regulated in a cell-type specific manner. Treatment of adult rats with a dopaminergic agonist decreases RESP18 mRNA levels in intermediate pituitary melanotropes. Dexamethasone treatment of AtT-20 cells increases RESP18 mRNA and protein levels. Insulin, estradiol, and epidermal growth factor treatment of rat somatotrope GH_3 cells lowers RESP18 mRNA levels and nerve growth factor treatment of PC12 rat pheochromocytoma cells increases RESP18 mRNA levels (Bloomquist *et al.* 1994*a*,*b*, Lee

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et al. 1995). RESP18 mRNA is localized to areas of the brain that control neuroendocrine and autonomic function, including the paraventricular, supraoptic, suprachiasmatic, dorsomedial, ventromedial and arcuate nuclei of the rat hypothalamus, and substantia nigra, ventral tegmental nucleus, amygdala and hippocampus (Darlington *et al.* 1996).

Although we have localized RESP18 to endocrine tissue in the pituitary and brain by *in situ* hybridization and Northern blot analyses (Bloomquist *et al.* 1994*a*), and we have characterized RESP18 expression in the testes in detail (Schiller & Darlington 1996), we have not examined the expression of RESP18 mRNA and protein in peripheral glands. In this study, we show that RESP18 is expressed in endocrine cells that produce bioactive peptides and is present in serum. By evaluating RESP18 expression using both *in situ* hybridization and immunocytochemistry, we identified cells containing RESP18 mRNA and protein along with cells containing RESP18 mRNA but lacking RESP18 protein, suggesting a role for a post-transcriptional step in regulating the function of RESP18. We previously observed 18, 19, 20, 22, and 24 kDa isoforms of RESP18 that result from expression of a single detectable mRNA species (Bloomquist *et al.* 1994*a*, Schiller & Darlington 1996). Subcellular fractionation analyses of anterior pituitary extracts suggest that the higher molecular mass RESP18 isoforms are indicative of RESP18 that has traversed the ER. The regulation, lack of endoproteolytic processing and widespread distribution of RESP18 suggest that RESP18, rather than acting as a prohormone, subserves a more general function in the secretory pathway of endocrine and neuroendocrine cells producing peptide hormones.

Materials and Methods

Animals

For immunohistochemistry/*in situ* hybridization experiments, 23 adult male, four adult virgin female, and two pregnant female Sprague–Dawley rats (300–350 g) were anesthetized with sodium pentobarbital (50 mg/kg) and perfused with 200 ml phosphate-buffered (0·05 M NaH_2PO_4) 4% formaldehyde solution (4 °C) via the left ventricle. Thyroid, adrenal, pancreas, testis, ovaries, mammary tissue, lung, thymus, pineal, liver, kidney, small intestine and pituitary were removed and fixed in phosphate-buffered 4% formaldehyde (4 °C) for 1 day followed by 25% sucrose (4 °C) for at least 2 days. Pregnant female rats were killed by halothane inhalation and the placentas were removed, placed in phosphate-buffered 4% formaldehyde for 1 day followed by 25% sucrose for at least 2 days. Plasma from trunk blood of four adult male or female rats was collected over a cocktail of protease inhibitors (5 μg/ml lima bean trypsin inhibitor, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 16 μg/ml benzamidine) as previously described (Mains & Eipper 1981). All animals were handled in accordance with the Johns Hopkins University and University of Maryland at Baltimore Institutional Animal Care and Use Committees.

Antibodies

Rabbit polyclonal antisera for RESP18 (JH1162, JH1163) (Bloomquist *et al.* 1994*b*), POMC (Eipper & Mains 1978), β-lipotropin (βLPH) (JH2) (Milgram & Mains 1994), PC1 (Milgram & Mains 1994), the trans-Golgi network marker (TGN38) (Milgram *et al.* 1996), growth hormone (GH) (JH89) (Dickerson & Mains 1990), prolactin (National Hormone and Pituitary Program, IC-5, AFP425–10-91), thyroid-stimulating hormone-β (TSH-β) (IC-1; AFP-1274789), somatostatin (SS 175c; Dr Brian Noe), calcitonin gene-related peptide (CGRP) (MU33; Dr Ian Dickerson), neuropeptide Y (NPY) (JH3) (Marek & Mains 1990), and dopamine β-mono-oxygenase (DβM) (JH2049 raised to recombinant rat DβM(217–327)) were used for immunocytochemistry. Mouse monoclonal antisera for insulin and glucagon (Sigma, St Louis, MO, USA; I-2018 and G-2654) were also used for immunocytochemistry. Heavy chain binding protein (BiP) antiserum was from Affinity BioReagents (Neshanic Station, NJ, USA) and α-synaptotagmin antiserum was a gift from Dr Richard Scheller (Elferink *et al.* 1993). Goat antirabbit and goat antimouse immunoglobulins linked to horseradish peroxidase were from Amersham Life Sciences (Arlington Heights, IL, USA).

Tissue extraction, subcellular fractionation and Western blot analysis

For Western blot analysis, tissues were extracted (10%, w/v) with 20 mM NaTES, 10 mM mannitol, 1% Triton X-100, pH 7·4 containing the above described protease inhibitor cocktail (Husten & Eipper 1994) supplemented with 100 μg/ml N-acetyl-leucine-leucine-norleucinal (ALLN). Extracts were frozen/thawed three times and centrifuged at 1000 ***g*** for 5 min to remove cell debris. The concentration of protein in supernatants was measured using the BCA assay (Pierce, Rockford, IL, USA). Western blot analysis was carried out as previously described (Bloomquist *et al.* 1994*a*). Plasma samples (5 μl) were prepared for electrophoresis by boiling in Laemmli sample buffer containing 4% SDS.

Subcellular fractionation of rat anterior pituitary tissue was carried out as previously described (Oyarce *et al.* 1996) with the following modifications. Briefly, a Teflon homogenizer was used to disrupt tissue (10%, w/v) in homogenization buffer (HB) containing the protease inhibitor cocktail (described above) with 100 μg/ml ALLN (Schiller *et al.* 1995). Cell lysis was monitored microscopically using trypan blue. Intact cells, nuclei, and

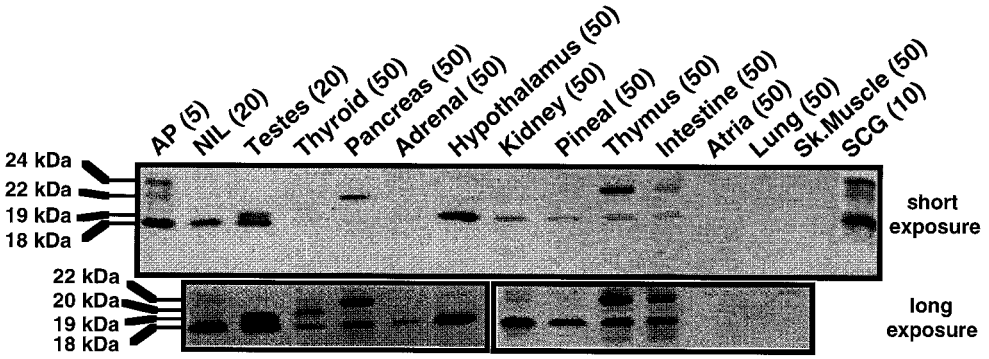


Figure 1 Forms of RESP18 protein in selected peripheral tissues. Particulate fractions prepared from anterior pituitary (AP), neurointermediate pituitary (NIL), testis, thyroid, pancreas, adrenal, hypothalamus, kidney, pineal, thymus, intestine, atrium, lung, skeletal (Sk.) muscle and superior cervical ganglion (SCG) were fractionated by SDS-PAGE, transferred and analyzed by Western blot with RESP18 antiserum JH1162 (1:2000). Numbers represent μg total protein loaded onto gel. The lower part of the figure shows a longer exposure. The figure represents part of the Western blot containing RESP18 bands. No other band was present on the blot.

debris were removed by centrifugation at 1100 ***g*** for 5 min (5 k; 5000 r.p.m.) in a Beckman TL-100 centrifuge at 4 °C. The supernatant was successively centrifuged for 15 min at 4400 ***g*** (10 k) and 17 400 ***g*** (20 k). Organelles in the 20 k pellet were further fractionated by sucrose density centrifugation. The pellet was resuspended in 170 μl HB buffer of which 150 μl was loaded onto a 2·05 ml sucrose step gradient prepared by layering buffered sucrose solutions as previously described (Oyarce *et al.* 1996). Density gradients were centrifuged at 214 000 ***g*** for 2 h and 150 μl fractions were removed sequentially from the top. Fractions were analyzed for RESP18, BiP, GH, synaptotagmin, TGN38 or PC1 by Western blot using the ECL system (Amersham Life Sciences).

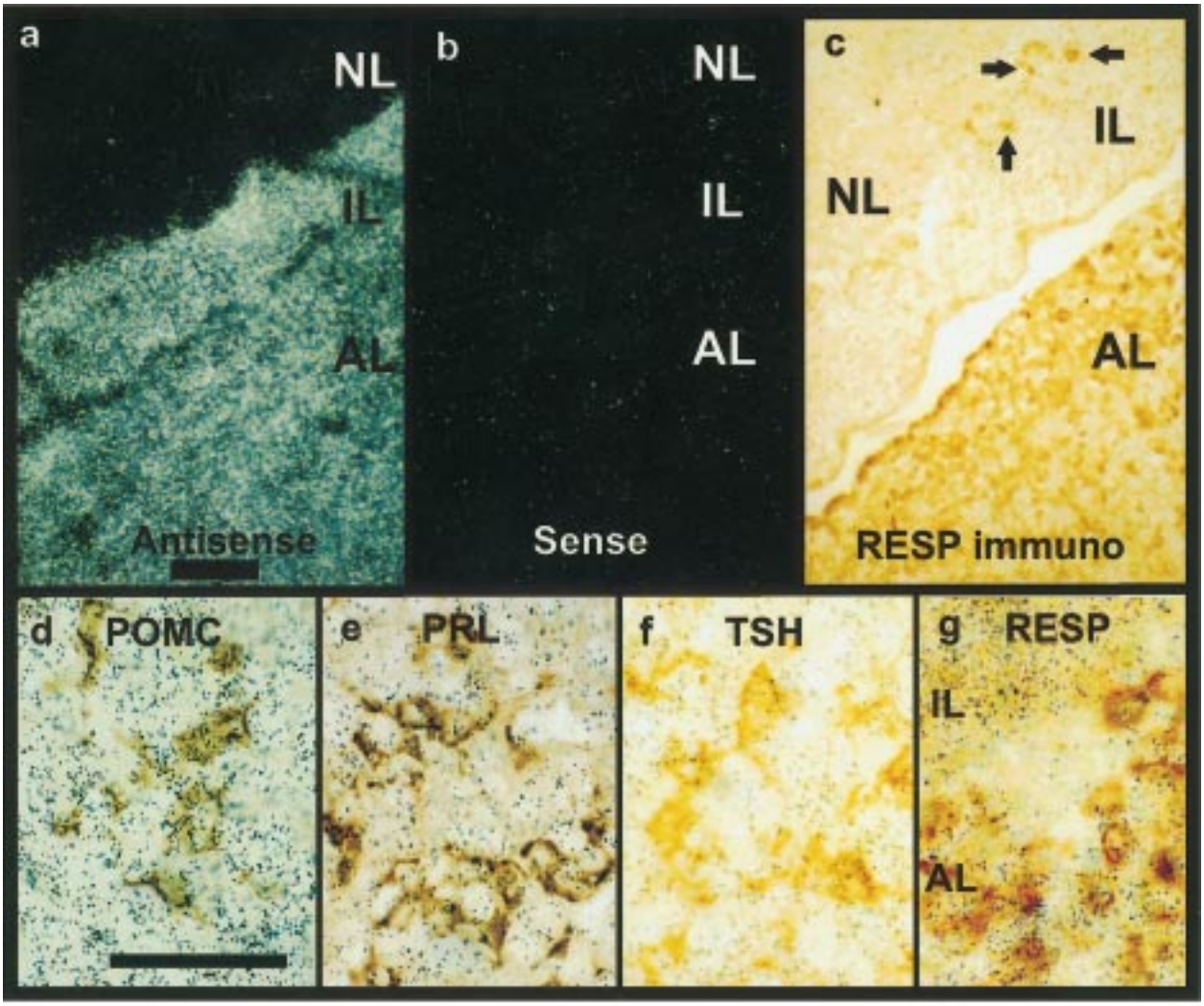
In situ *hybridization and immunocytochemistry*

Frozen sections (12 μm) were placed on gelatin-coated glass slides for *in situ* hybridization as previously described (Bhat *et al.* 1992, Bloomquist *et al.* 1994*a*, Darlington *et al.* 1996). After hybridization, the sections were subjected to immunocytochemical analysis by washing in PBS (5 min), blocking in 10% normal goat serum (NGS)/PBS (30 min), 3% H_2O_2/PBS (10 min), and rinsing in PBS for 10 min. Antiserum (l00 μl) to RESP18 (1:4000 dilution), POMC (1:2000), TSH-β (1:1000), insulin (1:1000), glucagon (1:000), somatostatin (1:5000), CGRP (1:2000), NPY (1:2000), or DβM (1:000) was diluted in NGS/PBS, placed on the tissue sections and incubated overnight at 4 °C. Sections were rinsed four times with PBS. Primary antisera were visualized using an avidin-biotin kit (Vector Laboratories, Burlingame, CA, USA). Slides were rinsed with double-distilled (dd) H_2O, dehydrated sequentially with 70 and 95% ethanol, air dried and exposed to film for 1–3 days. Slides were then dipped in Kodak NTB-3 photographic emulsion (Eastman Kodak, Arlington, VA, USA), exposed for 10–14 days, and developed. Slides were rinsed in ddH_2O and alternate sections were either coverslipped, or counterstained with 0·4% methyl green for l min, rinsed in ddH_2O, destained with 70% ethanol, and dehydrated sequentially with ethanol (95 and 100%), xylene and coverslipped.

As a control for non-specific binding, RESP18 antiserum (JH1162) was preincubated with purified recombinant RESP18 and used for immunohistochemistry; only background immunostaining was observed in the pituitary, adrenal, thyroid, pancreas, superior cervical ganglion and testis. No detectable binding of preimmune DβM serum was observed. As a control for non-specific binding of secondary antibodies, tissues were processed excluding incubation with primary antiserum; no distinct immunostaining remained in any of the tissues examined.

Biosynthetic labeling of primary anterior pituitary cultures

Primary anterior pituitary cultures prepared from adult female rats (n=8) as previously described were plated on four-well plates in Dulbecco's modified Eagles' medium (DMEM):F12 medium containing 10% NuSerum (Collaborative Research, Bedford, MA, USA) and 10% fetal calf serum (HyClone Laboratories, Logan, UT, USA). After a 5-day incubation, cells were incubated in CSFM-Air devoid of methionine and cysteine for 10 min and then incubated in 250 μl of the same medium supplemented with [^{35}S]Met/Cys (1 mCi/ml) for 1 h. Cells were then chased for 2 h in 250 μl CSFM-Air medium. Secretion was then stimulated by chasing the cells in CSFM-Air medium containing 0 or 1·0 μM phorbol–12-myristate-13–acetate (PMA) for 1 h. Media were harvested and briefly centrifuged to remove detached cells. Cells were extracted in 250 μl 5 M acetic acid/2 mg/ml BSA and lyophilized. Cell extracts were


a
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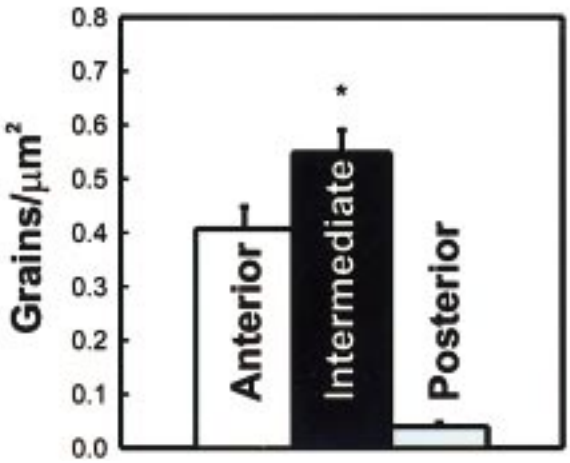
Grains/μm²
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Anterior
Intermediate
Posterior

resuspended in 50 mM sodium phosphate, 10 mM mannitol, 1% Triton X-100, pH 7·0. Aliquots of cell extract and medium were immunoprecipitated with antisera to RESP18 (JH1162) or β-endorphin (JH2) and analyzed as described (Zhou & Mains 1994).

Results

Forms of RESP18 protein expressed in selected peripheral tissues

Northern blot analysis identified a single 0·8 kb RESP18 transcript in anterior and neurointermediate pituitary, testis, and several peripheral endocrine tissues (Bloomquist *et al.* 1994*a*, Schiller & Darlington 1996). We analyzed particulate fractions prepared from peripheral endocrine tissues by Western blot to determine the levels of RESP18 protein, to identify the isoforms of RESP18 protein present and to provide a control for cross-reactive bands that might lead to false positive staining in subsequent immunohistochemical analyses (Fig. 1). Tissues characterized previously were included to provide accurate size measurements for the higher molecular mass immunoreactive bands cross-reactive with RESP18 antisera and to assess relative signal intensities (Schiller *et al.* 1995, Schiller & Darlington 1996).

An 18 kDa immunoreactive band was apparent in the anterior and neurointermediate pituitary, testis, hypothalamus, kidney, pineal, thymus, small intestine, and superior cervical ganglion. Note that the amount of protein loaded was adjusted to accommodate the different levels of RESP18 expression and varied from 5 μg to 50 μg for many of the other tissues. After a longer exposure, 18 kDa RESP18 was observed in the thyroid, pancreas and adrenal (lower part of Fig. 1). Taking into account the different amounts of sample analyzed (Fig. 1), RESP18 protein levels were highest in the anterior pituitary, followed by the superior cervical ganglion, neurointermediate pituitary and testis. No RESP18 protein was detectable in the atrium, lung, or skeletal muscle.

Higher molecular mass isoforms of RESP18 (24, 22, 20 and 19 kDa) were detectable in many of the tissues (Fig. 1). All of the bands detectable with antiserum JH1162, whose specificity is directed toward the NH_2-terminal region of RESP18, were also detected when the blot was stripped and reprobed with antiserum JH1163, whose specificity is directed toward the COOH-terminal region of RESP18 (not shown). An immunoreactive band of 24 kDa was observed in anterior pituitary and superior cervical ganglion. An immunoreactive band of 22 kDa was observed in anterior pituitary, pancreas, thymus, intestine, superior cervical ganglion and, after longer exposure, in neurointermediate pituitary, thyroid, and kidney. An additional 19 kDa isoform of RESP18 was found in testis, and a 20 kDa isoform was only prevalent in thyroid.

Localization of RESP18 in peripheral glands

We used *in situ* hybridization and immunohistochemistry to localize RESP18 mRNA in peptide-producing cells of several endocrine glands. RESP18 mRNA was found in all of the cells of the anterior and intermediate pituitary, with background levels in the neural lobe (Fig. 2a). The specificity of the signal was established with the sense riboprobe in the pituitary (Fig. 2b) and in all other tissues. Immunocytochemical staining of RESP18 identified positive cells throughout the anterior pituitary, with scattered positive cells in the intermediate lobe (Fig. 2c). As previously observed, RESP18 mRNA was found in anterior pituitary cells containing POMC (Fig. 2d), prolactin (Fig. 2e), TSH (Fig. 2f), and also in GH-, follicle-stimulating hormone- and lutenizing hormone-expressing cells (data not shown).

Quantification of silver grains revealed that RESP18 mRNA levels were significantly higher in the intermediate as compared with the anterior lobe (Fig. 2). Although RESP18 mRNA was highly expressed in both the anterior and intermediate pituitary, very few of the intermediate pituitary melanotropes could be immunostained for RESP18 protein (Fig. 2c). In contrast, strong immunostaining for RESP18 was observed in most of the anterior pituitary cells (Fig. 2g). Only background immunostaining was observed in the neural lobe. Similar results were observed using either JH1162 (directed against the N-terminal) or JH1163 (directed against C-terminal) RESP18 antiserum. The Western blots shown in Fig. 1 also indicate that levels of RESP18 protein in the neurointermediate lobe are much lower than in the anterior pituitary. The presence of intermediate pituitary melanotropes with high levels of RESP18 mRNA and low levels

Figure 2 Expression of RESP18 mRNA and protein in the pituitary. (a) Darkfield photomicrograph showing abundant RESP18 mRNA in the anterior lobe (AL) and intermediate lobe (IL), but not the neural lobe (NL) by *in situ* hybridization using a full-length antisense RESP18 riboprobe and (b) no significant signal using a sense RESP18 riboprobe. (c) Lightfield photomicrograph showing strong immunostaining (immuno) for RESP18 protein (JH1162) in most of the cells of the anterior lobe and in only a few intermediate lobe cells (arrows). The bar in (a)=200 μm and represents scale in (b) and (c). *In situ* hybridization combined with immunocytochemistry showing RESP18 mRNA in cells that contain (d) POMC, (e) prolactin (PRL), (f) TSH and (g) RESP18 (RESP). The bar in (d)=50 μm and represents scale in (e), (f), and (g). The graph shows the amount of RESP18 mRNA as silver grains/μm^2 in the pituitary. Values represent mean ± S.E. from 21 pituitary sections derived from four male rats. *$P<0{\cdot}05$ as compared with anterior pituitary by Student's *t*-test. Values for posterior pituitary were not different from background.

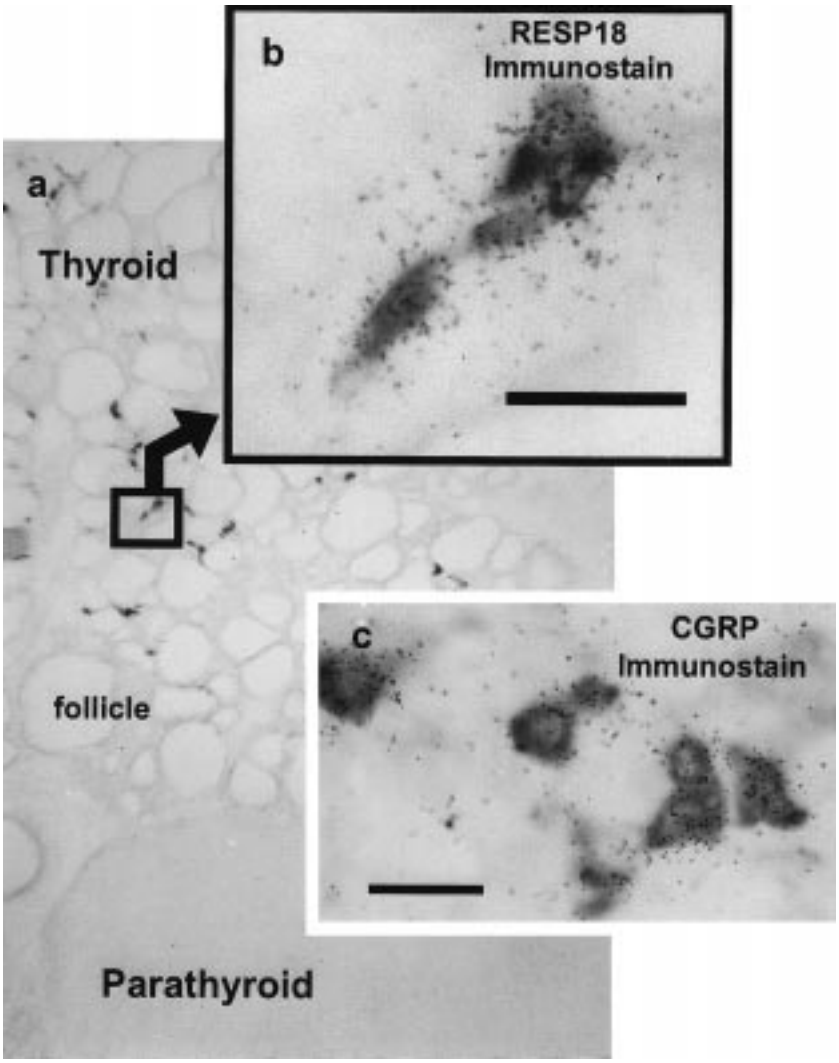

Figure 3 RESP18 expression in thyroid C-cells. Lightfield photomicrograph showing *in situ* hybridization for RESP18 mRNA in cells of the thyroid gland immunostained for (a and b) RESP18 or (c) CGRP. No RESP18 mRNA or protein was found in the parathyroid gland. Bars=100 μm.

of RESP18 protein suggests that RESP18 expression is controlled at the post-transcriptional level.

Analysis of the thyroid gland by *in situ* hybridization using an antisense RESP18 riboprobe showed silver grains concentrated in cells scattered between the follicles (Fig. 3a). RESP18 mRNA was located only in cells that immunostained for CGRP, suggesting that the calcitonin-secreting parafollicular cells express RESP18 (Fig. 3c). Immunostaining for RESP18 protein demonstrated the same cellular distribution as immunostaining for CGRP. Unlike the intermediate pituitary, RESP18 immunostaining was always observed in cells with silver grains representing RESP18 mRNA (Fig. 3b). RESP18 mRNA and protein were not detected in the parathyroid gland or in the follicular cells of the thyroid (Fig. 3a).

Analysis of the pancreas by *in situ* hybridization showed an abundant and uniform distribution of RESP18 mRNA throughout the islets of Langerhans, with no signal detectable in acinar cells or any other part of the exocrine pancreas (Fig. 4). RESP18 mRNA was identified in cells that immunostained for insulin, glucagon and somatostatin (Fig. 4a, b and c). Insulin immunostaining was found throughout the islet while glucagon and somatostatin immunostaining was found primarily at the periphery of the islet. Immunohistochemical staining for RESP18 identified positive cells in all parts of the islet and RESP18

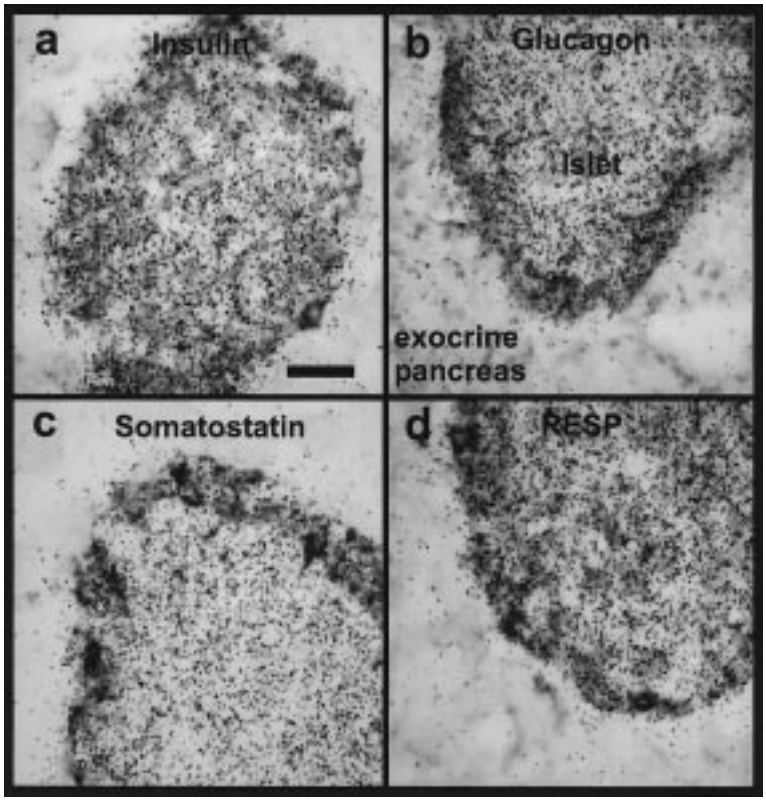

Figure 4 RESP18 expression in the pancreatic islets of Langerhans. Lightfield photomicrographs of *in situ* hybridization for RESP18 mRNA in the pancreas. RESP18 mRNA was found in cells that immunostain for (a) insulin, (b) glucagon, (c) somatostatin and (d) RESP18. The bar in (a)=100 μm and represents scale in (b), (c) and (d).

immunostaining was always observed in cells with RESP18 silver grains (Fig. 4d). Only background immunostaining for RESP18 was observed in the exocrine pancreas. The pattern of RESP18 immunostaining most closely resembled insulin immunostaining (Fig. 4a and d). Although RESP18 mRNA was uniformly distributed throughout the islet, the peripheral cells of the islet showed the most intense immunostaining for RESP18. As in the pituitary, the variable RESP18 protein/mRNA levels suggest that expression of RESP18 is controlled at the post-transcriptional level.

RESP18 mRNA and 18 kDa RESP18 protein were previously detected in the adrenal by Northern and Western blot respectively (Bloomquist *et al.* 1994*a*, Schiller *et al.* 1995). In this study, *in situ* hybridization of adrenal showed RESP18 mRNA uniformly distributed in the adrenal medulla, with background levels of silver grains in the adrenal cortex (Fig. 5a and b). RESP18 mRNA was located in cells that immunostained for DβM and in the subset of chromaffin cells that immunostained for neuropeptide Y (Fig. 5d and e). Similar to the pituitary and pancreatic islets, the adrenal medulla contained cells that had abundant RESP18 mRNA but barely detectable RESP18 immunostaining (Fig. 5c and f), suggesting post-transcriptional regulation of RESP18 expression.

Since RESP18 mRNA was previously observed in neurons of the diencephalon and hypothalamus (Darlington *et al.* 1996), we examined the expression of RESP18 in the superior cervical ganglion, part of the peripheral nervous system. All cells of the superior cervical ganglion expressed RESP18 mRNA and protein, including the subset of neurons that contain NPY (Fig. 6a and b). Unlike the pituitary, pancreatic islets and adrenal medulla, and similar to the thyroid, all of the superior cervical ganglion neurons that contained RESP18 mRNA exhibited similar staining intensity for RESP18 protein (Fig. 6a). Since the adrenal medulla and superior cervical ganglion are part of the peripheral autonomic nervous system, finding RESP18 expression in these organs complements our previous observation that RESP18 was located in autonomic and neuroendocrine areas of the brain (Darlington *et al.* 1996).

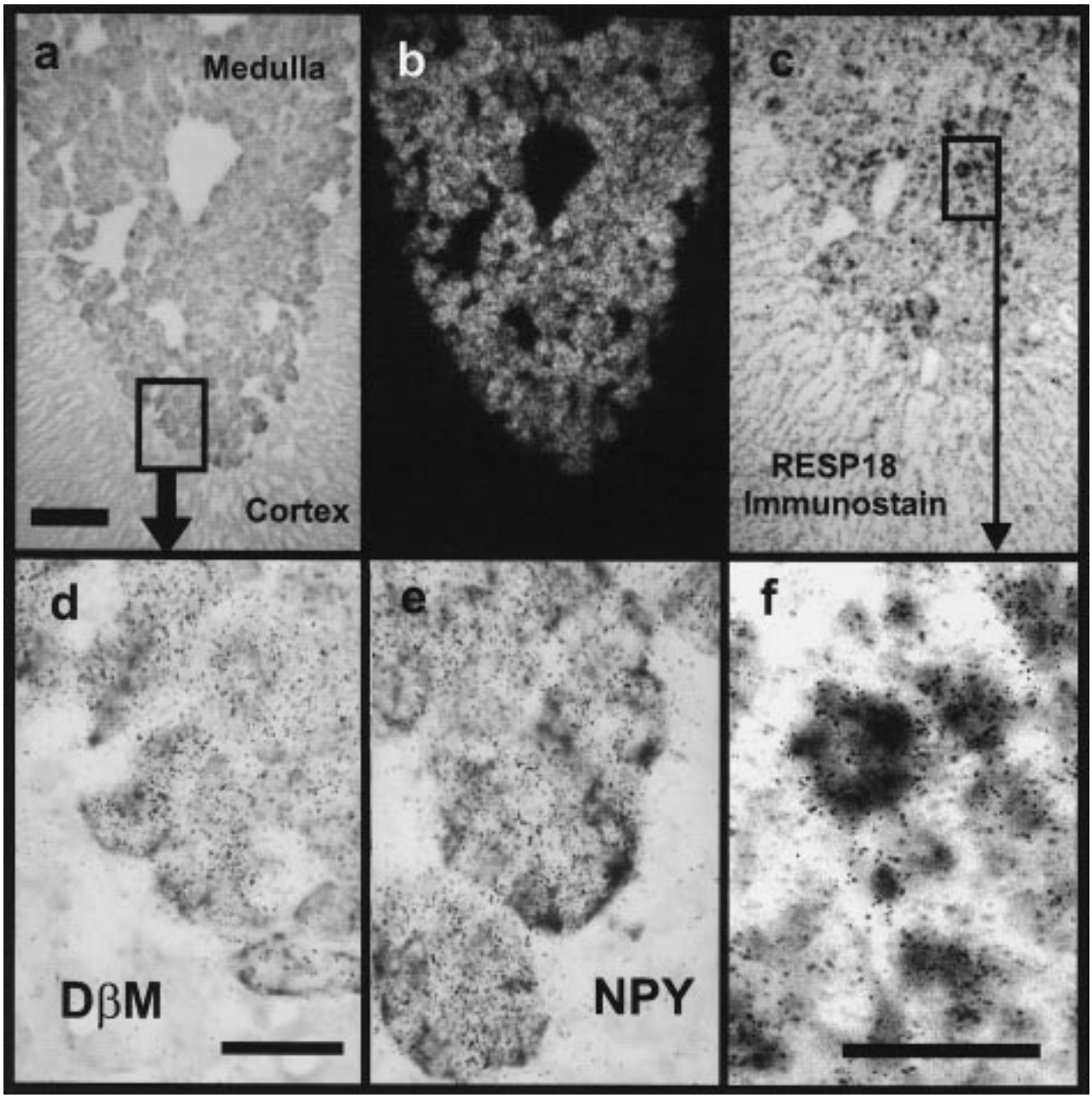

Figure 5 RESP18 expression in the adrenal medulla. (a and c) Lightfield and (b) darkfield photomicrographs of *in situ* hybridization for RESP18 mRNA in the adrenal medulla immunostained for (a and b) DβM or (c) RESP18. (d) A higher power representation of the inset in (a) shows RESP18 mRNA in medullary cells that contain DβM. (e) RESP18 mRNA was also located in medullary cells that immunostain for NPY. (f) Higher power representation of inset in (c) shows that RESP18 immunostaining varies between cells even though mRNA is present in fairly uniform levels. Bar in (a)=500 μm and represents scale in (b) and (c). Bar in (d)=100 μm and represents scale in (e). Bar in (f)=50 μm.

No RESP18 mRNA or protein expression was detected in the liver, lung, or heart atrium by *in situ* hybridization or immunostaining in male rats. No RESP18 mRNA or protein were identified in the ovaries, placenta or mammary tissue of female rats (data not shown).

RESP18 protein in plasma

In AtT-20 anterior pituitary corticotrope tumor cells, endogenous RESP18 protein was restricted to the endoplasmic reticulum and only the 18 kDa form of RESP18

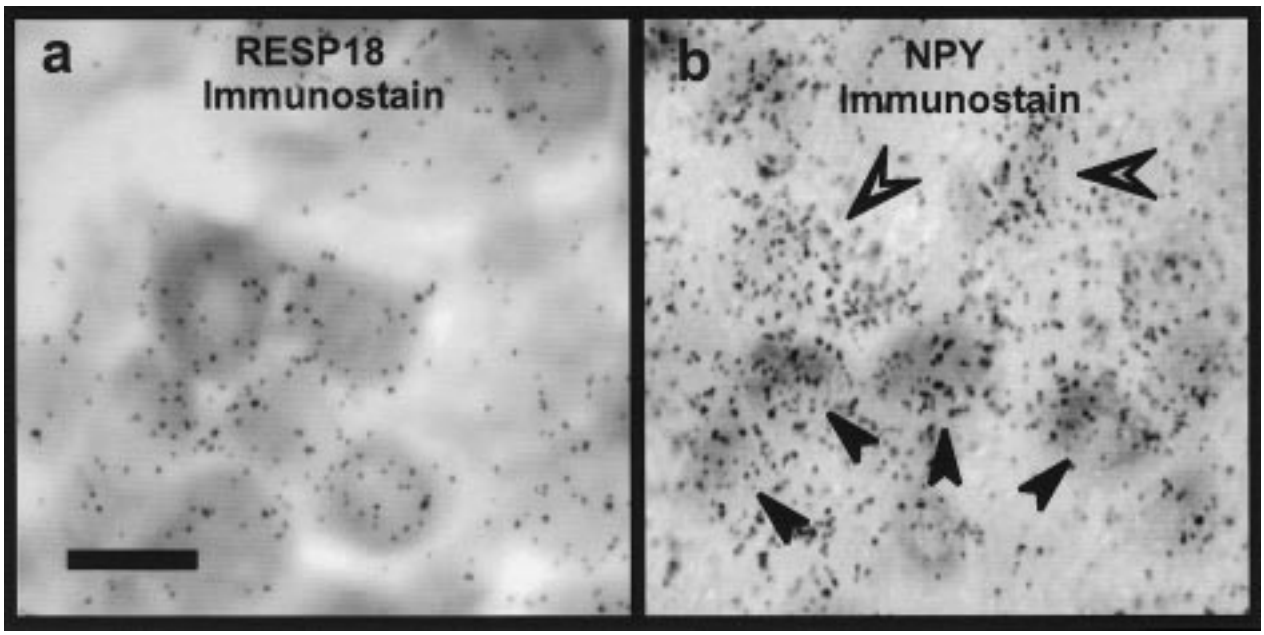

Figure 6 RESP18 expression in the superior cervical ganglion. Lightfield photomicrograph showing *in situ* hybridization for RESP18 mRNA in cells of the superior cervical ganglion that immunostained for (a) RESP18 or (b) NPY; solid arrowheads show RESP18 mRNA in NPY immunostained cells; open arrowheads show RESP18 mRNA in non-NPY immunostained cells. Bar=100 µm.

was observed on Western blots (Bloomquist *et al*. 1994*b*, Schiller & Darlington 1996). When RESP18 was over-expressed in AtT-20 cells or hEK-293 cells, a non-neuroendocrine cell type, the higher molecular mass 21–23 kDa RESP18 isoform(s) were secreted into the medium (Schiller *et al*. 1995). The observation that higher molecular mass RESP18 isoforms were found in peripheral endocrine glands compelled us to examine plasma for RESP18 protein. Analysis of plasma by Western blot with both JH1162 and JH1163 RESP18 antisera identified 18 and 20 kDa RESP18 isoforms in male and female rat plasma (Fig. 7). Figure 7*b* shows a comparison of RESP18 isoforms in blood and other tissues to determine accurate masses for the RESP18 isoforms in blood (18 and 20 kDa). The 20 kDa RESP18 isoform was prevalent in the thyroid suggesting that RESP18 in blood may come from the thyroid. However, the source of circulating RESP18 is not known. Western blot analysis of plasma from castrated or hypophysectomized rats showed RESP18 levels similar to control, suggesting that the testis, pituitary and pituitary target tissues may not be the source of RESP18 in plasma (data not shown). Quantitative Western blot analysis (n=4) using pure recombinant RESP18 as a standard and the JH1162 and JH1163 antisera was used to calculate the concentration of RESP18 protein in plasma. RESP18 levels in female plasma (0·34 nM) were double those in male plasma (0·17 nM).

Localization of RESP18 isoforms in the endoplasmic reticulum and distal parts of the secretory pathway

Since most tissues contained higher molecular mass isoforms of RESP18 (Fig. 1), we sought to identify the subcompartments of the secretory pathway that contained 18 kDa and higher molecular mass isoforms of RESP18. We chose the anterior pituitary for these studies because it contained the 18, 22 and 24 kDa isoforms of RESP18 and methods for subcellular fractionation of anterior pituitary are well developed (Oyarce & Eipper 1995). Anterior pituitary homogenates were fractionated by differential centrifugation at 5 k, 10 k and 20 k. The 20 k pellet was further fractionated by sucrose density centrifugation and analyzed by Western blot using antisera to RESP18 and several subcellular marker proteins (Fig. 8). A clear difference was observed in the distribution of 18 kDa and 24 kDa RESP. The 24 kDa isoform of RESP18 was concentrated in fractions 9 to 11, coinciding with the peak of GH and processed 66 kDa PC1 (Fig. 8); the small amount of 22 kDa RESP present exhibited a distribution similar to that of the 24 kDa RESP isoform. Based on its density and enrichment in GH and processed PC1, this region of the gradient contained mature secretory granules. Although some 18 kDa RESP18 was recovered in the secretory granule-enriched fractions, more of the 18 kDa isoform of RESP18 was found in fractions 4 through 8, mimicking the distribution of BiP, an endoplasmic reticulum marker. These less dense fractions also contained

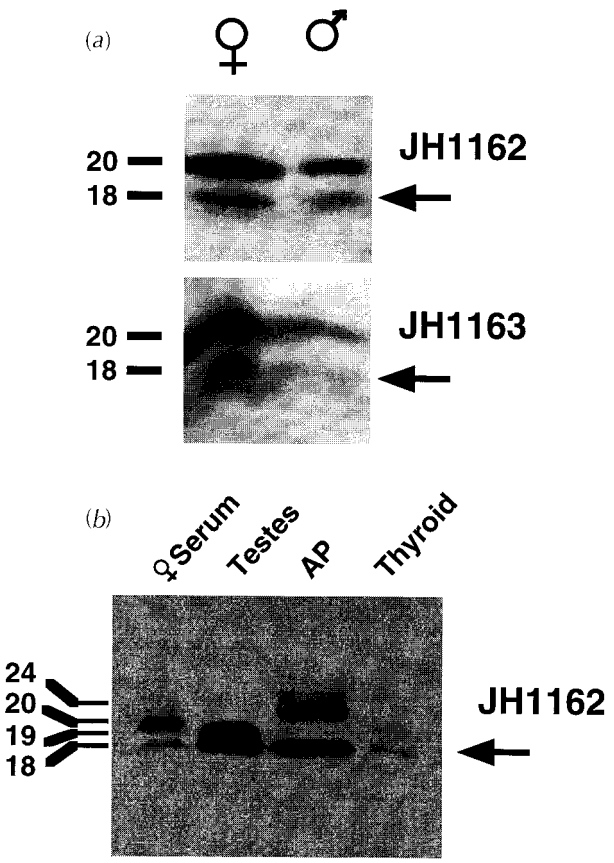

Figure 7 Expression of RESP18 protein in plasma. (*a*) Plasma samples pooled from four adult male and female rats were analyzed by Western blot using RESP18 antisera JH1162 (1:2000) or JH1163 (1:1000). Apparent molecular masses (kDa) were calculated based on the migration of marker proteins; arrows indicate 18 kDa RESP18. (*b*) A sample of female plasma was compared with samples prepared from anterior pituitary (AP), testes and thyroid.

TGN38, the trans-Golgi network marker, and synaptotagmin, a marker for secretory granules and synaptic vesicles (Calakos & Scheller 1996) The Western blot analysis shown in Fig. 8 was repeated using RESP18 antiserum JH1163 and yielded similar results. These results suggest that in the anterior pituitary, as in transfected AtT-20 cells, the 22 and 24 kDa isoforms of RESP18 are found only in later parts of the secretory pathway (Schiller *et al.* 1995).

Since subcellular fractionation of anterior pituitary suggested that the high molecular weight isoforms of RESP18 were concentrated in secretory granules and since RESP18 was present in plasma, we sought to determine if secretion of RESP18 from anterior pituitary cells could be stimulated by secretagogue. Primary cultures of rat anterior pituitary were incubated in medium containing [^{35}S]Met/Cys for 1 h; after a 2-h chase in medium lacking labeled amino acid, duplicate cultures were incubated with or without secretagogue (PMA) for 1 h and extracted for immunoprecipitation with an RESP18 antiserum (Fig. 9). Although newly synthesized 18 kDa RESP18 was readily detected in cell extracts, no radiolabeled RESP18 was detected in the medium even in the presence of secretagogue (Fig. 9). When the same samples were immunoprecipitated with β-endorphin antiserum, radiolabeled βLPH was detected in cell extracts and medium. Addition of secretagogue enhanced secretion of βLPH, demonstrating that the cells were capable of stimulated secretion. If newly synthesized RESP18 enters secretory granules within the 2-h chase time, it does not appear to be released in response to secretagogue. No 24 kDa RESP18 was detected in these metabolic labeling experiments.

Discussion

This study extends our previous observations and indicates that RESP18 is expressed in endocrine and neural cells that secrete peptide hormones. For clarity, sites of RESP18 expression observed in this and previous studies (Bloomquist *et al.* 1994*a*, Schiller *et al.* 1995, Schiller & Darlington 1996) are summarized in Table 1. Note that not all peptide-producing endocrine tissues express RESP18. In this study, we failed to detect RESP18 expression in the parathyroid gland, the lungs, liver, atrium, skeletal muscle, placenta or mammary tissue by *in situ* hybridization, immunocytochemistry or Western blot. Furthermore, RESP18 was not expressed in endocrine tissues that secrete steroid hormones, like the adrenal cortex, ovary or Leydig cells of the testis, nor in the thyroid hormone-producing follicular cells of the thyroid gland. RESP18 was expressed in the peripheral nervous system as represented by the superior cervical ganglion and in the adrenal medulla, complementing our previous study identifying RESP18 mRNA in autonomic and neuroendocrine regulating centers in the rat brain (Darlington *et al.* 1996).

The combined *in situ* hybridization/immunocytochemistry analyses carried out suggest the occurrence of post-transcriptional regulation of RESP18 protein levels in the intermediate pituitary, pancreatic islets and adrenal medulla. RESP18 protein expression was far greater in the anterior lobe than in the intermediate lobe of the pituitary even though RESP18 mRNA levels were slightly higher in the intermediate lobe (Fig. 2). Very few intermediate pituitary cells exhibited intense RESP18 immunostaining like that observed in the anterior pituitary. The results of Western staining of RESP18 (Fig. 1) suggest that more RESP18 protein may be present in the intermediate lobe than that suggested by immunostaining (Fig. 2). However, four times as much protein from the neurointermediate lobe was analyzed on the Western blot, and the signal from the neurointermediate lobe was still weaker (Fig. 1). It is more likely that the level of detectability of RESP18 protein by immunostaining is less than that by Western

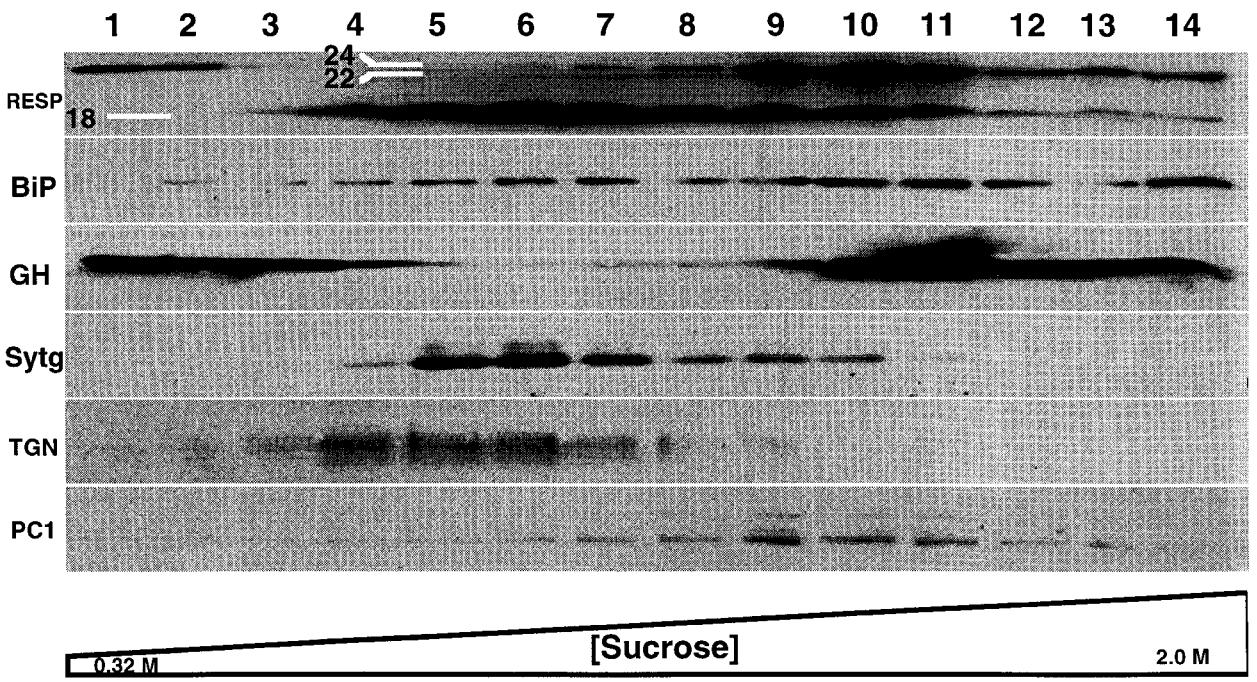

Figure 8 Sucrose density gradient fractionation of rat anterior pituitary. Pellets from differential centrifugation (10 k, 20 k) were subjected to sucrose density centrifugation; analysis of the 20 k pellet is shown. Aliquots of each fraction (50 µl) were analyzed by SDS-PAGE (peptide or 10% gels) and Western blot with RESP18 (RESP; JH1162; 1:2000), BiP/GRP78 (BiP; 1:2000), GH (1:2000), synaptotagmin (Sytg; 1:3000), TGN38 (TGN; 1:500), or PC1 (1:1000) antiserum. Markers for 18, 22 and 24 (kDa) RESP are on the top fractionation.

blot. In the endocrine pancreas, RESP18 mRNA expression was uniform throughout the islet and was identifiable in insulin, glucagon and somatostatin cells. However, RESP18 immunostaining most closely resembled insulin-producing cells. The difference in RESP18 protein expression between the three cell types of the islet may be due to their differential function and regulation (Yamaguchi 1992, Kabadi 1993). RESP18 mRNA was uniformly distributed in the adrenal medulla. However, RESP18 immunostaining was detectable in only a small percentage of the chromaffin cells scattered throughout the adrenal medulla (Fig. 6). This discrepancy could be the result of selective translation of RESP18 mRNA or it could be a post-translational effect, with RESP18 protein selectively and rapidly degraded, secreted or sequestered in a manner that allows it to escape detection with both of our RESP18 antisera.

Levels of RESP18 mRNA in the intermediate, but not the anterior lobe of the pituitary are sensitive to regulation by dopaminergic agonists and antagonists (Bloomquist *et al.* 1994*a*). It will be interesting to determine whether any translational and/or post-translational events that might affect expression of RESP18 protein are subject to regulation through the D_2 dopamine receptors that are present in the intermediate lobe (Pazos *et al.* 1985, Mansour *et al.* 1990, Pelletier 1993). Acute treatment of rats with bromocriptine or haloperidol has a translational effect on POMC and PC2 expression in the intermediate lobe of the pituitary (Oyarce *et al.* 1996).

In AtT-20 corticotrope tumor cells, endogenous RESP18 is localized to the endoplasmic reticulum and only 18 kDa RESP18 is present. When RESP18 is overexpressed in AtT-20 cells by stable transfection, 18 kDa and higher molecular mass RESP18 isoforms are localized to distal compartments of the secretory pathway (Schiller *et al.* 1995). Higher molecular mass isoforms of RESP18 have also been found in rat anterior and intermediate pituitary (Schiller *et al.* 1995) and testis (Schiller & Darlington 1996). In the anterior pituitary, the 24 kDa RESP18 isoform is sensitive to neuraminidase treatment, suggesting that the increased mass is due, at least in part, to O-glycosylation (Schiller *et al.* 1995). Because the anterior pituitary has both 22 and 24 kDa RESP18 isoforms, it can be used as a model to determine if RESP18 exits the endoplasmic reticulum by detecting higher molecular mass isoforms. Subcellular fractionation identified 22 and 24 kDa RESP18 only in fractions enriched with secretory granules; 18 kDa RESP was present in secretory granule-enriched fractions, but was also recovered in fractions enriched in endoplasmic reticulum. The higher molecular mass RESP18 isoforms appear to be reliable indicators of subcellular compartmentalization.

Although RESP18 was recovered from the secretory granule fraction, biosynthetic labeling of primary anterior

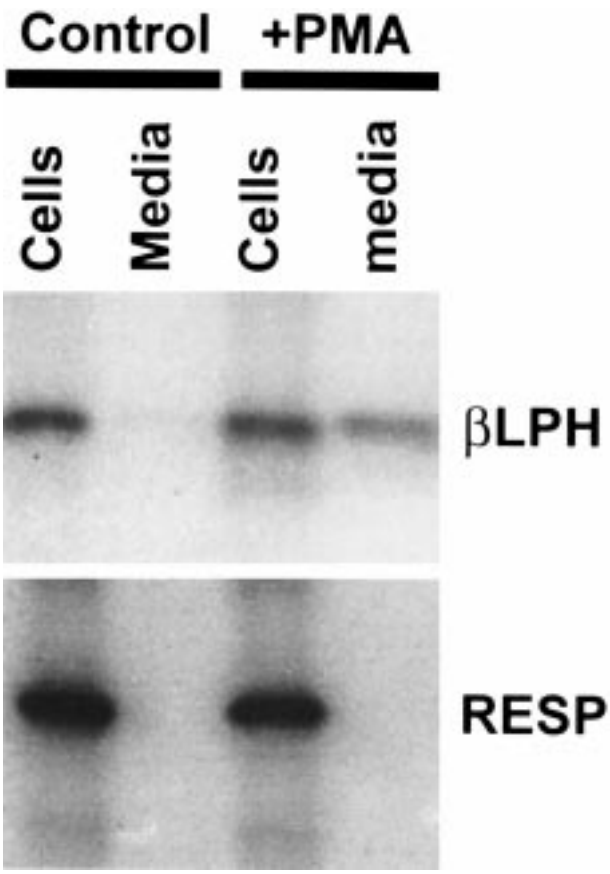

Figure 9 Metabolic labeling. Duplicate cultures of primary anterior pituitary cells were incubated in medium containing [^{35}S]Met/Cys for 1 h followed by unlabeled medium for 2 h. Media were collected over the next hour from control cells and from cells stimulated with 1 mM PMA (+PMA). Cell extracts and media were subject to immunoprecipitation with antisera to RESP18 or β-endorphin; the major product of POMC processing at this time in anterior pituitary is βLPH. Immunoprecipitates were fractionated by SDS-PAGE and newly synthesized proteins were visualized by fluorography. RESP18 was not detectable in the media of control or stimulated primary anterior pituitary cells; release of βLPH was stimulated by PMA.

Table 1 RESP18 is expressed in peptidergic endocrine cells

	RESP18 mRNA co-localized with[1]:	RESP18 isoforms[2]
Tissue/cell type		
Pituitary		18, 22, 24 kDa
Melanotropes	POMC	
Corticotropes	POMC	
Somatotropes	GH	
Lactotropes	Prolactin	
Gonadotropes	FSH and LH	
Thyrotropes	TSH	
Pancreas		18, 22 kDa
α-cells	Glucagon	
β-cells	Insulin	
δ-cells	Somatostatin	
Testes		18, 19 kDa
Spermatocytes, spermatids	Not determined	
Sperm	Not determined	18, 19 kDa
Thyroid		18, 20 kDa
C-cells	CRGP	
Adrenal		18 kDa
Medulla	DβM and NPY	
Pineal	Not determined	18 kDa
Thymus	Not determined	18, 22 kDa
Intestine	Not determined	18, 22 kDa
Superior cervical ganglion	NPY	18, 22, 24 kDa
Plasma	Not relevant	18, 20 kDa

No RESP18 is detected in the liver, lung, parathyroid, ovary, epididymis, testicular interstitial cells, exocrine pancreas, adrenal cortex, and thyroid follicular cells.
[1]Co-localization of RESP18 was performed by immunohistochemistry of protein indicated and *in situ* hybridization of RESP18 mRNA.
[2]RESP18 isoforms were determined by Western blot analysis using RESP18 JH1162 antisera.

pituitary cells in culture failed to demonstrate secretion of RESP18. Newly synthesized high molecular weight isoforms of RESP18 were not detected in culture; while a longer chase time might reveal synthesis of these isoforms of RESP18, cultured cells may lose the ability to produce high molecular weight isoforms of RESP18. Our inability to detect secretion of RESP18 suggests that this protein functions intracellularly, within the secretory pathway. The broad distribution of RESP18 in a wide variety of peptide-producing neuroendocrine cells is similar to that of the granins, carboxypeptidase E and peptidylglycine α-amidating mono-oxygenase, and may complement these proteins as a marker for the peptidergic phenotype.

Although secretion of RESP18 was not detected in anterior pituitary cultures, RESP18 was observed in plasma. At this time, we are not certain of the source of plasma RESP18. The 20 kDa RESP18 isoform is the major form in plasma and this isoform is not found in anterior pituitary extracts suggesting that the pituitary is not the source. Furthermore, hypophysectomy had no significant effect on RESP18 levels in blood (data not shown). Plasma levels of RESP18 were 0·17–0·34 nM, a level 100-fold higher than that of a typical peptide hormone like adrenocorticotropin, which is present in the plasma of resting rats in the 4–13 pM concentration range (Akana *et al.* 1986, Dallman *et al.* 1987, Darlington *et al.* 1992). The source of RESP18 in plasma and whether extracellular RESP18 serves a function remain to be determined.

In summary, RESP18 is expressed in peripheral endocrine glands that secrete peptide and protein hormones. RESP18 is also expressed in the peripheral nervous system

as demonstrated by the presence of mRNA and protein in the superior cervical ganglion and adrenal medulla. Several endocrine glands have cells with high RESP18 mRNA levels and low levels of RESP18 protein, suggesting an important post-transcriptional component to the regulation of RESP18 expression. Several distinct higher molecular mass RESP18 isoforms were observed in tissues expressing RESP18 protein. Subcellular fractionation data suggest that the presence of these higher molecular mass RESP18 isoforms can be used to locate RESP18 in organelles distal to the endoplasmic reticulum. These data support a role for RESP18 in production or regulation of peptide hormone synthesis, although an extracellular function cannot be ruled out considering the presence of RESP18 in plasma.

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