SYMPOSIUM

Proteomics of the Anterior Pituitary Gland as a Model for Studying the Physiology of a Heterogeneous Organ

CHARLES A. BLAKE*,[1] AND STEVE M. HELMKE†

Department of Cell and Developmental Biology and Anatomy, University of South Carolina School of Medicine, Columbia, South Carolina 29208; and †Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado 80045

The anterior pituitary gland (AP) secretes six established hormones that collectively control hundreds of biological and behavioral functions. Because of advances in mass spectrometry (MS), protein labeling, and bioinformatics, it is now possible to characterize, compare, and quantify the AP hormones together with large numbers of nonhormonal AP proteins. For example, by using high-performance liquid chromatography in line with tandem MS we characterized 145 proteins in subcellular fractions of the AP of young adult male Golden Syrian hamsters and 115 proteins in subcellular fractions of the AP of young adult male mice. These included hormones, proteins involved in hormone synthesis and release, and housekeeping proteins. We also used difference gel electrophoresis in conjunction with MS and peptide mass fingerprinting to quantify the effects of estrogen on the AP-soluble protein fraction in rats. Ovariectomized rats were administered 50 μg of estradiol valerate subcutaneously and studied 48 hrs later, before the onset of the anticipated surges of gonadotropins in blood. Following DeCyder image analysis, we identified by MS and peptide mass fingerprinting 26 protein spots that were upregulated and 19 protein spots that were downregulated. Estrogen increased levels of acidic isoforms of growth hormone and prolactin, several proteins involved in protein synthesis, folding and secretion, and several metabolic enzymes. Most of the downregulated proteins are involved in RNA or DNA interactions. We followed up on the results with RT-PCR and immunohistochemical techniques to demonstrate that one protein identified by MS in hamster AP, fertility protein SP22, is synthesized in the AP and localized primarily in somatotropes and thyrotropes. These experiments demonstrate the efficacy of our proteomics approach to characterize AP proteins and quantify changes in them. The approaches used to study the AP could serve as a model to investigate other heterogeneous organs. Exp Biol Med 230:793–799, 2005

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The Anterior Pituitary Gland

The anterior pituitary gland (AP) epithelium contains five major cell types that secrete six established hormones: somatotropes secrete growth hormone (GH), lactotropes secrete prolactin (PRL), gonadotropes secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), corticotropes secrete adrenocorticotropic hormone (ACTH), and thyrotropes secrete thyroid-stimulating hormone (TSH). Variants of all six AP hormones have been identified, and each form has distinct bioactivities and clearance rates from the circulation (1–13). Collectively, these hormones control hundreds of physiological and behavioral functions. The quantity and frequency of release of the AP hormones and their post-translational modifications (PTMs) are controlled both by hypothalamic factors that pass down the pituitary stalk within the long portal vessels and by target organ hormones that feed back at the level of the brain and/or AP.

The effects and interactions of hypothalamic factors and target organ hormones on the secretion of the AP

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[1] To whom correspondence should be addressed at Department of Cell and Developmental Biology and Anatomy, University of South Carolina School of Medicine, Columbia, South Carolina 29208. E-mail: blake@med.sc.edu

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hormones and their PTMs are not fully understood. In addition, little is known about the regulation of non-hormonal AP proteins, many of which mediate the synthesis and release of the AP hormones. When mass spectrometry (MS) is used to analyze the AP in appropriate animal models (e.g., Ref. 14), it is now possible to determine the response of the AP proteome to exogenously administered hypothalamic factors or target organ secretions, alone or in combination. Furthermore, such investigations open the door for discovery of novel AP secretions and intracellular proteins involved in regulating AP function. In spite of the potential of proteomics to contribute to this important area, there has been a paucity of such studies conducted on the entire pituitary gland (e.g., Refs. 15, 16) and specifically on the AP (17, 18).

Here we present, summarize, and compare some of the proteomic studies we have conducted on the AP of three rodent species. We discuss important decisions that needed to be made as first steps before employing high-performance liquid chromatography (LC) in line with tandem MS (MS/MS) to analyze subcellular fractions of the AP of Golden Syrian hamsters and mice. We then describe how difference gel electrophoresis (DIGE) technology was used in conjunction with matrix-assisted laser desorption ionization–time of flight MS (MALDI-ToF MS) and peptide mass fingerprinting (PMF) to quantify the effects of estrogen on the AP proteome in rats. Subsequently, we discuss the matching of proteomic results to specific cell types within the gland. Although the present overview focuses on the AP, these studies have the potential to serve as a model for investigation of other heterogeneous and multifunctional organs.

The AP Proteome and Proteomic Strategies

Proteomics is the study of the proteome, or the expressed protein complement of a biological tissue or fluid. Proteomics frequently includes the identification of the components, together with an assessment of the changes that occur in them in different states. The AP proteome is a dynamic entity composed of the individual proteins that are expressed in the gland, but it changes over life span, during alterations in physiological state, and with disease. The AP proteome changes during development, the female reproductive cycle, pregnancy, lactation, and aging; in response to hypothalamic factors and target organ secretions; with changes in the general health; and with the formation of adenomas. Posttranslational modifications of proteins are part of the changing proteome and may be especially important in determining function.

We have employed two different proteomic strategies to study the AP. These are illustrated in the flow diagrams in Figure 1. Procedures for subcellular fractionation of the AP do not necessarily differ between the two strategies, but they are distinguished by the timing of when the digestion is performed (i.e., trypsin digestion followed by peptide separation or protein separation followed by trypsin digestion). In the trypsin digestion and peptide separation strategy, peptides were separated and analyzed by LC-MS/MS using the database search software SEQUEST (19, 20). Because a mixture of proteins is digested in these studies, a peptide containing a PTM cannot be traced back to a specific protein form (i.e., a splice isoform or some other modified form of a protein). Although we did not quantify proteins in these studies, relative quantification of the proteins in two samples can be performed by using isotopic labeling strategies (e.g., Ref. 21). In the protein separation and trypsin digestion method, proteins are minimally labeled with cyanine dyes (CyDyes) and separated by 2D gel electrophoresis. Fluorescent gel images are acquired with a laser scanner and the gel spots quantified using DeCyder software (22). In our studies the gels were then poststained with Deep Purple and the spots excised robotically. The proteins in the gel spots were then digested with trypsin, and the mixture of proteolytic peptides was analyzed by MALDI-ToF MS. Proteins were identified by PMF using the database search software MASCOT.

Factors to Consider When Conducting Proteomics on the AP

We considered a number of factors before initiating proteomic analysis of the AP. These included the selection of species, gender, age, and physiological state of the animal to be investigated; determination of the time of day for collection of tissue and method of sacrifice; incorporation of

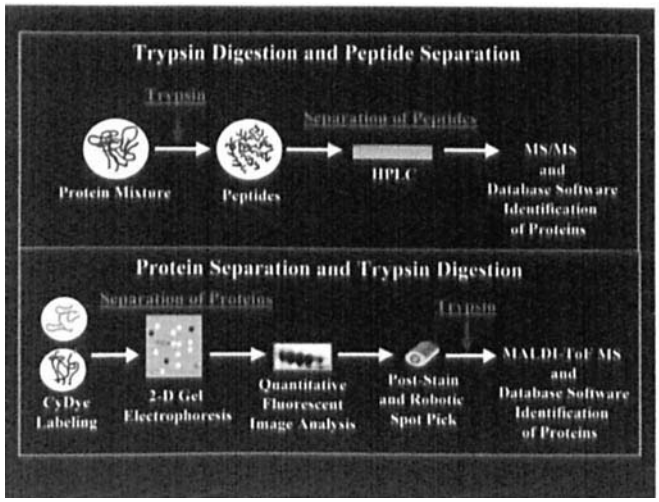

Figure 1. Comparison of the two proteomic strategies employed. In the protein separation and trypsin digestion strategy, an aliquot of a protein fraction from a control group was minimally labeled with Cy3 (green), and that of an experimental group was minimally labeled with Cy5 (red). An aliquot of a mixture of the two fractions was minimally labeled with Cy2 (blue; not shown) as an internal standard. Red spots on the 2D gel = upregulated proteins and green spots = down-regulated proteins (in the experimental relative to the control group). Yellow spots = proteins not differentially regulated. See Ref. 32 for details.

methods to enhance the purity of the tissue; fractionation of the gland into subcellular compartments to increase the number of proteins identified; and formulation of the proteomic strategy.

The decision was made to conduct initial proteomic analysis of the AP in Golden Syrian hamster despite the fact that its genome was only partially sequenced. It was selected because one of us (C.A.B.) has used this animal model extensively for neuroendocrine investigations. Subsequent studies were conducted on mouse and rat, species in which the genome has been sequenced. In our initial studies of the AP of the young adult male, we adopted hamster and mouse models. Thereafter, ovariectomized rats were used to assess the effects of estrogen on the AP proteome.

Rodents were kept in a room with controlled lighting conditions (12:12-hr light:dark cycle). They were killed during a specified time interval during the first half of the light period. This avoided any changes in the AP proteome that might occur in response to circadian fluctuations (23). In addition, this time interval preceded the time of the surge of gonadotropins in blood that starts during the second half of the light period in proestrous animals or ovariectomized rodents administered estrogen (24, 25).

Stress and anesthetics can influence proteomic analysis by affecting AP hormone release (e.g., Refs. 26–28) and possibly by altering the levels of nonsecreted AP proteins. Thus, it is of interest to avoid stress and to select an anesthetic agent that has minimal effects on AP hormone release. Two different methods of sacrifice were used in the hamster studies. Some hamsters were decapitated; others were anesthetized with sodium pentobarbital before perfusion (17). Decapitation minimizes stress but has the disadvantage of leaving substantial amounts of blood in the AP. Within minutes of injection, pentobarbital suppresses LH release (29) and causes only a small 2- to 4-fold increase in circulating PRL concentrations (30). The subsequent perfusion procedure removes large amounts of blood proteins that can interfere with and mask the analysis of AP proteins. Pentobarbital anesthesia and perfusion were employed in the mouse and rat studies.

Ideally, one would like to be able to expose, remove, and snap freeze or immediately use the AP or any other organ or tissue for proteomic analysis within a few seconds of touching the animal. This would minimize any proteolysis that might occur during the collection procedure. Any proteolysis compromises protein identification, especially when a gel-based, protein separation strategy is employed. Unfortunately, there is poor accessibility to the pituitary gland because of its location inside the skull. In addition, it takes time to separate the AP from the posterior lobe (pars intermedia [PI] and pars nervosa) along the line of Rathke's cleft (Fig. 2A). In rat, most all of the cells in the PI stain immunohistochemically for ACTH, but only selective cells in the AP stain for ACTH (31). After removal of the posterior lobe, staining for ACTH on horizontal sections of the AP can be used to assess contamination of the tissue

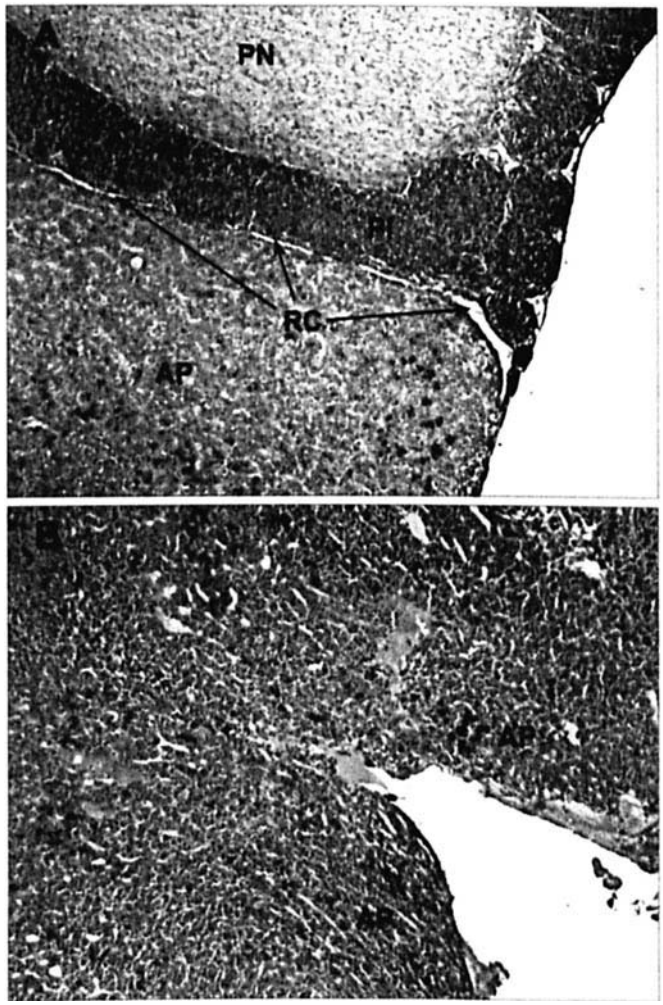

Figure 2. Photomicrographs (100×) of horizontal sections of rat pituitary gland stained immunohistochemically for ACTH and counterstained with hematoxylin. The section shown in (A) was taken from the dorsal third of the gland. It shows a portion of the left side of the anterior pituitary gland (AP) separated from the pars intermedia (PI) by Rathke's cleft (RC). The posterior surface of the gland is on the right. Selective cells in the AP stained brown for ACTH. Brown staining also can be seen over the entire PI but not in the pars nervosa (PN). The section shown in (B) was taken from the middle third of the gland after the posterior lobe was removed. Only AP tissue remains, as evidenced by selective cells staining for ACTH. The white area between the two AP labels represents missing posterior lobe tissue.

with cells of the PI (Fig. 2B). A major concern when analyzing the AP proteome is that it is free of pars nervosa tissue. Analysis of entire pituitary glands and the posterior lobe of mouse by LC-MS/MS has demonstrated that oxytocin and vasopressin neurophysins are excellent indicators of pars nervosa tissue in AP preparations. Others have reported vasopressin-neurophysin 2-copeptin precursor in postmortem human pituitary glands analyzed by LC-MS/MS (16).

Proteomic analysis of the AP will include some protein in addition to that in the glandular epithelium. Endocrine glands including the AP are highly vascular, and perfusion serves to remove most, but not all, of the blood protein.

Albumin and hemoglobin alpha and beta chains are excellent indicators of the degree of blood contamination. In perfused animals, almost all the AP tissue is the glandular epithelium; however, some endothelial cells lining the sinusoids are included. A thin, dense collagenous capsule encloses the external surface of the AP. It is included with the collection of the AP. This capsule, composed of collagenous fibers penetrating from the capsule to parts of the AP, and smaller reticular fibers, also consisting of collagenous fibrils that lie external to the epithelial cells and thin-walled sinusoids, are not included in the proteomic analyses. In the hamster, mouse, and rat studies (17, 18, 32), connective tissue was discarded by centrifugations during the preparation of subcellular fractions of the gland. In the rat studies, any residual connective tissue protein was not soluble to an appreciable extent in urea, thiourea, and CHAPS (32).

The weight of the AP in a young adult male is approximately 4 mg in hamster, 2 mg in mouse, and 7 mg in rat. Based on analyses of ovariectomized rat APs, the protein recovered in reaction buffer relative to tissue wet weight was approximately 8%. The distribution of protein in our subcellular fractions was approximately 50% soluble, 30% membrane, and 20% cytoskeleton/chromatin. Proteomic analysis can be performed on a single subcellular fraction of AP tissue by LC-MS/MS using approximately 10 μg of protein. The analyses are sensitive and can detect picomolar amounts of a protein. However, we combine APs to form a pool for two reasons. First, it enables us to prepare multiple protein fractions for replicate analyses, and, second, it ensures that the results we obtain are more representative of the proteome of the population in general rather than a single individual. A single hamster, mouse, or rat AP does not provide enough protein to allow direct analysis by DIGE. We therefore combined several glands, prepared subcellular fractions, used 500 μg of protein per gel, and performed analytical replicates.

To increase the total number of proteins identified, AP homogenates were separated into different fractions, and we treated and analyzed each fraction separately. We generated three subcellular fractions in our initial studies of the hamster and mouse AP: nuclear and nonnuclear 100,000 *g* and cytosolic fractions. In these studies, the trypsin digestion and peptide separation strategy was employed. In rat, the proteomes in two groups were compared. We prepared soluble protein fractions that were similar but not identical to the cytosolic fractions of hamster and mouse. We employed the protein separation and trypsin digestion strategy in these investigations.

LC-MS/MS Study of the AP of Young Adult Male Golden Syrian Hamster and Mouse

We identified 145 different proteins in hamster AP (17) and 115 different proteins in mouse AP (18) using similar subcellular fractionation procedures. These included hormones, proteins involved in hormone synthesis and release, and housekeeping proteins. In hamster, only 15 of the 145 proteins identified had amino acid sequences that matched to those of hamster proteins in the databases. This primarily reflects the incompleteness of the hamster protein databases at this time. However, because of considerable homology between hamster proteins and those of human, mouse, and rat, data derived from hamster proteins could be matched. It is also interesting to note that our successful identification of nearly 150 proteins in the hamster study indicates that the paucity of genome and proteome data on this species is not a major impediment. However, it is conceivable that a specific protein may lack homology with database entries for other species and will therefore be missed completely.

In hamster we identified the most proteins in specific fractions when perfusion was employed. Although these comparisons were not conducted under controlled conditions, we made the decision to adopt perfusion for collection of the glands in the subsequent studies of mouse and rat. However, perfusion did not totally eliminate albumin or hemoglobin chains from all the fractions in any of the three species, indicating that blood proteins and red blood cells were not completely washed out of the AP even when this step was incorporated. In hamster, mouse, and rat studies, there was no indication of any contamination of the APs with neurohypophysial tissue as evidenced by failure to detect neurophysins or any of a large number of proteins that have been described as specific to neural tissue.

The protein extraction and subcellular fractionation procedures employed to investigate the hamster (17) and mouse (18) AP proteomes were very similar, and the LC-MS/MS and SEQUEST analyses in the two studies were virtually identical. For these reasons and the fact that young adult males were used in both studies, it was possible to make some interesting comparisons and observations of the AP proteome in both species (18).

The proteins identified in the basic subcellular fractions of hamster and mouse are likely the more abundant proteins in the AP. Changes in these could be investigated in different treatment groups in future quantitative studies using isotope-coded affinity tags (21) and LC-MS/MS. It is also important to note that many of these same proteins were characterized in the AP of the ovariectomized rat using 2D-DIGE and MALDI-ToF MS (32). Proteins identified in the AP of hamster, mouse, and rat included some previously not reported in this tissue and that may be of interest for further study. A brief report of one such protein identified in the hamster AP is summarized at the end of this article.

DIGE and MALDI-ToF MS Study of the AP of the Estrogen-Treated Ovariectomized Rat

Estrogen has been shown to be involved in the regulation of all six of the established AP hormones (32);

however, little is known of the specific regulation of the AP hormones and nonhormonal AP proteins by this steroid. We have investigated the effects of estrogen on the AP of 2-month-old female rats that were ovariectomized and used at 6 months of age. They were injected subcutaneously with sesame oil vehicle or 50 μg estradiol valerate in vehicle. They were then studied 48 hrs later, approximately 3 hrs before the time of the anticipated onset of the estrogen-induced surges of LH and FSH in blood (33). Soluble, membrane, and cytoskeleton/chromatin fractions were prepared, and initial findings for the soluble fraction have been reported (32). Following DeCyder image analysis, we identified by MALDI-ToF MS and PMF 26 protein spots that were upregulated and 19 protein spots that were downregulated.

Estrogen treatment increased the levels of acidic isoforms of several proteins including GH and PRL; several proteins involved in protein synthesis, folding, and secretion; and several metabolic enzymes. The majority of the downregulated proteins were involved in RNA or DNA interactions, including five heterogeneous nuclear ribonucleoproteins. Detailed lists of the regulated proteins have been reported (32). These experiments demonstrate the efficacy of proteomics to identify AP proteins and perform relative quantifications on them. The results clearly indicated that estrogen acts *in vivo* to modulate the AP proteome (32), but the data do not enable us to determine whether estrogen acted directly on the AP or indirectly via the hypothalamus or by some other means. Further, because the homogenized AP tissue is a mixture of different hormone-secreting cells, these results do not determine the localization for changes in content of an individual protein.

Matching Proteomic Results to Specific Cell Types Within the AP and Ancillary Techniques

Use of MS for proteomic analysis of AP tissue is effective for identifying large numbers of proteins and their isoforms, but the results do not establish that the AP synthesizes the proteins identified, nor can it determine the cell types or individual cells that contain a particular protein. Many of the proteins we identified, such as histone 4 in hamster (17) and mouse (18), are housekeeping proteins, and one would expect them to be synthesized within all AP epithelial cell types. Other proteins, such as LHβ, are cell specific and are likely localized only in gonadotropes. Yet others involved in controlling hormone secretion might be in one or more cell types, depending on whether they are associated with the secretion of one or more hormones or all AP hormones in general. One can validate that the AP synthesizes the mRNA needed to synthesize a specific protein and localize the protein to specific cells within the gland. However, such studies are time consuming and expensive. Thus, one needs to be discriminating with regard to selecting proteins for study. The following summary of our initial studies on sperm protein 22 (SP22) illustrates some of the approaches we have employed.

Utilizing LC-MS/MS, we identified SP22 in cytosolic fractions of perfused hamster AP (17). This protein was of interest because it had not been localized specifically to the AP and it had been implicated in apoptosis, androgen receptor function, fertility, and ontogeny of early-onset Parkinson's disease.

As a first step, we cloned the cDNA for full-length SP22 from the AP in individual Golden Syrian hamsters and rats to confirm the ability of cells within the AP to synthesize SP22 and to eliminate the possibility that all the SP22 was synthesized elsewhere and stored in the AP. Individual tissue samples were placed into nuclease-free microfuge tubes containing Trizol reagent and homogenized. The RNA was extracted and used for reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA (1 μg) from AP, posterior lobe, and testis (positive control) was reverse transcribed using MuLV reverse transcriptase and amplified using Taq DNA polymerase (34). Primers were made using known homologous sequences for hamster and rat SP22 available in GenBank. Amplified cDNAs (570 base pairs) were obtained from all APs, posterior lobes, and testes. Amplicons were subcloned into plasmids and sequenced to confirm the identity of the SP22 cDNA. DNA sequencing confirmed the presence of the SP22 mRNA amplified in the AP and posterior lobe of the hamster and rat (34).

We then used standard peroxidase and fluorescence immunohistochemical techniques on Bouin's fixed tissue to determine which cell types contained SP22. Serial sections of the pituitary gland were mounted on slides in a paired flip-flopped orientation as previously described (31), resulting in the exposed surfaces of each pair of sections being mirror images of one another. Mirror image sections were labeled with SP22 and LHβ, TSHβ, PRL, ACTH, or GH using peroxidase conjugated secondary antibody. Additional sections were colabeled with SP22 and one of the AP hormones using fluorescent secondary antibodies. Immunostaining for SP22 revealed its presence in both species in numerous cells throughout the AP and the pars nervosa but not in the PI. The SP22 colocalized in somatotropes and thyrotropes in hamster and rat. We identified SP22 in a small percentage of corticotropes, gonadotropes, and lactotropes. This was the first report that SP22 was present specifically in the AP and localized primarily in somatotropes and thyrotropes, suggesting that SP22 may help regulate AP function and be particularly important for the control of GH and TSH secretion (34).

Other techniques in addition to RT-PCR and standard peroxidase and fluorescent immunohistochemistry can be used to compliment proteomic findings. Relative quantitative real-time RT-PCR can be used to determine or confirm that upregulation or downregulation of a specific protein under treatment conditions is accompanied by alterations in mRNA accumulation. However, changes in mRNA do not

necessarily reflect alterations in protein accumulation, and they do not determine PTMs. For this reason, we emphasize measurement of protein rather than mRNA in functional studies. Western blot analyses enable comparison of protein concentration in multiple animals comprising different groups at one time. The membrane also can be probed multiple times to study additional proteins. In this manner it is possible to compare relative changes in multiple proteins between individual AP samples. In studies using RT-PCR or Western blot analyses, data represent changes in the entire AP.

One can use immunohistochemical-stained sections of AP to determine cell volume density (35) and percent of different AP cell types (36). This information, coupled with AP weight, may help determine whether altered regulation of a protein is due to changes occurring within individual cells or a result of an increase in number of cells that express that protein. In cases where we have had difficulty in staining for nuclear proteins by standard peroxidase and fluorescent immunohistochemistry, we have used heat-induced antigen retrieval successfully (37). Although antisera can be specific with regard to binding to a particular protein, they often are of insufficient specificity to identify isoforms of the protein. The six established AP hormones and other AP proteins have been found to be located in select cell populations or subpopulations. It should be noted that species variations and variations due to physiological state have been observed (38–40).

The previously mentioned proteomic and accessory techniques address analysis of AP tissue and not dispersed AP cells or cell lines. It is important to note that normal function of the AP is often not maintained when the cytoarchitectural relationships within the AP are disrupted (41), and it is highly unlikely that dispersed AP cells or any of the limited number of AP cell lines that are available express the normal complement of proteins.

In summary, recent technological advances provide the opportunity to identify the AP proteome and large-scale changes in the proteome in response to changes in physiological state and experimental or disease conditions. Characterization of these changes, coupled with ancillary techniques, can elucidate changes in the AP that result in normal and altered secretion by this master gland. The procedures and techniques we employed to study the AP could be employed to investigate the proteome of other heterogeneous and multifunctional organs.

Additional information has been published on the LC-MS/MS studies conducted on young adult male hamsters and mice with David G. Kakhniashvili and Steven R. Goodman at the University of Texas at Dallas in Richardson, Texas (17, 18); the DIGE and MALDI-ToF MS studies conducted on estrogen-treated ovariectomized rats with Lewis M. Brown, Stephen W. Hunsucker, and Mark W. Duncan at the University of Colorado at Denver and Health Sciences Center in Aurora, Colorado (32); and the SP22 studies conducted on hamster and rat with Allison M. Benoit at the University of South Carolina School of Medicine and George L. McCoy at Benedict College in Columbia, South Carolina (34).

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