Review Article

Theme: Fishing for the Hidden Proteome in Health and Disease: Focus on Drug Abuse

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Fishing for the Hidden Peptidome in Health and Disease (Drug Abuse)

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Abstract. The proteome and peptidome are defined as the set of proteins and peptides present in a tissue or other biological sample. In most proteomic studies, only abundant proteins are detected and, although these are important molecules, they are often well-studied structural proteins. A number of approaches have been used to examine less abundant molecules that play roles in signaling or otherwise have regulatory functions, including peptides as well as proteins such as enzymes and receptors. The overarching goals of this special issue involve defining the peptidome, identifying the current state of the field, and discussing methods to characterize the peptides, their receptors, and future needs for such measurements.

KEY WORDS: biomarker; peptidase; peptides; peptidomics; proteomics.

INTRODUCTION

This special issue includes a number of articles based on work initially presented at a National Institute on Drug Abuse (NIDA)-sponsored workshop held May 21–22, 2009 that was focused on the “hidden” peptidome. As the proteome is defined as the set of proteins present in a tissue or other biological sample (serum, extracts of cell lines, etc.), a hidden proteome is one that is normally not characterized via conventional proteomics measurements. The most common method of analyzing proteins involves digestion of the proteins with trypsin and analysis of the products by liquid chromatography and mass spectrometry (LC/MS)(1). In most proteomic studies, information on the highly abundant proteins that are present in the sample extract is obtained. While these can be important proteins, they are often well-studied structural proteins. A number of approaches have been used to examine less-abundant molecules that play roles in signaling or otherwise have regulatory functions. This NIDA workshop was organized and conceived by Rao Rapaka specifically to address the brain peptidome as well as proteins important in peptide function (such as receptors). The overarching goals involved defining the peptidome, identifying the current state of the field, and discussing methods to characterize the peptides, their receptors, and future needs for such measurements.

A variety of methods have been used to characterize the hidden proteome, with these methods oftentimes divided into major categories; those that involve sample fractionation (separation techniques prior to proteomic analysis) and those that involve selective screening/data analysis to identify the relevant molecules from the complex tissue. Both approaches can reveal the hidden peptidome, the former by reducing the complexity of the sample prior to analysis, and the latter by identifying the functional molecules hidden within the large datasets. This meeting specifically highlighted a number of approaches to examine the endogenous peptides present in various samples, with these peptides being formed without the addition of trypsin or other proteolytic enzymes.

Why devote so much effort to characterize the peptidome? Peptides play many important roles in cell–cell signaling, with more than eighty known prohormones from most individual vertebrate species that encode more than a hundred known bioactive peptides, as well as hundreds of additional neuropeptide-like molecules. Several peptides are known to play critical roles in the response to drugs of abuse, as well as to contribute to other disorders/diseases, and therefore it is important to understand the identity and function of the numerous peptides present in an organism.

A critical step for successful peptidomic studies is sample preparation. Recent developments include selective sampling, optimized extraction, and approaches to selectively isolate peptides such as microfiltration and nanoparticle capture beads. The latter selectively binds peptides in a sample such as plasma and protects the peptide from proteolytic degradation. Another approach is the purification of secretory vesicles followed by proteomic analysis of their contents; this approach can detect both neuropeptides as well as other secretory pathway proteins. Neuropeptide-like molecules can

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also be identified from a bioinformatics screen of the genome, and then the predicted prohormone-processing products can be selectively analyzed for functional components. Lastly, combinatorial libraries can be used to identify novel molecules.

In this overview, we briefly outline the key questions in characterizing the peptidome and highlight the various approaches used to address them. New tools/methodologies are also discussed, along with the relevance to health and diseases such as drug abuse.

THE NEUROPEPTIDES

The term proteome is often used to refer to both proteins and protein-derived peptides, largely because the current proteomic techniques usually convert proteins into tryptic peptides prior to analysis and do not directly analyze the intact proteins. However, the natural peptidome of a cell, tissue, or other biological sample is quite different than the proteome, with the peptidome reflecting specific proteolytic events that occur within the cell. In the case of the enkephalins and other neuropeptides produced in the secretory pathway, endopeptidases and exopeptidases cleave the precursor at specific processing sites to generate the mature peptide (2). The peptides are then stored in secretory vesicles until stimulation of the cell causes the vesicles to be released. After release, neuropeptides and hormones can undergo further processing, both to create new biologically active peptides as well as catabolic nonactive products. Much of the research in the field has concentrated on peptides and the term neuropeptide has grown to include both the biologically active peptides from prohormones, and more loosely, other peptides derived from them during their processing.

Through the 1980s, only a small number of important biologically active peptides were characterized because of the large effort needed to purify and identify neuropeptides. As the field of peptidomics has matured, the ability to identify peptides has become extremely fast and sensitive. Now, one can take a brain region from a single mouse and obtain lists of hundreds of peptides derived from prohormones as well as other proteins that appear to be endogenous peptides. What are these peptides? Are they biologically active? Other questions involve prohormones expressed within non-neuronal cells. For example, most researchers would consider enkephalin found in a brain region a neuropeptide, but now we know that some of the peptide may be from glia cells (as their RNA is known to be in glia) in addition to neurons, which clearly produce enkephalin. Many texts define neuropeptides as derived from neurons only and so it may be that a fraction of the peptides in the brain are glia-derived, but the term, glia-peptides, is not common (although it is found). Regardless, we and others normally consider brain peptides to be neuropeptides, although this distinction according to cell type of origin may be functionally important. Peptides produced within the secretory pathway can be referred to as classical neuropeptides, by analogy with classical neurotransmitters such as acetylcholine, which are also stored in vesicles and released into the synapse upon stimulation. Non-classical neurotransmitters such as nitric oxide and anandamide are synthesized upon stimulation and then

constitutively released. Are there cytosol-derived peptides that function in cell-cell signaling? Here we term these "non-classical" neuropeptides. In this issue, Gelman and Fricker discuss the evidence for non-classical neuropeptides with a focus on peptides named hemopressins; these peptides bind to CB1 cannabinoid receptors and either function as agonists or inverse agonists/antagonists, depending on the length of the peptide (3). The hemopressins and other hemoglobin-derived peptides are also the focus of a review by Gomes *et al.* (4). In addition to the hemopressin and other hemoglobin-derived peptides, there are other examples of cytosolic peptides that have been reported to function in cell-cell signaling. Well-studied examples include interleukin 1 β and basic fibroblast growth factor, both of which clearly function in cell-cell signaling but are secreted via an unconventional mechanism from the cytosol (5). Beta thymosin is another cytosolic peptide/protein that is released from neurons in an activity-dependent manner and has several reported functions outside the cell (6). Another well-studied example of a biologically active cytosolic peptide is the yeast a-mating factor; this peptide is secreted from the cytosol by Ste6, an ATP-dependent membrane transporter (7). Therefore, there is considerable evidence that cytosolic-derived peptides and proteins can have extracellular functions in cell-cell signaling, and so the concept of non-classical neuropeptides is not as radical as it initially sounds.

In addition to potential functions in cell-cell signaling, intracellular peptides may also function in intracellular signaling; some peptides are known to be cleaved from proteins and enter the nucleus where they function in gene transcription (8). Other peptides may function in the cytosol, affecting protein/protein interactions; although there are no well-known examples of endogenous peptides playing this role, there are numerous examples where synthetic peptides corresponding to a protein-derived peptide influence the function of that protein (9).

FROM DISCOVERY TO FUNCTION

Although mass spectrometry has been used for several decades to study peptides, advances in the last 10–15 years has led to an explosion of data, with a typical peptidomics study generating long lists of peptides (10,11). Given the complexity of potential peptide function, it is helpful to have screening approaches that are targeted towards specific functions. In this issue, Lindberg, Roth, and colleagues (12) describe a bioinformatics approach to identify candidate prohormones based on similarities to known prohormones (i.e., the presence of a signal peptide to target the protein to the secretory pathway, specific cleavage sites recognized by the prohormone convertases and carboxypeptidases). Then, after verifying that the protein is processed by these enzymes into peptides, these peptides are screened for activity towards hundreds of known cell-surface G protein-coupled receptors. Hook and colleagues describe a related approach to identify novel neuropeptides by the isolation of neuropeptide-containing secretory vesicles followed by the proteomic analysis of the contents (13). While useful for identifying the presynaptic vesicle contents, this approach does not detect the forms of peptides produced by synaptic processing enzymes.

Unlike classical neurotransmitters such as acetylcholine, which are active until processing by extracellular enzymes that rapidly inactivate the molecule, peptides can be processed into forms that retain some biological activity, or even have distinct activities from the original form of the peptide. Therefore, knowledge of the extracellular processing events is important for fully understanding the forms of peptide that the receptor will be exposed to. Saghatelian and colleagues describe the peptidomics analysis of dipeptidyl peptidase 4 and other prolyl-directed peptidases (14). Extracellular processing is not limited to neuropeptides secreted via the regulated pathway, but can also occur for non-classical neuropeptides secreted from the cytosol via an unknown mechanism. In fact, in Saghatelian's analysis of mice lacking the extracellular processing enzyme dipeptidyl peptidase 4, several "cytosolic" peptides were found to be major substrates of this enzyme; this implies that either the extracellular enzyme is present and functional within the cytosol, or that the peptides are secreted and cleaved by the enzyme in the extracellular environment. The mechanism of secretion is not known, but peptidomics analysis done by Ferro and colleagues, suggest that the antigen peptide transporter TAP1 is not involved due to the similar levels of cytosolic peptides detected in brains of wild-type and TAP1 knock-out mice (15). Other possible mechanisms of peptide transport from the cytosol to the extracellular environment include the ATP-dependent transporters named p-glycoproteins, gap junction hemichannels, and direct insertion into the membrane; specific peptides are known to transit across membranes by all of these mechanisms. Further studies are needed to define the precise mechanism of peptide secretion from the cytosol.

While many peptides may be found in a tissue extract, unless they are found extracellularly, they cannot function in cell to cell signaling. Thus, measurement of the released component is critical. Several approaches have been demonstrated recently, including bead collection, and microdialysis. Analysis of peptide secretion is complicated by the extracellular peptide processing that occurs in most systems, and a potential solution is nanoparticle capture technology. Petricoin and colleagues describe a bead-based capture method that is specific for peptides and protects the molecule from further degradation (16). Although used for analysis of peptides in plasma as biomarkers for ovarian and prostate cancer, this approach should be easily adapted to use for analysis of the "secretome" of any cell or tissue.

Studies on the enzymes involved in the generation of non-classical neuropeptides and other cytosolic peptides would benefit from a nanoparticle capture technique that prevents secondary cleavages of peptides formed from proteases. There are a number of candidate cytosolic enzymes, based on the extensive literature on intracellular protein turnover. However, intracellular peptides are thought to be unstable, with half lives of less than 10 s (17), so either a subset of protein degradation fragments that are detected in peptidomics experiments are much more stable than others, or there is a selective production of this subset of peptides. Evidence for the former of these two possibilities has been provided from studies on antigenic peptides, and appears to involve binding to a heat-shock protein (18). Further studies are needed to determine whether a similar mechanism can

account for the selective detection of specific protein fragments seen in most peptidomics studies.

Other approaches for determining peptide function include determining if their levels change as a result of disease or drug exposure. Several methods of quantitative peptidomics have been developed. Many approaches involve the isotopic labeling of the samples, either by exposing cell lines to isotopically labeled amino acids so that the peptides are labeled *in situ*, or more commonly, by extracting peptides from cells or tissues and then labeling with chemicals containing various numbers of heavy isotopes. Then, samples are pooled and the peptides are purified and analyzed by LC/MS; the peak intensities reveal the relative levels in the original samples (11). Label-free methods that rely on spectral counts as a measure of protein/peptide levels are also being used (19), although these techniques have yet to be validated for peptidomics measurements; in this issue, Romanova *et al.* demonstrate the use of principle component analysis to measure peptide changes between treatments (20).

SCREENING/DATA ANALYSIS

Peptidomics approaches typically generate long lists of peptides, and it is important to screen these for functions. As mentioned above, Lindberg, Roth, and colleagues describe a high through-put approach to screen a large number of G protein-coupled receptors with putative neuropeptides (12). Devi and colleagues describe the discovery of the bioactive properties of the hemopressins by screening of G protein-coupled receptors with a novel antibody-based approach to detect activated receptors (4). Houghten and colleagues describe the screening of combinatorial libraries for compounds that show antinociceptive activity in a mouse model (21). Collectively, these approaches provide a better understanding of function as well as lead compounds for development of therapeutics.

RELEVANCE TO DRUG ABUSE

Given the research interest of NIDA, what is the relevance of peptidomics to drug addiction? Many of the drugs of abuse directly affect critical neurochemical pathways such as dopamine. Peptide receptors are the direct target of many drugs of abuse; well-known examples include heroin and related opiates. In addition, the hemopressins bind to CB1 cannabinoid receptors and may represent endogenous peptide endocannabinoids (4). Peptides are also clearly involved in the physiological responses to several drugs of abuse. For example, mice lacking mu opioid receptors do not show drug-reinforced behaviors for a number of addictive agents including nicotine, alcohol, cocaine, and tetrahydrocannabinol (22). Peptides also regulate mood, anxiety, depression, learning, and memory, and other behaviors that contribute to the addictive processes. Finally, some peptides are regulated by exposure to drugs of abuse; this is discussed by Sweedler and colleagues, who examined the effect of acute cocaine on levels of peptides in mouse brain (20). Understanding which peptides are regulated by drugs of abuse may provide additional clues as to the function of the peptides and/or biomarkers for drug exposure.

PERSPECTIVES/FUTURE DIRECTIONS

Considering the work illustrated here, what does the future bring? The new tools for peptide characterization have resulted in large increases in our knowledge of the peptidome—the peptide parts lists used by the brain. The rate of discovery has accelerated over the past decade, and may continue to accelerate as advances in mass spectrometry instruments allow for greater accuracy and sensitivity. An active area will be to understand the functions of the various peptides found in brain. Other major areas for research include understanding the routes to the formation and release of cell to cell signaling peptides (such as the non-classical secretion described above); new information on glia and neuron interactions; additional receptors for peptides that are not GPCR-based; and unusual or transient chemical modifications of peptides. Peptidomics discovery will certainly remain vigorous into the foreseeable future!

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