Tandem Mass Spectrometry in Physiology

Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) allows identification of proteins in a complex mixture without need for protein purification (“shotgun” proteomics). Recent progress in LC-MS/MS-based quantification, phosphoproteomic analysis, and targeted LC-MS/MS using multiple reaction monitoring (MRM) has made LC-MS/MS a powerful tool for the study of cell physiology.

A mass spectrometer is a device that measures the mass-to-charge ratio \((m/z)\) of charged chemical species. Modern mass spectrometers are capable of such high mass accuracies that, in principle, an investigator can identify charged species solely from the measured \(m/z\) or the derived mass. Two relatively recent developments have opened mass spectrometry to the identification of proteins and peptides. The first development was the invention of techniques that are capable of volatilizing peptide ions and delivering them to the vacuum environment of the mass spectrometer. These are the electrospray technique and matrix-assisted laser desorption and ionization (MALDI). The invention of these methods led to Nobel Prizes in Chemistry in 2002 for their inventors: John Fenn of Virginia Commonwealth University and Koichi Tanaka of the Shimadzu Corporation in Japan. The second development was the completion of genome projects for various animal, plant, and prokaryotic species. The nucleotide sequence data from these projects and the amino acid sequences of the corresponding proteins give the theoretical \(m/z\) values for proteins and peptides necessary to match to the measured \(m/z\) values. These matches identify the proteins (see LC-MS/MS Fundamentals below).

Tandem mass spectrometry (MS/MS) of proteins was initially developed as a qualitative technique capable of identifying large numbers of proteins in tissues, cells, and organelles but not necessarily quantifying them. The task of making MS/MS a quantitative approach has been a major area of focus in research laboratories throughout the world in recent years. The objective of this paper is to describe MS/MS with an emphasis on quantitative approaches in the context of cell physiology. We do not attempt an exhaustive review of applications thus far but rather focus on illustrative examples. Most of the examples presented here are from our own work using quantitative protein mass spectrometry to identify vasopressin signaling pathways involved in the regulation of the water channel aquaporin-2 in the renal inner medullary collecting duct (IMCD) cell. However, the principles are general and can be applied to a wide variety of physiological problems. A more extensive treatment of quantitative proteomics applied to physiology can be found in a paper by Hirsch et al. (9), with a number of examples in the area of lung physiology.

**LC-MS/MS Fundamentals**

**FIGURE 1** shows a simple view of the fundamental processes generally involved in most tandem mass spectrometry (MS/MS) experiments, where the investigator wishes to identify as many proteins as possible in a complex mixture (“shotgun” proteomics). Because the mass spectrometer is linked to a high pressure liquid chromatography (HPLC) unit, the overall approach is often referred to as LC-MS/MS. We are concerned here with the principles of the LC-MS/MS technique and the work flow needed to do shotgun proteomics and not with the workings of the mass spectrometer itself. The properties of various mass spectrometers in use for protein mass spectrometry have been reviewed recently (5).

The process described in **FIGURE 1** takes a mixture of proteins as the input and creates a list of identified peptides and their parent proteins as the outputs. The approach requires an initial proteolytic digestion step, usually with trypsin, which hydrolyzes peptide bonds following lysine (K) or arginine (R) moieties. These trypsinized samples, consisting of short peptides, are subjected to HPLC (usually reversed-phase chromatography), which is generally linked directly to the tandem mass spectrometer via Fenn’s electrospray technique (see Introduction). The HPLC stratifies the complex peptide sample, allowing new peptides to be continually delivered to the mass spectrometer over a relatively long period of time. (Alternatively, it is possible to interface between HPLC and MALDI via a robotic microfraction collector, which can be programmed to spot MALDI target plates.)

For MS/MS, processing occurs in two stages (**FIGURE 1**). The first stage (survey mass analysis) yields mass-to-charge \((m/z)\) spectra of parent peptide ions (MS1). Parent \(m/z\) peaks from the MS1 stage are selected for collision-induced dissociation (CID), which fragments the parent peptides, breaking the peptide backbone (MS2). This creates complementary b- and y-type ions as series of fragments (**FIGURE 1**.
(The b-ions contain the NH$_2$ terminus through
the cleavage site; the y-ions contain the COOH
terminus through the cleavage site.) The difference in $m/z$
between adjacent b- or y-series peaks is exactly
the residue mass of the amino acid present in one fragment
but absent in the other, thus allowing unique identification
of that amino acid. Thus the MS$^2$ spectrum can give
sequence information directly (de novo sequencing) or,
more commonly, assigns sequences by computerized
pattern matching against theoretical spectra of all possible
tryptic peptides (based on sequence data from
genome sequencing projects). In most cases,

**FIGURE 1. Approach to shotgun proteomics using LC-MS/MS**

The typical first step in the analysis of a complex mixture
of proteins is to carry out a global proteolysis with
trypsin. Trypsin cuts at peptide bonds of polypeptide
chains specifically following either an arginine (R) or a
lysine (K). Proteases with other specificities are less com-
monly used but can be helpful in increasing identification
coverage for a given protein in regions with inappropri-
ately spaced arginines or lysines. The mixture of tryptic
peptides is injected into the HPLC element of the LC-
MS/MS system. Most frequently, the HPLC separation
carried out using a reversed-phase medium that stratifies
the peptides according to hydrophobicity. The purpose
of the liquid chromatography step is to spread out the
delivery of peptides to the electrospray element over a
long period of time to increase the number of peptides
that can be identified. The peptides are delivered to the
mass spectrometer at low pH (e.g., in formic acid) to
convert them to cationic form. As the peptide ions are
delivered to the mass spectrometer, the first stage dis-
plays an ever-changing readout of $m/z$ of the various
tryptic peptides (the MS$^1$ spectrum). The peak heights
(currents) are functions of the abundances of the individ-
ual peptide ions but are influenced by other physico-
chemical properties. The $m/z$ information in the MS$^1$
spectra is generally not sufficient to identify specific pep-
tides. To accomplish this task, selected peptides under-
go another level of analysis initiated by targeted frag-
mentation of the selected "parent" peptide ion (in this
example, the red peak at $m/z = 860.1$). The most com-
monly used method for peptide fragmentation is colli-
sion-induced dissociation (CID), which generally breaks a
single peptide bond, fragmenting each peptide ion into
two pieces. The specific peptide bond broken in a given
parent peptide ion is variable, however, yielding a host
of fragments. Thus CID creates complementary b- and y-
type ions series of fragments. (The b-ions contain the
NH$_2$ terminus through the cleavage site; the y-ions con-
tain the COOH terminus through the cleavage site.) The
mass spectrometer displays the so-called "fragmentation
spectrum" (MS$^2$ spectrum; bottom). Here, the difference
in $m/z$ between adjacent b- or y-series peaks is exactly
the residue mass of the amino acid present in one fragment,
but absent in the other. Thus, in the difference
between the $y_{10}$ peak ($m/z = 1,276.4$) and the $y_{11}$ peak
($m/z = 1,147.4$) is exactly the residue mass of a glutamic
acid (E; 129.0 Da). In theory, the peptide sequence could
be read directly from the MS$^2$ spectrum. In practice,
however, a computer uses the MS$^2$ spectrum together
with the $m/z$ of the parent peptide ion to compare with
all of the theoretical spectra from the appropriate animal
species to identify the tryptic peptide and the protein of
origin. The database of theoretical peptide spectra is
generated from in silico tryptic digestion of all of the
amino acid sequences derived from the appropriate sin-
gle-species genome sequencing database. Note that
methods other than CID can be used for fragmentation,
such as electron transfer dissociation (ETD).
When multiple peptides corresponding to a given protein are identified, the confidence in the identification increases. However, when only one peptide can be identified, it is necessary to invoke reliable methods to limit the false-positive identification rate to a low value. Computational methods such as target-decoy analysis (6) provide effective means of doing this.

With extremely complex samples, an additional separation step is often required before the standard reversed-phase HPLC. This can be accomplished by adding another chromatography step online, e.g., strong cation exchange, as in the so-called MudPIT method (33). However, under most circumstances, stratifying proteins by molecular weight via one-dimensional SDS-PAGE analysis is the most practical approach to pre-separation before the formal LC-MS/MS analysis. The resulting gel can be sectioned into a series of gel pieces, and the trypsinization can be carried out in the gel. An advantage of this approach is that the protein identification receives another level of confirmation if the molecular weight of the identified proteins matches its location on the gel.

**Applications of qualitative LC-MS/MS in renal physiology**

To investigate intracellular compartments involved in vasopressin-regulated trafficking of aquaporin-2 (AQP2), LC-MS/MS was used for large-scale protein identification in intracellular vesicles immuno-isolated from rat renal inner medullary collecting ducts (IMCDs) using an anti-AQP2 antibody (1). The analysis revealed the presence of a large number of proteins associated with endosomal trafficking. For example, several endosomal proteins from the Rab small GTP-binding protein family were identified (FIGURE 2), including Rab GTPases 4, 5, 18, and 21 (associated with early endosomes); Rab7 (late endosomes); and Rab11 and Rab25 (recycling endosomes). In contrast, Rab3 isoforms (associated with secretory vesicles) were not found in the AQP2-vesicles. Infusion of vasopressin into the rats increased the amount of Rab11 and decreased the amount of Rab7 in the immuno-isolated AQP2 vesicles as determined by immunoblotting, consistent with increased AQP2 recycling.

Recently, a protein profile similar to that seen in AQP2-vesicles has been identified in intracellular tubulo-vesicles from human gastric parietal cells (16).

In another study aimed at identifying components of the protein network involved in AQP2-trafficking, Yu and colleagues (36) identified proteins associated with the apical plasma membrane of the rat IMCD. They used surface biotinylation to label proteins with extracellular lysines and followed by LC-MS/MS to identify the labeled proteins as well as peripheral membrane proteins bound to labeled proteins. Among the bound proteins that were identified were Rab GTPases and their effectors coordinate vesicle transport. They are involved in vesicle budding, transport, docking, and fusion. All Rab proteins listed were identified in the AQP2-containing vesicles except Rab3. CCV, clathrin-coated vesicle; EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; RE, recycling endosome.

**FIGURE 2.** Rab family small GTP-binding proteins identified in AQP2-vesicles from inner medullary collecting duct

Rab GTPases and their effectors coordinate vesicle transport. They are involved in vesicle budding, transport, docking, and fusion. All Rab proteins listed were identified in the AQP2-containing vesicles except Rab3. CCV, clathrin-coated vesicle; EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; RE, recycling endosome.
protein kinase A β-catalytic subunit, calcyclin, myosin Va, NOS1, Hermansky-Pudlak syndrome protein 6 (Hps6), Sipa1l1 (a Rap1-GAP containing a PDZ domain), Rab31, Wiskott-Aldrich syndrome interacting protein, bassoon, and piccolo. All have potential roles in AQP2 regulation and in mediation of cellular responses to vasopressin.

**Quantitative Mass Spectrometry**

The qualitative approach described in FIGURE 1 has been used many times with good success. However, for many physiological applications, a quantitative version of this approach is necessary. Not only must we identify proteins, but we must be able to compare the amounts of a given protein among two or more samples. There are at least two general strategies for protein and peptide quantification: 1) quantification techniques that use chemical labels to mark individual peptides as coming from one or another sample in a single LC-MS/MS run and 2) non-labeling (or “label-free”) methods that depend on comparison of individual MS1 peptide ion currents in two or more parallel LC-MS/MS runs.

**Label-based approaches**

Mass spectrometers can distinguish between two chemically identical molecules if one has additional neutrons added by introduction of stable isotopes in one or more of the atoms comprising the molecule (e.g., 2H replacing 1H, 13C replacing 12C, or 18O replacing 16O). If such molecules are covalently reactive with peptides, the two forms of the molecule can be used as isotopic tags that will discriminate between a given peptide in one sample and the same peptide in another by virtue of their different masses. A generalized example of such a procedure to quantitatively compare individual peptides from two samples is shown in FIGURE 3. The respective peak intensities can be compared to obtain a relative quantification of the peptide in sample 1 vs. sample 2 on the basis of the areas under the curve consisting of ion current plotted vs. time during the chromatographic separation. One limitation of the method is that many tryptic peptides will

enrichment of labeled peptides (after tryptic digestion) by avidin affinity chromatography. The labeling reaction is carried out in the two samples to be compared. One sample is derivatized with ICAT reagent with the light isotope and the other with the heavy ICAT reagent. Corresponding peptides are recognized at the MS2 level by the characteristic mass difference of 9 Da. Quantification is carried out at the MS1 level by numerically integrating under the extracted ion chromatogram, consisting of the curve of ion current vs. time during the chromatographic separation. One limitation of the method is that many tryptic peptides will
not contain cysteine and, therefore, will not be quantifiable by this method. For example, the water channel AQP2 was found to be totally invisible to this technique (21).

To increase coverage for quantification, labeling reagents that react with all peptides are desirable. An example of such a method is iVICAT (in vacuo isotope coded alkylation technique), which uses methyl iodide and deuterium-substituted methyl iodide to label the NH$_2$ termini of all tryptic peptides in two samples targeted for quantitative comparison (24). Carrying out the labeling reaction under a vacuum removes generated HI gas, driving the reaction to NH$_2$-terminal trimethylation rather than dimethylation, as would otherwise occur. The trimethylation results in a 9-Da mass difference between 1H- and 2H-derivatized peptides that can be detected and quantified at the MS$^1$ level as described for ICAT above. The trimethylation also creates permanent positive charge, namely a stable quaternary amine group, on all peptide ions, which amplifies the signal at the MS$^1$ level as well as the b-ion series at the MS$^2$ level, thus facilitating identification. One advantage of this method is the extremely low cost of the derivatization reagent methyl iodide. A drawback is the requirement for special vacuum equipment for the derivatization.

A third labeling technique that is effective for proteomics experiments in cultured cells is SILAC (stable isotope labeling by amino acids in cell culture) (19). In this method, isotopic labels are incorporated directly into proteins during cell culture by growing the cells in media containing two or more stable isotopic forms of an essential amino acid, e.g., leucine. After in vivo labeling and introduction of an experimental manipulation in the cultured cells, the cells are harvested, and proteins from the samples to be compared are mixed and analyzed by LC-MS/MS. As for the methods described above, peptide abundances in the two samples can be quantitatively compared at the MS$^1$ level.

A drawback of the quantitation methods described above is that they are limited in the number of samples that can be compared, most frequently to just two samples. This limitation is obviated in a technique called iTRAQ (isobaric Tags for Relative and Absolute Quantification), which can quantitatively compare up to four samples (four-plex format) as currently configured (23) and which is expected to be available soon in an eight-plex format. iTRAQ employs a set of four or eight amine-reactive isobaric tags to derivatize lysines and the NH$_2$ termini of tryptic peptides. The tags each contain a reporter group specific to the tag. At the MS$^1$ level, the peptides labeled with any of the isotopic tags are indistinguishable, since each has the same molecular mass. However, with CID fragmentation, the reporter groups generate four fragment ions differing sequentially by 1 Da. These appear in the low-mass region of the MS$^2$ spectrum and generally do not interfere with identification of ions from peptide backbone fragmentation seen at higher molecular weights. Thus the protein abundance of samples differentially labeled with each iTRAQ reagent can be compared by quantifying the MS$^2$ peak height (or peak areas over time) for each reporter group. Thus, unlike the other methods described earlier in this section, the quantification is done on the MS$^2$ level. This method requires a mass spectrometer with sufficient mass resolution in the second mass analyzer to resolve the characteristic 1-Da differences among peaks. An additional advantage is that one of the iTRAQ tags can be used for an added standard to allow absolute quantification rather than just relative quantification.

**Application of label-based quantitative LC-MS/MS to physiology.** To discover proteins whose abundances are altered by vasopressin in the renal inner medullary collecting duct (IMCD), Pisitkun and colleagues carried out ICAT analysis of IMCD cells isolated from the renal medullas of Brattleboro rats (21). This strain of rat has no circulating vasopressin, owing to a mutation in the neuropehin-vasopressin gene. The study compared IMCD proteins from Brattleboro rats treated with the vasopressin analog dDAVP for 3 days by osmotic-minipump infusion compared with control animals receiving the vehicle. The findings led to expansion of a core signaling network for vasopressin in the IMCD cell. Among the proteins found to be upregulated by vasopressin were cathepsin D (a renin-like asparyl protease) and syntaxin-7 (a SNARE protein involved in vesicle fusion in late endosomes). Cathepsin D was previously found to be upregulated in response to vasopressin in the IMCD in a prior study using two-dimensional electrophoresis (29).

An example of the application of the iTRAQ method can be found in a paper by Hirsch and colleagues, who investigated the effect of warm ischemia on the proteome of hepatic Kupffer cells (10). The authors successfully quantified more than 1,500 proteins and found upregulation of a large number of proteins normally associated with inflammation.

**Label-free approaches**

In label-free quantification of peptides, direct comparisons of peptide peak intensities (currents) can be made for the same peptide in two or more separate LC-MS/MS runs. This is generally done by peak-area integration (32). Overall reproducibility depends in part on the precision of the HPLC separation. A critical

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<td>ICAT</td>
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Several approaches for enrichment of phosphopeptides and cells has benefited from the development of proteomics laboratories. Proteomics is currently an important point of focus for events on a global scale. Consequently, phosphoproteomics is one of the most common post-translational modifications known. Phosphorylation is one of the most common post-translational modifications. Although the importance of protein phosphorylation, many laboratories have placed a priority on development of methods for detecting phosphorylation and have exploited them in a number of biological settings. Among the most important of these are protein posttranslational modifications.

**Detection and quantification of posttranslational modifications**

Posttranslational modification of proteins occurs through covalent attachment of a variety of small molecules to the peptide chain. Among the most common posttranslational modifications are phosphorylation, acetylation (including methylation, acetylation, myristoylation, prenylation, and palmitoylation), glycosylation, nitrosylation, ubiquitylation, and SUMOylation. In principle, all can be detected and quantified by LC-MS/MS techniques. Because of the importance of protein phosphorylation, many laboratories have placed a priority on development of methods for detecting phosphorylation and have exploited them in a number of biological settings. Consequently, we will focus on phosphoproteomics in this article.

**Phosphoproteomics using LC-MS/MS.** Protein phosphorylation is one of the most common post-translational modifications known. Phosphorylation can drastically alter protein conformation and activity and regulates a wide range of cellular processes including cell adhesion, cell division, hormone signaling, and membrane transport. Although the importance of phosphorylation to basic cellular physiology is clear, very little is known regarding phosphorylation events on a global scale. Consequently, phosphoproteomics is currently an important point of focus for many proteomics laboratories.

Large-scale phosphoproteomic analysis of tissues and cells has benefited from the development of several approaches for enrichment of phosphopeptides before LC-MS/MS identification. Phosphopeptide enrichment techniques include immunoprecipitation with phosphospecific antibodies, affinity purification via cationic metal (e.g., Ga³⁺) binding to the negative phosphate group (IMAC or “immobilized metal ion affinity chromatography”), chemical modifications via phosphoramidate chemistry, metal oxide affinity chromatography (MOAC, e.g., using TiO₂), and charge-based enrichment via strong cation exchange (SCX) chromatography. These methods can markedly reduce the complexity of the sample and may be complementary in the sense that different methods can enrich different subpopulations of phosphopeptides. Therefore, maximization of phosphopeptide identification in a given sample may require a multi-approach strategy that combines different enrichment techniques either in series or in parallel.

A typical workflow for phosphopeptide analysis using IMAC enrichment and neutral loss scanning (12) is shown in **FIGURE 4**. Phosphoproteins are digested with trypsin, yielding a mixture of both phosphorylated and nonphosphorylated peptides. This peptide mixture is then run on an IMAC column, which effectively binds phosphorylated peptides while allowing nonphosphorylated peptides to pass through. After elution from the IMAC column, phosphopeptides are analyzed by MS/MS. The initial spectrum that is acquired is a precursor ion scan that displays the m/z ratios of the intact phosphopeptides (MS²). After collision with an inert gas (CID), a given phosphopeptide loses the phosphate moiety along with a molecule of water resulting in a mass shift of ~98 Da (MS³ neutral loss peak). This identifies the parent peptide ion as being a phosphopeptide. Neutral loss peaks in MS³ spectra are selected for a further round of fragmentation (MS⁴). Since the peptide no longer contains a phosphate, backbone fragmentation can then occur, allowing the identification of the peptide as shown in **FIGURE 1**.

Generally, neutral loss of phosphoric acid occurs when the parent ion contains a phospho-serine or a phospho-threonine. However, phospho-tyrosines normally do not undergo neutral loss. Thus phosphotyrosines must be identified by recognition of the phosphotyrosine residues (residue mass = 243.0296 Da) on the MS² spectrum. Identification of phosphotyrosine-containing peptides, however, benefits greatly from the availability of excellent phosphotyrosine-specific antibodies allowing efficient affinity purification of these peptides. Consequently, strategies designed to carry out broad phosphoprotein profiling in a given tissue or cell type often employ dual parallel strategies involving phospho-tyrosine antibody-based affinity purification on one hand and IMAC (or similar) phosphopeptide enrichment on the other (30). Because identification is made based on analysis of a single peptide, error control is important.
to limit the false-positive identification rate. A convenient and effective means of doing this is target-decoy analysis (6).

A drawback of the reliance on phosphoric acid neutral loss to identify phosphorylated peptides is that the ion currents tend to be more and more attenuated with each round of CID, compromising the overall sensitivity. Consequently, alternative fragmentation methods are under development that do not result in the neutral loss of phosphoric acid and other labile posttranslational modifications (e.g., nitrosylation), namely electron capture dissociation (ECD) (37) and electron transfer dissociation (ETD) (17). These approaches induce fragmentation of the peptide backbone but at a different site, viz. the N-C bond rather than the peptide bond. This creates series of so-called c- and z-type ions instead of the b- and y-type ions generated with CID. In this case, phosphorylation or other posttranslational modifications can be recognized by identification of the residue mass for the modified amino acid in comparison of adjacent peaks from the c- and z-series. Another advantage of these newer fragmentation technologies is that they can be used to analyze larger polypeptides, permitting “top-down” analysis, done without the trypsinization step.

Despite challenges presented by the fact that the fraction of a particular protein that is phosphorylated is often small, a number of recent large scale phosphoprotein identification studies are yielding hundreds, or even thousands, of bona fide phosphorylation sites in a given cell type [see, for example, Villen et al (30) or Hoffert et al (12)]. In such studies, a majority of the identified phosphorylation sites have not been previously reported.

**LC-MS/MS based phosphoproteomics in physiology.** Vasopressin signaling in the renal IMCD involves increases in cyclic AMP and intracellular Ca^{2+} (4, 26), which are believed to exert their ultimate effects by activating various protein kinases and phosphatases, thereby altering the phosphorylation states of protein targets. A study aimed at identifying phosphorylation targets in the renal IMCD and quantifying the effects of vasopressin on protein phosphorylation was recently reported (12). A total of 714 phosphorylation sites on 223 distinct proteins were identified. Among the phosphorylation sites identified were four sites on the vasopressin-regulated urea transporter UT-A (all novel) and a polyphosphorylated region in the COOH-terminal tail of the vasopressin-regulated water channel AQP2 (three of four novel; **FIGURE 5A**). Quantification of AQP2 phosphorylation using label-free methods confirmed the previously demonstrated increase in Ser256-AQP2 phosphorylation in the IMCD in response to short-term exposure to vasopressin as well as a decrease in Ser261-AQP2 phosphorylation (**FIGURE 5B**), both of which were confirmed by immunoblotting with phospho-specific antibodies (11).

Another recent study investigated vasopressin-induced tyrosine phosphorylation in the IMCD using a phospho-tyrosine (pY) antibody to immunoprecipitate pY-containing peptides. The pY-containing peptides were quantified using IVCAT (Table 1) (25). This study identified 114 pY sites in the IMCD. Among these was phospholipase Cγ-1 (PLCγ-1), which underwent a twofold increase in phosphorylation at Y771 in response to vasopressin. This phosphorylation event was associated with translocation of the protein.
from a membrane-bound state to the cytosol, consistent with a net inhibitory action.

**Use of LC-MS/MS to study other regulatory processes in cells**

Aside from analysis of posttranslational modifications, LC-MS/MS can be used to investigate other regulatory processes in the cell. One such process is translocation of proteins from one cellular compartment to another. On activation, many signaling molecules undergo translocation from cytosol to a membrane-bound state, e.g., with Ca$^{2+}$-mediated activation of several protein kinase C isoforms or with activation of Rab-family small GTP-binding proteins in response to GTP-for-GDP exchange. Such events can be investigated in a large-scale discovery mode by combining differential centrifugation with quantitative LC-MS/MS analysis of the resulting fractions. Similarly, many transcription factors undergo translocation from cytosol to nucleus with activation, and this can be investigated by isolating nuclei and cytosol from cells and analyzing them with quantitative LC-MS/MS.

Another mode of regulation that can be investigated through LC-MS/MS methods is the binding of proteins to other proteins. MS-based large scale analysis of protein binding interactions has been successful in yeast, using tandem affinity purification/mass spectrometry (TAP/MS) (7, 15). This approach has not been employed globally in metazoan organisms. However, a large number of studies have been reported that employ targeted affinity purification coupled with MS identification of interacting proteins (see LC-MS/MS detection of protein-protein interactions in physiology for examples). In principle, analysis of protein-protein binding interactions could also be done globally in mammalian cells through the use of tagged bifunctional reagents that would allow cross-linking between two interacting proteins and identification of the cross-linked peptides via their mass spectra (13). At this point, however, such approaches have not been successful on a large-scale basis.

**LC-MS/MS detection of protein-protein interactions in physiology.** TonEBP is a transcription factor involved in osmotic regulation of organic osmolyte production and transport in the renal inner medulla. Chen and colleagues (3) recently investigated TonEBP binding partners by stably expressing amino acids 1–547 of TonEBP in cultured HEK 293 cells and immunoprecipitating it from the nuclei after exposure to a hypertonic culture medium. Using LC-MS/MS, they identified 14 proteins bound to TonEBP, including the DNA-dependent protein kinase Ku86, several RNA helicases, several small or heterogeneous nuclear
ribonucleoproteins (snRNPs or hnRNPs), two heat shock proteins (HSP90β and HSC70), and poly-ADP-ribose polymerase-1 (PARP-1). Follow-up studies showed that PARP-1 overexpression reduces TonEBP transcriptional and transactivating activity, whereas HSP90 overexpression enhances those activities. A similar immuno-affinity approach was employed by Noda and colleagues (18) in which they used antibodies to AQP2 to immunoprecipitate a protein complex containing SPA-1, β-actin, ionized calcium binding adapter molecule 2, myosin regulatory light chain, α-tropomyosin 5b, annexin A2 and A6, scinderin, gelsolin, α-actinin 4, α-II spectrin, and myosin heavy chain nonmuscle type A.

A different affinity approach was employed by Thelin and colleagues (28) who investigated binding partners for COOH-terminal tail of the cyclic AMP-regulated chloride channel CFTR. They synthesized the COOH-terminal tail of CFTR and used it to pull down proteins from cultured Calu-3 cells. Using LC-MS/MS, they identified the serine/threonine protein phosphatase PP2A as a binding partner and further showed that inhibition of this phosphatase results in increased CFTR channel activity. A similar approach using the NH2-terminal tail of CFTR led to the identification of both filamin A and filamin B as CFTR binding partners (27).

Targeted Proteomics Using MRM

In general, the LC-MS/MS techniques described above are optimal for discovery experiments, which are intended to identify previously unrecognized components of protein networks. However, such experiments must be followed up with targeted experiments aimed at confirming the observed events and placing the observed events into the context of causal models.

The LC-MS/MS techniques already described do not provide an optimal means of doing this, since the ability to observe a given peptide or protein cannot be controlled. In general, a particular peptide ion may be sporadically detected unless it is derived from one of the most abundant proteins in the sample mixtures. One solution is to use antibodies in "targeted studies" to investigate the roles of specific proteins and protein modifications discovered by MS/MS(14). However, this defeats the multiple-parallel strategy that makes LC-MS/MS such an appealing tool in systems biology applications. A promising solution to this problem is the development of approaches based on the multiple reaction monitoring (MRM) principle.

The MRM strategy is described in FIGURE 6. The peptide ion stream is mass filtered at the MS1 level, selecting a limited number of precursor ions with pre-specified m/z values for fragmentation. Meanwhile, the associated MS2 spectra are monitored for a specific pre-identified fragment ion associated with a given precursor, yielding a high degree of specificity in the identification. Many such transition pairs (precursor ion/fragment ion pairs) can be specified in a given LC-MS/MS run, allowing many proteins/peptides, corresponding to physiologically important targets, to be quantified in parallel. Since only predetermined precursor ions are selected for CID fragmentation, irrelevant peptide ions do not compete for CID fragmentation priority, allowing sufficient dwell times for generation of high-quality fragmentation spectra. In principle, the MRM approach can be used for physiological studies to target multiple proteins that are elements of a given signaling pathway, metabolic pathway, or trafficking mechanism. The parallel quantification of many proteins simultaneously is of obvious value in modeling of the complex systems that they comprise. Quantification can be conveniently achieved at the MS3 level using iTRAQ (35) (Table 1) or at the MS1 level with label-free methodology. Preliminary experiments must be carried out to determine optimal precursor/fragment ion pairs and to record the time frame when the precursor ions of interest can be expected to elute from the LC column. MRM requires a triple quadrupole-class tandem mass spectrometer because of the unique capability of these instruments for monitoring at both the MS1 and MS3 levels (5).

Outlook

Physiological systems are, by their very nature, complex. Traditionally, physiologists have dealt with complexity in a variety of ways. Some have used reductionist approaches and integrated information in an ad hoc manner using natural language-based models. Others have pursued intact integrative systems using clever experimental approaches to identify characteristics of the system of interest, often with...
mathematical modeling as an important adjunct. Now, in the 21st century, with new tools that could not have existed without comprehensive genomic and proteomic sequence information, physiology has another alternative, namely “data-rich systems biology” (34) in which it is possible to identify numerous components of a given biological system and measure them in a multiple-parallel manner. One such tool is LC-MS/MS, as described in this paper. We have focused mainly on discovery approaches in both qualititative and quantitative modes that can be used to identify new components of a given metabolic pathway, signaling network, or trafficking mechanism. However, the real promise in tandem mass spectrometry is beyond discovery. Specifically, targeted proteomics using MRM in tandem mass spectrometry is potentially capable of elucidating dynamics of a large number of pre-specified molecular entities, providing data needed for development of predictive mathematical models. In this manner, LC-MS/MS can provide a necessary foundation for large-scale hypothesis-driven research in cell physiology.

All four coauthors are supported by the intramural budget of the National Heart, Lung, and Blood Institute Project number HL-001285.

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