REVIEW

Global substrate specificity profiling of post-translational modifying enzymes

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Abstract: Enzymes that modify the proteome, referred to as post-translational modifying (PTM) enzymes, are central regulators of cellular signaling. Determining the substrate specificity of PTM enzymes is a critical step in unraveling their biological functions both in normal physiological processes and in disease states. Advances in peptide chemistry over the last century have enabled the rapid generation of peptide libraries for querying substrate recognition by PTM enzymes. In this article, we highlight various peptide-based approaches for analysis of PTM enzyme substrate specificity. We focus on the application of these technologies to proteases and also discuss specific examples in which they have been used to uncover the substrate specificity of other types of PTM enzymes, such as kinases. In particular, we highlight our multiplex substrate profiling by mass spectrometry (MSP-MS) assay, which uses a rationally designed, physicochemically diverse library of tetradecapeptides. We show how this method has been applied to PTM enzymes to uncover biological function, and guide substrate and inhibitor design. We also briefly discuss how this technique can be combined with other methods to gain a systems-level understanding of PTM enzyme regulation and function.

Keywords: post-translation modifying enzymes; proteases; kinases; substrate specificity; peptide libraries; peptide synthesis; mass spectrometry

Introduction

The primary mechanism by which the diversity of the proteome is increased is through the post-translational modification of proteins. PTM enzymes are responsible for over 200 kinds of modifications of protein substrates and can be divided into two distinct mechanistic categories: (1) enzymes that hydrolyze peptide bonds (proteases) and (2) enzymes that covalently modify amino acid side chains. PTM enzymes constitute over 5% of the human genome but most have yet to be fully characterized.[1] An important aspect of understanding the functions of these enzymes requires developing in vitro assays in which their specificity and activity can be monitored. Although a variety of assays exist for profiling PTM enzyme specificity, there is particular value in assays in which post-translational modifications of peptide substrates are quantitatively and directly measured. To facilitate this type of assay format, researchers have taken advantage of synthetic peptide chemistry to develop large and diverse peptide libraries.

Peptide synthesis was pioneered by the work of Emil Fischer and Ernest Fourneau who synthesized the dipeptide glycylglycine in 1901. This work laid the foundation for subsequent advances in peptide synthesis with the Nobel Prize in Chemistry being awarded to Bruce Merrifield in 1984 for the development of solid-phase peptide synthesis (SPPS).[2] Merrifield's strategy involved assembly of a peptide chain in a stepwise manner with one end of the nascent peptide anchored to a solid resin until completion of synthesis. Covalent attachment of the growing peptide chain to a solid support renders it insoluble, which facilitates easier transition between synthetic steps, such as washing away excess reactant and byproduct. SPPS has been further streamlined over the last several decades and Fmoc SPPS is currently the most widely used synthetic strategy.[3] Generating synthetic peptides using this technology gained popularity when biologists recognized that synthetic peptides could be used for antibody selection and production.[4] Fmoc SPPS is now easily accomplished using highly automated workflows.[5,6]

In this review, we first discuss how SPPS has been applied to generate large, highly diverse peptide libraries for the analysis of protease substrate specificity. We provide an overview of the various methods and describe several applications of how these methods have been applied to develop selective protease substrates and inhibitors. We extend this to a discussion of how SPPS has enabled the development of peptide libraries for determining the specificity of other types of PTM enzymes. Proteome-derived peptide libraries and phage and bacterial display have also been widely applied for analysis of protease substrate specificity; however, these technologies are not the focus of the current review and have been reviewed elsewhere.[7–9]

Peptide-Based Technologies for Analysis of Protease Specificity

Proteases are one of the largest classes of PTM enzymes, with over 550 encoded in the human genome.[10] These enzymes are essential for normal cellular functions and are implicated in a variety of diseases, such as cancer, neurodegeneration, and blood clotting disorders. Because of the size and importance of this enzyme class, substantial effort has been put into the development of peptide-based technologies for determining protease substrate specificity. Proteases generally recognize substrates in an extended linear conformation, making this class of enzymes particularly amenable to analysis with peptide-based profiling methods.[11]

Traditionally, identification of protease substrates relied on relatively small collections of synthetic peptides with sequences derived from proteins that were known to be proteolyzed. Peptides would be incubated with a target protease and their cleavage assessed, generally through high-performance liquid chromatography (HPLC) with mass spectrometry for cleavage site identification.[12] Once initial substrates were identified, new substrates with variations at select positions would be synthesized to explore subsite specificity. The development of colorimetric and fluorescent peptide substrates simplified cleavage assessment,[13–15] however, defining protease substrate specificity remained an iterative and tedious process. Over the last two decades, this process has been transformed by the development of large and highly diverse peptide libraries. Table I summarizes some of the peptide-based technologies for analysis of protease specificity. The information determined through these approaches can be used for a number of important applications. For example, selective substrates can be designed that enable the real-time monitoring of proteolysis in vitro and in vivo. Peptide substrates also can be converted into protease inhibitors through coupling to an electrophilic warhead. Furthermore, as proteolytic enzymes recognize their substrates as linear motifs of extended beta strands, specificity information can be used to prioritize potential endogenous substrates. For example, a number of computational approaches have been developed to predict caspase and granzyme B substrates using specificity data determined through peptide-based profiling methodologies.[16,17] Synthetic substrate synthesis, inhibitor design, and endogenous substrate identification are all critical steps in improving our understanding of the biological role of a given protease in both cellular function and pathogenesis.

Table I. *Peptide-Based Protease Activity Profiling Technologies*

| Peptide-Based Profiling Technology	Advantages	Disadvantages
On-bead FRET libraries	High sequence diversity. Useful for analysis of subsite cooperativity.	Requires prior knowledge of specificity. On-bead immobilization can produce artifacts. New libraries need to be synthesized for each protease.
Positional scanning-synthetic combinatorial libraries	High sequence diversity, especially for newer libraries incorporating unnatural amino acids. Simple and validated in-solution fluorescent assay.	Generally limited to nonprime specificity profiling. Does not provide information related to subsite cooperativity.
Electrophile-based libraries	High sequence diversity. Already contain electrophilic warhead for conversion into protease inhibitor.	Generally limited to nonprime specificity profiling. Primarily limited to serine, threonine, and cysteine proteases.
Peptide microarrays	Can be used to determine kinetic parameters for substrate hydrolysis. Able to analyze protease activity in complex biological samples. Useful for analysis of subsite cooperativity.	Limited sequence diversity. Immobilization of peptide substrates can produce artifacts. Limited to nonprime specificity profiling.
Mixture-based oriented peptide libraries	High sequence diversity. Can be used to profile prime and nonprime specificity.	New libraries often need to be synthesized for nonprime side profiling. Primarily limited to proteases with strong prime side specificity. Requires enrichment step.
Multiplex substrate profiling by mass spectrometry	Can profile prime and nonprime specificity. Can be used to determine kinetic parameters for hydrolysis of substrates. Useful for analysis of subsite cooperativity. Global activity in complex samples can be analyzed. Rationally designed peptide library.	Relativity limited sequence diversity. Activity is not monitored in real-time.

On-Bead Fluorescent Peptide Libraries

In the late 1980s, Kahne et al. developed a radioassay to monitor the hydrolysis of bead-bound peptides.[18] Although this assay was used to assess the half-life of an amide bond in neutral water, which remarkably was determined to be ~7 years, the authors suggested that this technique could also be used to monitor proteolysis. Meldal et al. were the first to carry that idea forward with the development of an on-bead, combinatorial peptide library for assessing protease specificity.[19] These combinatorial libraries were constructed through split peptide synthesis, leading to a single peptide sequence being present on each bead. The bead-conjugated peptides were constructed with a C-terminal fluorophore and N-terminal quencher, resulting in fluorescence quenching through Förster resonance energy transfer (FRET) prior to release of the fluorophore via proteolytic cleavage.[20,21] Positions C-terminal and N-terminal to the site of proteolytic cleavage are commonly referred to as prime (P') and nonprime

(P) positions, respectively.[22] Following treatment with a protease of interest, the fluorescent beads were isolated and subjected to Edman degradation where N-terminal residues are labeled and sequentially released to reconstruct the amino acid sequence. However, because each bead contained a mixture of intact and cleaved peptide, it was not possible to differentiate residues released from the native or neo-N-termini generated through proteolysis. This generally meant that the cleavage site needed to be predefined to accurately determine if amino acids detected during Edman degradation were from the intact or cleaved peptide. Once the ratio of intact to cleaved peptide was determined, this was used to calculate percent conversion and estimate catalytic efficiency.[19]

Positional Scanning Substrate Libraries

One of the more widely applied, fluorescence-based techniques for analysis of protease specificity involves the generation of positional scanning-
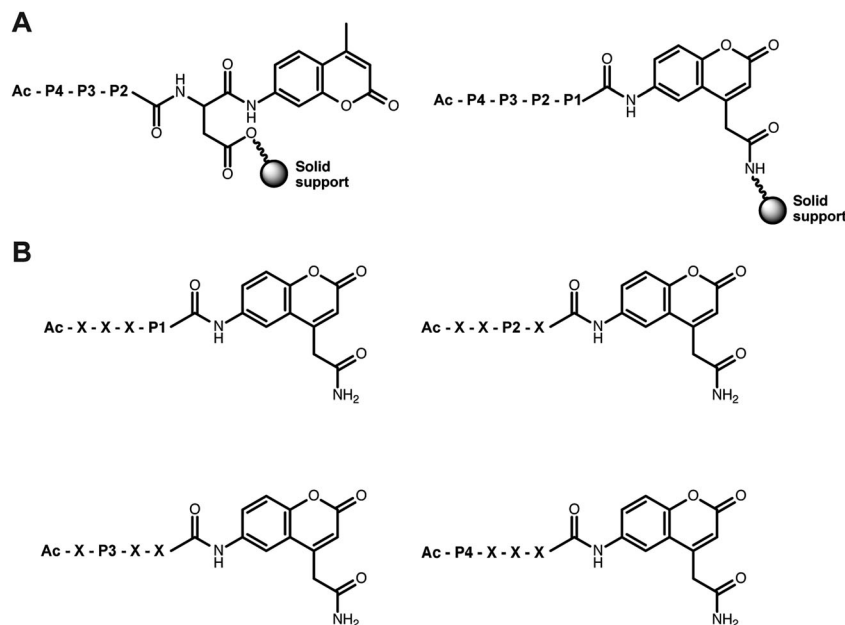
Figure 1. Construction of positional scanning-synthetic combinatorial libraries for analysis of prime side specificity. (A) During solid-phase peptide synthesis, 7-amino-4-methylcoumarin (AMC)-based positional scanning-synthetic combinatorial libraries are generally conjugated to the solid support via the side chain of the P1 amino acid, while 7-amino-4-carbamoylmethylcoumarin (ACC)-based libraries are conjugated directly through the fluorophore. (B) The four P_n sublibraries each contain 20 distinct pools of substrates, where one amino acid is fixed at P_n position and the remaining positions (X) contain an equimolar mixture of amino acids.

synthetic combinatorial libraries (PS-SCLs). PS-SCLs consist of distinct pools of peptides in which an amino acid in one position is fixed within each pool, while the other positions contain a mixture of amino acids. Initial PS-SCLs were used to determine the P4-P2 specificity of proteases and incorporated a 7-amino-4-methylcoumarin (AMC) fluorophore at the P1′ position [Fig. 1(A)].[23] These libraries were restricted to certain P1 residues, such as aspartic acid and lysine, because the SPPS protocol required attachment of the growing peptide chain to the solid support through the P1 amino acid side chain. The development of a bifunctional 7-amino-4-carbamoylmethylcoumarin (ACC) fluorophore, which can be directly attached to a solid support, enabled the development of PS-SCLs with diversity at the P1 position (Fig. 1).[24,25] P1 diversity significantly increased the number of proteases amenable to analysis through the PS-SCL approach. PS-SCLs have been used to profile the P4-P1 specificity of diverse proteases, including cysteine cathepsins,[26] kallikreins,[27] caspases,[28] and granzymes.[29]

Drag et al. recently reported the development of ACC positional scanning libraries incorporating up to 110 unnatural amino acids in the P1 to P4 positions.[30,31] These extended libraries, termed hybrid combinatorial substrate libraries (HyCoSuL), were used to identify a neutrophil elastase substrate with the highest reported catalytic efficiency.[30] The increased chemical space explored through HyCoSuL has also enabled the development of selective caspase substrates.[31] Furthermore, a Counter Selection Substrate Library (CoSeSuL) approach against caspases has been used to develop highly selective legumain probes.[32]

PS-SCLs have also been designed to profile prime side specificity through the incorporation of FRET-based quenching.[33–38] FRET-based PS-SCLs contain a fluorophore and quencher separated by several amino acids. Unlike AMC- and ACC-based PS-SCLs, fluorescence occurs following proteolytic cleavage between any of the amino acids in the peptide substrate. Therefore, mass spectrometry is required to validate the site of cleavage and reconstruct a specificity profile. Recently, Poreba et al. combined FRET- and non-FRET-based approaches to determine the optimal nonprime and prime side specificity of serine, cysteine, and metalloproteases.[39]

Electrophile-Based Libraries

An alternative design for positional scanning libraries incorporates electrophilic "warheads" that have been widely used for activity-based profiling of enzymes.[40,41] The electrophile is placed at the P1 position and covalently labels the active-site nucleophile of the target protease. Electrophile-based libraries contain diversity in the P4–P2 positions and generally use a radiolabel for quantitation of protease labeling.[42,43] These libraries have been successfully used to profile a number of proteases, including cysteine cathepsins, calpains,[42] and the proteasome subunits.[43] Electrophile-based libraries are currently limited to cysteine, serine, and

threonine proteases because of the requirement of an active site nucleophile and cannot be used to determine nonprime side specificity. However, a unique advantage of these libraries is that they can be readily converted into protease inhibitors, as specificity information is determined in the context of the electrophilic warhead that can be used in an inhibitor. Although beyond the scope of this review, large libraries of electrophile-containing compounds and fragments have been applied for identifying PTM enzyme inhibitors.[44,45]

Microarray Peptide Libraries

Fluorescent peptide libraries are also commonly used in microarray formats to profile protease substrate specificity.[46,47] Peptide microarrays consist of fluorescent substrates that are spatially separated on a microarray surface either by direct covalent attachment or through individual nanodroplets.[46,48,49] Unlike PS-SCLs, which use pools of fluorescent substrates, cleavage of individual substrate sequences can be directly assessed with microarrays. This enables the highly multiplexed determination of kinetic parameters for each of the typically hundreds of substrates that are evaluated in a given experiment. Moreover, proteases often exhibit subsite cooperativity due to shared determinants of substrate specificity among binding pockets and the optimal positioning of amino acids within the target sequence. Such subsite cooperativity information is lost when using techniques that rely on pools of substrates, but is readily assessable with microarrays because of the spatial separation of individual substrates. One drawback of the peptide microarrays developed to date is the relatively low sequence diversity as compared to positional scanning methods. With a few notable exceptions,[50] peptide microarrays have been most successful when exploring nonprime specificity and other methods are required to query prime side specificity.

Mixture-Based Oriented Peptide Libraries

Edman degradation of mixture-based oriented peptide libraries has been used to determine both prime side and nonprime side protease specificity within the same assay format. This profiling strategy developed by Turk et al. uses two separate peptide libraries.[51] First, a fully randomized 12-mer peptide library is synthesized with acetylated N-termini. This library is partially degraded with a protease of interest, releasing C-terminal cleavage products with free N-termini. Edman degradation of the C-terminal cleavage products is used to determine the frequency of each amino acid at each of the prime side positions. A second 12-mer peptide library is then synthesized that incorporates a randomized N-terminal 6-mer and the most favorable P1′ to P6′ amino acids for the C-terminal portion. The predefined prime side sequence directs cleavage to the middle of each peptide in the library. All peptides also contain a C-terminal biotin and free N-termini. Following protease treatment, C-terminal cleavage products are removed with immobilized avidin and the remaining N-terminal products are sequenced by Edman degradation to determine the nonprime side specificity. This approach has been successfully applied to a range of proteases, including matrix metalloproteases,[51,52] anthrax lethal factor,[53] and the serine proteases HtrA1/2.[54,55] Although this is a highly versatile technique, one limitation is that new peptide libraries generally need to be synthesized for each investigated protease. Furthermore, these libraries work best for proteases that have strong prime side specificity determinants, as a sequence is required to direct cleavage to the middle of each peptide in the second peptide library.

Multiplex Substrate Profiling by Mass Spectrometry

Mass spectrometry in combination with proteome-derived substrate libraries has been successfully applied to define protease specificity.[56–61] These "degradomics" methods use liquid chromatography with tandem mass spectrometry (LC-MS/MS) to identify the site of proteolytic cleavage within proteome-derived peptides and can define prime side and nonprime side specificity determinants in a single assay. These techniques are quite powerful, but require chemical labeling steps for enrichment and identification of neo-termini and present a challenge in extracting kinetic parameters.

MSP-MS was developed to provide simple, yet highly sensitive and quantitative assay for assessing the extended substrate specificity of proteases. This technique currently uses a library of 228 synthetic tetradecapeptides that contain maximal physicochemical diversity within a minimal sequence space.[62,63] This library was designed based on the observation that most proteases require two optimally positioned amino acids for substrate recognition and cleavage. This phenomenon is generally referred to as the "two-site hypothesis." For example, the specificity of granzyme B is dominated by a preference for isoleucine at the P4 position and aspartic acid at the P1 position.[24,62] As is evident in the crystal structure of granzyme B, two prominent cavities on the enzyme surface (S1 and S4) accommodate these residues and are the primary determinants of substrate recognition (Fig. 2).[64] Though these sites are not the only determinants of enzyme efficiency, they contribute to greater than 70% of the binding energy required for substrate recognition and turnover. Numerous proteases appear to follow the two-site hypothesis with the sites being juxtaposed, for example, on either side of the scissile
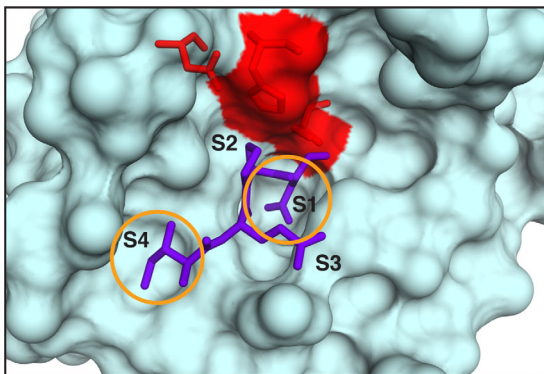
Figure 2. The ecotin peptide (purple) binds to the active site of granzyme B in a linear conformation.[64] The catalytic triad is shown in red. Recognition of the peptide, IEPD (written P4-P1), is dominated by the S4 and S1 pockets (circled in yellow).

bond or separated in space along the substrate by one or two amino acids. Therefore, physicochemical diversity in the MSP-MS library was generated through incorporation of all neighbor (XY) and near-neighbor (X*Y, X**Y) amino acid pairings. This simple and chemically defined library enables facile extraction of kinetic parameters for each substrate and is readily amenable to profiling the specificity of purified proteases or complex biological samples without the need for enrichment strategies.

For MSP-MS specificity determination, a recombinant protease or other biological sample of interest is incubated with the peptide library and aliquots are removed at multiple time points (Fig. 3). Cleavage sites within library peptides are then identified through LC-MS/MS analysis of each time point and specificity is visualized using a sequence logo, which displays protease amino acid preference relative to the site of cleavage. Label-free quantitation of both parent peptides and their corresponding cleavage products over time can be used to determine kinetic parameters for substrate hydrolysis. This

information is critical for the prioritization of optimal sequences for substrate and inhibitor design.

Substrate specificities of a wide range of proteases from all major classes have been interrogated using the MSP-MS assay. Furthermore, because the termini of the peptides are unmodified, the library is well suited for profiling exopeptidase specificity. In particular, the MSP-MS assay has been used to profile carboxypeptidases, such as PRCP,[62] which are generally not amenable to analysis with most of the previously discussed methods that use peptides modified with reporter groups. Furthermore, the MSP-MS assay has been used to identify the prime side specificity determinants of aminopeptidases, such as aminopeptidase N.[65]

The ability of the MSP-MS library to readily profile mixtures of proteases has transformed our ability to quantitatively characterize proteolytic activity in complex biological systems. The assay was recently used to profile the catalytic subunits of the *Plasmodium falciparum* proteasome.[66] The specificity differences between this and the human proteasome were then used to rationally design selective peptidic inhibitors that attenuated malaria development in vivo. The MSP-MS assay has been used with protease inhibitors, gene deletions, and immunodepletion in combination with traditional proteomic methods to identify component proteases that are highly active in complex biological samples. This has enabled the "deconvolution" of proteolytic signatures from fungal pathogens,[63,67,68] parasitic organisms,[69,70] cancer cell lines,[62,71] and patient samples,[72,73] and allowed for the prioritization of proteases based on their functional contribution to the global substrate specificity profile. For example, the MSP-MS assay was used to analyze the global activity signatures of the opportunistic fungal pathogen *Candida albicans* in the planktonic and biofilm states.[67] Comparison of the activity signatures from each state coupled to inhibitor and proteomic
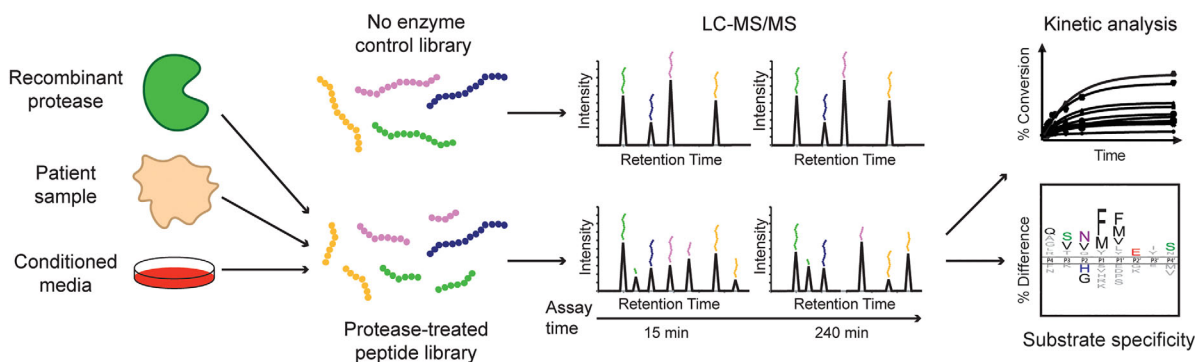
Figure 3. MSP-MS workflow for protease specificity determination. A recombinant protease, patient sample, conditioned media, or other complex, protease-containing biological sample is added to the MSP-MS peptide library. Aliquots are removed at specific time points and peptide cleavage is assessed through LC-MS/MS analysis. Cleavage-site identification can be used to construct a sequence logo representation of the global substrate specificity. Cleavage product quantification enables the kinetic analysis of individual substrate cleavage events.
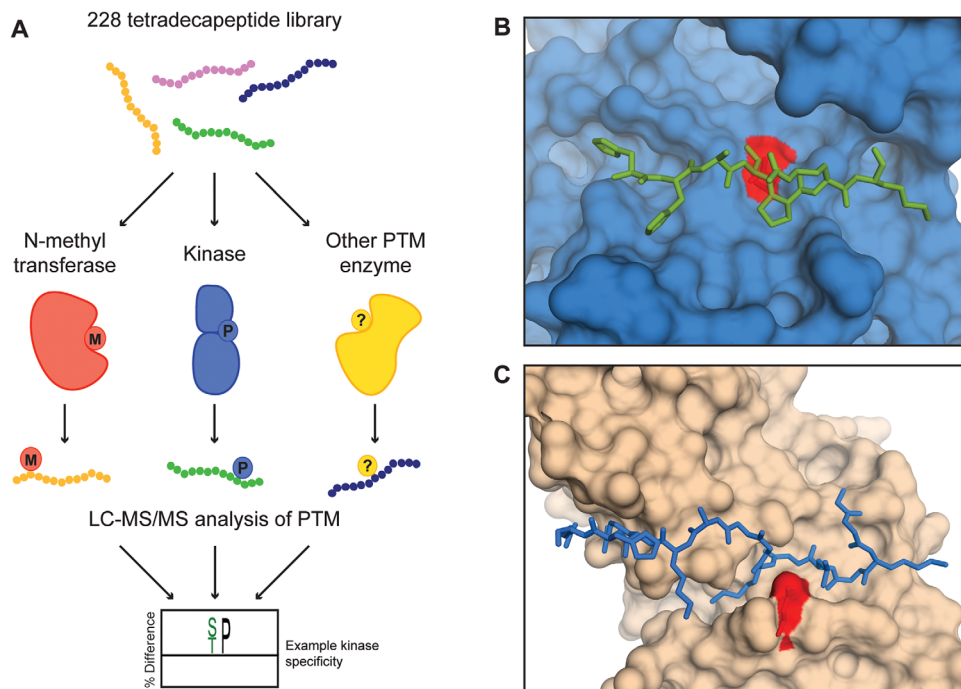
Figure 4. Application of the MSP-MS library to other PTM enzymes. (A) A general scheme for the specificity analysis of PTM enzymes using the MSP-MS assay. PTM enzymes are incubated with the peptide library and modification is detected through LC-MS/MS analysis. (B) The kinase CDK2/cyclin A (shown in blue) recognizes a peptide substrate (depicted as green sticks) in a linear conformation.[89] The active site lysine residue is colored red. (C) Similarly, the histone acetyltransferase HAT1 also binds a linear substrate.[88]

analysis revealed that two specific secreted aspartyl proteases (Saps) are upregulated during biofilm growth and are critical to biofilm formation in vitro and in vivo. MSP-MS was also recently used to identify two human aspartyl proteases that are selectively upregulated in cystic precursor lesions of pancreatic cancer.[73] This strategy enabled the development of a highly accurate diagnostic assay using fluorogenic substrates for differentiating benign and premalignant lesions within a cohort of patient cyst fluid samples.

Kinase Specificity Analysis Using Peptide Library-Based Methods

The MSP-MS assay was designed and validated using proteases primarily because they cleave linear peptides and predominately rely on primary amino acid sequence for substrate recognition. Similarly, kinases typically phosphorylate unstructured regions of proteins, and their specificity is strongly dependent upon the amino acid residues surrounding the phosphoacceptor site.[74] Available crystal structures of eukaryotic kinases reveal that many kinases, as with proteases, bind their substrates in an extended, linear conformation.[75,76] Computational efforts using these crystal structures have been able to successfully identify endogenous substrates, highlighting the importance of linear peptide sequences in kinase substrate specificity.[77]

There are numerous peptide-based approaches for profiling the substrate specificity of kinases.[77–82] These methods primarily employ fluorescence, radioactivity, or colorimetry to detect enzyme activity. For kinase-directed PS-SCLs, a series of biotinylated peptides are generated. The central phosphorylatable residue remains fixed and all other positions are varied to query amino acid preferences.[77] These peptide libraries are assayed in a multiwell format with the kinase of interest and radiolabeled ATP. Aliquots of the reaction are then transferred to a streptavidin-coated membrane, and phosphorylation of each peptide substrate is measured via radiography.[83] Quantification of the amount of phosphorylation is then used to determine the specificity of the kinase of interest.

The MSP-MS assay presents a significant improvement upon traditional methods available for profiling kinases. Reporter groups used in other techniques can interfere with kinase-substrate recognition and radioactivity-based methods have costly disposal and present health hazards. The label-free and unbiased design of the MSP-MS library has made it applicable to the analysis of a wide variety of kinases.[84]

Kinases without previously known substrate preference have been profiled using MSP-MS, allowing for the discovery of their key substrate specificity determinants. In addition, the high sensitivity of this assay can allow for profiling of picomolar

amounts of kinase.[84] This enables the profiling of a kinase from a single immunoprecipitation experiment, which is highly advantageous if the enzyme cannot be readily expressed and purified. The MSP-MS assay has also been used to obtain kinetic parameters for phosphorylation of individual peptides within the library. This capability has proven useful for analyzing the effect of interacting factors on kinase substrate specificity and catalytic efficiency. For example, MSP-MS was recently used to interrogate the P-TEFb–HIV-1 Tat interaction. P-TEFb, a human kinase that is integral to Tat's transactivation, phosphorylates RNA Polymerase II and two negative elongation factors to overcome the stalled RNA Pol II complex, which then allows transcription of the integrated viral genome to continue. There is controversy as to which site within RNA Pol II that P-TEFb phosphorylates, and what effect Tat has on specificity and phosphorylation rate.[85–87] MSP-MS analysis of recombinant and immunoprecipitated P-TEFb revealed that P-TEFb phosphorylates serine 5 within the RNA Pol II C-terminal domain. Analysis of P-TEFb with the addition of Tat revealed that Tat selectively increased the catalytic efficiency of P-TEFb toward peptides that most closely resembled RNA Pol II serine 5.[84]

MSP-MS Analysis of Additional PTM Enzymes

There are ∼200 types of PTM enzymes, and many of these enzymes have yet to be characterized.[1] In addition to being able to detect proteolytic cleavage and phosphorylation events, the MSP-MS assay could allow for profiling of a variety of other PTMs [Fig. 4(A)]. The mass spectrometry-based detection strategy enables high adaptability because most peptide modifications can be readily identified using existing MS data analysis packages. As described, this technology is particularly well suited for the specificity analysis of PTM enzymes that recognize peptide substrates in an extended, linear conformation. For example, crystal structures of the kinase CDK2/cyclin A and the histone acetyltransferase HAT1 bound to peptide substrates revealed that substrates adopt a linear conformation in the enzyme active site [Fig. 4(B,C)].[88,89] Initial MSP-MS experiments with other types of PTM enzymes have already been carried out. O-GlcNAc transferase (OGT) was profiled using the MSP-MS assay, resulting in "HexNAc" modifications at serine and threonine residues on five library peptides (unpublished data). Protein arginine N-methyltransferase (PRMT1), an arginine-specific histone methyltransferase, was also assayed using the MSP-MS library. PRMT1 methylated a single peptide in the library at an arginine residue adjacent to a glycine. This "RG" motif aligns with the known PRMT1 substrate motif, "RGG."[90] These preliminary results underscore that a simplified library of linear peptides can be used to obtain relevant PTM specificity information.

Conclusion

Peptide-based technologies have significantly expanded our ability to profile the substrate specificity of proteases and other PTM enzymes. The technologies discussed in this review can provide complementary information when applied together, enabling a more complete understanding of the specificity of a particular enzyme. The specificity information discovered can be used for a number of important applications. As mentioned previously, PTM enzyme specificity has facilitated the design of highly selective chemical probes, such as protease inhibitors or fluorescent substrates.[30,66] These types of probes have been particularly useful in enabling the noninvasive detection of a variety of cancers.[91,92] Specificity information can also be used to help better define the biological roles of a given PTM enzyme. For example, PTM enzyme specificity has been used to identify the likely site of post-translational modification within endogenous substrates.[16,17,93] This is particularly important when used in combination with degradomic-based methods or other techniques that identify large potential substrate repertoires. More generally, it is increasingly recognized that PTM enzymes, and particularly proteases, regulate biological systems through large, interconnected enzyme-inhibitor networks.[94–96] Determining specificity is a critical aspect of identifying likely interaction partners within the context of these networks. This, in turn, helps to define novel enzymatic cascades, allowing for the development of a systems-level understanding of PTM enzyme biology.

Peptide-based enzyme profiling technologies have progressed significantly since the advent of SPPS and we expect that they will continue to be critical for improving our understanding of the vast repertoire of cellular functions that PTM enzymes regulate.

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