Profiling the Serine Hydrolase Superfamily using Activity-based Probes

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Overview

Purpose: Profiling serine hydrolase expression and activity in mouse tissue lysates.

Methods: Fluorophosphonate (FP) activity-based probes were used to specifically label and enrich serine hydrolases for fluorescent gel imaging, Western blot and mass spectrometry (MS) analysis.

Results: Using FP probes to assess serine hydrolase activity, we profiled various serine hydrolase inhibitors. Additionly, we determined active-site labeling sites of 25 serine hydrolases and conclusively mapped the active-site serine using a combination of CID and ETD peptide fragmentation.

Introduction

The serine hydrolase superfamily is one of the largest, most diverse enzyme families in eukaryotic proteomes.[1] Serine hydrolases are generally grouped into two large 100+ member families: serine proteases (e.g., trypsin, elastase and thrombin) and metabolic serine hydrolases. Although many family members share a common catalytic active site, metabolic serine hydrolases are divided into multiple enzyme subclasses including esterases, lipases, amidases and peptidases based on differences in structure, catalytic mechanism and substrate preference. Because many proteins in this family are expressed as inactive proenzymes (i.e., zymogens), active-site probes are especially advantageous for activity assessment when compared to other expression profiling techniques that only measure abundance.

The Thermo Scientific ActivX Serine Hydrolase Probes enable selective labeling and enrichment of active serine hydrolases. The serine hydrolase probe consists of a tag linked to a fluorophosphonate (FP) group that specifically and covalently labels serines of enzymatically active serine hydrolases (Figure 1).[1-4] Depending on the active-site probe tag group used, FP probe-labeled enzymes can be detected and quantified by fluorescent gel imaging, Western blot or mass spectrometry (Figure 2). TAMRA-FP probes can label and detect serine hydrolase activity in samples using fluorescent gel imaging, capillary electrophoresis or MS.[4] Desthiobiotin-FP probes can enrich and detect active-site-labeled proteins by Western blot and MS.

Methods

Sample Preparation

Tissue protein labeling and profiling: Frozen mouse tissues (Pel-Freez Biologicals) were lysed using Thermo Scientific Pierce IP Lysis Buffer. Each lysate (50 µg) was labeled with 2µM TAMRA-FP probe for 1 hr at room temperature and separated by SDS-PAGE. Fluorescent gel scans were captured using a Typhoon™ 9410 Imager (GE Healthcare). For Western blot analysis, proteins were labeled with 2µM desthiobiotin-FP and denatured with 6M urea before capture with streptavidin agarose for 2 hr. Bound proteins were washed and eluted by boiling in sample buffer before SDS-PAGE separation and Western blotting.

Active-site peptide capture: Mouse tissue lysates were labeled with 2µM desthiobiotin-FP probe, denatured, reduced, alkylated and desalted into digestion buffer (20mM Tris pH 8.0, 2M urea). Samples were enzymatically digested before capture of active-site-labeled peptides using streptavidin agarose. Bound peptides were washed and eluted using 50% acetonitrile/0.1% TFA before MS analysis.

Liquid Chromatography and Mass Spectrometry

A NanoLC-2D™ UPLC (Eksigent) with a Magic™ C18 column 75µm ID x 20cm (Microhm) was used to separate peptides using a 10-45% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a flow rate of 300nL/min for 75 min. A Thermo Scientific LTQ Orbitrap XL ETD mass spectrometer was used to detect peptides using a data dependent decision tree (DDDT) to select peptides for collision induced dissociation (CID) or electron transfer dissociation (ETD) fragmentation.[5] For the DDDT, a top five experiment consisting of single stage MS followed by acquisition of five MS/MS spectra using CID for +2 peptides or ETD for peptides +3 (*m/z* 650), +4 (*m/z* 900) and +5 (*m/z* 950).

Data Analysis

MS spectra were searched using Thermo Scientific Proteome Discoverer software v1.2 with Mascot™ v2.3 and SEQUEST® search engines against a SwissProt database. Static modifications included carbamidomethyl (57.02 Da) with desthiobiotin-FP (443.29 Da) and methionine oxidation used as dynamic modifications.

Results

Serine hydrolase inhibitor profiling using FP probes

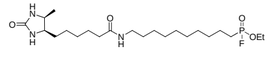
To demonstrate the utility and specificity of active-site probes for serine hydrolase activity profiling, mouse brain and liver tissue lysates were labeled with TAMRA-FP probe and analyzed by fluorescent gel scanning (Figure 3A). Using the fluorescent gel workflow described in Figure 2, we found that lysates pretreated with protease (AEBSF) or hydrolase (URB597 and CAY10401) inhibitors had different inhibition patterns of TAMRA-labeled serine hydrolases when compared to untreated control samples. Consistent with previously published reports, we found wide differences in active serine hydrolases expression between mouse brain and liver, while probe labeling also showed specificity for active serine hydrolases as heat denatured samples had signal similar to unlabeled control samples.[1-6]

Desthiobiotin-FP serine hydrolase probe was also used to enrich labeled proteins before Western blotting. The hydrolase inhibitors URB597 and CAY10401 were able to inhibit fatty acid amide hydrolase (FAAH), whereas the protease inhibitor AEBSF had no effect on enzyme activity (Figure 3B). As with our previous fluorescent gel imaging, these data confirmed inhibition of FAAH in mouse brain lysates using URB597 and CAY10401.

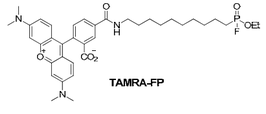
Beyond profiling inhibitor target specificity, active-site probes can be used to determine drug-binding constants and relative potency through inhibitor dose response curves (IC_{50}). Despite the ease of assessment of inhibitor targets by fluorescent gel scanning or Western blot, these workflows are limited by SDS-PAGE separation of proteins and the specific antibodies used. A more global approach to inhibitor assessment is required to identify drug targets and off-target effects. To this end, the MS workflow can identify protein targets by analyzing enriched serine hydrolase proteins or desthiobiotin-FP-labeled, active-site peptides.

FIGURE 1. Structures and labeling mechanism of fluorophosphate (FP) probes. Desthiobiotin-FP and TAMRA-FP probes (A) covalently label the active-site serine of enzymatically active serine hydrolases (B). The amino acids of the serine hydrolase active-site form a catalytic triad which changes the pKa of the serine hydroxyl. The orientation of these amino acids changes depending on the structural confirmation of the enzyme, which has different forms for the active and in-active states.

A



Desthiobiotin-FP



TAMRA-FP

B

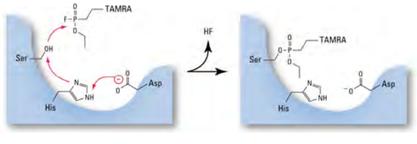


FIGURE 2. Schematic depicting three workflows for the profiling, capture, and detection of serine hydrolases with FP probes. Preincubation of enzymes with inhibitors enables the determination of inhibitor specificity, binding affinity and potency by Western blotting or fluorescent gel imaging of probe-labeled proteins or MS of probe-labeled peptides.

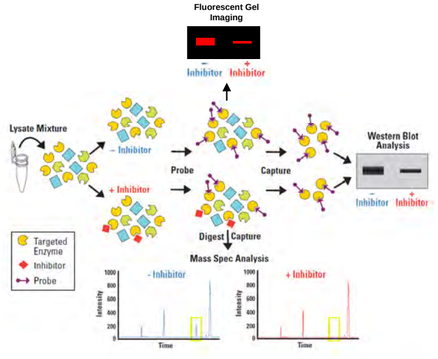
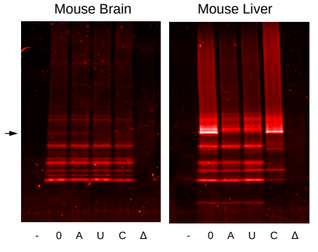
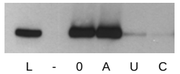


FIGURE 3. Using serine hydrolase probes for enzyme inhibitor profiling. A) Mouse brain or liver tissue lysates were pretreated with various inhibitors before labeling with TAMRA-FP probe. Unlabeled lysate (-) and heat denatured (Δ) lysate were used as controls to show probe labeling specificity. FAAH is indicated by arrow. B) Mouse brain tissue lysate was pretreated as in 3A and labeled with desthiobiotin-FP probe. Desthiobiotin-FP labeled proteins were denatured and enriched using streptavidin agarose before separation by SDS-PAGE and Western blotting with a specific FAAH antibody.

A



B

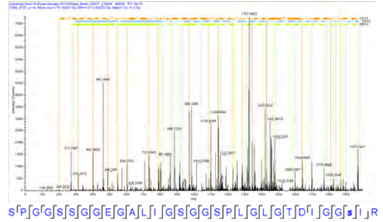


Legend
L: Lysate load
- : No probe
0: No treatment
A: 100µM AEBSF
U: 100µM URB597
C: 100µM CAY10401
Δ : Heat denatured

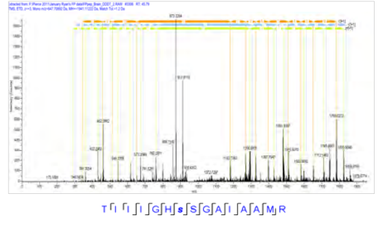
FIGURE 4. MS analysis of desthiobiotin-FP labeled peptides enriched from mouse tissue lysates. A) ETD MS/MS spectra of the FAAH and RBPP9 active-site-labeled peptides showing the desthiobiotin-FP modified serine (lower case "s") analyzed using an LTQ Orbitrap XL™ mass spectrometer. B) Venn diagram of total serine hydrolase active-site peptides identified using ETD and CID.

A

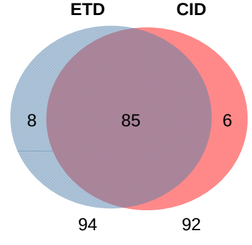
FAAH active-site peptide



RBPP9 active-site peptide



B



Total Identified Serine Hydrolase Active-site Peptides

Table 1. Serine hydrolases identified by MS with ActivX® Desthiobiotin-FP Serine Hydrolase Probe. Number of serine hydrolase family members from mouse brain and liver tissue extracts identified by MS after labeling and enrichment using the desthiobiotin-FP probe.

Serine hydrolase family	Number identified
Hydrolases	12
Esterases	3
Lipases	3
Peptidases	3
Other	2

Identification of serine hydrolase active-sites by MS

Using the MS Workflow (Figure 2), we determined active-site labeling sites for 25 serine hydrolase active-site peptides from mouse brain and liver tissues including FAAH and RBPP9 (Figure 4 and Table 1). To improve desthiobiotin-FP peptide fragmentation, we applied a data dependent decision tree (DDDT) to select for CID or ETD fragmentation. In this method, peptides with larger mass-to-charge ratios and higher charge states (e.g., +3, +4, +5) were fragmented with ETD, while smaller +2 peptides were fragmented with CID. Overall, this method combination increased the number of serine hydrolases identified (Figure 4B). In addition, ETD fragmentation typically resulted in better FP label assignment due to fewer neutral loss events. This was especially evident for peptides that contained multiple serines. The combination of CID and ETD spectra resulted in higher sequence coverage of labeled peptides, which resulted in higher scores in spectral library matching searches. In summary, FP activity-based probes facilitated the global profiling of serine hydrolase superfamily members in complex proteomes and the DDDT approach enabled identification of novel active-site serines.

Conclusions

- Thermo Scientific ActivX Serine Hydrolase Probes enable selective labeling and enrichment of active serine hydrolases in complex proteomes.
- Combining ETD and CID peptide fragmentation techniques increased the number of identified peptides, as well as peptide identification scores.
- ETD fragmentation resulted in unambiguous active-site serine modification assignment for peptides containing multiple serines.

References

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