Characterization of Adeno-associated viral proteins and related proteoforms using top-down approach on a LC-Orbitrap Tribrid MS platform

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ABSTRACT

Purpose: Develop a top-down AAV viral protein mass analysis method for the phospho localization of VP2 and protein sequence confirmation of VP3 clip.

Methods: An AAV6 sample was analyzed with a targeted top-down approach on a Thermo Scientific™ Orbitrap™ Accurate Mass MS platform equipped with a Thermo Scientific™ Vanquish™ Ultra UHPLC system using multiple fragmentation techniques. The top-down approaches were processed using the Thermo Scientific™ BioPharma Finder™ 5.1 Software.

Results: The developed top-down method was able to locate two phosphorylation sites (TPM1 and TPM2) by the multiple fragmentation techniques including EThcD, HCD and UVPD. The multiple fragmentation methods also allowed confident VP3 clip sequence confirmation with increased sequence coverage.

INTRODUCTION

AAV capsid viral proteins (VPs) are important constituents of AAV products and play an important role in immunogenicity and tissue tropism in gene therapy. In-depth characterization of viral proteins, including their truncated forms and PTMs, is critical for developing and manufacturing AAV products to ensure their safety, quality, and efficacy. A targeted top-down approach is one of the preferred methods for localizing post-translational modifications (PTMs) and identifying protein sequence. However, it was difficult to逵the accurate sites of the phosphorylation with the peptide mapping approach only because of the high sequence overlaps of the VP1 and VP2. Orbitrap-Accord Tribrid MS enables multiple fragmentation techniques with higher on-source isolation efficiency for the high m/z ions and provides an ideal platform for the top-down analysis of intact proteins. In this study, we performed targeted top-down analyses using EThcD, HCD and UVPD on the HPLC-Orbitrap Tribrid MS platform for the characterization of the proteoforms of VPs. For the targeted precursor ion selection, an additional intact protein analysis was carried out. The results are reported here.

MATERIALS AND METHODS

Sample Preparation

An AAV sample expressed in HEK293 cells was used. The AAV sample was buffer exchanged and concentrated to 80% in 20% acetonitrile containing 5 mM TCEP and 0.1% formic acid using 30K centrifugal filter (Amicon Ultra, 3.0 mL). The estimated protein concentration of the AAV sample was 0.2 μg/μL after buffer exchange and concentration.

HPLC conditions

For all experiments, chromatographic separations were carried out using a C4 stationary phase column (1.0 x 100 mm, 300 Å, 1.7 μm) on a Vanquish Horizon UHPLC System. The solvent A was water with 0.1% trifluoroacetic acid, and solvent B was acetonitrile with 0.1% trifluoroacetic acid. The column temperature was set at 50°C. The flow rate was 0.2 mL/min. The gradient condition used is listed in Table 1. The Orbitrap Accord Tribrid mass spectrometer equipped with the HPLC, EThcD and ETD options was used for MS data collection. The ESI source settings were shown in the Table 2. The MS settings for the intact mass analysis are shown in Table 3. The MS settings for the top-down mass analysis are shown in Table 4.

Data Analysis

Thermo Scientific™ BioPharma Finder™ 5.1 software was used for both intact protein and top-down data analysis.

RESULTS

Precursor ion selection for the targeted top-down experiments

An intact mass analysis was carried and the precursor ion selection. Figure 1 shows the TIC of the detected viral proteins and MS spectra of VP2 and VP3 clip. The enhanced mass range for the charge 46 of VP2 and 47 of VP3 clip shows the phosphorylation sites (Figure 1, inset). In order to generate the fragment ions directly linked to the two phospho-proteoforms, m/z 1441.5 (the second phospho-prototerm) was selected as the precursor ion for all targeted top-down experiments. By using the isolation window of 6, the m/z 1439.65 (representing the first phospho-prototerm) was also selected. The selected precursor ion with isolation window of 6 also covers the charge 30 (m/z 1441.68) of VP3 clip and the acquired top-down data can be used not only for the phosphosite localization of VP2, but also for the protein sequence verification of VP3 clip.

FIGURE 1. AAV viral protein intact mass analysis for precursor ion selection

Confident localization of two phosphorylation sites from VP2 using multiple fragmentation methods

EThcD, HCD and UVPD were performed for the targeted top-down MS experiments. A full MS scan was also performed in the same LC-MS run to ensure only the m/z data which are relevant to the VP2 or VP3 clip were used for data processing. Aimed to get more precursor ions into the e-Trap, high AGC value and up to 200 ms on injection time were used in all experiments, in order to get more fragment ions that are directly relevant the phosphorylated sites. We collected mass spectra data using three different mass ranges. Figure 2 shows the fragment ions of EThcD data with three mass range of m/z 460-1400 and the fragment pairs with and without phosphorylation in the specific amino acid and locations were identified clearly (Figure 2, inset).

FIGURE 2. Fragmented ions observed from EThcD with mass range of m/z 460 - 1400

A total of five top-down raw files (Three EThcD, one HCD and one UVPD) were processed using the top-down workflow of BioPharma Finder 5.1 software. The fragment ion data across the eluted VP2 peak was averaged per raw file and the averaged spectra were searched against three VP2 proteoforms, VP2 phospho-proteoform without phosphorylation, VP2 phospho-prototerm with phosphorylation on the 11S and 12V phospho-prototermions with use of PhosphoSite database. Figure 3 shows the full MS spectrum for VP2 (m/z 1441.5) and the observed phosphorylation localization confirmation. Figure 3 shows all three VP2 proteoforms were identified unambiguously with specific fragment ions using one fragmented ion (m/z 1439.65) and the full MSdata (Figure 3). The EThcD and UVPD data search results provided complementary fragment ions for the EThcD search results with increased sequence coverage. 28% sequence coverage was observed for the VP2 phospho-prototerm (11S) using UVPD (Figure 4). Overall, 30%, 43% and 47% sequence coverage were observed with the combined EThcD, HCD, UVPD data for the three VP2 proteoforms. Figure 5 shows the combined fragment map of the VP3 phospho-proteoform (with the 47% sequence coverage).

FIGURE 3. Fragment maps observed from EThcD with mass range of m/z 460 - 1400

REFERENCES

1. Thermo Scientific Application note # SW0036, Rafael Melani, et al., Highly sensitive intact mass analysis of viral protein phospho-proteoforms using Thermo Scientific™ Scientific™ BioPharma Finder™ 5.1 Software.
2. Thermo Scientific Application note # SW0037, Rafael Melani, et al., Adeno-associated virus capsid protein characterization and full NTA particle profiling using Thermo Scientific™ UHPLC Orbitrap MS.

CONCLUSIONS

A targeted top-down method that uses optimized mass spectrometer settings and multiple fragmentation techniques was developed to address the challenges of AAV viral protein PTM characterization and unambiguous protein sequence verification.

The developed method was applied to direct fragmentation of intact abundant VP2 phospho-proteoforms and VP3 phospho-proteoform with C-terminal trypsin (VP3 clip).

Two phospho-proteoforms were verified unambiguously from VP2.

The proposed VP3 clip sequence was confidently confirmed.

The multiple fragmentation methods provided complementary fragment ions and enabled increased sequence coverage.

TRADMARKS/LICENSING

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