Cysteine-Reactive Tandem Mass Tags for Subproteome Labeling, Enrichment and Quantitation

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Overview

Purpose: To develop a multiplexed, cysteine-reactive Tandem Mass Tag® (cysTMT™) reagent for subproteome peptide enrichment and quantification.

Methods: Cysteine-containing peptides labeled with cysTMTs were enriched using a novel, immobilized anti-TMT antibody resin.

Results: We developed a cysTMT reagent set to perform duplex isotopic or six-plex isobaric mass spectrometry (MS) quantification of cysteine-containing peptides. An anti-TMT antibody that recognizes the TMT reagent reporter region was characterized and assessed for immunoaffinity enrichment of peptides labeled with cysTMT reagents for multiplexed quantification.

Introduction

Tandem Mass Tag (TMT) reagents enable concurrent identification and multiplexed quantification of proteins in different samples using tandem mass spectrometry.[1,2] Isobaric TMT reagents share identical structures, including a peptide-reactive labeling group, a spacer arm, and an MS/MS reporter. During MS/MS analysis, each isobaric tag produces a unique reporter ion that makes quantification possible. Current amine-reactive NHS-ester isotopic duplex or six-plex isobaric TMT tags covalently attach to the free amino termini and lysine residues of peptides and proteins. We developed a novel set of cysteine-reactive Tandem Mass Tag (cysTMT) reagents and an anti-TMT antibody resin for capture of labeled peptides. The approach of targeted thiol labeling, affinity enrichment, and quantification is similar to Isotope Coded Affinity Tags (ICAT™). The cysTMT reagents can be used for functional studies of cysteine accessibility, oxidation, or nitrosylation state, and to reduce sample complexity and improve quantification dynamic range.[3-5]

Methods

Sample Preparation

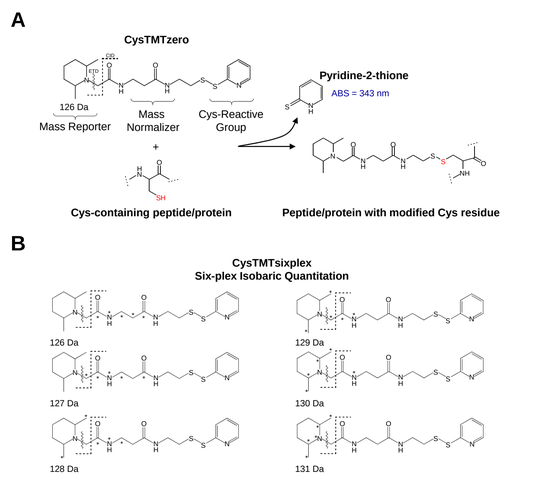
Preparation of cysTMT-labeled and ICAT-labeled proteins: Proteins were solubilized at 1 mg/mL in 6 M Urea/ 50 mM Tris/ 5 mM EDTA and reduced with 1 mM TCEP or DTT for 1 hour at 50˚C. Reductant was immediately removed using Thermo Scientific Zeba desalting columns, and the samples were combined with 20 µL of a 25 mM cysTMT solution. Each protein sample was labeled with the appropriate cysTMTzero™ or cysTMTsixplex™ reagents 126-131 for 2 hours at room temperature, combined and processed with Thermo Scientific Pierce Detergent Removal spin columns to remove excess tag. Proteins were digested at 37˚C overnight and desalted before liquid chromatography (LC)-MS/MS analysis or enrichment. For ICAT (Applied Biosystems) labeling, proteins were solubilized as above and processed according to manufacturer's protocol.

Preparation of cysTMT-labeled peptides: GLP-1 (7-17) (Anaspec) was reduced at 1 mg/mL with immobilized TCEP for 45 minutes at room temperature. Following this, the thiol concentration was determined using an Ellman's assay and the peptide was labeled for 2 hours with 0.85 equivalents of cysTMTzero reagent. Labeling efficiency was assessed by ultraviolet (UV) absorbance at 343 nm. The absence of free tag was confirmed by LC-MS/MS analysis.

Surface plasmon resonance (SPR) and Western blotting: Antibody affinity was determined using a TMT-derivatized CM5 chip on a Biacore™ 3000 instrument (GE Healthcare). TMT-labeled proteins were blotted using a purified mouse monoclonal anti-TMT antibody (0.1 µg/mL). Goat anti-mouse (H+L) HRP diluted 1:25,000 was used as the secondary antibody with Thermo Scientific Pierce SuperSignal West Dura Chemiluminescent Substrate for detection.

Enrichment of cysTMT-labeled peptides: Labeled peptides (5-200 µg) were resuspended in TBS at 0.5 µg/µL and allowed to incubate with an immobilized anti-TMT antibody column (20-200 µL of packed bed; 2.5 mg of antibody per mL of resin) overnight with end-over-end shaking at 4˚C. After collection of the unbound sample, the resin was washed 4 times with 4 M Urea/TBS, 4 times with 0.05% CHAPS, 4 times with TBS, and 4 times with water. Peptides were eluted 4 times with 50% acetonitrile, 0.4% TFA and then dried under vacuum before LC-MS/MS analysis.

FIGURE 1. cysTMT reagents and labeling reaction.



(A) Mechanism of cysTMT reaction with cysteine-containing peptides. (B) Structure of cysTMT isobaric six-plex reagents for cysteine-specific labeling, enrichment, and quantitation.

LC-MS Analysis

A NanoLC-2D™ high pressure liquid chromatography (Eksigent) with a Proteopep™ II C18 column 75 µm ID x 20 cm (New Objective) was used to separate peptides using a 4%-40% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 250 nL/min over 60 min. A Thermo Scientific LTQ Orbitrap XL ETD mass spectrometer was used to detect peptides using a top 2 x 3 experiment consisting of single stage MS, followed by acquisition of 3 MS/MS spectra with higher-energy C-trap dissociation (HCD) fragmentation, followed by 3 MS/MS with collision induced dissociation (CID) for protein identification.

Parameters in this mode were: Isolation width: *m/z* 3.0; collision energy: 50% (10% with two steps). Only doubly- and triply-charged peptides were selected for fragmentation. Dynamic exclusion parameters were set to the following: repeat count = 1; repeat duration = 30; exclusion list size = 500; exclusion duration = 20. Target values were as follows: MS = 5 e5; MS/MS (HCD) = 1 e5. Ion transfer times were set to 500 for Fourier transform mass spectrometry (FTMS) and 300 for MS/MS (HCD). Two microscans were required for HCD spectra.

Analysis of Quantification Data: Using Thermo Scientific Proteome Discoverer 1.2 software, mixed CID and HCD data were processed through a branched workflow that implemented a Reporter Ion Quantizer (20 ppm mass tolerance of fragment ions) to quantify the ratio. A separate segment processed the MS^2 spectra through the Spectrum Selector, Spectrum Normalizer, and Spectrum Grouper nodes. Data were then searched against both the SEQUEST® and Mascot™ search engines. In both cases, a 20 ppm mass tolerance was used. Static modifications were set to the cysTMTsixplex reagent (304.18 Da) and dynamic modifications included phosphorylation and methionine oxidation. The IPI human database was used in both cases.

FIGURE 2. Schematic of cysTMT workflow.

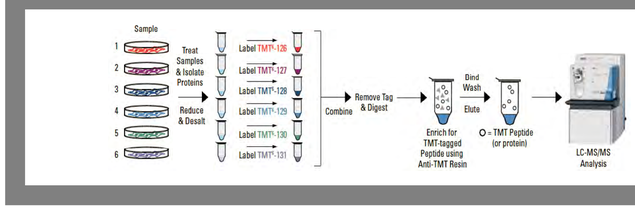
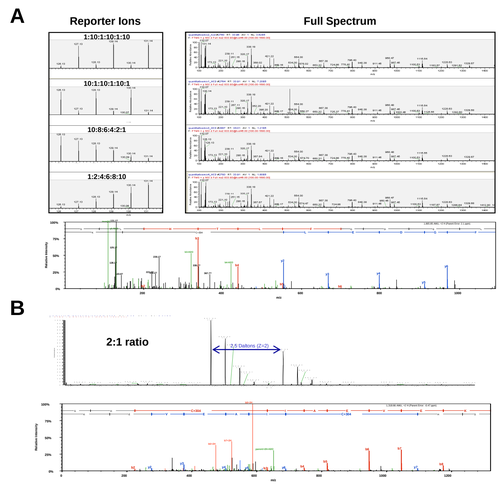
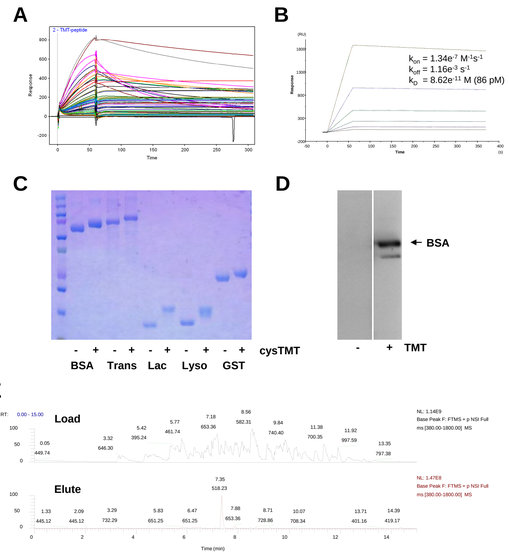


FIGURE 3. Quantification of BSA cysTMT-labeled peptides.



(A) Mass spectra of BSA peptides labeled with cysTMTsixplex tags mixed in different ratios and quantified using TMT reporter ions (*m/z* 126-131). (B) MS spectra of a BSA peptide labeled with cysTMTzero or cysTMT^6-127 mixed 2:1 to show isobaric quantitation.

FIGURE 4. Characterization of anti-TMT monoclonal antibody.



(A,B) SPR of anti-TMT hybridomas using a TMT-labeled surface. (C) SDS-PAGE of unlabeled (-) and cysTMT-labeled (+) proteins: BSA, transferrin (Trans), lactalbumin (Lac), lysozyme (Lyso) and GST. (D) Western blot of unlabeled (-) and TMT-labeled BSA (+) using anti-TMT antibody. (E) Chromatograms of a cysTMT-labeled GLP-1 peptide (1 nmol) spiked into 100 µg of a BSA digest and purified using anti-TMT resin.

Results

We have created a novel set of cysTMTzero and cysTMTsixplex reagents that contain a pyridyldithiol functional group for isotopic labeling of cysteine-containing peptides (Figure 1a). Unlike iodoacetamide labeling reagents, pyridyldithiols are not light-sensitive, have a high specificity for reduced thiols, and upon reagent labeling, release pyridine-2-thione which can be used to monitor reaction efficiency (Figure 1b).

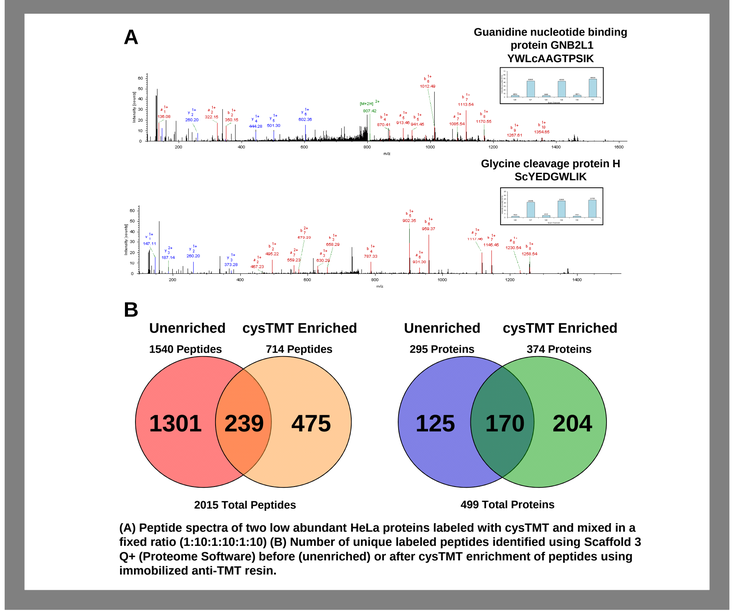
These reagents can be used to label cysteine residues of proteins pre- or post-digestion (Figure 2). Protein labeling with cysTMT reagents allows for labeling and mixing early in workflows for excellent quantification, and unincorporated tag can easily be removed using gel electrophoresis, size exclusion chromatography, or acetone precipitation. Alternatively, excess tag can be removed from peptides using strong cation exchange chromatography.

The cysTMT reagents are unique in that they can be used as heavy isotopic pairs or as six-plex isobaric tags for flexible quantification options (Figure 3). Since the reporter ions of the cysTMT tags are identical to amine-reactive TMT reagents, quantitative data analysis is supported by existing software.

In addition to novel chemical tags for quantification, we created an anti-TMT antibody developed against the reporter region of the TMT reagent. We have characterized the soluble antibody by surface plasmon resonance and Western blot detection of TMT- and cysTMT-labeled proteins (Figure 4), and assessed the immobilized anti-TMT antibody for multiplexed quantitative immuno-enrichment of proteins (not shown) and peptides labeled with TMT or cysTMT reagents (Figure 5).

This combination of cysTMT reagents with anti-TMT enrichment has several advantages over Isotope Coded Affinity Tags including: 1) more specific labeling of sulfhydryl groups; 2) a choice of six-plex isobaric multiplexing or duplex isotopic quantification using the same chemistry; 3) isobaric tagging for more efficient analysis of mass spectrometry data; and 4) a simpler workflow that does not require tag cleavage.

FIGURE 5. Capture of cysTMT-labeled HeLa peptides using immobilized anti-TMT antibody resin.



(A) Peptide spectra of two low abundant HeLa proteins labeled with cysTMT and mixed in a fixed ratio (1:10:1:10:1:10) (B) Number of unique labeled peptides identified using Scaffold 3 Q+ (Proteome Software) before (unenriched) or after cysTMT enrichment of peptides using immobilized anti-TMT resin.

Conclusions

- Cysteine-Reactive Tandem Mass Tags (cysTMT) are useful reagents for targeted labeling of cysteine residues pre- or post-digestion.
- cysTMT reagents can be used as either isotopic pairs or as an isobaric set for MS- or MS/MS-based multiplexed quantification.
- An antibody to the TMT reagent reporter region allows specific detection, capture, and enrichment of labeled proteins and peptides.
- cysTMT labeling and capture reagents are comparable to ICAT and allow for quantification of low abundance proteins.

References

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