Protein Quantitation of p53-Regulated Proteins using Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)

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Abstract
The p33 damage response pathway is critical in maintaining genome stability, and proteins within this pathway are commonly mutated or mis-regulated in cancer cells. In order to identify and characterize known and novel p53-regulated proteins, we employed a mass spectrometry (MS)-based proteomic approach using stable isotope labeling with amino acids in cell culture (SILAC). By comparing white cell lysates of heavy isotope-labeled A459 cells treated with camptothecin to control cells, MS spectra of isotope peptide pairs were compared and used in SEQUEST databases to identify proteins whose relative abundance was changed after treatment. This analysis revealed in the identification and quantification of over 200 proteins verified with at least two unique peptides. However, only changes in the most abundant p53-regulated proteins were observed. Using a sulphydryl-reactive biotin reagent, Bio-HPDP, samples were enriched for cysteine-containing proteins before MS analysis. This enrichment step resulted in the identification and quantification of additional p53-regulated proteins whose levels were altered after camptothecin treatment.

Introduction
Stable isotopic labeling with amino acids in cell culture (SILAC) is a method for quantification of differential changes in the proteome by mass spectrometry (MS). SILAC involves metabolic incorporation of heavy "L" or "H" labeled amino acids into proteins of actively growing cells using specifically modified media and unlabeled serum. Typical experiments involve growing two cell populations that are identical except that one of them contains a "light" and the other a "heavy" form of a particular amino acid (e.g., "C") and "L", or L-Lysine, respectively). Upon alteration of the proteome in one sample through chemical treatment or genetic manipulation, equal amounts of cell lysates from both cell populations are then compared for MS-based protein identification and quantitation. Multiple genoic and proteomic studies have identified roles for p53 in regulation of cell cycle arrest, DNA repair and apoptosis. Activation of the p33 DNA damage response pathway causes p33 stabilization through phosphorylation leading to transcription of various p53-regulated proteins (Figure 1). Camptothecin is an anti-cancer drug that inhibits topoisomerase II DNA unwinding and leads to DNA damage in cells undergoing DNA replication. Here, we studied the effects of camptothecin on protein levels in A459 lung carcinoma cells that have wild-type p53 expression. Using a Thermo Scientific Pierce® SILAC Quantitation Kit, A459 cells adapted to grow in Dulbecco’s Modified Eagle medium (DMEM) were labeled with "C", "L", or "G" to greater than 98% isotope incorporation. Heavy-labeled cells treated with camptothecin were lysed, mixed with control lysates, separated by SDS-PAGE and digested with trypsin before MS analysis. Cysteine-containing proteins present in whole cell lysates were also isolated using the reversible sulphydryl-reactive compound, Bio-HPDP. Peptides were then identified and quantitated using a Thermo Scientific Foreign LTO Orbitrap mass spectrometer. The SILAC method was able to successfully quantify changes in relative protein abundance after camptothecin treatment including some known to be up-regulated by p53 after DNA damage.

Model of p53 DNA Damage Response Pathway

Figure 1: Activation of the p53 DNA damage response pathway, results in p53 phosphorylation and stabilization through phosphorylation of the "activator" TAF1, which then complexes with ATM. This complex phosphorylates, and activates p53. This up-regulation of p53 results in transcription of p53-regulated genes, including repair, cell cycle arrest, and apoptosis. A549 cells treated with camptothecin with control cells. MS spectra of isopeptide peptide pairs were compared and used in SEQUEST databases to identify proteins whose relative abundance was changed after treatment. This analysis revealed in the identification and quantification of over 200 proteins verified with at least two unique peptides. However, only changes in the most abundant p53-regulated proteins were observed. Using a sulphydryl-reactive biotin reagent, Bio-HPDP, samples were enriched for cysteine-containing proteins before MS analysis. This enrichment step resulted in the identification and quantification of additional p53-regulated proteins whose levels were altered after camptothecin treatment.

Identification and Quantitation of p53 Peptides

Figure 2: (A) Identification of p53 peptide pairs in SILAC treated and control cell lysates. Quantitation of the p53-regulated genes was performed using the following protocol: (1) Figure 1: Activation of the p53 DNA damage response pathway, results in p53 phosphorylation and stabilization through phosphorylation of the "activator" TAF1, which then complexes with ATM. This complex phosphorylates, and activates p53. This up-regulation of p53 results in transcription of p53-regulated genes, including repair, cell cycle arrest, and apoptosis. A549 cells treated with camptothecin with control cells. MS spectra of isopeptide peptide pairs were compared and used in SEQUEST databases to identify proteins whose relative abundance was changed after treatment. This analysis revealed in the identification and quantification of over 200 proteins verified with at least two unique peptides. However, only changes in the most abundant p53-regulated proteins were observed. Using a sulphydryl-reactive biotin reagent, Bio-HPDP, samples were enriched for cysteine-containing proteins before MS analysis. This enrichment step resulted in the identification and quantification of additional p53-regulated proteins whose levels were altered after camptothecin treatment.

Identification and Quantitation of p53 Peptides

Figure 3: (B) Representative MS spectra of light and heavy isotope containing p53 peptide (7281429137359). The peptide has been deconvoluted into its components. In this example, the MS/MS spectrum shows up-regulation of the protein containing p53 peptide. Figure 1: Activation of the p53 DNA damage response pathway, results in p53 phosphorylation and stabilization through phosphorylation of the "activator" TAF1, which then complexes with ATM. This complex phosphorylates, and activates p53. This up-regulation of p53 results in transcription of p53-regulated genes, including repair, cell cycle arrest, and apoptosis. A549 cells treated with camptothecin with control cells. MS spectra of isopeptide peptide pairs were compared and used in SEQUEST databases to identify proteins whose relative abundance was changed after treatment. This analysis revealed in the identification and quantification of over 200 proteins verified with at least two unique peptides. However, only changes in the most abundant p53-regulated proteins were observed. Using a sulphydryl-reactive biotin reagent, Bio-HPDP, samples were enriched for cysteine-containing proteins before MS analysis. This enrichment step resulted in the identification and quantification of additional p53-regulated proteins whose levels were altered after camptothecin treatment.

Protocol for Using Bio-HPDP for Enrichment of Cysteine Containing Proteins

Figure 4: (A) Chemical structure of protein containing cysteine residue, e.g., L-DOPA. (B) Schematic representation of protein containing cysteine residue after labeling with Bio-HPDP. Bio-HPDP labels protein containing cysteine residues, e.g., L-DOPA. (C) Identification of Bio-HPDP labeled proteins. Bio-HPDP labeled proteins were enriched using the Bio-HPDP enrichment protocol (i.e., Bio-HPDP enrichment protocol as described in Figure 4A). Proteins were analyzed by LC-MS/MS and these were used to validate enrichment. (D) Enrichment of protein containing cysteine residue after labeling with Bio-HPDP. Bio-HPDP labels protein containing cysteine residues, e.g., L-DOPA. (E) Identification of Bio-HPDP labeled proteins. Bio-HPDP labeled proteins were enriched using the Bio-HPDP enrichment protocol (i.e., Bio-HPDP enrichment protocol as described in Figure 4A). Proteins were analyzed by LC-MS/MS and these were used to validate enrichment.

Table 1: Fifteen Most Up-regulated Proteins in A549 Whole Cell Lysates After Camptothecin Treatment

Table 2: Fifteen Most Down-regulated Proteins in A549 Whole Cell Lysates After Camptothecin Treatment

Table 3: Fifteen Most Up-regulated Proteins in A549 Whole Cell Lysates After Camptothecin Treatment with Biotin HPDP-Enrichment

Table 4: Fifteen Most Down-regulated Proteins in A549 Whole Cell Lysates After Camptothecin Treatment with Biotin HPDP-Enrichment

Conclusions
- SILAC method successfully quantitated over 350 proteins from only 100 μg of whole cell lysates.
- Average SILAC ratios of specific proteins were comparable to those determined by Western blot densitometry.
- Abundant p53-regulated proteins such as PCNA, SOD1, 14-3-3-δ and BAX were readily detected and quantified; however, lower abundant proteins such as p30, α2CD1 and p21 were not detected by MS and may require additional enrichment before MS analysis.

Biocytin HPDP labeling resulted in enrichment of cysteine-containing proteins which increased sample diversity, specifically proteins involved in cell signaling.

References

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Figure 5: (A) Representative MS spectra of light and heavy isotope containing peptide (7281429137359). The peptide has been deconvoluted into its components. In this example, the MS/MS spectrum shows up-regulation of the protein containing p53 peptide. Figure 1: Activation of the p53 DNA damage response pathway, results in p53 phosphorylation and stabilization through phosphorylation of the "activator" TAF1, which then complexes with ATM. This complex phosphorylates, and activates p53. This up-regulation of p53 results in transcription of p53-regulated genes, including repair, cell cycle arrest, and apoptosis. A549 cells treated with camptothecin with control cells. MS spectra of isopeptide peptide pairs were compared and used in SEQUEST databases to identify proteins whose relative abundance was changed after treatment. This analysis revealed in the identification and quantification of over 200 proteins verified with at least two unique peptides. However, only changes in the most abundant p53-regulated proteins were observed. Using a sulphydryl-reactive biotin reagent, Bio-HPDP, samples were enriched for cysteine-containing proteins before MS analysis. This enrichment step resulted in the identification and quantification of additional p53-regulated proteins whose levels were altered after camptothecin treatment.

Figure 6: (A) Schematic representation of protein containing cysteine residue after labeling with Bio-HPDP. Bio-HPDP labels protein containing cysteine residues, e.g., L-DOPA. (B) Identification of Bio-HPDP labeled proteins. Bio-HPDP labeled proteins were enriched using the Bio-HPDP enrichment protocol (i.e., Bio-HPDP enrichment protocol as described in Figure 4A). Proteins were analyzed by LC-MS/MS and these were used to validate enrichment.