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Utility of cleavable isotope coded affinity tagged reagents for quantification of low-copy proteins induced by methylprednisolone using liquid chromatography/tandem mass spectrometry

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Abstract

Gene expression changes underlie important biological and pharmacological responses. Although mRNA expression profiling is routine, quantification of low abundance proteins, which typically represent key effectors of responses, remains challenging. A novel strategy was developed for sensitive and accurate quantification of low abundance proteins in highly complex biological matrices. First, the cysteine specificity of cleavable isotope-coded affinity tags (cICAT) was employed to reduce the complexity of the digested proteome of tissue homogenates, and to improve the quantification of low abundance proteins. Second, cICAT treated tissue samples were analyzed on a capillary LC coupled to an ion-trap MS to screen for the subset of cICAT-peptides, derived from target proteins of interest, that was successfully labeled and retrieved. Third, putatively identified peptides derived from target proteins were synthesized, cICAT-labeled, and used both to optimize multiple reactions monitoring (MRM) analysis and to confirm chromatographic retention time and fragmentation pattern. Finally, batch quantification of target peptides was performed using MRM on a LC/triple-quad MS/MS using ^{12}C - (control) and ^{13}C (experimental) -cICAT labeled tissue mixtures. The utility of this method was demonstrated by elucidating the time course of tyrosine aminotransferase (TAT) induction in the liver of rats following treatment with the corticosteroid methylprednisolone (MPL). This approach significantly improved quantitative sensitivity, and the linear range was 10-fold greater than published previously. An additional advantage is that archived samples may be re-interrogated to investigate the regulation of additional targets that become of interest. Stored samples were successfully re-interrogated to monitor the induction of ornithine decarboxylase (ODC), which is also an MPL-induced protein. To our knowledge, this is the first report of an ICAT-based method capable of quantifying low abundance proteins in highly complex samples such as tissue homogenates. The approach enables simultaneous quantification of multiple effector proteins induced by biological or pharmacological stimuli, and the processed samples can be interrogated repeatedly as additional targets of interest arise.

INTRODUCTION

Changes in the expression of specific proteins not only regulate diverse biological and pathophysiological processes, but also underlie the pharmacological responses that are induced in tissues by many therapeutic agents. A variety of techniques have been developed to

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investigate gene expression changes at the transcriptional level^{1–3}, but quantification of mRNA often does not provide accurate information on the respective protein products^{4, 5} that are usually the effectors of biological action. Proteins are more difficult to analyze than mRNA owing to the extreme diversity of their chemical and physical properties. Furthermore, a handful of key proteins often are responsible for the overall regulation of important biological processes or drug responses; in tissues, these regulatory proteins may be present at relatively low copy numbers against an obscuring background of high-abundance “housekeeping” proteins⁵. Thus both high sensitivity and selectivity are necessary for the quantitative analysis of pharmaceutically- and biologically- important proteins.

Numerous approaches have been employed for protein quantification, including traditional electrophoretic and chromatographic methods^{6–10}, immune-based techniques^{11–13} and those based on mass spectrometric (MS) analysis^{5, 14–18}. Two dimensional electrophoresis combined with MALDI-TOF or ESI-MS/MS sequencing provides much better selectivity than traditional electrophoretic techniques^{19, 20}, but has poor quantitative accuracy²¹ and tends to discriminate against lower abundance proteins. A series of isotope-coded techniques has been developed for protein expression profiling^{21–24}, among which the isotope coded affinity tag (ICAT) is the most prevalent. The functional components of a cleavable ICAT (cICAT) reagent are shown in Scheme 1. The cICAT consists of a protein-reactive group, a retrieval ligand, and a linker region composed of 9 ¹²C or ¹³C atoms that constitute the heavy or light isotopic tag. In the example shown, the reactive moiety is specific for the thiol group of Cys residues, and the retrieval ligand is biotin. Proteins in experimental- and reference samples are separately labeled with the isotopically light or heavy cICAT reagent, and then are combined, digested with a protease, and retrieved using an avidin affinity column. After removal of the retrieval ligand, MS is used to screen for paired isotopic peaks that differ by 9 Da (or 4.5 for doubly charged MS peaks, etc.). Variations in the abundance ratio of the isotopic peaks reflect relative differences in the concentration of the target protein in the control and experimental samples.

ICATs enable accurate relative quantification of proteins and reduce the bias toward high-abundance proteins. Furthermore, this approach does not require the synthesis of specific isotopically labeled peptides for each target protein, as do some LC-MS/MS based methods such as AQUA¹⁷. Although the ICAT approach has enabled proteome-scale expression profiling^{21, 25}, several problems have hindered its use for the accurate quantification of lower abundance target proteins in complex biological matrices, which is essential for many applications in biochemical and pharmacological research. These challenges arise from the complexity of matrices such as animal tissue homogenates, the sensitivity of the method *vs.* the relative and absolute intensity of the targets, and the selectivity of the method for the specific target²⁵. A large number of cICAT-peptides are recovered after labeling and digestion of a tissue homogenate, but target peptides of interest may be present in a much lower relative abundance. ICAT-based methods frequently employ MALDI-TOF or automated LC-MS/MS strategies for the detection of target peptides, often combined with SCX chromatography^{22, 24, 25}. As a group, these strategies tend to discriminate against the lower abundance proteins in highly complex samples. With MALDI-TOF, low abundance ICAT-peptides often are masked by high abundance peptides of similar *m/z*, as well as chemical noise. Automatic LC-MS/MS strategies select a limited number of the highest intensity ions at each time point, and therefore may fail to interrogate the minor ions that could represent the low abundance effector proteins that are of greatest interest. Thus for highly complex samples such as tissue homogenates, these strategies are not suitable for the quantification of specific lower abundance proteins, unless extensive purification is employed before analysis²⁵. Furthermore, false-positive identification is a significant problem with low abundance peptides in complex mixtures²⁶. Finally, the linear range of ICAT-based methods employing MALDI-TOF or ion

trap MS for quantification is quite narrow (approximately 10-fold^{24, 25, 27}). A narrow dynamic range compounds the problem of monitoring larger protein expression level changes.

Here we describe an LC-MS/MS strategy that employs commercial iCAT reagents for the direct quantification in animal tissues of lower abundance target proteins that are important in the pharmacological response to corticosteroids, a prevalent, clinically-important class of drugs. Under optimized conditions, tissue homogenates are prepared and then proteins are extracted, iCAT-derivatized, digested, retrieved, and cleaved. The cysteine specificity of the ICAT reagent reduces the complexity of the digested proteome, thereby enabling better quantification of lower abundance proteins. Based on published sequence, the m/z for all possible tryptic iCAT peptides derived from each target protein of interest is predicted, and a capillary LC- ion trap MS is used to screen for any successfully labeled and retrieved targets in the peptide mixture. The identified target peptides are then synthesized and used both to confirm the identification of the target, and to optimize conditions for multiple reactions monitoring (MRM) of that peptide on a triple-quad MS/MS. The rationale is that the triple quad instrument is more sensitive in MRM mode and has better linearity for quantification^{28, 29}. Batch quantification of the tissue-derived target peptides is then performed using the same LC system coupled to the triple-quad MS/MS.

This approach was developed and optimized using low concentrations of bovine serum albumin (BSA), and then was employed to investigate the temporal expression pattern of tyrosine aminotransferase (TAT) in the livers of adrenalectomized (ADX) rats treated with methylprednisolone (MPL). TAT is a low abundance protein in liver, and is an important marker for the pharmacodynamic effects of corticosteroids such as MPL³⁰. Our previous studies demonstrated the induction of both TAT mRNA and enzymatic activity after administration of MPL^{31, 32}. However, the lack of a direct method for TAT quantification in biological samples has hindered the investigation of key steps in the cascade that links MPL receptor binding to the expression of the effector protein.

EXPERIMENTAL SECTION

Materials and Reagents

HPLC grade acetonitrile, methanol, and water were from B&J (Muskegon, MI). Trypsin (TPCK-treated) and the BCA protein assay kit were from Pierce (Rockford, IL). Trifluoroacetic acid (TFA), formic acid, triscarboxyethylphosphine (TCEP), phenylmethylsulfonyl fluoride (PMSF), leupeptin and bovine serum albumin (BSA) were from Sigma (St Louis, MO). Aprotinin was from ICN Biomedicals (Aurora, Ohio). The cleavable ICAT reagents and monolithic avidin cartridge were from Applied Biosystems (Foster City, CA). Metaflash cartridges tightly packed with strong cation exchange (SCX) adsorbent were from Varian (Palo Alto, CA). Methylprednisolone (MPL) was from Pharmacia-Upjohn Co. (Kalamazoo, MI).

Instrumentation

An Agilent 1100 capillary HPLC system (Palo Alto, CA), equipped with a flow rate sensor, microwell autosampler, and online degasser was used for all LC-MS assays. Peptide identification was performed using a Thermo Finnigan LCQ ion trap MS equipped with a nanospray ESI interface, and subsequent quantification was carried out on an Applied Biosystems (Foster City, CA) API 3000 triple-quadrupole mass spectrometer with a Turboionspray source. The original tubing in the Turboionspray source was replaced with 75 μ m I.D. fused silica capillaries in order to achieve optimal performance at low flow rates.

Sample preparation and cICAT derivatization

Rat livers that had been frozen rapidly in liquid nitrogen at the time of harvesting were pulverized under liquid nitrogen using a mortar and pestle. One gm of liver powder was suspended in 10 mL of buffer consisting of 30 mM Tris, 1.5% Triton X-100 (pH=8.2) and 1 mM Na_3VO_4 . TCEP (2 mM), PMSF (1 mM), leupeptin (10 $\mu\text{g}/\text{mL}$), and aprotinin (10 $\mu\text{g}/\text{mL}$) were then added. The suspension was homogenized on ice and centrifuged for 20 min at 10,000g. The concentration of total proteins in the supernatant was determined by the BCA method. The supernatant was diluted with 25 mM Tris, 0.1% SDS and 1 mM TCEP to achieve a final protein concentration of 1 mg/mL and the solution was boiled for 1 min. The ^{12}C - (control sample) or ^{13}C - (experimental sample) cICAT reagent was dissolved in the solution and incubated at 42 °C for 2.5 hr. The control and experimental samples were then combined and the mixture was digested with TPCK-trypsin at 37 °C for 16 hr. The mixture was loaded onto an SCX cartridge equilibrated with 10 mM KH_2PO_4 (pH 3.2), 30% acetonitrile, and eluted with 10 mM KH_2PO_4 (pH 3.2), 30% acetonitrile, 300 mM KCl. The cICAT-peptides recovered from SCX chromatography were retrieved using an avidin column. The biotin moiety was cleaved according to the manufacturer's instructions, except the incubation time was 1.2 hr. The cleaved peptides were then reconstituted in 50 μL of 0.1% TFA in 10% acetonitrile in water.

Qualitative LC-MS/MS and database searching

Screening of cICAT-peptides derived from target proteins of interest was performed using a capillary LC/ion trap MS to analyze a concentrated ^{12}C -cICAT labeled tissue extract (800 μg total proteins in 50 μL solvent). Separation of peptides was carried out on a 300 μm (i.d.) x 150 mm Zorbax C_{18} capillary column (particle size 3.5 μm). The injection volume was 8 μL , employing a peptide trap, and the flow rate was 5 $\mu\text{L}/\text{min}$. The mobile phases consisted of: (A) 0.1% formic acid and 0.02% TFA in 5:95 acetonitrile: water, and (B) 0.1% formic acid and 0.02% TFA in 60:40 acetonitrile: water. A gradient was then run to resolve the recovered cICAT peptides: 0% B held for 5 min, then to 14% B in 5 min, to 55% B in 95 min, to 83% B in 55 min, to 100% B in 10 min, and then 100% B held for 20 min. The ion trap was operated under positive ionization mode and employed data dependent scanning. For MS^1 scan events, the ion trap screened for MS peaks within ± 1.0 of the m/z values predicted for each cICAT peptide derived from the target protein. The flow rate of sheath and aux gas was set at 30 and 5 units, respectively. Source and capillary voltage was set at 5 kV and 34 V, respectively. The maximum ion time was 200 ms. For MS^2 events, the instrument was programmed to scan the fragments of candidate peptides, using an initial collision energy of 45 units and a parent ion isolation width of m/z 2.0. Database searching was performed using the MS-Tag online program (<http://prospector.ucsf.edu/ucshtml4.0/mstag.htm>) to interrogate the NCBI protein database. Parent and fragment ions were analyzed assuming a mass modification on each cysteine residue corresponding to the addition of the ^{12}C - or ^{13}C -cICAT moiety. Tentatively identified cICAT-peptides derived from target proteins were confirmed by synthesizing the peptide, derivatizing it with the cICAT reagent, and comparing its retention time, parent m/z , and fragmentation pattern with that of the cICAT-peptide putatively identified in the tissue homogenate.

Quantitative LC-MS/MS

The MRM conditions for peptide quantification were optimized by direct infusion of the synthesized cICAT-peptide at 1 $\mu\text{g}/\text{mL}$ into the triple-quad MS/MS. For liver-derived samples, separation was carried out on the same LC system as described above. The dwell time for each transition was 200 ms and the pause time for scan parameter changes was 8 ms. The flow rate of nebulizer (air) and curtain gas (N_2) was 0.3 L/min and 0.6 L/min, respectively. The pressure of the target gas (N_2) for collisionally activated dissociation (CAD) was 4.8 mTorr. The ion

spray voltage, orifice potential, and ring focus voltage was set at 5000 V, 60 V, and 280V, respectively.

Linearity and method validation

The cICAT-peptides derived from BSA were used to investigate the linearity of the proposed method. Standard solutions of BSA were prepared in 100 μ L 25 mM Tris/0.1% SDS and contained 0.075, 0.3, 0.6, 3.0, and 7.5 pmol protein, which is equivalent to 12–1200 fmol per injection (assuming 100% efficiency of the cICAT procedure). The control solution was 0.6 pmol BSA in 100 μ L Tris/SDS. Both standard- and control solutions were labeled, mixed, digested, and retrieved, and six selected cICAT-peptides (Table 2) were quantified simultaneously using the LC/triple-quad MS/MS. Calibration curves were prepared by plotting the extracting ion current (XIC) peak area ratios vs. protein concentration for the heavy/light cICAT labeled peptides. Linearity, sensitivity, and recovery were determined independently for each of the 6 cICAT-peptides of BSA.

Quantification of TAT induction by MPL. a. Animals

Adrenalectomized (ADX) male Wistar rats (210–275 g) were purchased from Harlan-Sprague-Dawley Inc. (Indianapolis, IN), and were allowed to acclimate for 1 week to a constant temperature environment and 12-h light/dark cycle. Free access was provided to standard rat chow and drinking water containing 0.9% NaCl.

Rats were treated with 50 mg/kg MPL sodium succinate via a cannula implanted into the femoral vein. Additional rats served as baseline controls. Groups of 3 rats were sacrificed at each of 5 time points that were chosen based on previous studies of MPL-induced changes in gene expression^{27, 30}. Animals were anesthetized with halothane and the thoracic cavity was opened. A blunt 18 ga. needle was inserted through the left ventricle into the aorta, and rats were perfused with 50 mL saline containing 5 U/mL heparin. The liver was removed, minced, frozen in liquid nitrogen, and stored at -80°C until analysis.

b. Quantification of TAT

Rat liver homogenates were prepared as described above and cICAT-derivatized. In order to screen for successfully derivatized and retrieved cICAT peptides from TAT using LC/ion trap MS, 800 μ g total liver proteins were treated with the ^{12}C -cICAT reagent. Identification of optimal TAT peptides, database searching, and peptide confirmation were performed as described above. The TAT peptide (R)VVITVPEVMMLEACSR(Q) (T7) was judged to be the optimal peptide for TAT quantification. A 1 μ g/mL solution of synthesized, cICAT-labeled T7 peptide was used for LC/triple-quad MS optimization. The transitions of m/z 1003.4/1494.2 and m/z 1007.8/1502.6 were selected for MRM of the ^{12}C and ^{13}C cICAT-peptides, respectively. The LC gradient for batch-wise TAT quantification was as follows: 0 min, 10% B; 5 min, 10% B; 10 min, 24% B; 50 min, 58% B. One hundred μ g of total liver proteins from both the experimental and control samples were analyzed for TAT quantification. A tissue sample harvested 4 h after MPL dosing was chosen as the ^{12}C cICAT control. TAT expression at each time point was determined by calculating the $^{13}\text{C}/^{12}\text{C}$ cICAT extracting ion current (XIC) peak area ratios.

c. Re-interrogation of samples for ODC quantification

The cICAT-derivatized liver homogenates used for TAT quantification, which were stored at -80°C after the initial analysis, were thawed and re-interrogated. Using the procedure described above for TAT, optimal tryptic cICAT-peptides derived from ODC were screened by LC/ion trap MS. Capillary LC gradient conditions were the same as used for TAT quantification. The peptide EEFDCHILDEGFTAK (O1) was selected, chemically synthesized, and cICAT-

labeled to permit optimization of MRM conditions. The transitions of m/z 991.4/319.0 and m/z 995.9.8/319.0 were used for MRM of the ^{12}C and ^{13}C cICAT labeled *O1*, respectively.

RESULTS AND DISCUSSION

Sample preparation

Procedures for sample preparation and cICAT derivatization were optimized for quantification of low abundance proteins in highly complex matrices such as tissue homogenates. Key elements of our strategy were to exploit the cysteine specificity of the ICAT reagent to reduce the complexity of the trypsin-digested proteome, employ an MS-ion trap instrument to screen a long, shallow LC gradient for predicted ICAT peptides that would be derived from target proteins of interest, and then utilize a triple quadrupole instrument, operating in MRM mode, to quantify target peptides with high sensitivity, selectivity, and wide dynamic range.

Several important steps in this strategy required detailed examination and optimization. They include efficiency of protein labeling with the ICAT reagent, trypsin digestion, and peptide recovery. Both SDS-PAGE and LC-MS/MS were used to monitor the completeness of reaction and to optimize conditions (data not shown). For the biotin moiety cleavage step, the optimal incubation time was 1.2 hr; noticeable degradation of target cICAT-peptides was observed with incubations ≥ 2 hr (data not shown).

A variety of separation procedures also were investigated. In preliminary experiments, SCX chromatography was used to pre-fractionate liver homogenates. However, considerable loss of some lower abundance target peptides was observed (data not shown). Therefore, the entire peptide mixture was eluted from the SCX column as a single fraction, and then a series of capillary HPLC gradient steps was used to resolve this complex sample.

LC-MS/MS strategy

Because animal tissues contain large numbers of proteins, and proteins of interest often are present at relatively low copy number⁵, high sensitivity and selectivity are essential to identify and quantify those target proteins in tissue homogenates. Furthermore, tryptic digestion of a tissue generates a large number of peptides, and the peptides derived from high-abundance proteins dominate the digested proteome. Therefore, strategies to reduce the complexity of the sample are essential to improve the quantification of lower abundance proteins. Because the ICAT approach is cysteine-selective, it can perform this essential function. To resolve ICAT peptides derived from trypsin digestion of cell or tissue lysates, two-D chromatography coupled with automatic MS² has been employed^{21, 22}. However, that approach was developed for relative expression profiling on the proteomic scale, and usually employs an automatic MS strategy. For quantification of specific target proteins of low relative abundance, the automatic MS strategy may discriminate against such peptides in highly complex samples. Furthermore, the fractionation of samples using 2-D chromatography is not only time-consuming, but also risks the loss of low abundance targets, as observed in preliminary experiments (data not shown).

Therefore, we devised a two-stage strategy in which optimal target peptides were first screened and identified by LC/ion-trap MS, which is sensitive in the full m/z range scan and product scan modes, and then quantified batchwise using LC/triple-quad MS/MS, which has a wider dynamic range and much higher sensitivity under MRM mode^{28, 29}.

a. Screening for the optimal target cICAT-peptides by capillary LC/ion-trap MS

Because the ICAT procedures and trypsin digestion can exhibit perplexing preferences (discussed below), prediction of the optimal cICAT peptides that would permit the

quantification of a specific target protein is virtually impossible. Without the assurance that a given cICAT peptide will be obtained and detected, the random selection of a cICAT peptide for synthesis as a standard for analytical optimization and quantification carries significant risk of failure. We regarded this approach to be prohibitive in cost, particularly when multiple target proteins are of interest. Many proteins of interest are not readily available in pure form, and therefore cannot be used to optimize quantification strategies. To circumvent these problems, cICAT-peptides suitable for the quantification of a specific target were identified by screening the entire cICAT-derivatized peptide mixture extracted from the tissue homogenate, searching for all predicted cICAT peptides that could be derived from the proteins of interest.

Although the specificity of the ICAT reagent for cysteine-containing peptides reduces the complexity of the digested tissue proteome considerably, a large number of cICAT-labeled peptides are nonetheless recovered from tissue homogenates. Therefore, the automatic MS/MS strategy that is often employed with the ICAT approach is not suitable for quantification of targets of low relative abundance in this situation; the bias toward higher abundance peptides decreases the probability of detecting lower-abundance targets of interest. A capillary HPLC step gradient was optimized to resolve the highly complex tissue homogenate matrix, which improved the ability to search for specific target peptides of interest. The screening and identification strategy consisted of three successive steps. First, an LC-MS¹ scan was programmed to search for candidate cICAT-peptides that were successfully tagged and retrieved by scanning narrow m/z windows ($m/z \pm 1.0$) around the m/z predicted for all possible cICAT peptides of the target protein, based on known sequence. Any detected candidate peptide was trapped and fragmented using LC-MS². Second, target peptides were identified by comparing the parent and fragment m/z against the NCBI protein database. If more than one peptide from a target protein was identified successfully, the selection of the optimal peptide for quantification was based on a compromise between two considerations: 1) the longer peptide would be preferable due to the inherently higher selectivity it would provide; 2) the more intense peptide would provide the greater sensitivity for quantification. Third, the optimal target peptide was then synthesized, and cICAT-derivatized. Its chromatographic retention time and fragmentation pattern was then compared to the putatively identified target peptide detected in the tissue homogenate. This step eliminated false-positive identification, which is problematic for low abundance peptides in complex samples. The synthesized peptide was also used to optimize conditions for MRM on the triple-quad MS/MS.

This strategy was developed and refined by screening for the predicted peptides derived from BSA in a tryptic digest of fetal bovine serum. Based on the amino acid sequence of BSA, 22 possible tryptic ¹²C cICAT-peptides in the m/z range of 500–2000 are predicted for the completely cleaved protein (Table 1; doubly charged peptides only). Fifteen of those peptides were successfully detected and identified in 0.1 μ g of total proteins from fetal bovine serum (Table 1). Pure BSA was used as a source of authentic cICAT peptides in order to confirm the identification of the putative target BSA peptides. This obviated the need for synthesis of the authentic target peptides. Figure 1 shows representative data obtained in the screening and identification of those BSA peptides, but omits data from the confirmation step.

b. Quantification of target proteins by capillary HPLC/triple-quadrupole MS/MS

Because of the need for high selectivity, sensitivity, and wide dynamic range, triple-quad MS/MS was used in MRM mode for quantification of the target peptides^{28, 29}. The triple-quad MS/MS has low sensitivity in full-scan and product-scan mode, and thus it is difficult to optimize MRM conditions for low abundance target cICAT-peptides using tissue homogenates. Therefore, synthesized target peptides tagged with the cICAT reagent were used for the selection of proper MRM pairs and the optimization of MRM conditions.

The dynamic range of LC/triple-quad MS/MS was investigated using low concentrations of BSA. The 6 most abundant cICAT-peptides derived from BSA were selected for quantification. Calibration curves for all peptides exhibited good linearity over a range of 12–1200 fmol BSA on-column (Table 2). Sufficient HPLC separation, coupled with MRM detection, resulted in an apparent dynamic range for protein quantification of approx. 100-fold (Table 2), significantly wider than the approx. 10-fold range reported for the ICAT approach in previous studies^{24, 25, 27}. The data in Table 2 also indicate that for BSA quantification, the LC/triple-quad MS/MS approach achieved much higher sensitivity than possible with LC/ion-trap MS². The mean quantitative recovery of BSA, based upon the 6 selected peptides, ranged from 79–108%. The injection-to-injection precision ranged from 3.9–8.2% (Table 3).

Fifteen of the 22 possible cICAT-peptides (68%) derived from BSA were detectable. Based on these peptides, the detection limit for BSA, defined as S/N 3, depended considerably upon the peptide chosen, and ranged from 0.5 to 800 fmol (30 pg to 50 ng) BSA on-column (data not shown). This result, along with the observation that not all predicted cICAT-peptides of BSA were successfully identified by LC/ion-trap-MS² (Table 1), suggests that the cICAT procedures and trypsin digestion exhibit significant bias among the possible cysteine-contained peptides of a given protein, and underscores the benefit of screening empirically for the optimal cICAT-peptides derived from proteins of interest.

Differences in chromatographic retention times between heavy and light ICAT-derivatized peptides have been observed when the non-cleavable ICAT reagent was employed²³. Such fractionation was not observed here using the cICAT reagent (data not shown).

Time course of TAT induction by MPL in rat liver

As a proof of concept, the induction of tyrosine aminotransferase (TAT) by corticosteroids was investigated. TAT is an important marker of pharmacological action for this class of drugs. Although the abundance of TAT in tissues has not been directly quantified previously, the quantitative results of the present study suggest that baseline expression of TAT is approx. 50 fmol per 100 μ g total liver proteins, or 20 ppm relative to total liver proteins, in terms of molar ratio. Based on this abundance, TAT would fit the definition of a low abundance protein¹⁷.

To screen for the optimal cICAT-peptide for TAT quantification, a relatively high amount of total liver protein (800 μ g) was ¹²C cICAT-derivatized. The fraction recovered and used for subsequent screening/identification of optimal cICAT-TAT peptides was reconstituted in 50 μ L.

Based on sequence information, 8 possible tryptic cICAT-peptides were predicted for completely-cleaved TAT (Figure 2). In the LC/ion trap-MS¹ screening step, well-defined XIC peaks were found within the anticipated m/z range for all 8 peptides (chromatogram not shown). However, LC-MS² analysis and database searching of each of the candidates revealed that most were not derived from TAT. Two cICAT-peptides, (R)EEVASYYHCHEAPLEAK(D) (*T1*) and (R)VVITVPEVMMLEACSR(Q) (*T7*), were preliminarily identified as TAT-derived by interrogating the NCBI nr protein database. The process for identification of the *T7* peptide, minus the confirmation step, is illustrated in Figure 3.

Because of its obviously more intensive MS signal under the conditions used, the *T7* peptide was selected for quantification of TAT. Its retention time was 88.1 min under the LC conditions described. Despite the reduction in sample complexity achieved by selecting only the cysteine-containing target peptides for analysis, the [M+2H]²⁺ ions of the *T1* and *T7* peptides were the 15th and 21st most intensive ions in the averaged MS¹ full-scan spectra (m/z 500–2000) acquired in their respective retention time windows (data not shown). Thus the target ions of the TAT cICAT-peptides would not have been identified if automatic MS² was employed.

Therefore, a search strategy that specifically seeks a limited number of predicted target peptides may yield a substantially higher success rate in screening and identifying lower abundance cICAT-peptides in a highly complex sample than would the use of automatic LC-MS².

The T7 peptide was synthesized and derivatized with the cICAT reagent. This synthetic peptide exhibited the same retention time, parent *m/z*, and ion trap MS² fragments as the corresponding T7 peptide detected in the liver homogenates (chromatogram not shown). The synthesized cICAT-T7 peptide was also used to develop optimal conditions for MRM. The CAD product spectrum of the ¹²C cICAT-T7 peptide produced by the triple-quad MS/MS is shown in Figure 4A. The y11 ion was the most abundant, and it consists of 11 amino acid residues. Thus, both sensitivity and selectivity were ensured by selecting the transition to this fragment for the quantification of TAT. The MRM conditions for this transition were optimized to obtain maximal signal intensities. Under the LC-MS/MS conditions selected, the cICAT-T7 peptide exhibited a good MRM signal in non-concentrated rat liver samples (consisting of 100 µg total proteins) and was well separated from the other isometric peptides (Figure 4B and 4C).

Analysis of numerous samples was necessary for adequate characterization of the time course of TAT induction, and MRM provided excellent selectivity for the target peptide. Therefore, assay throughput was improved by employing a shorter LC gradient for quantification of cICAT-T7. The synthesized cICAT-T7 peptide was spiked into liver homogenates in order to develop gradient conditions under which none of the previously detected isometric peptides overlapped the cICAT-T7 peptide (Figure 4D–4E). The standard curve for cICAT-T7 showed good linearity ($R^2=0.996$) over the range of 5–500 fmol on column. The detection limit (*S/N*=3) for this peptide was 0.1 fmol on-column, assuming 100% efficiency through the cICAT processing procedure. For all liver samples analyzed, the MRM signal of cICAT-T7 exhibited excellent *S/N* (>70), and the injection-to-injection reproducibility for quantification was 5.6% (*n*=4). Matrix effects that could severely impair LC-MS/MS quantification of the peptide in biological samples were not observed. This can be attributed to the use of the stable isotope internal standard, which is a fundamental feature of the ICAT method³³.

The time course of TAT induction by MPL was quantified in the livers of ADX rats (Figure 5a). The increase in TAT expression was significantly greater than baseline within 2 hr of MPL administration, peaked at approx. 4–5 hr post administration, and then declined toward baseline. This observation is in basic agreement with the time course of TAT mRNA induction observed previously^{30, 31}. TAT enzymatic activity was also analyzed in these samples. Interestingly, the magnitude of change in TAT protein concentration was considerably lower than the magnitude of change in TAT activity (Figure 5). Furthermore, the peak time of TAT protein induction preceded that of activity induction by approximately 1.5 hr (Figure 5b). The disproportionate induction of effector protein *vs.* activity suggests the possibility of post-translational mechanisms for TAT regulation, as suggested in previous studies of other glucocorticoids³⁴. Overall, this finding underscores the value of direct quantification of marker proteins.

Re-interrogation of the treated samples for new targets

Often, new targets of interest arise as additional experimental results are obtained. With many protein quantification strategies, particularly those that employ synthesized isotopically labeled peptides, specific internal standards must be added at the time of initial sample processing. To investigate the feasibility of quantifying additional targets in the peptide pool that was used for TAT quantification, we sought to characterize the induction of ornithine decarboxylate (ODC) by MPL in processed samples that were stored frozen (–80°C) after the analysis for TAT. Interest in ODC induction arose as a result of mRNA analysis³² that demonstrated the involvement of ODC in the pharmacological response to MPL.

Using the strategy described above for TAT, cICAT-peptides derived from ODC were identified (data not shown). The tryptic peptide EEFDCHILDEGFTAK (*O1*) was selected as optimal for ODC quantification. *O1* was synthesized and used to confirm the preliminary identification that was made by analysis of the liver sample extracts. The MRM conditions for batch quantification were also optimized using the synthesized, cICAT-derivatized *O1* peptide (data not shown). Typical MRM chromatograms are shown in Figure 6.

The time course of ODC induction by MPL is shown in Figure 7. Compared with mRNA expression data obtained previously³², the increase in ODC protein peaked significantly later than did ODC mRNA. To our knowledge, this is the first direct quantification of ODC induction in a whole tissue.

CONCLUSIONS

A strategy was developed for the quantification of pharmacologically important proteins that are present in low relative abundance in highly complex samples. This approach employs the ICAT strategy to reduce complexity of the sample and to introduce standards in the initial processing steps, LC/ion-trap MS² for screening and identification of the optimal cICAT-peptides from target proteins, the synthesis of the identified peptides to permit both target peptide confirmation and optimization of triple-quad MS/MS MRM conditions, and the use of LC/triple-quad MS/MS for batch-wise target quantification. This approach enables accurate, sensitive, and selective quantification of key effector proteins in biologically- or pharmacologically- important cellular response cascades. Compared to ICAT-based strategies reported previously, this approach improves significantly on both sensitivity and dynamic range for protein quantification (100-fold vs. 10-fold²⁵). The use of MRM for cICAT-peptide quantification offers much better selectivity than previous methods that rely upon the intensities of the parent ions (using MS¹) for quantification. MRM recently has been evaluated for the analysis of medium- and high-abundance serum proteins; a wide dynamic range and sensitivity sufficient for quantification of proteins in the low µg/ml range were reported²⁹. Here, MRM for quantification of validated peptide targets affords a sensitivity that enables quantification of proteins present in tissue homogenates at sub-fmol (10's of pg) levels.

For lower abundance peptides in highly complex samples, sequencing can yield false-positives caused by relatively poor quality of fragmentation spectra²⁶. The strategy described here eliminates false-positive identification by using synthesized target peptides for confirmation. The results also show clearly that the use of LC/automatic MS², which is commonly employed in ICAT approaches^{21–25} may have unacceptable bias against lower-abundance proteins, and would have prevented the detection of those of interest in the studies reported here. Screening instead for the specific m/z of putative target peptides can improve considerably the sensitivity for peptide identification, and eliminate discrimination against lower abundance proteins.

The utility of this strategy was illustrated by the successful determination of the time-course of expression changes in TAT, a low abundance effector protein that is induced in response to therapeutic concentrations of the corticosteroid MPL. Sub-fmol sensitivity and a quantitative linear range of 100-fold were achieved for the target TAT peptide, with a sample analysis time of 50 min.

To our knowledge, this is the first report of facile quantification of a low abundance protein in a matrix as complex as liver homogenates using an ICAT-based technique. Reduction in the complexity of the liver proteome by employing an ICAT method is one of several key components of our strategy. Two-dimensional chromatography coupled with automatic MS² for ICAT quantification^{21, 22} frequently has been used to reduce the complexity of samples, but would not have been suitable for this application, given the potential for loss of low

abundance proteins, as well as the bias against the detection of low abundance targets in highly complex matrices.

The strategy described here is also universal, in that it may be applied to a wide range of target proteins, and could achieve unparalleled high quantitative accuracy for low abundance proteins. Our results show that because the cICAT derivatizes the target proteins at the time of initial sample preparation, cICAT-treated tissue samples can be stored at -80°C and interrogated repeatedly as new targets of interest arise. This feature maximizes the utility of precious samples, saves the time and expense associated with sample preparation and cICAT labeling, and also permits correlation of the induction of different effector proteins in the same set of samples, even if those targets are not assayed simultaneously. Some other protein quantification strategies such as AQUA¹⁷ are not capable of this, because specific internal standards must be added at the point of initial sample preparation. Furthermore, the method described here does not require an expensive isotope-coded peptide for each target protein. Finally, multiple protein targets can be quantified simultaneously in a single LC/triple-quad MS/MS run, suggesting an application in high-throughput screening designed to reveal the state of complex signaling or response networks within cells or tissues.

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Figure 1.

Procedure for identifying successfully tagged and retrieved target cICAT-peptides using LC/ion trap MS². A ¹²C cICAT peptide (**MPCTEDYLSLILNR**) derived from BSA was identified in a mixture of 0.1 µg total fetal bovine serum proteins. (A) LC-MS¹: the extracting ion current (XIC) of the m/z range 947.0–949.0 (the expected m/z range of the doubly charged target peptide); (B) LC-MS² analysis: the averaged fragmentation spectrum of m/z 947.8 extracted within the retention time 71.7–72.5 min; (C) Database searching: the result by MS-Tag database searching using the m/z of the parent and fragment of the candidate peptide.

1 MDSYVIQTDV	11 DDSLSSVLDV	21 HVNIGGRNSV	31 QGRKKGRKAR
41 WDVRPSDMSN	51 KTFNPIRAIV	61 DNMKVQPNPN	71 KTVISLSIGD
81 PTVFGNLPTD	91 PEVTQAMKDA	101 LDSGKYNGYA	111 <i>T1</i> PSIGYLSSRE
121 <u>EVASYHCHE</u>	131 <u>APLEAKDVIL</u>	141 TSGCSQAIEL	151 CLAVLANPGQ
161 NILIPRPGFS	171 LYRTLAESMG	181 IEVKLYNLLP	191 EKSWEIDLKQ
201 <i>T2</i> LESLIDEK <u>TA</u>	211 <u>CLVVNNPSNP</u>	221 <u>CGSVFSKRHL</u>	231 <i>T3</i> QKILAVAER <u>Q</u>
241 <u>CVPILADEIY</u>	251 <i>T4</i> <u>GDMVFSDCK Y</u>	261 <u>EPLANLSTNV</u>	271 <u>PILSCGGLAK</u>
281 RWLVPGWRLG	291 WILIHDRRDI	301 FGNEIRDGLV	311 <i>T5</i> KLSQR <u>ILGPC</u>
321 <u>TIVQGALKSI</u>	331 LQRTPQEFYH	341 DTLSFLKSNA	351 DLCYGALAAI
361 PGLQPVRPSG	371 AMYLMVGIEM	381 EHFPEFENDV	391 <i>T6</i> EFTER <u>LIAEQ</u>
401 <u>AVHCLPATCF</u>	411 <i>T7</i> <u>EYPNFFR VVI</u>	421 <u>TVPEVMMLEA</u>	431 <i>T8</i> <u>CSR IQEFCEQ</u>
441 <u>HYHCAEGSQE</u>	451 <u>ECDK</u>		

Figure 2.

The sequence and predicted cICAT peptides of tyrosine aminotransferase (TAT).

Figure 3.

Identification of successfully tagged and retrieved TAT peptides from cICAT treated rat liver using LC/ion trap MS². Identification of the ¹²C cICAT labeled peptide **VVITVPEVMMLEACSR** (*T7*): (A) LC-MS¹: the XIC extracted in the predicted m/z range of ¹²C cICAT-*T7* (m/z 1002.9–1003.6); (B) LC-MS²: the fragmentation spectrum of m/z 1003.2 extracted within the retention time of 87.8–88.3 min; (C) The identification of ¹²C cICAT-*T7* by MS-Tag database searching.

Figure 4.

The quantification of TAT using LC/triple-quad MS/MS. (A) Fragmentation pattern of the synthetic ¹²C cICAT-VVITVPEVMMLEACSR (*T7*) peptide. (B) MRM chromatogram of ¹²C cICAT-*T7* and (C) ¹³C cICAT- *T7* in a rat liver sample collected at 2hr post-injection, under the same LC conditions used in the identification step; (D) MRM chromatogram of ¹²C cICAT-*T7* and (E) ¹³C cICAT- *T7* in the same liver sample using a shorter LC separation time; (F) calibration curve of *T7* within the range of 5–500 fmol on column (assuming 100% cICAT treating efficiency).

Figure 5. The normalized time course of (A) TAT expression level determined by LC-MS/MS and (B) TAT activity level determined by enzyme activity assay. ADX rats were treated with 50 mg/kg i.v. of MPL and sacrificed at the time points indicated.

Figure 6.

Representative MRM chromatogram for cICAT-derivatized ODC peptides in liver samples;
(a) ^{12}C cICAT treated *OI*; (b) ^{13}C cICAT treated *OI*

Figure 7.

Time course of ODC induction obtained by re-interrogating samples previously processed for TAT quantification samples. ODC levels were normalized to the concentrations present in the pre-treatment (time 0) sample.

Scheme 1.
The structure of cleavable ICAT reagents

Table 1Predicted ^{12}C cICAT-peptides derived from BSA

AA Sequence	[M+2H] ²⁺
(K)GLVLIAFSQYLQPCPFDEHVK (L)	1331.7
(K)TCVADESHAGCEK (S) *	902.4
(K)SLHTLFGDELCK (V) *	795.4
(R)ETYGDMADCCEK (Q) *	909.9
(R)NECFLSHK (D) *	602.8
(K)LKPDPTLTCDEFK (A) *	873.9
(K)YNGVFQECCQAEDK (G)	1044.5
(K)ECCHGDLLECADDR (A) *	1130.5
(K)YICDNQDTISSK (L) *	807.4
(K)ECCDKPLLEK (S) *	816.4
(K)SHCIAEVEK (D) *	621.8
(K)EYEATLEECCA (D)	921.9
(K)DDPHACYSTVFDK (L) *	862.9
(K)QNCDQFEK (L) *	619.7
(R)CCTKPESER (M) *	753.8
(R)MPCTEDYLSLILNR (L) *	947.9
(R)LCVLHEK (T) *	534.8
* (K)CCTESLVNR (R)	739.8
(R)RPCFSALTPDETYVPK (A) *	1026.0
(K)LFTFHADICTLPDTEK (Q) *	1039.5
(K)CCAADDK (E)	590.3
(K)EACFAVEGPK (L) *	639.3

* Denotes cICAT-peptides identified by LC-MS² and database searching in this study.

Table 2
Investigation of the Linearity, Recovery and Precision of LC/Triple-Quad MRM-MS/MS (LC/MRM) with Six cICAT-Peptides Derived from BSA and Comparison of the Sensitivity with that of LC/Ion Trap MS² (LC/MS2)

cICAT-Peptide from BSA	^a linearity (r ²)	recovery (SD)% ^b	precision (CV)% ^b	^c detection limit by LC/ MRM (fmol BSA)	^c detection limit by LC/ MS ² (fmolBSA)
TCVADESHAGCEK	0.990	79 (7), 96 (5)	6.1	6	200
SLHTLFGDELCK	0.998	85 (2), 104 (7)	5.5	0.5	4
YICDNQDTISSK	0.989	93 (11), 83 (4)	8.2	2	25
CCTKPESER	0.987	89 (6), 101 (9)	7.3	5	75
RPCFSALTPDETYVPK	0.993	108 (11), 104 (4)	3.9	0.75	10
LFTFHADICTLPDTEK	0.994	92 (5), 87 (9)	4.2	1.25	35

^aThe linearity for BSA quantification was calculated independently for the six cICAT-peptides over the range of 12–1200 fmol BSA on column (assuming 100% efficiency through cICAT procedures).

^bThe recovery and precision were measured with the same fetal bovine serum sample (0.1µg total proteins); the recoveries were determined in triplicate, by spiking respectively at the level of 48 and 480 fmol BSA (on column) into samples; to determine precision, aliquots of the serum sample stored at –80°C were injected twice on two different days (*n*=4).

^cDetection limits of both systems were defined as the BSA amount on column (assuming 100% efficiency through cICAT procedures) that gave a S/N of 3; the conditions for quantitative LC/MS² were optimized using cICAT-peptides derived from 50µg/mL BSA.