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Absolute Quantification of Specific Proteins in Complex Mixtures Using Visible Isotope-Coded Affinity Tags

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The identification of proteins in complex mixtures is most useful when quantitative information is also obtained. We describe a new type of protein tagging reagent called the visible isotope-coded affinity tag (VICAT) which allows the absolute amount of a target protein or proteins to be quantified in a complex biological sample such as a eukaryotic cell lysate. VICAT reagents tag thiol groups of cysteines or thioacetylated amino groups and introduce into the tryptic peptide a biotin affinity handle, a visible moiety for tracking the chromatographic location of the target peptide by a method other than mass spectrometry, a photocleavable linker for removing a portion of the tag, and an isotope tag for distinguishing sample and internal standard peptides. We demonstrate the use of VICAT reagents together with isoelectric focusing of peptides on an immobilized gel strip followed by combined microliquid chromatography/electrospray ionization mass spectrometry operating in selected reaction monitoring mode to determine the absolute abundance of a specific protein, human group V phospholipase A_2, in eukaryotic cell lysates. It is found that human lung macrophages contain 66 fmol of this protein per 100 μg of cell protein. Western blot analysis of human group V phospholipase A_2 in macrophages gave inconclusive data. VICAT reagents should be useful for numerous applications including the analysis of candidate disease markers in complex mixtures such as serum.

There is considerable current interest in developing reagents and methodologies for quantifying proteins present in complex biological samples. Although survey methods that strive to identify as many proteins as possible in a complex protein mixture provide biological insight and are progressing,[1,2] there is also a clear need for the development of reagents and accompanying methodologies that allow a *specific set of known proteins to be identified and their absolute abundance quantified*. Detection of specific proteins in complex mixtures is usually carried out by some form of immunological method involving a protein-specific antibody. The immunoblot (western blot) analysis is routinely used to detect specific proteins in a complex protein mixture such as a cell or tissue lysate. Although this technique is extremely important in biomedical research, it has limitations as noted below. It is highly desirable to develop new techniques for the quantitative analysis of specific proteins in complex mixtures to be used in cases where western blotting is problematic and for providing information not obtainable by immunological methods.

With recent advances in tandem mass spectrometry,[3−7] peptides derived from protein mixtures of considerable complexity can now be routinely identified and characterized in some depth. For example, MALDI combined with ion-trap mass spectrometry has been recently used to detect peptide pheromones of known amino acid sequence secreted from microorganisms.[8] Multidimensional chromatography coupled to tandem mass spectrometry has been useful for identifying hundreds to thousands of proteins in cell extracts (for example, ref 9). Unfortunately, different peptides analyzed in a mass spectrometer will produce different specific responses, which depend on their chemical composition, the matrix in which they are present, and other poorly understood variables. Therefore, the intensity of a peptide ion signal does not accurately reflect the amount of peptide in a sample. However, two peptides of identical chemical structure that differ in isotopic composition are expected, according to stable isotope dilution theory, to generate the same response in a mass spectrometer, such that their relative ion currents reflect the relative concentrations in the sample. We and others have combined stable isotope dilution with automated peptide tandem mass spectrometry. In

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one approach, stable isotopes are introduced at specific sites in proteins and peptides via chemical reagents we termed isotope-coded affinity tags (ICAT reagents).[10] ICAT reagents are useful for determining the *relative abundance* of proteins in two or more samples, for example, in yeast cells grown on different carbon sources[10] as well as in other mixtures of cellular proteins.[11−16] ICAT tags contain the affinity handle biotin, which allows the subset of peptides tagged to be selectively analyzed, for example, those containing cysteine in the case of the original ICAT reagents. The resulting simplification of the peptide mixture allows a higher fraction of proteins to be quantified. Other peptide tagging and enrichment schemes have been developed (for example, refs 17 and 18).

In the current study, we describe the design of a new generation of ICAT reagents that we call visible ICAT reagents (VICAT reagents) because they contain, as an additional feature, a probe that allows tagged peptides to be visualized. We illustrate the utility of VICAT reagents for determination of the absolute quantity of a specific protein in complex protein mixtures (cell lysates).

EXPERIMENTAL SECTION

Synthesis of Reagents. Full details for the synthesis of thiol-specific VICAT reagents ($VICAT_{SH}$) is published elsewhere.[19] Reagents are available by contacting M. H. Gelb.

Preparation of the Internal Standard and IEF Marker. The human group V secreted phospholipase A_2 (hGV) derived peptide SYNPQYQYFPNILCS (Biosynthesis Inc., 100 μL of 1 mM in 20% CH_3CN in water) was mixed with 2 μL of 1 M Tris-HCl, pH 8.3, and ^{14}C-$VICAT_{SH}$(+6) (3 μL of 14 mM in CH_3CN) and the mixture was stirred for 2 h (or overnight) at room temperature in the dark. Then, 13 μL of 250 mM DTT in water was added, the tube was incubated for 30 min in the dark, then 40 μL of 250 mM iodoacetamide in 50% CH_3CN was added, and the tube was incubated in the dark for 15 min. This latter step serves to cap the SH group of any remaining unreacted peptide since contamination of the biological sample with unreacted peptide would lead to false positive detection of the protein of interest. The internal standard was purified by HPLC (Vydac 218TP1010 column) with solvent A (water with 0.08% trifluoroacetic acid) and B (CH_3CN with 0.08% trifluoroacetic acid): 0−10 min, 0−25% B; 10−50 min, 25−45% B; 50−60 min, 45−80% B at 4 mL/min with monitoring at 240 nm (elution at 35% B, ESI-MS obs. $(M + 2H^+)^{2+} = 1239.3$). The IEF marker was made in the same way using 100 μL of 1 mM peptide stock, 2 μL of 1 M Tris-HCl, pH 8.3, and 1 μCi of ^{14}C-$VICAT_{SH}$(−28) (30 Ci/mol) Stock solutions of internal standard and IEF marker in 20% CH_3CN (stored at −20 °C) were quantified by scintillation counting, and structures were confirmed by ESI-MS.

Synthesis of C(*S*-$CH_2CONH(CH_2)_4NH_2$)DPGYIGSR. BOC−NH−$(CH_2)_4$−NH_2 (Fluka) was treated with iodoacetic anhydride (Aldrich) as described for iodoacetylation in the synthesis of $VICAT_{SH}$ reagents.[19] The laminin peptide CDPGYIGSR was treated with BOC−NH−$(CH_2)_4$−$NHCOCH_2I$ as described above for preparation of internal standards. Solvent was removed, and the residue was treated with 1 mL of trifluoroacetic acid:CH_2Cl_2 (1:1) for 30 min at room temperature. Solvent was removed, and the residue was resuspended in 20% CH_3CN and purified on a C18 reverse phase HPLC column. The structure of the product was confirmed by ESI-MS, and the concentration of tagged peptide in the stock solution was determined by OD280 nm using $\epsilon^{1\%} = 13.86$ (calculated from the amino acid sequence).

Cell Lysis and Preparation of $VICAT_{SH}$-Tagged Peptides. All steps with VICAT reagents were carried out under fluorescent tube room light and away from direct sunlight. *Spodoptera frugiperda* (Sf9) cells were grown as described,[20] pelleted by low-speed centrifugation, and washed twice with phosphate-buffered saline, and cell pellets were stored at −80 °C. To an Eppendorf tube containing 2 million Sf9 cells was added 200 μL of ice-cold lysis buffer (50 mM Tris, pH 8.3, 6 M urea, 2% (w/v) CHAPS, 5 mM EDTA), and cells were lysed with a vortex mixer (several 10−20-s bursts with intermittent cooling on ice). The sample was centrifuged at 4 °C at ∼12 000× *g*, the supernatant was transferred to a new tube, and the centrifugation and transfer were repeated. The protein concentration was determined using a small aliquot and the Bradford dye binding assay (BioRad) using bovine serum albumin as a standard. A 50-μL aliquot (100 μg protein) was transferred to a new tube, and the desired amount of recombinant hGV[21] internal standard (the hGV-derived, ^{14}C-$VICAT_{SH}$(+6)-tagged peptide), this peptide is unique to hGV protein when examined by BLAST analysis) were added. TCEP (0.5 μL) was added from a 250 mM stock in water to give 2.5 mM, and the sample was incubated at 37 °C for 30 min. $VICAT_{SH}$ (8 μL) was added from a 25.4 mM stock in CH_3CN to give 3.5 mM. The sample was incubated in the dark at room temperature for 3 h and diluted 3-fold with water, 6.5 μg of trypsin (modifed, sequencing grade, Promega) was added, and the sample was incubated for 15 h at 37 °C. The sample was concentrated back to its original volume (50 μL) in a Speed-Vac (Savant). Isoelectric focusing (IEF) marker (∼8900 dpm, 130 pmol) was added followed by 2.5 μL of IPG buffer (100X, Amersham Biotech. Cat. 17-6000-87) and 0.5 μL of bromophenol blue (saturated solution in ethanol). Finally, 6 M urea, 2% CHAPS in water was added to bring the sample volume to 250 μL.

The concentration of hGV in the stock solution was determined by submitting ∼10 μg for amino acid analysis (Protein Chemistry Laboratory, Department of Biochemistry and Biophysics, Texas

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A&M University). The nanomole amount of hGV was taken as the average of the individual nanomole values obtained by dividing the nanomole of each amino acid determined by the number of residues determined from the amino acid sequence.

For some experiments, a lysate of human lung parenchymal macrophages (obtained as a gift from M. Triggiani, University of Naples) (100 μg of protein in 10 μL of 0.5% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8) was mixed with an equal volume of water, and the desired amount of hGV protein was added to some samples. Trichloroacetic acid was added from a 50% (w/v) stock to give a final concentration of 20%. After incubation on ice for 45 min, the sample was centrifuged at 4 °C for 15 min. The supernatant was discarded, and the pellet was washed twice with 200 μL of ice-cold acetone with centrifugation as above. The pellet was air-dried and resuspended in 53 μL of Sf9 cell lysis buffer (see above). The hGV peptide internal standard (200 fmol, see above) was added, and tagging with VICAT$_{SH}$ and preparation for IEF was as above.

Preparative IEF. The commercial IPG strip (13 cm, linear pH range 3−10, Amersham Biotech.) was re-hydrated with sample and submitted to IEF using a Multiphor II device as described by the manufacturer: 0−100 V in 1 min, 100 V for 2 h, 100−500 V in 1 min, 500 V for 2 h, 500−3500 V in 6 h, and 3500 V for 10 h. The IEF unit was covered with aluminum foil to block light. The IPG strip was removed with tweezers, and excess mineral oil was removed by wiping the plastic backing with a tissue and tapping the edge several times on a tissue (avoid contacting the gel side of the strip with the tissue). The strip was laid gel-side up onto a piece of Whatmann 3MM paper, and the strip was covered with plastic (Saran Wrap). The plastic wrap above the desired region of the strip was marked with a pen (using a ruler as a guide). The desired region was cut into 10 ∼1-mm wide pieces using scissors (cutting through the paper, the IPG strip, and the plastic wrap), and individual IPG pieces were separated from the paper and plastic wrap with tweezers and transferred to individual Eppendorf tubes. More than 10 cuts of the IPG strip may be needed the first time a specific IEF marker is used since only the predicted migration position is known. Gel slices were subjected to continuous shaking on a vibrating platform in 200 μL of 1% NH_4OH in 20% CH_3CN for 1 h, and the liquid phases were transferred to new tubes. The extracts were concentrated to dryness (Speed-Vac). Gel slices were shaken for 1 h in 150 μL of 80% CH_3CN/0.1% trifluoroacetic acid water and extracts combined. After concentration to dryness, 100 μL of 20% CH_3CN was added, and a 5−10-μL aliquot was submitted to scintillation counting. Typically, >80% of the dpm along the entire strip were found in 1−2 tubes (i.e., ∼1−2 mm of the IPG strip). In subsequent runs, only 6 cuts of the IEF strip were more than sufficient.

Affinity Capture and Photocleavage of VICAT$_{SH}$-Tagged Peptides. Streptavidin-Agarose (50 μL of a 1:1 slurry as supplied by the manufacturer, Sigma S-1638) was placed in an Eppendorf tube, and the gel was pelleted by brief centrifugation. The gel was washed two times with 1-mL portions of phosphate-buffered saline. After removal of the final wash, 1 mL of phosphate-buffered saline was added followed by 200 μL of peptide sample (from two IEF combined eluant fractions). The sample was gently rocked for 1 h, the gel was washed as above two times with 1-mL portions of water, and 200 μL of 20% CH_3CN/5 mM Tris, pH 8.3, and 2 μL

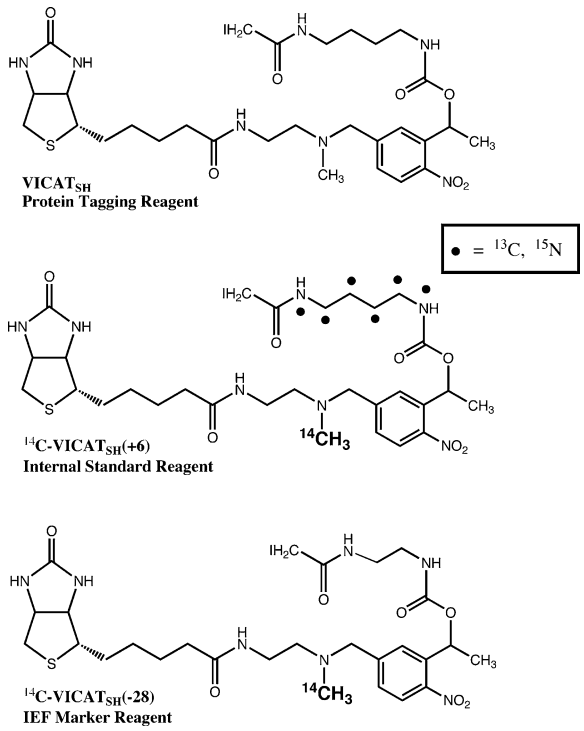

Figure 1. Structure of the three VICAT$_{SH}$ reagents.

of β-mercaptoethanol were added to the gel pellet. The tube was shaken for 16 min with a vibrating mixer platform (Eppendorf Thermomixer) with a UV lamp bulb (BLAK-RAY longwave model B-100AP, 100 W) held 6 cm from the top of the open tube. The gel was pelleted, and the supernatant was transferred to a new Eppendorf tube and concentrated to dryness (Speed-Vac).

Micro-Liquid Chromatography (Micro-LC)/ESI-MS. The dried sample was dissolved in 10 μL of 20% CH_3CN, and 8 μL was subjected to C18 micro-LC/ESI-MS using an automated system.[22] After sample loading and washing,[22] the micro-LC conditions were as follows: 0−5 min, 5−20% B (A is water with 0.1% formic acid, B is CH_3CN); 5−25 min, 20−40% B; 25−35 min, 40−80% B; 35−60 min, 80% B at a flow rate of 150 nL/min. Eluting peptides were analyzed in an ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, model LCQ Deca XP) using selected reaction monitoring (SRM) of 10 fragment ions from each of the two doubly charged, precursor ions (light and heavy tagged peptides). This was carried out using parameters provided in Tables 1−3 (Supporting Information). Data processing information is given in the legend to Figure 5 and as Supporting Information.

Western Blot Analysis of hGV in Human Macrophages. Full details are given as Supporting Information.

RESULTS

Overview of the Use of VICAT Reagents. Since the VICAT method involves a new set of reagents and methodologies, we first give a detailed description of the overall scheme for the absolute quantification of specific proteins in complex mixtures and then present the experimental data. The set of three VICAT$_{SH}$ reagents (Figure 1) are used in the following way to determine the absolute quantity of a specific protein in a complex protein

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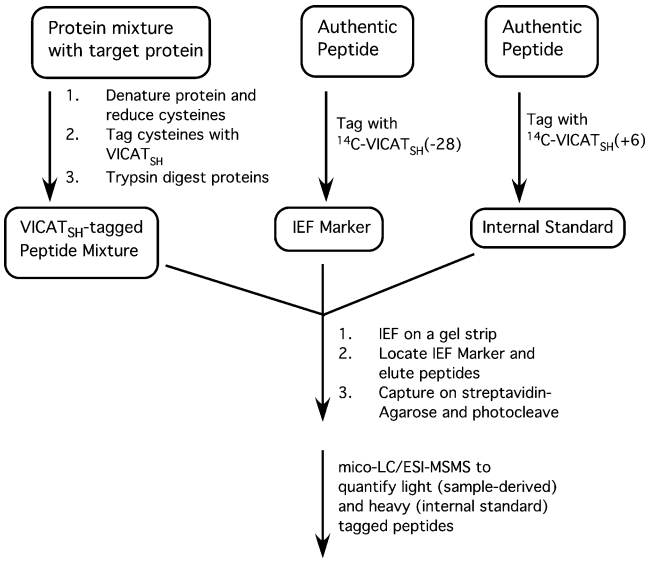

Figure 2. Schematic diagram of the absolute quantification of a target protein using VICAT$_{SH}$ reagents. Sample-derived, IEF marker, and internal standard tagged peptides are mixed together prior to IEF.

mixture. A flow diagram of the method is shown in Figure 2. Proteins in the biological sample are reduced, and cysteines are tagged with VICAT$_{SH}$. This reagent is referred to as the "protein tagging reagent". Proteins in the mixture are digested with trypsin (or other suitable protease) to generate a mixture of peptides; those containing cysteine are covalently tagged. A precise amount of internal standard is added to the mixture. The internal standard is the synthetic peptide, derived from the protein of interest, that has been tagged with the heavy reagent ^{14}C-VICAT$_{SH}$(+6), referred to as the "internal standard reagent". Finally, the biological sample is spiked with a desired amount of IEF marker. The latter is prepared by treating the same synthetic peptide as used for the internal standard with ^{14}C-VICAT$_{SH}$(−28), referred to as the "IEF marker reagent".

The mixture of sample-derived peptides, internal standard, and IEF marker is submitted to preparative IEF on a commercial gel strip containing immobilized ampholytes. After isoelectric focusing of the peptide mixture, the precise position of the tagged peptides of interest in the focusing strip is revealed by the radiolabeled IEF marker. Thus, the "visible" probe of VICAT reagents allows the electrophoretic position of the desired peptides to be determined by a method independent of mass spectrometry, which is important when using multidimensional separation required for highly complex peptide mixtures. The region of the gel strip containing the peptides of interest is sliced, peptides are eluted from the strip slices as separate fractions, and a small amount of each fraction is sacrificed for scintillation counting to locate the radioactive IEF marker. The internal standard also contains ^{14}C, which allows for simple and accurate quantification of stock solutions by scintillation counting. Most of the radioactivity in the IEF slices comes from the IEF marker. The tagged peptides are purified by a combination of affinity chromatography with streptavidin-Agarose and photocleavage with near-UV light (via the 2-nitro-benzyl linker as shown in Figure 3). This step serves to remove the large amount of ampholytes and other contaminants present in the IEF gel strip and results in highly efficient purification of tagged peptides since only those peptides containing both the biotin and 2-nitrobenzyl groups should survive the purification sequence.

The purified peptide mixture is submitted to micro-LC/ESI-MS in SRM mode. Several fragment ions derived from the lightly tagged peptide and the analogous fragment ions from the heavily tagged peptide are monitored, and the light-to-heavy peak area ratios are averaged to obtain the mole ratio of protein-derived peptide to internal standard (Supporting Information). Since the absolute mole amount of internal standard is known, one obtains the absolute mole amount of protein-derived peptide.

Typically, ∼100 pmol of IEF marker is used per sample to provide ∼5000 dpm of ^{14}C required to locate the marker following IEF. However, the amount of sample protein-derived, tagged peptide and internal standard are typically present at much lower abundance (sub-picomole, see below). After photocleavage and spontaneous decarboxylation of the formed carbamic acid (Figure 3), the tagged peptide derived from the IEF marker will contain the group $-CH_2CONH(CH_2)_2NH_2$ attached to the cysteine sulfur, whereas the protein sample-derived and internal standard-derived tagged peptides will contain the longer group $-CH_2CONH(CH_2)_4NH_2$. Thus, the former peptide will elute earlier than the latter two peptides during reverse-phase micro-LC. In addition, the peptide derived from the IEF marker is 28 mass units lighter than the peptide derived from the protein sample and is 34 mass units lighter than the internal standard-derived peptide. These features make it a simple matter to exclude, in the mass spectrometer, the much larger amount of the IEF marker-derived peptide from the peptides that need to be quantified (sample-derived and internal standard-derived peptides). This is the reason that ^{14}C-VICAT$_{SH}$(−28) contains the shorter linker. Other features of the VICAT$_{SH}$ reagents as well as justification for using IEF are given in the Discussion section.

Testing the Individual Steps of the VICAT Method. In this initial study of VICAT$_{SH}$ reagents, we determined the absolute quantity of a specific protein in a high complex mixture of proteins derived from a eukaryotic cell lysate. We chose *S. frugiperda* (Sf9) cell lysate since these cells are readily obtained in large numbers, and being of insect origin, they do not contain human proteins. Thus, we could spike the lysate with known amounts of a recombinant human protein, hGV, as a test case. Nonspiked lysate is useful for determining the background signal from a sample that is known to lack the analyte of interest.

We first evaluated the efficiency of protein tagging with VICAT$_{SH}$ reagents and subsequent trypsin digestion. Sf9 cells were disrupted in buffer containing 6 M urea and 2% CHAPS, and protein was tagged with excess VICAT$_{SH}$ after reduction of disulfides with TCEP. Small aliquots of the reaction mixture were taken before and after treatment with VICAT$_{SH}$ and submitted to SDS-PAGE with silver staining. As shown in Figure 4, the protein bands were completely gel-shifted, suggesting that tagging with VICAT$_{SH}$ proceeded to completion. Gel analysis of the trypsin digestion showed that all of the cell proteins were cleaved, within the detection range of silver staining (Figure 4). Efficient VICAT$_{SH}$ tagging and trypsin digestion were also observed when Sf9 cells were lysed in the presence of SDS (50 mM Tris, pH 8.3, 0.5%

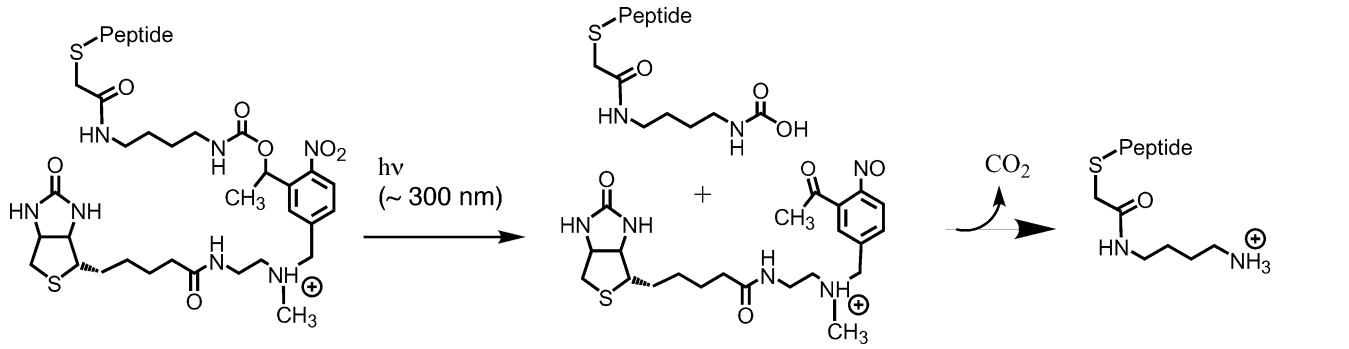

Figure 3. Shown is a peptide tagged with $VICAT_{SH}$ undergoing photocleavage to yield the initial photocleavage products. The carbamic acid present in the tagged peptide undergoes spontaneous decarboxylation to give the final photocleavage product. The biotin-containing photocleavage product is retained on the streptavidin-Agarose beads.

SDS, 5 mM EDTA), a buffer useful for the analysis of membrane proteins.[23]

To evaluate the IEF step, a mixture of $VICAT_{SH}$-tagged, tryptic peptides derived from an Sf9 cell lysate was spiked with 360 nCi of the IEF marker (prepared by reaction of laminin-derived peptide CDPGYIGSR with ^{14}C-$VICAT_{SH}(-28)$, and the mixture was submitted to IEF. As shown in Figure 4, a sharp band of radioactive material was seen when the IEF strip was exposed to X-ray film. In subsequent analyses, we used only ~3 nCi of IEF marker. In general, the position of the IEF marker cannot be predicted with millimeter resolution, and thus it makes sense to use the calculated p*I* value to approximate the position of the marker and to make several small cuts in the strip (Experimental Procedures). Thus, the appropriate region of the IEF strip was cut into six ~1-mm slices, peptides in each slice were eluted, and 5−10% of the eluant was sacrificed for scintillation counting. The IEF marker was localized to 1−2 IEF slices, and the yield of recovered IEF marker, and presumably sample-derived and internal standard tagged peptides, was 30−50%, based on dpm applied to the IEF strip (yield data based on more than 20 IEF runs). These results illustrate the high resolution of the IEF method due to the focusing of peptides into narrow bands. We also prepared a peptide conjugate IEF marker using an analogue of ^{14}C-$VICAT_{SH}(-28)$ that contains the 4-carbon 1,4-diaminobutane linker that is present in $VICAT_{SH}$ and ^{14}C-$VICAT_{SH}(+6)$ (Figure 1) and established that it comigrated during IEF with the marker prepared with ^{14}C-$VICAT_{SH}(-28)$ (Figure 4). Thus, as expected, the linker length does not alter the p*I* of the peptide conjugates.

The efficiency of photocleavage of the $VICAT_{SH}$-laminin peptide bound to streptavidin-Agarose beads to give Cys(*S*-CH_2CONH-$(CH_2)_4NH_2$)AspProGlyTyrIleGlySerArg was quantified by ESI-MS analysis of the supernatant containing the internal standard, angiotensin I (DRVYIHPFHL) after various irradiation times (synthesis of authentic photocleavage product given in the Supporting Information). Photocleavage reached a maximum recovery yield of 50% after 10−15 min, and the inclusion of β-mercaptoethanol, as a free radical trap, enhanced the yield. Since the light, protein-derived tagged peptide and the heavy internal standard undergo chemically identical photocleavage reactions, the less than quantitative photocleavage yield does not affect peptide quantification.

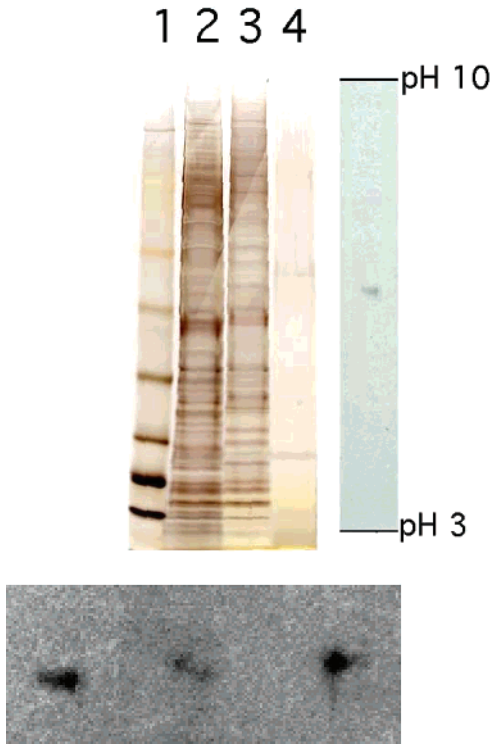

Figure 4. Sf9 cell lysate protein (200 μg) was tagged with $VICAT_{SH}$ and digested with trypsin, and peptides were submitted to IEF as described in the Experimental Section. Proteins were submitted to SDS−PAGE on a 4−12% gradient gel and visualized with silver staining (left side of top panel). Lane 1 is MW markers (14−250 kDa), lane 2 is a 0.5-μL aliquot of the reaction mixture after TCEP reduction, lane 3 is a 0.5-μL aliquot after $VICAT_{SH}$ tagging, and lane 4 is a 6.7-μL aliquot after trypsin digestion. The right panel shows a 15-min X-ray film exposure of the pH range 3−10 IEF strip after focusing of tryptic pepties derived from 200 μg of Sf9 protein spiked with 360 nCi of the laminin peptide IEF marker. The use of this relatively high amount of radioactivity permits band visualization by film exposure. The typical $VICAT_{SH}$ analysis is done with much smaller amounts of radioactivity, and the IEF strip position is determined by scintillation counting as described in the main text. The bottom panel is a portion of the X-ray film exposure of three individual IEF strips. The left strip is for 0.5 μCi of laminin peptide tagged with ^{14}C-$VICAT_{SH}(-28)$, the middle strip is 0.35 μCi of laminin peptide tagged with the ^{14}C-$VICAT_{SH}$ reagent with the four carbon linker (same structure as for ^{14}C-$VICAT_{SH}(+6)$ but without the heavy isotopes in the linker), and the right strip is the two tagged peptides applied to the same IEF strip. This figure shows that the different linker lengths do not noticeably change the IEF migration position.

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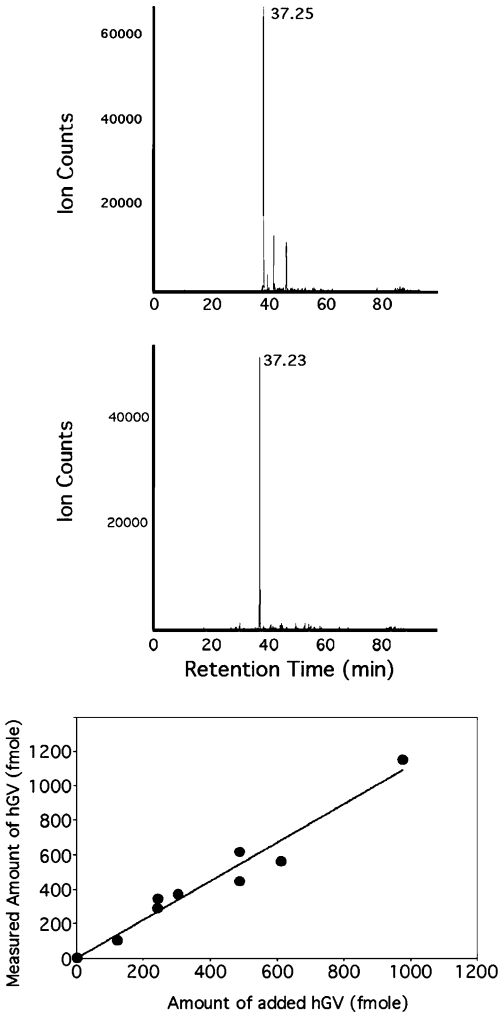

Figure 5. Quantification of recombinant hGV in Sf9 cell lysate. (Top panels) The ion chromatogram for the Y_{10}^{+} fragment ion derived from the lightly and heavy tagged peptide (SerTyrAsnProGlnTyrGlnTyr-PheProAsnIleLeuCys(*S*-$CH_2CONH(CH_2)_4NH_2$)Ser) for the light tagged peptide. The *Y*-axis is the ion counts versus the micro-LC time period (*X*-axis). (Bottom panel) ESI-MS in SRM mode was used to obtain the light-to-heavy ratio of the ion peak areas for 10 different fragment ions obtained from the hGV-derived tagged peptide and the internal standard (Supporting Information, Tables 1−3). The lowest and highest ratios were omitted, and the remainder was averaged to give the average light-to-heavy ratio per experiment. The average ratio of each experiment was then multiplied by the amount of internal standard added to sample to give the measured amountof hGV shown in the figure (*Y*-axis). The *X*-axis gives the amount of hGV added to the sample.

Absolute Quantification of a Specific Protein in a Complex Protein Mixture. A crude lysate derived from Sf9 insect cells was spiked with various amounts of recombinant hGV protein and a constant amount of internal standard (hGV peptide tagged with ^{14}C-VICAT$_{SH}$(+6)). Protein (100 μg) was subjected to VICAT$_{SH}$ tagging, trypsin digestion, IEF, streptavidin-Agarose affinity capture, photocleavage, and micro-LC/ESI-MS with SRM monitoring. Figure 5 shows the ESI-MS ion chromatograms for the lightly tagged peptide (hGV-derived) and the heavily tagged peptide (internal standard) for the sample containing 200 fmol of each. Figure 5 also shows that the absolute amount of hGV in the Sf9 lysates determined by the VICAT method varies linearly with the amount of hGV added (using a stock solution callibrated by amino acid analysis). The slope of this line is 1.12, close to the expected value of unity. We observed a signal-to-noise of 200 for the most abundant fragment ions (Y_{10}^{+} and Y_{12}^{2+}, see refs 3 and 4 for peptide fragment ion nomenclature) when 50 fmol of hGV protein was analyzed. Thus, the lower limit of detection of hGV is expected to be well below 50 fmol. This detection limit is expected to be dependent on the sequence of the peptide analyzed and on the instrumentation. The heavily tagged hGV peptide eluted ~1 s before the lightly tagged peptide (Figure 5), but this shift was not consistently observed and may be due to the selected reaction monitoring mass spectrometry method since we alternate between data collection for the lightly and heavily tagged peptides. The coelution of the lightly and heavily tagged peptides allowed us to integrate over the entire ion chromatogram peaks (lack of coelution means that the lightly and heavily tagged peptides may be coeluting with different contaminants, which may lead to differential suppression of detection of the target peptides).

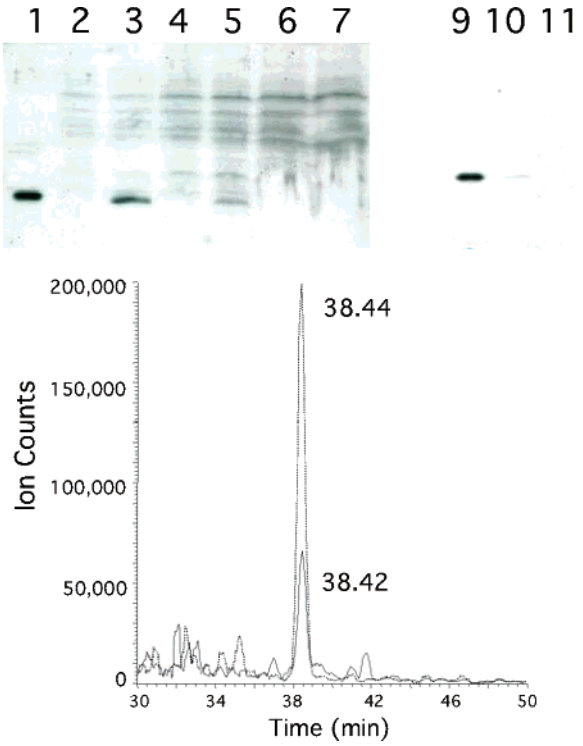

Figure 6. Analysis of hGV in macrophages. (Top panel) The western blot lanes are as follows: 1, 0.5 ng of hGV alone; 2, 10 μg of cell protein; 3, 10 μg of cell protein + 0.5 ng of hGV; 4, 30 μg of cell protein; 5, 30 μg of cell protein + 0.5 ng of hGV; 6, 100 μg of cell protein; 7, 100 μg of cell protein + 0.5 ng of hGV; 9, 1 ng of hGV; 10, 10 μg of cell protein + 1 ng of hGV; 11, 10 μg of cell protein. Lanes 1−7 were developed with a 1/750 dilution of anti-hGV polyclonal antiserum[28] and lanes 9−11 are with 1/300 of a commercial monoclonal anti-hGV antibody (Santa Cruz SC-18828). All lanes were probed with 1/4000 dilution of the ECL secondary antibody (see Supporting Information for other details). (Bottom panel) Macrophage protein (100 μg containing 240 fmol of hGV peptide internal standard) was subjected to VICAT analysis. Shown are the ion chromatograms for the Y_{10}^{+} fragment derived from the lightly tagged (solid line) and heavily tagged (dotted line) hGV peptides.

Western Blot and VICAT Analyses of hGV in Macrophages. Figure 6 illustrates the problems encountered in the detection of hGV in human lung macrophages using the western blot technique. Lane 1 of the blot shows that 0.5 ng (33 fmol) of

hGV loaded by itself on the gel is readily detected with the available polyclonal antiserum. However, the signal is dramatically suppressed by cell lysate proteins (when an equal amount, 33 fmol, of hGV is spiked into the macrophage cell lysate), and the suppression increases as the amount of macrophage protein is increased (lanes 3, 5, and 7 of Figure 6). Macrophages appear to contain a detectable band running just above the hGV standard (lanes 2, 3, 4, and 5), but it is not possible to tell if this is a post-translationally modified hGV or an unrelated protein. Also the detection of multiple high MW bands with this antiserum lessens one's confidence in the assignment of any detectable band in the hGV gel region to hGV. The use of a second antibody (a commercial monoclonal antibody) gives rise to a lower background, but the suppression of the signal of the spiked hGV by cell protein is still problematic (lanes 9−11).

We also analyzed for hGV in macrophages using the VICAT approach, and results are shown in Figure 6 for the analysis of 100 μg of cell protein containing 200 fmol of internal standard and with or without 240 fmol of added hGV protein. The detected hGV peptide showed the expected increase when hGV was added to the cell lysate (not shown). On the basis of the data, it is concluded that 100 μg of macrophage protein contains 66 fmol (0.9 ng) of hGV. The detection limit by western blot is about 33 fmol/30 μg of cell protein (Figure 6, lane 5), whereas the detection limit for the VICAT analysis (assuming a detection limit of $\ll$50 fmol/100 μg of protein, see above) is at least an order of magnitude more sensitive. The western blot result is inconclusive for the presence of hGV in macrophages; however, the VICAT method conclusively detected the protein.

DISCUSSION

VICAT reagents are expected to have the following advantageous features: (1) Absolute quantification of specific proteins with VICAT reagents is expected to be more accurate compared to western blotting which gives only semiquantitative detection of proteins. (2) Quantification of proteins by use of VICAT reagents makes use of a readily prepared internal standard made from commercially available peptides which do not have to be isotopically labeled. In contrast, quantification of proteins by radioimmunoassays requires a highly pure antigen and a method for quantification of the standard. (3) VICAT-based detection does not require the preparation of an antibody to detect the protein of interest, and protein identification is unambiguous. In contrast, immunological methods require an antibody and are only as good as the specificity of the antibody (which is difficult to judge a priori). (4) VICAT-based detection is based on mass spectrometry which is, in general, more powerful than immunodetection methods for the detection of post-translational protein modifications (i.e., phosphorylation, lipidation, and methylation). (5) VICAT-based analysis naturally looks at multiple segments of the target protein which is useful for the analysis of differential splice and translational forms, whereas immunodetection methods often require a specific antibody for each protein segment. (6) VICAT-based analysis is naturally multiplexable for the analysis of several proteins in a single sample. (7) VICAT technology allows multiple peptides from the same protein to be quantified, which allows the possibility of error analysis. (8) Detection of proteins with VICAT reagents does not suffer from the problems encountered with western blotting that are described above. It is our opinion that such problems are commonly encountered in western blotting, and the quality of the blots are often hard to judge since only a portion of the blot is typically given in publications. (9) The relatively large amount of IEF marker serves as a carrier to suppress nonspecific loss of the sample-derived and internal standard peptides during the multiple steps of the VICAT-based analysis. This is an important feature when working with small amounts of material, which is typical of proteomic analyses.

Additional important features of the VICAT reagents are as follows: (1) Removal of a major portion of the tag by photocleavage prior to micro-LC/ESI-MS ensures that the micro-LC retention time of the tagged peptide depends mainly on the physical properties of the peptide sequence and that maximum peptide bond fragmentation data is obtained. (2) $VICAT_{SH}$ reagents contain a tertiary amine (Figure 1), which is protonated below pH 10 and thus helps to ensure good solubility of these reagents in highly aqueous biological samples. Solubility becomes an issue as the molecular weight of the protein-tagging reagent is increased and when one considers that sub-millimolar to millimolar concentrations of reagent are needed to tag all of the cysteine-containing proteins in a complex sample such as a cell lysate. (3) $VICAT_{SH}$ reagents are synthesized by an efficient procedure, allowing introduction of the radiolabel in the last step from commercially available and inexpensive [^{14}C]HCHO.[19] The heavy atom-substituted linker of ^{14}C-$VICAT_{SH}$(+6) was made from inexpensive materials.[19]

Among the multitude of mature peptide separation methods available, IEF is particularly appealing. The method is robust, relatively inexpensive, highly reproducible, and easily multiplexed.[24] A nice feature of IEF for our purposes is that the isoelectric point (p*I*) of the peptides can be reasonably well calculated from their amino acid sequences.[25] Thus, the migration position of a specific $VICAT_{SH}$-tagged peptide can be predicted, allowing rational choice of the pH range of the IEF medium used and for determining the approximate location of the desired peptides for preparative isolation. Importantly, IEF is a very high resolution technique in which peptides are focused into narrow bands (as opposed to ion-exchange chromatography where the material elutes over a relatively broad peak). Also important is the fact that IEF and reverse-phase liquid chromatography separate peptides according to completely different physical properties (determined by the peptide sequence), and thus the combination of the two methods makes it possible to detect specific tagged peptides even in highly complex protein mixtures, for example, cell lysates. One factor to consider with preparative IEF is that it introduces a large amount of carrier ampholytes from the mobile phase buffer into the peptide mixture. However, these contaminants are readily removed prior to micro-LC/ESI-MS by affinity capture of $VICAT_{SH}$-tagged peptides.

One might make the argument that a visible probe in the tagging reagent is not required. The appropriate tagged peptide standard can be chromatographed alone to determine its elution coordinates, and these coordinates could be used in subsequent analysis of the sample of interest. However, on the basis of experience, we find that the chromatographic techniques are not sufficiently reproducible to ensure identical retention times of

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internal standard and sample-derived peptides in separate runs. The elution position may be dependent on the composition of the sample mixture (displacement effects) or on other factors that may be difficult to control. Furthermore, it is easier to use a visible tagging scheme to locate the peptides of interest following IEF than to carry out multiple micro-LC/ESI-MS runs on samples obtained from multiple IEF gel strips. The use of a visible tag also allows the IEF strip to be cut as narrowly as possible, which takes advantage of the extremely narrow focusing of peptides in this technique and minimizes the number of peptides submitted to micro-LC-ESI-MS. This in turn increases the chance of being able to specifically detect the tagged peptide of interest in the mass spectrometer.

The availability of absolute protein quantification data is useful for many purposes. One example is in functional proteomics. For example, if one knows the absolute amount of cytosolic phospholipase A_2 in platelets, the specific activity of the enzyme in vitro, and the amount of arachidonate release in thrombin-stimulated platelets, one could judge if there is sufficient amounts of this enzyme in platelets to account for the arachidonate release. If not, one would tend to hypothesize either that a different enzyme is responsible for the arachidonate release or that the cytosolic phospholipase A_2 is activated in the cell in some way that is not detected by the in vitro enzymatic assay. A second application is in the area of microarray analysis of mRNA levels, a technique that is generating vast amounts of information about possible disease markers. Often, the next step is to examine the levels of the corresponding proteins in a readily available biological fluid such as serum. The development of ELISA assays for all of the candidate proteins by a single laboratory is a daunting and expensive task. VICAT analysis would provide a method to rapidly and economically evaluate dozens of disease marker candidates. ELISA would then be developed for selected disease markers.

Recently, the AQUA method was described that allows the absolute quantification of specific proteins in complex mixtures to be determined.[26] The availability of two methods will increase the chance that the desired target protein will be detected. AQUA and VICAT both assume quantitative conversion of target protein to tryptic peptide. For both techniques one could prepare the internal standard peptide with N and C terminal extensions and allow the target peptide to be formed in the crude mixture via trypsin digestion. There are significant differences between the VICAT and AQUA methods that may be noted: (1) In the VICAT method, the internal standard is added earlier in the process than in the AQUA method, and thus all losses after the trypsinization/tagging steps do not affect quantification; (2) VICAT requires the extra step of protein tagging and assumes that the tagging is quantitative. However, the protein tagging is a simple step, and tagging of cysteines is thought to be a quantitative reaction for reduced and denatured proteins. For example, 2D gel analysis of ICAT tagged proteins shows quantitative gel shifts for all observable protein spots (see also Figure 4).[27] (3) $VICAT_{SH}$ reagents specifically tag cysteine-containing peptides which offers the advantage of simplifying the complex peptide mixture by an order of magnitude. There will be cases in which one desires to quantify a peptide that lacks cysteine (i.e., in a particular region of the protein that may contain a post-translational modification but that lacks cysteine). We have shown that the amino groups in tryptic peptides can be quantitatively converted to thioacetamido groups, which undergo quantitative $VICAT_{SH}$ tagging and photocleavage.[19] Thus, the VICAT method allows the choice of selective enrichment for cysteine-containing peptides versus detection of any peptide segment appropriate for mass spectrometry. (4) The VICAT method allows one to selectively follow the desired peptide through any chromatographic step. The use of techniques other than IEF, SDS-PAGE, and micro-LC may be required for a subset of highly complex peptide mixtures. The AQUA method requires that the migration position of the target protein in the SDS-PAGE gel be estimated based on the molecular weight of the protein. This is adequate for most proteins, but some proteins are well-known to run anomalously in SDS-PAGE gels. (5) The VICAT method allows the addition of a relatively large amount of IEF marker which can suppress nonspecific losses of internal standard and sample-derived peptides (carrier effect). In the AQUA method, nonspecific losses of femtomole amounts of internal standard and sample-derived peptide may be problematic for some peptides that bind to surfaces. (6) The AQUA method uses an internal standard that can be prepared on a peptide synthesizer, whereas the VICAT method requires a set of reagents that are prepared by solution phase chemistry. We anticipate that various forms of VICAT reagents will become commercially available soon.

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; hGV, human group V secreted phospholipase A_2; IEF, isoelectric focusing; micro-LC, micro-liquid chromatography; Sf9 cells, *S. frugiperda* cells; SRM, selected reaction monitoring; VICAT, visible isotope-coded affinity tag; $VICAT_{SH}$, thiol-specific VICAT.

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SUPPORTING INFORMATION AVAILABLE

Additional experimental details and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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