Isolation and Isotope Labeling of Cysteine- and Methionine-containing Tryptic Peptides

APPLICATION TO THE STUDY OF CELL SURFACE PROTEOLYSIS

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Inexpensive methods were developed for isolating and isotopically labeling tryptic peptides that contain either cysteine or methionine. After covalently capturing cysteine-containing peptides with pyridyl disulfide reactive groups on agarose beads, extensive wash steps were applied, and the attached peptides were released using a reducing agent. This approach results in less nonspecifically bound peptides and eliminates the possibility of generating avidin peptide background ions that can arise when using methods based on biotin and avidin (e.g. isotope-coded affinity tag). The thiols were alkylated using either N-ethyl- or N-D5-ethyl-iodoacetamide, both of which can be synthesized in a single step using inexpensive reagents. This isotopic labeling does not greatly increase the peptide mass, nor does it affect the peptide ion charge state in electrospray ionization. In addition, methionine-containing peptides were captured using commercially available methionine-reactive beads, and relative quantitation of peptides was achieved by isotopic labeling of amino groups using activated esters of either nicotinic acid or D4-nicotinic acid. These methods were used to study the metalloprotease-mediated shedding of cell surface proteins from a mouse monocyte cell line that had been treated with a phorbol ester and lipopolysaccharide. In addition to the identification of proteins previously determined to be inducibly shed, three new shed proteins were identified: CD18, ICOS ligand, and tumor endothelial marker 7-related protein. Molecular & Cellular Proteomics 2:315–324, 2003.

Quantitative proteomics usually involves separation of individual proteins using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)¹ and comparison of the staining density (1). Proteomic analyses using 2D-PAGE can be automated, but only at significant expense requiring automated gel staining and destaining devices, imaging equipment, imaging software, spot cutting robotics, automated in-gel digestion, robotic matrix-assisted laser desorption/ionization (MALDI) plate spotting, and mass spectrometry. Furthermore, it is not uncommon to identify several proteins in a single spot, which therefore makes quantitation unreliable. Even with an expensive high-throughput 2D-PAGE system, one has to be cognizant of the fact that these systems are known to have difficulties with higher molecular mass proteins, membrane proteins, and highly acidic or basic proteins. Despite the high resolution separations of proteins provided by 2D-PAGE, the method still suffers from a limited dynamic range in that low-abundance proteins are difficult to detect in the presence of high-abundance proteins (2). Nevertheless, 2D-PAGE has been the state of the art for making quantitative proteomic measurements.

An alternative to quantitative imaging of 2D-PAGE is to employ the concept of isotope dilution (3) in the context of proteomic analyses. Proteolytic peptides are labeled with different stable isotopes depending on the protein source (e.g. control cells versus stimulated cells). Because isotopic labeling of identical peptides will result in very similar chemical properties, pairs of peptides differing only in the label will elute approximately at the same time and exhibit nearly identical ionization efficiency. The first example of this was the use of metabolic ¹⁵N labeling to compare wild-type and mutant cell lines (4, 5). This approach is limited to studies of cultured cells, and the isotope coding involves the incorporation of varying numbers of nitrogen atoms in each peptide, hence mass differences between labeled peptides will vary, and the subsequent data analysis is more complicated. In contrast, isotope-coded affinity tag (ICAT) reagents, which employ a cysteine-specific chemical reactivity, an isotope-coded linker, and a biotin affinity tag (6), introduce a constant mass difference for each cysteine present in the peptide. An additional advantage of this method is that complex tryptic peptide

¹ The abbreviations used are: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ICAT, isotope-coded affinity tag; ICROC, isotope-coded reduction off of chromatographic support; TCEP, tris-(2-carboxyethylphosphine) hydrochloride; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; MS/MS, tandem mass spectrometry; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; SPE, solid-phase extraction; DRM, Dexter-ras-myc; ABC, ammonium bicarbonate; TFA, trifluoroacetic acid; PBS, phosphate buffered saline; BSA, bovine serum albumin; TEM7-R, tumor endothelial marker 7-related protein; ICOS, inducible costimulator; D0, D3, D4, D5, D8, designate the number of deuterium atoms that replace hydrogen atoms in various derivatization reagents.

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mixtures can be simplified by the selective isolation of peptides containing cysteine. Other approaches for proteomic isotope dilution have been described, including N-terminal labeling with deuterated nicotinic acid active esters (7) and proteolytic incorporation of $^{18}$O into the C-terminal carboxyl group (8, 9). Wang and Regnier (10) used thiopropyl Sepharose to isolate cysteine-containing peptides from several milligrams of protein, which were then acylated using isotopically labeled reagents. Cysteine-reactive beads with either a photocleavable (11) or acid labile (12, 13) linker have been described, and both leave isotopically tagged moieties attached to the cysteine residues.

In an earlier publication, we described an approach where cell surface proteolysis (a process sometimes termed “shedding”) was induced in two identical cell cultures, where the control supernatant was obtained by treating one of the cultures with a metalloprotease inhibitor (14). The cell supernatants were examined by SDS-PAGE, and differences in the gel staining pattern were located and the corresponding bands were excised for mass spectrometric analysis. One concern with this approach is that heavily stained proteins that do not change can obscure changes in proteins of low abundance that have similar electrophoretic mobility. Hence, it was of interest to find an alternative approach that did not depend on gel staining for locating differential changes in protein quantities.

Toward this end, we proceeded to test the first generation of ICAT reagents; however, initial results revealed a few problems that we found could be eliminated using an inexpensive alternative method that we named isotope-coded reduction off of a chromatographic support (ICROC). Peptides with cysteine are bound to pyridyl disulfide beads, washed, and cysteine-containing peptides are gently eluted using a reducing agent. The thiol of the released peptides are alkylated with either N-ethyl or N-D5-ethyl-iodoacetamide prior to mixing and mass spectrometric analysis. The cysteine-depleted pool of peptides that did not bind to the pyridyl disulfide beads was analyzed further using commercially available beads capable of selectively binding peptides containing methionine. Stable isotope labeling of the released methionine-containing peptides was achieved by acylating the amino groups with activated esters of either nicotinic acid or D4-nicotinic acid. These methods were applied to the identification of substrates of cell surface metalloproteases; proteins already known as substrates, plus some new ones, were identified.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Stimulation, and Lectin Affinity Purification**—Mouse monocytic Dexter-ras-myc (DRM) cells were cultured as described (14). Cell stimulation was performed in the same manner as before (14), except that serum-free cultures were incubated with 1 µg/ml lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0111:B4 (Sigma-Aldrich, St. Louis, MO) for 4 h. Phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) (ICN Biomedicals, Aurora, OH) was added following the LPS treatment, and the cultures were incubated for an additional 30 min. The control cells were treated identically except that 25 µg/ml of the metalloprotease inhibitor IC-3 (Immunex compound 3; Immunex, Seattle, WA) was added prior to addition of LPS and PMA (15). Supernatants were harvested, concentrated, and glycoproteins were isolated using a wheat germ agglutinin column (Vector Laboratories, Burlingame, CA) (14), followed by protein precipitation to remove lipids and salts (16). The protein pellet was solubilized in 25 µl 8 M urea and 1 µl was used to measure the total protein content using a Micro BCA kit (Pierce, Rockford, IL). From 1.6 × 10^5 cells, the total lectin-purified protein from the IC-3-treated cells was ~50 µg, and 40 µg was obtained from the cells not treated with the inhibitor.

**Protein Standards**—Protein standards, including total *E. coli* proteins, were purchased from Sigma. Two protein mixtures were made containing hen ovalbumin (430 and 215 µg/ml), bovine β-lactoglobulin (80 and 20 µg/ml), rabbit glyceraldehyde-3-phosphate dehydrogenase (49 and 98 µg/ml), rabbit phosphorylase b (97 and 323 µg/ml), and bovine α-lactalbumin (120 and 120 µg/ml). Approximately 50 µg of each mixture was used to test the ICAT reagent and the ICROC procedure.

**ICAT Procedure**—The first generation ICAT reagents were a gift from Applied Biosystems (Framingham, MA). The dried protein (50 µg) was solubilized in 100 µl 50 mM Tris pH 8.5 plus 0.1% SDS and boiled for 5 min. The denatured proteins were reduced by the addition of 1 µl 1 M tris(2-carboxyethylphosphine) hydrochloride (TCEP) for 10 min at 37 °C. Reducing agent was removed by overnight acetone precipitation at −20 °C. The pellet was suspended in 100 µl Tris/SDS (above) and treated with 100 µg of either D0 or D8 ICAT reagent for 90 min at room temperature in the dark. The reaction was quenched by the addition of 1 µl mercaptoethanol for 30 min at room temperature, and separate D0 and D8 reactions were mixed together following the quench. Excess ICAT reagent was removed by acetone precipitation, and the pellet protein was suspended in 50 mM ammonium bicarbonate and digested with 2 µg trypsin overnight at 37 °C with constant vortexing. The sample was boiled for 10 min, 100 µg 2× phosphate-buffered saline (PBS) was added, and the pH was adjusted to 5 with 5 µl 3 M sodium acetate prior to loading on the monomeric avidin column.

Two hundred microliters of a 50% slurry of monomeric avidin beads was placed in a BioRad mini column, washed twice with 1 ml of 30% acetonitrile with 0.4% trifluoroacetic acid (TFA), followed by 1.2 ml of 2× PBS, pH 7.2. To block tetrameric avidin sites, the column was washed with 0.6 ml 2× PBS with 0.6 µl 100 mM glycine pH 2.8, and washed further with 1.2 ml 2× PBS to return the column to pH 7.2. After plugging the column, the sample (300 µl total volume) was incubated with the beads for 20 min with occasional mixing. The column was drained, washed with 1 ml 2× PBS, then 1 ml 1× PBS, followed with a wash of 1.25 ml 50 mM ammonium bicarbonate in 20% methanol. The retentate was eluted with 0.8 ml of 30% acetonitrile containing 0.4% TFA.

**Synthesis of N-ethyl and N-D5-ethyl-iodoacetamide—**Ethyl-D5-amino hydrochloride (0.85 g, 9.81 mmol) was suspended in a solution of iodoacetic anhydride (3.47 g, 9.81 mmol) in dichloromethane (40 ml) and cooled to 0 °C. A solution of 3.6 ml, 20.6 mM of *N,N*-disopropylethylamine in dichloromethane (10 ml) was added dropwise over 20 min. The reaction was stirred at 0 °C for 30 min then allowed to stir at room temperature for 4 h. The solvent was evaporated to give a yellow syrup. The syrup was dissolved in ethyl acetate (100 ml) and washed sequentially with 1N hydrochloric acid (2 × 50 ml), saturated sodium bicarbonate solution (2 × 50 ml), and saturated sodium chloride solution (50 ml). After drying over anhydrous magnesium sulfate, the organic solution was filtered and evaporated to give a light yellow solid. Purification by flash chromatography on silica gel (50 g) using 1:1 ethyl acetate/hexane for the elution gave the...
desired product as a white solid (446 mg, 21%). $^1$H-NMR: (CDCl$_3$) $\delta$ 6.04 (1H, br s), 3.68 (2H, s). $^{13}$C-NMR: (CDCl$_3$) $\delta$ 166.6, 34.6, 13.3, –0.3. MS: 219 (M+H$^+$). N-ethyl-iodoacetamide was prepared in a manner analogous to that described above, using ethylamine hydrochloride. $^1$H-NMR: $\delta$ (chloroform-d) 6.37 (1H, br s), 3.67 (2H, s), 3.28 (2H, m), 1.13 (3H, t). $^{13}$C-NMR: $\delta$ (chloroform-d), 166.9, 35.3, 14.3, –0.3. MS: 214 (M+H$^+$).

Preparation of pyridyl disulfide beads—Diaminodipropylamine gel slurry (4 ml; Pierce) was placed into an empty column and washed five times with 2 ml of water. The gel was then washed five times with 2 ml of 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2 (PBS). The column was drained until the liquid level reached the top of the gel, and then the bottom of the column was capped. The pyridyl disulfide groups were attached to the amino groups on the beads using 16 mg/ml sulfo-LC-SPDP (Pierce) in PBS. This solution was freshly prepared and 1 ml was immediately pipetted into the center of the column. The top of the column was capped, and the column was gently and repeatedly inverted for 1 h at room temperature, and the gel was subsequently washed with 15 ml of PBS. The amount of pyridyl disulfide on the beads was determined by ultraviolet spectrophotometry of the supernatant following treatment with a reducing agent (pyridine-2-thione extinction coefficient is 8.08 x $10^3$ M$^{-1}$ cm$^{-1}$ at 353 nm). Approximately 5–10 nmol of disulfide was present per microliter of beads. The beads were stored at 4 °C as a 50% slurry in 0.05% sodium azide.

**ICROC method**—The following procedure is suitable for samples containing 25–100 μg total protein; for samples outside of this range, volumes and quantities of reagents may need to be adjusted accordingly. An overview of the method is shown in Fig. 2.

**Step 1: Reduction and Digestion**—Samples were solubilized in 25 μl 8 M urea, and reduced in 10 μl of 1 M ammonium bicarbonate (ABC) pH 8, 1 μl 500 mM EDTA, and 1 μl 1 M TCEP at 37 °C for 1 h. To avoid carbamylation, 50 ml of urea solution was freshly prepared and continuously mixed for 30 min with a 10 ml bed volume of AG-501 X8 ion exchange resin (Bio-Rad, Hercules, CA) prior to use. After reduction of the disulfide bonds, samples were diluted with 100 μl water, vortexed, and trypsin was added (5% by weight). Samples were incubated at 37 °C for 1 h, followed by the addition of another 5% trypsin (optional) and incubation for an additional 3–4 h. The digestion was stopped by acidification to a final concentration of 0.1% TFA. At this point, samples can be stored overnight at –20 °C.

**Step 2: Attachment of Cysteine-containing Peptides to Beads**—Excess reducing agent in the peptide mixture needs to be removed before attaching to the beads, and this was achieved using a Peptide MacroTrap solid-phase extraction (SPE) trap (Michrom Bioresources, Auburn, CA). The trap cartridge was wetted using 80% acetonitrile, and then equilibrated using 0.1% TFA containing 1 mM EDTA. The samples were repeatedly loaded to ensure binding of peptides, and the salts and reducing agents were washed out using 300 μl 0.1% TFA, 1 mM EDTA. It is critical that all of the reducing agent be removed, which can be verified using Ellman’s reagent to test the final washes for the presence of reducing agent. After washing the trap cartridge, the peptides were eluted using 50 μl each of 20%, 40%, and 60% acetonitrile in 1 mM EDTA plus 0.1% TFA, followed by a final elution of 100 μl 80% acetonitrile. The combined eluates were dried using a vacuum centrifuge and were solubilized in 25 μl 100 mM ABC 5 mM EDTA, pH 8, by repeated sonication and vortexing.

Pyridyl disulfide beads (10–15 μl wet bead volume) were placed in the bottom of a Macro Spin Column (The Nest Group, Southboro, MA), where care was taken not to let the beads stick too far up on the side of the column. A large excess of pyridyl disulfide over peptide thiol (>25-fold) is necessary for good recoveries, so the quantity of beads might need to be adjusted, depending on the concentration of pyridyl disulfide on the beads. The beads were washed repeatedly first with 500 μl 100 mM ABC, 5 mM EDTA, 500 mM NaCl, and then several more times using the same buffer without NaCl. The bottom of the column was sealed shut using parafilm, and a sample was added to the washed beads. The column top was sealed with parafilm and the sample plus beads were incubated at room temperature for 90 min with constant and gentle shaking. Holes were punched in the top parafilm seal using a syringe needle, the bottom parafilm seal was removed, and the contents were gently spun out using a centrifuge. This flow-through was the cysteine-depleted peptide pool, which can be saved and analyzed separately.

**Step 3: Washing and Eluting**—The beads were washed four times with 500 μl of 500 mM NaCl, 100 mM ABC, 5 mM EDTA using a slow gravity flow over a period of 30 min. Four additional washes used 500 μl of the same buffer without salt and were gently forced through the column using air pressure applied to the top using a 5-ml syringe. The bottom of the column was sealed with parafilm, and 25 μl of 10 mM TCEP in 100 mM ABC and 5 mM EDTA (reducing solution) was added to the washed beads. The top of the column was sealed with parafilm to prevent evaporation, and the tube was gently flicked to ensure that the bead bed formed a slurry with the reducing solution. The column was gently shaken at 37 °C for 60 min to release the bound peptides. A needle tip was used to punch holes in the top parafilm seal to relieve air pressure before removing the bottom seal, and then the liquid was gently forced out into a clean Eppendorf tube to collect the eluate. The eluate was temporarily stored in a freezer, while a second aliquot of 25-μl reducing solution was added to the beads as described above. Following 60 min of incubation at 37 °C, this second aliquot of reductant was spun out and combined with the first. The beads were washed with 25 μl of 100 mM ABC and 5 mM EDTA, extensively vortexed, and then the supernatant from this wash was combined with the saved reducing solution.

**Step 4: Isotopic Labeling**—At this point, peptides containing cysteines with free thiols were in the combined reducing solutions plus final bead wash (total volume of about 75 μl). This sample was alkylated by the addition of 75 μl of freshly prepared 50 mM N-ethyl-iodoacetamide (deuterated or undeuterated) in 100 mM ABC, pH 8, and 5 mM EDTA. The reaction was allowed to proceed for 1 h at room temperature in the dark. Alkylation was stopped by acidification with TFA (final concentration of 0.1%), and the samples were stored at –20 °C.

**Methionine Capture and Isotopic Labeling**—Peptides that did not bind to the pyridyl disulfide beads were isotopically labeled using N-hydroxysuccinimide active esters of nicotinic acid (Advanced Chemtech, Louisville, KY) or D4-nicotinic acid (prepared as described (7)). Peptides were repeatedly vacuum centrifuged to remove the ABC buffer prior to dissolution in 0.1 M sodium phosphate buffer, pH 8. N-hydroxysuccinimide -nicotinic ester reagent was solubilized in dimethyl sulfoxide at a concentration of 20 μg/μl. One microliter of the reagent was added to the solubilized peptides every 10 min over the course of 1 h, followed by one additional hour of incubation at room temperature. The reaction mixtures (D0 and D4 labeled) were combined and dried on a vacuum centrifuge. The O-esters were hydrolyzed by the addition of 20 μl of 20 mg/ml hydroxylamine hydrochloride, pH 10 (pH adjusted using ammonium hydroxide) for 1 h at room temperature, followed by acidification using TFA. Methionine-containing peptides were isolated using commercially available beads using the instructions provided (BioMolecular Technologies, Sunnyvale, CA).

**Liquid Chromatography (LC)/Mass Spectrometry (MS)**—Alkylated samples were directly loaded onto 50-μm inner diameter packed capillaries, and LC/MS was performed as described previously (14) except that in some cases data-dependent acquisition of MS/MS spectra was carried out at a reduced flow rate over a prolonged period of time. This was accomplished by adjusting the pre-injector
isotope peak heights. For higher molecular mass peptides, the 13C native method named ICROC was developed. The first step in tered when using the first generation ICAT reagents, an alter-
specific fragment ions for low-intensity precursors (Fig. 1). ions, which may adversely affect the number of sequence-
Fifth, the ICAT label produces intense ICAT-specific fragment charge state precursor ions produced from ICAT labeling. spectra of doubly charged precursor ions generally provide the MS/MS spectra. It is our experience that the MS/MS ions, which may adversely affect the information content of reagent tends to increase the charge state for many peptide increase in molecular mass can adversely affect the quality of peptides containing several cysteines, the substantial in-
base match was correct. Manual inspection certified that an extensive tandem mass spectra were manually inspected to verify that the data base match was correct. Manual inspection certified that an extensive and contiguous series of y-type ions (18) was present, and that it was possible to account for the more intense ions. For proteins not previously known to be shed that were identified by single peptides, there was an additional requirement that the identifying peptide be at least 10 residues in length.
Quantitation was achieved by summing all of the LC/MS scans that contain the high- and low-mass isotope labels, and intensity ratios were calculated from these summed mass spectra using the 12C isotope peaks. For higher molecular mass peptides, the 13C isotope peaks were included in the determination of relative ratios. For low-intensity ions, it was necessary to subtract background ion intensity before calculating the ratios.

RESULTS

Problems Encountered Using ICAT Reagents—While testing the first generation of ICAT reagents on standard protein mixtures (see “Experimental Procedures”), several problems were encountered. First, we confirmed by MS/MS the presence of four peptides that did not contain cysteine (data not shown). The ion signals of these nonspecifically bound pep-
tides were of significant intensity and were comparable to what was observed for the ICAT-labeled peptides that had cysteine in their sequence. Second, significant avidin tryptic peptide signals were found in the ICAT preparation when we omitted the strong cation exchange fractionation step prior to loading onto the avidin column (data not shown). Trypsin-specific inhibitors and boiling do not completely eliminate activity, and although loading and washing the avidin column at low pH (pH 5) reduced the avidin peptide background, proteolysis was still evident. Third, the ICAT reagent adds ~450 Da for each cysteine present in any given peptide. For peptides containing several cysteines, the substantial in-
crease in molecular mass can adversely affect the quality of their tandem mass spectra. Fourth, the addition of the ICAT reagent tends to increase the charge state for many peptide ions, which may adversely affect the information content of the MS/MS spectra. It is our experience that the MS/MS spectra of doubly charged precursor ions generally provide more complete sequence coverage compared with higher charge state precursor ions produced from ICAT labeling. Fifth, the ICAT label produces intense ICAT-specific fragment ions, which may adversely affect the number of sequence-specific fragment ions for low-intensity precursors (Fig. 1).
The ICROC Method—To overcome the problems encountered when using the first generation ICAT reagents, an alternative method named ICROC was developed. The first step in the ICROC method (Fig. 2) is to solubilize, denature, and reduce the disulfide bonds in the two protein samples to be compared. Trypsin is active in the presence of up to 2 M urea plus reducing agent, so proteolysis can be achieved simply by diluting out the urea prior to the addition of enzyme. Following digestion, the excess reducing agent used to break protein disulfide bonds must first be removed using a reverse-phase SPE peptide trap. Care must be taken at this step to ensure that the SPE trap has sufficient capacity, and that the reduc-
ting agent has been removed. The cysteine thiols then undergo disulfide exchange with the pyridyl disulfide groups attached to the beads. Once the cysteine-containing peptides are attac-
ted to the beads, they are washed extensively to remove any peptides not covalently bound. The bound peptides are eluted using a reductant dissolved in the same buffer used to wash the beads, and the released cysteines are subsequently alkylated with the isotope-labeled reagents. Here we used N-ethyl-iodoacetamide and N-D5-ethyl-iodoacetamide, which provides a 5-Da mass difference for each cysteine. An alternative is to alkylate using commercially available acrylamide and D3-acylamide; however, for higher molecular mass pep-
tides, given a 3-Da shift, one would need to account for the contribution of 13C isotope peaks. Following the alkyla-
tion step, the peptides labeled with the different reagents are combined for further HPLC fractionation and mass spectrom-
etry analysis.
The ICROC method exhibited few of the problems encountered when using the ICAT reagents. Nonspecifically bound peptides were not observed when using the ICROC procedure on the same protein test mixture (e.g. Fig. 3), and potential problems with contaminating avidin trypsic peptides were eliminated. Alkylation using N-ethyl- or N-D5-ethyl-iodoacetamide adds only 85 or 90 Da per cysteine to the peptide mass, respectively, and the charge state of the labeled peptides is not altered. When performing data-dependent LC/MS/MS on the ICAT-labeled peptides from the standard mixture, there was a tendency to acquire MS/MS spectra of higher charge state precursor ions as compared with the same experiment performed on N-ethyl-iodoacetamide-labeled peptides. For example, using the ICAT-labeled protein standard mixture, data-dependent acquisition selected 11, 51, and 33 precursor ions with four, three, and two charges, respectively. In contrast, the identical sample alkylated with N-ethyl-iodoacetamide yielded of 2, 25, and 67 MS/MS spectra for precursor ions with four, three, and two charges, respectively. Also, ICAT has an ether linker that is susceptible to fragmentations that produce abundant ICAT-specific fragment ions (Fig. 1a) (19). In contrast, the N-ethyl-iodoacetamide modification is stable upon collisional activation, and MS/MS spectra derived from lower charge state precursor ions typically provides more sequence-specific fragment ions (Fig. 1b).

To test the yields of the ICROC procedure, 100 μg bovine serum albumin (BSA) was reduced and digested with trypsin. The sample was split, and one of the aliquots was subjected to the ICROC procedure, where the cysteine-containing peptides were labeled with the light alkylating reagent (N-ethyl-iodoacetamide). The other aliquot was not subjected to the ICROC procedure, but was only alkylated using the heavy reagent. The samples were mixed and analyzed by MALDI-MS. Numerous pairs of cysteine-containing BSA tryptic peptides were observed, and, depending on the peptide sequence, the intensity of the light isotope-labeled peptides ranged from 60 to 80% relative to the heavy isotope-labeled counterpart. Variability in these yields did not seem to corre-

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**Fig. 2. Isotope-coded reduction off of a chromatographic support.** Sample proteins are reduced and digested with trypsin. Next, the cysteine thiols are covalently attached to the pyridyl disulfide beads, and the unbound peptides are washed away. The bound peptides are released using a reductant (TCEP), and the thiols on the cysteines are alkylated with either a light reagent (N-ethyl-iodoacetamide) or a heavy reagent (N-D5-ethyl-iodoacetamide), and then the two samples are mixed prior to any further high-performance LC fractionation or MS.

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**Fig. 3. Nonspecifically bound peptides.** a. Mass chromatogram of m/z 655.4 obtained from an ICAT preparation of a protein standard mixture (see "Experimental Procedures"). The peak at 39 min is the quadruply charged ion, (M + H)\(^+\), of the peptide VIHDHFGIVEGLMTVHAITATQK from rabbit glyceraldehyde-3-phosphate dehydrogenase. b. Mass chromatogram of m/z 655.4 obtained from an ICROC preparation of the same protein mixture. The lower abundance chromatographic peaks are either due to ions of different charge states or to \(^{13}\)C isotopes of ions unrelated to the peptide described above. Three other noncysteine-containing peptides were found in the ICAT preparation that produced a sufficiently intense ion to trigger MS/MS data acquisition: NVLQPSSVDSQAMVILNIVFVK and ILELPFASGTMSMLVPDEVSGLEQLSIFEK from chicken ovalbumin and VAGTWYSLMAASISLDDLQAPL from bovine beta-lactoglobulin. These peptides were not found in the ICROC preparation.
late with the number of cysteines present in the peptide. No cysteine-containing BSA peptides were found in the pyridyl disulfide flow-through, indicating that the beads captured them all. HPLC ultraviolet chromatograms of a BSA tryptic digest were nearly identical to the same sample following desalting on the SPE trap, indicating that the yield from the trap was better than 90% for most peptides. A couple of the most hydrophilic peptides, however, had reduced signals, perhaps due to isocratic elution during the wash step. To test the alkylation reaction, synthetic peptides containing cysteine were reacted with N-ethyl-iodoacetamide, and no unmodified peptide ions were observed in their MALDI mass spectra. From these results, we estimate that the alkylation yield is greater than 95%. Furthermore, because these reagents are inexpensive and are soluble at concentrations over 100 mM, one should be able to achieve a nearly quantitative alkylation reaction. Hence, the overall estimated yield for the ICROC method is ~55–75%, depending on the peptide sequence.

The precision of the ICROC procedure was estimated using proteins obtained from an acetone precipitate of E. coli. A sample containing proteins that could be solubilized in 8 M urea was split into equal portions, and each one was worked up separately (i.e., separate reduction, digestion, thiol isolation, and alkylation steps). The cysteine-containing peptides from each aliquot were alkylated with either the heavy or light reagent, and the samples were recombined to give a theoretical 1:1 ratio. Ratios for 76 pairs were measured and exhibited an average intensity ratio of 1.03 with a standard deviation of 0.10 (Fig. 4a). Measurements made from lower intensity ions, however, are subject to greater error (Fig. 4b). No claim is being made that ICROC has superior precision compared with ICAT; however, these results demonstrate that it is sufficient for most purposes.

Studies of Cell Surface Proteolysis—Two identical groups of cultured mouse monocytes were stimulated to induce cell-surface proteolysis using a combination of LPS and PMA, where one group was pretreated with a metalloprotease inhibitor (IC-3) as a control and the other was not. The culture supernatants were collected and the glycoproteins isolated using a lectin column. The glycoproteins were analyzed using the ICROC method, and the cysteine-depleted peptide pool was subsequently analyzed using the methionine capture method. Light isotope reagents were always used to label supernatant proteins obtained from cell stimulation occurring in the presence of IC-3, a metalloprotease inhibitor, whereas the heavy isotope reagent was used to label proteins obtained in its absence. Peptides derived from the cysteine- and methionine-capture procedures were analyzed using single reverse-phase LC/MS/MS experiments.

Using the Mascot search program, 55 proteins were identified from 265 MS/MS spectra, where only two were from peptides that did not contain a modified cysteine (i.e., less than 1% of the peptides from the ICROC procedure were nonspecifically bound). All cysteines found were modified with alkylating agent. The vast majority of peptide signals from the 55 identified proteins exhibited a pattern similar to that shown in Fig. 5, a and b, where the ratio of heavy to light isotope ion intensity is about 0.56. This is likely a reflection of the ratio of total protein present in the two samples. In a few cases, the ratio of heavy to light isotope ion intensity was significantly different (Fig. 5, c and d, and Table I), suggesting that these peptides were from proteins that can be inducibly shed by metalloproteases upon LPS and PMA stimulation.

In addition to selection and quantitation of cysteine-containing peptides, we utilized another method to enrich and quantitate methionine-containing peptides. Peptides that did not bind to the pyridyl disulfide beads (peptides lacking cysteine) were amino-acylated using D0- or D4-nicotinic acid prior to mixing. The differentially labeled peptides that contained methionines were isolated using commercially available methionine capture beads and analyzed by LC/MS (Table I). It is important to note that there were several interesting cases where ICROC alone provided only one peptide, or none, and that for some of these proteins, the additional peptides found in the methionine capture experiment greatly increased the confidence that they are inducibly shed. In this experiment, ~37% of the identified peptides did not have...
methionines and were due to nonspecific binding. At this point, it is not known if this percentage can be reduced by additional washes. In contrast to ICAT and ICROC, one can nevertheless obtain quantitative information from nonspecifically bound peptides, because the isotopic labeling occurs on the amino groups. The fact that the quantitation results obtained from the cysteine-containing peptides are generally consistent with the results from the methionine-containing peptides provides independent validation for each method.

Among the proteins identified as inducibly shed (Table I and Fig. 6), Alzheimer’s disease amyloid A4 protein homolog, AXL receptor tyrosine kinase, macrophage colony-stimulating factor 1 receptor, SHP substrate 1 protein, and CD14 were identified in our previous gel-based analysis (14). Two of them—tumor necrosis factor and tumor necrosis factor receptor 2—are known from the work of others to be shed proteins (20, 21). The remaining three proteins from Fig. 6—inducible costimulator (ICOS) ligand, CD18, and tumor endothelial marker 7-related protein (TEM7-R)—have not previously been identified as proteins subject to inducible shedding by metalloproteases.

**DISCUSSION**

The approach used here for isolating cysteine-containing peptides and measuring their relative quantitation is conceptually similar to that described by Wang and Regnier (10); however, there are differences in bead chemistry and isotopic labeling, which result in greater simplicity and sensitivity. Wang and Regnier first introduce the isotopic label via succinylation of amino groups (D0 or D4 succinic anhydride), thereby replacing basic sites with acidic carboxyl groups. To overcome the poor solubility of the pyridyl disulfide reagent, these authors bind the succinylated peptides to a reverse-phase cartridge, which serves as a reaction chamber while a dilute solution of the pyridyl disulfide is continuously passed over the immobilized peptides. The peptides are eluted from the reverse-phase cartridge and mixed with a thiopropyl Sepharose resin, where the pyridyl disulfide-labeled peptides form mixed disulfides with the resin. The resin is washed, and the cysteine-containing peptides are eluted using a reducing agent and are alkylated using iodoacetic acid. In contrast, we combine the peptide solution with beads already derivatized with pyridyl disulfide; thereby eliminating the need for a dedicated apparatus for forming mixed disulfides. Following the release of the cysteine-containing peptides, isotopic labeling and cysteine alkylation is achieved in one step by using D0- or D5-N-ethyl-iodoacetamide. The amino groups remain unmodified, positive ion production is not compromised, and sensitivity is not adversely affected. These modifications to the original method may be responsible for increased sensitivity. Whereas 40–50 µg total protein from cell supernatants was more than sufficient for the work described here, 10–30 mg of total protein was used in the *E. coli* lysate experiment described by Wang and Regnier.
In addition to reagent costs, the ICROC method appears to have some advantages over the first generation of ICAT reagents. First, we noted fewer nonspecifically bound peptides (those lacking cysteine) using the ICROC method. It is not clear if this is due to reduced nonspecific binding to the pyridyl disulfide beads or less release of nonspecifically bound peptides due to the gentle pH 8 elution. In either case, the significance of this is that singlet ions produced by nonspecifically bound peptides cannot be differentiated from ICAT-labeled peptides that have been completely up- or down-regulated. Sequencing the peptide is the only way to make this distinction. Second, labeling peptides with N-ethyl-iodoacetamide does not greatly increase the peptide mass, nor is there an increase in the peptide ion charge state. Furthermore, the most abundant ions in MS/MS spectra of ICAT-labeled peptides tend to be ICAT-related fragment ions, which are absent from peptides labeled with N-ethyl-iodoacetamide. For higher intensity precursor ions, these ICAT fragments are not a problem and can be construed as a benefit when trying to establish that a peptide is in fact labeled with ICAT and not nonspecifically bound. However, for low intensity precursor ions, shunting a significant portion of this current into the production of ICAT fragment ions rather than sequence-specific fragments could reduce the information content of the MS/MS spectra.

One disadvantage of ICROC compared with ICAT is that the isotopically labeled peptides are not mixed together until late in the procedure. Of course, this adds to the amount of work required—two samples must be separately digested with trypsin, attached to pyridyl disulfide beads, released, and labeled prior to mixing. In contrast, the ICAT procedure introduces the isotopic labeling early in the experiment, and then the combined samples are worked up as a single mixture. In practice, we do not find the additional work onerous, and, in

### Table I

| Protein | Peptide | Heavy | Light | Ratio |
|---------|---------|-------|-------|-------|
| SHPS-1  | VICEAHITLDR +3 | 1597  | 503   | 3.2   |
|         | VICEAHITLDR +2 | 561   | 184   | 3.0   |
|         | NNMDFSIR\* +2 | 1197  | 425   | 2.8   |
|         | VVLNSMDVHSK\* +2 | 1157  | 370   | 3.1   |
|         | LLYSFTGEHFPR\* +2 | 343   | 106   | 3.2   |
| C-FMS   | VLDSNTYVCK +2 | 5328  | 3340  | 1.6   |
|         | KLEFITQR\* +2 | 894   | 372   | 2.4   |
|         | VIIGSQLPIGTLK\* +2 | 541   | 319   | 1.7   |
|         | ASEAGQYFLMAQNK\* +2 | 981   | 450   | 2.2   |
| AmylA4  | SQVMTHLR\* +2 | 699   | 310   | 2.3   |
|         | QQLVETHMAR\* +2 | 373   | 159   | 2.3   |
| AXLr    | TSSFSCEAHNAK +2 | 209   | 96    | 2.2   |
| CD14    | NAGMETPSGVCSALAAR +2 | 441   | 436   | 1.0   |
| TNF     | GGGCPDYVLLHTVSRR +3 | 773   | 82    | 9.4   |
| TNFr2   | VCACEAGR +2 | 418   | 91    | 4.6   |
| IcosLig | NVTPQDQFETFTR +2 | 811   | 301   | 2.7   |
|         | TYTCMSK +2 | 1137  | 258   | 4.4   |
|         | LGLYDVISTLR\* +2 | 264   | 50    | 5.3   |
|         | VFMTATELVK\* +2 | 226   | 49    | 4.6   |
| CD18    | STTGCLNAR +2 | 717   | 217   | 3.3   |
|         | YNSQVCGGGSR +2 | 557   | 139   | 4.0   |
|         | SRGDCDGVQINNPVTPQ +3 | 311   | 135   | 2.3   |
| TEM7-R  | HRQDWDGSQEHQK +3 | 555   | 218   | 2.5   |
Studies of Shedding by ICROC

Fig. 6. Proteins that were found to be inducibly shed via a metalloprotease. To obtain the relative change in protein quantities for the inducibly shed proteins, the ratios in Table I were normalized by the average D5/D0 ion intensity ratio (i.e. 0.56) observed for the secreted or constitutively shed glycoproteins (Fig. 5, a and b). Error bars were obtained from cases where multiple peptides were observed for the same protein. SHPS-1 denotes SHP substrate 1 protein (GenPept: JC5289), C-FMS denotes macrophage colony-stimulating factor 1 receptor (GenPept: P09581), AmylA4 denotes Alzheimer’s disease amyloid A4 protein homolog (GenPept: P12023), AXL denotes AXL receptor tyrosine kinase (GenPept: NP 033491), CD14 has the GenPept accession number BAB8578, TNF denotes tumor necrosis factor (GenPept: AAA40457), TNFr2 denotes tumor necrosis factor receptor 2 (GenPept: P25119). IcosLig denotes ICOS ligand (GenPept: NP 056605), CD18 has a GenPept accession number of S04847, and TEM7-R protein). Likewise, six proteins (interleukin 1 receptor type 2, interleukin 6 receptor, low-density lipoprotein receptor, l-selectin, met proto-oncogene, and SorLA) were identified using the gel-based approach that were not found in the current experiment even though all of them contain several cysteines and methionines. Only five were found using both methods (Alzheimer’s disease amyloid A4 protein homolog, AXL receptor tyrosine kinase, macrophage colony-stimulating factor 1 receptor, SHP substrate 1 protein, and CD14).

One might expect somewhat different results using different methods, but it should also be pointed out that the earlier data was obtained on cells that were stimulated only by PMA, whereas the current experiments were carried out by stimulating the same cells using a combination of LPS and PMA. Beyond this, another explanation is that the current results were obtained from a single dimension of HPLC; the dynamic range of protein abundance would doubtless increase if multidimensional HPLC was used instead (22). Also, given equimolar quantities, higher molecular mass proteins stain more intensely on a gel compared with lower molecular mass ones. Hence, observation of high molecular mass proteins might be favored in the gel-based approach, and the no-gel approach might be skewed toward the observation of lower molecular mass proteins. Finally, there were probably proteins whose differential staining on a SDS-PAGE gel was obscured by abundant proteins that did not change; hence, those proteins were not selected for analysis in our earlier experiments.

Validation of these methods is provided by the identification of proteins previously known to be shed, which in turn gives confidence that the three new proteins are also shed molecules. One of these new proteins, CD18, is the beta-subunit of a B7 family coreceptor such as CD28. Recently, a new member of this coreceptor family, called ICOS, was identified, along with its ligand (28). This coreceptor is expressed as a transmembrane protein on activated T cells, whereas the ICOS ligand, also a transmembrane protein, is
expressed on a variety of antigen presenting cells, as well as in some nonlymphoid tissues such as muscle and kidney. Engagement of ICOS with its ligand, in conjunction with T cell receptor binding, results in an augmented proliferative response and cytokine production (29). This is the first report demonstrating that the ICOS ligand is shed from a cell membrane, and although the exact function of the shedding event is unknown, one would expect that a soluble form of the ligand would exhibit considerable immunological effects. For all three proteins, the biological significance of the shedding event is not obvious; however, implementation of amino acid-specific capture methods in conjunction with isotope dilution resulted in new information and raised new questions that can now be addressed using more standard hypothesis-driven research.

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