Selective Detection of Membrane Proteins Without Antibodies

A MASS SPECTROMETRIC VERSION OF THE WESTERN BLOT*

David Arnott‡§, Adrianne Kishiyama‡, Elizabeth A. Luis‡, Sarah G. Ludlum‡, James C. Marsters, Jr.¶, and John T. Stults‡

A method has been developed, called the mass western experiment in analogy to the Western blot, to detect the presence of specific proteins in complex mixtures without the need for antibodies. Proteins are identified with high sensitivity and selectivity, and their abundances are compared between samples. Membrane protein extracts were labeled with custom isotope-coded affinity tag reagents and digested, and the labeled peptides were analyzed by liquid chromatography-tandem mass spectrometry. Ions corresponding to anticipated tryptic peptides from the proteins of interest were continuously subjected to collision-induced dissociation in an ion trap mass spectrometer; heavy and light isotope-coded affinity tag-labeled peptides were simultaneously trapped and fragmented accomplishing identification and quantitation in a single mass spectrum. This application of ion trap selective reaction monitoring maximizes sensitivity, enabling analysis of peptides that would otherwise go undetected. The cell surface proteins prostate stem cell antigen (PSCA) and ErbB2 were detected in prostate and breast tumor cell lines in which they are expressed in known abundances spanning orders of magnitude. *Molecular & Cellular Proteomics 1:148–156, 2002.

Tools for the measurement and analysis of gene and protein expression patterns are at the core of several recently defined disciplines, functional genomics, transcriptomics, proteomics, and subfields such as pharmacogenomics and pharmacoproteomics. Among these tools, differential display of mRNA is performed routinely using cDNA microarrays (1, 2). Fluorescence detection, together with the amplification of DNA using the polymerase chain reaction, allows such experiments to be performed with exquisite sensitivity, and the parallel detection of thousands of gene products enables high throughput measurements. For protein measurement, 2D PAGE is capable of resolving 2500 or more distinct protein spots (3), making it the highest resolution protein separation experiment yet devised. This venerable technique has undergone a rebirth because of advances in reproducibility and automation and the ability to identify most detectable proteins using mass spectrometry and sequence data base searching (4, 5).

More recently, a particularly powerful technique to emerge is the combination of liquid chromatography and mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS). Because spectra from only a few peptides (or even a single peptide) can be sufficient to identify a protein, multiple components of a protein mixture can be identified (6). Several groups have used this technology to identify hundreds of proteins from the tryptic digests of crude cellular extracts (7–12). The isotope-coded affinity tag methodology (ICAT) first described by Gygi and colleagues (13, 14) has extended such experiments to allow relative quantitation of proteins between two samples. This technique involves differential labeling of proteins in two samples with affinity (e.g. biotinylation) reagents differing slightly in mass. After mixing and digestion of the samples the labeled peptides are isolated by affinity chromatography and analyzed by mass spectrometry. Each peptide is detected as two peaks in an LC-MS experiment. Tandem MS is used to identify the protein from which each peptide is derived, and the relative abundances of corresponding peaks reflect the amounts of protein in each sample from which they were derived.

As powerful and complementary as current genomic and proteomic tools are, they nevertheless suffer several shortcomings. Although tools such as cDNA microarrays are extraordinarily powerful for the simultaneous detection of thousands of gene products, mRNA levels do not necessarily correlate with protein expression levels (15, 16). 2D PAGE is of limited use in verifying DNA microarray results, because it is difficult to predict in advance which of the potentially thousands of spots corresponds to a given protein because of the spectrum of possible post-translational modifications. Alternatively, fluorescence-activated cell sorting, immunohisto-

* The abbreviations used are: 2D, two-dimensional; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; CID, collision-induced dissociation; ICAT, isotope-coded affinity tag; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PSCA, prostate stem cell antigen; HPLC, high pressure liquid chromatography.
chemistry, Western blots, enzyme-linked immunosorbent assays, and other antibody-based approaches can be used to explore the expression patterns and biological function of proteins. These powerful, but often time-consuming, techniques are currently the methods of choice to expand on the results of mRNA-based experiments. Reliance on antibodies, however, makes this difficult to do quickly, because antibodies must first be generated for each target protein. Furthermore, an antibody that binds a native protein (as in immunoprecipitation) may not be useful for detecting the denatured protein on a Western blot. Thus, a technique is needed that is similar to a Western blot but does not require an antibody to each protein of interest. Such a technique should be rapid, sensitive, quantitative, and capable of identifying a specific protein out of extremely complex mixtures without bias or need for extensive purification of intact proteins.

We have developed an analytical procedure with the potential to meet these requirements based on the ICAT methodology of Gygi et al. (13). This experiment, dubbed the mass western in analogy to the Western blot, was applied to the detection of proteins from plasma membrane preparations of human cells without electrophoresis or other initial purification steps. Proteins assayed included the prostate stem cell antigen (PSCA) and the receptor tyrosine kinase ErbB2, which are over-expressed in significant numbers of prostate tumor and breast tumors, respectively.

**Experimental Procedures**

**Purification of Membrane Proteins**—Breast tumor cell lines SK-BR-3 and MCF-7, or prostate tumor cell line PC3 transfected with the full-length sequence of PSCA, were used in these experiments. Eighty million of the transfected PC3 cells (designated clone 11) were used in the PSCA experiment. Fifty million MCF-7 cells and approximately half as many SK-BR-3 cells, normalized by total protein content, were used in the ErbB2 study. Cells cultured in flasks (175 cm²; Falcon) were harvested and disrupted with a Dounce homogenizer (17). In the ErbB2 experiment the cell lysate was centrifuged at 1000 × g for 5 min to remove intact cells and nuclei. The resulting post-nuclear supernatant was centrifuged at 16,000 × g for 15 min to produce a crude membrane pellet. In the PC-3 experiment the post-nuclear supernatant was layered over a 35% (w/v) sucrose solution and centrifuged in a SW55Ti rotor at 38,000 × g for 45 min. The membrane layer was collected, pelleted, and washed successively for 30 min with each of the following: 1) ice-cold 25 mM Na₂CO₃, pH 11, to remove cytoskeletal proteins (16), 2) 0.5% Tween 20 (Calbiochem) at 4 °C, and 3) cold phosphate-buffered saline, with each step followed by centrifugation at 16,000 × g for 15 min. Protein concentrations were determined by bicinchoninic acid assay.

**d₃ ICAT Reagent Synthesis**—a-N-iodoacetyl-e-N-biotinyl-L-lysine trideuteromethyl ester was prepared in a two-step synthesis. e-N-biotinyl-L-Lysine (biocytin, 100 mg; Sigma) was suspended in methanol (62 mg; M + H) = 557.8 (558.2 calculated). Incorporation of the label increases the residue mass of cysteine by 426.2 Da. d₃ ICAT Reagent Synthesis—a-N-iodoacetyl-e-N-biotinyl-L-lysine trideuteromethyl ester was prepared similarly using methanol (62 mg; M + H) = 557.5 (555.1 calculated). Incorporation of the label increases the residue mass of cysteine by 426.2 Da.

**Labeling and Digestion of Proteins**—Membrane pellets were solubilized in 0.5% SDS in 100 mM HEPES, pH 8.5. Disulfide bonds were reduced by the addition of tributyl phosphate to a 1 mM concentration with incubation for 5 min at 90 °C. Cysteine residues were biotinylated using either polyethyleneoxide-iodoacetyl biotin (Pierce) or the ICAT reagents synthesized as above. These sulfhydryl-reactive reagents were added to a concentration of 5 mM and incubated at room temperature in the dark for 1 h. Excess reagents were removed by chloroform/methanol precipitation of the proteins (19), which were resuspended by sonication in digestion buffer (50 mM HEPES, pH 8.5), followed by stepwise addition of SDS and Triton X-100 to concentrations of 0.5 and 1%, respectively. 500 units of PNGase F (New England Biolabs) were added to deglycosylate proteins for 1 h at 37 °C. Modified trypsin (Promega) was added in a 1:50 weight ratio, and digestion was allowed to proceed overnight at 37 °C.

**Avidin Purification of Biotinylated Peptides**—Monomeric avidin affinity columns with a 1-ml bed volume (Pierce) were packed according to the manufacturer’s instructions. Protein digests were heated to 90 °C for 1 min and treated with AEBSF (Roche Molecular Biochemicals) to inhibit residual trypsin activity and loaded onto the column in phosphate-buffered saline adjusted to pH 6.5. The column was washed with 20 ml of phosphate-buffered saline to remove unlabeled peptides. An extra wash of 3 ml of deionized distilled water removed excess sodium. Biotinylated peptides were then eluted onto a polysulfoethyl aspartamide ion exchange column (PolyLC Inc.) with 5 ml of 50 mM trifluoroacetic acid in 25% acetonitrile. The ion exchange column was washed with 750 μl of 0.1% formic acid/25% acetonitrile to remove residual detergent. The peptides were eluted with five 100-μl fractions of 500 mM sodium chloride/0.15% acetic acid, pH 4.1/25% acetonitrile. The fractions were then diluted to 500 μl with 0.1% heptfluorobutryric acid. The fractions were loaded onto a C18 cartridge (1 × 8 mm; Michrom BioResources) and washed with 250 μl of 0.1% heptfluorobutryic acid to remove sodium. The peptides were eluted with 20 μl of 75% acetonitrile/0.1% trifluoroacetic acid. LC-MS/MS—Peptide mixtures (2 μl diluted to 50 μl with 0.1% heptfluorobutryic acid) were loaded onto a 0.25 × 30-mm trapping cartridge packed with Vydac 214MS low trifluoroacetic acid C4 beads. This cartridge was placed in-line with a 0.1 × 100-mm resolving column packed with Vydac 218MS low trifluoroacetic acid C18 beads. The resolving column was constructed using a PicoFrit™ (New Objective) fused silica capillary pulled to a 30-μm metal-coated tip, which formed a microelectrospray ionization emitter. Peptides were eluted with a 2-h gradient of 5–80% (v/v) acetonitrile containing 0.1% formic acid/0.005% trifluoroacetic acid at a rate of 0.5 μl/min. Tandem mass spectrometry was performed using an ion trap instrument (LCQ DECA; ThermoFinnigan). For the mass western experiment, three or four selected precursor ions, chosen from the predicted tryptic peptides of the protein of interest, were subjected to collision-induced dissociation (CID) one after the other, cycling repeatedly throughout the LC gradient. Doubly charged ions were assumed for peptides of 8 to 20 residues. A precursor isolation window of 3 Da was used for polyethyleneoxide-iodoacetyl labeled peptides. Heavy and light ICAT-labeled peptides were simultaneously trapped and fragmented by using a 5-Da isolation window. The Sequest data
base-searching program was used to generate cross-correlation scores for each CID spectrum versus the predicted peptides.

RESULTS AND DISCUSSION

Principles of the Technique—The mass western experimental scheme is illustrated in Fig. 1A. It is similar to the LC-MS-based approaches described above in that relatively crude cellular extracts are labeled with a cysteine-reactive biotinylation reagent and digested without prior separation followed by isolation of the cysteine-containing peptides and analysis by LC-MS/MS. But whereas the object of those experiments was to identify and quantify as many proteins as possible, a more directed approach has been taken to verify the presence of one or a few specific proteins. Given the sequence of a protein, the masses and CID fragmentation patterns of its tryptic peptides are predictable. An LC-MS/MS experiment can therefore be devised that monitors only the predicted tryptic peptides from the protein(s) of interest. The selectivity of tandem mass spectrometry allows all peptides with the wrong precursor masses to be ignored, and the specificity of the fragmentation patterns allows each chosen peptide to be distinguished from all others with the same nominal precursor mass. The number of peptides that can be analyzed in one LC-MS/MS experiment is a function of chromatographic peak widths and the duty cycle of the instrument. With chromatographic peak widths of 20 to 30 s and scan times of 5 s per CID spectrum, three or four ions can be monitored with a strong likelihood of acquiring several spectra for each of the selected ions. Wherever possible, peptides with molecular masses of 1000 to 2500 Da and lacking methionine, tryptophan, or likely sites of post-translational modification are chosen to be assayed.

The ICAT methodology was used to achieve quantitative comparisons of samples, but the reagents and scan modes were adapted to optimize sensitivity. Previously reported ICAT experiments used isotope ratios detected in full mass range scans for quantitation, with MS/MS performed in separate scans for protein identification (13, 14). A characteristic of ion trap mass spectrometers is extraordinarily high sensitivity for MS/MS experiments because of the ability to trap and accumulate precursor ions. Under the conditions used in these experiments high quality CID spectra of peptides can be obtained at the 250 attomole level, whereas the limit of detection in full mass MS mode is on the order of 5 to 10 femtomoles. Peptides can therefore be detected by MS/MS that would otherwise be lost in the background noise of full mass range MS. This property of ion traps has been exploited, for example, to obtain MS/MS data on peptides initially detected by matrix-assisted laser desorption-ionization-time-of-flight MS (20, 21). To take advantage of the sensitivity of the ion trap for MS/MS new ICAT reagents were synthesized that incorporate three (rather than eight) deuterium atoms in the heavy version (Fig. 1B) so that the multiply charged precursor ions of both heavy and light ICAT-labeled peptides are simul-
taneously trapped and subjected to CID. Fragment ions in the CID spectrum that contain cysteine thus appear as doublets separated by 3 Da allowing both quantitation and identification to be derived from a single scan function performed at maximal sensitivity.

**Sample Handling Optimization**—The intrinsic sensitivity of capillary HPLC microelectrospray ionization MS and MS/MS on ion trap mass spectrometers is extremely high; efficient sample preparation is therefore a key factor to success. The losses associated with several steps outlined in Fig. 1 were assessed. The initial protein extraction appears robust, as evidenced by minimal residue after solubilization. The extent of labeling with the ICAT reagents is difficult to determine on the membrane preparations themselves, but bovine serum albumin labeled for 30 min followed by addition of excess iodoacetamide revealed no incorporation of the second reagent (data not shown).

The removal of excess alkylating reagents after labeling is essential, because they are present in excess over proteins and otherwise dominate the mass spectra. These reagents are not removed by reverse phase, avidin, or size exclusion (nominal 10-kDa cutoff) chromatography. Reaction with tributyl phosphate yields a positively charged molecule, so cation exchange chromatography is only partially effective, although use of this method has been reported (22). Chloroform-methanol precipitation, however, effectively removed the reagents. Resolubilization of the precipitated proteins was efficient, with amino acid analysis of the labeled membrane proteins indicating 92% recovery for this procedure. Acid hydrolysis of the labeled proteins cleaves the amide bonds internal to the biotinylation reagents converting each labeled cysteine to carboxymethyl cysteine, which serves as a marker to track the abundance of labeled peptides through the analysis.

The use of SDS facilitates solubilization of the membrane proteins but poses a problem for enzymatic digestion. Trypsin activity is almost undetectable at SDS concentrations above 0.25% as measured by a synthetic substrate \( p \)-nitroaniline assay; even at 0.05% SDS, 25% of activity is lost. But if a zwitterionic or nonionic detergent like Triton X-100 is first added to an equal or greater concentration than the SDS, trypsin retains its full activity (Fig. 2A). The bands between 10 and 20 kDa observed post-digest are consistent with the presence of trypsin, and the high molecular mass smear is also found in control samples; essentially complete digestion is inferred. Purification of the labeled peptides on the avidin column is another step where sample can be lost. As determined by amino acid analysis, 82% of labeled peptides were recovered when eluted from the column. Of the peptides recovered, over 90% eluted in the second and third fractions collected (1 ml = 1 column volume per fraction), and 99% eluted in the first four fractions.

**Mass Western for Selective Detection of PSCA from Plasma Membranes**—Prostate stem cell antigen was detected in a cell line engineered for its expression. PSCA, a 123-amino acid glycoprotein in the family of glycosylphosphatidylinositol-anchored cell surface antigens, is predominantly expressed in prostate epithelium and is overexpressed in a majority of human prostate cancers (23). PSCA is a very hydrophobic protein, having a grand average hydropathy score of 0.48, typical of membrane proteins (24). Grand average hydropathy scores in several model organisms range from \(-2.2\) (most hydrophilic) to 1.7 (most hydrophobic) (25). Despite progress (26–28), only a few proteins with positive grand average hydropathy scores have been identified by 2D electrophoresis (29, 30). PC3 cells otherwise lacking PSCA were stably transfected with the PSCA gene. An expression level of \(~200,000\) copies per cell as estimated by Scatchard analysis (data not shown) was obtained in the cell line designated clone 11. A subcellular fraction enriched in plasma membrane was prepared from \(~80\) million cells by differential sedimentation; one-third of the total PSCA was recovered in this fraction, with the remainder found among the cellular debris pellet as determined by Western blot (data not shown).

Proteins in the clone 11 membrane pellet were solubilized, disulfide bonds were reduced, and cysteines were labeled with commercially available polyethyleneoxide-iodoacetyl biotin. Following removal of excess reagents and lipids, proteins were deglycosylated and digested with trypsin. The course of these steps was assessed by SDS-PAGE (Fig. 2B). A Western blot probed with anti-PSCA before deglycosylation (lane 1) and after (lane 2) demonstrates consolidation of a diffuse 25–30-kDa band to a compact band at 14 kDa. Precipitated protein was efficiently recovered (lane 3 versus lane 2). Native PSCA resisted trypsin digestion but was effectively cleaved following deglycosylation (lane 4). A silver-stained gel
Fig. 2A illustrates the complexity of PC3 membrane fractions (lane 1) and completeness of digestion (lane 2).

The presence of PSCA in the clone 11 sample was proven by detection of the peptide GCSLNCVDDSQDYYVGK in the avidin-purified tryptic digest. An aliquot (10%) of the peptide mixture was analyzed by capillary reverse phase LC-microspray ion trap mass spectrometry. The instrument data system was programmed to alternately collect full mass range spectra and product ion spectra of ions with m/z 1347, the anticipated doubly charged ion of the labeled PSCA peptide. Although unnecessary for the mass western experiment, the full mass range spectra were acquired so that the overall complexity of the sample could be judged. The results are diagrammed in Fig. 3. As expected, the full mass range spectra are exceedingly complex (Fig. 3A), with many coeluting peptides at every time point. An extracted ion chromatogram for ions with m/z 1347 is also complex, with over 50 discrete peaks apparent (Fig. 3B). The PSCA peptide was distinguished from all others by its fragmentation pattern. A reconstructed ion chromatogram for ions with m/z 1347 that fragment to form a product with m/z 1189, corresponding to the y_{10} ion of the PSCA peptide, shows only two peaks, a small one with a retention time of 55 min and a larger peak at 64 min (Fig. 3C). The Sequest algorithm (31) was used to compare every CID spectrum to the calculated product ions of the PSCA peptide by searching a data base consisting only of the sequence of PSCA. When the cross-correlation score reported by Sequest is plotted for each CID spectrum (Fig. 3D) it is apparent that both major and minor peaks match PSCA. Examination of the complete CID spectra for both peaks revealed them to be qualitatively identical and containing extended series of ions matching those predicted for the PSCA peptide (Fig. 3E corresponds to the larger peak). The reason for the chromatographic splitting of the peptide is unknown but may be because of secondary structure effects in this relatively large and doubly-biotinylated peptide.

Validation of Quantitation from MS/MS Spectra—Reproducible quantitation of proteins by ICAT methodology was demonstrated by Gygi et al. (13) by comparison of peptide MS signals, but our approach of quantifying proteins from MS/MS data introduces new considerations. To illustrate these, lysozyme samples were labeled with the d_{0}/d_{3} ICAT reagents and combined in ratios of 1:5, 1:1, and 3:1, digested, and analyzed by LC-MS/MS. The CID spectrum of the labeled tryptic peptide GYSLGNWVCAAK is displayed in Fig. 4A and is qualitatively identical for each sample. Each of the y ions beginning with y_{4} contains the labeled Cys, and these peaks appear as doublets from which quantitative data can be extracted. Panels B, C, and D from Fig. 4 detail the y_{10} ion from each sample. No chromatographic separation was observed between d_{0}- and d_{3}-labeled peptides, and isotope ratios were consistent across the peaks, a result consistent with all peptides studied to date. This is significant, because a shift in elution times between heavy and light isotopic forms would have a deleterious effect on quantitation. Zhang et al. (32) have measured retention time differences among d_{0}, d_{3}, d_{4}, and d_{8} isotope labels, with the finding that d_{0}- and d_{4}-labeled peptides were sufficiently resolved to introduce substantial worst case errors in quantitation, with smaller deviations for d_{2}- and d_{3}-labeled peptides (32). Their predicted shift of about 1 s for peak maxima of d_{0} versus d_{3} would likely go...
Reliable quantitation requires that the light and heavy ICAT-labeled forms of a peptide are both trapped and fragmented in the ion trap mass spectrometer. Expanding the precursor isolation window can accomplish this, at the cost of some increase in background noise. A window of 5 Da was empirically determined to be sufficient for trapping two isotope clusters separated by 1.5 Da, the difference between doubly charged ions of d_0/d_3-labeled peptides that contain one cysteine. If a peptide contains more than one cysteine the ion selection window is insufficiently wide. In that case either a label with fewer deuterium atoms should be used, or another peptide should be selected.

Each product ion in a CID spectrum that contains cysteine can be used to calculate relative abundance; these are averaged to obtain a more accurate measurement. In the lysozyme examples, the calculated ratios d_3/d_0-labeled peptides and standard deviations are 5.0 ± 0.6 for the first sample, 0.9 ± 0.1, and 0.29 ± 0.05. The relative standard deviations are 11, 9, and 18%, respectively. More precise values could be obtained by averaging the results from multiple peptides from the same protein, but even the single peptide considered here yielded precisions adequate for most biological experiments.

It is necessary that the isotope clusters of the labeled product ions are sufficiently resolved for extraction of quantitative data. The ion trap mass spectrometer operates at unit resolution, so the isotope patterns of singly charged product ions are readily apparent. A more serious complication is overlap of the second ^13C isotope of a d_0 ICAT-labeled product ion with the ^12C isotope of the corresponding d_3 ICAT-labeled product ion, skewing the apparent intensities of these peaks. The natural isotopic abundance of carbon is such that the second ^13C peak is less than 10% of the ^12C isotope in peptides and fragment ions below 1,000 Da, increasing to about 35% at 2,000 Da. This contribution can generally be ignored when the d_3-labeled peak is more abundant than the d_0-labeled peak or when the two peaks are of similar abundance. When the d_3-labeled peak is small compared with the d_0-labeled peak low mass product ions can be used for quantitation, because they contain little ^13C, or a correction can be made based on theoretical isotope ratios. If the intention is to test hypotheses such as "protein X is more abundant in sample A than sample B," one would choose to label the putatively more abundant sample with the heavy ICAT reagent.

**Quantitative Comparison of ErbB-2 Expression between Breast Tumor Cell Lines**—The 185-kDa receptor tyrosine kinase ErbB2 is overexpressed in 15–30% of invasive ductal breast cancers (33). A humanized monoclonal antibody raised against ErbB2 has proven an effective therapeutic for relapsed, refractory metastatic breast cancer both as a single agent and in conjunction with chemotherapy (34). Expression levels of ErbB2 have been quantified in a variety of breast tumor cell lines including MCF-7 and SK-BR-3. MCF-7 cells express 15,000 receptors per cell (clinically normal expression level) whereas SK-BR-3 cells can express as many as 2 million copies per cell, depending on culture conditions (35). Comparison of the cells used for our experiment shows an ErbB2 expression ratio of between 1:15 and 1:20 (MCF-7:SK-BR-3) by Western blot (data not shown). The quantitative version of the mass western experiment was performed to compare the relative abundance of ErbB2 in these cell lines. Membrane proteins from ~50 million MCF-7 cells were labeled with the d_0 ICAT reagent, and proteins from the membranes of SK-BR-3 cells were labeled with the d_3 ICAT reagent. Because of the much different expression levels expected, twice as much protein as determined by bicinchoninic acid assay from the MCF-7 membrane preparation was used as from the SK-BR-3 cells. The samples were mixed,
Selective Detection of Membrane Proteins without Antibodies

A Proteomic Approach to Hypothesis-driven Science—The mass western experiment described here represents the use of proteomic techniques for testing hypotheses about protein expression. This is in contrast to most proteomic investigations to date, which have used one- or two-dimensional electrophoresis, multidimensional protein chromatography, or ICAT LC-MS for the wholesale, non-directed collection of protein expression data (see Refs. 12, 22, and 36–41 for examples). As with traditional Western blots, the mass western technique can detect a specific protein from a highly complex mixture. Rather than using an antibody for the chemical recognition of a three-dimensional epitope, physical detection of a peptide is accomplished by mass spectrometry. The specificity of the CID fragmentation pattern of a peptide is such that false-positive results are very unlikely provided the peptide chosen for detection is unique to the protein of interest, a question that can be answered by a BLAST search of the peptide sequence(s) under consideration (42). Just as blotted proteins can be probed sequentially with several antibodies, peptides from multiple proteins can be detected in subsequent LC-MS/MS experiments, provided sufficient sample is available. This experiment differs from true Western blots in that no indication of the size of a protein is obtained, because gel electrophoresis is not performed.

An important advantage of this experiment compared with techniques such as 2D electrophoresis is its general applicability. Even previously unknown proteins predicted on the basis of expressed sequence tags or genomic DNA should be identifiable if the gene sequence is correct and translated in-frame. Almost any protein that can be solubilized with SDS and reducing agents is potentially detectable. These include very large (e.g. ErbB2) and/or hydrophobic proteins such as PSCA. Some proteins (less than 10% of all proteins with molecular mass over 15 kDa) lack any cysteine residues, and some further proteins do not contain suitable Cys-containing amine-reactive) chemistries e.g. amine-reactive) chemistries. As with traditional Western blots, the mass western technique can detect a specific protein from a highly complex mixture. Rather than using an antibody for the chemical recognition of a three-dimensional epitope, physical detection of a peptide is accomplished by mass spectrometry. The specificity of the CID fragmentation pattern of a peptide is such that false-positive results are very unlikely provided the peptide chosen for detection is unique to the protein of interest, a question that can be answered by a BLAST search of the peptide sequence(s) under consideration (42). Just as blotted proteins can be probed sequentially with several antibodies, peptides from multiple proteins can be detected in subsequent LC-MS/MS experiments, provided sufficient sample is available. This experiment differs from true Western blots in that no indication of the size of a protein is obtained, because gel electrophoresis is not performed.

An important advantage of this experiment compared with techniques such as 2D electrophoresis is its general applicability. Even previously unknown proteins predicted on the basis of expressed sequence tags or genomic DNA should be identifiable if the gene sequence is correct and translated in-frame. Almost any protein that can be solubilized with SDS and reducing agents is potentially detectable. These include very large (e.g. ErbB2) and/or hydrophobic proteins such as PSCA. Some proteins (less than 10% of all proteins with molecular mass over 15 kDa) lack any cysteine residues, and some further proteins do not contain suitable Cys-containing amine-reactive) chemistries e.g. amine-reactive) chemistries. As with traditional Western blots, the mass western technique can detect a specific protein from a highly complex mixture. Rather than using an antibody for the chemical recognition of a three-dimensional epitope, physical detection of a peptide is accomplished by mass spectrometry. The specificity of the CID fragmentation pattern of a peptide is such that false-positive results are very unlikely provided the peptide chosen for detection is unique to the protein of interest, a question that can be answered by a BLAST search of the peptide sequence(s) under consideration (42). Just as blotted proteins can be probed sequentially with several antibodies, peptides from multiple proteins can be detected in subsequent LC-MS/MS experiments, provided sufficient sample is available. This experiment differs from true Western blots in that no indication of the size of a protein is obtained, because gel electrophoresis is not performed.
background noise in CID spectra. At low signal-to-noise ratios the lower abundance ICAT-labeled ion series may not be detected, placing a limit on the calculable abundance ratio. ErbB2 expressed at 15,000 copies per cell was detected, and significantly lower amounts are likely tractable with our current procedure, but many important proteins are present at copy numbers of 1,000 per cell or less. Detection of such proteins will require additional fractionation of peptides, either by selection of different peptide subsets (e.g. histidine-containing sequences) or tandem chromatographic separations (8, 11, 12).

Acknowledgments—We thank Tony Moreno and Wendy Shillinglaw in the laboratory of William J. Hnzel for performing the amino acid analysis and Susan Spencer for providing the PC3 clone 11 cell line. Professor Ruedi Aebersold kindly provided advice on the avidin purification of ICAT-labeled peptides.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated asadvertisement.

To whom correspondence should be addressed: Genentech, §

REFERENCES

42. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410